

Renewable Biomass as a Platform for Preparing Green Chemistry

Edited by Qiaoguang Li, Puyou Jia, Ying Luo, Yue Liu Printed Edition of the Special Issue Published in Journal of Renewable Materials

https://www.techscience.com/journal/jrm



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Editors

Qiaoguang Li

Puyou Jia

Ying Luo

Yue Liu



Editors

Qiaoguang Li Associate Professor Zhongkai University of Agriculture and Engineering Guangzhou China

Puyou Jia Associate Professor Chinese Academy of Forestry Beijing China

Ying Luo Associate Professor South China Agricultural University Guangzhou China

Yue Liu Associate Professor Qingdao University of Science and Technology Qingdao China

Editorial Office Tech Science Press 871 Coronado Center Drive Suite 200, Henderson, Nevada 89052, USA

This is a reprint of articles from the Special Issue published online in the open access journal *Journal of Renewable Materials* (ISSN 2164-6325 (print); ISSN 2164-6341 (online)) (available at: https://www.techscience.com/journal/jrm).

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Author Surname, Author Initial. (Year Published). Title. *Journal Full Name, Volume Number (Issue Number)*, Page Number.

ISBN 978-1-7340206-9-4 (PDF)

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DOI: 10.32604/jrm.2023.044083

EDITORIAL





Renewable Biomass as a Platform for Preparing Green Chemistry

Qiaoguang Li^{1,*}, Puyou Jia^{2,*}, Ying Luo³ and Yue Liu⁴

¹College of Chemistry and Chemical Engineering, Zhongkai University of Agriculture and Engineering, Guangzhou, 510225, China
 ²Institute of Chemical Industry of Forestry Products, Chinese Academy of Forestry, Nanjing, China
 ³College of Materials and Energy, South China Agricultural University, Guangzhou, 510642, China
 ⁴College of Chemical Engineering, Qingdao University of Science and Technology, Qingdao, 266061, China
 ^{*}Corresponding Authors: Qiaoguang Li. Email: liqiaoguang8799@163.com; Puyou Jia. Email: jiapuyou@icifp.cn
 Received: 01 July 2023 Accepted: 13 July 2023

1 About the Special Issue Editor

Qiaoguang Li is an associate professor and master's supervisor in the Department of College of Chemistry and Chemical Engineering, Zhongkai University of Agriculture and Engineering. He received his PhD from Institute of Chemical Industry of Forestry Products, Chinese Academy of Forestry in 2018. He has been focusing his research on the chemical basis and application of natural resources. He has published nearly 30 international peer reviewed papers and applied for 10 patents. He is now responsible for more than 8 items of research projects competitively granted from natural science foundation of China, Department of education of Guangdong Province and other institutions.

He has received the third prize of Guangdong Provincial Agricultural Technology Promotion Award (first) and appointed as a young editorial board member for the Sustainable Structures journal and guest editor in chief for Journal of Renewable Materials, and Frontiers in Environmental Chemistry journals.

Puyou Jia completed Ph.D. in 2016 at the Beijing Forestry University. Then, he joined Institute of Chemical Industry of Forestry Products, Chinese Academy of Forestry (CAF) as an associate professor. Puyou Jia's work broadly concentrates on the chemical transformation and utilization of biomass resources. He has published more than 100 papers on the atom-efficient, ecofriendly, highly efficient chemical transformations of a variety of biomass into sustainable intermediates, polymer auxiliaries, bioplastics, elastomers and advanced bio-based materials.

Ying Luo, Associate Professor, College of Materials and Energy, South China Agricultural University, China. Dr. Ying Luo received her doctoral degree of Polymer Chemistry and Physics from Sun Yat-sen University, China, in 2005. Her research interests have been focused on the preparation & functional modification of polymer or bio-based polymer materials, and degradable plastics. She has authored or co-authored more than 30 international peer reviewed papers and 1 book chapters. She has applied 4 patents.

Yue Liu is currently an associate professor and master supervisor at the College of Chemical Engineering, Qingdao University of Science and Technology. He received his PhD degree from Qingdao University of Science and Technology in 2017. His research interests include environment-friendly catalysis, fine chemical industry, biomass conversion, core-shell composite materials, and the preparation, modification, characterization and formation mechanism of core-shell catalysts. He has published 23 SCI



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papers as the first author or corresponding author. He has presided over one project of Natural Science Foundation of Shandong Province, one project of Open Fund Project of Jiangsu Key Laboratory of Biomass Energy and Material, and three enterprise projects.

2 Special Issue Introduction

This, combined with the associated environmental issues around global warming and environmental pollution, has resulted in a drive for scientific research to alleviate the dependence of society on chemical compound/polymer derived from fossil fuels. The biomass (such as cellulose and lignin) of carbohydrates are rich in hydroxyl makes it through the hydrothermal catalytic hydrogenation, dehydration and oxidation etc directly into polyols, lactic acid and 5-HMF important ideal oxygen chemical raw material. Cellulose, sugar, starch, natural fiber and vegetable oil in these renewable resources have been used as green monomers or intermediates for polymer preparation and polymer materials. Moreover, the development and application of these renewable biomass as a platform for preparing of green chemistry will improve their utilization, contribute to the protection of the environment and reduce dependence on petrochemical products. This special issue, which consists of **14 articles, including one review article**, focuses on the latest advances in green chemistry and bio-based polymer materials.

Yuan et al. researched the thermal decomposition process and kinetics behavior of oleuropein from the olive resource. For the first and second thermal decomposition stages, the Kissinger, Friedman, Flynn-Wall-Ozawa, and Coats–Redfern four methods were applied to determine the activation energy (E = 143.72 and 247.01 kJ mol⁻¹) and Arrhenius preexponential factor (ln A = 26.34 and 42.45 min⁻¹), respectively.

Li et al. reported that the regenerative electrochemical system is a microbial electrolytic cell improvement system for methane gas produced by biological carbon sequestration technology using renewable energy sources to provide a voltage environment. The methane production rate of regenerative electrochemical system is increased by 2 times and the start-up time is reduced to 10 days. This study can obtain a theoretical basis and technical reference for the early industrial application of microbial CO_2 methanation technology based on renewable energy.

Fu et al. studied the overall safety assessment of eucommia ulmoides leaf extract (ELE) was performed, including genotoxicity and long-term toxicity. Plasma triglycerides and low-density lipoprotein cholesterol levels significantly decreased, and plasma high-density lipoprotein cholesterol levels significantly increased with ELE treatment. Not only ELE did not cause genotoxicity, but it possessed good bioactivities and health-promoting. Therefore, we affirmed ELE is safe to consume as a traditional Chinese health food.

Lai et al. successfully synthesized copper nanoparticles in Masson's Pine as a preservative process for sawn timber by situ generation. The results showed that the samples treated by heating without copper salt treatment showed poor suppression of fungal growth, the copper-impregnated heat-treated wood suppressed (100%) the growth of Botryodiplodia theobromae Pat., Aspergillus niger V. Tiegh., Penicillium citrinum Thom, and Trichoderma viride Pers. This study has demonstrated an effective method of increasing low-grade wood's utility and commercial value.

Wang et al. fabricated cationic lignin hydrogels for dye adsorption. The maximum Congo red removal efficiency was obtained at the initial concentration of Congo red of 50 mg/L, pH 7, and 5 mg dosage of cationic lignin hydrogel with 20% cationic lignin content. After five cycles of adsorption, the adsorption efficiency of the hydrogel for Congo red still reached more than 80%. These results demonstrate the potential of the CKLA hydrogel as an adsorbent for water treatment.

Yan et al. prepared of vegetable-oil-based thioether polyol and ethyl cellulose supramolecular composite films. Pure ethyl cellulose is fragile, and the addition of Ethyl cellulose makes the ethyl cellulose films more flexible. The application of bio-based material is environmentally friendly, and the novel DATP can be used as a special and effective plasticizer to prepare flexible ethyl cellulose films.

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Chu et al. studied phosphorus containing rubber seed oil as a flame retardant plasticizer for polyvinyl chloride via epoxidation reaction and ring opening addition reactions. When Dioctyl Phthalate was replaced with flame-retardant rubber seed oil-based plasticize, the torque of PVC blends increased from 11.4 to 18.4 N·m, the LOI value increased from 24.3% for PVC-FRP-0% to 33.1% for PVC-FRP-20, suggesting a new way to prepare flame retardant plasticizer using rubber seed oil as raw material.

Zhao et al. reviewed research progress of tung oil/ultraviolet photocomposite curing material. Firstly, the chemical structure and application of tung oil and ultraviolet Photocatalysis Technology were briefly introduced. Secondly, the research status of novel tung oil/ultraviolet photo-composite curing materials developed by the Diels-Alder reaction was discussed. Finally, the research and application prospects of TO/UV photo-composite curing materials were presented.

Yao et al. specially designed and synthesized a tung oil-based boron-nitrogen coordination polymer (TWE-BN) as a highly efficient water-based lubricant additive for investigating its hydrolysis stability and tribological properties. TWE-BN was better than nitrogen-free tung oil-based lubricant additive and remained non-hydrolyzed for at least 15 days, implying the feasibility of tung oil-based boron-nitrogen coordination as highly effective and hydrolytic stability lubricant additives.

Tang et al. used rosin and choline as raw materials to prepare a bio-based CO_2 responsive surfactant rosin acid dimaleimide choline (R-BMI-C) with an extremely rigid skeleton by Diels-Alder addition reaction and acid-base neutralization reactions. The internal aggregation structure of R-BMI-C aqueous solution changed from spherical micelles to laminar micelles.

Wang et al. researched the promoting effect of multifunctional groups on the thermal and mechanical properties of PVC Materials. Myrcene with double bonds, amino groups, ester groups, and phospholipid groups was introduced into the chains of PVC to improve the thermal stability of PVC. The double bonds trapped the unstable chlorine atoms originated from the degradation of the PVC chain and reacted with the labile macromolecular radicals originated from PVC, thus inhibiting the radical degradation of the PVC chain. Furthermore, the amino groups absorbed the HCl produced in the degradation of PVC, inhibiting the adverse effects of HCl.

Chang et al. synthesised the antioxidant activity of (E) ω -formylcamphene-based thiazole hydrazone derivatives. The results show that 14 (E) ω -formylcamphene-based thiazole hydrazone compounds exhibited good scavenging effects on the two free radicals, especially when the concentration of the drug solution was 125 and 62.5 mg/L; most compounds exceeded the scavenging efficiency of Trolox and L-ascorbic acid.

Huang et al. successfully prepared fosthiazate-stearic acid/expanded perlite sustained-release particles by vacuum impregnation using expanded perlite (EP) as carrier, fosthiazate (FOS) as model pesticide and stearic acid (SA) as hydrophobic matrix. With the mass ratios of FOS to SA decreasing from 7:3 to 3:7, the 24 h release rate of FOS-SA/EP decreased from 18.77% to 8.05%, and the drug loading decreased from 461.32 to 130.99 mg/g.

Wang et al. assessed their effects on H_2O_2 -induced oxidative damage model of human umbilical vein endothelial cell (HUVEC) by selecting four characteristic components (chlorogenic acid, geniposidic acid, aucubin, quercetin). Cell cycle, cell apoptosis, cell senescence, and their related proteins under characteristic components treatment exhibited a better effect than under H_2O_2 treatment, implying the characteristic components could participate in anti-aging via multiple pathways.

In summary, green chemistry and bio-based polymer materials made from various renewable feedstocks represent one of the most promising directions due to their sustainability. These field have attracted extensive attention and achieved significant progress during the past few decades. The field of green chemistry and bio-based polymer materials is experiencing rapid growth, which should continue for the coming decade.

Acknowledgement: This special issue would not have been come out without the authors' insightful contributions and reviewers' professional work, which we gratefully acknowledge for the wonderful and productive relationship we had. We would also like to express our gratitude to the editorial team of Journal of Renewable Materials for all of their assistance and support.

Funding Statement: This work was supported by the National Natural Science Foundation of China (Grant No. 32101475), Scarce and Quality Economic Forest Engineering Technology Research Center (Grant No. 2022GCZX002) and the Key Lab. of Biomass Energy and Material, Jiangsu Province (Grant No. JSBEM-S-202305).

Author Contributions: All authors contributed equally to this work. The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Availability of Data and Materials: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.



DOI: 10.32604/jrm.2023.028046

ARTICLE





Investigation of the Thermal Decomposition Behavior of Oleuropein with Many Pharmacological Activities from Olive by Thermogravimetry

Jiaojiao Yuan¹, Su Tuo¹, Yangyang Liu¹, Jing He¹, Shao-Hwa Hu¹ and Junling Tu^{2,*}

¹School of Business and Trade, Dongguan Polytechnic, Dongguan, 523808, China
 ²School of Chemical Engineering and Energy Technology, Dongguan University of Technology, Dongguan, 523808, China
 ^{*}Corresponding Author: Junling Tu. Email: tujl@dgut.edu.cn
 Received: 28 November 2022 Accepted: 30 January 2023 Published: 26 June 2023

ABSTRACT

Due to the existence of poly-hydroxyl structures, the temperature may have an effect on the thermal stability of oleuropein for its applications. In the current study, the thermal decomposition process and kinetics behavior of oleuropein from the olive resource were researched by thermogravimetric theoretical analysis methods and nonisothermal kinetics simulation. The results of thermogravimetry analysis showed the whole thermal decomposition process of oleuropein involved two stages, with 21.22% of residue. It was also revealed that high heating rates of more than 20 K min⁻¹ led to significant thermal hysteresis and inhibited the whole thermal decomposition behavior of oleuropein. Moreover, an investigation of the thermal decomposition kinetics indicated that the non-isothermal decomposition behavior followed a D3 model during the first stage (three-dimensional diffusion, Jander equation) and a D1 model in the second stage (one-dimensional diffusion). For the first and second thermal decomposition stages, the Kissinger, Friedman, Flynn-Wall-Ozawa, and Coats–Redfern four methods were applied to determine the activation energy (E = 143.72 and 247.01 kJ mol⁻¹) and Arrhenius preexponential factor ($\ln A = 26.34$ and 42.45 min⁻¹), respectively. Therefore, the study will provide good theoretical guidance for thermal stability and thermal transformation application of oleuropein. It will be suitable for low-temperature applications in the cosmetic, food supplement and pharmaceutical industries.

KEYWORDS

Oleuropein; thermal decomposition behavior; kinetics process; thermogravimetry analysis



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Graphic Abstract



Nomenclature

dx/dt A rate of conversion;

- *x* Conversion of the reaction, which is defined as $x = (w_0 w_t)/(w_0 w_f)$, where w_0 and w_f stand for the initial and final masses of the sample at each stage of decomposition (mg), respectively, and w_t stands for the mass of the sample at time *t* (mg);
- f(x) Differential mathematical model function of kinetics, which depends on the reaction type and behavior;
- g(x) Integral mathematical model function of kinetics, which depends on the reaction type and behavior;
- k(T) Temperature-dependent rate constant, which can be described by the Arrhenius equation;
- A Preexponential factor (\min^{-1}) ;
- *R* Gas constant 8.314 J mol⁻¹ K⁻¹;
- *E* Apparent activation energy (kJ mol⁻¹);
- *T* Absolute temperature (K);
- *B* Heating rate (K min⁻¹);
- T_p Absolute thermodynamic temperature at which the mass loss rate reaches to a maximum value (K).

1 Introduction

As a cooking oil, the widespread use of olive oil has relations with low rates of cardiovascular disease in Mediterranean countries. Due to its high levels of polyphenols, olive oil has presented cardiovascular disease-preventing function. Oleuropein and hydroxytyrosol are the most representative polyphenols.

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Oleuropein, which is a natural secoiridoid glycoside [1], is the primary active compound of olive resources [2] and the chemical structural formula is presented in Fig. 1. Owing to hydroxyl active functional groups in chemical structure, oleuropein exhibits a variety of pharmacological activities. Based on the angle of the structure–activity relationship, its main pharmacological activities are closely related to its hydroxyl and aglycone structures. Extensive research efforts in the fields of medicine, food industry, and cosmetics have recently unveiled new pharmacological activities and mechanisms of action of oleuropein, mainly including antitumor [3,4], antioxidation [5], antimicrobial [6], antibacterial [7,8], hypoglycemic [9], and antihypertension activities, and coronary heart disease and atherosclerosis prevention function [10], among other biological activities. Used in cosmetics, food supplements and pharmaceuticals, olive leaf extract contains 15% to 30% oleuropein generally.



Figure 1: The chemical structural formula of oleuropein

Temperature and other processing conditions of olive leaves, such as drying treatment, extraction process, and storage after extraction, are known to affect the oleuropein stability on account of its multiple hydroxyl groups. Malik et al. [11] found that the original oleuropein level was preserved during the natural drying process of fresh olive leaves at 25°C, whereas drying at 60°C reduced. The active component of the oleuropein aqueous extract remained relatively stable for 7 days at room temperature; however, the degradation rate accelerated after 17 days. In addition, Xie et al. [12] discussed the effects of the temperature factor on oleuropein stability, finding that the degradation rate of oleuropein increased gradually with time. After 27 days, the degradation rate of oleuropein was 95.24% at 25°C and 38.1% at 4°C. This degradation was found to be related to the β -glucosidase contained in oleuropein.

Considering the important factors affecting the thermal stability of oleuropein for its related applications, gaining a deeper understanding of the specific degradation mechanisms is very significant. At present, the thermal analysis method has emerged as a promising technology to study drug thermal stability characteristics and kinetics decomposition behavior [13,14]. Furthermore, as determined by theoretical experimental studies, decomposition kinetics characteristic factors analysis has attracted much attention [15–18], mainly emphasizing on predicting material lifetimes and describing the thermodynamic properties in practical applications. The dynamic change of material weight with temperature can be determined using thermogravimetry (TG), and decomposition behavior and kinetics characteristic parameters are examined by different heating rates. Thus, the thermal decomposition stability and kinetics process of oleuropein could be investigated. According to a previously reported TG analysis [12], the

decomposition of oleuropein began at 232.3°C. However, the specific thermal decomposition behavior and kinetics process remained unexplored.

In this current study, the oleuropein thermal stability was investigated by the thermal analysis method that involved weight change with temperature and the decomposition process. The effect of different heating rates on the oleuropein degradation kinetics behavior was examined, and the kinetics reaction behavior was simulated, and the relevant characteristic parameters were obtained to evaluate the degradation process. The current study would provide a systematic method for the analysis of the decomposition behavior and kinetics mechanism of oleuropein, and a theoretical basis to unveil its stability, quality control, and thermal processing as a drug.

2 Materials and Methods

2.1 Materials

Oleuropein (standard substance, purity > 98%, molecular weight 540) was purchased from Sigma Chemicals (St Louis, MO, USA).

2.2 Instrument and Methods

The thermogravimetric analyzer (NETZSCH TG 449C; Selb, Germany) was performed to investigate the oleuropein thermal stability, and the TG measurements were completed using about 10 mg sample in a nitrogen atmosphere of 313 to 1173 K (35 mL min^{-1}) at different heating rates (5, 10, 20, and 40 K min⁻¹). The kinetics decomposition behavior was studied by obtained data.

2.3 Kinetics Theoretical Analysis Methods of Thermal Decomposition

Eq. (1) is usually used to analyze the thermal decomposition kinetics theory.

$$\frac{\mathrm{d}x}{\mathrm{d}t} = k(T)f(x) \tag{1}$$

where k(T) is rate constant, which is dependent only on the temperature, and it is usually calculated by the Arrhenius Eq. (2) as follows:

$$k(T) = Ae^{-E/RT}$$
⁽²⁾

where E is the activation energy and A is the Arrhenius preexponential factor.

When the heating rate (β) is dT/dt under non-isothermal condition, the mathematical expression of the thermal decomposition kinetics parameters can be expressed by Eq. (3) with substituting Eqs. (1) into (2) [19], and the corresponding kinetics parameters can be obtained on the basis of the TG result.

$$\frac{\mathrm{d}x}{\mathrm{d}t} = \beta \frac{\mathrm{d}x}{\mathrm{d}T} = Ae^{-E/RT} f(x) \text{ or } \frac{\mathrm{d}x}{\mathrm{d}T} = \frac{A}{\beta} e^{-E/RT} f(x)$$
(3)

By integrating the above equation, it is expressed by Eq. (4):

$$g(x) = \frac{A}{\beta} \int_0^T e^{-E/RT} dT$$
(4)

and when

$$g(x) = \int_0^x \frac{1}{f(x)} \mathrm{d}x \tag{5}$$

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2.3.1 Kissinger Method

The mathematical theoretical expression of Kissinger method [20] is described as follows by Eq. (6):

$$\ln\left(\frac{T_P^2}{\beta}\right) = \ln\left(\frac{E}{R}\right) - \ln A + \frac{E}{RT_P} \tag{6}$$

By fitting (T_p^2/β) against $1/T_p$, two constants of the slope (E/R) and intercept ln (E/AR) of the line can be obtained. Then, *E* and *A* can be calculated according to the binary first-order equation.

2.3.2 Flynn-Wall-Ozawa Method

The mathematical theoretical expression of the Flynn-Wall-Ozawa (F-W-O) method is expressed as follows by Eq. (7) [21]:

$$\ln \beta = \ln \left(\frac{AE}{R}\right) - \ln g(x) + 5.3305 - 1.052 \frac{E}{RT}$$
(7)

When the conversion rate (x) value is fixed, the value of $\ln (AE/R g(x))$ can be regarded as a constant, and there is a linear relationship between $\ln \beta$ and 1/T. Thus, with a given conversion rate, *E* can be calculated by the slope (-1.052 *E/R*) of the line between $\ln \beta$ and 1/T.

2.3.3 Friedman Method

The mathematical theoretical expression of the Friedman method [22] is represented as follows by Eq. (8):

$$\ln\left(\frac{\mathrm{d}x}{\mathrm{d}t}\right) = \ln[Af(x)] - \frac{E}{RT} \tag{8}$$

By plotting $\ln(dx/dt)$ against 1/T, the slope (-E/R) of line is obtained, and E can be calculated.

2.4 The Most Probable Kinetics Reaction Behavior Model

In the study for a thermal decomposition behavior, choosing the probable kinetics reaction behavior model is important to reduce the difference between the mathematical models of theoretical kinetics reaction behavior and the actual kinetics reaction behavior. In order to select the most possible mathematical model of oleuropein dynamic behavior, the specific method is to combine the above four methods. Firstly, the integral forms of the 15 theoretical dynamic kinetics behavior functions [23] are respectively substituted into Eq. (9) (seen in Table 1), and the Coats-Redfern function is used to calculate the corresponding E value. Then, the range of E values closest to those calculated by Kissinger, F-W-O and Friedman methods is selected, and the corresponding theoretical mathematical model and reaction behavior is considered as the most possible kinetics behavior analysis of oleuropein.

The mathematical theoretical expression of the Coats-Redfern method [24] is represented as follows by Eq. (9):

$$\ln\frac{g(x)}{T^2} = \ln\frac{AR}{\beta E} - \frac{E}{RT}$$
(9)

The integral expressions g(x) of different reaction models are respectively substituted into Eq. (9), and two constants of the slope (-E/R) and intercept $(\ln (AR/\beta E))$ of the fitting line are obtained by fitting $\ln[g(x)/T^2]$ and 1/T. Thus, according to the two equations, *E* and $\ln A$ of corresponding models are calculated.

Number	Model	Reaction behavior	g(x)	f(x)
1 (F1)	Chemical reaction	n = 1	$-\ln(1-x)$	1 <i>x</i>
2 (F2)		n = 2	$(1-x)^{-1}-1$	$(1-x)^2$
3 (F3)		<i>n</i> = 3	$[(1-x)^{-2}-1]/2$	$(1-x)^3$
4 (D1)	Diffusion	One-dimensional diffusion	x^2	$1/2x^{-1}$
5 (D2)		Two-dimensional diffusion	$(1-x)\ln(1-x)+x$	$[-\ln(1-x)]^{-1}$
6 (D3)		Three-dimensional diffusion (Jander equation)	$[1-(1-x)^{1/3}]^2$	$3/2(1-x)^{2/3}[1-(1-x)^{1/3}]^{-1}$
7 (D4)		Three-dimensional diffusion (Ginstling-Brounshtein equation)	$(1-2x/3)-(1-x)^{2/3}$	$3/2[(1-x)^{-1/3}-1]^{-1}$
8 (A2)	Random nucleation and growth	Two-dimensional	$[-\ln(1-x)]^{1/2}$	$2(1-x)[-\ln(1-x)]^{1/2}$
9 (A3)		Three-dimensional	$[-\ln(1-x)]^{1/3}$	$3(1-x)[-\ln(1-x)]^{2/3}$
10 (R1)	Interfacial reaction	One-dimensional	x	1
11 (R2)		Cylindrical symmetry	$[1-(1-x)^{1/2}]$	$2(1-x)^{1/2}$
12 (R3)		Spherical symmetry	$[1-(1-x)^{1/3}]$	$3(1-x)^{2/3}$
13 (P2)	Exponential nucleation	Power function, $n = 1/2$	<i>x</i> ^{1/2}	$2x^{1/2}$
14 (P3)		Power function, $n = 1/3$	<i>x</i> ^{1/3}	$3x^{2/3}$
15 (P4)		Power function, $n = 1/4$	$x^{1/4}$	$3x^{3/4}$

Table 1: Kinetics behavior functions of pyrolysis

2.5 Calculation of InA

The *A* is largely related only to the reactant (oleuropein) properties, not to the temperature parameters. Therefore, the calculation of *A* value is very important to determine the kinetics characteristic of oleuropein. Since *E* and ln *A* exist compensation effect, ln *A* is calculated usually by Eq. (10):

$$\ln A = aE + b$$

(10)

Firstly, the slope (a) and intercept (b) of the fitting line are obtained by fitting E and $\ln A$ under different β according to the data of the most suitable kinetics degradation reaction model. Then, the average E is substituted into Eq. (10) to calculate $\ln A$.

3 Results and Discussion

3.1 Thermal Stability of Oleuropein

The thermogravimetry and differential thermogravimetry (TG/DTG) results for the oleuropein thermal decomposition were presented at 5, 10, 20, and 40 K min⁻¹ heating rates in Figs. 2 and 3. As shown in Figs. 2, 3 and Table 2, the thermal decomposition process of oleuropein was comprised of two stages based on the data of 10 K min⁻¹ heating rate. From Fig. 2 we can see that oleuropein was stable with little mass loss below 230.7°C, however, it started to decompose severely above this temperature. This result agreed with that reported by Xie et al. [12]. At approximately 339.2°C, the first stage of oleuropein decomposition was

almost finished with 59.18% of residue. The second stage occurred from 339.2°C to 504.7°C, with 21.22% of residue. From Fig. 3 we can conclude that the first peak of the DTG curve appeared at 254.2°C with -4.36% min⁻¹ maximum loss ratio. Furthermore, the second peak of the DTG curve and the maximum loss were 368.5°C and -3.27% min⁻¹, respectively.



Figure 2: TG curve of oleuropein at different heating rates



Figure 3: DTG curve of oleuropein at different heating rates

Furthermore, the TG and DTG curves were presented basically at the same tendency with different heating rates of the oleuropein thermal decomposition. With the increase of heating rate, the thermal hysteresis phenomenon became more and more obvious, and the beginning and end decomposition temperature also increased, and the temperature peak corresponding to the maximum weight loss rate also shifted to a higher temperature region. This tendency was reported to agree with previous research [25].

Especially in the DTG curve, the temperature with the maximum loss ratio moved obviously to the right, which meant that the pyrolysis temperature required would be higher under the condition of the same weight loss rate. From a kinetics point of view, the thermal decomposition behavior had no connection with the heating rate, and it just depended on the temperature [26]. Too high heating rates would lead to an incomplete spectrum of part intermediate products, which had not enough time to separate each other. The effects of heating rates on thermal decomposition were mainly as follows: (1) With the heating rate increased, the time to obtain pyrolysis temperature was shortened, but the stopping time of volatiles was insufficient. In order to achieve full thermal decomposition, a higher temperature was needed. (2) Too high a heating rate would lead to the increase of temperature ladder inside and outside the raw material particles, and reduce the heat transfer efficiency of the thermal decomposition process. However, the heating rate was too low, which would lead to spectral discontinuity of the separated products and even serious trailing phenomenon. By fitting the data of T_m and β [27], the equations were obtained as follows: $T_m = 1.1426\beta + 511.58$ ($R^2 = 0.9493$) for the first stage and $T_m = 1.0419\beta + 630.19$ ($R^2 = 0.9923$) for the second stage, which showed that T_m were both closely related to β with a good positive correlation.

Pyrolysis	Heating rate $\beta(\text{K}\cdot\text{min}^{-1})$	Beginning temperature (°C)	End temperature (°C)	<i>T</i> _m (°C)	Max. loss ratio $(\% \cdot \text{min}^{-1})$	Residue (%)
First	5	215.4	331.2	239.2	-2.23	58.39
stage	10	230.7	339.2	254.2	-4.36	59.18
	20	238.7	345.4	263.4	-8.44	58.52
	40	257.3	374.3	282.6	-20.61	53.03
Second	5	331.2	491.3	360.6	-1.27	25.50
stage	10	339.2	504.7	368.5	-3.27	21.22
	20	345.4	509.1	379.2	-6.47	20.11
	40	374.3	517.3	398.0	-14.08	19.54

 Table 2: The oleuropein thermal decomposition characteristic factors

Note: $T_{\rm m}$ means the temperature with the maximum loss ratio.

3.2 Non-Isothermal Kinetics Analysis of Oleuropein

The oleuropein non-isothermal thermal decomposition kinetics was calculated by the Kissinger, Friedman, F-W-O, and Coats–Redfern four theoretical analysis methods.

3.2.1 Kissinger Method

The kinetic factors of oleuropein thermal decomposition, calculated by Kissinger method, were presented in Table 3. According to Eq. (6), *E* and ln *A* of the first stage pyrolysis were 107.14 kJ mol⁻¹ and 23.76 min⁻¹, respectively, and the linear correlation coefficient (R^2) was 0.9835. Furthermore, those of the second stage pyrolysis were 181.67 kJ mol⁻¹ and 33.33 min⁻¹, with R^2 value of 0.9625. According to the obtained *E* values, the first stage of oleuropein decomposition was more favored than the second stage.

Table 3: Thermal decomposition kinetics factors of oleuropein by Kissinger method

Pyrolysis	$E/(kJ \cdot mol^{-1})$	ln A	R^2
First stage	107.14	23.76	0.9835
Second stage	181.67	33.33	0.9625

3.2.2 Flynn-Wall-Ozawa Method and Friedman Method

Based on the Friedman and F-W-O methods, *Es* were obtained by Eqs. (6) and (7), and the corresponding kinetics decomposition data were presented in Tables 4 and 5. It was found that good R^2 values were obtained in all cases, and the *E* value increased with the conversion ratio from the kinetics data, which suggested that oleuropein decomposition was more difficult. As the conversion rate increased, the reaction activities of the products were more sensitive to temperature. The initial activation energy was lower, mainly because there were a large number of weak bonds (hydroxyl, hydrocarbon chain or carboxyl groups) in oleuropein during the initial pyrolysis, which were prone to fracture. With the increase in conversion rate, it was mainly the thermal decomposition of the benzene ring or glycosidic bond, in which the fracture of strong bonds needed more energy consumption, manifested by higher activation energy. Therefore, the *E* mean values were needed to calculate, and were 168.98 and 142.30 kJ mol⁻¹ in the first stage and 299.06 and 236.38 kJ mol⁻¹ in the second stage using Friedman and F-W-O methods, respectively.

Pyrolysis	Conversion <i>x</i>	β							
		5 °C/min		10 °C/min		20 °C/min		40 °C/min	
		dx/dt	t	dx/dt	t	dx/dt	t	dx/dt	t
First stage	0.1	-2.030	230.43	-4.036	246.72	-7.667	253.77	-17.943	272.05
	0.15	-2.222	241.11	-4.328	256.72	-8.405	265.98	-20.579	283.75
	0.2	-1.892	253.33	-3.642	269.22	-7.414	279.21	-17.925	295.46
	0.25	-1.565	268.09	-3.371	284.22	-6.865	293.97	-15.328	310.21
	0.3	-1.435	285.39	-3.214	299.22	-6.603	308.73	-14.795	324.46
	0.35	-1.290	303.71	-2.787	316.72	-6.458	324.50	-14.422	338.71
	0.4	-1.207	324.06	-2.547	334.22	-6.330	340.28	-14.206	353.47
Second stage	0.1	-1.255	355.62	-2.465	364.22	-6.400	364.20	-14.181	389.60
	0.15	-1.247	366.81	-2.442	376.72	-6.450	372.85	-14.091	397.24
	0.2	-1.153	378.52	-2.337	386.72	-6.443	382.01	-13.900	404.87

Table 4: Kinetics data of oleuropein thermal decomposition by Friedman and F-W-O methods

Table 5: Kinetics factors of oleuropein thermal decomposition by Friedman and F-W-O methods

Pyrolysis	Conversion (<i>x</i>)	$\frac{\text{Friedman method}}{E/\text{kJ mol}^{-1}} R^2$		F-W-O r	nethod
				$E/kJ mol^{-1}$	R^2
First stage	0.1	122.01	0.9842	111.33	0.9766
	0.15	126.44	0.9907	113.02	0.9884
	0.2	134.97 0.987		119.26	0.9917
	0.25	144.70	0.9931	125.89	0.9906
	0.3	174.12	0.9951	143.27	0.9931
	0.35	211.98	0.9862	169.62	0.9895
	0.4	268.65	0.9763	213.70	0.9832
	mean	168.98		142.30	

(Continued)

Table 5 (continued)											
Pyrolysis	Conversion (x)	Friedman	method	F-W-O method							
		$E/kJ mol^{-1}$	R^2	$E/kJ mol^{-1}$	R^2						
Second stage	0.1	251.14	0.9851	200.67	0.9847						
	0.15	289.95	0.9954	231.72	0.9999						
	0.2	356.08	0.9932	276.74	0.9988						
	mean	299.06		236.38	_						

Note: "-" means without this value.

The average *E* values for the first and second stages obtained by the Friedman method were a litter higher than those obtained by the F-W-O method in Table 5. Furthermore, combined Table 3, it was shown that the Kissinger method was the lowest value among the three methods. The significant difference was due to the fact that the equation of the Kissinger method assumed that the kinetic factors of temperature and *E* were independent of the conversion degree [28,29]. In conclusion, it could be concluded that the *E* calculation results of these three methods, with oleuropein thermal decomposition in the first and second stages, were in the range of 107.14–168.98 and 181.67–299.06 kJ mol⁻¹, respectively.

3.2.3 Coats-Redfern Method

In Table S1, the main kinetics factors of oleuropein thermal decomposition by the Coats–Redfern method were presented with the heating rates (5–40 K min⁻¹), and it was concluded that E values increased with 5–40 K min⁻¹, because more energy was required to decompose oleuropein at higher heating rates. Moreover, except for the P3 and P4 models, the fitting results of 13 models had good linear correlation, and E and ln A all showed clear differences. Therefore, a good linear dependence was not sufficient to select the most appropriate reaction model. The current study combined the Kissinger method, Friedman method, F-W-O method, and Coats-Redfern method, the four methods were used to screen and verify the E value and the most suitable kinetics degradation reaction model for the thermal decomposition process of oleuropein [30].

3.2.4 Calculation of ln A

The *E* values, obtained using the above-mentioned three methods for the first stage, ranged between 107.14 and 168.98 kJ mol⁻¹. Considering the linear dependence of the fitting results, D3 was the best model, which afforded *E* values of 131.73–151.86 kJ mol⁻¹. In consequence, the diffusion model and three-dimensional diffusion Jander equation reaction behavior (D3) could describe exactly the first stage of oleuropein thermal decomposition. The integral function for the reaction model was $g(x) = \left[1 - (1-x)^{\frac{1}{3}}\right]^2$, and the differential function was $f(x) = \frac{3}{2}(1-x)^{\frac{2}{3}}\left[1 - (1-x)^{\frac{1}{3}}\right]^{-1}$.

As shown in Fig. 4, $\ln A = 0.20E - 2.4$ was obtained by fitting the data calculated from the D3 model, and it was used to resolve the kinetics compensation effect for the first stage of oleuropein pyrolysis. Firstly, corresponding E_0 was calculated as the mean of E with different heating rates in model D3, and was obtained for 143.72 kJ mol⁻¹. Secondly, $\ln A$ was 26.34 min⁻¹ through E_0 based on the above-mentioned compensation

effect fitting the linear equation. Finally, The differential form $f(x) = \frac{3}{2}(1-x)^{\frac{2}{3}} \left[1-(1-x)^{\frac{1}{3}}\right]^{-1}$, E_0 , and A

were substituted into Eq. (3), resulting in the following kinetics expression for the first stage of oleuropein thermal decomposition: $\frac{dx}{dT} = \frac{2.75 \times 10^{11}}{\beta} \exp\left(-\frac{1.44 \times 10^5}{8.314 \times T}\right) \times \frac{3}{2}(1-x)^{\frac{2}{3}} \left[1-(1-x)^{\frac{1}{3}}\right]^{-1}$.



Figure 4: Kinetics compensation effect of the first stage of oleuropein pyrolysis by different heating rates

In the second stage of oleuropein pyrolysis, the kinetics characteristic factors, which were summarized in Table S1, were calculated by the Coats–Redfern method. The above-mentioned three methods afforded a range of *E* values for the second stage of 181.67–299.06 kJ mol⁻¹, and model D1 was the closest with the range of *E* (195.53–328.24 kJ mol⁻¹). Therefore, the one-dimensional diffusion reaction behavior (D1 model) could describe exactly the second stage of the oleuropein thermal decomposition process. The integral function for the reaction model was $g(x) = x^2$, and the differential function was $f(x) = 1/2x^{-1}$.

By fitting the data of *E* and ln *A* calculated from the D1 model, ln A = 0.19E - 4.48 was obtained. For the second stage of oleuropein pyrolysis, the kinetics compensation effect at different heating rates was presented in Fig. 5. Then, according to the *E* calculated by model D1 at different heating rates, the mean E_0 value was 247.01 kJ mol⁻¹, and ln *A* was calculated to 42.45 min⁻¹ by the above-mentioned fitting linear equation. Finally, the kinetics function was obtained by substituting f(x) = 1/(2x), E_0 , and *A* into Eq. (3), and the final equation for the second stage of oleuropein thermal decomposition was expressed as follows: $\frac{dx}{dT} = \frac{2.23 \times 10^{18}}{\beta} \exp\left(-\frac{2.47 \times 10^5}{8.314 \times T}\right) \times \frac{1}{2x}$.

3.2.5 Proposed Mechanism of Oleuropein Thermal Decomposition

From the TG/DTG results for the oleuropein thermal decomposition (the rate was 10 K/min), we could see that oleuropein was degraded from 230.7°C to 339.2°C firstly, with 59.18% of residue. The second stage occurred at 339.2°C, and was almost finished with 21.22% of residue at approximately 504.7°C. Due to multiple hydroxy groups, it produced water at the beginning. According to the oleuropein acidic and enzymatic hydrolysis processes and products, the main chemical bond ruptures existed at the ester bond and glycosidic bond in the chemical structure, presented in Fig. 6.



Figure 5: Kinetics compensation effect of the second stage of oleuropein pyrolysis by different heating rates



Figure 6: Oleuropein thermal decomposition

From the reference [25], we could conclude that the main decomposition process of hydroxytyrosol started at 262.8°C, and ended at 409.7°C. A solid residue of 2.68% was observed, and the final products were CO₂, water, and 2.68% solid char. According to the TG diagram of hydroxytyrosol pyrolysis, the residual weight was about 13% at 339.2°C. As reported in reference [31], the glucose was decomposed from 190°C, and the weight loss rate gradually decreased from about 379°C until the end of the pyrolysis process. At the end of the 600°C pyrolysis reaction, the total weight loss of the sample was 85% of the initial weight. The primary products of glucose pyrolysis were water, furfural, CO₂ and other products.

According to the TG diagram of glucose pyrolysis, the residual weight was about 34% at 339.2°C. Therefore, at the end temperature of the first stage of oleuropein decomposition (339.2°C), the weights of volatile substances were calculated by hydroxytyrosol molecular weight multiply (1%–13%) and glucose molecular weight multiply (1%–34%), and the result (253) was close to oleuropein experimental value (221). Moreover, at the end temperature of the second stage of oleuropein decomposition (504.7°C), the hydroxytyrosol had 2.68% residue and glucose had 18% residue. In the experimental result, the second stage of oleuropein was with 21.22% of residue, and it could be inferred that elenolic acid was nearly decomposed completely in this stage. So, oleuropein was firstly decomposed for hydroxytyrosol, elenolic acid and glucose, and these substances were further degraded. In summary, the pyrolysis products of oleuropein mainly consisted of solid char, water, CO_2 and furfural, according to pyrolysis products of hydroxytyrosol and glucose.

4 Conclusion

The thermal decomposition stability and kinetics behavior of oleuropein from olive plants were studied by TG analysis and non-isothermal theoretical method. The TG-DTG results revealed that oleuropein decomposition consisted of two stages, the first of which started at 230.7°C and the second at 339.2°C. The oleuropein decomposition process of the first stage followed a non-isothermal kinetics behavior described with a D3 model (three-dimensional diffusion, Jander equation), and the second stage with a D1 model (one-dimensional diffusion). In addition, by combining the abovementioned four methods, *E* and ln *A* were both calculated. It was inferred that the pyrolysis products of oleuropein mainly consisted of solid char, water, CO_2 and furfural. Therefore, this present research would provide new theoretical information on the thermal decomposition behavior of oleuropein, and suggests its suitability for lowtemperature applications in cosmetic, food supplement and pharmaceutical industries.

Funding Statement: This work was funded by Guangdong Basic and Applied Basic Research Foundation (No. 2019A1515111159), Characteristic Innovative Projects for Education Department of Guangdong Province 2021 Year (No. 2021KTSCX302).

Conflicts of Interest: The authors declared that they had no conflicts of interest to report regarding the present study.

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Supplementary Materials

Pyrolysis	No.	$\beta = 5$	K min	-1	$\beta = 10$) K min	-1	$\beta = 20$) K min	-1	$\beta = 4$	0 K mi	n ⁻¹
		$E/kJ mol^{-1}$	ln A	R^2	$E / kJ mol^{-1}$	ln A	R^2	$E/kJ mol^{-1}$	ln A	R^2	$E/kJ mol^{-1}$	ln A	R^2
First	F1	63.45	11.04	0.9828	68.78	12.51	0.9746	70.34	13.39	0.9871	73.44	14.14	0.9923
stage	F2	69.28	12.57	0.9854	75.07	14.11	0.9779	78.10	14.84	0.9893	80.10	15.75	0.9939
	F3	75.41	14.17	0.9876	81.70	15.78	0.9806	84.16	16.36	0.9912	87.11	17.43	0.9953
	D1	124.44	23.95	0.9825	134.48	26.06	0.9747	140.76	26.52	0.9867	143.52	27.94	0.9916
	D2	128.02	24.17	0.9833	138.34	26.32	0.9757	140.29	27.70	0.9874	147.62	28.21	0.9922
	D3	131.73	23.62	0.9842	142.34	25.81	0.9767	148.95	26.89	0.9882	151.86	27.70	0.9927
	D4	129.25	22.99	0.9836	139.38	25.15	0.9760	144.50	26.49	0.9877	149.03	27.04	0.9924
	A2	27.45	2.71	0.9768	29.97	3.81	0.9666	30.69	4.51	0.9822	32.09	5.31	0.9898
	A3	15.44	-0.36	0.9674	17.04	0.62	0.9542	17.80	0.98	0.9740	18.31	2.08	0.9858
	R1	57.94	9.58	0.9797	62.82	10.99	0.9709	65.89	10.99	0.9844	67.14	12.61	0.9903
	R2	60.66	9.61	0.9813	65.76	11.05	0.9729	67.58	11.05	0.9858	70.24	12.67	0.9914
	R3	61.58	9.44	0.9818	66.76	10.90	0.9735	68.49	11.52	0.9862	71.30	12.52	0.9917
	P2	24.69	1.92	0.9720	26.99	2.99	0.9607	27.96	3.68	0.9778	28.94	4.48	0.9868
	P3	13.61	-0.94	0.9590	15.05	0.02	0.9442	15.98	0.82	0.9662	16.21	1.48	0.9811
	P4	8.06	-2.60	0.9351	9.08	-1.67	0.9156	9.50	-1.09	0.9434	9.84	-0.24	0.9710
Second	F1	100.97	15.21	0.9896	105.98	16.59	0.9997	135.41	23.10	0.9888	172.56	29.72	0.9923
stage	F2	109.96	17.07	0.9915	115.32	18.49	0.9999	147.21	25.47	0.9907	187.36	32.54	0.9938
	F3	119.45	19.02	0.9930	125.14	20.48	0.9999	159.63	27.95	0.9923	202.94	35.50	0.9951
	D1	195.53	31.63	0.9887	205.05	33.60	0.9994	259.23	44.79	0.9877	328.24	56.28	0.9912
	D2	201.05	32.05	0.9894	210.78	34.05	0.9995	266.47	45.52	0.9884	337.32	57.57	0.9917
	D3	206.79	31.71	0.99	216.73	33.73	0.9996	273.98	45.50	0.9891	346.74	57.57	0.9923
	D4	202.97	30.93	0.9896	212.76	32.94	0.9995	268.97	44.51	0.9886	340.47	56.39	0.9919
	A2	45.16	4.85	0.9869	47.60	5.90	0.9996	62.33	9.65	0.9868	80.71	13.41	0.9912
	A3	26.56	1.13	0.9830	28.14	2.08	0.9995	37.97	4.93	0.9841	50.09	7.75	0.9898
	R1	92.44	13.44	0.9874	97.13	14.79	0.9993	124.24	20.86	0.9866	158.55	27.04	0.9905
	R2	96.64	13.62	0.9885	101.50	14.98	0.9995	129.75	21.27	0.9878	165.46	27.67	0.9915
	R3	98.07	13.51	0.9889	102.98	14.88	0.9996	131.62	21.24	0.9881	167.80	27.71	0.9917
	P2	40.90	3.91	0.9837	43.18	4.94	0.9990	56.75	8.48	0.9839	73.70	12.02	0.9890
	P3	23.72	0.45	0.9784	25.19	1.39	0.9987	34.25	4.11	0.9804	45.42	6.78	0.9871
	P4	15.13	-1.45	0.9701	16.20	-0.55	0.9981	23.01	1.78	0.9755	31.28	4.03	0.9847

Table S1: Kinetics parameter of oleuropein thermal decomposition by coats-redfern method



DOI: 10.32604/jrm.2023.027749

ARTICLE





Optimisation Strategy of Carbon Dioxide Methanation Technology Based on Microbial Electrolysis Cells

Qifen Li, Xiaoxiao Yan*, Yongwen Yang, Liting Zhang and Yuanbo Hou

College of Energy and Mechanical Engineering, Shanghai University of Electric Power, Shanghai, 200090, China ^{*}Corresponding Author: Xiaoxiao Yan. Email: yanxiao4231@163.com Received: 13 November 2022 Accepted: 13 January 2023 Published: 07 June 2023

ABSTRACT

Microbial Electrolytic Cell (MEC) is an electrochemical reaction device that uses electrical energy as an energy input and microorganisms as catalysts to produce fuels and chemicals. The regenerative electrochemical system is a MEC improvement system for methane gas produced by biological carbon sequestration technology using renewable energy sources to provide a voltage environment. In response to the influence of fluctuating disturbances of renewable electricity and the long system start-up time, this paper analyzes the characteristics of two strategies, regulating voltage parameter changes and activated sludge pretreatment, on the methane production efficiency of the renewable gas electrochemical system. In this system, the methane production rate of regenerative electrochemical system is increased by 1.4 times through intermittent boosting start-up strategy; based on intermittent boosting, the methane production rate of regenerative electrochemical system is increased by 2 times through sludge pyrolysis pretreatment start-up strategy, and the start-up time is reduced to 10 days. Meanwhile, according to the simulation test results of power input fluctuation and intermittency, the stability standard deviation of its system operation is 75% of the original one, and the recovery rate is about 1 times higher. This study can provide a theoretical basis and technical reference for the early industrial application of microbial CO_2 methanation technology based on renewable energy.

KEYWORDS

Carbon sequestration; CO₂ methanation; gap boosting strategy; sludge pretreatment strategy

1 Introduction

In 2020 General Secretary Xi proposed at the 75th United Nations General Assembly that China would ensure that it would achieve peak carbon emissions by 2030 and strive to become carbon neutral by 2060 [1]. This commitment has given a strong impetus to CO_2 reduction efforts in China and globally. As a resource, the production of chemicals and fuels from CO_2 through the use of physical, chemical and biological technologies is one of the most effective ways to control and reduce CO_2 emissions. As CO_2 is a chemically inert and thermodynamically stable gas, the key to its resource conversion is the effective activation of CO_2 , which breaks down its chemical structure and transforms it into other usable substances. At present, there are two main types of CO_2 resource utilization: physical utilization and chemical utilization, and the main methods include biological fixation, electrochemical reduction, photocatalytic conversion and catalytic hydrogenation reduction. After a long period of research and



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progress, electrochemical reduction technology is an ideal way to reduce CO_2 to high value-added products with low energy consumption, while the choice of catalysts for redox reactions using biocatalyst has the advantages of environmental friendliness, mild reaction conditions (atmospheric pressure and room temperature) and high product selectivity [2]. Therefore, Biological carbon sequestration, as one of the highly promising CO_2 capture and utilization technologies, has gained much attention in recent years.

Microbial electrocatalytic reduction of CO_2 to produce methane system (MEC) can produce hydrogen or reduce CO_2 to produce methane by treating wastewater through the microbial electrocatalytic process. This, this system coupled energy, municipal environment, biology, and other fields. The, the process operation is simple, energy saving, low investment cost, and operation cost. The, environmentally friendly methane gas application technology field is more mature and, has great potential for development [3]. Microbial electrocatalytic reduction of CO₂ to produce methane is a highly efficient and low-cost carbon-neutral energy storage technology that can store excess renewable energy and convert CO2 into easily stored methane through the catalytic action of anaerobic microorganisms in wastewater sludge. It, which not only effectively reduces CO₂ emissions, but also converts them it into another important energy material, and treats organic wastewater and organic solid waste. The conversion of CO2 into easily stored methane is not only effective in reducing CO₂ emissions but also in converting it into another important energy source and treating organic wastewater and solid waste. However, there are still many challenges that need to be solved, and the main technical bottlenecks are the need to significantly improve the operational performance and the lack of practical technology research. The operational performance of the dual-chamber MEC methanogenic system is mainly limited by the biocathode environment, which has a long start-up time, low current density and methane production rate, and poor operational stability. For example, the low methane production rates reported in the literature for CO₂ methanogenic systems based on microbial electrolysis cells and the difficulty in rapidly enriching efficient and electrically active methanogenic biofilms at the cathode [4]. In addition, since the electrical input is the only source of energy to produce the reduction equivalent at the cathode, one of the greatest challenges for this technology is to improve its stability in the face of fluctuations and interruptions in the electrical input from renewable energy sources. Instability in power input may lead to changes in the structure and metabolic pathways of the biocathode microflora, resulting in reduced methane production [5]. Therefore, a large number of studies have been conducted to review the factors affecting the performance of microbial electrolytic cell CO2 methanogenesis systems, such as: electrochemical start-up strategies, cathode materials and reactor structures, pretreatment methods, voltage, temperature, pH, etc., to clearly identify the influence of these factors on the promotion of efficient microbial methane synthesis, with the aim of finding effective and easy-to-implement electrochemical start-up strategies to overcome these problems [6].

Cheng et al. set a potential of -0.5 V by the constant potential method and spent approximately one month to complete the initiation of a methanogenic biocathode in a single chamber EMG reactor with a methane production rate of $0.034 \text{ m}^3\text{m}^{-3}\text{d}^{-1}$ [7]; Riedl et al. found that a continuous change in potential from -0.6 to 0.4 V with a continuous change in scan rate of (5 mVs^{-1}) resulted in an increase in bioelectrode current density and turnover, mainly due to the enrichment of a more efficient electroactive biofilm [4]; Li et al. converted the electrode potential by polarity reversal from bioanode of -0.4 to -0.7 V at the cathode by polarity reversal. This was found to promote direct electron transfer and increased biomass in the biofilm, thereby promoting direct electron transfer and increased biomass in reducing the start-up time by approximately 40% [8]. Wang et al. found that intermittent power supply was more beneficial in promoting methane production than the commonly used continuous power supply, mainly because the intermittently applied electric field increased the biofilm charge storage capacity and electron mobility [9]. Xiang et al. operated at different voltages microbial electrosynthesis systems using bioenergy from wastewater to convert carbon dioxide to acetic acid, with high biomass on the anode and cathode surfaces of the bipolar membrane (BPM) and the relative abundance of acetic acid bacteria in the cathode [10]. Fu et al. applied a resting potential of 0.5 V to SHE in a two-chamber reactor and increased CH4 productivity of the biocathode by approximately 6-fold after 160 h of incubation, showing that the ability of the biocathode to catalyze electrogenic methanogenesis via direct electron transfer was enhanced during the adaptation process [11]. Borole et al. measured changes in anode, cathode, and solution/membrane impedance during enrichment of anodic microbial consortia and found that biological systems have the unique ability to reduce the electron transfer resistance of MFCs and their potential for stable energy production over long periods of time [12]. In addition, some researchers have tried to artificially lower the cathode potential during start-up to up-regulate the input power and found that dense biofilms with high metabolic and electrical activity were enriched at the cathode, allowing the biocathode to maintain stable coulombic efficiency despite fluctuations in electrode potential or current [13–15]. Rossi et al. also found that up-regulating the current was able to reduce the charge transfer impedance and diffusion impedance, fundamentally altering the performance of the bioelectrode [16]. In summary, the performance and stability of the biocathode can be significantly improved by adjusting the cathodic potential and current.

In order to speed up the anaerobic digestion of activated sludge and to shorten the digestion reaction time, a pre-treatment of the sludge is necessary. Zhang et al. used a high-pressure homogenization method to pretreat residual sludge and investigated the effects of homogenization pressure, homogenization cycle, and total sludge solids concentration on the sludge cracking rate [17]. Dohányos et al. used centrifugal lysis for anaerobic digestion of residual sludge after pretreatment for methane production, with methane yields ranging from 8.1% to 86.4% for different properties of sludge [18]. Jeong et al. used high-efficiency pulse technology for pretreatment of residual sludge, and the values of SCOD/TCOD and the content of extracellular polymers were increased compared to the untreated sludge by 4.5-fold and 6.5-fold [19]. Baier et al. increased the percentage of SCOD from 5% to 47% after pretreatment of the residual sludge using the rotating grinding ball method [20]. Boungier et al. used pyrolysis to pretreat the residual sludge, and the results showed that the cracking rate, protein and carbohydrate release rate, and methane yield of the sludge were more beneficial under 190°C compared to 135°C [21]. Feng et al. investigated the effect of heat treatment on the physical properties and dewatering properties of municipal residual sludge, and the results showed that: after heat treatment, the results showed that the dewatering properties, particle size and viscosity of the sludge decreased after heat treatment [22]. Tang et al. investigated the effect of three different garden wastes on the methanogenic effect of anaerobic digestion of kitchen waste using freeze-thaw pretreatment and found that freeze-thaw pretreatment could increase the gas yield by 25.0% and 85.4%, respectively [23]. Tang et al. performed anaerobic fermentation of mixed sludge from primary and secondary settling tanks after freeze-thaw pretreatment to produce methanogenic firing, and the gas yield was 1.31 m³/kgVS, which was 1.5 times higher compared to the original sludge [24]. In summary, sludge pretreatment technology can effectively promote the release of organic matter from sludge flocs and microbial cells into the liquid phase, improve sludge's biochemical properties of sludge, accelerate the rate of sludge digestion rate, and increase the energy recovery rate. In practical engineering applications, the sludge should be pretreated according to the nature of the sludge, and the appropriate pretreatment method should be selected.

According to the literature review, better operational performance can be achieved when the biocathode has a variable potential and current, and its two electrochemical parameters are able to respond to changes in cathode kinetics during start-up and change simultaneously, but further studies are lacking. Therefore, this paper proposes an intermittent boosting strategy to increase the electrical output based on simultaneous changes in current, and potential is expected to further improve the methane production performance and its stability of renewable electrochemical systems; meanwhile, among the many foundations of sludge pre-treatment, there is a lack of a certain degree of research on the way in which pre-treatment

technologies combine voltage and current influencing factors for incubation [25]. Therefore, this paper uses three sets of comparative experimental start-ups of reduced CO_2 methanogenic systems using constant voltage start-up and intermittent boost start-up [26] and intermittent boost start-up after sludge pyrolysis treatment to investigate the changes in methane production rate, pH of the cathode chamber solution, current density, and the stability and recovery rate analysis of operation under fluctuating and intermittent power input under the three start-up methods. The aim is to reveal the effects of intermittent boost startup strategies and activated sludge pyrolysis pretreatment processes on the performance of CO_2 biomethanation, thus providing a theoretical basis and technical reference for the application of CO_2 methanation engineering based on microbial electrolysis cells.

This paper presents the following innovations: (1) For the first time, the intermittent boosting strategy is combined with sludge thermal treatment to analyze the biofilm formation and enrichment characteristics of bacterial strains, which in turn affects the performance and stability of methane production in regenerative gas electrochemical systems. (2) The methanogenic performance and stability of the regenerative electrochemical system is characterized by simulating fluctuations in power input and intermittency to further validate the practical application results.

2 Experimental Materials and Methods

2.1 Construction of Reactor

The reactor used in this experiment is an H-type double-chamber reactor, the main body of which is made of Plexiglas material and Its physical diagram is shown in Fig. 1. Three sets of control experiments were set up as shown in Table 1. three sets of single-chamber type H-type double-chamber reactors of 100 mL, the effective volume of both cathode and anode chambers is 155 mL, and the cathode and anode chambers are filled with 110 mL and 120 mL of electrolyte solution (PH = 7), respectively. The first set of two-chamber reactors provided an applied voltage of 0.8 v and was inoculated with 35 g of centrifuged sludge; the second set of two-chamber reactors provided a changing applied voltage and was inoculated with about 35 g of centrifuged sludge and the third set of two-chamber reactors provided a changing applied voltage and was inoculated with about 35 g of sludge that had been pretreated by pyrolysis and then centrifuged to extract water. The anode was made of platinum sheet electrode, and the reference electrode was Agcl electrode. The cathode was made of a 1 mm diameter titanium wire connected to a high-purity graphite felt.



Figure 1: Three sets of reactors during start-up

Experimental group	Voltage range	On and off power duration	Pre-processing methods
Constant voltage group	0.8 v	Non-stop	-
Intermittent booster group	0.2–0.8 v, 0.2 v per power-up	Power on for 20 h, power off for 4 h	-
Intermittent booster group after sludge	0.2–0.8 v, 0.2 v per	Power on for 20 h,	Sludge pyrolysis
heat treatment	power-up	power off for 4 h	treatment

Table 1: Comparison of three experimental groups

2.2 Pretreatment Types and Conditions

The thermal pretreatment method refers to the heating of the residual sludge, where the microbial cells are ruptured by the pressure difference generated in the heat process, and the organic matter inside the cells is released and rapidly hydrolyzed and dissolved under the action of high temperature. The temperature range of sludge heat treatment is generally in the range of 60°C–270°C, and the most commonly used temperature range is 60°C–180°C. Take 38.76 g of sludge in a beaker, add 1.2979 g of NaOH solids, stir well, and then place the beaker in a water bath at 65°C for 30 min to complete the pretreatment. The purpose of adding NaOH solid is to increase the degree of hydrolysis of sludge, reduce the temperature tolerance of biological cell walls in sludge, and promote the efflux of cellular organism inclusions, which improves good conditions for accelerating the process of anaerobic digestion of colonies.

The activated sludge was treated by pyrolysis, and after centrifugation at 5000 r/min for 10 min, the sludge obtained was 35.5624 g, and finally, the treated sludge was put into the reactor for methane production experiment.

2.3 Reactor Operation

Three start-up strategies, constant voltage, intermittent boosting, and intermittent boosting after pyrolysis pretreatment, were used to study the effect of the start-up process of the microbial electrolysis cell system on the operational performance of the methanogenic biocathode. The constant voltage start-up group was set at 0.8 v; the intermittent boost start-up group was increased from 0.2 to 0.8 v, and the voltage was increased by 0.2 v at an interval of 24 h; the intermittent boost start-up group after pyrolysis pretreatment was operated in the same way as the second group. Other conditions were kept constant, and after normal operation of the reactor, 1 ml of gas was extracted from the sampling place with a sampling needle at every measurement interval of 24 h and injected into the gas phase spectrometer for gas detection.

At the same time, aeration treatment was carried out, and CO_2 with a flow rate of 0.3 L/min was passed into the cathode chamber for aeration, in which the aeration time was 30 min and the period was 24 h. During the period, the appropriate amount of cathode chamber solution was excluded using the air pressure difference for PH measurement, and the solution was reinjected into the cathode chamber at the end of the test.

2.4 Analysis and Calculation Methods

2.4.1 Calculation Method

After the MEC reactor started operation, 1 ml of gas produced in the cathode chamber was removed every 24 h with a feeder and injected into a gas chromatograph (GC9800, Shanghai Tech Chromatography Instruments Co., Ltd., China) for detection. The different gases were distinguished according to their peak times, and the yields of each gas component were calculated from the peak areas. The equation for the production rate of methane gas is shown below:

$$V_{(CH_4)}(A/m^2) = \frac{\Delta_{CH_4}}{T}$$
(1)

Among them, V_{CH4} is the volume change of methane produced by the reactor (m³) and T is the time of reactor operation (S).

During the operation of the H-type dual-chamber reactor, the current variations of three different starting strategies were recorded in real time with a data logger, and the desired current values were selected from the logger at the end of the experiment. During the operation of the MES reactor, the current density is the magnitude of the current generated per unit area of the cathode electrode and is calculated as follows:

$$I_A(A/m^2) = \frac{I}{A} \tag{2}$$

Among them, I is the circuit current (A), and A is the surface area of the cathode graphite felt (m^2) .

2.4.2 Solution PH Test Method

During the experiment, the pH value of the cathode chamber solution was measured every 48 h with a pH meter (PHS-3E type, Shanghai Yidian Scientific Instruments Co., Ltd., China). This was done by withdrawing a small amount of cathode chamber solution with a syringe before CO_2 aeration at the end of each experimental cycle, and then measuring and recording it with a pH meter, and re-injecting the withdrawn solution into the cathode chamber after the measurement.

2.4.3 Stability Simulation Test

The effect of fluctuations in electrical power input on the operational stability of the regenerative gas electrochemical system was simulated using an electrochemical workstation by increasing the current density from 0.5 to 5 A/m^2 (0.5 A/m^2 every 2 h, with re-exposure to CO₂ for about half an hour at the end of each current density plateau).

Intermittent power input was simulated by interrupting the power input for 20, 40, 60 and 80 h after 2 h of stable operation, and then reconnecting the power to test the system's recovery rate of the system. The recovery time was defined as the time to restore the methane production rate to 90% of that before the power outage after the power input was restored, which was used to evaluate the recovery performance of the regenerative gas electrochemical system.

3 Experimental Results and Discussion

3.1 Comparative Analysis of Methane Production Performance under Different Conditions

3.1.1 Analysis of Methane and Hydrogen Production Rates under Different Conditions

The start-up process of the three groups of biocathodes is shown in the Fig. 2a. After using constant voltage start-up (0.8 v), the methane production rate of the cathodes increased slowly throughout the start-up period, and the methane production rate was stable at $6.5 \pm 0.03 \text{ m}^3 \text{d}^{-1}$ after 15 days. After using intermittent boost start (0.2–0.8 v), the methane production rate of the cathode increased faster in 0–15 days compared with the constant voltage group, and also the methane production rate was stable at $15.66 \pm 0.02 \text{ m}^3 \text{d}^{-1}$ after 15 days, and the start-up time of the methanogenic biocathode was about 15 days. After using activated sludge after heat treatment with intermittent boost start (Pretreatment, 0.2–0.8 v), methane production gradually increased from 1 to 5 days, and methane production rate continued to accelerate from 5 to 10 days, and methane production rate stabilized at 20.18±0.01 m³d⁻¹ after 10 days methane production start time was about 10 days.



Figure 2: (a) Methane production rates for the three groups of experiments. (b) Hydrogen production rates for the three sets of experiments

The methane production of the cathode in the constant voltage start group and intermittent boost start group increased continuously during the start and tended to be stable after 15 days and the methane production of cathode in the intermittent boost start group after activated sludge was heat treated increased continuously during the start and tended to be stable after 10 days and the start time of biological cathode was significantly shortened after the activated sludge was heat treated; meanwhile, the methane production of activated sludge after heat treatment in The methanogenic rate of the intermittent boost start group was stable at 20.18 ± 0.01 m³d⁻¹ about three times higher than that of the constant voltage start group. It is inferred that the methanogenic bacteria in the sludge rupture under the effect of pressure difference generated during the heat treatment, and the organic matter inside the cells is released, which is rapidly hydrolyzed and dissolved under the effect of high temperature, which is favorable to the formation of methanogenic bacteria biofilm on the cathode surface. The methanogenic rate of the intermittent boost start group was stable at $15.66 \pm 0.02 \text{ m}^3 \text{d}^{-1}$, which was 2.4 times higher than that of the constant voltage start group, and the cathode was enriched with dense methanogenic bacteria biofilm with high metabolic and electrical activity by adjusting the cathode potential and current [13], while the constant voltage start group, in which the hydrogen-producing bacteria grew more vigorously at the beginning of the start, consumed excessive reduction equivalents, and the shear force generated by the hydrogen bubbles had an adverse effect on The shear force generated by the hydrogen bubbles also had a negative effect on biofilm attachment [27–29].

The process of H_2 production from the three groups of biocathodes is shown in the Fig. 2b after starting with constant voltage, the amount of hydrogen produced by the biocathodes increased continuously from day 0 to day 5, and decreased continuously with the gradual increase of methane from day 5 to day 10, and the amount of hydrogen produced was stable at 5.13 m³d⁻¹ after day 15. After the start-up of the activated sludge with heat treatment, the hydrogen production from the biocathode increased slowly from day 0–5 and decreased from day 5–10, stabilizing at 3.13 m³d⁻¹ after day 15.

The production of by-product H_2 is unavoidable for mixed strain inoculated biocathodes, and the variation of by-product H_2 production during the three groups of the biocathode start-up is shown in the graph. The peak H_2 production in the start-up phase (day 5) was six times higher in the constant voltage

start-up group compared to the intermittent boost start-up group, and 2.5 times higher in the stabilization phase. It can be seen that this method significantly reduces the hydrogen production during the bioinitiation and stabilization phases while increasing the methane production from the biocathode during the same period. So the intermittent ramp-up strategy improves the product selectivity of the methanogenic biocathode, which is probably due to the incomplete enrichment of the methanogenic biofilm in the constant voltage start-up group, resulting in a lower utilization of CO_2 and reducing equivalents and the escape of H_2 before it is utilized by methanogenic bacteria utilize it in time. The peak H_2 production rate in the start-up phase and the steady-state H_2 rate in the intermittent boost start-up group, it is possible that not only the methanogenic bacteria are more active in the activated sludge pretreated by pyrolysis, but also other microorganisms (e.g., hydrogen-producing bacteria) are more active in reproduction and metabolism.

In previous studies of methane production from CO_2 reduction in bioelectrochemical systems, hydrogen and methane have always co-existed in a single system therefore, to increase the efficiency of methane production, the amount of hydrogen produced must be minimised.

3.1.2 Analysis of the Variation of PH Values under Different Conditions

The pH of a solution is a key factor in the growth and metabolism of microorganisms. Therefore, it is essential to control the pH in solution. As shown in Fig. 3, the pH of the medium solution was adjusted to about 7 (phosphate buffer was added) when the medium was prepared before the start of each batch of experiments. The change in pH decreased and then increased in all three experimental groups. The activity of the biological strains was low at the beginning of the start-up phase and the H^+ from the anode chamber reached the cathode through the ion exchange membrane but was not yet fully utilised, resulting in a low PH in the cathode chamber. The constant voltage start-up group had a PH value of 6.68 on day 4 (the lowest value) and the intermittent boost start-up group had a PH value of 6.17 on day 4. The reason for this may be that the intermittent boost strategy favours the enrichment of the methanogenic biofilm, with lower H⁺ utilization and lower H₂ content compared to the constant voltage group. In contrast, the activated sludge was heat-treated to a PH value of 6.28 on day 4 for the intermittent boost start-up group, which was pre-treated by pyrolysis to improve the sludge cracking rate, giving the reactor better electrochemical performance and enriching the more intensive hydrogenproducing bacteria biofilm to synthesize lower H₂ content at the biocathode. After day 4, the PH of all three sets of experiments increased at an accelerated rate, while the methane production of all three sets of experiments also increased at an accelerated rate. The reason for this may be that with the enrichment of the bioactive biofilm at the biocathode, H^+ was used to synthesize methane and the rate at which H^+ was utilized in the cathode chamber was much higher than that passed from the anode chamber, so H^+ was consumed in large quantities and methane was generated at the cathode leading to a rise in pH of the solution. After the stabilization phase of the three sets of experiments, the pH is still slowly increasing, and their methane production remains essentially constant, which will decrease in the long term. The higher pH may also be one of the reasons for the inhibition of methane production, this is because the optimum pH environment for methanogenic strains is between 7 and 8, and high pH (>8.5) increases hydrogen production, as the alkaline environment induces the formation of an enrichment of the dominant hydrogen-producing strains and the same reduction equivalents are used more for hydrogen production, resulting in lower methane production of the target gas. At the same time the activity of the methanogenic archaea was affected and consequently, the overall reaction equilibrium of the fermentation system was disrupted, severely affecting the activity of the microorganisms and resulting in their inability to efficiently convert H_2 and CO_2 to CH_4 [30]. Therefore, ensuring that the ph of the solution was maintained at a relatively stable level was more conducive to the stable synthesis of methane in the MES.



Figure 3: Solution pH of cathode chamber for three groups of experiments

3.1.3 Analysis of Current Density under Different Conditions

As shown in Fig. 4, the constant voltage starter group showed a sharp and unstable drop in current during the first 4 days of operation when the reactor was connected to voltage, probably due to the rapid loss of protons and electrons from the culture solution after being connected to voltage, with the current density gradually decreasing and slowly stabilizing after the 4th day. The change in methane production was also observed to accelerate from day 4 and then slowly stabilize. It can be seen that the decrease in current is due to the formation of a bio-cathode methanogenic biofilm resulting in an increase in the internal resistance of the bio-electrosynthesis system, as the applied voltage provided is constant.



Figure 4: Variation of current density in three groups of experiments

The intermittent ramp-up starter group also goes through an unstable phase and the current density starts to decrease gradually. However, the voltage in this experimental group changed in stages from small to large, with the current density increasing again after day 10 and then gradually decreasing towards stability. In the microbial electrochemical system, the magnitude of the current density also reflects, to some extent, the rate

of electron transfer between the methanogenic bacteria and the electrode [14]. The cathode of the intermittent boost group was probably enriched with highly efficient electroactive biofilm, which reduced the internal resistance of the cathode. The reason why the intermittent boosting strategy results in a lower resistance of the biocathode may be the method's ability to achieve simultaneous changes in cathode potential and current. By giving the flexibility that these two parameters can be adjusted simultaneously, the intermittent boosting method buffers the drop in potential and current and achieves a relatively constant current during start-up, thus steadily supplying the cathode biofilm with the appropriate reduction equivalents to promote its enriched growth [28]. The current density in the stable phase of the heat-treated activated sludge in the intermittent boost start group was 3.573 A/m², slightly higher than the current density in the intermittent boost start group. This may be due to the fact that the pyrolysis pretreatment allows for an effective cracking of the activated sludge and a more complete release of organic matter, leaving the methanogenic microorganisms in a dominant position and more active, using a large amount of organic simple small molecules in the system for metabolism, synthesis of their own nutrients and methanogenic activities.

3.2 Stability Simulation Test Analysis

3.2.1 System Stability Testing during Fluctuations in Power Input

The results of the test to characterize the stability of the methanogenic performance of the microbial electrolyzer system through fluctuations in electrical input are shown in Fig. 5. As the input current density I increased from 0.5 to 5 A/m², the methane production rate of the constant voltage start-up group started to decrease after I reached 3.5 A/m^2 , when the hydrogen production rate was gradually increasing, while the methane production rate of the intermittent boost start-up group increased continuously from 2.23 m³d⁻¹. The methane production rate of the intermittent boost start group increased continuously from 2.23 to 16.84 m³d⁻¹ and then started to decrease slowly; the methane production rate of the intermittent boost start group increased continuously from 3.34 to 21.47 m³d⁻¹ after the activated sludge was heat-treated, and then also started to decrease slowly. The analysis of the graph shows that the standard deviation of stability of the intermittent boosting group and the intermittent boosting group with sludge heat treatment is 75% of that of the constant voltage group.



Figure 5: Impact of power input fluctuations on system operational performance
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The stability standard deviation of the intermittent boost group and the intermittent boost group of sludge heat treatment was 85% of that of the constant voltage group. The intermittent boost starting strategy significantly improved the stability of methane production performance and effectively suppressed the by-product H₂ production at high current density input. This may be attributed to the fact that the intermittent boosting strategy induced the construction of multiple extracellular electron transfer pathways for CO_2 reduction methanogenesis through cathodic potential changes and increased the supply rate of reduction equivalents through intermittent steps, so that the biofilm could obtain higher electron mobility and electrical activity, thus forming an efficient and stable methanogenic biofilm [13].

Meanwhile, the intermittent step-up start-up strategy of activated sludge after thermal treatment was tested with fluctuation of electric power input, and the change trend was consistent with the intermittent step-up start-up strategy, except that the methanogenic production was high in all stages. The main reason may be that the biological community has been treated by pyrolysis at high temperature and the methanogenic bacteria are more active [31], meanwhile, the efficiency of biofilm using electrons has been enhanced by adjusting the cathode potential in the start-up stage, so that the methanogenic performance and stability of this group can reach the best.

3.2.2 System Stability Testing during Intermittent Power Input

The results of the intermittent test to characterize the stability of the methane production performance of the microbial electrolytic cell system by the power input (20, 40, 60, 80 h power outages in sequence) are shown in Fig. 6. Compared with the constant voltage starter group, the intermittent boost starter group showed a lower drop in methane production per power outage and a faster recovery rate, which was about twice as fast as the constant voltage starter group, and the methane production during the stabilization phase (13.84 m^3d^{-1}) was basically the same as that before the power outage (15.66 m^3d^{-1}) as the power outage time increased. In contrast, the constant voltage starter group had not only difficulty in recovering the methane rate before power failure after each power failure, but also its methanogenic capacity gradually weakened with the increase of power failure time until the methanogenic rate decreased to 0.43 m³d⁻¹ at 80 h of power failure, because most of the methanogenic strains had lost their activity, and thus the methanogenic biofilm formed by the colony was judged to be basically ineffective in methanogenic function. Thus, the intermittent ramp-up strategy shortens the recovery time after power input interruptions and enhances the stability of the methanogenic performance of the microbial electrolyzer system, allowing it to withstand the adverse effects of power outages and maintain an excellent recovery capability [32]. This may be due to the enrichment of the cathode with highly stable methanogenic biofilm, which has a more stable extracellular electron transfer pathway compared to the constant voltage group and is more resilient to the periodic polarization of the cathode caused by repowering after a power outage [33,34].

Compared with the intermittent boost-start group, the activated sludge heat-treated intermittent booststart group had higher methanogenic production after each power outage and, subsequently higher stabilization phase of about 18.44 $m^3 d^{-1}$. Therefore, the heat-treated intermittent boost-start strategy improved the methanogenic performance of its stabilization phase. The heat-treated intermittent boost start strategy enriched biofilms in the start-up phase with more stable metabolic pathways (including energy acquisition and carbon consumption) and a microbial community highly adapted to the cathodic environment.



Figure 6: Impact of intermittent power input on system operational performance

4 Conclusions

In this experiment, an H-type dual-chamber MEC reaction system with anaerobic activated sludge from Shanghai Bailonggang Wastewater Treatment Plant as the inoculated sludge and CO_2 storage bottle as the sole carbon source was constructed to investigate the effects on the performance of CO_2 methanation based on microbial electrolysis cell under three sets of strategies: constant voltage, intermittent boosting and intermittent boosting after sludge thermal treatment, as well as system stability tests under fluctuating and intermittent power input. The study showed that:

- (1) The cathode potential, the regulation of current, and the pretreatment method of activated sludge all affect the methane production by CO₂ by the cathode-functional microorganisms in the MEC reactor. Under the same conditions, the intermittent boosting strategy cathode enriched dense methanogenic bacteria biofilm with high metabolic and electrical activity, which significantly increased methane production; the sludge pyrolysis treatment on the basis of intermittent boosting accelerated the formation of methanogenic bacteria biofilm on the cathode surface, which not only increased methane production but also significantly shortened the whole start-up time. In this experiment, the cumulative amount of methane material in the constant voltage experimental group, the intermittent boosting experimental group, and the intermittent boosting experimental group with sludge pretreatment were 6.52, 15.66, and 20.18 m³d⁻¹, respectively, and the start-up times were about 15, 15, and 10 days, respectively.
- (2) Instability of power input can potentially lead to changes in the structure and metabolic pathways of the biocathode microflora, which can result in reduced methane production. The intermittent boosting strategy reduces the dependence on external energy and carbon sources by building a stable and efficient biofilm. In this experiment, during the power input fluctuation test, the

stability standard deviation of its system operation is 75% of that of constant voltage; during the power input intermittent test, the intermittent boost experimental group recovers faster, and its recovery speed is nearly one times higher relative to the constant voltage start-up group.

Funding Statement: This paper is supported by Shanghai Science and Technology Development Fund, China, No. 19DZ1205604.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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DOI: 10.32604/jrm.2023.026689

ARTICLE





Comprehensive Assessment of the Safety of *Eucommia ulmoides* Leaf Extract for Consumption as a Traditional Chinese Health Food

Huiling Fu¹, Mijun Peng², Qiuwen Tang², Haojun Liang², Yanli Liang², Jiali Fang^{2,*} and Xuesong Wang^{2,*}

¹School of Chemical and Environmental Engineering, Hunan Institute of Technology, Hengyang, 421002, China

²Guangdong Provincial Key Laboratory of Chemical Measurement and Emergency Test Technology, Guangdong Provincial Engineering Research Center for Ambient Mass Spectrometry, Institute of Analysis, Guangdong Academy of Sciences (China National Analytical Center, Guangzhou), Guangzhou, 510070, China

*Corresponding Authors: Jiali Fang. Email: callyfangjl@163.com; Xuesong Wang. Email: wlwxs57813@163.com

Received: 20 September 2022 Accepted: 07 November 2022 Published: 07 June 2023

ABSTRACT

To ensure the export quality of Eucommia ulmoides leaf extract (ELE) and facilitate E. ulmoides leaf inclusion in the directory of traditional Chinese health foods, an overall safety assessment of ELE was performed, including genotoxicity and long-term toxicity, according to the national food safety standards of China. No variations in the reverse mutation number of the nominal bacterial strains were observed under ELE treatment in comparison with the solvent control. Additionally, the micronucleus rates of in vivo mammalian erythrocytes and in vitro mammalian cells under ELE treatment were equivalent to or significantly lower than those of the solvent control. The fold change in the trifluorothymidine resistance mutation frequency of the thymidine kinase gene under ELE treatment was less than three times in comparison with the solvent control, suggesting that ELE did not cause genotoxicity. Moreover, animal experiments showed that the growth performance of rats under ELE treatment was enhanced because the body weights of rats increased. No oxidative injury or inflammatory responses were induced and no histopathological lesions of tissues were detected under ELE treatment. In addition, plasma triglycerides and low-density lipoprotein cholesterol levels significantly decreased, and plasma high-density lipoprotein cholesterol levels significantly increased with ELE treatment, suggesting that ELE was health-promoting. Furthermore, moderate to excellent antimicrobial activities, a favorable anticancer capacity, and superior antioxidant abilities of ELE were found, implying ELE possesses good bioactivities. Therefore, we affirmed ELE is safe to consume as a traditional Chinese health food.

KEYWORDS

Eucommia ulmoides leaf extract; genotoxicity; long-term toxicity; growth performance; bioactivity



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Graphic Abstract



1 Introduction

Eucommia ulmoides (E. ulmoides) Oliver, a traditional Chinese medicinal plant generally known as "duzhong" in China [1,2], is a monotypic deciduous and dioecious tree and is the only species of the genus *Eucommia* [3,4]. *E. ulmoides* originated in China during the glacial movement in the third century and is currently cultivated at an altitude range of 300-1300 m in China, such as in the Hunan, Henan, and Yunnan provinces [5]. *E. ulmoides* has a long application history, particularly the raw bark. As recorded in the Chinese medical classics *Shen Nong Ben Cao Jing* and *Ben Cao Gang Mu*, the raw bark of *E. ulmoides* has been used as traditional Chinese medicine and has been included in the Chinese Pharmacopoeia [6]. Based on its pharmacological activities, *E. ulmoides* bark is commonly used as a tonic for organs (liver and kidney) and has been found to prevent miscarriage, regulate blood pressure, and act as an anti-aging agent [7–10]. Although *E. ulmoides* bark has shown multiple pharmacological effects, the bark is not conducive to large-scale development and utilization due to resource constraints. Therefore, a new renewable resource is urgently needed to compensate for the shortage of bark.

To find an alternative to the bark, an analysis of the chemical components of the bark was necessary. The chemical components of the bark were separated, purified, identified, and found to be lignans, iridoids, flavonoids, phenolics, steroids, terpenoids, and gutta-percha [5]. Of these, lignans and iridoids were identified as the two most important constituents, and are usually denoted as key chemotaxonomic markers. These two constituents were shown to exhibit excellent pharmacological activities, such as the lignans liridondrin, (+)-pinoresinol di-O- β -D-glucopyranoside, and (+)-syringaresinol, which were found to have a satisfactory antihypertensive effect, and aucubin, genipin, and geniposidic acid of iridoids,

which displayed remarkable antihypertension and anti-aging effects. Recently, evidence has shown that the chemical components of the bark are similar to those of the leaves, including lignans, iridoids, phenols, steroids, triterpenes, polysaccharides, and flavonoids [11,12], suggesting that the leaves of *E. ulmoides* can solve the dilemma of the bark shortage. In addition, numerous studies have reported that *E. ulmoides* leaves were found to increase bone strength, reduce fattiness, and increase energy metabolism, thus resulting in longevity and improved fertility in humans [13,14]. Moreover, flavonoids of *E. ulmoides* leaves (e.g., chlorogenic acid (CGA), rutin, ferulic acid, and caffeic acid) displayed good antioxidant activity [15]. Taken together, *E. ulmoides* leaves are a promising replacement for bark [16]. Furthermore, due to the process of peeling will cause trunk damage (even death) and the bark regeneration speed being slower than leaves, the *E. ulmoides* leaves may be more suitable for commercialization based on their advantages of picking easily, large amount, and growth every year. Therefore, *E. ulmoides* leaves are a better renewable material in comparison with the bark, which can meet the ever-increasing demand.

Because the utility and value of E. ulmoides have been widely recognized, it has attracted much attention from abroad and has been successfully traded with other countries, such as the United States, Russia, Britain, and France [17]. In 2019, we formulated the group standards for E. ulmoides leaf extract at the invitation of the China Chamber of Commerce for Import and Export of Pharmaceutical and Health Products, which ensured the export quality of E. ulmoides leaf extract. Furthermore, although the chemical components of the bark are similar to the leaves, some composition differences were determined between them, such as the characteristic component, CGA, which is highly enriched in leaves, whereas its concentration in the bark is below the detection limit. Simultaneously, divergent in other characteristic components composition is observed between leaves and barks, e.g., rutin is only determined in leaves, whereas geniposide and pinoresinol diglucoside are only detected in barks. In addition, apart from the composition difference, the concentrations of the same components (aucubin, geniposidic acid) in leaves also distinct from that of in barks, and their concentrations are significantly higher in barks [18]. Previous studies have demonstrated that CGA induces an antihypertensive effect [19] and significantly reduced the systolic and diastolic blood pressure of hypertensive patients and rats [20,21], and can potentially serve as an antihypertensive drug [22]. Meanwhile, geniposidic acid and aucubin show the abilities of free radical scavenging, anti-inflammatory, and anti-cancer [23]. Therefore, the composition and concentration difference of these characteristic components between the bark and leaves might lead to bioactivity variations, and an overall assessment of the safety of E. ulmoides leaf extract is urgently needed to ensure the continued trade of E. ulmoides leaf extract around the world.

In the present study, genotoxicity tests, cytotoxicity assay, and *in vivo* long-term animal toxicity studies of *E. ulmoides* leaf extracts were performed according to the national food safety standards of China. Furthermore, the bioactivity of *E. ulmoides* leaf extracts was also explored, including analysis of antimicrobial activity, anticancer capacity, and antioxidant abilities. We hypothesized that *E. ulmoides* leaf extract was safe for serving as a traditional Chinese health food based on its bioactivities. Our study will examine the safety of *E. ulmoides* extract, which contributes to the application of *E. ulmoides* leaf extracts.

2 Materials and Methods

All of the experiments regarding animals (rats) were approved by the Animal Welfare and Ethics Committee of the Institute of Analysis, Guangdong Academy of Sciences (China National Analytical Center, Guangzhou, China). The permission number is No. 2021002.

2.1 Standards and Reagents

The chemical standards (chlorogenic acid, geniposidic acid, aucubin, geniposide, pinoresinol diglucoside, and rutin) were provided by Yuanye Technology Co., Ltd. (Shanghai, China) with a purity ≥99%. Methanol and phosphoric acid were provided by Merck (Darmstadt, Germany), while hexane, petroleum ether, and

chloroform were provided by Guangzhou Chemical Reagent Factory (Guangzhou, China). All of the chemical standards and solvents were commercially available and used without dilution unless otherwise specified. A Milli-Q filter system (Millipore, Bedford, MA, USA) was used to prepare ultrapure water. The cells and bacteria trains used in the present study were provided by iPhase (Beijing, China).

2.2 Preparation of E. ulmoides Leaf Extract (ELE)

The dry leaves of *E. ulmoides* were provided by Hengxin Co., Ltd. (Zhangjiajie, China). After extraction twice with ultrapure water (liquid:solid = 1:10) under the condition of 80°C for 1 h, the extracted liquid was concentrated to 20 Brix under vacuum rotary evaporation. Subsequently, the obtained concentrated liquid was dried by spray drying. During spray drying, the inlet air temperature was $150^{\circ}C-170^{\circ}C$ and, while the outlet air temperature was $90^{\circ}C-95^{\circ}C$. The extraction yield of ELE is about 25%.

2.3 Composition Analysis of ELE

The chemical composition analysis of ELE was performed on an LC-20A HPLC system (Shimazu, Japan) equipped with a diode array detector. The standard curves of the six chemical standards were plotted first. A Thermo BDS HYPERSIL C-18 analytical column (250 mm× 4.6 mm, 5 µm) was used for target component separation. For separation, the mobile phase solutions consisting of solvent A (0.2% phosphoric acid in water) and solvent B (MeOH) were prepared. A linear gradient elution program was designed as follows: 95% A from 0–17 min; 95%–86% A from 17–45 min; 86%–65% A from 45–55 min; and 65%–55% A from 55–70 min. For analysis, a 10 µL sample was injected into the column, and then the column temperature was maintained at 30°C. The flow rate was set at 1 mL/min. Four characteristic components (geniposidic acid, chlorogenic acid, geniposide, and pinoresinol diglucoside) were detected at 238 nm, while the other two components (aucubin and rutin) were monitored at 208 and 254 nm, respectively. The analysis of physicochemical parameters in ELE (moisture and ash), pesticide residues (hexachlorocyclohexane (HCH), dichlorodiphenyl trichloroethane (DDT), quintozene (PCNB)), microbial content (aerobic bacterial count, coliforms, molds and yeasts, Salmonella, *Staphylococcus aureus*), and mineral elements were carried out following GB 5009.3-2016, GB 5009.4-2016, GB 4789.4-2016, GB 4789.10-2016, and GB 5009.268-2016, respectively.

2.4 Genotoxicity Studies

The bacterial reverse mutation assay, mammalian erythrocyte micronucleus test, *in vitro* mammalian cell micronucleus test, and *in vitro* thymidine kinase (TK) gene mutation test on ELE were conducted following GB 15193.4-2014, GB 15193.5-2014, GB 15193.28-2020, and GB 15193.20-2014, respectively. All of these standards were in line with the OECD guidelines with appropriate modification, including OECD 471 bacteria reverse mutation test, OECD 474 mammalian erythrocyte micronucleus test, OECD 487 *in vitro* micronucleus test, and OECD 490 *in vitro* mammalian cell gene mutation tests using the thymidine kinase gene.

For bacterial reverse mutation assay, the plate incorporation technique was used. Briefly, five nominal bacterial strains were adopted, including four *Salmonella typhimurium* strains (TA1535, TA100, TA98, TA97a) and one *Escherichia coli* strain (WP2uvrA). Four dose solutions of ELE (5, 2.5, 1.25, 0.625 mg/dish) were set with fold changes of two. Pure water was selected as solvent control. 2-aminoanthracene (2-AA), sodium azide (NaN₃), 2-aminofluerene (2-AF), methyl methanesulfonate (MMS), and sodium p(dimethylamino) benzenediazo sulfonate (Dexon) were used as the positive control. The culture medium (25 mL/dish) was prepared and stored at 37°C in dark for 24 h. Then, a mixture consisting of agar medium (2 mL), bacteria solution (0.1 mL), ELE solution (0.1 mL), and phosphate buffer (0.5 mL) was added. If the experiment was conducted under metabolic activation conditions, S9 solution (0.5 mL) was added too and denoted as $+S_9$; otherwise, it was defined as $-S_9$. After

solidification, the plates were turned upside down and cultivated at 37°C for another 72 h. The reverse mutation number of bacterial strains was recorded.

For the mammalian erythrocyte micronucleus test, three dose solutions of ELE (10000, 5000, 2500 mg/kg) were prepared with a fold changes of two. The 10000 mg/kg was selected as the highest dose based on the rule of 50% lethal dose higher (LD50) than 10 g/kg. Pure water was selected as solvent control. Cyclophosphamide (CTX, 40 mg/kg) was used as the positive control. Ten rats (half male and half female) were used for each dose and ELE solution was given twice in 30 h by the method of gavage. Then, marrow fluids were harvested at 36 h and mixed with fatal bovine serum (FBS) on a slide. After smearing and drying, the slide was fixed with methanol for 5 min and stained with Giemsa for 15 min. Consequently, the slide was washed with phosphate buffer saline (PBS, 1/15 mol/L) and sealed. Two thousand cells were counted and the micronucleus rate (‰) was therefore calculated.

For *in vitro* mammalian cell micronucleus test, three dose solutions of ELE (5.0, 2.5, 1.25 mg/mL) were prepared with a fold changes of two. The highest dose was set based on the maximum concentration (5.0 mg/mL) without cytotoxicity. Pure water was selected as solvent control. CTX (20 ug/mL) and mytomycin-C (MMC, 0.25 ug/mL) were used as the positive control. If the experiment was conducted under metabolic activation conditions, S9 and CTX solutions were added and denoted as $+S_9$; otherwise, MMC was added and denoted as $-S_9$. Cells with a density of 1×10^5 cells/mL were cultured in a tissue culture flask under the condition of 5% CO₂ and 37°C. After removing the medium, ELE (1%) and S9 (10%) solutions were added, together with the complete medium (exclusion FBS). These cells were cultured for 4 h at the same condition and were washed with PBS three times. Consequently, a complete medium was added and cultured for another 24 h. After washing with PBS twice and digestion with 0.25% trypsin solution for 5 min, the cells were centrifuged under 1000 r/min for 5 min. The cells were harvested by discarding supernate and then fixed with stationary liquid (2 mL) three times. After natural drying, the cells were stained with Giemsa for 15 min. Two thousand cells were counted and the micronucleus rate (‰) was therefore calculated.

For *in vitro* TK gene mutation test, three dose solutions of ELE (5.0, 2.5, 1.25 mg/mL) were prepared with a fold changes of two. The highest dose was set based on the maximum concentration (5.0 mg/mL) without cytotoxicity. Pure water was selected as solvent control. CTX (50 ug/mL) and methyl methanesulfonate (MMS, 5.0 ug/mL) were used as the positive control. If the experiment was conducted under metabolic activation conditions, S9 and CTX solutions were added and denoted as $+S_9$; otherwise, MMs were added and denoted as $-S_9$. Cells with a density of 5×10^5 cells/mL were used and ELE solution (1%) was added. These cells were shaken under 37°C for 3 h and centrifuged to remove supernate. The obtained cells were washed with PBS twice and resuspended by 10% RPMI 1640 medium. The cell density was adjusted to 2×10^5 cells/mL. The plating efficiency at 0 day (PE0) was measured. A cell density of 8 cells/mL (0.2 mL) was inoculated into a 96-well plate and cultured under the condition of 37°C and 5% CO2 for 12 d. For expression, the cell suspension was cultured for 2 d and the cell density was kept below 10^6 cells/ml. The plating efficiency at the second day (PE2) was determined as the same procedure as PE0. Meanwhile, the trifluorothymidine (TFT) resistance mutation frequency (MF) was detected. The expression cells of the second day were adopted and the cell density was adjusted to 1×10^4 cells/mL. TFT (3 µg/mL) was added and inoculated into a 96-well plate (0.2 mL). The prepared plate was cultured under the condition of 37°C and 5% CO₂ for 12 d. The number of wells with mutant colony growth was counted.

2.5 Cytotoxicity Assay

The cell viability (%) of CHO, CHL, V79, and human hepatocellular liver carcinoma (HepG2) cells under ELE treatment was determined based on a methyl thiazolyl tetrazolium (MTT) cell proliferation assay. A cell density of 1×10^5 /mL was prepared and then 100 µL of the cell suspension was added to the wells of 96-well plates. Medium alone (100 µL) was prepared as a negative control. The prepared plates were incubated at 37°C and 5% CO₂ for 12 h. Subsequently, MTT solution (5 mg/mL, 10 µL) was added to each well and incubated for

another 6 h. After incubation, the medium of each well was removed and 100 μ L of dimethyl sulfoxide (DMSO) was added. The obtained reaction system was mixed thoroughly and the OD value of each well was measured at 490 nm. Three concentrations of ELE (25, 12.5, and 6.25 mg/mL) were used for analyzing the cell viability of CHO, CHL, and V79 cells, while six concentrations of ELE (40.0, 20.0, 10.0, 5.0, 1.0, 0.1 μ g/mL) were used for analyzing the cell viability of HepG2 cells. Cisplatin was chosen as the positive drug for evaluating an anticancer effect against HepG2 cells.

2.6 Long-Term Toxicity Testing

2.6.1 90-Day Repeated Dose Toxicity Study with the Following Subsections

Long-term toxicity testing of ELE was conducted following GB15193.13-2015. Sprague-Dawley rats, about 100 g each (n = 80, half male and half female), were randomly assigned to four groups: A group was the basal diet; B, C, and D groups were the basal diet supplemented with 1000, 500, or 200 mg/kg of ELE, respectively. The basal diet was provided by Jiangsu Xietong Shengwu Co., Ltd. (Nanjing, China). All of the rats were allowed free access to water and were fed under constant conditions of a 12 h light and 12 h dark cycle in the China National Analytical Center, Guangzhou (Zhongshan, China). The room temperature ($23 \pm 1^{\circ}$ C) and relative humidity ($50\% \pm 5\%$) were constant during the entire experimental period of 90 days.

2.6.2 Sample Collection

Blood samples of each group were obtained as eptically from the abdominal aortic vein of rats after 90 days of feeding. Then, the blood samples were immediately centrifuged at 3000 r/min for 15 min. All of the blood samples were stored at -80° C before use. Additionally, seven organs (brain, liver, spleen, heart, kidney, adrenal glands, and testis) were collected and weighed individually. Two intestinal tissues (duodenum and ileum) were also collected and stored in 4% paraformal dehyde until further use.

2.6.3 Biochemical and Hematological Analysis

Blood routine indexes (white blood cell (WBC), neutrophil (Neu), lymphocyte (Lym), monocyte (Mon), eosinophil (Eos), basophil (Bas), red blood cell (RBC), hemoglobin (HGB), hematocrit value (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpusular hemoglobin concerntration (MCHC), red blood cell distribution width-coefficient of variation (RDW-CV), red blood cell distribution width-standard deviation (RDW-SD), platelet (PLT), mean platelet volume (MPV), platelet distribution width (PDW), thrombocytocrit (PCT)) and blood biochemical indexes (albumin (ALB), alkaliphosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA-S), glucose (Glu-G), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total phosphorus (TP), UREA, yglutamyl transpeptidase (y-GT)) were determined by using relevant detection kits (Mindray, Shenzhen, The blood antioxidant indexes (superoxide dismutase (SOD), glutathione (GSH), China). molondialdehyde (MDA), catalase (CAT)) and inflammatory factors (tumor necrosis factor- α (TNF- α), Interleukin-1 β (IL-1 β), Interleukin-6 (IL-6)) were also determined by using relevant detection kits from GuangZhou KYDbio Technology Co., Ltd. (Guangzhou, China). All of the analytical procedures were strictly in accordance with the instruction manual of each kit.

2.6.4 Histopathological Examination

Five organs (liver, spleen, kidney, stomach, and pancreas) and two intestinal tissues (duodenum and ileum) were dehydrated with graded alcohol and xylene, and the tissues were embedded in paraffin. After solidification, the obtained paraffin block was cut into 4- μ m sections and stained with hematoxylin and eosin. An inverted microscope (ECHO, Chicago, Illinois, USA) was used to observe the tissue morphology. The villus height and the crypt depth of the duodenum and ileum were measured by using an automatic image analyzer (Olympus DP73 camera, Japan). A minimum of six villi and 10 crypts that were well-oriented were obtained from different parts of each intestinal tissue sample.

2.7 Antimicrobial Activity

The antimicrobial activities of ELE against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Aspergillus niger* were determined following GB 15979-2002 with minor modifications. Two concentrations of each microbe $(1 \times 10^3 \text{ and } 1 \times 10^4 \text{ CFU/mL})$ and two concentrations of ELE (25.0 and 5.0 mg/L) were prepared. The prepared microbial suspension (100 µL) and ELE solution (0.5 mL) were added to a petri dish with 15 mL Mueller Hinton/potato dextrose agar medium. After solidification, the petri dish was turned over and incubated for 36 h. The incubation temperature of *E. coli*, *S. aureus*, and *P. aeruginosa* was 36°C, while the incubation temperature of *A. niger* was 15°C. The experiment was repeated three times. Pure DMSO was used as a negative control. The bacterial suspension was used as a blank control. The antimicrobial activity was then calculated.

2.8 Free Radical Scavenging Capacity

Four typical free radicals (hydroxyl radical (OH), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide radical (PTIO·), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid radical (ABTS)) were selected to assess the *in vitro* free radical scavenging rate of ELE. The detection process of the four free radicals was accorded to the method reported by Li et al. [24].

2.9 Statistical Analyses

All data were analyzed by using GraphPad 8.0 (Diego, California, USA) and SPSS 19.0. One-way analysis of variance (ANOVA) and the Tukey test were performed to conduct multiple comparisons. All data were expressed as mean \pm standard deviation (SD), with P < 0.05 considered statistically significant.

3 Results and Discussions

3.1 Composition of ELE

The composition of ELE is listed in Table 1. The concentrations of crude polysaccharide, total polyphenol, total flavonoid, and protein in ELE were 2.06%, 17.35 g/100 g, 5.27 g/100 g, and 1.62 g/100 g, respectively. Four major characteristic components of ELE (chlorogenic acid, aucubin, geniposidic acid, and rutin) were determined and their concentrations were 81.73, 55.51, 86.55, and 7.55 mg/g, respectively. Based on these active ingredients, ELE likely confers multiple pharmacological activities, such as antiviral, anti-inflammatory, and antioxidant [25–28]. Physiochemical properties, pesticide residues, microbiological components, and mineral element levels in ELE were also measured and all met the group standards for *E. ulmoides* leaf extract. ELE underwent a safety assessment and bioactivity analysis without findings unless otherwise specified. The chromatographic peaks of standards and ELE are shown in Fig. S1.

Category	Index	Content	Category	Index	Content
Functional	Crude polysaccharide (%)	2.06 ± 0.18		K (mg/kg)	28994.45 ± 234.67
composition	Total polyphenol (g/100 g)	17.35 ± 0.61		Ca (mg/kg)	13187.64 ± 121.87
	Total flavonoid (g/100 g)	5.27 ± 0.06		Na (mg/kg)	2550.56 ± 25.89
	Protein (g/100 g)	1.62 ± 0.01	Mineral	Mg (mg/kg)	4008.76 ± 33.48
	Chlorogenic acid (mg/g)	81.73 ± 3.73	elements	P (mg/kg)	3169.25 ± 20.96
	Aucubin (mg/g)	55.51 ± 0.43		Fe (mg/kg)	39.74 ± 2.67
	Geniposidic acid (mg/g)	86.55 ± 0.56		Zn (mg/kg)	20.23 ± 1.81
	Rutin (mg/g)	7.55 ± 0.08		Cu (mg/kg)	0.85 ± 0.23

 Table 1: The composition of E. ulmoides leaf extract (ELE)

(Continued)

Table 1 (continued	Table 1 (continued)						
Category	Index	Content	Category	Index	Content		
Physico- chemical tests	Moisture (%)	3.39 ± 0.05		Mn (mg/kg)	191.88 ± 4.43		
	Ash (%)	11.6 ± 0.50		B (mg/kg)	25.16 ± 1.83		
Pesticide residue	Hexachlorocyclohexane (mg/kg)	ND		Sr (mg/kg)	30.45 ± 2.10		
	Dichlorodiphenyl Trichloroethane (mg/kg)	ND		Al (mg/kg)	9.88 ± 0.67		
	Quintozene (mg/kg)	ND		Cr (mg/kg)	2.37 ± 0.13		
Microbiological Tests	Aerobic bacterial count (CFU/g)	<10		Ni (mg/kg)	1.70 ± 0.18		
	Coliforms (MPN/g)	< 0.3		Se (mg/kg)	0.33 ± 0.02		
	Moulds and Yeasts (CFU/g)	<10		As (mg/kg)	0.22 ± 0.02		
	Salmonella (25 g)	ND		Pb (mg/kg)	0.38 ± 0.03		
	Staphylococcus aureus (25 g)	ND		Cd (mg/kg)	0.08 ± 0.01		

3.2 Bacterial Reverse Mutation Assay of ELE

The bacterial reverse mutation assay is a fundamental genetic toxicology test, which is essential in assessing the genotoxic capabilities of food and drugs [29]. In this study, the reverse mutation number of the five nominal bacterial strains (TA1535, WP2uvrA, TA100, TA98, TA97a) under positive control $(+/-S_9)$ were either three or two times higher than that under solvent control (Table 2), suggesting that mutagenesis was triggered under positive control. Interestingly, the reverse mutation numbers of the five nominal bacterial strains under ELE treatment were equivalent to that under solvent control, and the fold changes of the reverse mutation numbers between ELE treatment and solvent control were less than two, no matter if the concentration of ELE was low or high, indicating that ELE did not cause mutagenicity.

Table 2: The bacterial reverse mutation assay of *E. ulmoides* leaf extract (ELE). 2-AA represents 2-aminoanthracene; NaN₃ represents sodium azide; 2-AF represents 2-aminofluerene; MMS represents methyl methanesulfonate; Dexon represents sodium p(dimethylamino) benzenediazo sulfonate. Different small letters indicate significant differences at P < 0.05 level of tukey test under different treatments

Strain Po				Concentration of ELE			
	Positive c	ontrol	(Pure water)	5000 μg/dish	2500 μg/dish	1250 μg/dish	625 μg/dish
TA1535	+S ₉ (2-AA, 20 ug/mL)	176.45± 5.67 a	38.23 ± 4.03 b	36.43 ± 3.91 b	$\begin{array}{c} 36.25 \pm \\ 4.12 \text{ b} \end{array}$	39.23 ± 3.17 b	38.28± 1.72 b
	-S ₉ (NaN ₃ , 15 ug/mL)	152.67± 4.32 a	29.14 ± 2.22 b	28.29 ± 2.92 b	31.45 ± 5.43 b	29.46 ± 2.59 b	26.45 ± 2.95 b

(Continued)

Table 2 (co	ontinued)							
		Positive control			Concentration of ELE			
Strain	Positive co			5000 μg/dish	2500 μg/dish	1250 μg/dish	625 μg/dish	
TA100	+S ₉ (2-AF, 200 μg/mL)	429.34± 8.51 a	78.45 ± 3.74 b	$76.44 \pm 3.75 \text{ b}$	73.64± 4.57 b	78.36± 5.72 b	78.43 ± 8.67 b	
	-S ₉ (MMS, 10 uL/mL)	$\begin{array}{c} 293.65 \pm \\ 6.62 \ a \end{array}$	97.98 ± 3.48 c	117.78± 6.72 b	81.28± 5.86 d	83.48± 4.23 d	86.29± 3.88 d	
TA98	+S ₉ (2-AF, 200 μg/mL)	198.85± 4.44 a	33.67 ± 3.36 b	37.20± 1.96 b	34.16± 2.22 b	37.25± 1.92 b	37.66± 2.15 b	
	-S ₉ (Dexon, 6 μg/dish)	121.36± 7.06 a	47.84 ± 3.19 b	46.35± 2.80 b	45.87± 2.85 b	49.65± 2.24 b	38.29± 2.63 c	
TA97a	+S ₉ (2-AF, 200 μg/mL)	369.78± 6.72 a	151.23 ± 4.23 c	144.66± 4.90 c	150.49± 3.86 c	165.78± 3.36 b	146.33± 5.52 c	
	-S ₉ (Dexon, 500 ug/mL)	352.24± 8.43 a	127.64 ± 7.04 c	160.79± 9.02 b	129.77± 7.37 c	162.66± 4.55 b	134.82± 5.97 c	
WP2uvrA	+S ₉ (2-AF, 200 μg/mL)	401.33± 9.72 a	148.66 ± 6.32 b	145.64± 8.64 b	146.77± 5.94 b	$\begin{array}{c} 150.45 \pm \\ 6.38 \text{ b} \end{array}$	$143.44 \pm 5.04 \text{ b}$	
	-S ₉ (MMS, 10 uL/mL)	361.23± 6.08 a	158.33 ± 5.31 b	151.58± 9.53 b	138.28± 3.95 c	154.78± 3.8 b	136.67± 1.25 c	

3.3 Mammalian Erythrocyte Micronucleus Test of ELE

Currently, the in vivo mammalian erythrocyte micronucleus test is the most commonly performed regulatory test in the genotoxicity study [30]. Therefore, the mammalian erythrocyte micronucleus test (Table 3) was carried out on ELE. The micronucleus rates of mammalian erythrocyte under positive control was significantly higher than that under solvent control, indicating that elevated mammalian erythrocyte micronucleus rate was observed under positive control. In contrast to the positive control, the mammalian erythrocyte micronucleus rate under ELE treatment was close to the rate under solvent control, no matter if the concentration of ELE was low or high, implying that ELE did not cause an elevated mammalian erythrocyte micronucleus rate.

Table 3: The mammalian erythrocyte micronucleus test of *E. ulmoides* leaf extract (ELE). CTX represents cyclophosphamide. Different small letters indicate significant differences at P < 0.05 level of tukey test under different treatments

	Desitive control (CTV	Colourst control (Dome	Concentration of ELE			
Item	40 mg/kg)	water)	10000 mg/ kg	5000 mg/ kg	2500 mg/ kg	
Cell number	2000	2000	2000	2000	2000	
Micronucleus number	479.88±23.24 a	17.66 ± 3.45 b	18.78± 2.61 b	17.28± 2.83 b	19.29± 1.69 b	
Micronucleus rate (‰)	239.94	8.83	9.39	8.64	9.65	

3.4 In Vitro Mammalian Cell Micronucleus Test of ELE

Similar to mammalian erythrocyte micronucleus test, the *in vitro* mammalian cell micronucleus test is one of the tests most often used and required by regulations targeting food safety [31] and the results of *in vitro* mammalian cells micronucleus test on ELE is listed in Table 4. The micronucleus rates of mammalian cells under positive control $(+/-S_9)$ were all significantly higher than those under solvent control, indicating that elevated mammalian cells' micronucleus rates were determined under positive control. In contrast to the positive control, the micronucleus rates of mammalian cells under ELE treatment were close to or significantly lower than the rates under solvent control, no matter if the concentration of ELE was low or high, suggesting that ELE did not cause an elevated mammalian cells micronucleus rate.

Table 4: The *in vitro* mammalian cells micronucleus test of *E. ulmoides* leaf extract (ELE). CTX represents cyclophosphamide; MMC represents mytomycin-C. Different small letters indicate significant differences at P < 0.05 level of tukey test under different treatments

	Positive control			Concentration of ELE		
Item			solvent control (Pure water)	5.0 mg/mL	2.5 mg/mL	1.25 mg/mL
Cell number	2000		2000	2000	2000	2000
Micronucleus number	+S ₉ (CTX, 20 ug/mL)	56.48± 6.62 a	8.33 ± 2.34 b	8.82± 1.16 b	4.66± 2.17 c	8.58± 2.24 b
Micronucleus rate (‰)		28.24	4.17	4.41	2.33	4.29
Micronucleus number	-S ₉ (MMC, 0.25 ug/mL)	56.89± 5.84 a	6.67 ± 1.85 b	2.34± 1.26 c	3.66± 1.43 c	6.29± 1.62 b
Micronucleus rate (‰)		28.45	3.34	1.17	1.83	3.15

3.5 In Vitro TK Gene Mutation Testing of ELE

To better understand the mechanistic implications regarding *in vitro* genotoxicity test, the TK gene mutation assay was developed to provide higher specificity toward DNA-damaging reagents [32]. The TFT resistance mutation frequency (T-MF) of the TK gene under positive control $(+/-S_9)$ was three times higher than under solvent control (Table 5), indicating that the TK gene mutagenicity was confirmed for the positive control. Although the T-MF of the TK gene under ELE treatment was higher than under solvent control, the fold changes of the T-MF under ELE treatment were less than three times the fold changes of the solvent control, no matter if the concentration of ELE was low or high, indicating that ELE did not cause TK gene mutations.

Evaluation of genotoxicity is a fundamental part of food safety assessment due to the potential health problems caused by genotoxicity. According to the national standards of food safety (GB 15193.4-2014, GB 15193.5-2014, GB 15193.28-2020, and GB 15193.20-2014), four genetic toxicity tests (including the bacterial recovery mutation test, mammalian erythrocyte micronucleus test, *in vitro* mammalian cells micronucleus test, and *in vitro* TK gene mutation test) were conducted in the present study. Interestingly, the bacterial reverse mutation assay results showed that the reverse mutation numbers of the tested bacterial strains under ELE treatment were equivalent to that under solvent control, suggesting that ELE does not cause genetic toxicity. Accordingly, the micronucleus rates of mammalian erythrocytes and *in vitro* mammalian cells under ELE treatment were close to or significantly lower than those of the solvent

control, showing ELE did not cause chromosomal aberrations. In addition, the fold changes of the T-MF mutation frequency of the TK gene under ELE treatment were no greater than three times in comparison to solvent control, indicating that ELE did not cause TK gene mutation. Therefore, we concluded that ELE does not cause genotoxicity. Since these genotoxicity studies were in keeping with the OECD guidelines, the results of no genotoxicity could not only meet the demand of China, but also be admitted by other counties, which benefited the normal trade of ELE.

Table 5: The *in vitro* TK gene mutation test of *E. ulmoides* leaf extract (ELE). CTX represents cyclophosphamide; MMS represents methyl methanesulfonate; PE0 represents plating efficiency at 0 day; PE2 represents plating efficiency on the second day; RS represents relative survival; DSG represents relative suspension growth; RTG represents relative total growth; T-MF represents TFT resistance mutation frequency. Different small letters indicate significant differences at P < 0.05 level of the tukey test under different treatments

	Group		PE0 (%)	PE2 (%)	RS (%)	RSG (%)	RTG (%)	T-MF (×10 ⁻⁵)
+S ₉	Positive control 50 ug/mL)	(CTX,	$47.02 \pm 1.21 \ d$	96.28± 2.54 a	59.12± 1.23 d	79.70± 2.34 c	47.10± 1.69 e	150.45 ± 6.41 a
	Solvent control water)	(Pure	79.54± 1.81 a	91.81 ± 2.16 b	100.00± 0 a	100.00± 0 a	100.00± 0 a	5.48± 1.23 d
	Concentration of ELE	5.0 mg/mL	47.08 ± 2.32 d	55.73 ± 2.15 d	59.15 ± 2.44 d	97.32 ± 2.37 b	57.00± 1.62 d	11.08 ± 1.62 b
		2.5 mg/mL	58.80 ± 0.94 b	68.62± 2.44 c	$\begin{array}{c} 73.94 \pm \\ 2.33 \text{ b} \end{array}$	97.44 ± 2.18 b	71.39± 1.95 b	9.54± 1.23 c
		1.25 mg/ mL	52.76± 1.66 c	58.86± 1.83 d	66.33 ± 1.85 c	98.04± 1.87 b	65.15 ± 2.26 c	8.42± 1.54 c
-S ₉	Positive control 5 ug/mL)	(MMS,	31.60 ± 2.12 d	100.00± 0 a	37.86± 1.62 d	70.09 ± 1.57 d	26.50 ± 2.31 d	100.88± 4.54 a
	Solvent control water)	(Pure	83.50± 2.43 a	96.22 ± 2.37 b	100.00± 0 a	100.00± 0 a	100.00± 0 a	4.23 ± 1.09 c
	Concentration of ELE	5.0 mg/mL	72.15 ± 2.23 c	79.57± 1.64 d	86.44 ± 1.64 c	95.36± 2.36 b	82.10± 2.33 b	6.25± 1.22 bc
		2.5 mg/mL	72.13 ± 2.74 c	87.54± 1.95 c	86.45 ± 1.45 c	90.37 ± 2.74 c	77.78 ± 2.82 c	7.36± 1.33 b
		1.25 mg/ mL	79.54± 2.32 b	96.21± 2.06 b	95.34± 2.26 b	88.45± 2.33 c	84.18± 1.84 b	5.19± 1.67 bc
Blar	ık		58.81 ± 2.25	91.82± 1.53	$\begin{array}{c} 70.42 \pm \\ 1.81 \end{array}$	$\begin{array}{c} 96.70 \pm \\ 2.16 \end{array}$	$\begin{array}{c} 68.10 \pm \\ 1.47 \end{array}$	5.65 ± 1.12

3.6 Cytotoxicity Testing of ELE

To license a functional food, new material must undergo a cytotoxicity test. In this study, three typical cells (CHL, CHO, V79) were used to evaluate cytotoxicity [33]. The cell viabilities of CHL, CHO, and V79 cells under ELE treatment are listed in Table 6. The cell viability of V79 cells under ELE treatment was in a range of 65.22%–81.32%, indicating that ELE slightly induced V79 cell death. Although slight growth inhibition of ELE-treated V79 cells was determined, the inhibitory effect of ELE on CHL and CHO cells was acceptable as the cell viability of these ELE-treated cells surpassed 85%, no matter if the

concentration of ELE was low or high, suggesting that ELE did not induce significant cytotoxicity. Since checking the cytotoxicity is a prerequisite to deciding the concentrations to be tested in a genotoxicity assay and also aids in the interpretation of positive results, our results can certify the concentrations used in genotoxicity tests are acceptable based on the standards and can support the obtained negative results from each test to some extent, which co-verify that ELE has no genotoxicity.

		Cell viability (%)	
Concentration of ELE	CHL	СНО	V79
25 mg/mL	85.03 ± 2.6 c	85.31 ± 2.3 c	65.22 ± 5.2 c
12.5 mg/mL	$94.46 \pm 1.4 \text{ b}$	$96.46 \pm 1.2 \text{ b}$	$74.20\pm4.4~b$
6.25 mg/mL	99.77 ± 0.2 a	99.87 ± 0.6 a	81.32 ± 3.8 a

Table 6: The cytotoxicity test of *E. ulmoides* leaf extract (ELE). Different small letters indicate significant differences at P < 0.05 level of the tukey test under different treatments

3.7 Growth Performance of Rats under Long-Term ELE Treatment

Long-term toxicity testing is essential to evaluate the *in vivo* safety of plant extracts for serving as functional foods [34]. It can determine whether long-term administration induces tissue and organ damage, and also can determine the safe dose of administration. In the present study, no mortality in rats was observed during the entire long-term toxicity experiment. The results of ELE on the growth of rats are listed in Table 7. Rat body weight increases under ELE treatment were observed in comparison with the control, and the body weights of male rats significantly increased (P < 0.05), indicating that ELE was growth-promoting. Additionally, the heart and kidney weights of both male and female rats increased under ELE treatment, especially under high-level treatment (B group). Surprisingly, the brain weights of male rats significant variations were detected in the weights of other tissues (liver, spleen, adrenal gland, and testis) in both male and female rats among the four tested groups.

		А	В	С	D
	Body weight (g)	334.20 ± 14.94 c	$359.80 \pm 13.07 \ b$	375.80 ± 20.87 ab	390.60 ± 19.97 a
	Brain (g)	$1.49\pm0.02\ b$	1.83 ± 0.23 a	1.78 ± 0.20 a	$1.95 \pm 0.14 \ a$
	Liver (g)	$7.91\pm0.52\ b$	$7.90\pm0.47~b$	$7.96\pm0.48\ b$	$8.83 \pm 0.60 \ a$
Male	Spleen (g)	0.54 ± 0.07 a	0.61 ± 0.07 a	$0.59\pm0.06~a$	0.66 ± 0.14 a
	Heart (g)	$0.89\pm0.04\ b$	$1.03 \pm 0.05 \ a$	$0.97\pm0.03~a$	$0.99\pm0.04~a$
	Kidney (g)	$1.87\pm0.13\ b$	2.11 ± 0.17 a	$2.23\pm0.22~a$	2.31 ± 0.20 a
	Adrenal gland (g)	0.05 ± 0.01 a	$0.05\pm0.01~a$	$0.06\pm0.01~a$	$0.06 \pm 0.01 \ a$
	Testis (g)	3.52 ± 0.04 a	3.59 ± 0.17 a	3.60 ± 0.10 a	3.74 ± 0.16 a

Table 7: Effects of dietary supplementation *E. ulmoides* leaf extract (ELE) on the growth of rats. Different small letters indicate significant differences at P < 0.05 level of Tukey test under different treatments

(Continued)

Table 7 (continued)							
		А	В	С	D		
	Body weight (g)	243.80 ± 16.10 a	258.40 ± 18.97 a	260.80 ± 10.71 a	255.60 ± 12.10 a		
	Brain (g)	1.77 ± 0.11 a	$1.73 \pm 0.01 \ a$	1.76 ± 0.36 a	1.71 ± 0.16 a		
	Liver (g)	6.59 ± 0.47 a	$7.58\pm0.92~a$	6.93 ± 0.41 a	6.93 ± 0.36 a		
Female	Spleen (g)	0.50 ± 0.05 a	$0.57\pm0.09~a$	$0.59\pm0.11a$	0.53 ± 0.05 a		
	Heart (g)	$0.78\pm0.02\ b$	$0.87\pm0.04~a$	$0.87\pm0.04~a$	$0.80\pm0.02\ b$		
	Kidney (g)	$1.52\pm0.10\ b$	1.71 ± 0.08 a	1.63 ± 0.09 ab	$1.64 \pm 0.04 \text{ ab}$		
	Adrenal gland (g)	0.06 ± 0.01 a	0.05 ± 0.01 a	0.05 ± 0.01 a	0.05 ± 0.01 a		

Many studies have reported that dietary medical plant extracts promoted animal growth [35,36], such as *Piper sarmentosum* extract for weaned piglets [37], *Moringa oleifera* extract for goats [38], *Crataegus pinnatifida* extract for juvenile golden pompano [39], and *Cassia abbreviata* extract for chickens [40]. Our previous work also showed that dietary supplementation with *E. ulmoides* leaf powder enhanced the growth performance of weanling piglets [41]. Similar to weanling piglets, the body weights of rats (both male and female) under ELE treatment increased in comparison to the control group, indicating that the growth performance of rats was strongly associated with ELE. The effects of ELE on elevated animal growth performance may be ascribed to numerous active ingredients of ELE belonging to phytoestrogens, such as aucubin and wogonin [42], which can serve as estrogen receptor modulators in animals and strengthen the growth and development of animals, such as diet supplementation with ELE at a relatively high level shown to heighten the laying performance of hens [43].

3.8 Blood Index Analysis of Rats under ELE Treatment

No significant alterations in the routine blood indexes of rats (both male and female) were observed under ELE treatment when compared to the control (Table S1). An evaluation of the blood biochemistry indexes (Table 8) showed that the AST concentration in rats significantly increased under dietary supplementation with ELE when compared to the control (P < 0.05), no matter if the rats were male or female, implying that ELE influenced liver function. The blood TG and LDL-C concentrations significantly decreased and HDL-C concentrations significantly increased under high-level ELE treatment (B group), indicating that dietary addition of ELE at a relatively high level contributed to lowering the blood lipids of rats. Interestingly, the blood TC concentrations in male rats significantly decreased under high-level ELE treatment in comparison with the control, whereas the TC concentrations in female rats significantly increased, indicating a divergent mechanism of rats to cope with ELE. Additionally, four typical antioxidant indexes (SOD, GSH, MDA, CAT) and three typical inflammatory factors (TNF- α , IL- 1β , IL-6) were analyzed in both male and female rats. No significant difference in antioxidant indexes or inflammatory factors was determined among the four tested groups (Table S2), suggesting that ELE did not induce oxidative injury or an inflammatory response in rats.

Research on hyperlipidemia amelioration generally focuses on plant extracts or products that positively participate in lipid metabolism, such as *Ampelopsis grossedentata* extract and its characteristic component (dihydromyricetin) that was found to significantly lower plasma TG, TC, and LDL-C levels and increase plasma HDL-C levels in mice and hens [43–46]. In addition, evidence has shown that *E. ulmoides* can also improve blood lipid levels [4]. In this research, plasma TG and LDL-C concentrations significantly decreased and the plasma HDL-C concentration significantly increased in both male and female rats

under ELE treatments. Although a dose-response was not determined, the highest dose of ELE exerted a slightly stronger effect on HDL-C and LDL-C contents alterations if compared with the other two doses, which is in accordance with the findings in hens [43], indicating that ELE is beneficial for lowering blood lipid of rats. Previous research showed that the active ingredients of *E. ulmoides* (flavonoids and phenolics) ameliorated hypertriglyceridemia by upregulating the expression of genes related to hepatic α -, β -, and ω -oxidation [47] and thus participated in regulating blood lipid metabolism [48]. Since the complete mechanism of the lipid-decreasing effects of *E. ulmoides* is a complex network, the specific mechanisms should be further studied based on the abundant composition data of ELE.

		А	В	С	D
	ALB (g/L)	33.37 ± 1.43 a	32.92 ± 1.77 a	34.96 ± 0.39 a	35.02 ± 2.89 a
	ALP (U/L)	113.36 ± 8.09 a	117.32 ± 20.68 a	108.68 ± 12.27 a	106.80 ± 16.55 a
	ALT (U/L)	35.31 ± 5.82 a	29.96 ± 4.17 a	30.48 ± 4.35 a	31.84 ± 7.82 a
	AST (U/L)	88.34 ± 3.43 b	98.76 ± 4.38 a	99.58 ± 3.17 a	97.88 ± 2.44 a
	CREA-S (µmol/L)	41.32 ± 11.34 a	33.52 ± 3.93 a	34.38 ± 5.44 a	43.20 ± 6.64 a
Mala	Glu-G (mmol/L)	9.17 ± 1.03 a	$6.94\pm1.10\ b$	$7.17\pm0.87\ b$	$7.60\pm0.51~b$
Male	TC (mmol/L)	$2.45\pm0.28~a$	$1.97\pm0.24\ b$	$2.02\pm0.25\ ab$	$2.43\pm0.23~a$
	TG (mmol/L)	0.68 ± 0.10 a	$0.44\pm0.10\ b$	$0.43\pm0.11\ b$	$0.47\pm0.11\ b$
	LDL-C (mmol/L)	$2.57\pm0.09~a$	$2.10\pm0.10\ c$	$2.15\pm0.12\ c$	$2.43\pm0.05\ b$
	HDL-C (mmol/L)	$1.40\pm0.10\ c$	1.79 ± 0.07 a	$1.65\pm0.16\ ab$	$1.54\pm0.13~bc$
	TP (g/L)	57.20 ± 2.16 a	56.81 ± 2.22 a	58.98 ± 1.48 a	60.75 ± 4.86 a
	UREA (mmol/L)	5.95 ± 2.10 a	6.06 ± 0.43 a	6.59 ± 0.38 a	6.53 ± 0.69 a
	γ-GT (U/L)	1.06 ± 0.93 a	$-0.76 \pm 0.55 \ b$	-1.50 ± 0.65 b	-1.88 ± 1.10 b
	ALB (g/L)	37.82 ± 1.63 a	40.10 ± 4.43 a	38.20 ± 0.67 a	37.84 ± 1.89 a
	ALP (U/L)	76.31 ± 32.70 a	85.58 ± 27.82 a	57.90 ± 9.84	78.94 ± 34.64 a
	ALT (U/L)	25.60 ± 4.22 a	30.28 ± 8.69 a	28.94 ± 5.64 a	28.78 ± 6.67 a
	AST (U/L)	86.86 ± 13.22 b	102.46 ± 18.01 a	105.58 ± 10.99 a	106.58 ± 12.10 a
	CREA-S (µmol/L)	51.15 ± 12.26 ab	65.50 ± 13.13 a	$42.54 \pm 4.50 \ b$	$45.08\pm3.50\ b$
	Glu-G (mmol/L)	$7.23\pm1.39~b$	10.60 ± 1.46 a	$6.51\pm0.23~b$	$6.56 \pm 1.72 \text{ b}$
Female	TC (mmol/L)	$1.96\pm0.24~b$	2.28 ± 0.26 a	2.27 ± 0.37 a	$1.95\pm0.32~b$
	TG (mmol/L)	0.82 ± 0.11 a	$0.53\pm0.15\ b$	$0.47\pm0.10\ b$	$0.40\pm0.10\ b$
	LDL-C (mmol/L)	2.62 ± 0.09 a	$2.13\pm0.10\ b$	$2.25\pm0.12\ b$	$2.23\pm0.05\ b$
	HDL-C (mmol/L)	$1.51\pm0.10\ c$	1.89 ± 0.07 a	$1.74\pm0.04\ b$	$1.58\pm0.11\ c$
	TP (g/L)	63.32 ± 2.56 a	68.60 ± 8.01 a	64.85 ± 0.94 a	65.27 ± 4.08 a
	UREA (mmol/L)	7.38 ± 1.43 a	8.59 ± 1.43 a	7.19 ± 1.53 a	$7.25\pm0.48\ a$
	γ-GT (U/L)	1.03 ± 1.06 a	$-1.30 \pm 0.83 \ b$	-1.30 ± 0.88 b	-1.62 ± 1.65 b

Table 8: Effects of dietary supplementation *E. ulmoides* leaf extract (ELE) on the blood biochemistry indexes of rats. Different small letters indicate significant differences at P < 0.05 level of the Tukey test under different treatments

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3.9 Histopathological Analysis of Rats under ELE Treatment

Five tissues (liver, spleen, kidney, stomach, and pancreas) of rats were selected for conducting histopathological examinations (Fig. 1). Among the four groups, no significant changes or cytoplasmic vacuolation were observed. The intercellular spaces of the liver, spleen, kidney, stomach, and pancreas revealed no significant differences. The cell morphology of the liver, spleen, kidney, stomach, and pancreas under ELE treatment was regular without abnormal cellular changes when compared to the control; for example, the glomerulus was intact without distension. No inflammatory infiltration was observed in the cells of tissues under ELE treatment. Therefore, it was concluded that ELE did not cause a histopathological lesion in rats.



Figure 1: Effect of dietary supplementation *E. ulmoides* leaf extract (ELE) on the selected tissues of rats. (A–D) Representative photomicrographs of liver ($20\times$), spleen ($10\times$), kidney ($20\times$), pancreas ($10\times$), and stomach ($20\times$) among the 4 tested groups

In our study, the long-term toxicity tests showed that the growth performance of rats (both male and female) were unaffected. The routine blood indexes, the antioxidant indexes, and the inflammatory factors were consistent between the ELE treatment and the control, indicating that long-term ingestion of ELE was harmless to the body because oxidative injury and inflammatory responses were not induced. No visible difference in the liver, spleen, kidney, stomach, or pancreas was identified between the ELE treatment and the control, which indicated that ELE did not cause histopathological lesions in rats. Taken together, we can therefore conclude that ELE does not cause long-term *in vivo* toxicity.

The duodenum villus heights of rats in the A group (both male and female) were significantly lower than those of rats under ELE treatment (Fig. 2), especially in female rats (P < 0.05), indicating that ELE promoted intestinal villus growth. No significant differences in the duodenum crypt depth of rats (both male and female) were observed between the ELE treatment groups and the A group (P > 0.05). A slight downregulation in the ileum villus height and crypt depth of female rats was observed under ELE treatment in comparison with the control condition (A group), without a significant difference (P > 0.05). In addition, the ileum villus height and

crypt depth of male rats showed no difference among the four tested groups. In accordance with the alterations in villus height and crypt depth, the villus height to crypt depth ratio of the duodenum and ileum also changed. The ratios of the A group were slightly lower than the ratios of the B, C, and D groups without significant differences (P > 0.05). These results implied that ELE was beneficial to intestinal morphology.



Figure 2: Effect of dietary supplementation *E. ulmoides* leaf extract (ELE) on the selected intestinal of rats. (A–D) Representative photomicrographs of villus in the duodenum (10×) and ileum (20×) of the 4 tested groups. (E) Villus height, crypt depth, and villus height to crypt depth ratio of duodenum and ileum in the 4 tested groups. Different small letters indicate significant differences at P < 0.05 level of tukey test under different treatments

Based on intestinal histomorphology, a higher villus height, as well as a higher villus height to crypt depth ratio, contribute to nutrient absorption [49,50]. Consistently, a higher villus height together with a higher villus height to crypt depth ratio of the duodenum and ileum were found under ELE treatment in comparison with the control condition, suggesting that dietary supplementation with ELE at a relatively high level was beneficial to nutrient absorption, as determined by intestinal histology changes. Therefore, another reason the growth performance was elevated may be attributed to ELE-mediated enhanced nutrient absorption via intestinal morphological changes. Furthermore, the alterations in villus height and crypt depth of female rats were more obvious than those of male rats, suggesting that ELE functioned as a phytoestrogen in stimulating the growth performance of rats.

3.10 Antimicrobial Testing of ELE

To further investigate the bioactivities of ELE, the antimicrobial activities, anticancer capacity, and antioxidant abilities of ELE were explored. The inhibition rates of ELE against *E. coli*, *S. aureus*, *P. aeruginosa*, and *A. niger* are listed in Table 9. Based on the results, ELE exhibited good to excellent antimicrobial activities against *S. aureus* and *P. aeruginosa* as the inhibition rate against *S. aureus* reached above 80% and the inhibition rate against *P. aeruginosa* ranged from 58.93%–98.19%. A moderate inhibition rate of ELE against *E. coli* and *A. niger* was noted as the inhibition rates were in a range of 34.08%–58.30%. These results imply that ELE has certain antimicrobial activity, which is in accordance with the former report [4].

Concentration of microbial Concentration of ELE	Inhibition rate (%)				
	1×10^3 (CFU/mL	1×10^4 CFU/mL		
	25 mg/mL	5 mg/mL	25 mg/mL	5 mg/mL	
Escherichia coli	58.30 ± 2.81 a	$34.08\pm2.23~b$	44.10 ± 2.55 b	40.50 ± 2.27 a	
Staphylococcus aureus	83.60 ± 1.92 a	80.68 ± 2.47 a	84.95 ± 2.84 a	80.20 ± 2.12 a	
Pseudomonas aeruginosa	98.19 ± 1.32 a	97.91 ± 1.63 a	64.01 ± 2.92 a	$58.93 \pm 1.76 \ b$	
Aspergillus niger	50.40 ± 2.46 a	48.46 ± 2.66 a	55.67 ± 2.02 a	50.47 ± 2.35 b	

Table 9: The antimicrobial test of *E. ulmoides* leaf extract (ELE). Different small letters indicate significant differences at P < 0.05 level of the tukey test under different treatments

3.11 Potential Anticancer Capacity of ELE

The cell viability of HepG2 cells under different ELE treatments was evaluated (Fig. 3). As the concentration of ELE increased, the viability of HepG2 cells decreased, which was in agreement with the effects of ELE on V79 cells. Besides, the IC50 of ELE against HepG2 was 3.98 mg/L, which was significantly lower than the IC50 of cisplatin (12.52 mg/L). Although the experimental design was the same as in cytotoxicity assays and the treatment time was only 12 h, a down-regulation in cells viability of HepG2 was observed along with ELE concentration increasing, suggesting ELE had the potential in inhibiting HepG2 cells proliferation based on the numerous active components.

3.12 Free Radical Scavenging Rate of ELE

Four typical free radicals (·OH, DPPH·, PTIO·, ABTS·) were chosen to assess the *in vitro* free radical scavenging rate of ELE (Fig. 4). Although scavenging rates (%) of ELE against the selected free radicals were lower than that of vitamin C, the free radical scavenging rate of ELE against ·OH, DPPH·, PTIO·, and ABTS· all increased as the ELE concentration increased, indicating that ELE at a relatively high

concentration was beneficial for free radical scavenging. Due to flavonoids of *E. ulmoides* leaves (e.g., CGA, rutin, ferulic acid, and caffeic acid) possessing good antioxidant activity [15], our results certified the ELE could also reveal preferable antioxidant activities, which had benefits in anti-aging [23].



Figure 3: The cell viability of HepG-2 cells under different *E. ulmoides* leaf extract (ELE) treatment. DDP represents cisplatin



Figure 4: The free radical scavenging rates of E. ulmoides leaf extract (ELE). VC represents vitamin C

Because several types of active compounds were identified in *E. ulmoides* (either the bark or leaves) [5], *E. ulmoides* has been found to exhibit multiple pharmacological activities [4,51]. Numerous studies have shown that CGA, the most important characteristic component of *E. ulmoides* leaves, protected mice against Cd-induced hepatorenal injury [52], and the other characteristic components with high levels in *E. ulmoides* leaves, such as geniposidic acid (GPA) and aucubin (AU), caused free radical scavenging and

anticancer effects [53-55]. Therefore, these results are corroborative evidence showing that ELE has significant prospects for antimicrobial, anticancer, and antioxidant uses, based on the active ingredients. Meanwhile, our previous studies had proved *E. ulmoides* leaf extract revealed higher antihypertension effect than bark extract, and could enhance the body health and production of hens [18,43]. Together with our previous research showing that GPA and AU have tremendous potential for their anti-aging effects [23], ELE also shows outstanding potential in traditional Chinese health food fields, such as food, medicine, feeding, and daily chemical product. Many types of products derived from ELE have emerged, such as tea [56], beverages [57], feed additives [43], and healthy food [58]. Our work can facilitate *E. ulmoides* leaf inclusion in the directory of traditional Chinese health food as a new resource and can ensure the export quality of ELE.

4 Conclusion

In the present study, a comprehensive assessment of the safety of *E. ulmoides* leaf extract was conducted through a combination of genotoxicity tests, cytotoxicity assay, and *in vivo* long-term animal toxicity analysis. The conclusions from the preliminary data and discussion were as follows:

- 1. The reverse mutation number of nominal bacterial strains, the micronucleus rates of *in vivo* mammalian erythrocyte and *in vitro* mammalian cells, and the TFT resistance mutation frequency of the TK gene under ELE treatment were less than the rated fold change of revealing genotoxicity in comparison with the solvent control, indicating that ELE had no genotoxicity.
- 2. The growth performance of rats under ELE treatment was enhanced and no oxidative injury, inflammatory response, as well as histopathological lesions of tissues were detected between ELE-treated and control rats, indicating that ELE also had no *in vivo* long-term toxicity.
- 3. The blood lipid levels were ameliorated under ELE treatment, suggesting that ELE could potentially improve body health.
- 4. Good to excellent antimicrobial, anticancer, and antioxidant activities of ELE were determined, implying that ELE possessed preferable bioactivities.

These results could deepen our understanding of ELE as a traditional Chinese health food in addition to supporting the use of ELE in actual applications, such as the food, drug, and cosmetics industries.

Acknowledgement: This study was supported by the National Natural Science Foundation of China (Grant No. 42107020) and the Scientific Research Start Funds, Hunan Institute of Technology (No. HQ20014). We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

Funding Statement: The authors received no specific funding for this study.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix



Figure S1: Chromatograms of standards. (A) and characteristic components in *E. ulmoides* leaf extract (ELE) (B) using HPLC system.

Table S1: Effects of dietary supplementation E. ulmoides leaf extract (ELE) on the blood routine inde	xes
of rats. Different small letters indicate significant differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at $P < 0.05$ level of Tukey test under differences at	ent
treatments	

		А	В	С	D
	WBC (10 ⁹ /L)	$4.88 \pm 1.48a$	$4.60 \pm 1.68a$	$4.82 \pm 1.11a$	$5.46 \pm 1.51a$
	Neu (10 ⁹ /L)	$0.65\pm0.17b$	$1.10\pm0.20a$	$0.67\pm0.15b$	$0.61\pm0.09b$
	Lym (10 ⁹ /L)	$3.38 \pm 1.60a$	$3.22\pm1.17a$	$3.88\pm0.97a$	$4.55 \pm 1.48a$
	Mon (10 ⁹ /L)	$0.24\pm0.12a$	$0.25\pm0.12a$	$0.25\pm0.12a$	$0.27\pm0.04a$
	Eos (10 ⁹ /L)	$0.03\pm0.02a$	$0.03\pm0.03a$	$0.02\pm0.01a$	$0.02\pm0.02a$
	Bas (10 ⁹ /L)	$0.01\pm0.01a$	$0.01\pm0.01a$	$0.01\pm0.01a$	$0.01\pm0.01a$
	Neu (%)	$17.13\pm6.43ab$	$23.98\pm3.48a$	$14.02\pm4.77b$	$11.78\pm2.83b$
	Lym (%)	$76.22\pm6.92ab$	$70.00\pm 2.74b$	$80.32\pm6.72a$	$82.26\pm5.23a$
	Mon (%)	$5.60\pm0.57a$	$5.12 \pm 1.16a$	$5.18\pm2.12a$	$5.24\pm2.29a$
	Eos (%)	$0.88\pm0.59a$	$0.72\pm0.50a$	$0.46\pm0.32a$	$0.42\pm0.31a$
	Bas (%)	$0.17\pm0.11a$	$0.18\pm0.22a$	$0.02\pm0.04b$	$0.30\pm0.16a$
Male	RBC (10 ¹² /L)	$9.15\pm0.66a$	$9.52\pm0.17a$	$9.45\pm0.41a$	$9.36\pm0.26a$
	HGB (g/L)	$163.09\pm8.73a$	$173.40 \pm 6.31a$	$170.80\pm5.97a$	$167.80 \pm 4.15a$
	HCT (%)	$46.70\pm2.55a$	$47.72\pm1.38a$	$48.96 \pm 1.22a$	$47.74 \pm 1.41a$
	MCV (fL)	$51.10 \pm 1.40a$	$50.10\pm0.99a$	$51.84 \pm 1.05a$	$51.02\pm0.94a$
	MCH (pg)	$17.85\pm0.51a$	$18.20\pm0.44a$	$18.08\pm0.49a$	$17.92\pm0.25a$
	MCHC (g/L)	$349.55\pm5.16b$	$362.80\pm7.66a$	$348.60\pm5.27b$	$351.40\pm3.78b$
	RDW-CV (%)	$12.70\pm0.35a$	$12.78\pm0.13a$	$12.58\pm0.18a$	$12.72\pm0.33a$
	RDW-SD (fL)	$26.35\pm0.39a$	$26.82\pm0.53a$	$26.66\pm0.44a$	$26.50\pm0.42a$
	PLT (10 ⁹ /L)	$894.18 \pm 115.78a$	$1052.20\pm58.90a$	$1018.00\pm94.14a$	$946.40 \pm 73.62a$
	MPV (fL)	$6.22\pm0.33a$	$6.40\pm0.29a$	$6.50\pm0.29a$	$6.66\pm0.26a$
	PDW (%)	$15.30\pm0.14a$	$15.34\pm0.09a$	$15.14\pm0.11a$	$15.20\pm0.14a$
	PCT (%)	$0.56\pm0.09a$	$0.67\pm0.03a$	$0.66\pm0.04a$	$0.63\pm0.06a$
	WBC (10 ⁹ /L)	$4.73 \pm 1.19a$	$4.23\pm0.62a$	$5.29 \pm 1.40a$	$5.75\pm2.59a$
	Neu (10 ⁹ /L)	$0.55\pm0.16a$	$0.67\pm0.08a$	$0.52\pm0.20a$	$0.94\pm0.56a$
	Lym (10 ⁹ /L)	$3.86 \pm 1.02a$	$2.86 \pm 1.16a$	$4.47 \pm 1.08a$	$3.21\pm2.06a$
	Mon (10 ⁹ /L)	$0.28\pm0.07a$	$0.21\pm0.10a$	$0.27\pm0.12a$	$0.27\pm0.19a$
	Eos (10 ⁹ /L)	$0.04\pm0.02a$	$0.02\pm0.02a$	$0.02\pm0.02a$	$0.03\pm0.04a$
Female	Bas (10 ⁹ /L)	$0.01\pm0.01a$	$0.01\pm0.01a$	$0.01\pm0.01a$	$0.01\pm0.02a$
	Neu (%)	$11.50 \pm 1.94 ab$	$16.82 \pm 4.11a$	$9.56 \pm 1.38b$	$13.36\pm3.85ab$
	Lym (%)	$81.28\pm2.63ab$	$75.98\pm6.46b$	$84.94 \pm 2.26a$	$79.02\pm3.58ab$
	Mon (%)	$6.07 \pm 1.64a$	$6.32\pm1.54a$	$4.98\pm0.84b$	$6.68 \pm 1.84a$
	Eos (%)	$0.81\pm0.41a$	$0.54\pm0.26a$	$0.42\pm0.37a$	$0.62\pm0.41a$
	Bas (%)	$0.34\pm0.13a$	$0.34\pm0.41a$	$0.30\pm0.07a$	$0.32 \pm 0.16a$

(Continued)

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Table S1 (continued)				
	А	В	С	D
RBC (10 ¹² /L)	$8.46\pm0.33a$	$8.24\pm0.60a$	$8.48\pm0.38a$	$8.61\pm0.54a$
HGB (g/L)	$157.90 \pm 5.76a$	$156.80 \pm 8.14a$	$155.80\pm3.77a$	$158.80\pm8.35a$
HCT (%)	$44.46 \pm 1.68a$	$44.16\pm2.52a$	$44.74\pm0.95a$	$45.72\pm2.86a$
MCV (fL)	$52.55\pm0.91a$	$53.64 \pm 1.70a$	$52.78 \pm 1.65a$	$53.10\pm0.60a$
MCH (pg)	$18.67\pm0.33a$	$19.06\pm0.71a$	$18.38\pm0.64a$	$18.44\pm0.21a$
MCHC (g/L)	$355.40\pm3.78a$	$355.20\pm2.86a$	$348.20\pm1.92b$	$347.20\pm5.63ab$
RDW-CV (%)	$12.13\pm0.39a$	$12.20\pm0.07a$	$12.08\pm0.45a$	$12.28\pm0.43a$
RDW-SD (fL)	$26.05\pm0.81a$	$26.72\pm0.68a$	$26.18 \pm 1.28a$	$26.64\pm0.89a$
PLT (10 ⁹ /L)	$1080.90 \pm 143.44a$	$968.00\pm42.08a$	$1082.40 \pm 135.73a$	$1114.20 \pm 98.58a$
MPV (fL)	$6.37\pm0.17a$	$6.66\pm0.17a$	$6.30\pm0.17a$	$6.46\pm0.29a$
PDW (%)	$15.27\pm0.18a$	$15.32\pm0.13a$	$15.10\pm0.19a$	$15.18\pm0.08a$
PCT (%)	$0.69\pm0.09a$	$0.64\pm0.01a$	$0.68\pm0.09a$	$0.72\pm0.07a$

Table S2: Effects of dietary supplementation *E. ulmoides* leaf extract (ELE) on the antioxidant indexes and inflammatory factors of rats. Different small letters indicate significant differences at P < 0.05 level of Tukey test under different treatments

			А	В	С	D
Male	Antioxidant index	SOD (U/ml)	$107.31 \pm 17.70a$	113.77±17.16a	$121.81 \pm 31.57a$	$111.81\pm32.02a$
		GSH (umol/L)	$1.95\pm0.32a$	$2.07\pm0.31a$	$2.21\pm0.57a$	$2.03\pm0.58a$
		MDA (nmol/L)	$8.43 \pm 1.56a$	$7.89 \pm 1.26a$	$7.60 \pm 1.87a$	$8.34\pm2.10a$
		CAT (U/ml)	$15.61 \pm 2.57a$	$16.55\pm2.50a$	$17.72\pm4.59a$	$16.26 \pm 4.66a$
	Inflammatory factor	TNF- α (ng/L)	$378.84\pm36.92a$	$379.84\pm32.47a$	$388.47 \pm 39.01 a$	$397.25\pm17.78a$
		IL-1 β (ng/L)	$42.04\pm3.85a$	$41.59\pm2.37a$	$43.54\pm2.37a$	$43.83\pm5.00a$
		IL-6 (pg/ml)	$122.95\pm10.75a$	$128.95\pm7.32a$	$120.21\pm8.62a$	$114.03\pm6.17a$
Female	Antioxidant index Inflammatory factor	SOD (U/ml)	$150.83\pm40.62a$	$108.42\pm25.05a$	$128.12 \pm 21.33a$	$124.01\pm27.19a$
		GSH (umol/L)	$2.74\pm0.74a$	$1.97\pm0.46a$	$2.33\pm0.39a$	$2.25\pm0.49a$
		MDA (nmol/L)	$6.19 \pm 1.56a$	$8.51\pm2.20a$	$7.03 \pm 1.21 a$	$7.36 \pm 1.59a$
		CAT (U/ml)	$21.94\pm5.91a$	$15.77\pm3.64a$	$18.64\pm3.10a$	$18.04\pm3.96a$
		TNF- α (ng/L)	$385.96 \pm 26.09a$	$406.50 \pm 27.83 a$	$386.29\pm28.64a$	$377.87 \pm 21.99a$
		IL-1 β (ng/L)	$41.95\pm3.24a$	$41.80\pm3.65a$	$43.59 \pm 2.96a$	$40.71 \pm 1.06a$
		IL-6 (pg/ml)	$119.73\pm10.61a$	$115.07\pm7.68a$	$119.66 \pm 7.50a$	$132.09\pm5.95a$



DOI: 10.32604/jrm.2023.027441

ARTICLE





In Situ Generation of Copper Nanoparticles in Heat-Treated Copper-Containing Masson's Pine as a Preservative Process for Sawn Timber

Minting Lai, Guijun Xie^{*}, Wanju Li, Lamei Li and Yongjian Cao

Guangdong Provincial Key Laboratory of Silviculture, Protection and Utilization, Guangdong Academy of Forestry, Guangzhou, 510520, China

*Corresponding Author: Guijun Xie. Email: xgj80@126.com Received: 30 October 2022 Accepted: 30 November 2022

ABSTRACT

Heat-treated wood has good dimensional stability, durability, and color, but its susceptibility to fungal growth affects its commercial value. In this study, lumber harvested from mature Masson's pine (*Pinus massoniana* Lamb.) was vacuum impregnated with a basic copper salt solution (copper hydroxide, diethanolamine, and poly-ethylene glycol 200) prior to heat-treatment at 220°C for 3 h. Antifungal properties, surface chemistry, crystal structure and sugar contents were tested, compared with heat treatment alone. The results showed that the samples treated by heating without copper salt treatment showed poor suppression of fungal growth, the copper-impregnated heat-treated wood suppressed (100%) the growth of *Botryodiplodia theobromae* Pat., *Aspergillus niger* V. Tiegh., *Penicillium citrinum* Thom, and *Trichoderma viride* Pers. The combined results of X-ray photo-electron spectroscopy, X-ray diffraction and sugars analysis suggested that fungal inhibition by the heat-treated copper-bearing Masson's pine was mainly due to the reduction of the metal salt by PEG200 at high temperature to generate copper nanoparticles. In addition, the reduced sugar content of the treated timber, and hence the nutrient substrate for spoilage microbes, reduced in the presence of the metal salts at high-temperature. This study has demonstrated an effective method of increasing low-grade wood's utility and commercial value.

KEYWORDS

Heat treatment; Masson's pine; sawn timber; inhibition of fungal growth; copper nanoparticles

1 Introduction

Wood has good strength-to-weight and visual characteristics. Consequently, it is an important and widely used renewable resource. However, because wood is vulnerable to biological invasion and physical defects such as cracking and deformation, it is usually heat treated to improve its utility. High-temperature heat treatment is an attractive process that can improve the dimensional stability, durability, and color of wood [1-6]. Consequently, heat-treated wood products are popular materials and widely used in various settings such as decorative wallboard. Although heat-treated wood is generally considered to have good preservative properties, it can be susceptible to fungal growth [7,8].

The specialized extendible cells of fast-growing bamboo are expanded with sugar and water during growth, and the high nutrient content of its tissues promotes insect damage and microbial spoilage [9]. Because high temperatures can accelerate the modification of wood components, heat treatment can



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influence changes in the fungal resistance properties of timber. Kamdem et al. [6] analyzed the decomposition resistance of heat-treated wood using improved soil block or agar block test methods and concluded that the decomposition resistance of heat-treated wood required improvement. Sivonen et al. [10] studied the spoilage of heat-treated pine (*Pinus spp.*) by brown rot fungus (*Coriolus versicolor*) and proposed that timber treated at a temperature above 220°C could attain high resistance to fungi. Zhu et al. [11] followed changes in the sugar contents of heat-treated wood from *Pinus spp.*, *Fraxinus mandshurica* and *Betula spp.* at 200°C, and found that the heat-treated wood was more prone to spoilage than the untreated control wood: When wood from *P. sylvestris* and *Betula spp.* was heat-treated at 180°C and 200°C, respectively, their sugar contents increased, and their contents in the surface layer were higher than that of core layer. In another study, heat treatment of timber from *P. sylvestris* var. *mongolica* and *Quercus mongolica* Fisch. At 185°C and 205°C respectively for 1.5 h, inhibited the damage caused by cyanobacteria, but did not inhibit or reduce surface fungal growth [7].

Nanomaterials impregnated wood can obtain better properties [12]. Photocatalytic nano-TiO₂ can partially inhibit *Aspergillus niger*, reduce the growth of harmful mold, and better protect wood-based artifacts [13]. However, *Pinus nigra* L. was treated by vacuum impregnation with nano zinc oxide, zinc borate and copper oxide, and the mildew test was carried out on three kinds of molds, namely *Aspergillus niger*, *Penicillium chrysogenum* and *Trichoderma viride*. It was found that nano zinc borate had a low control effect on mildew, and its infection value was 4, while the others had no control effect on mildew [14].

More recently, researchers have explored green methods by combining thermal modification with mineralization to improve the physico-mechanical properties and microbial resistance of wood. For example, wood impregnated with a silver nano-suspension (400 ppm) and heat-treated between 145°C and 165°C showed improvements in its physico-mechanical properties [15]. Xie et al. [16] impregnated wood samples with a copper-containing solution at high pressure, and the subsequent high-temperature treatment produced copper nanoparticles which have good antifungal properties.

Due to practical limitations, small sections of wood are often used in the research environment. However, wood is a naturally variable material, and studies of small specimens may not accurately reflect the behavior of the full-size material.

In this study, lumber from Masson's pine was impregnated with a basic copper salt solution and subjected to high-temperature treatment. Surface sections of the treated timber were tested to determine their antifungal properties, chemistry and crystal structure, and sugar contents using conventional methods. Large-size timber was chosen for the investigation to reduce the time between experimental research and production practice. Superior quality anti-mildew heat-treated wood can be obtained directly by cutting rough wood.

2 Materials and Methods

2.1 Materials

Wood was harvested from *P. massoniana* Lamb. (average diameter at breast height, 24 cm; average height, 16 m; age, 25 years) at the Xinyi Forestry Research Institute (Guangdong Province, China), and was without decay or microbial growth.

3,5-dinitrosalicylic acid (DNS), copper hydroxide, diethanolamine, glucose, polyethylene glycol 200 (PEG200), phenol, potassium sodium tartrate ($C_4H_4O_6KNa\cdot 4H_2O$), sodium hydroxide, sodium metabisulfite ($Na_2S_2O_5$), were from Guangzhou, China. Arabinose, fucose, galactose, glucose, mannose, rhamnose, and xylose were purchased from Hubei Weishi Chemical Reagent Co., Ltd., China.

2.2 Preparation of Wood and Impregnation Solution

The timber was processed into six sawn sections measuring 600 mm \times 220 mm \times 30 mm shown in Fig. 1. The sections taken for analysis post-treatment are indicated by S1–S3 and CS1–CS3, which represent the different treatments applied (Table 1).

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Figure 1: Dimensions of the lumber obtained from *P. massoniana* showing the sampling sections S1–S3 and CS1–CS3 (designating different treatments) taken for analysis post-treatment

Table 1: Experimental processing scheme for the heat-treated Masson's pine lumber (CS is non-impregnated control, S is the impregnated samples, 1–3 denotes the 5 mm sections taken for analysis)

Comm10	Process				
Sample	[CuG] (%)	[CuG] (%) θ heat treatment (°C)			
CS1	-	220	3		
CS2	-	220	3		
CS3	-	220	3		
S 1	6.35	220	3		
S2	6.35	220	3		
S3	6.35	220	3		

The impregnation solution was composed of copper hydroxide, diethanolamine, polyethylene glycol 200 (PEG200) and water: Diethanolamine and copper hydroxide were mixed in a molar ratio of 2:1, and water was added with stirring until copper ammonia solution was formed, and then PEG200 is added to give the copper-containing impregnation solution (CuG).

2.3 Vacuum Impregnation

Freshly sawn timber samples were dried at 60° C to constant weight using a DHG-9051 kiln (Shanghai Bluepard Instruments Co., Ltd., China). Dried timber was then immersed in the CuG solution contained in a vacuum dipping tank (Self-Assembled Equipment, China) maintained at -0.09 MPa for 30 min, followed by 1.5 MPa for 40 min. Following treatment, the timber was dried to a constant weight at 60° C in the kiln.

2.4 Heat Treatment

Vacuum impregnated timber was heat treated in a customized (Jiangxi Meilong Wood Protection Co., Ltd., China) vacuum steam chamber under the following conditions: Steam treatment at 100 kPa for 20 min; thermal (electric) heat treatment at 220°C for 3 h; controlled cooling to 140°C under steam.

2.5 Processing Sequence for Masson's Pine Lumber

The sequence of processing treatments for the Masson's pine lumber is given in Table 1.

2.6 Fungal Inhibition Test

The fungal resistance of the treated samples was determined by the method given in Chinese standard GB/T 18261–2013 using *Botryodiplodia theobromae* Pat., *Aspergillus niger* V. Tiegh., *Penicillium citrinum* Thom and *Trichoderma viride* Pers., which were all purchased from the Chinese General Microbiological Culture Collection Center (Beijing, China). The test was repeated six times for each set of samples. Briefly, culture medium (2% maltose and 1.5% agar) was inoculated with the chosen fungal strain and cultivated at 28°C and 85% relative humidity for 7 d. Two sterilized glass rods were placed the culture medium, followed by the sterilized wood samples to be tested. After one month, the discoloration grade (0–4) of the wood sample was recorded: 0, no mold growth; 1, mold area <25%; 2, mold area 25%–50%; 3, mold area between 50%–75%; 4, mold area >75%. The efficacy of fungal growth control (E) was calculated from Eq. (1).

$$E = \left(1 - \frac{D_1}{D_0}\right) \times 100\% \tag{1}$$

where D_1 is the average discoloration grade, and D_0 is the average discoloration grade of the control sample.

2.7 Scanning Electronic Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS)

The heat-treated copper-bearing wood samples were ground into powders that were subsequently analyzed using SEM (Zeiss SUPRA 40, Oberkochen, Germany) and EDS (ZEISS SUPRA 40).

2.8 X-Ray Photoelectron Spectroscopy (XPS)

Two samples from each treatment group were selected at random, and reduced to a fine powder using a 425 and a 250 µm sieve, and a 20 mg aliquot was analyzed using an Escalab 250XI Instrument (Thermo Fisher Scientific, USA).

2.9 X-Ray Diffraction Analysis (XRD)

The crystal form and particle size of the metal species were characterized using powdered samples (100 mg, see above) on a D8 diffractometer (Bruker, USA).

2.10 Analysis of Sugars

2.10.1 Sample Extraction

Samples of powdered wood (1.5 g) were combined with distilled water (30 mL) in a hydrolysis bottle, shaken at 50°C for 0.5 h, quickly filtered, and the filtrate (2 mL) taken for the analysis of total reducing sugars and total sugars.

2.10.2 Total Reducing Sugars

DNS (7.5 g) and sodium hydroxide (14.0 g) were dissolved in hot water (1000 mL; added 10 min after boiling) and allowed to cool. Potassium sodium tartrate (216.0 g, phenol (5.5 mL, melted in a water bath at 50° C) and sodium metabisulfite (6.0 g) were then added, and the solution was stored in an amber glass bottle and used within 5 d.

Glucose reference standard was dried at 105° C for 3 h and used to prepare a stock standard solution (100 mL of 1 g L⁻¹) in distilled water. Glucose calibration standards were prepared over 0.2–0.02 mg mL⁻¹ from aliquots of the stock standard solution according to the dilutions given in Table 2.

To each calibration standard or sample extract (2 mL), contained in 25 test tubes, DNS (1.5 mL) was added, shaken to mix, and incubated in boiling water for 5 min. The absorbance of the cooling solutions was recorded at 520 nm. The amounts of reducing sugars in the samples were obtained from the absorbance/glucose mass standard curve.
Target concentration (g L ⁻¹)	Dilution factor	Volume of stock standard (mL)	Added water (mL)
0.20	5.00	1.0	4.00
0.18	5.55	1.0	4.55
0.16	6.25	1.0	5.25
0.14	7.14	1.0	6.14
0.12	8.33	1.0	7.33
0.10	10.00	1.0	9.00
0.08	12.50	1.0	11.50
0.06	16.66	0.5	7.83
0.04	25.00	0.5	12.00
0.02	50.00	0.2	9.80

Table 2: Glucose calibration standards: Stock standard solution (1 mg L^{-1}) dilutions

2.10.3 Total Sugars

A glucose stock standard solution (2 mg L^{-1}), prepared from the dried reference material above, was used to prepare calibration standards over 0.1–0.02 mg L^{-1} according to the dilutions given in Table 3.

Target concentration (g L^{-1})	Dilution factor	Volume of stock standard (mL)	Added water (mL)
0.20	10.00	0.50	4.50
0.18	11.11	0.50	5.05
0.16	12.5	0.50	5.75
0.14	14.29	0.50	6.64
0.12	16.67	0.50	7.83
0.10	20.00	0.40	7.90
0.08	25.00	0.30	7.20
0.06	33.33	0.20	6.47
0.04	50.00	0.10	4.90
0.02	100.00	0.09	8.91

Table 3: Total sugars glucose calibration standards: Stock standard solution (2 mg L^{-1}) dilutions

To each calibration standard or sample solution (2 mL), contained in 25 mL glass tubes, sulfuric acid (5.0 mL) was added dropwise, shaken to mix, and incubated at 50°C for 30 min. The absorbance of cooling solutions was recorded at 490 nm, and the total sugar contents of the samples were obtained from the absorbance/glucose mass standard curve. Distilled water was used as the blank control.

2.10.4 Analysis of Individual Reducing Sugars by LC-MS

A mixed stock standard solution of each monosaccharide (rhamnose, fucose, xylose, arabinose, glucose, mannose, galactose), prepared at 50 mg mL⁻¹ in deionized water (4 mL), was serially diluted to given calibrations standards over the range 5–500 ppm; solutions were passed through a 0.22 μ m membrane filter prior to LC-MS.

Powdered wood extracts (2 mL) were vacuum dried at 50°C to near dryness, reconstituted with deionized water (1 mL), and passed through a membrane syringe filter (0.22 um) prior to analysis by LC-MS.

Calibration standards and sample extracts were analyzed with a LCMS8040 LC/MS system (Shimadzhu, Tokyo, Japan). Sugars in the injection solutions (2 μ L) were separated on an InertSustain NH2 column (5 um, 4.6 mm × 250 mm; GL Sciences (Shanghai) Ltd., China) maintained at 35°C: The analytical column was protected by an InertSustain NH2 guard column (5 um, 4.0 mm × 10 mm); the mobile phase was 25% water 75% acetonitrile using isocratic elution at 1.1 ml min⁻¹. Eluting sugars were ionized in the ESI source operating at 3.5 KV, and atomizing and drying gas flow rates of 3 and 15 L min⁻¹, respectively. The desolvation line and heating module temperatures were 250°C and 400°C. Monosaccharide precursor to product ion transitions was recorded in the negative ion multiple reaction monitoring (MRM) mode at a collision energy of 35 eV.

3 Results and Discussion

3.1 Antifungal Properties of Heat-Treated Masson's Pine

The control materials (CK, no treatment), and heat-treated timber with copper (S1–S3) and without copper (CS1–CS3) exhibited different inhibitory effects towards each fungal species (Fig. 2). As expected, CK prepared with each treatment group exhibited no inhibition, thereby confirming the validity of the test results (Figs. 2A, 2H).



Figure 2: Inhibition of fungal growth by treated Masson's pine lumber (A). Photographs that are some examples show inhibitory zone of the prepared wood against *Aspergillus niger* V. Tiegh. and *Trichoderma viride* Pers. for four weeks. In which CS1 (B), CS2 (C), CS3 (D), S1 (E), S2 (F), S3 (G), CK (H)

Sections of the heat-treated lumber (CS1–CS3) showed some inhibition towards *Botryodiplodia theobromae* Pat. (8.25%) and *Penicillium citrinum* Thom (12.55%) only, while CS2 and CS3 had no inhibition activity. In contrast, all surface sections of the heat-treated copper-bearing Masson's pine (S1–S3) exhibited good inhibition properties: 67%–100% for S1; 100% for S2; and 92%–100% for S3: the lowest antifungal activity was observed for S1 towards *Trichoderma viride* Pers. (67%).

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3.2 SEM, EDS and Surface Chemistry (XPS)

Fig. 3 shows the SEM and EDS test results of heat-treated copper-containing Masson's pine lumber (S) and untreated Masson's pine control wood (CK). On the whole, the inner surface of CK is relatively smooth. After heat treatment, the inner surface of Cu-impregnated Masson's pine wood was rough with many small cracks by SEM photos, and copper element was observed on the inner surface by EDS photos. Among them, EDS shows that S2 has the strongest copper signal, which is as well as the work reported by Aguayo et al. [12], corresponding to the best mildew resistance in Section 3.1, which supports that copper in wood can inhibit the growth of mold.



Figure 3: SEM and EDS results for heat-treated wood samples dipped in solutions of copper. S is the impregnated samples, 1–3 denotes the 5 mm sections taken for analysis. CK is the samples with no treatment

The C1 S peaks of wood fiber can usually be deconvoluted into four peaks (C1, C2, C3 and C4; Table 4): C1 can be assigned to lignin and surface contamination; C2 is largely due to carbon atoms in cellulose; C3 mainly arises from oxidation products containing ketone and aldehyde groups, or from the cellulose molecules within lignin; C4 is due to the carbonyl group attached to a heteroatom [17,18].

Peak	Binding energy (eV)	Symbol	Assignment
C1s	285 ± 0.4	C1	С–С, С–Н
	286.5 ± 0.4	C2	С–ОН, С–О–С
	288.0 ± 0.4	C3	С=О, О–С–О
	289.0 ± 0.4	C4	O-C=O
Ols	533.0 ± 0.4	01	С–О, С–О–С, О–С=О, С=О

Table 4: Peak assignments for XPX C1s and O1s in wood [17]

Fig. 4 shows the high-resolution XPS C1s obtained from the heat-treated copper-bearing lumber. The absence of C4 differed from literature reports [17–19]: The peak at 285.0 eV agreed with reported values for the binding energy of C1 (284.6–285.0 eV) [19,20], and was attributed to the C–C bonding; 286.5 eV was assigned to C2, and may be due to C–O bonds [21]; 288.0 eV agreed with C3, and a C=O structure was inferred. A peak due to C4 was not observed, indicating that its content was very small [22].

The XPS spectra of Cu 2p from the heat-treated copper-bearing samples (S1–S3) are shown in Fig. 4B: The peaks between 932.8 and 952.3 eV, were assigned to Cu0; while the peak between 934.2 and 954.4 eV agreed with Cu⁺. This suggested that divalent copper ions undergo bond cleavage or reduction reactions during heating and are reduced to Cu0 and Cu⁺. Because the heat-treatment conditions for the lumber (220°C, 3 h) were below the thermal decomposition properties of the copper salt (Cu(OH)₂), this indicated that high-temperature bond cleavage was unlikely. However, residual PEG200 (from the CuG) can initiate the reduction of Cu²⁺ in wood, generating Cu0 and some Cu⁺ [23,24].

The XPS N1S spectra suggested the presence of amino groups arising from the wood and introduced in the pretreatment process (Fig. 3C). The single peak in the O1S spectra implied that there is only one O state in the heat-treated copper-containing Masson's lumber (Fig. 4D).

Table 5 gives the surface chemical composition of the untreated control and treated samples measured by XPS. The observed decrease in the O/C ratios for CS1–CS3 agreed with a decrease in the carbohydrate content with increasing surface depth in the lumber [25], probably due to their migration to the surface with the movement of water during heating [26].

The relative C1s peak intensities of the untreated control and treated Masson's pine lumber are given in Table 6. Compared with CK, the intensity of the C1s peaks in S1 showed the following trend: C1 decreased from 55.8% to 39.0%; C2 increased from 32.2% to 56.5%; C3 decreased from 6.5% to 4.6%, and C4 disappeared. The decrease in C1 and simultaneous increase in C2 peak intensities implied an increase in OH groups in the surface layer, which would facilitate the adsorption of water vapor from the air. The antifungal effects of heat-treated copper-bearing Masson's pine were much higher than that of CK, which could be attributed to the in situ Cu0 and Cu⁺. The intensity of the C1S peaks showed the following trends with increasing surface depth (S1–S3): C1 decreased and increased: C2 and C3 both increased and decreased. Since the sawn section of S2 exposes more cellulose to the atmosphere, the increased oxygen functionality also increases the wettability of the material. However, increased wettability enhances the antifungal properties of the copper nanoparticles [27].



Figure 4: High resolution XPS spectra of copper-containing heat-treated wood. (A) C1s spectra, subpeak separated by deconvolution. (B) Cu spectra. (C) N spectra. (D) O1s spectra

C		Surface composition (atom %)					
Samples	С	0	O/C	Cu			
СК	53.71	46.29	0.86	/			
CS1	44.02	55.98	1.27	/			
CS2	48.07	51.93	1.08	/			
CS3	50.34	49.66	0.99	/			
S1	44.28	50.94	1.15	4.78			
S2	40.92	54.69	1.34	4.39			
S3	45.59	50.19	1.10	4.22			

Table 5: Surface chemical composition of the untreated control and treated samples measured by XPS

Comula	Relative C1s peak intensities (%)						
Sample	C1 (285 eV)	C2 (286.5 eV)	C3 (288 eV)	C4 (289 eV)			
CK	55.8	32.2	6.5	5.5			
CS1	46.4	49.1	4.5	-			
CS2	50.8	44.8	4.4	-			
CS3	57.2	39.1	3.7	-			
S 1	39.0	56.5	4.6	-			
S2	30.5	64.1	5.4	-			
S3	37.0	58.3	4.7	-			

Table 6: Relative C1s peak intensities, calculated from the XPS spectra of untreated control and treated Masson's pine lumber

3.3 Crystalline Structure (XRD)

Fig. 5 shows the XRD patterns obtained from the analysis of heat-treated and heat-treated coppercontaining Masson's pine lumber. The absence and presence of peaks due to Cu crystals in samples CS1– CS3 and S1–S3 respectively agreed with the XPS analysis (Table 5). Cu0 and Cu1 (Cu₂O), identified by high-resolution XPS, can both form crystal structures. However, the peaks at 43.29°, 50.51° and 74.15° correspond to the (111), (200), and (220) planes of CuO, which conformed to PDF#065-9026 for copper and a centered cubic crystalline structure. However, Cu₂O did not appear to form crystal structures in the treated wood, or it formed too few to be observed. The results differed from a similar investigation that involved the hydrothermal carbonization of wood containing zinc ions, which showed no change in the oxidation state of the metal [28]. In this study, the presence of residual PEG200 induced the reduction of Cu²⁺ to Cu⁺ and Cu0 during hydrothermal carbonization [29], where the average particle size of the crystalline copper particles was 100 nm [30].



Figure 5: XRD patterns obtained from the heat-treated, and heat-treated copper-containing lumber

3.4 Sugar Profiles

There is a correlation between the sugar content of wood and the mildew-prone property of wood. The mechanism of wood mildew can be inferred by studying the changes in the sugar content of wood after heat treatment [31].

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Fig. 6 shows the total sugar and total reducing sugar contents in the untreated and treated Masson's pine samples. Total sugars measurement refers to the water-soluble mono- and disaccharides. Reducing sugars comprise the aldose and ketose saccharides which convert to the open-chain form with an aldehyde group, e.g., mainly glucose, fructose, xylose, arabinose, etc.



Figure 6: Total sugars and total reducing sugar contents of samples taken from the treated Masson's pine lumber

The total sugars and total reducing sugar contents of samples taken from the treated Masson's pine lumber are shown in Fig. 6. Compared with CK, the total sugars and total reducing sugars were all higher in the treated samples. Among the treated samples, the lowest and highest total sugar contents were measured in S2 and S1, respectively, which agreed with the results from the fungal control experiments (Fig. 2). The relatively low concentrations of reducing sugars may also account for the reduction of Cu^{2+} to Cu^{+} , and the low detectability of the monovalent ion [32].

Table 7 shows the concentrations of individual reducing sugars in the untreated and treated Masson's lumber obtained using LC-MS: Arabinose, and glucose + mannose $(34.25-30.96 \ \mu g \ g^{-1})$ were found in CK; small amounts of xylose, and glucose + mannose $(0.64-1.82 \ \mu g \ g^{-1})$ were present in S1–S3; and xylose and arabinose $(2.33-9.33 \ \mu g \ g^{-1})$ were present in CS1–CS3. A previous study showed that the content of arabinose gradually increases from sapwood to heartwood, while xylose is related to hemicellulose [33]. Thermal degradation of hemicellulose may lead to the formation of xylose in the Masson's pine lumber. Since xylose and arabinose are degraded at temperatures above 220°C, their contents increase with increasing depth in the surface layers of after heat treatment [34,35]. The trace amounts of xylose, and glucose + mannose in the S1–S3 could be attributed to their increased degradation by metal salts in the wood.

Overall, the results showed that the sugar content of heat-treated wood was much higher than that of its copper-bearing counterpart. Hence CS1–CS3 were more susceptible to fungal growth.

Comm1a	Sugar ($\mu g g^{-1}$)						
Sample	Rhamnose	Fucose	Xylose	Arabinose	Glucose + mannose	Galactose	Sum
СК	/	/	/	34.25	30.96	/	65.21
S 1	/	/	/	/	/	/	/
S2	/	/	0.64	/	/	/	0.64
S3	/	/	/	/	1.82	/	1.82
CS1	/	/	2.33	4.65	/	/	6.98
CS2	/	/	2.68	7.01	/	/	9.69
CS3	/	/	3.52	9.33	/	/	12.85

Table 7: Individual reducing sugar contents of untreated and treated Masson's lumbar measured by LC-MS

4 Conclusion

The heat-treated copper-bearing Masson's pine lumber samples, after removing the surface layer, showed good antifungal properties: The middle surface layer (5–10 mm) totally suppressed (100%) the growth of *Botryodiplodia theobromae* Pat., *Aspergillus niger* V. Tiegh., *Penicillium citrinum* Thom, and *Trichoderma viride* Pers. The equivalent samples subjected to heat treatment alone showed virtually no inhibition of fungal growth (0%–12.5%). The antifungal properties of the heat-treated of copper-containing lumber could be attributed to two processes: i) The reduction of residual copper salts from the impregnation process by PEG200 at high temperature to generate antifungal copper nanoparticles; ii) the degradation of reducing sugars influenced by metal salts during heat treatment. Hence, the immersion of timber in a CuG dipping solution followed by high-temperature treatment may be an effective method of increasing the utility and value of low-grade lumber.

Acknowledgement: The authors would like to express their gratitude to EditSprings (https://www.editsprings.cn) for the expert linguistic services provided.

Funding Statement: This research was sponsored by the Guangdong Forestry Science and Technology Innovation Project "Research on the Thermal Modification of Eucalyptus and Spingbract Chinkapin Wood and the Key Technologies of Their Wood Flooring Preparation" (No. 2018KJCX006).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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DOI: 10.32604/jrm.2023.024521

ARTICLE





Fabricating Cationic Lignin Hydrogels for Dye Adsorption

Chao Wang, Xuezhen Feng, Wanbing Li, Shibin Shang^{*} and Haibo Zhang^{*}

Institute of Chemical Industry of Forest Products, CAF; National Engineering Laboratory for Biomass Chemical Utilization; Key Laboratory of Chemical Engineering of Forest Products, National Forestry and Grassland Administration; Key Laboratory of Biomass Energy and Material, Jiangsu Co-Innovation Center of Efficient Processing and Utilization of Forest Resources, Nanjing, 210042, China

*Corresponding Authors: Shibin Shang. Email: shangsb@163.com; Haibo Zhang. Email: shdzhanghaibo@163.com Received: 01 June 2022 Accepted: 03 August 2022

ABSTRACT

Due to the low content of adsorption-active groups in lignin, its application in the field of adsorption is limited. Herein, we first prepared cationic kraft lignin acrylate, from which a cationic lignin (CKLA) hydrogel was further prepared by cationic kraft lignin acrylate, acrylamide, and N, N'-methylenebisacrylamide. The morphology, compression properties and swelling properties of CKLA hydrogels were investigated. The prepared CKLA hydrogel was applied as an adsorbent for Congo red. The effect of CKLA hydrogel dosages, initial concentration of Congo red, and pH on adsorption efficiency was investigated. The maximum Congo red removal efficiency was obtained at the initial concentration of Congo red of 50 mg/L, pH 7, and 5 mg dosage of CKLA hydrogel with 20% cationic lignin content. After five cycles of adsorption, the adsorption efficiency of the hydrogel for Congo red adsorption. These results demonstrate the potential of the CKLA hydrogel as an adsorbent for water treatment.

KEYWORDS

Adsorption; lignin; congo red; hydrogel

1 Introduction

With the discharge of a large amount of dye wastewater, the textile, printing and dyeing industries have caused serious water pollution [1,2]. Anion dyes are widely used in the dyeing process due to their simple synthetic process, low cost, and better staining performance [3,4]. During the process of anionic dyeing, the fabric needs to be washed several times to improve its dyeing fastness, resulting in the generation of a large amount of anionic dye wastewater [5], causing oxygen deficiency and jeopardizing the viability of aquatic animals and plants [6,7].

Several methods such as chemical methods (flocculation [8], advanced oxidation [9], etc.), biological methods (anaerobic, aerobic [10], etc.), membrane separation methods [11], and physical methods (adsorption, etc.) have been developed to treat dye wastewater. Adsorption [12] stands out among the above-mentioned methods because of its many advantages, e.g., low cost, easy operation, less secondary pollution, and good treatment effect. Hydrogels have a 3D cross-network structure, containing hydrophilic and reactive functional groups [13]. Classic superabsorbent hydrogels composed of polymerized acrylic acid and acrylamide are gradually being replaced due to being non-sustainable, non-biodegradable, and costly [14]. Compared to conventional adsorbents, biomass-based adsorption materials such as cellulose [15],



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lignin [16], corn [17], and sugarcane [18] have the advantages of wide source, low price, easy availability, and biodegradability, providing the excellent prospect for developing environmentally friendly materials [16].

Many bio-based materials, including lignin, cellulose and chitosan, have been used as adsorbents [19,20]. However, cellulose and chitosan are expensive and difficult to modify due to poor solubility in solvents. Lignin is the second most abundant bioresource on our planet [21], which has phenylpropane nits with methoxyl, phenolic and aliphatic hydroxyl, and carboxyl groups [22,23]. Therefore, lignin has been used to prepare different value-added products, including functional hydrogels, due to its rich availability, renewable source, low cost, and unique physiochemical properties. Lignin hydrogels are significantly porous with a larger specific surface area and exhibit high mechanical strength and chemical stability [24,25].

Considering the fast-swelling properties and porous structure of lignin hydrogels, they can absorb dye molecules through chemical, electrostatic interactions and hydrogen bonding [26]. A based biosorbent hydrogel (AML) was prepared by grafting alkali lignin with methylamine and formaldehyde via Mannich reaction, which displayed with fast adsorption of Pb^{2+} and adsorption capacity of 60.5 mg/g [27]. Domínguez-Robles et al. Crosslinked lignin with a mixed solution of ammonium hydroxide, methyl vinyl ether, and maleic acid through ester bonds to obtain superabsorbent lignin hydrogels, showing good adsorption efficiency for methylene blue [28]. Noteworthy, lignin does not contain cationic groups and is only used as a crosslinking agent while preparing hydrogels.

In this work, a functional cationic lignin hydrogel (CKLA) has been prepared from the polymerization of cationic lignin acrylate, acrylamide and N, N'-methylenebisacrylamide, and the adsorption properties of the hydrogels for Congo red (Fig. 1) were investigated. The effect of the amount of lignin used for preparing hydrogel on the swelling rate of hydrogels was investigated. The optimized hydrogel was assessed at different time intervals for its adsorption under different pH and congo red initial concentrations. Furthermore, kinetics and isotherm studies were conducted to assess the viability of CKLA (biomasses cationic lignin hydrogel) as an adsorbent for the removal of congo red.



Figure 1: The chemical structure of congo red

2 Materials and Methods

2.1 Material

Kraft lignin was provided by Nanjing Shanhu Chemical Co., Ltd. (Nanjing, China). Glycidyl trimethyl ammonium chloride, N, N'-methylenebisacrylamide were obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Acrylylchloride, acrylamide (AM), and Congo red were supplied by Aladdin Reagent (Shanghai) Co., Ltd. (Shanghai, China). Sodium bicarbonate was provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All the solvents and chemicals used in the syntheses and analyses were of analytical grade, obtained from commercial suppliers, and used without further purification.

2.2 Preparation of Cationic Lignin Acrylate

First, Kraft lignin (15 g), 2,3-epoxypropyl ammonium chloride (7.8 g) and benzyltrim-ethy-lammonium chloride (0.3 g) were dissolved in 30 g of N, N'-dimethylformamide, heated to 140°C and kept for 2 h, followed by cooling to room temperature. Next, NaHCO₃ (7.77 g) was added and mixed evenly. Acryloyl

chloride (8.38 g) was added dropwise to the above solution over 4 h. After the reaction, the mixture was poured into ethyl acetate for precipitation. Finally, the precipitate was vacuum-dried to obtain 19.70 g of cationic lignin acrylate.

2.3 Preparation of Cationic Lignin-Based Hydrogel

Different masses of cationic lignin acrylate, acrylamide and N, N'-methylenebis(2-propenamide) (MBA) were dissolved in water with respect to the V-50 catalyst, retaining the total mass of monomers be 10 wt%, under a nitrogen atmosphere for 15 min to remove oxygen. The mixture was reacted at 50°C for 4 h, affording the CK cationic lignin-based hydrogel (CKLA hydrogel).

2.4 Characterization

FTIR spectra were obtained using a spectrometer (Nicholas iS50, U.S.) in the scanning range of 4000– 500 cm⁻¹. ³¹P NMR spectra were recorded using a Bruker 500 MHz NMR spectrometer. UV-vis spectral investigations were carried out using a UV-vis spectrometer (UV-2450, Shimadzu). Elemental analysis was performed using an elemental analyzer (2400, PerkinElmer).

2.5 Adsorption Experiment of Congo red

Congo red aqueous solutions of various concentrations (10, 30, 50, 70, 100, and 150 mg/L) were prepared. The prescribed amount of CKLA hydrogels was added in 25 mL congo red aqueous solutions, and the resulting mixture was shaken for 24 h. Once the adsorption equilibrium was reached, the absorbance of the supernatant was measured by UV-vis spectroscopy, and the adsorption efficiency was calculated as follows:

$$\eta = \frac{C_0 - C_e}{C_0} \times 100\%$$
(1)

where C_0 and C_e are initial and equilibrated dye concentrations (mg/L), respectively.

The amount of adsorption was calculated as follows:

$$q_e = \frac{V \times (C_0 - C_e)}{m} \tag{2}$$

where C_0 and C_e are initial and equilibrated dye concentrations in the solution (mg/L), respectively; V is the volume (L) of the congo red solution, and M is the weight (g) of the adsorbent.

2.6 Adsorption Kinetics

The adsorption kinetics was studied to investigate can reveal the equilibrium state of the adsorption process and inspect the important parameters that provide the suitability of the CKLA hydrogel as an adsorbent. Therefore, during the adsorption kinetics experiment, the following optimal reaction conditions were used: the initial concentration of the CR solution was 50 mg/L, the pH was 7, and the dosage was 5 mg/25 mL for a time of 1000 min. The adsorption experiments were evaluated using the adsorption capacity, and the adsorption kinetic models were analyzed by the pseudo-first-order adsorption kinetic model, the Pseudo-second-order adsorption kinetic model, the Webber-Morris intraparticle diffusion model, and the Elovich kinetic model.

3 Results and Discussion

3.1 Characterization of Kraft Lignin and Cationic Lignin-Based Acrylates

Various hydroxyl groups on lignin were studied by the 31P NMR spectrum. The various hydroxyl groups on lignin and their concentrations are shown in Fig. 2 and Table 1, respectively. They are aliphatic

OH, 5-substituted OH, guaiacyl OH, p-hydroxy OH, and carboxylic acid, and the peaks appearing were observed in 149.3–145.2, 144.4–140.2, 140.2–138.7, 138.7–136.6, 135.8–133.9 ppm, the corresponding contents were 2.42, 1.69, 1.89, 0.17, and 0.39 mmol/g, respectively.



Figure 2: ³¹P NMR spectrum of Kraft lignin

Fable 1:	Hydroxyl	content in	Kraft lignin	(mmol/g)
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Sample	Aliphatic OH	5-Substituted OH	Guaiacyl OH	p-Hydroxy OH	Carboxylic acid
Kraft lignin	2.42	1.69	1.89	0.17	0.39

The cationic lignin was obtained by reaction between the hydroxyl group in Kraft lignin and 2,3epoxypropyl ammonium chloride, resulting in an epoxy ring-opening reaction to introduce a quaternary ammonium salt structure. Subsequently, the CKLA was prepared by the reaction of cationic lignin and acryloyl chloride (Fig. 4). The FTIR spectra of kraft lignin and CKLA are shown in Fig. 3. Compared with lignin, CKLA exhibited a noticeable decrease in the OH absorption peak at 3308 cm⁻¹. After the reaction of Kraft lignin with 2,3-epoxypropylammonium chloride and acryloyl chloride, an ester group peak at 1724 cm⁻¹ and an unsaturated double bond peak at 1650 cm⁻¹ appeared in the FT-IR spectra of CKLA. Furthermore, through elemental analysis (Table 2), the N content in kl was obtained to be 0, while it was 3.15% in CKLA, proving the successful synthesis of CKLA.

3.2 Preparation and Swelling Properties of CKLA Hydrogel

The preparation route of the cationic lignin-based hydrogel is delineated in Fig. 4. Concisely, CKLA hydrogels were prepared by copolymerization of cationic lignin-based acrylate, acrylamide, and N, N'-methylene bisacrylamide in an aqueous solution [29].

The swelling properties of CKLA hydrogels with different lignin content are shown in Fig. 5. The swelling ratio of all CKLA hydrogels increases gradually, then slowly reaches the swelling equilibrium. It is evident that the hydrogels reached the swelling equilibrium (the equilibrium swelling ratios of

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hydrogels with different lignin contents are abbreviated as Seq-x, where x is the lignin content) after soaking in water for 13 h, and their trend was as follows: Seq-25 > Seq-20 > Seq-5 > Seq-15 > Seq-10 (Fig. 5). With the increased CKLA content, the quaternary ammonium salt group content also increased, resulting in higher equilibrium swelling ratios of hydrogels. However, Seq-5 is higher than Seq-15 and Seq-10; this is because the content of CKLA is low, leading to a low cross-link density [30].



Figure 3: FT-IR spectra of Kraft lignin and CKLA

Table 2:	Elemental analysis of Kraft lignin CKLA	

	С	Н	Ο	Ν
KL	61.74%	5.65%	28.97%	0
ACKL	57.77%	5.77%	22.92%	3.15%



Figure 4: Synthetic route of CKLA hydrogels

3.3 Effect of the Dosage of CKLA Hydrogels on CR Adsorption

The effect of the dosage of various CKLA hydrogels on the adsorption of CR is shown in Fig. 6. The CR removal efficiency of hydrogels initially increased with the increasing amount of cationic lignin-based hydrogel and then reached the maximum. The adsorption efficiency obtained was the best for 20% lignin content, while the hydrogel dosage was 5 mg. The main reason affecting the adsorption is that with the

increase of the content of CKLA, the quaternary ammonium salt on the CKLA hydrogel also increases, which provides more adsorption sites and thus reduces the required dosage of CKLA hydrogels. A decrease in adsorption efficiency was observed for the CKLA hydrogel with 25% CKLA content due to the increase of quaternary ammonium content, which causes electrostatic repulsion [31].



Figure 5: (a) and (b) The SEM images of CKLA hydrogel; (c) Compression stress-strain curves; (d) Swelling curves of CKLA hydrogels

3.4 Effect of CR Initial Concentration on Adsorption Efficiency

The effect of the initial concentration of Congo red on the removal efficiency of the prepared hydrogels is shown in Fig. 7. Upon increasing the concentration of Congo red solution progressively, the removal efficiency of cationic lignin-based hydrogel first showed an increasing trend. It reached the maximum for 50 mg/L Congo red aqueous solution, and further concentration increments resulted in the decline of the removal efficiency. A higher initial dye concentration will provide an enhanced propulsion force to accelerate the mass transfer resistance of dye molecules from the liquid phase to the solid phase (adsorbent), thereby improving the adsorption capacity of the hydrogel [32]. At the beginning of the adsorption process, there is a mass of vacancies on the surface of the adsorbent. The repulsive force between the Congo red molecules adsorbed on the surface of the adsorbent and present in the solution phase gradually weakens the effective adsorption sites of the adsorbent [33]. Thus the CKLA hydrogels

reach adsorption saturation at high Congo red concentrations, resulting in a decrease in the adsorption efficiency.



Figure 6: Effect of dosage of CKLA hydrogels on the adsorption of CR



Figure 7: Effect of initial concentration of CR on removal efficiency of hydrogels

3.5 Effect of pH on Adsorption Efficiency

The effect of pH on the adsorption efficiency of cationic lignin-based hydrogels is shown in Fig. 8. It was observed that with the increase of pH value, the removal efficiency for Congo red by CKLA hydrogels first increased and then showed a decreasing trend. The maximum adsorption efficiency was obtained at pH 7. Under acidic conditions, the conversion of sodium sulfonate in Congo red to sulfonic acid groups weakens the electrostatic interaction with quaternary ammonium salt groups, resulting in low adsorption efficiency [34,35]. At pH > 7, the electrostatic repulsion between hydroxide ions and Congo red molecules [36], reduces removal efficiency.



Figure 8: Effect of pH on the adsorption efficiency of CKLA hydrogels

3.6 Adsorption Kinetics

During the adsorption process, the diffusion of the adsorbate from the solution to the adsorbent and the rate at which the adsorbate accumulates on the surface of the adsorbent determine the kinetics of the adsorption process and the adsorption efficiency [37]. The adsorption time can indicate whether the whole adsorption process has reached an equilibrium state and evaluate the adsorption capacity of the adsorption process [38]. The effect of adsorption time on the adsorption of CR by CKLA hydrogels is illustrated in Fig. 9. Evidently, CKLA hydrogels reached adsorption equilibrium at 600 min.



Figure 9: Effect of time on the adsorption efficiency of CKLA hydrogels

To describe the adsorption process more clearly, the adsorption kinetics of CR for CKLA hydrogel was investigated by various kinetic models: pseudo-first-order model: i.e., pseudo-second-order model, Webber-Morris Intral-particle diffusion model, and the Elovich model.

The pseudo-first-order model can be expressed by Eq. (3):

$$\ln(q_e - q_t) = \ln q_e - k_1 t \tag{3}$$

The pseudo-second-order model can be described by Eq. (4):

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e}$$
(4)

 q_e is the amount of CR adsorbed at equilibrium (in mg/g), qt represents the CR adsorbed at time t (in mg/g), and k_1 and k_2 are the pseudo-first-order model and pseudo-second-order model constants, respectively.

The Webber-Morris Intral-particle diffusion model can be given by Eq. (5):

$$q_t = k_i t^{1/2} + C \tag{5}$$

The Elovich model can be given by Eq. (6):

$$q_t = \frac{1}{\beta} \ln(\alpha\beta) + \frac{1}{\beta} \ln t \tag{6}$$

where q_e and q_t are the same as previous. k_i is the rate constant for the Webber-Morris Intral-particle diffusion model, whereas α and β are the Elovich model rate constants.

Table 3 summarizes the three kinetic model fitting parameters and mass transfer process models for the adsorption of CR by CKLA. The linear fitting form of these models is displayed in Fig. 10. The pseudo-second-order kinetic model fitted the maximum adsorption capacity of 125.63 mg/g, close to the experimental value. Moreover, the pseudo-second-order kinetic model R2 had the best coefficient. Therefore, the adsorption process of CKLA hydrogel for Congo red was more consistent with the pseudo-second-order kinetic model, showing that the adsorption process is mainly chemical adsorption. Furthermore, the intra-particle diffusion model demonstrated that the adsorption process was the internal mass transfer.

 Table 3: Kinetics parameters for CR adsorption by CKLA hydrogel

	Pseud	lo-first-order	Pseudo-	second-order	Elovic	h model	Intra-part	icle diffusion model
Parameter	$q_{ m e}$	k_1	$q_{ m e}$	k_2	α	β	k _i	С
value	69.14	$5.26 * 10^{-3}$	125.63	$1.21 * 10^{-4}$	17.11	0.057	2.1128	61.2766
r^2		0.9818	().9868	0.9	9780		0.9228

3.7 Reusability of CKLA Hydrogel

The reusability of CKLA hydrogel is shown in Fig. 11. The results showed that after five cycles of adsorption, the adsorption efficiency of the hydrogel for Congo red still reached more than 80%. This proved that the hydrogel had excellent reusability.

3.8 Possible Mechainism for the Adsorption of Congo Red by CKLA Hydrogel

The adsorption mechanisms tend to be electrostatic interactions between the quaternary ammonium group in the hydrogel and the sulfonate in Congo red [39]. Besides, Congo red is adsorbed through hydrogen bonding, van der Waals forces and hydrophobic interactions [40,41].



Figure 10: (A) Pseudo-first-order, (B) Pseudo-second-order, (C) Elovich model, and (D) Intra-particle diffusion models



Figure 11: The reusability of CKLA hydrogel

4 Conclusion

In this work, cationic kraft lignin acrylate (CKLA) was prepared by reacting Kraft lignin with 2,3epoxypropyl ammonium chloride and acryloyl chloride. Cationic lignin hydrogels were further synthesized by polymerization of acrylamide, MBA, and CKLA. The effect of dosage of CKLA hydrogels, initial concentration of Congo red, and pH on the adsorption efficiency of the prepared hydrogels was investigated. The optimum Congo red removal efficiency was obtained for the hydrogel with 20% lignin content, 5 mg hydrogel dosage, 50 mg/L concentration of Congo red aqueous solution, and pH=7. Adsorption kinetics studies revealed that the adsorption of CR by CKLA hydrogels followed pseudo-second-order kinetics.

Funding Statement: This study was supported by Fundamental Research Funds of CAF (CAFYBB2020MB002).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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DOI: 10.32604/jrm.2023.025126

ARTICLE





Preparation and Properties of Vegetable-Oil-Based Thioether Polyol and Ethyl Cellulose Supramolecular Composite Films

Ruyu Yan^{1,2,3,4}, Jian Fang^{1,*}, Xiaohua Yang^{2,3,4,5,6}, Na Yao^{2,3,4,5,6}, Mei Li^{2,3,4,5,6}, Yuan Nie^{2,3,4,5,6}, Tianxiang Deng^{2,3,4,5,6}, Haiyang Ding^{2,3,4,5,6}, Lina Xu^{2,3,4,5,6} and Shouhai Li^{2,3,4,5,6,*}

¹College of Materials Science and Technology, Beijing Forestry University, Beijing, 100083, China

²Institute of Chemical Industry of Forestry Products, CFA, Nanjing, 210042, China

³National Engineering Lab. for Biomass Chemical Utilization, Nanjing, 210042, China

⁴Key Lab. of Chemical Engineering of Forest Products, National Forestry and Grassland Administration, Nanjing, 210042, China

⁵Key Lab. of Biomass Energy and Material, Nanjing, 210042, China

⁶Jiangsu Co-Innovation Center of Efficient Processing and Utilization of Forest Resources, Nanjing, 210042, China

*Corresponding Authors: Jian Fang. Email: fj515@sina.com; Shouhai Li. Email: lishouhai1979@163.com

Received: 23 June 2022 Accepted: 04 August 2022

ABSTRACT

Ethyl cellulose (EC), an important biomass-based material, has excellent film-forming properties. Nevertheless, the high interchain hydrogen bond interaction leads to a high glass transition temperature of EC, which makes it too brittle to be used widely. The hydroxyl group on EC can form a supramolecular system in the form of a non-covalent bond with an effective plasticizer. In this study, an important vegetable-oil-based derivative named dimer fatty acid was used to prepare a novel special plasticizer for EC. Dimer-fatty-acid-based thioether polyol (DATP) was synthesized and used to modify ethyl cellulose films. The supramolecular composite films of DATP and ethyl cellulose were designed using the newly-formed van der Waals force. The thermal stability, morphology, hydrophilicity, and mechanical properties of the composite films were all tested. Pure EC is fragile, and the addition of DATP makes the ethyl cellulose films more flexible. The elongation at the break of EC part (60/40) and EC/DATP (50/50) was up to 40.3% and 43.4%, respectively. Noticeably, the thermal initial degradation temperature of the film with 10% DATP is higher than that of pure EC, which may be attributed to the formation of a better supramolecular system in this composite film. The application of bio-based material (EC) is environmentally friendly, and the novel DATP can be used as a special and effective plasticizer to prepare flexible EC films, making it more widely used in energy, chemical industry, materials, agriculture, medicine, and other fields.

KEYWORDS

Ethyl cellulose; dimeric fatty acid based thioether polyol; supramolecular system; composite films



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Graphic Abstract



1 Introduction

Film materials play an increasingly important role in life and industrial production following the development of society. However, most of the films originated from petroleum and coal chemical products, and these fossil resources are facing the risk of depletion [1]. Meanwhile, most of these fossilbased films cannot be recycled or naturally degraded, so the waste film materials will severely threaten the environment [2,3]. In pace with people's improved awareness of environmental protection and resource preservation, the development of renewable resources-based films has attracted great attention [4-7].

Starch, cellulose, chitosan, vegetable oil, and their derivatives are commonly-used raw materials for preparing biomass-derived products, including films. These renewable biomass resources, which are highly potential to replace traditional petrochemical products in daily life and industrial fields, have gained extensive attention [8–10].

As the most widely-sourced natural resource, cellulose has generated a large number of derivatives and is used in medicine, coatings, membrane technology, construction, and other fields. For example, bacterial cellulose (BC), which is usually used as a wound dressing for repairing human skin and tissue, is characterized by good biocompatibility, adaptability, and air permeability [11]. Zhao developed a stiffness-changing material composed of cellulose (Cel) and PAA, the biomimetic Cel-PAAm has a unique self-healing behavior and a self-regulating capability between soft state and reinforced state [12]. Ethyl cellulose (EC), an important cellulose derivative, has high plasticity, biocompatibility, and heat and light resistance [13–16]. Remarkably, EC possesses excellent film-forming performance like other cellulose derivatives [17-20]. However, the brittleness of EC films restricts their applications in many fields. Consequently, plasticizers are needed as additives to strengthen the flexibility and processability of EC films [21,22]. Some petroleum-based plasticizers of EC films include dibutyl sebacate, citrate, triacetate, triethylacetylated monoglyceride, and diethyl phthalate [23,24], but the effect on improving the performance of ethyl cellulose films is not significant [25]. In addition, owing to toxicity, most petroleum-based plasticizers are strictly forbidden in medicine and children's products. Thus, many ecofriendly bio-based plasticizers are continuously explored for sustainable development. Triethyl citrate (TEC) can considerably enhance flexibility and reduce the glass transition temperature of EC films.

Additionally, many common plasticizers such as dioctyl-phthalate (DOP), dioctyl terephthalate, and biobased TEC were used to plasticize EC materials. They can easily migrate out from composite films without forming supramolecular systems with EC. Therefore, high-efficiency and low-toxicity plasticizers shall be prepared and used to form supramolecular systems with EC.

Noncovalent bonds (e.g., hydrogen bonds, metal coordination, hydrophobic or hydrophilic bonds, π - π interactions, van der Waals forces) can construct supramolecular systems [26]. Ethyl cellulose, an amphiphilic polymer, can form many hydrogen bonds as well as hydrophobic or hydrophilic interactions, and easily form supramolecular systems. Li et al. synthesized and used a new type of ricinoleic acid mercaptotriol (STRA) to plasticize EC films [27]. A series of composite films with different proportions of STRA and EC based on van der Waals forces were prepared, and their flexibility and hydrophilicity were improved to a certain extent. Lin combined bio-based small-molecule lipoic acid (LA) and EC to produce supramolecular composite films [28]. LA weakened the hydrogen bond and hydrophobic binding in EC molecular chains, which remarkably improved the flexibility and processability of ethyl cellulose films. When the LA content was 20%–50%, the elongation at break of EC composite films rose by 5.5–11.1 times.

Dimer fatty acid is a widely-used vegetable-oil-based derivative, which molecularly contains two carboxyl groups, more than one unsaturated double bond, and long fat chains. It can be used to prepare many chemical products. Lee prepared dimer acid (DA) esters with different short to long alkyl chains via the hydrolysis of waste vegetable oil, Diels-Alder reaction, and esterification of fatty acids [21]. The DA esters had higher thermal stability than DOP, a petroleum-based plasticizer. The optical clarity and SEM images of the composite films composed of DA esters and EC showed high miscibility on a micro-scale [29].

In this study, the method to synthesize a novel bio-based thioether polyol plasticizer from dimer fatty acid was first introduced. Thus, the plasticizer was named dimer-fatty-acid-based thioether polyol (DATP). Then various supramolecular films of EC and DATP were prepared. The thioether group and hydroxyl group in DATP can be combined with the hydroxyl group of EC when composite systems are regenerated [30]. The morphology, hydrophilicity, thermal stability, and mechanical properties of the composite films were all tested. This study provides a distinct guide for the preparation of such materials.

2 Experimental

2.1 Raw Materials

NaOH (AR, 97.0%), and benzyl triethyl ammonium chloride (98%) were obtained from Aladdin Industrial Co., Ltd., China. Dimer fatty acids (stabilized, 91.5%) were provided by Jiangxi Yichun Yuanda Chemical Industry Co., Ltd., China. The epichlorohydrin (AR, \geq 99.0%) was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd., China), the diatomaceous earth (Shanghai Aladdin Biochemical Technology Co., Ltd., China), acetic acid (\geq 99.5%, Chinasun Specialty Products Co., Ltd., China), and mercaptoethanol (AR, 99%, Xiya Reagent, China). Ethyl cellulose M70 (EC, CP) was provided by the Sinopharm Chemical Reagent Co., Ltd., China. Calcium oxide (Xilong Chemical Company, China). All materials were used as received without further purification.

2.2 Synthesis of Dimer-Fatty-Acid-Based Thioether Polyol

Dimer-fatty-acid-based epoxy (DAE) resulted from dimer fatty acid and epichlorohydrin by ringopening and the closing reaction of epoxy groups and then was converted to the DATP. Scheme 1 displays the synthesis of DATP.

Firstly, 84.15 g (0.15 mol) of dimer fatty acid, 139.50 g of epichlorohydrin, and 1.40 g of benzyl trimethyl ammonium chloride were put into a 500 mL flask containing a magnetic stirrer, a thermometer, and a reflux condenser. Then the mixed reactants were heated to 100°C and held for 3 h. After cooling to 60°C, 12.00 g (0.3 mol) of NaOH and 16.80 g (0.3 mol) of CaO were added under stirring for 3 h. After the reaction, the product was filtered with a Buchner funnel covered with diatomite, and the filtrate was

collected. A light orange viscous liquid DAE was formed after excessive epichlorohydrin was distilled via rotary vacuum evaporation. The epoxy value of DAE was 0.241 mol/100 g.



Scheme 1: Synthetic route of DATP and films of EC and DATP

Then 10.00 g of DAE, 1.76 g of mercaptoethanol, and 0.12 g of DMP-30 were added to a 100 mL flask containing the three devices mentioned above. After stirring at 120°C and 400 r/min for 2 h, DATP was obtained.

2.3 Preparation of the EC and DATP Composite Films

Scheme 1 plots the two-step synthesis of EC composite films. First, EC was dissolved in acetic acid and stirred at 80°C for 1 h to form 7.3% (w/w) solutions. Second, supramolecular composite films with different DATP contents were made by casting (the preferred method to prepare films). DATP was blended with the 7.3% EC solutions and stirred at 80°C for 0.5 h. Next, the solution was removed into a clean polypropylene mold with a casting area of 70 × 100 mm² and dried at 120°C for 2 h. Films containing 0, 10, 20, 30, 40, or 50 wt% DATP were obtained and named as EC/DATP (x) (x = 100/0, 90/10, 80/20, 70/30, 60/40, 50/ 50, respectively). The compositions of EC/DATP are listed in Table 1.

Formulations	DATP (g)	EC (g)	EC acetic acid solution 7.3% (w/w) (g)
EC/DATP (90/10)	0.25	2.25	33.07
EC/DATP (80/20)	0.50	2.00	29.40
EC/DATP (70/30)	1.00	2.33	34.30
EC/DATP (60/40)	1.00	1.50	22.05
EC/DATP (50/50)	1.00	1.00	14.70

Table 1: Formulation of EC composite membranes with different proportion

2.4 Characterization

2.4.1 Epoxy Value

The epoxy values were characterized according to Chinese Standards GB/T 1677-2008 and calculated by the following formula: EPV = $\frac{(V_o - V) \times C}{10 \times w}$

 V_0 : volume of standard sodium hydroxide solution consumed in blank test (57.10 ml)

V: volume of standard sodium hydroxide solution consumed by the sample (42.40 ml)

C: concentration of sodium hydroxide standard solution (0.0984 mol/L)

W: sample quality (0.600 g)

2.4.2 Nuclear Magnetic Resonance (¹H NMR)

The ¹H NMR of DA, DAE, and DATP were recorded using an AVANCE III HD 400 MHz spectrometer at room temperature, CDCl₃ was employed as a deuterated solvent.

2.4.3 Attenuated Total Reflectance–Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The ATR-FTIR of DA, DAE, DATP, and composite films were recorded by Nicolet IS10 spectrometer (Thermo-fisher, USA). Each sample was recorded in the range of 4000-500 cm⁻¹ at a resolution of 4 cm⁻¹.

2.4.4 Scanning Electron Microscope (SEM)

The micrographs of the supramolecular composite films were scanned by Hitachi S3400-N (Japan). For the sake of avoiding electrostatic charging during the examination, the exposed fracture surface cryofractured by liquid nitrogen was coated with gold.

2.4.5 Thermal Gravimetric Analysis (TGA)

A NETZSCH STA 409 PC (Netzsch Instrument Crop., Germany) was used to perform TGA tests. The samples were heated from 40°C to 800°C under a nitrogen atmosphere at a rate of 15 °C/min.

2.4.6 Dynamic Mechanical Analysis (DMA)

A dynamic mechanical analyzer (Rheometric Scientific IV) with stretching mode at an oscillatory frequency of 1 Hz was employed for DMA testing. The DMA test specimens with a size of approximately 40.0 mm (L) \times 6.0 mm (W) \times 0.3 mm (T) were cut with a utility knife. The measurements were carried out at a heating and cooling rate of 3 °C/min from -80°C to 60°C in a liquid N₂ atmosphere.

2.4.7 Mechanical Properties

Tensile properties of the samples were measured by an E43.104 Universal Testing Machine (MTS Instrument Crop., China) with a crosshead speed of 5 mm/min. The test of tensile strength and elongation at break is in the light of GB/T 1040-2006 (China). The dumbbell-shaped samples for tensile tests were obtained by a manual punching machine (Yangzhou Yuanfeng Testing Equipment Co., Ltd., China), and the specimens' size was approximately 50.0 mm (L) $\times 4.0 \text{ mm}$ (W) $\times 0.3 \text{ mm}$ (T). Under a fixed test temperature of 25°C, five specimens were tested at a crosshead speed of 5 mm/min, and the average values were obtained.

2.4.8 Water and Oil Contact Angle

The water and oil contact angles of EC films were tested by instrument (DSA 100, Krüss, Hamburg, DE) to evaluate the hydrophilicity of EC films. 5 μ L of water and oil were separately dripped on the film surface and stabilized for 60 s, all measurements were carried out at 20°C, and the final water and oil contact angle values are the average value of three replicates.

3 Results and Discussion

3.1 FTIR and ¹H NMR of DA, DAE, and DATP

ATR-FTIR spectra of DA, DAE, and DATP are shown in Fig. 1a. There are two identical characteristic peaks in all three spectra. The peaks at 2921 and 2852 cm⁻¹ confirm the presence of methyl and methylene groups, respectively. The peak around 1706 cm⁻¹ is attributed to the carboxyl group, which fully disappeared in other spectra.



Figure 1: FTIR and ¹H NMR spectra of DA, DAE and DATP

In comparison with the spectrum of DA, the peak at 910 cm^{-1} corresponds to the epoxy group, and the ester group peak at 1740 cm⁻¹ exists in DAE. The FTIR above suggests the successful epoxidation of DA. In the FTIR spectrum of DATP, the peak of the epoxy group is almost absent and two new peaks caused by primary hydroxyl appear at 3408 and 1062 cm⁻¹. These results demonstrate the successful reaction between S-H and the epoxy groups. All the FTIR spectra indicate the successful synthesis of DATP.

Fig. 1b exhibits the ¹H-NMR spectra of DA, DAE, and DATP, with detailed attributions of chemical shifts of protons. On the DA spectrum, the peak around 11.3 ppm corresponds to the hydroxy of the carboxyl group, which is almost absent in DAE. Besides, new peaks at 2.5–2.8 ppm, 3.1 ppm, and 3.8 –4.4 ppm in the ¹H-NMR spectrum of DAE are ascribed to the epoxy group. Compared with the ¹H-NMR spectrum of DAE, the peak standing for the proton of CH-O-CH₂ around 3.0 ppm is fully unseen on the spectrum of DATP. Moreover, the peak at about 2.3 ppm indicates the presence of the hydroxy group, and the peaks at 2.6–2.7 ppm ascribed to the proton of $-S-CH_2-$ are weakened. All these ¹H-NMR spectra prove the successful synthesis of DATP [31].

3.2 FTIR of EC and EC/DATP Films

Fig. 2 depicts the FTIR spectra of supramolecular EC composite films with varying proportions of DATP. The pure EC film shows distinct peaks at around 3474 and 1051 cm⁻¹, which represent O-H and C-O-C stretching, respectively. With the addition of DATP, the new peak at 1738 cm⁻¹ ascribed to the carbonyl group appeared. In addition, as can be seen from the FTIR spectra of different EC films, the peaks (3474–3459 cm⁻¹) belonging to the stretching vibration of O-H shifted to lower frequency and the width of which increased, due to the formation of hydrogen bonds between DATP and ethyl cellulose, and the force constant lowering in the bound state [32]. Therefore, it can be observed that good supramolecular systems were formed by DATP and EC.



Figure 2: ATR-FTIR spectra of different EC films

3.3 Morphology of EC and EC/DATP Films

The photos of EC films on the black paper are shown in Fig. 3. We can see that the EC films are relatively transparent compared with pure EC, indicating that EC and DATP are very compatible. Pure EC film is very brittle and divided into two parts when it bends. EC films with DATP remain intact when bent by tweezers. DATP can improve the flexibility of EC film to some extent.



Figure 3: Photos of EC films

Fig. 4 displays the SEM images of the films at the exposed section (magnification 2000 and 5000 times). Many holes exist on the fractured surface of pure EC film, owing to the relatively poor hydrogen bonding system caused by the large steric hindrance of EC molecules. With more DATP added to the EC films, fewer holes appear on the cross-section of EC composite films. When there are a lot of holes on the fracture surface of the film, it is brittle. With the addition of DATP, the surface presents a lamellar structure, which indicates that the flexibility of the film has been improved, when the surface is relatively smooth, the flexibility of the film is best. Hence, DATP can be well embedded into EC molecular chains to form novel supramolecular systems through hydrogen bonding.



Figure 4: SEM images of fractured surface in EC films

3.4 TGA

TGA was used to evaluate the thermal stability of different EC films. The thermal data of special temperatures with 5%, 10%, and 50% mass losses (marked T_5 , T_{10} , and T_{50} respectively), the fastest decomposition temperature T_{max} and carbon yield at 600°C (CY600) of EC films were listed in Table 2. The thermal degradation curves of composite films are exhibited in Fig. 5. Apparently, the initial thermal degradation of pure EC (T_5) occurred at 301.2°C, but dropped to 293.7°C and 276.3°C in the composite

films with 30% and 50% DATP, respectively. The reason is that heat destroys the van der Waals interactions [31] so DATP molecules are released from the composite films during the heating. Significantly, the initial degradation temperature of the composite film with 10% DATP is higher than that of pure EC, which may result from a better supramolecular system formed in this composite film. Thermal degradation of EC started at 300°C and ended at 390°C. For composite films, major decomposition occurred within 320°C–380°C, which was related to the thermal degradation of EC. When at above 380°C, the weight percentages of all composite films were higher than that of pure EC, which means relatively higher thermal stability of composite films. This evidence suggests that DATP can be used as an effective additive to maintain thermal stability for EC films.

Formulations	T_5^a (°C)	T_{10}^{a} (°C)	T_{50}^{a} (°C)	T_{max}^{a} (°C)	CY ₆₀₀ ^b (%)
Pure EC	301.2	323.7	361.2	365.5	6.3
EC/DATP (90/10)	316.2	338.7	366.2	368.2	6.4
EC/DATP (70/30)	293.7	331.2	366.2	366.1	4.5
EC/DATP (50/50)	2763	321.3	368.8	362.5	41

Table 2: TGA data of EC films



Figure 5: TGA curves of EC films

3.5 DMA

DMA was used to detect the dynamic mechanical thermal properties of the EC films. The storage modulus and loss factor (tan δ) of EC composite films are depicted in Fig. 6. The newly-formed complex compositing system consists of rigid EC units, flexible aliphatic chains, and elastic -S-. Hence, the rich DATP phase, rich EC phase, and EC/DATP phase may all exist in the compositing system. The FTIR and SEM data indicate the EC/DATP films are homogeneous.



Figure 6: DMA curves of EC films

The storage modulus of all samples declines with the increasing temperature. Generally, with the presence of DATP, the storage modulus of EC films is smaller. For all composite systems, the rigid interchain hydrogen bond of pure EC is destroyed because of the addition of DATP, decreasing the storage modulus of films [33]. However, at below -50° C, the EC/DATP (50/50) system shows a relatively higher storage modulus. The reason is that the rich DATP phase in this composite can be frozen more easily at certain temperatures.

The loss factor analysis curves become increasingly irregular with the rise of DATP content, which may be because the molecular movement of DATP in the composite system becomes freer at high temperatures. The pure EC film possesses a relatively higher T_g of 146.7°C, which is close to the data reported before [21]. The T_g of EC composite films drops from 144.1°C to 88.2°C with the increasing content of DATP. This is because the introduction of the oxhydryl, thioether, polar groups carbonyl, and long alkyl chain structures of DATP can damage the hydrogen bonding system of EC. When the temperature is near or above T_g , the supramolecular films start to creep with the structure lost, and the van der Waals force is weakened, which accounts for such a change trend on the loss factor curves.

3.6 Mechanical Properties

Fig. 7 shows the representative stress-strain curves of the composite films. The detailed mechanical properties were summarized in Table 3, including film thickness, tensile strength, elongation at break, and elastic modulus. The pure EC film has an elongation at a break of only 6.16% and thus is brittle. With the presence of DATP, the elongation at break is strengthened, but the tensile strength and elastic modulus decline. Elongations at break of EC/DATP (60/40) and EC/DATP (50/50) are up to 40.3% and 43.4%, respectively.

In contrast, the composite films added with 20% DATP are improved in elongation at a break from 0.77% to 2.19% [25]. Triethyl citrate and epoxidized soybean oil, two other industrial plasticizers, cannot significantly enhance the flexibility of the EC films like that [22,34]. The elongations at break of EC/STRA (60/40) and EC/STRA (50/50) increase by nearly 7.0 times from that of pure EC film.

The possible reasons are stated below. Firstly, the original hydrogen bond in EC molecular chains can be destroyed due to the introduction of DATP, and a new non-covalent bond interaction is formed between DATP and EC. Thus, a supramolecular system is established between DATP and EC [27,28]. Secondly, the existence of elastic –S– and flexible aliphatic chains on DATP can enhance the ductility of the newly-prepared composite films [30]. Thirdly, the large atom radius and low electronegativity of sulfur result in

its weak bonding with EC molecules, which can modulate the interaction of EC molecules and DATP to a suitable level.



Figure 7: Stress-strain curves of different EC films

Formulations	Films thickness (mm)	Tensile strength (MPa)	Elongation at break (%)	Elasticity modulus (MPa)
Pure EC	0.32	33.00 ± 1.32	6.16 ± 2.40	419.06 ± 23.25
EC/DATP (90/10)	0.21	24.07 ± 2.44	5.48 ± 0.24	426.13 ± 61.92
EC/DATP (80/20)	0.25	11.66 ± 0.32	6.53 ± 2.55	427.63 ± 38.22
EC/DATP (70/30)	0.24	7.19 ± 1.04	11.38 ± 2.63	316.00 ± 29.53
EC/DATP (60/40)	0.30	4.51 ± 0.63	40.28 ± 6.53	124.27 ± 8.44
EC/DATP (50/50)	0.40	2.32 ± 1.09	43.37 ± 4.39	42.99 ± 20.37

Table 3: Mechanical properties of pure EC and EC/DATP films

3.7 Contact Angle

Fig. 8 shows the contact angles of the composite films. The water contact angles of EC films (a) are ranked as: EC/DATP (100/0) [85.46°] > EC/DATP (90/10) [73.61°] > EC/DATP (70/30) [50.09°] > EC/DATP (50/50) [37.39°]. The oil contact angles of EC films (b) are ranked as: EC/DATP (100/0) [1.67°] < EC/DATP (90/10) [3.32°] < EC/DATP (70/30) [23.91°] < EC/DATP (50/50) [29.93°]. The analysis of contact angle shows that the composite films become more hydrophilic and oleophobic with more DATP added. It was because the introduction of DATP leads to an increased amount of free hydrophilic hydroxyl group on the surface of composite films. Hence, the compositing films can be used as hydrophilic films.



Figure 8: Contact angles of the various EC formulations

4 Conclusions

A novel special plasticizer for EC named dimer-fatty-acid-based thioether polyol (DATP) was successfully prepared, and incorporated into EC systems to form novel supramolecular composite systems. The thermal stability test demonstrates the EC/DATP (90/10) system has a higher initial temperature of thermal degradation than that of pure EC, indicating the formation of a better supramolecular system in this composite film. The incorporation of DATP strengthens the flexibility of the EC films. With the addition of DATP, the elongation at the break of EC films increases, but the tensile strength and elastic modulus decline. The elongations at break of EC/DATP (60/40) and EC/DATP (50/50) increase by nearly 7.0 times from that of pure EC film. The EC/DATP films possess a lower storage modulus and T_g than pure EC. The T_g of the EC films will have broad applications because of their controllable mechanical properties and flexibility. These supramolecular films can partially substitute traditional fossil-based thermoplastic films and are expected to be used in packaging, energy, chemical industry, materials, agriculture, medicine, and other fields.

Funding Statement: This work was supported by Jiangsu Province Biomass Energy and Materials Laboratory, China (Grant No. JSBEM-S-202007).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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DOI: 10.32604/jrm.2022.024160

ARTICLE





Phosphorus Containing Rubber Seed oil as a Flame Retardant Plasticizer for Polyvinyl Chloride

Hongying Chu^{1,2,*}, Huabei Li^{1,2}, Xiaoyan Sun¹ and Yaowang Zhang^{1,2}

¹Yellow River Conservancy Technical Institute, Kaifeng, 475004, China
²Henan Engineering Technology Research Center of Green Coating Materials, Kaifeng, 475004, China
^{*}Corresponding Author: Hongying Chu. Email: hongyingchu@163.com
Received: 28 May 2022 Accepted: 18 July 2022

ABSTRACT

The application of phthalate plasticizers has been restricted around the world due to their poor migration and potential harm to the human body. Hence, producing functional bio-based plasticizers via exploiting clean and reusable resources meets the satisfaction of current demands. In this study, flame-retardant rubber seed oil-based plasticize (FRP) was prepared via epoxidation reaction and ring opening addition reactions, which was used as a flame-resistant plasticizer for polyvinyl chloride to replace petroleum-based phthalate plasticizer. When DOP was replaced with FRP, the torque of PVC blends increased from 11.4 to 18.4 N·m, the LOI value increased from 24.3% for PVC-FRP-0% to 33.1% for PVC-FRP-20. The THR value diminished from 39 MJ/m² (pertaining to PVC-FRC-0) to 22 MJ/m² Tg increased from 23°C to 47°C, the weight loss of plasticized PVC blends significantly reduced from 22.6% to 2.8% in leaching tests. The study provided a new way to prepare flame retardant plasticizer using rubber seed oil as raw material.

KEYWORDS

Plasticizer; rubber seed oil; polyvinyl chloride; vegetable oil modification



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Graphic Abstract



Nomenclature

DOP	Dioctyl Phthalate
FRC	Flame-retardant rubber seed oil-based plasticizer
PVC	Polyvinyl chloride
RSO	Rubber seed oil
LOI	Limiting oxygen index
ERSO	Epoxy rubber seed oil
HRR	Heat release rate
THR	Total heat release (THR)
T _d	Temperature at 5% weight loss
T ₁₀	Temperature at 10% weight loss
T ₅₀	Temperature at 50% weight loss
Tg	Glass transition temperature

1 Introduction

Plasticizer has been widely used in the processing of polymers such as polyvinyl chloride (PVC) plastics. The most studied, most abundant, and perhaps most well-known plasticizers that are also emerging contaminants are phthalate plasticizer. The phthalate plasticizer derived from petroleum is the

most widely used plasticizer in industrial production, but its application is increasingly limited in view of its potential menace to the surrounding environment even human health [1-3]. With the increasingly stringent global environmental protection regulations, environmental protection and safety have become a significant trend in the development of the plasticizer industry. Bio-based plasticizers specially used in the fields of medicine or health equipment, packaging, as well as environmentally friendly plasticizers with enhanced mechanical properties have been continuously developed [4–8]. Many bio-based plasticizers have been reported including isosorbide [9], cardanol [10–12], castor oil [13–15], lactic acid [16,17], and many kinds of vegetable oils [18–20].

Rubber seed oil (RSO) is extracted from rubber seed. The main component of RSO is triglyceride [5,21]. Bio-based plasticizer from RSO can be synthesized via esterification, epoxidation, and halogenation hydrolyzation reactions. Not only can the polar groups inside, including ester groups and ether groups, enhance the compatibility of plasticized PVC materials, but they also optimize physical property of PVC products in some respects [22,23]. Thus, reactions on RSO are carried out in order for the bio-based plasticizers synthesis, which can be taken as a promising and strategic research.

In this study, the rubber seed oil with rich reserves, wide sources and low price was selected as the raw material. The rubber seed oil was modified by epoxidation and ring opening addition reaction to synthesize the bio-based plasticizer products, which were used to replace the petroleum-based phthalate plasticizer; The mechanical properties, thermal properties, processability, flame-retardancy and extraction resistance of plasticizer for phthalate plasticizers is studied to reduce the dependence of the plastic additives industry on petrochemical products.

2 Experimental

2.1 Materials

Dioctyl phthalate (DOP, 98%), diethyl phosphite (99%), N-hexane (97%), toluene (98%), triphenyphosphine (95%), dichloromethane (99%), ethyl acetate (99%), calcium stearate (Zn 10%–12%) and sodium chloride (99.5%) were bought from Aladdin reagent (Shanghai) Co., Ltd. (China). S-1100 PVC resin (no additives) was provided by Jinling Chemical Industry Co. (Nanjing, China). Rubber seed oil (RSO) was provised by Kuming Jinning Chemical Industry Co., Ltd. (China).

2.2 Synthesis of Epoxy Rubber Seed Oil (ERSO)

RSO was dissolved in dichloromethane and put in the a round bottom flask, which was put in the ice bath. 3-Chloroperoxybenzoic acid was dropped in the mixture (mole ratio of double bonds to 3-Chloroperoxybenzoic acid is 1:0.5). The reaction was kept stirring for 5 h at room temperature to obtain the raw product. The obtained raw product was dissolved in dichloromethane and washed with brine and distilled water, then dried by anhydrous magnesium sulfate (MgSO₄) and purified using silica gel column chromatography to obtain ERSO.

2.3 Synthesis of Flame-Retardant Rubber Seed Oil-Based Plasticizer (FRP)

ERSO (20 g) was dissolved in toluene (16 g), all of which were blended in a three-necked round-bottom flask. The mix of diethyl phosphate (6 g), triphenyphosphine (0.02 g) and toluene (16 g) were also put in the flask within 40 min at room temperature. Then, a 4 h-stir at 75°C was carried out. The reaction mixture was dropped to room temperature with sodium hydroxide (NaOH) solution and washed until pH = 7. FRP was obtained via removing water by rotary-evaporation, after a 3-time-wash through distilled water. The synthesis of FRP was showed in Fig. 1.



Figure 1: Synthesis of FRP

2.4 Preparation of FRP-Plasticized PVC Blends

FRP-Plasticized PVC blends were prepared by melt processing and the formulations were illustrated in Table 1. Then, further extrusion into strips like dumbbell or rectangular was necessary for subsequent tests.

Table 1: Detailed formulations of FRP-plasticized PVC blends

PVC blends	PVC (g)	DOP (g)	FRP (g)	Calcium stearate (g)
PVC-FRP-0	100	40	0	2
PVC-FRP-5	100	35	5	2
PVC-FRP-10	100	30	10	2
PVC-FRP-15	100	25	15	2
PVC-FRP-20	100	20	20	2

2.5 Characterization

RSO, ERSO and FRP were respectively illustrated through Fourier transform infrared spectrometer (FT-IR) (Nicolet 6700), with the chosen wavelength between 4000 and 500 cm⁻¹.¹H nuclear magnetic resonance (¹H NMR) spectrums were presented with the solvent of CDCl₃ and internal standard of tetramethylsilane (TMS), respectively. Combustion tests were carried out according to GB/T 2406.1-2008. The FRP-modified PVC samples were designed into 80 mm × 10 mm × 4 mm. FTT200 cone calorimeter was used to analyze the combustion properties of samples that were cut into 100 mm × 100 mm × 2 mm, and ISO-5660 standard procedures would be referenced. The thermogravimetric analysis (TGA) was detected with surrounding N₂ in the flow rate of 50 mL/min. The heating rate of PVC blends was set to 10 °C/min from the beginning of 50°C to the final 600°C. The leaching tests related to plasticizers were examined in term of ASTMD1239-98 with N-hexane as the chosen solvent. The PVC blends were submerged into solvents at the temperature of 30°C for one week. Thereafter, the samples were removed and the surface solvent was wiped off. Then further drying was conducted at 50°C for whole day. The migration rate was calculated in term of the following Eq. (1):

Migration rate = $(W_1 - W_2)/W_1$

Thereinto, W₁ and W₂ represent the front and rear weight, respectively.

In addition, the tensile rate of electronic tensile testing machine was set to 20 mm/min, and the dynamic mechanical analysis (DMA) was brought into effect to investigate the specific glass transition temperature (T_g) , from -80° C to 100° C at a heating speed of 3 °C/min. Limiting oxygen index (LOI) values were measured on a JF-3 oxygen index measuring instrument.

3 Results and Discussions

3.1 Synthesis of FRP

FTIR spectra of RSO, ERSO and FRP were shown in Fig. 2. The characteristic peaks around 3007 cm⁻¹ were associated with -CH=CH- in RSO [24,25]. As Compared with the spectra of ERSO, the characteristic peak of CH=CH- vanished, opposite to the -C-O-C vibration which could be observed at approximately 972 and 1062 cm⁻¹. All mentioned indicated that the -C=C- double bonds were converted into epoxy groups [26,27]. The peak at 1032 cm⁻¹ was attributed to -P-O-CH- symmetric bending vibration, and -OH was observed at 3470 cm⁻¹ in the FT-IR of FRP. The epoxy group at 972 and 1062 cm⁻¹ cannot be observed in the FT-IR of FRP. The epoxy group at 972 and 1062 cm⁻¹ cannot be observed in the FT-IR of FRP.



The ¹H NMR spectrums pertaining to RSO, ERSO and FRP are exhibited in Fig. 3. The strong peak at δ = 5.28 ppm is attributed to the protons of -CH=CH- in RSO [25,26]. When epoxidation was finished, the peak at δ = 5.28 ppm became weak in the ¹H NMR spectrum of ERSO, because the epoxidation reaction was not complete, and an emerged peak at 2.95–3.20 ppm was assigned to protons on the epoxy groups of ERSO [26,27], which indicated that some -CH=CH- bonds of RSO were converted into epoxy groups. In the ¹H NMR spectrum of FRP, a new proton signal of -P-O-CH₂- appeared at δ = 2.3 ppm [28,29], and the protons signals in the 5.28 ppm associated with -C-O-C- bonds showed weaker than ERSO, which indicated that chemical structure of FRP contains phosphate groups, which indicated that FRP was synthesized successfully.





3.2 Performance of Plasticized PVC Blends

The melting behavior was confirmed in the Haake chamber by gathering the processing data during the mix. Fig. 4 presents the torque values relevant to the proportion of plasticizer for the 5 min processing time. When more DOP was replaced with FRP, the torque of PVC blends increased from 11.4 to 18.4 N·m, respectively, corresponding to PVC-FRP-0 and PVC-FRP-20. In other words, 61% augmentation could be achieved, showing disadvantages in conduction of thermoplastic processing pertains to PVC blends.



Figure 4: The torque data of plasticized PVC blends

FRP contains many flame-resistant phosphate groups, which could be used as a alternative to DOP as the flame-resistant plasticizer. The flame-resistance of plasticized PVC blends was conducted by the LOI tests and the variation with regard to FRP content was described in Fig. 5. When DOP was replaced with FRP in plasticized PVC blends, the LOI value increased from 24.3% for PVC-FRP-0% to 33.1% for PVC-FRP-20. Only the latter one exhibited greater value than 26.0%, to a certain extent, displaying self-blackout capacity. Generally, FRP is not suitable to be considered as a preferable flame retardant plasticizer in the PVC blends [30,31].



Figure 5: LOI data for plasticized PVC blends

PVC chemical structure contains a large amount of chlorine, which makes it hard to burn. However, it will combust and release much heat when PVC products plasticized with DOP, being blended into PVC. Fig. 6 showed the heat release rate (HRR) curves of plasticized PVC blends. There exists a notable difference in HRR curves between PVC-FRC-0 and PVC-FRC-20. Almost all HRR curve of PVC-FRC-0 is higher than PVC-FRC-20, which indicats that PVC-FRC-0 released heat faster than PVC-FRC-20.

Once ignited, PVC-FRC-0 produced flammable volatiles and released a lot of heat. However, the PPVC-FRC-20 was harder to burn than PVC-FRC-20. Thermal degradation of phosphate ester groups containing FRP produces a large amount of phosphoric acid and polyphosphate [31–33], which can promote carbon formation by removing water from FRP. The char residue will block heat transfer and the pyrolysis process. In addition, the total heat release (THR) curves of plasticized PVC blends were shown in Fig. 7. It can be found that the whole THR curve of PVC-FRC-0 was higher than PVC-FRC-20, which indicated that PVC-FRC-0 released more heat than PVC-FRC-20. The THR value decreased from 39 MJ/m² (pertaining to PVC-FRC-0) to 22 MJ/m² (pertaining to PVC-FRC-20) when 20 phr DOP was replaced with FRP in the plasticized PVC blends. All mentioned above revealed that FRP had capacity of improving the flame retardancy.



Figure 6: HRR curves of plasticized PVC blends



Figure 7: Total heat released of FRP-plasticized PVC blends

The thermogravimetric loss data of plasticized PVC blends with different mass of plasticizers were shown in the Figs. 8 and 9, and Table 2. As is demonstrated in the Fig. 8, the degradation temperature ($T_{d:}$ Temperature at 5% weight loss) of PVC blends increased with more DOP replaced by FRP, from the

initial content of 5% FRP. When PVC blends plasticized with 40 phr DOP, T_d is 252°C, while the T_d reached 272°C when PVC blends plasticized with 20 phr DOP and 20 phr FRP. In addition, the temperature at 10% weight loss (T_{10}) and the 50% one (T_{50}) showed the same increasing trend when DOP was replaced with FRP. This indicated that FRP can improve thermal stability of PVC blends. All plasticized PVC blends presented 3 stages in N₂ with regard to thermal degradation curves. None of the samples illustrated conspicuous weight loss until approaching 150°C. At stage one (from 150°C to 320°C), the weight loss process could be ascribed to the FRP migration together with the PVC dechlorination [34,35]. The temperature range belonging to the second stage was 320°C–450°C, which is mainly understood as the conversion process of conjugated olefins to aromatics. Finally curves between 450°C and 600°C corresponding to the fact that polyenes were cracked into olefin, as the third stage came out [34,35]. FRP had a considerable effect on preventing the weight loss of plasticized PVC blends. There exist numerous polar groups in plasticizer molecules that form hydrogen bonds with α -H of the PVC chain, deferring the PVC dechlorination [36,37].



Figure 8: TGA curves of FRP-plasticized PVC blends



Figure 9: DTG curves of FRP-plasticized PVC blends

PVC blends	T_d (°C)	T ₁₀ (°C)	T ₅₀ (°C)	T_{g} (°C)
PVC-FRP-0	252	261	292	23
PVC-FRP-5	256	267	301	29
PVC-FRP-10	261	274	304	36
PVC-FRP-15	266	278	310	39
PVC-FRP-20	272	281	318	47

Table 2: The TGA data of PVC blends

 $T_{\rm g}$ partly mirrors the plasticizing property of the plasticizers. Fig. 10 shows the DMA curves of plasticized PVC blends containing different masses of FRP. Each curve has only a single peak, corresponding to a homogeneous system. It has been reported that the T_g value of the original PVC is approximately 85.8°C, indicating that adding DOP or FRP to PVC can prominently reduce the T_g value. When DOP was replaced with FRP gradually, T_g increased from 23°C to 47°C, which indicated that plasticized ability of FRP was lower than DOP.



Figure 10: DMA curves of FRP-plasticized PVC blends

Extraction resistance is an important quality in evaluating the migration property of plasticizers. The excellent extraction resistance can maintain the long-term stability of mechanical, thermal, electrical properties and flame-retardancy of plasticized polymer products, and prevent the potential threat to human health caused by the migration of plasticizers in the use of food and medical devices. Fig. 11 manifests the weight loss of plasticized PVC with DOP and FRP in N-hexane, the advantage of using N-hexane as solvent is that organic solvent can accelerate the migration of plasticizer, and then its migration performance can be evaluated in a short time [5]. With the gradual decrease of DOP and increase of FRP in PVC blends, the weight loss of plasticized PVC blends significantly reduced from 22.6% to 2.8%. FRP showed superior solvent extraction resistance than that of DOP. FRP with a larger relative molecular weight and higher branching degree has better migration resistance than DOP.

The tensile test was conducted at the temperature of 25°C to appraise FRP's plasticizing effect, with the detailed results being exhibited in Table 3. The affixion of plasticizer usually increases elongation at break and decreases the tensile strength of plasticized PVC materials. Furthermore, when DOP was replaced gradually with FRP, the elongation at break of plasticized PVC blends decreased from 450% to 19%,

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seeing an opposite trend to the tensile strength, from 12.3 to 28.9 MPa. The results indicated though the ester groups and phosphate groups in the structure of FRP have the lubrication effect on PVC chains, making plasticized PVC blends more flexible [38,39], but FRP showed a lower plasticizing effect than DOP.



Figure 11: Migration property of FRP

Table 3: The detailed mechanical parameter of PVC blends with different contents of

PVC blends	Tensile strength (MPa)	Elongation at break (%)
PVC-FRP-0	12.3 ± 2.0	450 ± 25
PVC-FRP-5	16.8 ± 1.5	238 ± 13
PVC-FRP-10	21.0 ± 2.1	189 ± 9
PVC-FRP-15	23.1 ± 1.5	89 ± 6
PVC-FRP-20	28.9 ± 2.3	19 ± 3

4 Conclusions

In this study, phosphorus-containing rubber seed oil was prepared via epoxidation reaction and ring opening addition reactions, which was used as a flame-retardant polyvinyl chloride plasticizer to replace petroleum-based phthalate one. In plasticized PVC blends, the torque of PVC blends increased from 11.4 to 18.4 N·m after DOP was replaced by FRP at the content of 20 phr, which indicate the addition of FRP is unfavorable to the thermoplastic processing process. But the LOI value, at the same time, increased from 24.3% to 33.1%, and the THR data decreased from 39 to 22 MJ/m², which indicated that FRP in the plasticized PVC blends reduced the flammability. As a result of hydrogen bond formation, with the coaction of α -H in PVC molecular chains and polar groups in plasticizers, the dichlorination of PVC will be intervened to delay. With the addition of DOP or FRP, PVC can significantly raise the T_g value from 23°C to 47°C. It means the plasticized ability of FRP was lower than DOP, though FRP still has the potential to be a flame retardant and secondary plasticizer for PVC materials. FRP with the larger relative molecular weight and higher branching degree has better migration resistance than DOP. When DOP was replaced gradually with FRP. In all, the bio-based plasticizer from RSO can be used as a flame-retardant plasticizer to partly replace DOP in PVC blends.

Funding Statement: This work was funded by the Science and Technology Project of Henan Province (202102310593) and Science and Technology Project of Kaifeng City (2002003).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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DOI: 10.32604/jrm.2022.023669

REVIEW





Research Progress of Tung Oil/UV Photocomposite Curing Material

Zicheng Zhao^{1,2}, Zhihong Xiao², Xudong Liu², Desheng Kang³, Wenzheng Dong¹, Qiquan Lin^{1,*} and Aihua Zhang^{2,*}

¹College of Mechanical Engineering and Mechanics, Xiangtan University, Xiangtan, 411105, China
²State Key Laboratory of Utilization of Woody Oil Resource, Hunan Academy of Forestry, Changsha, 410000, China
³Hunan Xiangchun Agricultural Technology Co., Ltd., Changsha, 410000, China
^{*}Corresponding Authors: Qiquan Lin. Email: xtulqq@163.com; Aihua Zhang. Email: zhangaihua909@163.com
Received: 09 May 2022 Accepted: 18 July 2022

ABSTRACT

Tung oil/UV photocomposite curing material has the advantages of low curing temperature, low material shrinkage and low environmental pollution. Therefore, it is potentially significant to be developed and utilized. Using the main structure of refined tung oil α -chemical structure characteristics of conjugated double bonds contained in the molecular long chain of tung acid, it can be modified by many chemical methods to develop a new Tung oil/UV photocomposite curing material. This paper reviews the research progress of Tung oil/UV photocomposite curing materials in recent years. Firstly, the chemical structure and application of tung oil and the UV Photocatalysis Technology were briefly introduced. Then, the research status of novel Tung oil/UV photocomposite curing materials developed by Diels-Alder reaction was discussed. The method and curing effect of UV curing system constructed by other chemically modified tung oil were also discussed. Next, the application of tung oil in industrial production is introduced from four directions: the application of tung oil in biodiesel, the application in synthetic resin, the application in self-healing coating and microcapsules and other applications. Finally, the research and application prospect of Tung oil/UV photocomposite curing materials were viewed.

KEYWORDS

Biomass; UV-curing; tung oil; diels-alder reaction; chemical modification

Nomenclature

Term 1	Interpretation 1
Term 2	Interpretation 2
Ø	Porosity
s	Skin factor

1 Introduction

With the increasing depletion of global petrochemical resources, new biomass energy is gradually becoming an important strategic puzzle in the field of resources and energy in various countries. As an important renewable biomass energy, woody oil has gradually replaced the traditional petrochemical products and become the raw material for producing many chemical products. There are many kinds of



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woody oils, and its fatty acid chains usually contain some functional groups that can give them special chemical properties, such as conjugated double bonds, ester groups and hydroxyl groups [1,2] For example, castor oil contains multiple hydroxyl groups, flax oil and tung oil contain unsaturated double bonds. Over the years, the continuous advancements of woody oil have developed many new application fields of woody oil and obtained a variety of new derivatives. These new fields and products continue to make up for the shortage of many non-renewable resources in social development. China is rich in woody oil-based resources. Over the years, predecessors have carried out a lot of basic research on biomass materials on some natural renewable resources, such as soybean oil, castor oil and tung oil. These renewable resources have long become a hot spot in the research of biomass materials. Tung oil, as a rich and unique woody oil in China, has high development and production value. Tung oil has higher chemical activity than other woody oils because of its unique multi chain conjugated triene structure. Over the years, researchers have mainly carried out anti-corrosion paint or chemical grafting on tung oil to fabricate low molecular weight polymer, most of them are processed and utilized by thermal curing technology [3]. Compared with SLA (Stereo Lithography Appearance) light curing technology, thermal curing technology requires a large amount of chemical reagents and energy consumption in the reaction process, which does not meet the requirements of green development in the current ecological environment. At the same time, the development of photosensitive materials is also the core of SLA technology. Based on the above background, the combination of renewable tung oil with the efficient and environmentally-friendly UV curing technology can effectively open up a "green" application road of tung oil. On account of the basic research of tung oil and its derivatives at home and abroad, the research progress of new UV curing materials based on tung oil was introduced in detail.

2 The Overview of Tung Oil

Tung oil, is a plant of the genus Aleurites in Euphorbiaceae, which is the seed of Vernicia fordii (Hemsl.) Airy Shawy are pressed to get refined vegetable oil. Tung oil has been used as a protective coating for wood for thousands of years, which were widely used in varnish [4], resin [5,6], polyurethane [7] and other aspects. Tung oil has the characteristics of fast drying speed, light specific gravity, good gloss, strong adhesion, acid resistance, alkali resistance, heat resistance, rust resistance, corrosion resistance, and non-conductive [8,9]. Tung oil is also an economic value, high application value of degradable biomass resources, in biodiesel [10-12], tougheners [13], self-healing coatings [14–19], self-healing microcapsules [20–24], solubilizers [25], adhesives [26] and other processing and manufacturing have its wide applications, can also be processed into films [27], soaps, pesticides, pesticides and so on. Tung oil as the main raw material for the manufacture of grease paint. Such as alkyd resin paint, epoxy resin paint, phenolic resin paint modifier. For example, tung oleic acid and epoxy resin E-20 or E-12 reaction can produce growth, medium and short oil degree of epoxy resin, for electrical insulation metal primer, equipment plant anti-corrosion and so on. It has good weather resistance, corrosion resistance, can be white drying, strong adhesion to metals and other advantages. Tung oil modified unsaturated polyester (UPR) has good air-drying. It overcomes the defect that the surface of general unsaturated polyester products or coatings cannot be dried due to the resistance of O₂ in the air, can be used in FRP, coatings and atomic ash basal resin. Under alkaline conditions, tung oil modified phenolic resin and cotton or wood pulp cellulose paper made of laminate material, the product has high heat resistance, low water absorption, excellent insulation properties and high mechanical strength and easy processing performance, can be applied to the coated board industry. In addition, tung oil also has good, ink, ship, cardboard waterproof performance, widely used in construction [28], coatings, inks, ships, cardboard [29,30], nano composite materials [31] and furniture wood [32] and other aspects.

The main component of tung oil is triglyceride [33], it mainly contains carbon double bond, ester group [34]. Its structural formula is shown in Fig. 1, it has the structural basis for chemical reactions such as

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Diels-Alder reaction [35,36], Foucault alkylation reaction [37], transesterification reaction, etc. Berhoic acid is an unsaturated long-chain fatty acid with three conjugated double bonds, systematically named 9,11,13-octadecatrienoic acid. According to the difference in the spatial configuration of the three double bonds, the α -niamic acid of the tung acid and the β -niamic acid of the cis, trans, trans-configuration of the α -niacin and the trans, trans-configuration of the tung acid can be identified by infrared, ultraviolet, Raman and other means to identify the conjugate structure, spatial configuration and number and position of the double bonds [38], in addition, the chemical properties of α -niacin acid are relatively active, and it is easy to isomerize into β -niamic acid under heating conditions.

 $\begin{array}{c} CH_2-OOCH_2-(CH_2)_7-CH \equiv CH-CH \equiv CH-CH \equiv CH-(CH_2)_3-CH_3 \\ | \\ CH-OOCH_2-(CH_2)_7-CH \equiv CH-CH \equiv CH-CH \equiv CH-(CH_2)_3-CH_3 \\ | \\ CH_2-OOCH_2-(CH_2)_7-CH \equiv CH-CH \equiv CH-CH \equiv CH-(CH_2)_3-CH_3 \end{array}$

Figure 1: Chemical structure of the triglyceride in tung oil

The structural formula of berhoic acid is shown in the Fig. 2:



Figure 2: Molecular formula of berhoic acid

China has always been in the forefront of the world in the production and exportation of tung oil. According to the data of China's National Bureau of Statistics, the output of tung oil seeds in China reached more than 450,000 tons in 2018. According to the annual statistics of China Customs in 2017, the exportation volume of China's tung tree was more than 6000 tons, and the export trade volume are as high as US \$1.9 million. Compared with that in 2016, the export volume increased by approximately 45% and the trade volume increased by approximately 30%. However, at this stage, most of China's export and processing trade products are mainly low-cost crude oil, and the yield quality between plants is very different, the yield and quality of tung oil are significantly affected [39]. In view of the above situations, efficient processing and utilization of tung oil to improve its valorization potential and economic benefits is a problem to be solved at this stage.

Tung oil is a kind of dry oil. It is also a kind of vegetable oil, rich in unsaturated fatty acids. It could be self-oxidized at room temperature or heated to achieve drying and curing [40]. The self-oxidation mechanism of unsaturated oil was in the initial stage of reaction, where allyl position on fatty acid chain α -H was easily removed by hydrogen extraction reaction to form free radicals, which then react with oxygen to form hydroperoxides [41]. These hydroperoxides were unstable and easy to be decomposed to form stable alkoxy radicals. In particular, metal driers can also be used to promote the formation and decomposition of hydroperoxides. Finally, the free radical in the system initiate oxidative polymerization, which further increase the molecular weight of the system, so as to realize curing [42]. For a long time in the past, raw tung oil and boiled tung oil were widely used as protective coatings. Boiled tung oil was made by boiling Raw tung oil at high temperature. At high temperature, tung oil changed chemically and increased its chemical activity. Therefore, the cured film of cooked tung oil has good heat resistance and corrosion resistance [43]. When boiled tung oil was used as an anti-corrosion material for wood, it can not only maintain excellent gloss, but also play a good moisture-proof role. Therefore, cooked tung oil was one of the important coatings for the protection of Chinese ancient buildings and cultural relics.

Tung oil has conjugated double bonds and is prone to undergo oxidative polymerization. It was found that it cannot only cure and crosslink at room temperature, but also undergo free radical polymerization and cationic

polymerization under UV light [44,45], especially in light curing coatings [46,47]. It was found that when the addition amount of tung oil was 20%, adding 50% epoxy resin, 25% active diluent, acryl morpholine, 5% three aryl Sulphur salt and 1% free radical photo initiator, benzoin two ether, the film prepared by UV curing had a gel rate of 93.25% and excellent mechanical properties [48]. In addition, tung oil can also be used to prepare plastics [49], biodiesel [50], microcapsule [51] and so on. Tung oil can also be used as the matrix of resin modification and mixed with other raw materials to prepare tung oil based coatings by chemical modification [52]. Wang et al. [53] first prepared hydroxyl derivatives from tung oil. Then through the reaction with toluene diisocyanate (TDI) and polyester polyol, and dihydroxymethylpropionic acid (DMPA) as chain extender, tung oil based waterborne polyurethane with excellent water resistance, thermal properties and mechanical properties was prepared. In conclusion, tung oil has been widely used in paint and other related fields [54]. Most of these applications use high-temperature heating and chemical modification to make tung oil participate in curing, while there are few studies on curing with environment-friendly and efficient UV light [55].

3 Introduction to UV Photocatalysis Technology

Photocatalysis referred to the process of using light as a source of energy, the photocatalyst absorbs a certain range of wavelengths of energy, converts from the ground state to the excited state, and then triggers a series of chemical reactions to generate new compounds, or converts into intermediates to catalyze the progress of the reaction.

3.1 Introduction to UV Curing

Ultraviolet curing technology (UV curing) is a kind of advanced material surface treatment technology originated in the 1960s, generally using high-energy ultraviolet light (325 nm) as a curing energy source, the photo initiator in the liquid system absorbs a certain range of ultraviolet wavelengths of light energy, cracking to produce free radicals or cationic radicals, and then trigger a series of chain chemical reactions between the system containing unsaturated double bonds, epoxy groups, vinyl ethers and other substances, so that liquid materials with reactive activity were rapidly crosslinked and polymerized , a new technology that instantly solidifies into solid materials, with broad application prospects [56]. UV curing technology is because of its fast curing, energy saving, environmental protection, economy, wide applicability and other characteristics, has been widely used in printing, packaging, advertising, building materials, electronics, communications, home appliances, automotive, aerospace and other industries.

3.2 Characteristics of UV Light Curing

Ultraviolet light curing technology was summarized as -5E green environmental protection industrial new technology, that is:

Efficiency (fast curing rate, can be cured in a few seconds to tens of seconds, or even as fast as 0.1 s to complete the curing), Energy (room temperature curing, the principle is that only need to be used to excite the photo initiator excitation of radiation energy can be, do not need high temperature heating, its energy consumption is only between one-tenth to one-fifth of the heat curing product), Enabling (light curing products can be applied to a variety of matrix materials, such as paper, wood, plastic, metal, leather, stone, glass, etc., some heat-sensitive materials such as electronic components are also suitable for them. And the scope of application of its products is also very extensive, such as wood, anti-corrosion, electronics, construction, etc.), Ecology (traditional coatings in order to reduce viscosity generally contain volatile organic compounds, and most of them are carcinogenic, and the reason why the light curing products are in line with green environmental protection is because it basically does not contain volatile solvents, most of the active diluents used are compounds with high boiling points, and when curing, they can participate in crosslinking polymerization and become part of the cross-networked structure. Greatly reducing environmental pollution and harm to the human body), Economy (light curing products have high production efficiency, low energy consumption, low equipment investment, small construction

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footprint, easy to achieve flow-through production; at the same time, due to their low pollution, the cost of processing pollution is relatively low)

Although UV coatings have many advantages, but there are also its shortcomings, such as: UV paint curing speed was fast, cured into a film within a few tenths of a second, its film-forming shrinkage rate was larger, the internal stress was correspondingly larger, so the adhesion of the coating is poor. In addition, ultraviolet curing also has certain damage to the human body, ultraviolet radiation converts oxygen in the air into ozone, which in turn causes headaches and fatigue.

3.3 Composition of the UV Light Curing System

In the UV curing system, the main components generally include: photo initiators, reactive diluents (also known as monomers or functional monomers), reactive oligomers (oligomers), pigment fillers and various additives.

- (1) Photo initiators can be divided into two categories: one is the photo initiator of free radical types, such as acetophenone, benzophenone, thiazone, benzother, etc.; the other is the photo initiator of the ionic type, mainly cationic type, such as aromatic diazole salts, ferrocene aromatic hydrocarbon complexes, iodine salts and sulfur salts, etc. [57,58], among which the most representative of the gilded salt photo initiator, especially important is that the cationic photo initiator is basically not affected by the inhibition of oxygen, and can be quickly polymerized and cured, while the cationic photo initiator has the advantages of small volume shrinkage, strong adhesion, fast curing speed, good depth curing, low toxicity and other advantages in recent years [59]. In addition, free radicals and cationic intercompatibility also have certain advantages, free radicals can promote cation polymerization, further enhance the depth and strength of curing [60,61], while the cationic photo initiator involved in the ring-opening polymerization reaction so that many monomers that could not have polymerization, such as cyclic vinyl ethers, oxygen compounds, etc., can also undergo photopolymerization reactions [62], greatly broadening the scope of application of light-curing materials.
- (2) Reactive diluent commonly referred to as monomers or functional monomers, it is an organic small molecule substance containing polymerizable functional groups, mainly used to dissolve the solid components in the light curing system (such as photo initiators), and to adjust the viscosity of the system. According to the number of functional groups contained in its molecules, the reactive diluent can be divided into monofunctional groups, bifunctional groups and polyfunctional groups.
- (3) Reactive oligomer also known as photosensitive resin, it is a relatively low molecular weight photosensitive material, with a variety of high curing activity of the reactive group, such as a variety of unsaturated double bonds, hydroxyl and epoxy groups. In the various components of the light curing system, oligomer is the most important body, its performance determines the main performance of the cured product, so the selection and design of oligomers is the most important part of the light curing product formula [63].
- (4) Additive such as Pigmen is a colored substance with a fine powder, uniformly dispersed in the binder of the coating or ink, coated on the surface of the substrate to form a color layer, thereby presenting a certain color. Additives used to improve the performance of photosetting coatings and inks in manufacturing, construction applications, and transportation and storage. The additives commonly used are defoamers, leveling agents, dispersants, matting agents, etc. [64].

4 Study on Tung Oil/UV Photocomposite Curing Material

4.1 Study on Catalyst-Free UV Curing of Tung oil

It was found that [65] tung oil was affected by oxygen concentration in the process of light curing. Under normal temperature and heating conditions, tung oil could not be solidified into film without oxygen; with

UV light, free radical photo-initiator could not be solidified tung oil into film isolated from oxygen, while cationic photo-initiator can solidify tung oil into film. As for the effect of oxygen, it was mainly because tung oil was prone to undergo hydrogenation reaction after being exposed to light. The free radical formed after dehydrogenation forms hydroperoxide with oxygen, which promotes the oxidative polymerization of tung oil and further promotes the curing. Some results showed that tung oil can form a large number of free radicals and initiate the polymerization of double bonds and other UV curing components under the environment of aerobic and UV light irradiation. Further analysis showed that the cross-linking reaction (double bond and free radical) and oxidative polymerization of tung oil mainly occurred in the process of photopolymerization (α -H), the polymerization mechanism was shown in Fig. 3.



Figure 3: A schematic showing the mechanism of UV-induced polymerization of tung oil [65]

The results showed that the cured film formed by direct UV curing of tung oil will produce wrinkles on the surface, which was mainly caused by the rate difference in the curing process, and the curing of the surface layer and the bottom layer was not synchronous, so the rate difference was generated. It was further found that the UV curing material obtained by chemical modification has excellent film-forming properties by mixing tung oil with epoxy acrylate (EA) and adding measured acryloylmorpholine (AC-MO), triaryl sulfonium salt (TAS) and benzoin dimethyl ether (PI-BDK). SEM scanning electron microscope observation of the cured material showed that the surface of the composite UV curing film is glossy and smooth. It was found that the main reason is that epoxy acrylate (EA) promotes the reaction at the bottom of the coating, making the previous rate difference disappear. Therefore, the combination of epoxy acrylate and tung oil can improved the UV curing effect of tung oil. In order to keep the original chemical structure of tung oil, physical blending was used in the experiment. Without chemical change, the operation is simple and the product can be used directly. This method undoubtedly provides a new idea for the application of tung oil in the field of UV curing coatings. Zhang et al. [66] found that tung oil has light curing activity and can achieve light curing and curing time under ultraviolet light irradiation much faster than the thermal curing and room temperature curing of tung oil, and tung oil was affected by oxygen concentration in the process of light curing. Under normal temperature and heating conditions, without oxygen, and tung oil cannot be cured into a film; under UV light without oxygen and free radical photo initiators cannot solidify tung oil into a film, while cationic photo initiators can promote tung oil to cure into a film. Epoxidation of tung oil referred to the reaction of tung oil with epoxylating reagents under certain conditions, forming an oxygenated ternary ring in the position of double bond, but there may be other side reactions in the process of reaction, such as open ring, acidolysis and so on. Tung oil treated with epoxidation can be reacted with a variety of amine compounds to open the ring, and then prepare tung oil-based polyols containing functional groups such as amino or hydroxyl groups in the structure. Cao et al. [67] developed a new epoxy modified tung oil water-based insulation paint, which uses a closed hexamethylene diisocyanate as a curing agent. Zhao [68] prepared methyl tungate by two

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steps using tung oil as raw material, first using p-methylbenzenesulfonic acid as a catalyst and tetrahydrofuran as a solvent, and synthesizing methyl tungate through the transesterification reaction of tung oil and methanol; then using concentrated sulfuric acid as the catalyst, with H_2O_2 solution and glacial acetic acid as the oxidant, methyl oxytung acid was prepared.

It can be seen from the above that in the catalyst-free reaction system, the directly cured film will have problems such as size and smoothness, and the influence of side reaction products in the reaction needs to be considered. In the follow-up work, it is necessary to comprehensively strengthen the consideration of experimental factors, such as reaction substrate, oxygen conditions, etc.

4.2 Study on Diels-Alder Modified Tung Oil/UV Photocomposite Curing Material

The D-A reaction, also known as the diene addition reaction, refers to a compound with a conjugated olefin structure reacting with an olefin or alkyne compound to form a hex acyclic ring structure. Since the molecular long chain of tung oil has a structure of trans-anti-conjugate double bonds, the Diels-Alder reaction can occurred with the dipolefins with the double bond structure [69].

Since tung oil is an unsaturated triglyceride with three long chains, each long chain contains three conjugated double bonds, so the Diels-Alder reaction in which it participates is a very complex process.

Its reaction types are roughly divided into three categories:

- (1) Tung oil itself occurs as a pro-dipene and conjugate dienes between molecules Diels-Alder reaction;
- (2) Tung oil, as a dipienogen, reacts with another conjugated dienes-Alder;
- (3) Tung oil acts as a conjugated diene and reacts with other dipienogens.

The conjugated double bond in triglyceride of tung oil is very easy to undergo Diels-Alder addition reaction with dienophiles and addition reaction with dienophiles containing different active groups, which can produce tung oil Diels-Alder composite products with different types of properties, so as to develop various types of Diels-Alder modified tung oil based UV curing materials. Narin et al. [70] prepared the alkyd resin by trimethylol triacrylate with tung oil and tung oleic acid monoglyceride by Diels-Alder addition reaction to prepared two kinds of photocurable resin UVTO and UVTA, and then added two resins to the photo initiator to make a photocuring resin, and studied the curing process with a light differential thermal analyzer. The results showed that the introduction of three double bonds of tung oilbased conjugate improves the addition rate of the Diesel-Alder reaction and shortens the curing time. Wutticharoenwong et al. [71] synthesized three kinds of tung oil-modified active diluents TOMAS, TOF and TOTAE through the Diesel-Alder reaction, and then combined with drying agents, humectants, and long oleic alkyd resins to obtained a variety of modified alkyd resins. The results showed that the addition of three kinds of tung oil modified active diluents reduced the viscosity of the alkyd resin, reduced the amount of organic solvent in the alkyd resin, and improved the pencil hardness, tensile strength and heat resistance of the coating film. Liu et al. [72] used tung oil as an active toughener to synthesized tung oil-based bicyclopentadiene unsaturated polyester resin (DCPD-UPR-TO) by Diels-Alder reaction and bicyclopentadiene unsaturated resin, and then used styrene to obtained the thermoset polymer by free radical polymerization crosslinking reaction. Experiments showed that when the amount of tung oil is added at 20%, the impact strength and tensile failure strain of DCPD-UPR-TO reached the maximum 373% and 875%, respectively. When the amount of tung oil is added to 10%, the hardness and flexibility of the polymer reach a flat equilibrium value. Li et al. [73] compounded and modified the five derivatives of tung oil with unsaturated polyester resin, and the study showed that the hardness and adhesion of the UPR coating film modified by the five tung oil-based derivatives were significantly improved compared with the unmodified UPR, while the tung oil derivative tung oleic acid had better compatibility with UPR, and the flexibility, tensile strength and impact resistance of the coating film were greatly improved. Yang et al. [74] synthesized a new type of UV photocurable vinyl ester resin through a

series of reactions such as Diels-Alder reaction of laurene and tung oil, glycidyl esterification reaction and open-ring esterification.

As one of the most important means of forming carbon bonds in organic chemical synthesis, the Diels-Alder reaction is also one of the reactions commonly used in modern organic synthesis. The reaction has rich stereochemical properties and occupies an important proportion in various basic synthesis studies.

4.2.1 Modification of Tung Oil by Diels-Alder Addition Reaction Used Maleic Anhydride

Maleic anhydride, is a Diels-Alder reaction with conjugated double bonds in tung oil. The main methods of synthesizing tung oil anhydride were emulsification heating method [75] and microwave method [76]. New composites can be effectively developed by introducing anhydride groups with high chemical activity into tung oil through Diels-Alder reaction.

Huang et al. [77] used Diels-Alder reaction to obtained two kinds of tung oil maleic anhydride (MAT1 and MAT2), as shown in Fig. 4. After obtaining the two composites, under the action of catalyst, the two composites react with hydroxy acrylate HEA and PETA respectively, and then continue to add glycidyl acrylate (GMA) to the reaction. The reaction process is shown in Fig. 5. Next, darocur 1173 photo initiator and 1,6-hexanediol diacrylate (HDDA) diluent were added to obtained HG-MAT and PG-MAT composites. By verifying the FTIR images before and after UV light irradiation, the author confirmed that the composites were cured after UV light irradiation; The UV curing kinetics and film thermodynamic properties of HG-MAT and PG-MAT composites were characterized by photo DSC and TG. The characterization results show that the two composites have fast film-forming speed and excellent thermal stability, and the thermogravimetric temperature is more than 358°C.



Figure 4: Synthesis and theoretical structure of MAT1 and MAT2 [77]

Huang et al. [78] explored another synthesis path of composites constructed by mat and tung oil base. Firstly, piperazine-1,4 dicarboxylic acid bis-(2-hydroxyethyl) ester (PCD) and isophorone diamine-1,4 dicarboxylic acid bis-(2-hydroxyethyl) ester (ICD) was synthesized by the reaction of piperazine and isophorone diamine with ethylene carbonate (EC). Then, PCD and ICD were esterified with two configurations of mat respectively, and the obtained product was reacted with GMA to obtained GMA-PCDMAT and GMA-ICDMATUV light curing matrix, as shown in Fig. 6. Then, 68% (mass ratio) of light curing resin, 2% (mass ratio) of Darocur-1173 photo-initiator and 30% (mass ratio) of HDDA constituted the synthesis path of light curing material. The UV curing kinetics and film thermodynamic properties of the two composites were characterized by photo DSC and TG. The results showed that the introduction of non-cyanate polyurethane (NIPU) structure (PCD and ICD) greatly enhanced the thermal stability of the membrane and the degree of cross-linking between substances. Zhang et al. [79] modified mat with hydroxyethyl methacrylate (HEMA), and compounded the modified product (HEMA-MAT) with resin, photo initiator and light curing monomer to obtained a new tung oil UV curing material, and measured the emulsification critical micelle concentration (CMC) of the system. The results showed that HEMA-MAT composites not only have excellent UV curing properties, but also can be used as emulsifier of acrylic esters. Li et al. [80] studied boric acid and tung oil modified phenolic resins. Boric acid first reacted with phenol, and then reacted with tung oil and formaldehyde to form a resin, the reaction can be expressed in Fig. 7.



Figure 5: Synthesis and theoretical structure of HG-MAT and PG-MAT [77]



Figure 6: Synthesis and structure of GMA-PCDMAT and GMA-ICDMAT [77]



Figure 7: Boric acid, tung oil modified phenolic resin [80]

4.2.2 Modification of Tung Oil by Other Dienophile Diels-Alder Addition Reaction

As a natural substance, rosin, like tung oil, also has special functional groups (conjugated double bonds and carboxyl groups) and high chemical reaction activity. Combined with the special structure of tung oil, both rosin and tung oil can be used as conjugated doublets or dienes in Diels-Alder addition reaction system, and some studies have showed that the unique ring structure of rosin can reduced the stress generated in the curing process of buffer film and make the surface of buffer film smooth after curing. Wu et al. [81] used the above characteristics to modified tung oil with rosin. After hydroquinone was added into the container, Diels-Alder reaction occurred between tung oil and rosin in different proportions to obtained tung oil rosin D-A product, as showed in Fig. 8. The UV curing material system of tung oil rosin was composed of epoxy acrylate, isobornyl acrylate, 1,6-hexanediol diacrylate and Irgacure-184 photo initiator. On this basis, it was found that when the mass ratio of rosin to tung oil is 1:3, the composites with low volume and shrinkage could be obtained, and have good thermal stability.

In an oxygen-free environment, Thanamongkollit et al. [70] used the unique Diels-Alder reaction of trimethylolpropane trimethylacrylate (TMPTMA) with tung oil to obtained tung oil based phenolic resin (UVTO). Then UVTO was evenly mixed with 10% TPGDA and 5% Irgacure-2100 photo-initiator to obtained tung oil based phenolic resin UV curing composite. This study based on tung oil, TMPTMA with multi-functional groups was added. The composite system can promote the crosslinking and curing of tung oil under UV light. At the same time, this method also strengthens the hardness and wear resistance of tung oil based composite coating.

4.3 Study on Transesterification Modified Tung Oil/UV Curing Material

The main component of tung oil is triglyceride oleate which contains three ester groups. This structure has a good basis for transesterification reaction and is also an excellent raw material for transesterification reaction. When alcohol was used as solvent in the reaction system, the other two ester bonds are partially replaced or removed to change the triester with large molecular weight into the monoester with smaller molecular weight and spatial resistance, so as to improve the reaction activity. Liu et al. [82] used tung oil and methanol as raw materials, NaOH as catalyst, prepared methyl tungate by transesterification reaction, and then used silver silica gel column nitrate to carry out gradient elution of methyl berlate, separation and purification, and obtained pure methyl berlate. The products were analyzed and characterized by infrared, ultraviolet, and the results showed that the purity of methyl beetlate was as

high as 95%. Lianhua et al. [83] used tung oil and methanol as raw materials, solid acids as catalysts, and using fixed-bed reactors, they discussed the effects of the molar ratio, temperature and spatial velocity of methanol to oil on the esterification of tung oil. Thanamongkollit et al. [70] first proposed UV curing system of tung oil based alkyd resin. Firstly, the transesterification reaction of tung oil and glycerol catalyzed by lead (IV) oxide was carried out to obtained glycerol monotung oleate. Then, the alkylated monotung oil is esterified with phthalic anhydride to obtained tung oil alkyd. The structural formula is shown in Fig. 9. Finally, through Diels-Alder reaction between tung oil alkyd and TMPTMA monomer, UVTA was obtained. The process was shown in Fig. 10. They used ¹H NMR and ¹³C NMR to characterized the structure of UVTA, used photo DSC to studied the UV curing kinetics of UVTA, and also compared the photo DSC analysis curves of UVTA and UVTO mentioned earlier. The results showed that UVTA has better performance than UVTO in light curing speed and light curing efficiency. The alkyd resin prepared by traditional method contains more hydroxyl and carboxyl groups, which has poor water resistance and thermal stability; TMPTMA active crosslinker has the characteristics of rapid curing because of its high double bond content. At the same time, it has low viscosity and low volatility. Many characteristics make it the most commonly used active crosslinker in the curing field. Based on the characteristics of TMPTMA, Thanamongkollit et al. modified tung oil-based alkyd resin with TMPTMA. The research results showed that the modified resin has been greatly strengthened in terms of crosslinking density and curing time. The long-chain conjugated triene bond of tung oil-based alkyd could also react with highly active pentaerythritol tetra acrylate at 150°C to form a highly polymerizable alkyd resin. At the same time, tung oil-based alkyd plastics with excellent mechanical properties are synthesized. The storage modulus of the plastics can reach 3.19 GPa and the glass transition temperature is 55°C, These studies provide new ideas and methods for the research of tung oil-based alkyd resin UV curing materials.



Figure 8: Idealized Diels-Alder addition reaction scheme between rosin and tung oil [78]



Figure 9: The synthesis route of tung oil alkyd [70]



Figure 10: The synthesis of UV-curable tung oil alkyd (UVTA) system from tung oil alkyd [70]

Chittavanich et al. [84] further developed and applied the above UVTA composite system to the field of colored coatings. A UV curable tung oil-based alkyd yellow paint was prepared by mixing caterpillar yellow pigment with UVTA, photo-initiator, dispersion additive and silicone additive. By exploring the physical properties of the coating, it is found that the addition of monomer makes the coating not only have good flexibility, but also take impact resistance and adhesion into account.

Although the transesterification has advantages such as reducing the viscosity of the reaction system, improving the reactivity, mild reaction conditions, and fast reaction speed. However, higher requirements are also put forward for the quality of raw materials and oils before exchange. It is a good process route to use a solid acid catalyst to pre-esterify vegetable oil with a high acid value, and then to prepare biodiesel by exchanging alkali-catalytic esters.

4.4 Study on Tung Oil Based Hyperbranched Ultraviolet Photo-Solidification Material

Hyperbranched polymers (HBPS) are chain structured polymers composed of many tree branches [85], it is widely used in biomedical materials [86] and other areas. The main characteristics of HBPS are compact structure, many branches, low viscosity, and can combine various active functional groups.

Through a series of tests, Mirshahi et al. [87] showed that HBPS improved the light curing level and conversion of monomers and oligomeric systems. At the same time, HBPS increased the crosslinking density of UV curing system and increased the glass transition temperature in UV curing coatings. Zhou et al. [88] also explored the tung oil based hyperbranched UV curing material composite system. Firstly, they used tung acid methyl ester as reaction raw material to prepare epoxy tung acid methyl ester, and reacted it with diethanolamine under UV light to prepare tung oil-based polyol. Then, tung oil-based polyols were reacted with isophorone diisocyanate (IPDI) and HEMA under the catalysis of dibutyltin dilaurate (DBTDL) to obtain tung oil based hyperbranched polyurethane acrylate. Finally, a certain amount of the above products, monomers and photo initiators were mixed in a certain proportion to develop tung oil based hyperbranched UV curing materials. The results showed that the thermal stability and elongation at break of the film formed by the UV curing system are comparable to the commercial No. 2665-3. This showed that tung oil based hyperbranched UV curing materials are expected to replace traditional petroleum based materials [89].

5 Research on the Application of Tung Oil in Industrial Production

Tung oil has undergone some chemical modification and can be applied to various aspects, such as biodiesel [90,91], resins, coatings, construction, preservatives, foams, etc. [92].

5.1 Application of Tung Oil in Biodiesel

Biodiesel [93] is a synthetic oil converted from biomass materials, generally chemically converted from vegetable oils, which as a biomass material, with non-toxic, harmless, low exhaust emissions, sustainable use and other characteristics, is a substitute for traditional petrochemical diesel, so it has attracted more and more attention and attention. At present, there are four main methods for preparing biodiesel: physical method, chemical method, biological method and chemical physical method. Biodiesel prepared from tung oil-based biomaterials has the characteristics of high flash point, good fluidity, safe use, low sulfur content, etc., so it meets the requirements of green development and has extremely broad application prospects [94]. Zhao et al. [95] prepared tung oil-based biodiesel by transesterification reaction between tung oil and methanol, and the optimal reaction conditions were: the molar ratio of tung oil and methanol was 1:6, the amount of catalyst NaOH was 1.0% of the quality of tung oil, the optimal temperature of the reaction was 30°C, the optimal reaction time was 20 min, and the average conversion rate was 82.8%.

5.2 Application of Tung Oil in Resin

Based on the characteristics of the molecular structure of tung oil, it is generally modified by addition, substitution, polycondensation and other reactions to synthesize tung oil-based polymer resin materials [96,97]. Polyurethane (PU) is a class of polymers containing NHCOO repeating structural units, is an isocyanate (monomer) isocyanate and polyhydroxy compounds after a series of polymerization reactions to obtain polymer materials, polyurethane resin modified with tung oil as the matrix can be applied to

coatings, adhesives, preservatives and the like [98]. First of all, tung oil as raw material, the double bond is hydroxylated into a polyol, and then the alcohol reaction with triethanolamine was carried out, and the rigid polyurethane is prepared as a nucleus, which improves its flexibility and increases its elongation at break. Epoxy resin generally refers to an oligomer containing two or more epoxy groups in the molecular structure, which was characterized by a plurality of active epoxy groups in the molecular chain, which can be located at the end of the molecular chain, can also be in the middle, or a ring-like structure. After epoxidation modification, tung oil can improve its curing activity, achieve the purpose of self-crosslinking curing, and can also be used as a curing agent for epoxy resins [99]. An important curing agent for manifold alkali-based epoxy resins. Its synthetic reaction formula is shown in Fig. 11. The phenolic skeleton structure with it at the same time further improved the heat deformation temperature and improves the shortcomings of the resin itself with insufficient heat resistance and insufficient corrosion resistance. The curing speed can be adjusted by adjusting the dosage of the ratio, and the epoxy resin can be cured in normal temperature, low temperature (0°C-5°C), humidity and underwater environment. However, there are shortcomings such as fast resin curing speed, uneven internal stress distribution, large brittleness of resin cured matter, and weak adhesion, and the application is limited. In order to overcome this defect, by using tung oil with many long flexible carbon chains, which can be substituted with phenol as a modifier, it participates in the structure of the curing agent, so that its molecular weight is increased, the sensitivity to water and vapor is reduced, and the brittleness of the epoxy resin cured was improved and the flexibility was improved, so that there is higher adhesion and adhesion. According to organic chemistry, olefins with conjugated double bonds are prone to Fourier substitution reaction, phenol aromatic ring on the phenol hydroxyl ortho position, one of the three active hydrogen positions in the position can be electrophilic substitution reaction with the double bond under the action of an acid catalyst, the introduction of flexible long-chain compound tung oil with conjugated double bond, the remaining two active hydrogen positions can still react with aldehydes and amines Manicheher, the specific reaction formula is shown in Fig. 12. The product obtained by the reaction steps greatly improves the brittleness and flexibility of the resin cured substance, and has lower toxicity, higher mechanical strength, adhesion and adhesion characteristics compared with the usual low-grade lapfruites. It is worth noting that the Fu's substitution reaction of phenol and tung oil is extremely sensitive to water, and the water content in the reaction system exceeds 3.7%, which will prevent the reaction from proceeding. In addition, this reaction was extremely fast. The absorption peak on the infrared spectrum of the conjugated double bond of active hydrogen and tung oil on the phenol ortho-paradectron disappears 15 min in after the start of the reaction, and a new absorption peak of the substitution product appears. Under heating conditions, the phenolic resin itself can be directly cured, and at high temperature, the phenolic alcohol in the phenolic resin can be dehydrated to form a hypomethylquinone containing co-choke double bonds, which can be combined with the double bond in the tung oil coolimide Diels-alder addition reaction to generate tung oil imide phenolic resin, thereby combining the advantages of the heat resistance of the imide and the phenolic resin, and curing under certain conditions to form a tung oleimide phenolic resin. Shang et al. [100] synthesized the inexpensive new bismaleimide (ABMI) as shown in Fig. 13. Synthetic ABMI has a cost advantage.



Figure 11: Manich alkali-based epoxy resin synthesis reaction path



Figure 12: Manich alkali tung oleoresin synthesis reaction path

5.3 Application of Tung Oil in Self-Healing Coating and Microcapsules

Tung oil has become a promising self-healing core material due to its natural source and environmental friendliness. Tung oil contains a large number of unsaturated fatty acids, which can be hardened to form corrosion-resistant polymers under oxygen-containing conditions. Sharma et al. [101] modified the epoxy coating of steel bars by adding nano-clay and tung oil microcapsules, and the microcapsule-based epoxy coating demonstrated outstanding corrosion resistance performance.

Zhang et al. [24] used microwave technology to get novel UV-curable oligomer (TMG) from renewable tung oil (TO), and a biobased reactive diluent (MM) was derived from malic acid under ambient conditions. Afterward, a set of UV-curable coatings containing plentiful hydroxyl and ester groups were prepared by photo-polymerizing the TMG oligomer with the MM diluent. The resulting coatings demonstrated a high biobased content (49.2%–58.1%), good mechanical and thermal properties (e.g., Tg of 70.6°C–78.6°C), and excellent coating adhesion and flexibility. Furthermore, the TMG material containing 10% of MM demonstrated a crack repair efficiency of 92.5%, a welding efficiency of tensile strength up to 171.8%, a recycling efficiency of tensile strength up to 404.3%, and a shape fixity ratio of 98.2%.



Figure 13: Chemical synthesis pathway of ABMI

Zhu et al. [102] used tung oil as the core material, and Ca-Al LDH was adopted as corrosion inhibitors to hybridize ethyl cellulose, and the solvent evaporation method was adopted to prepare the microcapsules. The synthesis mechanism diagram is shown in Fig. 14 and Fig. 15. Ca-Al LDH was loaded on the surface of hybrid microcapsules. The layered Ca-Al LDH was stabilized by the physical preparation method of microcapsules, and the chemical structure was not destroyed during the violent preparation of microcapsules. They found that the nano-layered Ca-Al LDH within the hybrid wall provided additional nucleation sites for cement hydration. The chlorine adsorption capacity of cement paste doped with 3 wt% and 6 wt% microcapsules was 14.7% and 26.7% higher than that of pure cement paste in SCPS, respectively. Also, the highest corrosion inhibition efficiency of hybrid microcapsules in SCPS is 94.12%, and the corrosion inhibition efficiency of self-healing microcapsules in SCPS is less than 75%. The hybrid microcapsules improved the corrosion resistance in SCPS via the synergistic effect of self-healing and chlorine adsorption.

Wang et al. [103] used tung oil (TO) as core, layered double hydroxides (LDH) as corrosion inhibitor and ethyl cellulose (EC) as wall, to synthesized the self-healing and corrosion resistance microcapsule (S/CMC). They found Microcapsules respond to microcracks in coating by releasing core material rapidly after cracking or releasing core material slowly via slow-release pores to repair coating microcracks. LDH embedded in EC to form stable three-dimensional wall structure which could capture the chloride ion by hybrid wall through surface adsorption and ion exchange, corrosion resistance of epoxy resin coating was improved by S/CMC, the mechanism of synthesis is shown in Fig. 16.



Figure 14: Preparation process and formation mechanism of microcapsule



Polymer cured film

Figure 15: Oxidation polymerization mechanism of tung oil



Figure 16: Formation mechanism of S/CMC



Figure 17: The schematic of microencapsulation mechanism

Li et al. [104] used insitu polymerization method to prepared Tung oil-loaded microcapsules, as can be seen in Fig. 17. The average diameter was approximately 105 μ m. Microcapsules keep the spherical shape with core content of more than 80.0 wt%. Microcapsules exhibit excellent thermal stability. Dualfunctional coatings were prepared by incorporating tung oil-loaded microcapsules into epoxy. Self-healing and self-lubricating function of coatings were realized by the releasing of tung oil from microcapsules under the scarp or wear condition. Tung oil has excellent film-forming property when they contact with oxygen. There were 17.3% and 78.6% decrease respectively compared to the pure epoxy. They also discussed the self-healing and self-lubricating mechanism (Fig. 18).



Figure 18: Schematic diagram of self-healing mechanism

5.4 Application of Tung Oil in Other Aspects

With the continuous development of society, tung oil-based modified products and their application fields have broken through their traditional coatings, varnishes and other manufacturing fields, and are also widely used in the electronics industry, plastics industry, printing ink, rubber industry, polymer materials and other chemical industries [105]. Tung oil can be used as a reaction diluent in resin synthesis to reduce the change in viscosity during the reaction [71], and can also be used as a composite material to participate in film-forming curing, improving its mechanodynamic properties and thermodynamic properties.

Wutticharoenwong et al. [106] used tung oil and unsaturated Diels-alder reaction prepared three reaction diluents, the dipenosome is methacryloyloxypropyl trimethoxysilane (MAS), methacrylic acid 2,2,2-trifluoroethyl ester (TFM) and acrylate trialelyl ether (TAEA), and then formulated with thiol resin in a certain proportion to make a cured sample, The results showed that the viscosity of the alkyd resin with the addition of tung oil-based reactive diluent was reduced to 70%, and its drying and curing time was 1.8 times faster than that of the alkyd resin alone.

6 Conclusion and Prospect

Tung oil has UV curing activity and can be used as the matrix of UV curing coating reaction, but the film-forming effect of tung oil alone is poor and needs to be further improved. Combined with the main structure of tung oil α -the chemical structure of conjugated double bonds contained in the molecular long chain of tung acid can be effectively improved by chemical modification. After modification, tung oil can be combined with the characteristics of other substances to construct UV curing materials with good film-forming quality, good mechanical properties and special properties.

Tung oil/UV photocomposite curing products can be widely used in many fields. For example, tung oil based waterborne hyperbranched resin has the characteristics of low viscosity and high solubility. It can be used as raw material for the preparation of waterborne ink and reduce the generation of volatile organic solvents [107]; Tung oil based unsaturated resin has high strength and acid resistance, and can be used to prepare corrosion-resistant FRP products [108]; Tung oil based phenolic resin can improve the mechanical strength and electrical properties of tung oil by using phenolic resin. Tung oil based phenolic resin can also be used in wear-resistant materials [109]. In addition, the UV curing materials chemically modified by
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organic substances such as tung oil Diels-Alder and transesterification are the most studied at present. Considering that the conjugated diene structure of tung oil can react with different dienophiles, and the ester group of tung oil can be trans esterified with different alcohols, so as to obtained modified products with a variety of functional groups and different chemical properties, Therefore, a variety of UV curing materials can be developed. To sum up, with the increasing depletion of global petrochemical resources, deepening the research on Tung oil-based UV curing materials can enrich the theoretical system of woody oil reaction, reduce the excessive dependence on fossil resources and avoid environmental pollution caused by petrochemical resources, broaden the application scope of woody oil and improve its practical value.

In addition, there are some deficiencies in the field of woody oil-based UV curing. Most woody oils are difficult to showed strong material properties after chemical modification and polymerization, and could not replace petrochemical polymerization products accordingly; Secondly, there is a certain gap between light curing coatings and heat curing coatings in resistance and mechanical properties [110]. To sum up, there is still a long way for researchers to explore the construction of UV curing material system by modifying tung oil with inorganic materials. Tung oil is expected to become the most "changeable" vegetable oil in the 21st century because of its long-chain structure of conjugated polyene triesters. The application of UV curing of tung oil has changed the traditional chemical research of multi reagent and multi pollution of vegetable oil, and realized the goal of green chemical and carbon-neutral development of tung oil. Therefore, today, when environmental protection and energy saving are advocated, more in-depth research should be carried out on the development of tung oil in the field of UV curing.

Funding Statement: This research was funded by Major Landmark Innovation Demonstration Project, 2019XK2002; Changsha Functional Oil Technology Innovation Center, KH2101007; Hunan Forestry Bureau Outstanding Training Research Project, XLK202108-2.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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DOI: 10.32604/jrm.2022.023411

ARTICLE





Insight into Hydrolytic Stability and Tribological Properties of B-N Coordination Tung Oil-Based Lubricant Additive in Water

Na Yao^{1,2}, Haiyang Ding^{1,2,*}, Mei Li², Pengcheng Wang³, Shouhai Li^{1,2}, Lina Xu² and Xiaohua Yang²

¹Institute of Ecological Conservation and Restoration, CAF, Beijing, 100091, China

²Institute of Chemical Industry of Forest Products, CAF; National Engineering Laboratory for Biomass Chemical Utilization; Key Laboratory of Chemical Engineering of Forest Products, National Forestry and Grassland Administration; Key Laboratory of Biomass Energy and Material, Jiangsu Province; Jiangsu Co-Innovation Center of Efficient Processing and Utilization of Forest Resources, Nanjing, 210042, China

³School of Chemical Engineering, Nanjing University of Science & Technology, Nanjing, 210094, China

*Corresponding Author: Haiyang Ding. Email: dinghaiyang2008@163.com

Received: 25 April 2022 Accepted: 21 June 2022

ABSTRACT

A tung oil-based boron-nitrogen coordination polymer (TWE-BN) was specially designed and synthesized as a highly efficient water-based lubricant additive, which has been beneficial to both energy conservation and conducive to environmental protection. Its hydrolysis stability and tribological properties in water were investigated. To better research the lubricating properties, and thus to understand the interaction between the surface and the lubricating additives. Herein, both experimental and theoretical computations based on density functional theory (DFT) were performed. The addition of TWE-BN reduces the water friction coefficient and wear scar diameter, and the maximum non-seizure load increased from 93 to 726 N. Moreover, the anti-corrosion ability on copper was classified as 1b level. The stainless-steel surface was analyzed using scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS). In hydrolytic stability testing, TWE-BN was better than nitrogen-free tung oil-based lubricant additive (TWE-B) and remained non-hydrolyzed for at least 15 days, implying the feasibility of tung oil-based boron-nitrogen coordination as highly effective and hydrolytic stability lubricant additives.

KEYWORDS

Tribological properties; tung oil; borate; hydrolytic stability; boron-nitrogen coordination



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Graphic Abstract



1 Introduction

In recent years, with the rapid increase in the global high-end equipment manufacturing capacity and the whole society attaching high attention to environmental protection issues, people have higher requirements for the high efficiency, degradability, harmless, and other operational performance for lubricating oil [1–3]. The traditional petroleum-based mineral oil has weakened cost advantages and limited biodegradation and service performance, causing it to face the pressure of resources and environmental protection [4–9]. Therefore, there is an urgent need for an alternative environmentally friendly and low-polluting lubricant. Water-based lubricants are a valid candidate for liquid lubricants because of their low cost, high fire resistance, good storage stability, and excellent biocompatibility [10–12].

At present, many types of research have shown that water-based lubricants were capable of forming lubricating films on metal surfaces and improving the friction properties of water-based lubricants. Fernando et al. synthesized a series of alkyl glyceryl ethers and it was demonstrated that these ethers have a good performance as an additive for water-based lubricants when formulated with Xanthan gum, and the coefficient friction decreased from 0.34 to 0.06 [13]. Hu et al. [14] prepared graphene-based material to be used as a nano additive a water-based lubricant. The results indicated that the nano additive could stably disperse in water for a long time and exhibit excellent friction-reducing and anti-wear properties. Zhang et al. [15] modified polytetrafluoroethylene micro powder with dopamine as a water-based lubricating additive, research shows that the additive as water-based lubricant additives is very scarce. Besides, the use of water-based lubricants that exist disadvantages of poor lubrication performance and corrosion equipment [16–18]. One of the effective ways to improve the performance of water-based lubricants is to add additives containing extreme pressure elements and anti-corrosion properties [19–22].

Tung oil is a non-edible oil, mainly grown in China, which primarily contain unsaturated fatty acids with the following average molar distribution, 77.2% α -eleostearic acid, 7.6% linoleic acid, 5.9% oleic acid, 4.2%

palmitic acid, 2.3% stearic acid, and 0.4% linolenic acid. As an excellent drying oil, tung oil has long fatty acid chains and polar groups, and this amphiphilicity enables them to have a good film/force relationship, which can act as a boundary lubricant [23-26]. However, due to the high degree of unsaturation and low oxidation stability of tung oil, it is not suitable for direct use as lubricating oil [27]. Based on the improved performance and environmental consciousness of water-based lubricants, this work envisages the introduction of extreme pressure element boron into tung oil to prepare water-based extreme pressure lubricant additives. Despite the fact that boronic ester has been successfully used in the preparation of various lubrication additives, designing boronic ester with enhanced hydrolytic stability and superior extreme pressure performance remains extremely challenging due to the fact that the electron-deficient boron sites linked by boronate esters are vulnerable to attack by nucleophiles such as water molecules [28,29]. As a result, exposure to water, even vapor in the air, could potentially hydrolyze the boronate ester and compromise the integrity of lubricant additives [30,31]. Given the problem of easy hydrolysis of borate esters, we postulated a novel strategy for synthesizing stable nitrogen-coordinating cyclic boronic ester compounds by introducing an N-B coordination bond and then using them to construct a lubrication additive. Different from most studies on borate extreme pressure lubricant additives, the introduction of tung oil makes up for the shortcomings of water-based lubricants that are easy to corrode. In addition, explore through a combination of experimental and theoretical calculations design and characterization of the tung oil-based lubricant additive, and discuss the inherent law of the structurefunction relationship between microscopic molecular structure and macroscopic properties. To provide a reference for pain point relief of water-based lubrication.

2 Experimental Sections

2.1 Materials

All chemicals we used were of analytical grade without further purification. The tung oil was purchased from the Luodian County Anling Vegetable Oil Plant (China). Methoxypolyethylene glycol (Relative molecular weight of 1000, Shanghai Aladdin Biochemical Co., Ltd., China), Diethanolamine (AR, \geq 99%), Carbon tetrachloride (AR, \geq 99%), Boric acid (AR, \geq 99.5%), Trimethylolpropane (AR, 98%), ethylene glycol (AR, \geq 99%) all purchased from the Sinopharm Chemical Reagent Co., Ltd. (China).

2.2 Preparation of Tung Oil-Based Waterborne Extreme Pressure Lubrication Additives

Tung oil-based waterborne extreme pressure lubrication additive (TWE-BN) was fabricated as illustrated in Scheme 1. In order to compare the effect of the nitrogen coordination on the properties of the lubrication additives, especially hydrolysis stability, the nitrogen-free lubrication additive (TWE-B) was also synthesized. Synthesis details are described below.

2.2.1 Preparation of TWE-BN

Preparation of Tung Oil Acid Diethanolamine (TODN)

Tung oil acid diethanolamine (TODN) was synthesized by the amidation reaction between tung oil and diethanolamine, following specific methods are adopted. Firstly, transferred tung oil, diethanolamine, and alkaline catalyst into four flasks with a mixer, thermometer, and condensate tube under nitrogen protection this mixed solution reacts 4 h, 90°C–120°C to prepare TODN. After this period, the resulting reaction product is a mixture containing glycerol, diethanolamine, tung oil, and TODN. After the product is purified and distilled, a pure TODN product can be obtained.

Preparation of Tung Oil-Based Nitrogen-Containing Borate (TO-BN)

Tung oil-based nitrogen-containing borate (TO-BN) was synthesized by the esterification method [32,33]. Details are as follows, methylbenzene as a water-carrying agent, TODN, boric acid, and a few catalysts placed on three flasks with 110°C condensation reflux reaction 5 h. After the product is purified and distilled, a pure TO-BN product can be obtained.

Preparation of Tung Oil-Based B-N Coordination Waterborne Extreme Pressure Lubrication Additives (TWE-BN)

TWE-BN was synthesized by the esterification method. Details are as follows, methoxypolyethylene glycol of 1000 relative molecular weight, TO-BN, and a few catalysts placed on three flasks with 110°C condensation reflux reaction 5 h. After the product is purified and distilled, a pure TWE-BN product can be obtained.



Scheme 1: Synthesis routes of TWE-BN and TWE-B

2.2.2 Preparation of TWE-B

Preparation of Trihydroxymethyl Tung Oil Ester (TO-tOH)

Trihydroxymethyl tung oil ester (TO-tOH) was synthesized by the transesterification between tung oil and trimethylolpropane, following specific methods are adopted. Firstly, transferred tung oil, trimethylolpropane, and acid catalyst into four flasks with a mixer, thermometer, and condensate tube under nitrogen protection this mixed solution reacts 4 h, 90°C–120°C to prepare TO-tOH. After the product is purified and distilled, a pure TO-tOH product can be obtained.

Preparation of Tung Oil-Based Borate (TO-B)

Tung oil-based borate (TO-B) was synthesized by the esterification method. Details are as follows, methylbenzene as a water-carrying agent, TO-tOH, boric acid and a few catalysts placing to three flasks with 110°C condensation reflux reaction 5 h. After the product is purified and distilled, a pure TO-B product can be obtained.

Preparation of Tung Oil-Based Waterborne Extreme Pressure Lubrication Additives (TWE-B)

TWE-B was synthesized by the esterification method. Details are as follows, methoxy polyethylene glycol of 1000 relative molecular weight, TO-B, and a few catalysts placed on three flasks with 110°C condensation reflux reaction 5 h. After the product is purified and distilled, a pure TWE-B product can be obtained.

2.3 Characterization Methodologies

Characterizations of the obtained lubrication additives and steel ball obtained after friction include identification of chemical structure (by infrared spectroscopy (FTIR), ¹H Nuclear magnetic resonance (¹H NMR), X-ray photoelectron spectroscopy (XPS)), observation of microscopic morphology (by scanning electron microscope (SEM)). FTIR was recorded on the attenuated total reflection mode spectrometer. ¹H NMR measurement was performed on an AV ANCE 400 Bruker, samples were first dissolved in CDCl₃. Mestre Nova software (Santiago de Compostela, Spain) was used to handle the NMR data. Steel ball obtained after friction was analyzed by XPS (PHI 5000 Versa Probe. The tribological properties were analyzed in terms of coefficient of friction (COF), wear scar diameter (WSD) of the ball, and the worn track in the disk. The worn surface morphologies of the worn surface were investigated with SEM (FEI Co., Ltd., Hillsboro, USA).

2.4 Hydrolytic Stability Test

Hydrolysis stability adopts the wet-heat treatment to accelerate the hydrolysis method (patent USP 4892670): add 150 g of sample (1.0% additive in water) into a 200 ml beaker and put it in a moist heat oven (temperature $50 \pm 2^{\circ}$ C, relative humidity greater than 95%). Monitor samples every hour and records the time. If precipitation is observed in the sample or the sample is no longer transparent, this means that the additive has been hydrolyzed [29].

2.5 Measurement of Tribology Properties

The maximum non-seize load (P_B value), and friction performances of the samples were measured by the four-ball tribometer (MRS-10). Its measurement method is similar to ASTM D2783. The test conditions for P_B were 556 mm/s (1450 r/min) for 10 s at room temperature, and the coefficient of friction and wear scar diameter tests conditions were 1450 rpm for 1800 s under an applied load of 200 N [10]. Each measurement was triplicated.

2.6 Anticorrosion Tests

The anticorrosion performance test is carried out in accordance with the GB/T 5096–2017 standard for TWE-BN and TWE-B in water. The bright-treated copper bars were dipped into the prepared samples. After that, they were transferred to a constant temperature of $100 \pm 2^{\circ}$ C for 3 h. After the test, the copper strip is taken out for cleaning, dried at room temperature, and then compared with the corrosion standard color plate to determine the corrosion level [34].

2.7 Computational Details for DFT Simulations Studies

In order to thoroughly analyze the lubrication mechanism, we explore the lubrication mechanism using theoretical calculation as auxiliary instructions. Density Functional Theory (DFT) calculations were performed using the Gaussian16 software. All calculations were performed using the M06–2X functional. For geometric optimization and frequency calculations, using the def2-TZVP basis set. The optimal geometry for each compound was determined [35].

3 Results and Discussion

3.1 Characterization of Tung Oil-Based Waterborne Lubrication Additives

Fig. 1b displays the FTIR spectra of Tung oil, TWE-BN, and TWE-B. A series of similar strong and broad bands at approximately 3470 cm^{-1} are ascribed to the stretching vibrations of hydroxide groups (-OH) and/or H-bonding for TWE-BN, and TWE-B, while the peak did not appear in the tung oil. The characteristic peak of carbonyl stretching vibration appears at 1740 cm^{-1} attributable to the glycerides in the structure of tung oil. The peaks at 989 cm⁻¹ were ascribed to the conjugate double bond of tung oil. These position peak also occurs on both TWE-BN and TWE-B. The symmetric stretching vibrations with

a new peak of the B-O bond appear at 1348 and 1344 cm^{-1} , and the formation of the B-O bond in borates indicates that the hydroxyl of methoxy polyethylene glycol has been connected by esterification reaction [36]. The peak at 1644 cm⁻¹ represents C-N of TWE-BN, which is not shown on the spectrum of TWE-B. The ¹H-NMR spectra of tung oil, TWE-BN, and TWE-B are shown in Fig. 1c and the attributions of the chemical shifts of the protons are labeled. Multiple peaks assigning to the protons of conjugated double bond appeared at 5.3–6.6 ppm, the protons attributing to the methyl appeared at 0.9 ppm, the signals ascribing to protons of methylene appeared at 1.2–1.5, 2.0–2.5 ppm. It also could be seen that the peaks associated with double bonds in the spectrum of TWE-BN and TWE-B, indicated that the double bonds remained after the esterification. The FTIR and 1H-NMR spectra validate the successful preparation of TWE-BN and TWE-B.



Figure 1: FTIR (a) and ¹H-NMR (b) spectra of tung oil, TWE-BN, and TWE-B

3.2 Evaluation of Hydrolytic Stability

To investigate the impact of $B \rightarrow N$ coordination on the electronic structures of the lubricant additives, the density functional theory calculations were carried out using a suite of Gaussian 16 programs [37–39]. Since the hydrolytic process of borate ester has little relationship with the structure of tung oil and methoxy polyethylene glycol. A simplified model was chosen for two lubricant additives (TWE-BN and TWE-B) by comprehensively considering computational cost and accuracy. The TWE-BN and TWE-B molecule full geometry optimization of the transition state (TS) was carried out. Reactant and product complex geometries were found by the IRC descend in the B3LYP method and reactant complexes were subsequently optimized Fig. 2 shows the calculated energy profiles of hydrolytic processes of TWE-BN and TWE-BN and TWE-B in the presence of water molecule. The hydrolysis of the boronate ester linkage is a

three-step reaction. In the first step, one H₂O molecule reacts with the B-O bond outside the ring in TWE-BN, the coordination between boron and nitrogen alleviates the occurrence of this hydrolysis process. In the following step, another H₂O molecule reacts with the hydrolysis with one of the B-O bonds on the seven rings. These two steps have energy barriers of 166.45 and 170.31 kJ/mol, respectively. It shows that the resistance of the second step of the hydrolysis process is larger. On the one hand, it needs to destroy the coordination between B and N even the ring structure also needs to be destroyed. Last step, an additional H₂O molecule is found to greatly assist the hydrolysis by lowering the reaction barriers to 134.94 kJ/mol. This is attributed to the seven rings being completely destroyed, and the coordination between B and N almost disappears, which makes the hydrolysis reaction easier. For TWE-B, the total energy barrier for the second hydrolysis process is below TWE-BN by approximately 22.46 kJ/mol. This indicates that the presence of N and B can alleviate the hydrolysis of borate lubrication additives, TWE-BN showed higher hydrolysis stability than TWE-B because of the intramolecular coordination of $N \rightarrow B$ in the TWE-BN linkages, which is probably easier to maintain extreme pressure performance. Lubrication performance will be discussed in detail in the following section. Fig. 3 shows the changes in the ring where the B element is located before and after the two lubrication additives bind to the water to demonstrate the contribution of the N in the hydrolysis process. As shown in Fig. 3a, the distance between B and N is 1.723 Å before water molecules attack borate, which forms a strong coordination effect. After the water molecules attack, the distance between B and N is further shortened and the coordination effect becomes stronger, that is, N exhibits further hydrolysis inhibition of organic borate.

In contrast to TWE-BN, the distance between the B atom and its para-atom is large for TWE-B. Para-atom of B has a similar position to N in the TWE-BN and the interatomic distance is further increased after the water molecules attack. It can also be seen from the comparison figure before and after 7 days that there is a turbid precipitate in TWE-B, but there is almost no change in TWE-BN. This further indicates that the hydrolysis stability of TWE-BN is stronger than that of TWE-B. In addition, we performed hydrolysis stability monitoring for the two additives. The experimental results are listed in Table 1. TWE-BN remained unhydrolyzed for at least 15 days compared to TWE-B, suggesting that the formation of inter-molecular-coordination of nitrogen to boron $N \rightarrow B$ (TWE-BN) that could decrease the possibility of the attack on water.

3.3 Tribological Properties

3.3.1 Maximum Nonseizure Load

The load-carrying properties with varying concentrations of TWE-BN and TWE-B additives are marked with the maximum non-seizure load (P_B), and the experimental results are shown in Fig. 4. The P_B value of water is 93 N, indicating it is difficult to form an effective lubrication film on a metal surface. Nevertheless, the addition of TWE-BN and TWE-B significantly improved the load-carrying capacity of water, which is related to the formation of protective film on the friction surface. This may be due to long-chain molecules, complex structures, and polar groups, which can be firmly adsorbed on the metal surface to form an aligned molecular layer. As the content of additives increases, the mono-molecular layer changes to the multi-molecular layer. Furthermore, the intermolecular Van der Waals force makes the molecular layers tightly combined to form a physical adsorption film. Under the conditions of high-speed friction and instant high temperature, it is easy to form on the metal surface with low shear strength and good thermal stability, and the chemical adsorption membrane with strong bearing capacity. It is noteworthy that the TWE-BN and TWE-B copolymer concentration exceeds 1.0 wt%, and it is found that in maximum non-seizure loads the P_B value does not change much. Further, the higher P_B value of TWE-BN than TWE-B indicates that the coordination effect between N and B alleviates the hydrolysis of boric acid ester, making the lubrication film more stable and having a higher P_B .



Figure 2: DFT-calculated hydrolytic process of TWE-BN and TWE-B in the presence of water molecule

3.3.2 Wear Resistance and Frictional Properties

The coefficient of friction (COF) and wear scar diameter (WSD) of TWE-BN and TWE-B were carefully studied to examine the lubrication effect. Under the same test conditions, we also tested the friction coefficient of water, but only 141 s before the steel ball stopped moving due to a serious seizure. The test was forced to stop and cannot continue, which indicates that the carrying capacity of water is very weak. It is clearly seen from Fig. 5a that COF decreases with the addition of TWE-BN, while the regular pattern of the friction coefficient of TWE-B is not obvious and fluctuates greatly. As TWE-BN copolymer content increases, the COF decreases insignificantly. The lowest COF values (0.062) are obtained at

1.0 wt% TWE-BN, which is below the value TWE-B (0.075, 1.0 wt%). Fig. 5b illustrates the WSD of the balls after friction with TWE-BN and TWE-B (four-ball tester; load: 200 N, speed: 1450 rpm, duration: 1800 s). It can be seen that the introduction of TWE-BN and TWE-B in water leads to a sharp decrease of WSD when the concentration is less than or equal to 1.0 wt%. When the concentration of TWE-BN and TWE-B additive is greater than 1.0 wt%, the WSD varies slightly increased. And the smallest wear scar diameter of 0.63 mm was obtained at a concentration of 1.0 wt% for TWE-BN. This indicates that the addition of TWE-BN and TWE-B at a concentration of 1.0 wt% makes the lubricant produce a stable protective lubricating film on the contacting metal surfaces and improves the water carrying capacity.



Figure 3: Most stable conformations of TWE-BN (a) and TWE-B (b) according to DFT calculation results, appearance change of TWE-BN and TWE-B (additive concentration: 1.0 wt%, normal pressure and temperature)

Borate additives	Structural formula	Hydrolytic stability (h)
TWE-BN	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	>360
TWE-B		96

Table 1: The hydrolytic stability of TWE-BN and TWE-B in water

3.4 Mechanism Analysis of as-Synthesized Borate Ester

3.4.1 SEM Analysis

The worn surface of steel ball was studied by SEM. SEM micrographs of the worn steel surface TWE-BN and TWE-B (1.0 wt%) are shown in Fig. 6. The size of the wear scar corroborates the order of tribological performance obtained experimentally. In the steel surface lubricated by TWE-B, the surface

was damaged and deformed due to grain wear, indicating that severe wear and abrasion occurred during the test, which was due to the poor strength of the lubricating film formed by TWE-B. However, the wear surface of TWE-BN is smoother with no signs of severe wear along the sliding direction, and the wear scar is relatively narrow and shallow when compared to the surface with TWE-B lubrication. The observations indicate that TWE-BN reduces steel ball wear to some extent, in relative agreement with the corresponding friction wear test data in Fig. 5.



Figure 4: Maximum non-seizure loads (P_B values) of the TWE-BN and TWE-B in water (four-ball tester, rotation velocity 1450 rpm, 10 s)



Figure 5: Variation of coefficient of friction (COF) and wear scar diameter (WSD) with the additive concentration of TWE-BN and TWE-B (four-ball tester; load: 200 N, speed: 1450 rpm, duration: 1800 s)

3.4.2 XPS Analysis

A comparative analysis of the worn surfaces of the TWE-BN 1.0 wt% and TWE-B 1.0 wt% lubricated steel balls was performed with XPS to obtain more information on the mechanism of action in the lubrication process. As shown in Fig. 7, the XPS spectrum of the wear-out steel surface was lubricated with TWE-BN 1.0 wt%. The

B1s peaks of 187.5 and 189.7 eV belong to B-N and Fe-O-B, respectively, suggesting that borates react with nitrogen on the friction surface during friction. This suggests that boron is involved in the frictional chemical reaction during sliding. The N1 s peak appears at 397.8 and 398.6 eV the former corresponding to B-N and the latter corresponding to the organic nitrogen compound of diethanolamine. Furthermore, these compounds were also supported by the binding energy of B1s at around 187.5 eV. This suggests that N also participates in frictional chemical reactions during its physical and/or chemical adsorption on frictional metal surfaces. The C1s peaks at 284.7, 285.8, and 288.1 correspond to the C-H, C-C, and C-O bonds of organic compounds, suggesting that TWE-BN is adsorbed on the steel surface. Combining the binding energies of 710.6 and 709.8 eV in the O1 s and Fe2p spectra can further confirm that iron oxide should be the component of the friction film [40]. The binding energies of the O1 s were 530.1, 531.8, and 532.6 eV, which can be attributed to ferrous oxide, C-O bonds, and Fe-O-B, respectively, further confirming that the TWE-BN additive underwent frictional chemical reactions in this process. Fig. 8, the XPS spectrum of the worn steel surface is lubricated with 1.0 wt% TWE-B. The B1s peak at 189.8 eV belongs to the B-OH or B-O bond, indicating that the TWE-B has been decomposed to form the B-O and B-OH groups during the friction process. The peaks of the B1s spectra are not obvious, indicating that the XPS is less detectable of the boron elements adsorbed on the worn surface, possibly due to the hydrolysis of the borate interfering with the B1s spectra. Spectra of the other elements are similar to the TWE-BN.



Figure 6: SEM images of the worn surfaces lubricated at concentrations 1.0 wt% for TWE-BN and TWE-B

For the above reason, it can be deduced that TWE-BN 1.0 wt% and TWE-B 1.0 wt% easily form lubricating films containing various compounds of carbon, oxygen, iron, and boron elements on the surface of steel balls through adsorption and/or friction reactions. The tribological properties of water can be significantly improved.

3.5 Anticorrosion Properties

Fig. 9 shows the photographs of the copper sheet after the immersion. It is clearly seen that the TWE-BN and TWE-B sample had slightly tarnished the copper sheet surface, and all samples of different concentrations were at the 1b level of anti-corrosion ability. The process of corrosion inhibition involved releasing the tung oil chain segment which protects the opened copper sheet surface from the surrounding environment through protective film formation by oxidative polymerization of tung oil. This could be explained by the presence of a thicker protecting layer on the copper sheet surface during the test, which impedes the corrosion process. Therefore, the addition of a suitable amount of Tong oil-based modified lubricant additives into the water has successfully improved the corrosion behavior of the water-based lubricant.



Figure 7: XPS spectra of worn steel surface lubricated with TWE-BN 1.0 wt% (four-ball friction and wear tester)



Figure 8: XPS spectra of worn steel surface lubricated with TWE-B 1.0 wt% (four-ball friction and wear tester)



Figure 9: Photographs of the corrosion test of the lubricant samples on copper sheets. From left: Blank copper sheet, TWE-BN 0.5 wt%~2.0 wt%, TWE-B 0.5 wt%~2.0 wt%

4 Conclusions

Based on developing high-performance and environmentally friendly lubricant additives. We synthesize tung oil-based heterocyclic boron-nitrogen coordination polymer (TWE-BN) as highly effective and hydrolytic stability extreme pressure lubricant additives in the water. When the TWE-BN concentration was 1.0 wt%, the P_B value reached 726 N, which was 6.8 times that of water. The hydrolytic stability and lubrication performance of TWE-BN are higher than that of without nitrogen (TWE-B) because of the coordination effect between N-containing heterocyclic rings and borate in TWE-BN acting an important role. Furthermore, DFT studies gave the conformation of coordination B and N on the adsorbing surface, the energy required for TWE-BN to destroy the boronic ester on the ring is larger in the three-step hydrolysis simulation reaction and the distance between B and N is further shortened (from 1.723 to 1.719) after water molecules attack the boronate ester, which indicates the formation of a strong coordination effect. The XPS analysis results show that TWE-BN reacts with the metal surface to form a chemical adsorption film during the friction process, which plays a crucial role in reducing friction and anti-wear. Paving the way to an understanding of the lubrication and hydrolysis mechanism of waterbased lubricant.

Funding Statement: This work was supported by Special Fund of Chinese Central Government for Basic Scientific Research Operations in Commonweal Research Institutes [No. CAFYBB2019SY037] and National Natural Science Foundation of China [No. 31901260].

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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DOI: 10.32604/jrm.2022.022809

ARTICLE





CO₂-Responsive Smart Foams Stabilized by an Extremely Rigid Bio-Based Surfactant

Weishan Tang, Xin Feng, Caiyun Lin and Xiaoping Rao*

College of Chemical Engineering, Huaqiao University, Xiamen, 361021, China ^{*}Corresponding Author: Xiaoping Rao. Email: raoxp@hqu.edu.cn Received: 27 March 2022 Accepted: 19 May 2022

ABSTRACT

Environment friendly and intelligent surfactants have attracted great attention in recent years. A bio-based CO_2 responsive surfactant rosin acid dimaleimide choline (R-BMI-C) with an extremely rigid skeleton was prepared using rosin and choline as raw materials by Diels-Alder addition reaction and acid-base neutralization reactions. Its structure was confirmed by IR and ¹H NMR spectra. The foams' properties of R-BMI-C could be adjusted by bubbling CO_2/N_2 to change the structure of the surfactant. At pH 10.4, R-BMI-C forms an unstable foam with a half-life of 1.5 h. When the pH was reduced to 7.4 by bubbling CO_2 , R-BMI-C forms an extremely stable foam with a half-life of 336 h. The surfactant R-BMI-C changed from bola type to conventional type when bubbling CO_2 . And the internal aggregation structure of R-BMI-C aqueous solution changed from spherical micelles to laminar micelles according to the cryogenic-transmission electron microscope. We know that the lamellar structure tends to adsorb at the air/water interface or is trapped in the foam film, which slows down the foam coarsening and agglomeration process, resulting in a significant increase in foam stability. R-BMI-C could be used in oil extraction, fire-fighting and chemical decontamination due to its excellent foaming, stabilization and defoaming properties.

KEYWORDS

Rosin; bio-based; surfactant; CO2-responsive; foams

1 Introduction

Foams are a dispersion of an insoluble gas in a liquid or solid, which has the excellent properties of light mass, large specific surface area and high mobility [1,2]. Therefore, foams are used in numerous industrial processes, such as oil recovery, mineral flotation, cosmetics, fire-fighting and chemical decontamination [3–6]. As we all know, foams are sub-stable systems where the dispersed gas is wrapped in a liquid film. Over time gas transfer between foams occurs, the average foams particle size increases and the liquid film breaks down [7]. To effectively stabilize foams, stabilizers such as proteins, surfactants, polymers and nanoparticles are usually added [1,8,9]. Stabilizers stabilize foam by slowing down the three foam destabilization mechanisms of foams' drainage, coarsening and coalescence [10,11].

However, in some industrial production such as wastewater treatment and material recovery, the presence of stable foams is destructive and needs to be defoamed promptly at the end of use [2]. The most common method currently used is the addition of a defoamer, but the defoamer is difficult to separate from the components and causes contamination of the original system. And its addition hinders



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the product from foaming again, making it impossible to be used twice [12-14]. To solve this problem, stimuli-responsive surfactants have attracted a lot of attention from researchers [15]. Through certain triggering mechanisms, such as pH [3], temperature [16,17], CO₂/N₂ [18–20] and light [21], structural changes are induced in the surfactant molecules in solution. The reversible transition between "surface-active" and "non-surface active" surfactant results in responsive foams that can switch between stable and unstable states. In fact, pH-sensitive surfactants generally contain ionizable organic groups such as carboxyl, phenylhydroxyl and amino groups [3]. Their solubility or ionization state changes with the pH of the solution, leading to a shift in their macroscopic properties. Usually, researchers have used the addition of acids or bases to change the pH of the solution. However, they generate by-products that disturb the original system. CO₂, being an environmentally friendly gas, does not accumulate in the system when used as a trigger. After bubbling CO₂ into the solution, it forms carbonates with water and lowers the pH of the solution. The surfactant molecules in the solution are protonated. Since the carbonate is very unstable, the original system can be restored to its initial state by simply passing N₂ under heated conditions.

Most surfactants are currently produced from petrochemical resources, which is not conducive to green and sustainable development. The use of natural products as raw materials instead of traditional petroleum resources to synthesize green surfactants has become an industry trend. Rosin is a forest resource with abundant production and is one of the main raw materials for the preparation of green surfactants [7,22]. Its unique tricyclic diterpene rigid skeleton structure can be arranged more regularly at the gas/liquid interface, increasing the strength of the interfacial film and forming stable foams [23–25]. Lei et al. [12] synthesized an azo-benzene surfactants from dehydrofirmanoic acid, which can be adjusted to the transcis isomerization of the azo-benzene moiety using UV light. Zhai et al. [7] prepared a pH-responsive surfactant from rosin, which can adjust the pH of the solution by adding acid and base to regulate the foaming performance of the surfactant. However, the structural modification of surfactants containing rigid structure and their conformational relationships are still under-studied.

In this article, we synthesized an extremely rigid rosin-based amphiphile surfactant rosin acid dimaleimide choline (R-BMI-C). By Diels-Alder addition and acid-base neutralization reactions, we obtained a large rigid skeleton and introduced CO_2/N_2 responsive groups. The strongly responsive surface activity and self-organized system were realized. In addition, the CO_2 responsive surfactants formation was constructed by forming carboxylic acid choline salt expands the controllable range of surfactants.

2 Experimental Section

2.1 Sample Preparation and Characterization

Abietic Acid (purity >95%) was purchased from Westech Chemical Co. Ltd., Guangzhou, China. N, N'-(4,4'-Methylenediphenylene) dimaleimide (BMI), p-Toluenesulfonic acid and choline (44% weight in water) were obtained from Maclean's Chemistry Company, Shanghai, China. Acetic acid (AR), acetone (AR) and petroleum ether (AR) were purchased from Sinopharm Chemical Reagent Co. Ltd., Shanghai, China. All reagents were used as received.

Fourier transform infrared (FT-IR) spectra were recorded by Thermo Scientific Nicolet and tested in the wavenumber range of 400~4000 cm⁻¹. ¹H NMR was measured by 500 MHz Nuclear Magnetic Resonance Spectroscopy using deuterated dimethyl sulfoxide as the solvent.

2.2 Synthetic Methods

2.2.1 Synthesis of Rosin Acid Dimaleimide (R-BMI)

10 g of Abietic Acid and 4.3 g of N, N'-(4,4'-Methylene-diphenyl) bismaleimide were dissolved in 30 mL of acetic acid and 0.46 g of p-toluenesulfonic acid was added as catalyst. The mixture was stirred at 120°C for 15 h. After cooling to room temperature, the crystalline R-BMI was obtained by filtration.

The crystals were recrystallized using acetone as solvent. Finally, the resulting solid was dried under vacuum at 70°C for 12 h.

2.2.2 Synthesis of Rosin Acid Dimaleimide Choline (R-BMI-C)

Firstly, choline solution with a concentration of 0.02 mM was prepared in ultrapure water, and then the choline solution was mixed with R-BMI in the ratio of molar ratio $R = \frac{n_{choline}}{n_{rosin acid dimaleinide}} = 2.4$, and the reaction was carried out at a temperature of 75°C for 1 h. When the reaction solution was cooled to room temperature at the end, it was extracted with petroleum ether three times and freeze-dried for 12 h to obtain R-BMI-C. The synthetic route of R-BMI-C was shown in Fig. 1.



Figure 1: Synthesis of R-BMI-C

2.3 CO_2/N_2 Responsive

Aqueous solutions of surfactants of different concentrations were bubbled with CO_2 until the solutions obtained the desired pH. CO_2 can be removed by passing N₂ into the solution at 65°C for 1–2 h.

2.4 pH Measurement

To confirm the responsiveness of the rosin-based amphiphile surfactant, the pH of the surfactant solution (Sigma pH test pen) was measured under the conditions of alternate passage and removal of CO_2 . The pH of each sample was measured three times and the average value was taken.

2.5 Surface Tension Measurement

The surface tension of R-BMI-C was measured by the SDC-100S contact angle meter. The dynamic surface tension of the R-BMI-C aqueous solution at different concentrations and pH were determined by the suspension drop method. R-BMI-C was prepared as two series concentration surfactant solutions, and the pH of prepared surfactant solutions were adjusted to10.4 and 7.4 by bubbling CO₂, respectively. All samples were performed at room temperature, and each sample was tested three times and averaged, with negligible differences between measurements.

2.6 Foaming and Foam Stabilization

Add 4 mL of surfactant solutions of different concentrations at different pH values to a 10 mL stoppered measuring cylinder, respectively. The surfactant solutions were shaken vigorously by hand for 30 s. All foams were prepared by the same operator to avoid excessive errors. To characterize the foaming properties of the surfactant solutions, we recorded the volume of foams formed. And the foams' stabilization was determined by recording the change in foams volume with time.

2.7 Fluorescence Microscope Observation

The morphology of the foams was obtained by a ZYF-2000E inverted fluorescence microscope (Shanghai Zhaoyi Photoelectric Technology Co., Ltd.). The microscope is equipped with a color camera to take digital images (4632×3488). The foams with different pH were prepared according to the method in 2.6 and tested after standing for 5 min. A drop of foam was placed on a clean slide (24×60 mm) and covered with another slide (18×18 mm), and then the micrographs were recorded.

2.8 Cryogenic-Transmission Electron Microscope (Cryo-TEM)

The microscopic morphology of the rosin-based surfactant solution was observed by Cryo-TEM. 5 μ L of the solution to be measured was added dropwise to the copper mesh using a pipette, then the copper mesh was quickly placed in liquid propane, and the prepared samples were placed in liquid nitrogen for storage. The frozen sample rod was Gatan 626, tested by applying a JEOL JEM-1400 transmission electron microscope, and the images were collected and stored by Gatan 831 CCD.

3 Results and Discussion

3.1 Synthesis and Structure Characterization of R-BMI-C

The structure of rosin acid contains two reactive centers, double bonds and carboxyl group, which are easy to be chemically modified. In this study, the rigid structure rosin acid was used as the raw material, and with the intervention of p-toluenesulfonic acid, the rosin acid underwent isomerization to generate the more reactive levopimaric acid. The conjugated double bonds of levopimaric acid underwent Diels-Alder addition reaction with the diolefins hydrocarbon of bismaleimide to produce rosin acid bismaleimide. Finally, the desired product R-BMI-C was obtained by a simple acid-base neutralization reaction of rosin acid bismaleimide with choline.

The chemical structures of R-BMI-C were characterized by FT-IR and ¹H-NMR. FT-IR spectra of R-BMI and R-BMI-C were shown in Fig. 2. ¹H NMR spectrum of R-BMI and R-BMI-C were shown in Figs. 3 and 4, respectively. It indicates that the intermediate product R-BMI and the target product R-BMI-C have been successfully synthesized.

The absorption peaks at 3444.73 cm⁻¹ are stretching vibrational peaks of hydroxyl groups (-OH), 2935.13 and 2868.60 cm⁻¹ for methyl and methylene (CH₃-, -CH₂-), 1770.82 cm⁻¹ for amide (-CO-N-OC-), and 1698.98 cm⁻¹ for carboxylic acid (-COOH). After the reaction with choline, a new stretching vibration peak of carboxylic acid ion (-COO⁻) appeared at 1560.07 cm⁻¹, and it can be inferred that the target product R-BMI-C has been synthesized.

¹H NMR (500 MHz, DMSO) δ 7.30 (m, 4H, C3-4H), 6.97 (m, 4H, C2-4H), 5.50 (s, 2H, C6-2H), 3.98 (s, 4H, C1-4H), 3.43 (m, 2H, C5-2H), 2.70 (d, 2H, C4-2H), 2.43-2.40 (m, 4H, C9-2H, C20-2H), 1.73-1.36 (m, 26H, C14-4H, C10-4H, C11-4H, C15-4H, C16-4H, C19-4H, C12-2H, C13-6H), 1.05 (s, 2H, C18-2H), 0.94-0.91 (m, 18H, C7-6H, C8-6H, C17-6H).



Figure 2: FT-IR spectra of R-BMI and R-BMI-C



Figure 3: ¹H-NMR spectrum of R-BMI

¹H NMR (500 MHz, DMSO) δ 7.30 (m, 4H, C3-4H), 6.97 (m, 4H, C2-4H), 5.50 (s, 2H, C6-2H), 3.98 (s, 4H, C1-4H), 3.43 (m, 2H, C5-2H), 3.13 (s, 18H, C21-18H), 3.09 (s, 4H, C22-4H), 2.64 (d, 2H, C4-2H), 2.43-2.40 (m, 4H, C9-2H, C20-2H), 1.81-1.15 (m, 26H, C14-4H, C23-4H, C10-4H, C11-4H, C15-4H, C16-4H, C19-4H, C12-2H, C13-6H), 1.05-0.83 (m, 22H, C18-2H, C24-2H, C7-6H, C8-6H, C17-6H).



Figure 4: ¹H-NMR spectrum of R-BMI-C

The number of hydrogens of the synthesized product was the same as that of the target product, and the chemical shifts of the characteristic hydrogens were similar to those of the target product. It showed that the intermediate product R-BMI and the target product R-BMI-C were successfully synthesized.

3.2 Determination of Ionization Equilibrium Constants

R-BMI-C (1 g/L) was dissolved in 0.1 M NaOH solution and titrated with 0.1 M HCl solution. Determine the pH at different HCl volumes and make an acid-base titration curve with pH as the y-axis and HCl volume as the x-axis.

Fig. 5 shows the acid-base titration curve of R-BMI-C. Initially, the added HCl reacts with the NaOH in the solution in a neutralization reaction, at which time the pH of the solution slowly decreases. When NaOH is finished being reacted, the pH drops faster. When the pH of the solution is 10.17, the pH drop slows down. This indicates that part of the $-COO^-$ of R-BMI-C starts to combine with H⁺ to convert to -COOH. At this time, R-BMI-C transforms from a bola-type surfactant containing a hydrophobic backbone with two hydrophilic groups to a conventional-type surfactant with a single head and a single tail. As can be seen from the figure, when the pH was further reduced to 7.42, the $-COO^-$ at the other end of R-BMI-C also started to combine with H⁺ to convert to -COOH. Until the pH of the solution was reduced to 5.39, the solution R-BMI-C are 10.17 and 7.42, respectively.

3.3 The Transmittance of R-BMI-C Aqueous Solution

The transmittance of R-BMI-C was measured using a UV 2450 UV spectrophotometer with a test wavelength of 200–800 nm. The aqueous solution of R-BMI-C was added to a quartz cell with a 1 cm optical path length at 25°C, with ultrapure water as the background.



Figure 5: Variation curve of pH with the volume of HCl in the R-BMI-C aqueous solution

First, we bubbled CO_2 into the R-BMI-C aqueous solution (2 mM) to regulate the pH of the surfactant solution. The transmittance was also measured to determine the dissolution of R-BMI-C at different pH values, and the results are given in Fig. 6. The transmittance of R-BMI-C aqueous exceeded 99.17% when the pH was large than 10.3, at which time R-BMI-C was completely dissolved in water. The pH value of the solution decreased by bubbling CO_2 into the aqueous surfactant solution. The transmittance decreased with the decrease of pH value, and the transmittance was 2.38% when the pH value decreased to 6.3. It indicates that lots of R-BMI-C precipitated from the solution. During the bubbling of CO_2 , H⁺ was produced in the solution and R-BMI-C was protonated. When the pH was in the range of 6.3 to 10.3, both protonated (-COOH) and deprotonated (-COO⁻) forms of R-BMI-C coexist in the solution. And at pH less than 6.3, R-BMI-C is completely protonated and converted to its acid form. Therefore, the pH of the aqueous R-BMI-C solution was adjusted to greater than 6.3.



Figure 6: (a) Variation of transmittance of R-BMI-C with wavelength at different pH values; (b) transmittance of R-BMI-C at different pH values at 600 nm

3.4 Surface Tension and Critical Micelle Concentration of R-BMI-C

R-BMI-C has an excellent ability to reduce surface tension. The surface tension of R-BMI-C at pH 7.4 and pH 10.4 is shown in Fig. 7. The surface tension of the R-BMI-C aqueous solution showed two inflection points with increasing concentration. This is consistent with the properties of bola-type surfactants, which contain a hydrophobic backbone with two hydrophilic groups at both ends [26]. The second inflection point was the critical micelle concentration (cmc) [27], and the value of the cmc was 0.55 mM. When the pH was reduced to 7.4, some of the carboxylate ions of R-BMI-C were converted to carboxyl groups, and the transformation of the surfactant from bola type to conventional type was achieved. At this time, the cmc value of R-BMI-C was 0.09 mM, exhibiting strong aggregation ability. In the intervention of CO₂, some of the carboxylate ions of the surfactant were protonated (-COOH). The solution pH was changed and the cmc was also changed about 6-fold, exhibiting significant aggregation differences before and after the response. Sanchez-Fernandez et al. reported phosphocholine and sulfobetaine surfactants [28]. They both had cmc values of about 1 mM, when the sulfobetaine surfactant could reduce the surface tension to $32.79 \text{ mN} \cdot \text{m}^{-1}$, exhibiting excellent surface tension reduction. The fully rigid backbone of R-BMI-C has relatively fixed positions of carbon atoms, which cannot resist structural changes caused by environmental changes by twisting or bending like flexible chains. This property allows it to maximize the property changes caused by molecular structure changes triggered by external stimuli [12]. However, the large rigid structure of R-BMI-C hinders its ability to reduce surface tension. At pH 10.4 and pH 7.4, the surface tensions of R-BMI-C at cmc were 42.79 and 47.83 mN·m⁻¹, respectively, which were significantly higher than those of surfactants containing flexible chains in general. The surface tensions were significantly higher than those of the general surfactants containing flexible chains. It shows that R-BMI-C is more capable of aggregation but less capable of reducing surface tension.



Figure 7: Surface tension as a function of concentration for R-BMI-C aqueous solutions at pH 7.4 and pH 10.4

3.5 The Effect of Concentration on Foams

To evaluate the foams' performance of R-BMI-C, we bubbled CO_2 into R-BMI-C aqueous solution to adjust its pH to 7.4 and studied the foam produced by R-BMI-C aqueous solution at this time. Firstly, we prepared a series of aqueous solutions of R-BMI-C with a concentration gradient. Stable foams were

formed by vigorous shaking for 30 s. As shown in Fig. 8a, the foams volumes were 2.6, 4.0, 5.2 and 6.2 mL for concentrations of 0.1, 0.5, 1 and 2 mM, respectively. The initial volume of foams increased with the increase of R-BMI-C aqueous solution concentration. The stability of the foams was commonly judged by their half-life time, which is the time required to reduce the volume of the foam to half of the initial volume. Fig. 8b records the variation of the volume of the foam formed by the R-BMI-C aqueous solution at each concentration with time. This resulted in foams half-lives of 3, 196, 310, and 336 h for R-BMI-C aqueous solutions with concentrations of 0.1, 0.5, 1, and 2 mM, respectively. As far as we know, Lei et al. [12] reported a half-life of 976 min for almost completely rigid R-AZO-Na at a concentration of 4 mM. Zhai et al. [7] reported a half-life of 2145 min for the bola-type surfactant Na-MPA-AZO-Na at 2 mM. In conclusion, the completely rigid surfactant R-BMI-C has excellent foams stabilization properties. This is attributed to its large rigid backbone structure, which is more regularly arranged at the gas-liquid interface than the flexible hydrophobic chains. Thus, increasing the strength of the interfacial film and forming a more stable foam.



Figure 8: (a) Digital photographs of aqueous foams (b) Variation of foams volume by R-BMI-C aqueous solutions with concentrations of 0.1, 0.5, 1, and 2 mM at pH 7.4

In this paper, we used the shaking hand method to characterize the foaming properties of the surfactant solutions. Each set of experiments was repeated three times. Even though the experiments were operated by the same person, there were still errors. The error bars of foaming and foam stabilization tests are shown in Fig. 9.

3.6 CO_2/N_2 Responsive Aqueous Foams

R-BMI-C is highly soluble in aqueous solutions at pH 10.4. By bubbling CO_2 into the solution, R-BMI-C was protonated and the pH of the solution was then reduced. In this process, aqueous solutions of R-BMI-C with pH 10.4, 9.2, 8.4, and 7.4 were taken and their foams' properties were explored. As shown in Fig. 10, the initial volume of foams was 7.0, 6.8, 6.6, and 6.2 mL at pH 10.4, 9.2, 8.4 and 7.4, respectively. The initial foams volume decreased slightly with increasing CO_2 content, but the foams' stability increased. The foams half-lives of the aqueous R-BMI-C solutions at pH 10.4, 9.2, 8.4 and 7.4 were 1.5, 13, 37, and 336 h, respectively. The error bars of foaming and foam stabilization tests are shown in Fig. 9. As mentioned above, the foams' stability was related to the structure of the surfactant skeleton, and the rigid structure was more favorable than the flexible chain to stabilize the foams. In addition, the foams' stability is closely related to the foams' particle size. We further observed the foams'

size by fluorescence microscopy and found from Fig. 11 that the foams' particle size decreases with increasing pH. It is well known that the smaller the foams particle size, the stronger the resistance to coarsening process and the stronger the foams' stability [29]. In summary, the foams formed by the aqueous solution of R-BMI-C have excellent CO₂ responsiveness.



Figure 9: (a) Error bars of foaming tests for different concentrations of R-BMI-C aqueous solution; (b) error bars of foam volume at half-life for R-BMI-C aqueous solutions of different concentrations; (c) error bars of foaming tests for different pH values of R-BMI-C aqueous solution; (d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life for R-BMI-C aqueous solution; d) error bars of foam volume at half-life foa R-BMI-C aqueous solution; d) error bars of foam volume

We also investigated the responsiveness of R-BMI-C (2 mM) aqueous solutions to CO_2/N_2 . As shown in Fig. 12, the bubbling CO_2 modulates the pH of the solution to 7.4 and the transmittance is 76.09%. At this time, R-BMI-C aqueous solution could form abundant and stable foams with an initial volume of 6.2 mL and a foams half-life of up to 336 h. At 65°C, N₂ was introduced into the above solution to blow away the CO_2 in the solution. The pH of the solution then rose to 10.4 and the transmittance of the R-BMI-C aqueous solution was close to 100%. At this point, the initial volume of foams formed by R-BMI-C aqueous solution increased to 7 mL, but the foams collapsed completely within 2 h. In addition, continuing to blow CO_2 into the R-BMI-C aqueous solution to adjust the pH to 7.4, stable foams with an initial volume of 6.2 mL could be formed again. The process of changing the foaming and defoaming properties of R-BMI-C aqueous solution by bubbling CO_2/N_2 can be cycled at least three times, indicating that

R-BMI-C aqueous solution has excellent CO_2/N_2 responsiveness. The changes in molecular structure and macroscopic self-assembly before and after the response of R-BMI-C are shown in Fig. 13. By bubbling CO_2 into the R-BMI-C aqueous solution, some of the carboxylate ions of R-BMI-C were protonated to form carboxylic acids. The macroscopic scale shows the transition from a clarified aqueous solution to a turbid state. By bubbling N_2 into the solution, the carboxylic acid in the R-BMI-C molecule is deprotonated back to the initial state.



Figure 10: (a) Digital photographs of aqueous foams (b) Variation of foams volume by R-BMI-C aqueous solutions with pH 7.4, 8.4, 9.2 and 10.4 at a concentration of 2 mM



Figure 11: Foam micrographs of foams by R-BMI-C aqueous solutions at (a) pH 10.4, (b) pH 9.2, (c) pH 8.4 and (d) pH 7.4 at a concentration of 2 mM (All foam micrographs were taken at 5 min of foam preparation)



Figure 12: (a) Digital photographs of aqueous foams, (b) pH and transmittance for three responsive of R-BMI-C aqueous solutions at 2 mM



Figure 13: CO_2/N_2 -responsive R-BMI-C structural reversible transition; The inset corresponds to the schematic diagram of the CO_2 -induced self-assembly transition of R-BMI-C in aqueous solution

3.7 Mechanism of CO₂ Responsive and Photoresponsive Foams

Various properties of surfactants, such as emulsification, solubilization, foaming, and foams stabilization, are closely related to surfactant self-assembled structures [30,31]. The self-assembly morphology can be described by the molecular arrangement parameter $p = v/a_0 l$, where v and l are the volume and maximum effective extension length of the hydrophobic group of the surfactant, respectively, and a_0 is the effective area occupied by the hydrophilic group of the surfactant on the surface of the aggregates. When p < 1/3, the surfactant tends to form spherical micelles, self-aggregates to form worm micelles at 1/3 . For <math>1/2 , a vesicle structure is formed in solution and obtains a bilayerstructure at $p \approx 1$ [32,33]. The self-assembled structure of surfactants depends on the interaction forces between surfactant molecules, including hydrogen bonding, electrostatic interactions, van der Waals forces, and hydrophobic forces. Among them, hydrophobic forces are the key driving force for the aggregation of surfactants to form different aggregates [30,34]. Currently, the most common method to increase the hydrophobic interaction of surfactants is to increase the hydrophobic group of surfactants, thus enhancing surfactant self-assembly [35]. In addition, the electrostatic interaction between the charge ion head groups of surfactants is also an effective means to enhance the intermolecular forces of surfactants. The self-assembled structure of surfactants can be changed by adjusting the composition or concentration of anionic and cationic surfactants.

To investigate the structure of R-BMI-C aqueous solution aggregates, we obtained their internal structure diagrams by Cryo-TEM, as shown in Fig. 14. Before bubbling CO_2 , R-BMI-C had strong hydrophobic interactions between the bulky rigid groups and electrostatic interactions between the charge ion head groups. Both interactions lowered the packing parameters and formed spherical micelles in the solution (Fig. 14a at the red arrow). The foams produced by spherical micelles are unstable but the foams produced by laminar or vesicular micelles are extremely stable [15]. The intervention of either CO_2

provides H⁺, which protonates some of the carboxyl groups of R-BMI-C. At this point, the electrostatic force between surfactant head groups decreases. When both forms of R-BMI-C protonated and deprotonated coexist, hydrogen bonds can be formed between the molecules of both forms. As a result, the effective headgroup area decreases while the packing parameter increases and R-BMI-C self-assembles to form lamellar micelles (Fig. 14b at the yellow arrow). Fig. 15 shows the transition of the self-assembled microstructure from spherical micelles to lamellar micelles [36]. The lamellar structure tends to adsorb at the air/water interface or is trapped in the foams film. And they avoid the too fast exchange of surfactant molecules at the interface or between monomers. As a result, foams' coarsening and coalescence processes are slowed down, and foams' stability is significantly increased [37].



Figure 14: Cryo-TEM images of R-BMI-C aqueous solution (a) at pH 10.4 and (b) after bubbling CO₂



Figure 15: Schematic diagram of microstructure changes in CO₂ responsive of R-BMI-C aqueous solution

4 Conclusion

Most reported CO_2 -responsive surfactants mainly focus on surfactants with straight-chain alkyl groups as lipophilic groups, and responsive surfactants with larger rigid groups have rarely been studied. In this work, the rosin-based surfactant R-BMI-C was synthesized by simply mixing R-BMI with choline hydroxide in a molar ratio of 1:2.4. This process avoided complex synthetic reactions and can reduce the reaction cost. And the clean response of bio-based surfactant was achieved by using CO_2 as triggering agents. The foams prepared by R-BMI-C at pH 10.4 were unstable with a half-life of only 1.5 h. The foams stability can be enhanced by lowering the pH of the solution by bubbling CO_2 . According to the results, the foams prepared at pH 7.4 were exceptionally stable with a half-life of up to 336 h. In addition, unstable foams can be formed again by blowing away CO_2 by bubbling N_2 into the solution. The intervention of CO_2/N_2 enables at least three transitions between stable and unstable foams. The CO_2 content controls the structure change of the surfactant from bola type to conventional type. The coexistence of both protonated and deprotonated forms of R-BMI-C increases the packing parameters to form a bilayer structure and shows excellent foam stabilization. It is shown that the rigid surfactant R-BMI-C has excellent foams performance and responsiveness and could be widely used in oil extraction, fire fighting, or chemical decontamination.

Acknowledgement: The authors would like to thank the Analytical Instruments Testing Center of Huaqiao University for allowing us to use their instrumentation to analyze our data.

Funding Statement: This research is supported by the National Natural Science Foundation of China (32171734) and the Scientific Research Funds of Huaqiao University (20BS201).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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DOI: 10.32604/jrm.2022.022996

ARTICLE





The Promoting Effect of Multifunctional Groups on the Thermal and Mechanical Properties of PVC Materials

Mei Wang^{1,*}, Xinzhu Fan¹, Xianghai Song² and Quan Bu^{1,*}

¹School of Agricultural Engineering, Jiangsu University, Zhenjiang, 212013, China

²Institute of the Green Chemistry and Chemical Technology, School of Chemistry and Chemical Engineering, Jiangsu University, Zhenjiang, 212013, China

^{*}Corresponding Authors: Mei Wang. Email: 1000004927@ujs.edu.cn; Quan Bu. Email: qbu@ujs.edu.cn Received: 04 April 2022 Accepted: 06 June 2022

ABSTRACT

The development of PVC materials grafted with mannich base originated from myrcene (P-MAM-g, where the mannich base derived from myrcene is abbreviated as MAM) via green and effective synthetic methods is a good strategy to avoid unacceptable discoloration and deterioration of thermal and mechanical properties caused by autocatalytic dehydrochlorination (DHC) during PVC processing. In this study, MAM with double bonds, amino groups, ester groups, and phospholipid groups was introduced into the chains of PVC to improve the thermal stability of PVC. The experimental results showed that the covalent attachment of MAM to PVC enhanced both the initial and the long-term stability of PVC. The enhanced performance of P-MAM-g compared with unmodified PVC is attributed to the simultaneous introduction of double bonds and amino groups into the PVC structure. The double bonds trapped the unstable chlorine atoms originated from the degradation of the PVC chain and reacted with the labile macromolecular radicals originated from PVC, thus inhibiting the radical degradation of the PVC chain. Furthermore, the amino groups absorbed the HCl produced in the degradation of PVC, inhibiting the adverse effects of HCl. P-MAM-g displayed better intrinsic flexibility and anti-migration ability of organic functional components compared with the control PVC materials. A possible stabilizing mechanism of the P-MAM-g was also presented.

KEYWORDS

Myrcene; poly(vinyl chloride); covalent grafting; self-stabilization

Nomenclature

Term 1:	Interpretation 1
Term 2:	Interpretation 2
e.g.	
Ø:	Porosity
s:	Skin factor



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1 Introduction

Poly(vinyl chloride) (PVC) has been widely applied in various fields; however, it degrades easily at processing temperatures due to autocatalytic dehydrochlorination (DHC) [1,2]. This degradation occurs because of the elimination of HCl and the production of conjugated unsaturated double bonds [3], which remarkably decreases the color, thermal and mechanical properties [4,5]. Previous studies have shown that adding thermal stabilizers is an effective and practical method to inhibit the degradation of PVC [6].

Metallic soaps, organic tin/lead salts, and nitrogenous compounds have been recently used as thermal stabilizers and color retention reagents for PVC [7–9]. Though lead salts and organic tin can effectively stabilize PVC, the toxicity limits their application in PVC products. CaSt₂/ZnSt₂ (calcium stearate/zinc stearate) composite heat stabilizers have been extensively investigated attributing to their synergistic effect in stabilizing PVC. However, zinc chloride (ZnCl₂) with strong Lewis acidity is formed in the manufacturing process of PVC materials thermally stabilizers show great potential attributing to their advantages including high efficiency, low environmental impact, good compatibility, and strong universality. However, existing organic thermal stabilizers fail to inhibit long-term discoloration of PVC and need to be used in conjunction with other thermal stabilizers for further enhancing thermal stability [11].

PVC materials are currently prepared by physically blending thermal stabilizers with PVC, causing uneven dispersion of the thermal stabilizers and the migration of organic functional components [12]. This process deteriorates their thermal stability [13]. Therefore, the development of non-toxic and green, uniformly dispersed and anti-precipitated organic thermal stabilizers is of great significance to the development of thermally stable PVC materials.

In comparison with physical blending, the introduction of organic functional groups into the structure of PVC via chemical grafting is expected to significantly improve the migration resistance abilities and mechanical properties of PVC. For example, Jia et al. [14] prepared cardanol butyl ester mannich base with cardanol and grafted it onto PVC via a substitution reaction. The as-obtained PVC sample displayed good plasticizing and mechanical properties and a migration rate almost of 0. Although PVC materials grafted with organic compounds present improved long-term thermal stability compared with pure PVC, it is still difficult to simultaneously delay "zinc-burning" and inhibit the initial degradation and coloring of PVC materials. For instance, Zhu et al. [15] successfully inhibited the first stage of thermal degradation of PVC by grafting phenol to PVC under microwave irradiation, resulting in improved long-term thermal stability of the PVC. However, the initial color and prolonged long-term thermal stability of the PVC sample was not satisfactory due to the rigid structure of phenol, thus the sample exhibited poor mechanical properties. Therefore, it is still urgent to develop novel organic thermal stabilizers by means of a grafting strategy to further facilitate the initial stability, long-term stability, and mechanical properties of PVC materials.

Recently, it was reported that Ca/Zn stabilizers with multifunctional groups such as amino groups, double bonds, and ester groups were reported to endow PVC with good long-term stability [16,17]. These functional groups can trap the labile chlorine atoms removed from the PVC chain, react with the unstable macromolecular radical of PVC, and act as HCl absorbers at subsequent stages of degradation, demonstrating a significant thermal stabilizing effect on PVC. Thus, PVC samples grafted with organic compounds containing amino groups, double bonds, and ester groups are expected to display enhanced stability and thus improved mechanical properties. Myrcene, an oily liquid, contains conjugated double bonds in its structure and thus is an ideal raw material for the preparation of multifunctional PVC materials. The conjugated double bond of myrcene enables it to react easily with dienophiles via the Diels–Alder reaction to introduce other functional groups into its structure. The as-obtained products

from this reaction of myrcene have been widely applied in coatings, epoxy curing agents, and vinyl ester resin [18–20]. Similarly, the introduction of lauraldehyde derivatives into the PVC chains via covalent bonds is a promising choice for constructing self-stabilized PVC polymers.

Accordingly, this study describes the development of a myrcene-based organic thermal stabilizer containing double bonds, amino groups, ester groups, and phospholipid groups in its structure and its application in stabilizing PVC via chemical grafting. A series of MAM grafted PVC samples (P-MAM-g, where the mannich base derived from myrcene is abbreviated as MAM) were successfully prepared by the replacement reaction of the chlorine group in PVC with a mannich base derived from myrcene. To date, there are no reports of PVC materials simultaneously incorporating double bonds, amino groups, ester groups, and phospholipid groups into their structure.

2 Experimental Section

2.1 Materials

Myrcene was purchased from Jiangxi Global Natural Spices Co. (China), containing about 79% of myrcene. Acrolein (98%, Aladdin), diethyl phosphite (99%, Aladdin), dioctyl phthalate (DOP, 98%, Aladdin) diethylenetriamine (99%, Aladdin), pure PVC (suspension grade, S-1000 ZnCl₂ (95%, Nanjing Chemical Reagent), sodium chloride (99%, Nanjing Chemical Reagent), sodium bicarbonate (99%, Nanjing Chemical Reagent), anhydrous magnesium sulfate (99%, Nanjing Chemical Reagent), tetrahydrofuran (THF, 99%, Nanjing Chemical Reagent), N,N-dimethyl-formamide (DMF, 99%, Nanjing Chemical Reagent), methanol (99%, Nanjing Chemical Reagent), and n-hexane (97%, Nanjing Chemical Reagent) were used as purchased.

2.2 Synthesis of Myrac Aldehyde (MA)

First, myrcene (55.85 g, 0.410 mol) and zinc chloride (3.41 g, 0.025 mol) were added into 250 mL flask equipped with the magnetic stirrer and reflux condenser. After the temperature was raised to 55°C, acrolein (27.47 g, 0.49 mol) was added drowsily and, maintained the temperature at approximately 55°C. The resulting suspension was aging at 60°C for 6 h. The obtained product was washed by 10% sodium chloride solution, 5% sodium bicarbonate, dried with anhydrous magnesium sulfate and removed the excess acrolein by a rotary evaporator to get a light yellow liquid (MA, Fig. 1).

2.3 Synthesis of Mannich Base Derived from Myrcene (MAM)

First, MA (28.85 g, 0.15 mol) was added dropwise to a flask containing diethylenetriamine (15.48 g, 0.15 mol) solution. The mixture was stirred at 20°C for 2 h and then raised to 90°C. Thereafter, the resulting mixture was raised to 90°C. Then diethyl phosphite (20.72 g, 0.15 mol) was added drop-wise to the above mixture and kept at 90°C for 4 h. After the reaction, the product was raised to 120°C to remove water. Finally, a light-yellow slimy product was obtained (MAM).

2.4 Synthesis of MAM Grafted PVC (P-MAM-g)

A mixture (7 g) of PVC and MAM was dissolved in 80 mL of DMF, the resulted solution was washed with methanol several times and dried in a drybox at 60°C. The obtained product (5 g) was dissolved in THF (60 mL), then poured into a glass dish and dried at room temperature for 2 days to get the self-plasticization PVC materials (P-MAM-g) [21]. All the P-MAM-g (P-MAM-1, P-MAM-2, P-MAM-3, and P-MAM-4) were synthesized by the same reaction conditions as described above, except that the mass ratios of PVC and MAM were 5:1, 5:2, 5:3, 5:4, respectively. Pure PVC and PVC/40DOP [m(PVC):m(DOP) = 2.5] samples were prepared by the same method.



Figure 1: Preparation of mannich base originated from myrcene (MAM) grafted PVC (PVC-MAM-g)

2.5 Molecular Structure Characterization

Fourier transform infrared (FT-IR) spectra were achieved from Nicolet iS10 FT-IR (Nicolet Instrument Crop., USA) infrared spectrophotometer by the KBr disc method.

¹H NMR test was performed on a Bruker 400 MHz spectrometer (Bruker, Rheinstetten, Germany). CDCl₃ was used as a solvent to dissolve the samples of MA and MAM, respectively. Deuterated dimethyl sulfoxide (DMSO) was used as a solvent to dissolve the samples of PVC P-MAM-2 and P-MAM-4, respectively.

Weight-average molecular weight and dispersity were measured using a gel permeation chromatograph (GPC) made by Waters, USA at 30°C (flow rate: 1 mL min⁻¹, column: mixed PL gel 300 × 718 mm and 25 μ m, HPLC-grade DMF as solvent) using. All PVC materials were dissolved in THF solutions to form a concentration of 1~5 mg mL⁻¹.

2.6 Thermal Stability Analysis

The static thermal aging test was performed according to ISO 305-1990 (E) (Plastics. 1990). The PVC sheet was cut into 20 mm \times 20 mm pieces and put in a thermal aging test box at 180°C. The color of the sample was recorded every 5 min, and then the thermal stability of the sample could judge by the degree of color change.

The thermogravimetric analysis (TGA) was also performed by a TA Q600 instrument (NetzschInstrument Crop., Germany). Typically, 4 mg of samples were heated from 40 to 600°C at a heating rate of 10 °C min⁻¹, under nitrogen flow (100 mL min⁻¹).

2.7 Mechanical Properties Analysis

Differential scanning calorimetry (DSC) measurement was measured on a TA Instrument (NETZSCCH DSC 200 PC) in a nitrogen atmosphere. The measurement temperature range is from -50 to 120° C with a heating rate of 20° C min⁻¹. Approximately $5 \sim 10$ mg of PVC and self-plasticization PVC materials were

weighed and sealed in a 40 μ L aluminum crucible and immediately detected using DSC measurement. The DSC data was collected from the first cycle of heating.

Tensile strength and elongation at break were carried out according to the American Society for testing and materials (ASTM D638-03), and recorded on a CMT4303 universal test machine (Sans, China). The cross head speed was 50 mm min⁻¹. The samples used in the tensile test were 0.47 mm thick, 3.98 mm wide and 25 mm long. At least five tests were repeated for each sample.

DMTA Q800 (TA Instruments, USA) was used to analyze the dynamic mechanical behavior of the PVC sample under the tension mode. The oscillatory frequency used in the dynamic test was 1 Hz. The temperature range was within $-80-120^{\circ}$ C with a temperature rate of 3 °C min⁻¹.

2.8 Migration Properties Analysis

The migration resistance ability of the samples was investigated via the leaching experiment according to the ASTM D5227. Typically, the PVC sample $(2 \times 2 \times 0.45 \text{ cm}^3)$ with precise mass was immersed in n-hexane and kept at 50°C for 2 h. And PVC sample $(2 \times 2 \times 0.45 \text{ cm}^3)$ with precise mass was immersed in deionized water and kept at 25°C for 24 h. Subsequently, the above sample was thoroughly washed with deionized water, dried and weight for a second time. Degree of migration (%) = $\frac{W_{before} - W_{after}}{W_{before}} \times 100$. W_{before} is the weight of the sample before test. W_{after} is the weight of the sample after the test. Besides, the T_g of extracted samples were determined by DSC from -50 to 120°C at a heating rate of 20 °C min⁻¹.

3 Results and Discussion

3.1 Synthesis and Characterization

The structures of the as-prepared products were characterized and confirmed by FT-IR and ¹H-NMR spectroscopy. Fig. 2a shows the FT-IR spectra of myrcene, MA, and MAM. After the reaction of myrcene and acrolein, the absorption peaks representing conjugated double bonds (1594 cm^{-1}) and the terminal vinyl group of myrcene (3086 and 1796 cm⁻¹) disappeared. In addition, the resulting product showed the peaks for aldehyde groups (1725 cm⁻¹) and C-H bonds in the hexatomic ring (2706 and 1311 cm⁻¹). The double bond absorption peak (1644 cm⁻¹) was retained, indicating the successful preparation of MA. In the FTIR spectrum of the mannich reaction adduct, the peaks representing aldehyde groups (1725 cm^{-1}) disappeared and the peaks representing P=O, C-N and P-O (1439, 1027 and 950 cm⁻¹) appeared after the reaction of MA, diethyl phosphite and diethylenetriamine, suggesting successful preparation of MAM. A deep insight into the chemical structures of MA and MAM was further obtained by ¹H NMR characterization [Fig. 2b]. In comparison with MA, the MAM spectrum showed several new peaks in the approximately 4.5-2.5 ppm range (peaks 11, 12, 14, and 16-19), attributing to the hydrogen in the methylene connected to N or O containing groups such as -CH-NH-, P-O-CH2-, and -NH -CH2-CH2-NH-. This indicates that diethyl phosphite and diethylenetriamine were successfully introduced into the structure of MA. Moreover, other characteristic peaks of MA such as methyl (peaks 1 and 2) and methylene (peaks 3 and 6) were well-preserved. The FT-IR and ¹H NMR results demonstrate successful preparation of MAM.

MAM was covalently bonded to PVC via nucleophilic replacing the unstable chlorine atoms of PVC chains. The functional groups of the as-prepared samples were investigated by FT-IR spectroscopy. The peaks at 2977, 2912, 2837, 1668, 1026, 950, and 690 cm⁻¹ (Fig. 3a) were assigned to the C–H (sp), C–H (sp³), C–H (sp²), N–H stretching vibrations, C–N, P–O, and N–H rocking vibrations, respectively. The intensity of the peaks at 1668, 1026, 950, and 690 cm⁻¹ increased with the increasing replacement of chlorine atoms with MAM in PVC. In addition, the intensity of the peaks at approximately 627 cm⁻¹, assigned to the C–Cl stretching vibration of PVC, gradually decreased with increasing replacement of chlorine atoms with MAM, indicating successful preparation of P-MAM-g. The chemical structures of

PVC and P-MAM-g were also investigated by ¹H NMR. As shown in Fig. 3b, P-MAM-2 and P-MAM-4 show several new peaks in the ranges of 2.0–5.5 ppm (peaks 11, 12, 14, and 16–19) in comparison with PVC. These typical peaks are also be found in MAM, indicating the successful introduction of MAM into the PVC structure. Moreover, the intensity of these signals increased with the increasing displacement of chlorine in the PVC structure, while the intensity of other characteristic peaks such as -CH-Cl (peak 1') and CH_2 (peak 2') gradually decreased, indicating successful preparation of P-MAM-g.



Figure 2: (a) FT-IR spectra of myrcene, MA, and MAM; (b) ¹H NMR spectra of MA and MAM



Figure 3: (a) FT-IR spectra of pure PVC and P-MAM-g; (b) ¹H NMR spectra of pure PVC, P-MAM-2, and P-MAM-4

The number average molecular weight (M_n) of the as-prepared P-MAM-g samples was studied by gel permeation chromatography (GPC), as displayed in Fig. S1 and Table S1. The M_n increased from 28,048 to 28,851 g/mol with increasing replacement of chlorine atoms. These results demonstrate that the chlorine atoms in PVC were successfully substituted with mannich base derived from myrcene.

3.2 Thermal Stability Evaluated by Discoloration

In the process of heating treatment, the DHC of PVC unavoidably results in unacceptable discoloration. To comprehensively investigate the thermal stability of the PVC materials described above, the color stability was measured with a thermal aging test at 180°C (Table 1). The pure PVC strips initially exhibited excellent color, but turned dark brown in 5 min, and absolute black within 15 min. This phenomenon is derived from the degradation of pure PVC in the thermal treatment process. During this process, the chloride anions originating from the C-Cl bond breaking can extract protons from the adjacent C-H bond, eliminating HCl from the PVC chains. The loss of HCl forms conjugated unsaturated double bonds sequences, causing the color change of the polymer from white to slight yellow, dark brown, and finally absolute black [22]. The initial color of the P-MAM-g materials was tan or light brown. The thermal aging tests of the P-MAM-g samples show that at 5 min, all four P-MAM-g materials had a tawny brown color, lighter than the dark brown displayed by pure PVC at 5 min. The sample with the smallest change in color was P-MAM-3. Moreover, after 15 min, P-MAM-3 remained dark brown instead of turning completely black, unlike pure PVC or the other P-MAM-g materials. These results indicated that the covalent attachment of MAM to PVC improved the initial and long-term stability of PVC. This phenomenon can potentially be attributed to the double bonds of P-MAM-g, replacing active chlorine atoms and reacting with the unstable macromolecular radical of PVC, thus enhancing stability. In addition, the multiple double bonds of P-MAM-g assisted the formation of stable conjugated polyene compounds, further restraining the deterioration of PVC samples.



Table 1: Color changes of pure PVC and P-MAM-g samples under thermal aging at 180°C

3.3 Thermal Stability Evaluated by TGA

The additional insight into the thermal stability and thermal degradation behavior of pure PVC and P-MAM-g was obtained by thermogravimetric analysis (TGA) in an N₂ atmosphere. Fig. 4 shows that the thermal decomposition behavior of all PVC materials can be divided into two stages in good agreement with previously reported results [23,24]. The first stage (200–400°C) is mainly attributed to the release of hydrogen chloride leading to the generation of conjugated polyene structures. The second stage (400–560°C) is caused by the formation of aromatic compounds due to the cyclization of conjugated polyene sequences. All the relevant information is listed in Table 2. T_5 is the temperature at 5% weight

loss, T_{max1} and T_{max2} correspond to the temperatures at the maximum weight loss rate in the first and second degradation stages, respectively, while W_{300} and W_{600} are associated with the yields of residue at 300 and 600°C, respectively. The T_5 and W_{600} values of pure PVC were 154.2°C and 10.53%, respectively, while the T_5 and W_{600} values for P-MAM-g were 179.2–194.2°C and 15.44%–16.23%, respectively. Compared to the PVC samples, the grafted PVC samples show higher T_5 , the maximum weight loss rate of the first degradation, and W_{600} . These results demonstrate that P-MAM-g has enhanced initial and long-term thermal stability compared with pure PVC. Moreover, P-MAM-3 demonstrates enhanced thermal stability among the P-MAM-g materials. These results match those of the thermal aging test at 180°C.



Figure 4: TGA curves of pure PVC and P-MAM-g

Sample	<i>T</i> ₅ (°C)	T_{max1} (°C)	W ₃₀₀ (%)	$T_{\rm max2}$ (°C)	W ₆₀₀ (%)
Pure PVC	154.2	270.3	43.89	464.9	10.53
P-MAM-1	179.2	238.9	45.71	459.7	16.09
P-MAM-2	189.2	233.9	47.85	464.0	15.44
P-MAM-3	194.2	228.7	52.17	463.6	16.23
P-MAM-4	191.7	206.8	54.16	462.7	15.52

Table 2: Eigenvalues of the TGA curves of pure PVC and P-MAM-g

3.4 Thermal Stabilization Mechanism of P-MAM-g

Pure PVC sample tends to degrade under the processing temperature of 180°C. A potential degradation mechanism can be described as follows: the chloride anion produced by the C–Cl bond breaking extracts hydrogen from the adjacent C–H bond, eliminating HCl from the PVC chain. The loss of HCl results in the formation of conjugated double bonds sequences, causing the color change of the polymer from white to light yellow, dark brown, and finally completely black. As illustrated in Fig. 5, the generally accepted stabilization mechanism is based on the phenomena of P-MAM-g double bonds acting as radical trap reagents. In addition, the amino groups can absorb HCl to improve the stabilization of P-MAM-g. During the induction period of degradation (i.e., 0, 10, 20, 30, and 40 min), the Cl radicals originated from P-MAM-g degradation react with the double bond of P-MAM-g to form the radical III. Next, III captures the P-MAM-g radicals (I) to produce IV. Subsequently, IV captures the HCl eliminated from PVC. The process of capturing radicals and HCl continues until large amounts of conjugated polyene appear in the P-MAM-g molecules.



Figure 5: Proposed stabilization mechanism of P-MAM-g

3.5 Mechanical Properties

In addition to thermal aging, the thermal DHC of PVC materials can also deteriorate their mechanical properties [25]. The T_g of P-MAM-g was studied by DSC and compared with the T_g of pure PVC and a non-covalent mixture of PVC/40DOP. As displayed in Fig. 6, the T_g values of PVC/40DOP, P-MAM-1, P-MAM-2, P-MAM-3 and P-MAM-4 were 39.2°C, 36.4°C, 32.7°C, 28.5°C, and 33.0°C, respectively, far lower than the T_g value of pure PVC (84.4°C) [17]. P-MAM-g demonstrated an improved plasticization ability in comparison to PVC/40DOP and pure PVC. Grafting ester group, hexatomic ring and longer alkyl chains simultaneously onto PVC decreased the polymer-polymer interaction and enhanced the free volume between polymer molecules, which is helpful for decreasing the T_g of P-MAM-g [26–28]. The above analysis verifies that the introduction of multifunctional mannich base derived from myrcene into PVC is an effective strategy for providing PVC with intrinsic plasticization abilities.

The tensile strength of P-MAM-g was characterized to evaluate its mechanical properties. Fig. 7 shows that the tensile strength of P-MAM-g tends to decrease with increasing levels of MAM. This phenomenon may originate from the fact that MAM acts as a lubricant in PVC, weakening the internal friction between the PVC chains. Among the grafted PVC, P-MAM-2 exhibited the largest elongation at break (304.1%), which is 1.54 times higher than that of pure PVC. This may be attributed to the covalent bond between MAM and PVC, which can maintain the uniform and optimal structure of the material under tensile, preventing crack propagation and enabling the molecular chains to extand maximally [29]. These results further indicate that the covalent bond between MAM and PVC plays an important role in obtaining PVC



materials with high flexibility and extensibility. This characterization is in strong agreement with the DSC results.

Figure 6: DSC curves of P-MAM-g



Figure 7: Tensile properties of P-MAM-g

3.6 Migration Resistance Properties

The migration resistance ability is an important criterion for assessing the performance of PVC materials [30] and was investigated via the leaching test in this study according to ASTM procedures. As illustrated in Fig. 8, PVC/40DOP displayed 13.21% and 0.22% of the migration ability of compounds, respectively, indicating its poor anti-migration ability. However, the leaching tests showed that the migration of all P-MAM-g samples was almost negligible, indicating the outstanding anti-migration ability of P-MAM-g ascribed to the covalent bond between PVC and MAM [31]. As shown in Fig. 9, no considerable changes in the T_g of the P-MAM-g were observed, and the T_g values were below 38°C after the leaching experiment. Under the same experimental conditions, the T_g of PVC/40DOP remarkably raised from 39.2 to 49.9°C. Therefore, compared with PVC/40DOP, P-MAM-g films demonstrated improved organic functional component anti-migration ability and retained their flexibility at room temperature.



Figure 8: Weight loss of PVC/40DOP and P-MAM-g migration after the leaching test



Figure 9: T_g of the P-MAM-g before and after leaching test in n-Hexane

4 Conclusion

A series of self-stabilized PVC materials (P-MAM-g) were obtained by replacing chlorine in PVC with a mannich base derived from myrcene. The thermal stability, mechanical properties, and migration resistance ability of P-MAM-g were determined by TGA, thermal aging, DSC, and tensile tests. The thermal aging tests demonstrate that the best-performing P-MAM-3 material showed significantly less discoloration than the pure PVC after 15 min at 180°C, turning brown instead of completely black. In the TGA experiments, the temperature at 5% weight loss was 154.2°C and the residue yield at 600°C was 10.53%, whereas the two parameters for P-MAM-g were in the range 179.2–194.2°C and 15.44%–16.23%. These results demonstrate that covalent attachment of MAM to PVC improved the initial and long-term stability of PVC materials. This enhanced performance was attributed to the grafting of the double bonds and amino groups, that can simultaneously trap PVC radicals and HCl resulting from PVC deterioration onto the architecture of P-MAM-g. The DSC results revealed that P-MAM-g had improved plasticization performance compared with a non-covalent mixture of PVC and DOP. P-MAM-g also demonstrated an improved anti-migration ability of organic functional components compared with PVC/40DOP and retained their flexibility at room temperature. In the tensile tests, P-MAM-2 exhibited the highest elongation at break value (304.08%), 1.54 times higher than that of pure PVC. The tensile experiments

further showed that the covalent bond between MAM and PVC plays an important role in obtaining PVC materials with self-stabilization, high flexibility, and stretch ability.

Acknowledgement: This study was financially supported by the National Natural Science Foundation of China (21905117) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Funding Statement: This work was subsidized for the National Natural Science Foundation of China (21905117) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Supporting Information



Figure S1: GPC spectra of pure PVC and of P-MAM-g

PVC material	Number average molecular weight $(M_n, g/mol)$	Weight-average molecular weight $(M_w, g/mol)$	Z-average molecular weight (<i>M</i> _z , g/mol)	Dispersity $(M_z/M_w, g/mol)$
PVC	19381	22087	25362	1.1
P-MAM-1	28048	30598	33430	1.1
P-MAM-2	28548	30787	33908	1.1
P-MAM-3	28669	30952	34719	1.1
P-MAM-4	28851	31242	35885	1.1

Table S1: Relative molecular mass and distribution of pure PVC and -MAM-g



DOI: 10.32604/jrm.2022.022535

ARTICLE





Synthesis and Antioxidant Activity of (Ε) ω-Formylcamphene-Based Thiazole Hydrazone Derivatives

Jiayu Chang¹, Qingying Ding¹, Chong Zhong¹, Yao Long², Zhuanquan Xiao³, Hai Luo¹, Yuling Yang¹, Zongde Wang¹, Shengliang Liao¹, Jinzhu Chen², Shangxing Chen^{1,*} and Ji Zhang^{1,*}

¹College of Forestry, Jiangxi Agricultural University, East China Woody Fragrance and Flavor Engineering Research Center of National Forestry and Grassland Administration, Camphor Engineering Research Center of NFGA, Nanchang, 330045, China

²College of Science, Jiangxi Agricultural University, Nanchang, 330045, China

³College of Chemistry and Chemical Engineering, Jiangxi Normal University, Nanchang, 330027, China

*Corresponding Authors: Shangxing Chen. Email: csxing@126.com; Ji Zhang. Email: ex1990111@163.com

Received: 15 March 2022 Accepted: 29 April 2022

ABSTRACT

(E) ω -formylcamphene was synthesized from α -pinene, the main component of turpentine, and then reacted with thiosemicarbazide to obtain (E) ω -formylcamphene thiosemicarbazide **3**, which was reacted with 14 α -bromoace-tophenone compounds to obtain 14 (E) ω -formylcamphene thiazole hydrazone compounds **5a–5n**; the yields were all above 80%. The structures of the target compounds were characterized by IR, ¹H-NMR, ¹³C-NMR, and HR-MS analyses. Then, 500, 250, 125, 62.5, and 31.25 mg/L drug solutions were prepared. Free radical scavenging experiments of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2, 2-bis (3-ethyl-benzothiazole-6-sulfonic acid) diammonium salt (ABTS) were carried out with Trolox and L-ascorbic acid as the control samples. The scavenging rates of 14 compounds for DPPH and ABTS free radicals were obtained; the IC₅₀ values of scavenging free radicals were fitted using SPSS software. The results show that 14 (E) ω -formylcamphene-based thiazole hydrazone compounds exhibited good scavenging effects on the two free radicals, especially when the concentration of the drug solution was 125 and 62.5 mg/L; most compounds exceeded the scavenging efficiency of Trolox and L-ascorbic acid.

KEYWORDS

(E) ω-formylcamphene; thiosemicarbazone; thiazohydrazone; structural analysis; antioxidant activity

1 Introduction

In a biological system, oxidative stress is a state of imbalance between the oxidation and antioxidation in the body, and a negative effect produced by free radicals in the body, which is considered to be an important factor leading to aging [1,2] and disease [3,4]. A large number of reactive oxygen species (ROS) generated by oxidative stress will destroy the antioxidant defense system. Free radicals such as superoxide anion radical, hydroxyl radical, and hydrogen peroxide radical can cause oxidative damage to nucleic acids, proteins, and biofilms, leading to hypertension, diabetes, heart disease, and Alzheimer's disease [5–7]. Antioxidants can interact with free radicals to prevent the destruction of reactive oxygen species; therefore, many studies have been conducted on the design and development of new and effective antioxidants [8,9]. In recent years, the research and development of antioxidants has become an important research topic worldwide.



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However, synthetic chemical antioxidants have some potential health hazards; therefore, the antioxidants prepared by the modification of natural products have attracted much attention [10-12].

Camphene, a dicyclic monoterpene compound, exists in a variety of natural volatile oils, such as camphor oil, citronella oil, turpentine oil, and cedar oil [13], and it can be prepared from the main component of turpentine α -pinene [14]. It is a renewable raw material in the fine chemical industry and can participate in many chemical reactions [15]. (E) ω -formylcamphene is synthesized by the *Vilsmeier–Haack* formylation reaction of camphene, and a series of camphene derivatives such as ω -acyl camphene, internal isocamphene alkyl methanol and alkyl ethers, alcohol acetate, and isocamphene alkyl ketone oxime can be synthesized from (E) ω -formylcamphene [16–19]. Some of these derivatives exhibit good biological activities. Thiazole is a five-atom heterocyclic compound and a very important scaffold in the pharmaceutical chemistry [20,21]. Many compounds with this structure often have antioxidant [22], antibacterial [23,24], antitumor [25], and other biological activities. The derivatives of thiazolylhydrazone compounds contain two active groups of thiazolyl ring and hydrazone (R–C=N–N–R), which may produce new compounds with higher biological activities. Therefore, they have received much attention in medicine, pesticides, and materials [26].

In this study, (E) ω -formylcamphene was prepared by the Vilsmeier-Hack formylation reaction of camphene with thiosemicarbazide to obtain (E) ω -formylcamphene thiosemicarbazide derivatives. DPPH and ABTS methods were used to determine the free radical scavenging capacity of the compounds, and the antioxidant activity of the derivatives was analyzed to provide a reference for the development of new antioxidants.

2 Experimental

2.1 Materials

All the chemicals are commercially available (Aladdin Biochemical Technology Co., Ltd., Shanghai, China). All the solvents were distilled and dried according to standard procedures. Melting points were determined using a WRS-2 melting point apparatus (Shanghai Precision & Scientific Instrument Co., Ltd., Shanghai, China). FT-IR spectra of the compounds were recorded using a Nicolet IS10 FT-IR spectrometer. Proton nuclear magnetic resonance spectra (¹H NMR and ¹³C NMR) were obtained at 400 MHz using a Bruker DPX 400 spectrometer (Bruker, Germany). Spectra were recorded in CDCl₃ and DMSO solutions, and TMS was used as the internal standard. ESI-MS were recorded on a Hybrid Quadrupole-TOF Mass Spectrometer. Multifunctional Enzyme Marker (SpectraMax M2, Meigu Molecular Instruments Co., Ltd., Shanghai, China) was selected to measure the activities during the experiment.

2.2 Synthesis

2.2.1 Formylcamphene 2 was Synthesized by Vilsmeier–Haack Formylation Reaction

In a three-necked flask, DMF (90–100 mL) was added at low temperature and then POCl₃ (74 mL) was dropped through a drip funnel under stirring conditions to maintain the temperature below 10°C. After dropping, the temperature was increased to 60° C–70°C, and 105 g camphene dissolved in 1, 2-dichloroethane solution (40 mL) was slowly dropped into the bottle through the drop funnel under stirring conditions. The mixture was stirred at 80° C–85°C for additional 8 h under condensation and circumfluence conditions. At the end of the reaction, 200–300 g cold water was added and cooled to separate the organic layer. Then saturated NaOH was added to neutralize the water layer to pH 7–8. The organic layer was extracted with toluene, and washed with saturated salt water (3 ×, 100 mL). (E) ω -formylcamphene **2** was obtained by vacuum distillation.

2.2.2 Synthesis of (E) ω -Formylcamphene Thiosemicarbazone 3

In a ground Erlenmeyer flask, a solution of thiosemicarbazide (0.05 mol) was prepared in an ethanol aqueous solution (60 mL, V_{ETOH} : V_{H2O} =1:1). The resulting solution was stirred for 15 min at 50°C until the solid was

dissolved. Then, the corresponding formylcamphene **2** (0.05 mol) was added slowly, and the mixture was stirred at 40°C–45°C for additional 24 h under condensation and circumfluence conditions. The reaction mixture was cooled and stand for 1–2 h. The reaction mixture was filtered to obtain a solid and washed with petroleum ether (3 ×, 100 mL). The crude product was recrystallized from ethanol to afford compound **3**.

2.2.3 Synthesis of (E) ω-Formylcamphene-Based Thiazole Hydrazone Derivatives 5a-5n

In a ground Erlenmeyer flask, the appropriate (E) ω -formylcamphene-based thiosemicarbazone **3** (0.05 mol) and substituted 2-bromoacetophenone **4** (0.05 mol) were added to an absolute alcohol solution (60 mL). The resulting mixture was stirred at room temperature for 5–6 h. The reaction mixture was filtered to obtain a solid. The resulting solid was washed with petroleum ether (3 ×, 100 mL), filtered, and dried under vacuum to afford (E) ω -formylcamphene-based thiazole hydrazone derivatives **5**.

The characterization data of the compounds (3, 5a–5n) are shown in Table 1.

Table 1: Structural analysis of (E) ω -formylcamphene-based thiazole hydrazone deriv	atives
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Compounds	Characterization data
3 S	white solid, yield 86.2%, m.p. 129.7°C; IR (cm ⁻¹): 3425, 3257, 3151, 3022, 1642, 1530, 1193; ¹ H NMR (CDCl ₃ , 400 MHz) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃ , 400 MHz) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃ , 400 MHz) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃ , 400 MHz) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃ , 400 MHz) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃ , 400 MHz) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃ , 400 MHz) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃ , 400 MHz) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃ , 400 MHz) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃ , 400 MHz) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃ , 400 MHz) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.99 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.90 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.90 (s, 1H, NH), 7.85 (d, J = 9.6 Hz, 1H, CDCl ₃) δ_{H} : 9.90 (s, 1H, NH), 7.85 (s, 2H, NH), 7.85 (s, 2H, NH), 7.85 (s, 2H, NH)
N NH2	¹¹ -CH), 7.06, 6.36 (2 s, 2H, NH ₂), 5.71 (d, $J = 9.6$ Hz, 1H, ₁₀ -CH), 3.16 (s, 1H, ₁ -CH), 1.97 (s, 1H, ₄ -CH), 1.73 (m, 2H, ₅ -CH ₂), 1.64 (m, 1H, ₆ -CH), 1.44 (m, 1H, ₆ -CH), 1.32 (m, 1H, ₇ -CH), 1.19 (m, 1H, ₇ -CH), 1.07 (s, 3H, ₈ -CH ₃), 1.04 (s, 3H, ₉ -CH ₃); ¹³ C NMR
	(CDCl ₃ , 100 MHz) δ_C : 176.80 (S=C), 172.65(C ₋₂), 145.05 (C ₋₁₁), 111.95 (C ₋₁₀), 47.22 (C ₋₁), 43.44 (C ₋₃), 42.35 (C ₋₄), 37.37 (C ₋₇), 28.29 (C ₋₈), 28.00 (C ₋₆), 25.17 (C ₋₉), 23.50 (C ₋₅): ESI-MS <i>m/z</i> : 236.12 [M-H] ⁻ , 238.17 [M+H] ⁺ .
5a	light yellow solid, yield 85.6%, m.p.152°C; IR (cm ⁻¹): 3369, 3056, 1626; ¹ H NMR (CDCl ₃ , 400 MHz) δ_{H} : 12.91 (br, 1H, N–H), 8.19 (d, J =10 Hz, 1H, ₁₁ -CH), 7.70 (d, J =7.6 Hz, 2H, _{2'} -CH, _{6'} -CH), 7.45 (m, 3H, _{3'} -CH, _{4'} -CH), 6.75 (s, 1H, ₁₃ -CH), 5.83
	(d, $J = 9.6$ Hz, 1H, ₁₀ -CH), 3.27 (m, 1H, ₁ -CH), 2.00 (s, 1H, ₄ -CH), 1.51 (m, 3H, 5-CH ₂ , ₆ -CH), 1.48 (m, 1H, ₆ -CH), 1.37 (m, 1H, ₇ -CH), 1.21 (d, $J = 21.2$ Hz, 1H, 7-CH), 1.11 (s, 3H, ₈ -CH ₃), 1.09 (s, 3H, ₉ -CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_C :
	1/6.05 (C ₋₁₂), 168.05 (C ₋₂), 151./1 (C ₋₁₁), 140.59 (C ₋₁₄), 150.39 (C ₋₄ ·), 129.55 (C ₋₂ ·, C ₋₆ ·), 127.31 (C ₋₁ ·), 125.74 (C ₋₃ ·, C ₋₅ ·), 111.47 (C ₋₁₀), 100.90 (C ₋₁₃), 47.41 (C ₋₁), 44.69 (C ₋₃), 42.72 (C ₋₄), 37.53 (C ₋₇), 28.31 (C ₋₈), 28.12 (C ₋₆), 25.26 (C ₋₉), 23.58 (C ₋₅); ESI-MS <i>m/z</i> : 336.16 [M-H] ⁻ , 338.17 [M+H] ⁺ .
5b	white solid, yield 86%, m.p. 156.9°C; IR (cm ⁻¹): 3379, 3048, 1629, 740; ¹ H NMR
S H H Br	(DMSO-d6, 400 MHz) $\delta_{H^{\circ}}$ 11.88 (br, 1H, N–H), 7.95 (m, 1H, ₁₁ -CH), 7.83 (m, 1H, _{4'} -CH), 7.42 (s, 1H, ₁₃ -CH), 7.38 (m, 2H, _{6'} -CH, _{5'} -CH), 5.77 (m, 1H, ₁₀ -CH, ₁₃ -CH), 3.13 (s, 1H, ₁ -CH), 1.95 (s, 1H, ₄ -CH), 1.72 (m, 3H, ₅ -CH ₂ , ₆ -CH), 1.43 (m, 1H, ₆ -CH), 1.33 (m, 1H, ₇ -CH), 1.14 (m, 1H, ₇ -CH), 1.08, 1.05 (2 s, 6H, ₈ -CH ₃ , ₉ -CH ₃);
	¹³ C NMR (DMSO-d6, 100 MHz) δ_C : 168.57 (C ₋₁₂), 167.90 (C ₋₂), 147.99 (C ₋₁₄),
	144.08 (C ₋₁₁), 136.71 (C _{-3'}), 131.27 (C _{-2'}), 128.64 (C _{-4'}), 127.09 (C _{-1'}), 125.00 (C _{-5'}), 122.53(C _{-4'}), 113.11 (C ₋₁₀), 105.34 (C ₋₁₂), 47.39 (C ₋₁), 43.36 (C ₋₂), 42.16 (C ₋₄), 37.44
	(C_{-7}) , 28.66 (C_{-8}) , 28.27 (C_{-6}) , 25.54 (C_{-9}) , 23.76 (C_{-5}) ; ESI-MS m/z : 414.07 $[M-H]^-$, 416.08 $[M+H]^+$.

Table 1 (continued)	
Compounds	Characterization data
5c N N H S N N N N N N N N N N	light yellow solid, yield 85.3%, m.p. 169.1°C; IR (cm ⁻¹): 3362, 3046, 1618, 817, 795, 517; ¹ H NMR (DMSO-d6, 400 MHz) δ_H : 12.32 (br, 1H, N–H), 8.05(d, J = 10 Hz, 1H, ₁₁ -CH), 7.76 (d, J = 8.4 Hz, 2H, _{3'} -CH, _{5'} -CH), 7.63 (s, 1H, ₁₃ -CH), 7.62 (d, J = 8.4 Hz, 2H, _{2'} -CH, _{6'} -CH), 5.74 (d, J = 10 Hz, 1H, ₁₀ -CH), 3.20 (s, 1H, ₁ -CH), 1.96 (s, 1H, ₄ -CH), 1.68 (m, 3H, ₅ -CH ₂ , ₆ -CH), 1.43 (m, 1H, ₆ -CH), 1.34 (m, 1H, ₇ -CH), 1.11 (m, 1H, ₇ -CH), 1.07, 1.05 (2 s, 6H, ₈ -CH ₃ , ₉ -CH ₃); ¹³ C NMR (DMSO-d6, 100 MHz) δ_C : 169.47 (C ₋₁₂), 168.49 (C ₋₂), 146.90 (C ₋₁₄), 145.36 (C ₋₁₁), 132.58(C _{-4'}), 132.07 (C _{-3'} , C _{-5'}), 130.29 (C ₋₁₃), 128.17 (C _{-2'} , C _{-6'}), 121.61 (C _{-1'}), 112.73 (C ₋₁₀), 47.32 (C ₋₁), 42.19 (C ₋₄), 43.39 (C ₋₃), 37.38 (C ₋₇), 28.55 (C ₋₈), 28.24 (C ₋₆), 25.45 (C ₋₉), 23.66 (C ₋₅); ESI-MS <i>m</i> /z: 414.07 [M–H] ⁻ , 416.06 [M+H] ⁺ .
$ \underbrace{Sd}_{N} \underbrace{S}_{N} \underbrace{S}_{N} \underbrace{Sd}_{N} \underbrace{Sd}_{N$	yellow solid, yield 87%, m.p. 153.2°C; IR (cm ⁻¹): 3432, 3140, 1627, 1526, 1340, 759; ¹ H NMR (CDCl ₃ , 400 MHz) $\delta_{H^{:}}$ 12.32 (br, 1H, N–H), 8.16 (d, <i>J</i> = 10 Hz, 1H, 11-CH), 8.08 (d, <i>J</i> = 8 Hz, 1H, 3·-CH), 7.73(m, 3H, 4·-CH, 5·-CH, 6·-CH), 6.71 (s, 1H, 13-CH), 5.83 (d, <i>J</i> = 10 Hz, 1H, 10-CH), 3.32 (s, 1H, 1-CH), 2.02 (s, 1H, 4-CH), 1.74 (m, 3H, 5-CH ₂ , 6-CH), 1.48 (m, 1H, 6-CH), 1.40 (m, 1H, 7-CH), 1.16 (m, 1H, 7-CH), 1.12, 1.09 (2 s, 6H, 8-CH ₃ , 9-CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_{C} : 176.26 (C ₋₁₂), 167.63 (C ₋₂), 151.86 (C ₋₁₁), 148.10 (C ₋₁₄), 134.98 (C ₋₂ ·), 133.87 (C ₋₃ ·), 131.84 (C ₋₆ ·), 125.30 (C ₋₄ ·, C ₋₅ ·), 122.46 (C ₋₁ ·), 111.41 (C ₋₁₀), 106.30 (C ₋₁₃), 47.33 (C ₋₁), 43.93 (C ₋₃), 42.68 (C ₋₄), 37.60 (C ₋₇), 28.28 (C ₋₈), 28.09 (C ₋₆), 25.28 (C ₋₉), 23.56 (C ₋₅); ESI-MS <i>m/z</i> : 381.14 [M–H] ⁻ , 383.15 [M+H] ⁺ .
5e $M_{\rm NO_2}$	yellow solid, yield 86.3%, m.p. 172.7°C; IR (cm ⁻¹): 3426, 3077, 1617, 1531, 1350, 738; ¹ H NMR (CDCl ₃ , 400 MHz) δ_{H} : 12.42 (br, 1H, N–H), 8.57 (s, 1H, ₂ ·.CH), 8.29 (d, J = 8 Hz, 1H, ₁₁ -CH), 8.20 (d, J = 8 Hz, 1H, ₄ ·.CH), 8.15 (t, J = 9.6 Hz, 1H, ₅ ·.CH), 7.74(d, J = 10 Hz, 1H, ₆ ·-CH), 7.11 (s, 1H, ₁₃ .CH), 5.85(d, J = 10 Hz, 1H, ₁₀ .CH), 3.26 (m, 1H, 1.CH), 2.02(s, 1H, ₄ .CH), 1.75 (m, 3H, ₅ .CH ₂ , ₆ .CH), 1.50 (m, 1H, ₆ .CH), 1.39 (m, 1H, ₆ .CH), 1.42 (m, 1H, ₇ .CH), 1.20 (d, J = 20 Hz, 1H, ₇ .CH), 1.13 (s, 3H, ₈ .CH ₃), 1.10 (s, 3H, ₉ .CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_C : 176.83 (C ₋₁₂), 168.37 (C ₋₂), 152.13 (C ₋₁₁), 148.66 (C ₋₁₄), 138.05 (C ₋₃), 131.37 (C ₋₂), 131.14 (C ₋₄), 128.91 (C ₋₁), 124.66 (C ₋₆), 120.77 (C ₋₅), 111.30 (C ₋₁₀), 104.10 (C ₋₁₃), 47.36 (C ₋₁), 44.04 (C ₋₃), 42.76 (C ₋₄), 37.55 (C ₋₇), 28.28 (C ₋₈ , C ₋₆), 25.26 (C ₋₉), 23.57 (C ₋₅); ESI-MS <i>m</i> /z: 383.14 [M–H] ⁻ , 383.15 [M+H] ⁺ .
5f $\downarrow \qquad \qquad$	yellow solid, yield 86.7%, m.p. 188.1°C; IR (cm ⁻¹): 3411, 3054, 1623, 1525, 1344, 853; ¹ H NMR (CDCl ₃ , 400 MHz) $\delta_{H^{\circ}}$: 11.93 (br, 1H, N–H), 8.30 (d, J = 8.4 Hz, 2H, $_{3^{\circ}}$ CH, $_{5^{\circ}}$ CH), 8.16 (d, J = 8.4 Hz, 2H, $_{2^{\circ}}$ CH, $_{6^{\circ}}$ CH), 7.98 (d, J = 9.6 Hz, 1H, $_{11}$ CH), 7.63 (s, 1H, $_{13}$ CH), 5.77 (d, J = 7.6 Hz, 1H, $_{10}$ CH), 3.14(s, 1H, $_{1}$ CH), 1.95 (s, 1H, $_{4}$ CH), 1.71 (m, 3H, $_{5}$ CH ₂ , $_{6}$ CH), 1.43 (m, 1H, $_{6}$ CH), 1.33 (m, 1H, $_{7}$ CH), 1.15 (m, 1H, $_{7}$ CH), 1.08 (s, 3H, $_{8}$ CH ₃), 1.05 (s, 3H, $_{9}$ CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_{C} : 168.84 (C. ₁₂), 167.40 (C. ₂), 146.91 (C. ₁₄), 144.32 (C. ₁₁), 140.22 (C. ₁), 129.16 (C. ₃ °, C. ₅ °), 124.05 (C. ₂ , C. ₆ °), 112.98 (C. ₁₀), 47.41 (C. ₁), 43.36 (C. ₃), 42.20 (C. ₄), 37.44 (C. ₇), 28.65(C. ₈), 28.24 (C. ₆), 25.52 (C. ₉), 23.74 (C. ₅); ESI-MS <i>m</i> / <i>z</i> : 381.14 [M–H] ⁻ , 383.15 [M+H] ⁺ .

Table 1 (continued)

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Compounds	Characterization data
$\frac{5g}{H}$	yellow solid, yield 83%, m.p. 135.8°C; IR (cm ⁻¹): 3432, 3072, 1615, 1248, 1017, 781; ¹ H NMR (CDCl ₃ , 400 MHz) $\delta_{H^{:}}$ 13.47 (br, 1H, N–H), 8.19 (d, J =9.6 Hz, 1H, 11.CH), 7.82 (d, J =7.6 Hz, 1H, 3·.CH), 7.61 (t, J =7.2 Hz, 1H, 4·.CH), 7.41 (m, 2H, 13.CH, 5·.CH), 6.93 (d, J =6.8 Hz, 1H, 6·.CH), 5.82 (d, J =9.6 Hz, 1H, 10.CH), 4.10 (s, 3H, OCH ₃), 3.26 (m, 1H, 1.CH), 2.00 (s, 1H, 4.CH), 1.72 (m, 3H, 5.CH ₂ , 6.CH), 1.40–1.35 (m, 2H, 6.CH, 7.CH), 1.23 (d, J =23.2 Hz, 1H, 7.CH), 1.11 (s, 3H, 8.CH ₃), 1.08 (s, 3H, 9.CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_C : 175.23 (C ₋₁₂), 167.13 (C ₋₂), 155.92 (C ₋₁₄), 151.16 (C ₋₁₁), 147.32 (C ₋₁₃), 137.59 (C ₋₂ ·), 134.83 (C ₋₃ ·), 131.68 (C ₋₄ ·), 128.06 (C ₋₆ ·), 121.32 (C ₋₅ ·), 115.67 (C ₋₁ ·), 111.78 (C ₋₁₀), 56.23 (OC), 47.35 (C ₋₁), 43.80 (C ₋₃), 42.60 (C ₋₄), 37.59 (C ₋₇), 28.33 (C ₋₈), 28.30 (C ₋₆), 25.32 (C ₋₉), 23.58 (C ₋₅); ESI-MS <i>m/z</i> : 366.17 [M–H] ⁻ , 368.17 [M+H] ⁺ .
5h $S \to S $	white solid, yield 82.6%, m.p. 128.5°C; IR (cm ⁻¹): 3426, 3076, 1617, 1512, 1257, 1028, 831; ¹ H NMR (CDCl ₃ , 400 MHz) δ_{H} : 12.52 (br, 1H, N–H), 8.17 (d, J = 10 Hz, 1H, ₁₁ .CH), 7.64 (d, J = 8.4 Hz, 2H, _{3'} .CH, _{5'} .CH), 6.98 (d, J = 8.4 Hz, 2H, _{2'} .CH, ^{6.} CH), 6.58 (s, 1H, ₁₃ .CH), 5.83 (d, J = 10 Hz, 1H, ₁₀ .CH), 3.84 (s, 3H, OCH ₃), 3.26 (s, 1H, ₁ .CH), 2.01 (s, 1H, ₄ .CH), 1.82–1.68 (m, 3H, ₅ .CH ₂ , ₆ .CH), 1.48 (m, 1H, ^{6.} CH), 1.39 (d, J = 10 Hz, 1H, ₇ .CH), 1.23 (m, 1H, ₇ .CH), 1.11 (s, 3H, ₈ .CH ₃), 1.08 (s, 3H, ₉ .CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_C : 175.88 (C ₋₁₂), 168.09 (C ₋₂), 161.09 (C ₋₁₄), 151.51 (C ₋₁₁), 140.41 (C _{-1'}), 127.28(C _{-3'} , C _{-5'}), 119.91 (C _{-4'}), 114.88 (C _{-2'} , C _{-6'}), 111.47 (C ₋₁₀), 98.72 (C ₋₁₃), 55.48 (OC), 47.39 (C ₋₁), 43.93 (C ₋₃), 42.69 (C ₋₄), 37.53 (C ₋₇), 28.31 (C ₋₈), 28.29 (C ₋₆), 25.29 (C ₋₉), 23.60 (C ₋₅); ESI-MS <i>m/z</i> : 366.17 [M–H] ⁻ , 368.18 [M+H] ⁺ .
5i	light pink solid, yield 84%, m.p. 145.1°C; IR (cm ⁻¹): 3432, 3027, 1616, 1511, 818; ¹ H NMR (CDCl ₃ , 400 MHz) δ_{H} : 13.40 (br, 1H, N–H), 8.15 (d, $J = 10$ Hz, 1H, ¹¹ .CH ₂), 7.58 (d, $J = 8$ Hz, 2H, 2·.CH, 6·.CH), 7.24 (d, $J = 7.6$ Hz, 2H, 3·.CH, 5·.CH), 6.76 (s, 1H, 13.CH), 5.83 (d, $J = 9.6$ Hz, 1H, 10.CH), 3.25 (s, 1H, 1.CH), 2.53 (s, 3H, 4·.C–CH ₃), 2.00 (s, 1H, 4.CH), 1.76 (m, 3H, 5.CH ₂ , 6.CH), 1.48 (m, 1H, 6.CH), 1.39 (m, 1H, 7.CH), 1.21 (d, $J = 21.2$ Hz, 1H, 7.CH), 1.11 (s, 3H, 8.CH ₃), 1.08 (s, 3H, 9.CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_C : 175.77 (C.12), 168.07 (C.2), 151.36(C.11), 140.64 (C.14), 140.38 (C.1·), 130.20 (C.2·,C.6·), 125.56 (C.3·,C.5·), 124.44 (C.4·), 111.46 (C.10), 100.34 (C.13), 47.35 (C.1), 43.89 (C.3), 42.66 (C.4), 37.51 (C.7), 28.30 (C.8), 28.27 (C.6), 25.27 (C.9), 23.57 (C.5), 21.43 (C.4-CH ₃); ESI-MS <i>m/z</i> : 350.17 [M–H] ⁻ , 352.18 [M+H] ⁺ .
s_{j}	yellow green solid, yield 85%, m.p. 79.4°C; IR (cm ⁻¹): 3432, 3052, 1616, 758; ¹ H NMR (CDCl ₃ , 400 MHz) δ_{H} : 12.70 (br, 1H, N–H), 8.20 (d, $J = 10$ Hz, 1H, ₁₁ -CH), 7.86 (d, $J = 8$ Hz, 1H, ₃ ·-CH), 7.44 (t, $J = 7.2$ Hz, 1H, ₅ ·-CH), 7.31 (d, $J = 7.6$ Hz, 1H, ₆ ·-CH), 7.21 (t, $J = 8.8$ Hz, 1H, ₄ ·-CH), 7.06 (s, 1H, ₁₃ -CH), 5.84 (d, $J = 10$ Hz, 1H, ₁₀ -CH), 3.27 (s, 1H, ₁ -CH), 2.01 (s, 1H, ₄ -CH), 1.73 (m, 3H, ₅ -CH ₂ , ₆ -CH), 1.48 (m, 1H, ₆ -CH), 1.40 (m, 1H, ₇ -CH), 1.20 (d, $J = 21.2$ Hz, 1H, ₇ -CH), 1.18 (s, 3H, ₈ -CH ₃), 1.09 (s, 3H, ₉ -CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_C : 176.06 (C ₋₁₂), 167.66 (C ₋₂), 158.32 (C ₋₁₄), 151.76 (C ₋₁₁), 134.03 (C ₋₂ ·), 131.77 (C ₋₃ ·), 127.90 (C ₋₅ ·), 125.44 (C ₋₆ ·), 116.77 (C ₋₄ ·), 115.67 (C ₋₁ ·), 111.43 (C ₋₁₀), 105.69 (C ₋₁₃), 47.34 (C ₋₁), 43.91 (C ₋₃), 42.68 (C ₋₄), 37.51 (C ₋₇), 28.29 (C ₋₈), 28.26 (C ₋₆), 25.25 (C ₋₉), 23.56 (C ₋₅); ESI-MS <i>m/z</i> : 354.15 [M–H] ⁻ , 356.15 [M+H] ⁺ .

Table 1 (continued)	
Compounds	Characterization data
5k	white solid, yield 85.8%, m.p. 125.2°C; IR (cm ⁻¹): 3411, 3078, 1625, 1511, 838; ¹ H NMR (CDCl ₃ , 400 MHz) δ_{H} : 12.77 (br, 1H, N–H), 8.19 (d, J = 8.4 Hz, 1H, ₁₁ .CH), 7.72 (s, 2H, _{3'} .CH, _{5'} .CH), 7.16 (s, 2H, _{2'} .CH, _{6'} .CH), 6.78 (s, 1H, ₁₃ .CH), 5.83 (d, J = 8.4 Hz, 1H, ₁₀ .CH), 3.26 (s, 1H, ₁ .CH), 2.02 (s, 1H, ₄ .CH), 1.74 (m, 3H, ₅ .CH ₂ , 6.CH), 1.45 (m, 2H, ₆ .CH, ₇ .CH), 1.18 (m, 1H, ₇ .CH), 1.113, 1.09 (2 s, 6H, ₈ .CH ₃ , ₉ .CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_C : 169.24 (C ₋₁₂), 168.15 (C ₋₂), 162.36 (C ₋₁₄), 147.73 (C ₋₁₁), 139.40 (C ₋₄), 127.86 (C ₋₃ , C ₋₅), 127.67 (C ₋₁₃), 123.64 (C ₋₁), 116.86 (C ₋₂ °, C ₋₆ °), 111.41 (C ₋₁₀), 47.34 (C ₋₁), 43.94 (C ₋₃), 42.70 (C ₋₄), 37.61 (C ₋₇), 28.28 (C ₋₈), 28.09 (C ₋₆), 25.26 (C ₋₉), 23.26 (C ₋₅); ESI-MS <i>m</i> / <i>z</i> : 354.15 [M–H] ⁻ , 356.16 [M+H] ⁺ .
51	white solid, yield 86.1%, m.p. 111.5°C; IR (cm ⁻¹): 3424, 3074, 1623, 1509, 845; ¹ H NMR (CDCl ₃ , 400 MHz) δ_{H} : 12.35 (br, 1H, N–H), 8.23 (d, $J = 10$ Hz, 1H, ₁₁ .CH), 7.90 (m, 1H, ₃ ·_CH), 7.06 (m, 1H, ₅ ·_CH), 7.00 (s, 1H, ₁₃ _CH), 6.96 (d, $J = 8.8$ Hz, 1H, 6·_CH), 5.83 (d, $J = 10$ Hz, 1H, ₁₀ .CH), 3.27 (s, 1H, ₁ -CH), 2.01 (s, 1H, ₄ .CH), 1.74 (m, 3H, ₅ -CH ₂ , ₆ .CH), 1.49 (m, 1H, ₆ .CH), 1.38 (m, 1H, ₇ .CH), 1.21 (d, $J = 20$ Hz, 1H, ₇ .CH), 1.12 (s, 3H, ₈ .CH ₃), 1.09 (s, 3H, ₉ .CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_{C} : 176.16 (C ₋₁₂), 167.69 (C ₋₂), 161.35 (C ₋₄ ·), 158.81 (C ₋₁₄), 151.84 (C ₋₁₁), 133.50 (C ₋₂ ·), 129.44 (C ₋₃ ·), 112.98 (C ₋₅ ·), 112.95 (C ₋₆ ·), 112.41 (C ₋₁ ·), 111.43 (C ₋₁₀), 105.26 (C ₋₁₃), 47.37 (C ₋₁), 43.91 (C ₋₃), 42.70 (C ₋₄), 37.50 (C ₋₇), 28.26 (C ₋₈ ,C ₋₆), 25.22 (C ₋₉), 23.55 (C ₋₅); ESI-MS <i>m/z</i> : 372.14 [M–H] ⁻ , 374.15 [M+H] ⁺ .
5m	pink solid, yield 85.7%, m.p. 125.9°C; IR (cm ⁻¹): 3427, 3032, 1619, 1507, 760; ¹ H NMR (CDCl ₃ , 400 MHz) δ_{H} : 13.01 (br, 1H, N–H), 8.24 (d, $J = 10$ Hz, 1H, ₁₁ .CH), 7.49 (m, 1H, ₃ ·.CH), 7.41 (m, 3H, ₄ ·.CH, ₅ ·.CH, ₆ ·.CH), 7.02 (s, 1H, ₁₃ .CH), 5.84 (d, $J = 10$ Hz, 1H, ₁₀ .CH), 3.26 (s, 1H, ₁ .CH), 2.00 (s, 1H, ₄ .CH), 1.76 (m, 3H, ₅ .CH ₂ , ₆ .CH), 1.48 (m, 1H, ₆ .CH), 1.36 (m, 1H, ₇ .CH), 1.17 (m, 1H, ₇ .CH), 1.11, 1.08(2 s, 6H, ₈ .CH ₃ , ₉ .CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_C : 179.70 (C ₋₁₂), 175.99 (C ₋₂), 167.43 (C ₋₁₄), 151.78 (C ₋₁₁), 136.56 (C ₋₂ ·), 132.21 (C ₋₁ ·), 131.36 (C ₋₃ ·), 131.09(C ₋₆ ·), 130.27 (C ₋₄ ·), 127.94 (C ₋₅ ·), 111.48 (C ₋₁₀), 106.58(C ₋₁₃), 47.35 (C ₋₁), 43.89 (C ₋₃), 42.68 (C ₋₄), 37.60 (C ₋₇), 28.31 (C ₋₈), 28.10 (C ₋₆), 25.26 (C ₋₉), 23.57 (C ₋₅); ESI-MS m/z : 370.12 [M–H] ⁻ , 372.13 [M+H] ⁺ .
$\sum_{i=1}^{n} \sum_{j=1}^{n} \sum_{i=1}^{n} \sum_{i$	yellow solid, yield 86.2%, m.p. 130.8°C; IR (cm ⁻¹): 3432, 3053, 1624, 1492, 831; ¹ H NMR (CDCl ₃ , 400 MHz) δ_{H} : 12.82 (br, 1H, N–H), 7.82 (d, J =9.6 Hz, 1H, ₁₁ .CH), 7.67 (d, J =8 Hz, 2H, _{3'} .CH, _{5'} .CH), 7.37 (s, 1H, ₁₃ .CH), 7.35 (m, 2H, _{2'} .CH, _{6'} .CH), 5.78 (d, J =10 Hz, 1H, ₁₀ .CH), 2.79 (s, 1H, ₁ .CH), 1.95 (s, 1H, ₄ .CH), 1.65 (m, 3H, ₅ .CH ₂ , ₆ .CH), 1.42 (m, 1H, ₆ .CH), 1.26 (m, 1H, ₇ .CH), 1.20 (m, J =24 Hz, 1H, ₇ .CH), 1.13 (s, 3H, ₈ .CH ₃), 1.07 (s, 3H, ₉ .CH ₃); ¹³ C NMR (CDCl ₃ , 100 MHz) δ_C : 171.39 (C ₋₁₂), 168.90 (C ₋₂), 147.95 (C ₋₁₁), 144.36 (C ₋₁₄), 135.62 (C ₄ ·), 135.01 (C ₋₁ ·), 129.34 (C _{-3'} , C _{-5'}), 127.10 (C _{-2'} , C _{-6'}), 111.95 (C ₋₁₀), 102.37 (C ₋₁₃), 47.43 (C ₋₁), 43.57 (C ₋₃), 42.30 (C ₋₄), 37.49 (C ₋₇), 28.42 (C ₋₈), 28.19 (C ₋₆), 25.28 (C ₋₉), 23.62 (C ₋₅); ESI-MS <i>m/z</i> : 370.12 [M–H] ⁻ , 372.13 [M+H] ⁺ .

Note: Abbreviations: m.p., melting point; IR, infrared; HR-MS, high-resolution mass spectrometry.

2.3 Antioxidant Studies

2.3.1 DPPH Radical Scavenging Activity

The DPPH assays of compounds **5a–5n** were performed using a method reported previously by Moussa et al. [27] with some modifications. DPPH[•] has an intense violet color with a maximum absorbance at 517 nm, but turns colorless as unpaired electrons are scavenged by antioxidants. Ethanol was used as the solvent to dissolve compound **5**, and the final concentration was 500 μ M concentration. Then, compound **5** was diluted twice to prepare five groups of concentrations. Reaction mixtures containing 50 μ L of sample and 200 μ L of 100 μ M DPPH[•] (prepared in ethanol) were incubated in a dark place at 37°C for 30 min. The absorbance was measured at 517 nm (A₁), and the percentage inhibition was calculated against a control. A blank sample containing 200 μ L of ethanol in the DPPH[•] solution was prepared, and its absorbance was measured (A₀). This assay uses Trolox and L-ascorbic acid as positive controls. The experiment was carried out in triplicate. The activity was determined using a microplate reader and analyzed using SPSS software to obtain IC₅₀. Radical scavenging activity was calculated using the following formula:

(1)

inhibition(%) = $(A_0 - A_1)/A_0 * 100\%$

2.3.2 ABTS Radical Scavenging Activity

The ABTS⁺ radical scavenging assays of compounds **5a–5n** were performed using a previously reported method by Wołosiak et al. [28] with some modifications. ABTS was dissolved in deionized water to a concentration of 7 mM. ABTS radical cation (ABTS^{+*}) was produced by reacting ABTS solution with 1.4 mM potassium persulfate and allowing the mixture to stand in the dark at 4°C for 12–16 h before use. For the study, the ABTS^{+*} solution was diluted in ethanol to an absorbance of 0.70 ± 0.02 at 734 nm to form the test reagent. Ethanol was used as the solvent to dissolve compound 5, and the final concentrations. Reaction mixtures containing 50 µL of sample and 200 µL of reagent were incubated at room temperature for 30 min, and the absorbance reading was taken after the initial mixing (A₁). A sample blank reading using ethanol was also taken (A₀). All the solutions were used on the day of preparation, and all determinations were carried out in triplicate. This assay used Trolox and L-ascorbic acid as positive controls. The percentage of inhibition of ABTS^{+*} was calculated using Eq. (1). The activity was determined using a microplate reader and analyzed using SPSS software to obtain IC₅₀.

3 Results and Discussion

3.1 Structural Characterizations

The synthetic route of (E) ω -formylcamphene-based thiazole hydrazone derivatives is shown in Fig. 1. In this experiment, (E) ω -formylcamphene 2 obtained by the *Vilsmeier–Haack* formylation reaction of camphene 1 was used as the raw material, and (E) ω -formylcamphene-based thiosemicarbazone 3 was obtained by condensation reaction. Then, the condensation of compound 3 with differently substituted 2-bromoacetophenone 4 afforded the target (E) ω -formylcamphene-based thiazole hydrazone derivatives 5a–5n.

The structure of synthesized compounds (**5a**–**5n**) was determined by IR, ¹H NMR, ¹³C NMR, and HR-MS analyses. IR spectrum of compound 3 showed a strong vibration absorption peak at 3425, 3257, and 3131 cm⁻¹, which can be attributed to N-H and NH₂ groups. In the IR spectra of 14 compounds **5a–5n**, only one absorption peak was observed in the range of 3362–3432 cm⁻¹, which is the vibrational absorption of NH bond in these compounds, and the peak for the absorption of NH₂ group completely disappeared. All the 15 compounds have C=N structure with strong absorption peaks near 1620 cm⁻¹ and weak absorption peaks from 3020 cm⁻¹ to 3150 cm⁻¹. They are the vibrational absorption of ₁₀-CH, ₁₁-CH, ₁₃-CH, and benzene ring CH. Because a nitro (NO₂) group is present on the benzene ring of **5d**, **5e**, and **5f** molecules, two absorption peaks at 1526 and 1345 cm⁻¹ appeared on the IR spectrum; **5g** and **5h** showed absorption peaks at 1250 and 1020 cm⁻¹, which are the characteristic peaks of methoxy (OCH₃) group attached to a benzene ring; 13 compounds **5b–5n** showed a clear absorption peak in the range of 740–850 cm⁻¹, indicating the presence of one (or two) substituent(s) on the benzene ring of thiazole hydrazone compounds. In the ¹H NMR spectra, each compound showed the signals for $_{10}$ -CH, $_{11}$ -CH, and NH groups (5.8, 8.2, 12–13 ppm), two single-peak absorption signals for $_{8}$ -CH₃ and $_{9}$ -CH₃ (1.05–1.20 ppm), and proton signals of benzene ring at 6.9–8.6 ppm. The displacement values were different with different substituents on the ring. In the ¹³C NHR spectra, different carbon atoms in each compound showed different absorption signals. In the HR-MS spectra, all compounds showed M+H and M–H values, consistent with the molecular formula.



Figure 1: Synthesis of (E) ω-formylcamphene-based thiazole hydrazone derivatives

3.2 Antioxidant Activity

The scavenging rates of compounds **3**, **5a–5n** and the control samples Trolox and L-ascorbic acid on DPPH and ABTS free radicals at five different concentrations are shown in Tables 2 and 3.

Compounds	R	scavenging rate (%) at a concentration (µmol/L)				
		500	250	125	62.5	31.25
3	-	25.70 ± 1.71	11.84 ± 1.92	8.02 ± 1.11	6.83 ± 0.67	4.4 ± 0.91
5a	Н	88.2 ± 0.21	88.02 ± 0.78	77.29 ± 1.22	44.14 ± 1.42	26.82 ± 1.2
5b	3-Br	91.58 ± 0.2	90.53 ± 0.9	87.8 ± 2.49	55.33 ± 2.74	37.22 ± 1.47
5c	4-Br	87.76 ± 0.88	87.27 ± 0.1	74.85 ± 0.6	39.95 ± 1.32	21.18 ± 1.69
5d	2-NO ₂	93.16 ± 0.18	92.22 ± 0.94	74.42 ± 1.37	45.59 ± 4.12	25 ± 3.36
5e	3-NO ₂	91.01 ± 0.82	89.77 ± 0.51	80.56 ± 0.42	45.57 ± 1.96	26.73 ± 1.39
5f	$4-NO_2$	91.62 ± 0.23	86.04 ± 0.23	66.14 ± 1.37	41.83 ± 0.1	23.65 ± 1.86
5g	2-OCH ₃	91.46 ± 0.06	79.61 ± 0.1	59.28 ± 2.01	27.14 ± 2.84	16.14 ± 1.98
5h	4-OCH ₃	85.78 ± 1.72	84.42 ± 1.63	83.74 ± 2.39	49.27 ± 2.79	27.85 ± 2.9

Table 2: Determination of different concentrations of (E) ω -formylcamphene-based thiazole hydrazone derivatives for DPPH scavenging assay

Table 2 (continued)						
Compounds	R		scavenging rate (%) at a concentration (µmol/L)			
		500	250	125	62.5	31.25
5i	4-CH ₃	86.01 ± 0.55	85.5 ± 0.17	85.31 ± 0.23	51.78 ± 1.77	27.51 ± 2.19
5j	2-F	92.11 ± 0.28	91.06 ± 0.25	61.33 ± 1.16	34.67 ± 0.36	21.9 ± 0.21
5k	4-F	86.14 ± 0.35	87.22 ± 0.46	73.26 ± 1.17	33.42 ± 3.28	25.31 ± 1.57
51	2, 4–2F	89.9 ± 0.22	87.91 ± 0.76	61.1 ± 0.63	33.29 ± 2.86	18.2 ± 3.49
5m	2-C1	91.95 ± 0.75	91.21 ± 1.04	65.09 ± 7.05	31.5 ± 7	14.12 ± 5.8
5n	4-C1	87.69 ± 0.71	87.12 ± 0.44	78.31 ± 1.36	42.91 ± 1.18	24.89 ± 0.99
Trolox	-	97.23 ± 0.12	97.12 ± 0.19	67.22 ± 1.99	36.65 ± 3.8	19.18 ± 4.26
L-ascorbic acid	-	97.29 ± 0.36	97 ± 0.16	77.37 ± 1.04	40.06 ± 2.61	23.04 ± 1.98

Table 3: Determination of different concentrations of (E) ω -formylcamphene-based thiazole hydrazone derivatives for ABTS scavenging assay

Compounds	R	5	Scavenging rate (%) at a concentrations (µmol/L)			
		500	250	125	62.5	31.25
3	-	76.2 ± 1.93	59.95 ± 7.27	59.87 ± 8.83	32.67 ± 4.84	28.38 ± 5.06
5a	Н	100.00	99.11 ± 0.97	83.19 ± 2.60	30.9 ± 6.74	18.18 ± 4.39
5b	3-Br	99.8 ± 0.12	99.72 ± 0.14	94.31 ± 0.66	37.21 ± 1.09	20.89 ± 2.53
5c	4-Br	99.87 ± 0.02	99.81 ± 0.09	84.99 ± 2.08	35.98 ± 1.58	18.52 ± 3.24
5d	2-NO ₂	100.00	98.78 ± 0.83	64.18 ± 0.98	31.97 ± 4.56	30.19 ± 5.72
5e	3-NO ₂	99.95 ± 0.06	99.63 ± 0.08	95.67 ± 2.93	59.14 ± 5.28	32.99 ± 4.57
5f	$4-NO_2$	99.83 ± 0.17	99.74 ± 0.28	87.75 ± 6.49	50.27 ± 8.16	29.44 ± 4.94
5g	2-OCH ₃	100.00	99.83 ± 0.13	73.79 ± 9.14	27.86 ± 6.19	11.25 ± 5.08
5h	4-OCH ₃	99.57 ± 0.69	98.13 ± 1.55	88.97 ± 2.47	30.15 ± 6.70	20.69 ± 0.72
5i	4-CH ₃	100.00	99.63 ± 0.22	99.03 ± 0.77	43.62 ± 2.48	12.26 ± 1.52
5j	2-F	100.00	98.69 ± 1.36	68.61 ± 2.37	33.32 ± 6.86	19.96 ± 7.43
5k	4- F	99.96 ± 0.22	99.64 ± 0.1	82.42 ± 3.74	31.39 ± 3.67	15.31 ± 2.37
51	2, 4–2F	99.85 ± 0.18	99.72 ± 0.09	72.42 ± 0.70	33.23 ± 1.33	18.54 ± 2.54
5m	2-C1	100.00	99.95 ± 0.11	81.22 ± 3.15	40.99 ± 1.91	27.06 ± 7.68
5n	4-C1	99.88 ± 0.09	99.81 ± 0.09	87.22 ± 4.24	41.67 ± 5.55	20.54 ± 2.30
Trolox	-	99.96 ± 0.15	98.28 ± 0.97	57.40 ± 2.96	28.31 ± 2.95	17.34 ± 1.26
L-ascorbic acid	-	99.96 ± 0.02	99.58 ± 0.19	66.19 ± 2.31	32.23 ± 1.59	14.91 ± 0.61

3.2.1 DPPH Radical Scavenging Activity

Table 2 shows that the DPPH radical scavenging rates of (E) ω -formylcamphene-based thiazole hydrazone compounds at the concentrations of 500 mg/L and 250 mg/L are slightly lower than those of the control samples, but both were above 85% (except **5g** and **5h**: 79.6% and 84.4%). When the drug

concentration decreased to 125 mg/L, the scavenging rates of **5b**, **5e**, **5 h**, and **5i** were still above 80%, higher than L-ascorbic acid (77.3%). The scavenging rates of **5a**, **5c**, **5d**, **5k**, and **5n** were also over 73%, close to L-ascorbic acid and higher than Trolox (67%). At a concentration of 62.5 mg/L, the DPPH free radical scavenging rates of eight compounds (**5a**, **5b**, **5d**, **5e**, **5f**, **5 h**, **5i**, and **5n**) were still above 40%, higher than those of L-ascorbic acid (40%) and Trolox (36.6%). These results indicate that the synthesized thiazolylhydrazone compounds exhibited good scavenging effect on DPPH free radical.

3.2.2 ABTS Radical Scavenging Activity

Table 3 shows that when the concentration of the drug solution was 500 and 250 mg/L, the scavenging rates of 14 thiazolylhydrazone compounds against ABTS free radicals were all above 98%, most of them were higher than 99.6%. Among them, the scavenging rates of **5a**, **5d**, **5g**, **5i**, **5j**, and **5 m** at 500 mg/L were as high as 100%, better than those of the two control samples. At a concentration of 125 mg/L, the scavenging rates of **5b**, **5i**, and **5e** were still over 94%, and the scavenging rates of **5a**, **5e**, **5f**, **5 h**, **5k**, **5 m** and **5n** were still above 80%. The scavenging rates of the remaining four compounds were higher than those of two control samples (66% and 57.4%).

According to the free radical scavenging rates listed in Tables 2 and 3, the IC₅₀ values of the two free radical scavenging effects of the compounds fitted using SPSS software are shown in Table 4. The IC₅₀ values of **5a**, **5b**, **5d**, **5e**, **5** h, and **5i** were less than 65.2 μ mol/L in the DPPH radical scavenging test, better than L-ascorbic acid (66.27 μ mol/L). Also, **5e**, **5f**, **5k**, and **5n** were superior to Trolox (75.74 μ mol/L). Among the ABTS radical scavenging activities, compounds **5e** (45.8 μ mol/L) and **5f** (52.82 μ mol/L) had the lowest IC₅₀ values, while compounds **5b**, **5i**and **5 m** had lower IC₅₀ values than 61 μ mol/L. Moreover, other compounds were also better than Trolox (86.21 μ mol/L) and L-ascorbic acid (79.70 μ mol/L).

Compounds	R	IC ₅₀ (IC ₅₀ (μmol/L)		
		DPPH	ABTS		
3	-	>1000	114.91		
5a	Н	65.21	70.07		
5b	3-Br	45.52	60.74		
5c	4-Br	74.92	66.27		
5d	2-NO ₂	65.19	70.82		
5e	3-NO ₂	62.00	45.80		
5f	4-NO ₂	75.89	52.82		
5g	2-OCH ₃	103.56	80.36		
5h	4-OCH ₃	58.67	66.93		
5i	4-CH ₃	56.31	60.36		
5j	2 - F	83.11	74.84		
5k	4-F	77.58	71.64		
51	2, 4–2F	90.39	73.52		
5m	2-C1	89.53	60.38		
5n	4-C1	67.63	61.54		
Trolox	-	75.74	86.21		
L-ascorbic acid	-	66.27	79.70		

Table 4: Half maximal inhibitory concentration (IC₅₀) of (E) ω -formylcamphene-based thiazole hydrazone derivatives evaluated on DPPH and ABTS radicals

The results of antioxidant activity test show that the thiazole hydrazone derivatives that were synthesized by the blend of thiazolyl ring and hydrazone exerted better antioxidant activity on DPPH and ABTS free radical scavenging. All thiazole hydrazone derivatives have stronger antioxidant activity than (E) ω -formylcamphene thiosemicarbazone 3. Some derivatives with different substituents exhibited moderate-to-significant antioxidant activity. For DPPH free radical, the four most potent compounds in the increasing order were 5e < 5h < 5i < 5b. For ABTS free radical, the four most potent compounds in the increasing order were 5m < 5i < 5f < 5e. This result was similar to our previous research, which demonstrated that thiazolyl ring structure and hydrazone structure are key structures for exerting antioxidant activity [29,30], and the substituted aromatic groups can capture free radicals through the degradation of potential aromatic structures [31].

4 Conclusions

In this study, 14 (E) ω -formylcamphene thiazolylhydrazone compounds were synthesized by the condensation of (E) ω -formylcamphene with thiosemicarbazide and reaction with 14 α -bromoacetophenones. The structures of all compounds were characterized by IR, ¹H NMR, ¹³C NMR, and HR-MS analyses. The scavenging rates of 14 compounds against two free radicals were determined by DPPH and ABTS free radical scavenging tests, and the IC₅₀ values for scavenging the two free radicals were fitted using SPSS software. The results of antioxidant activity tests show that 14 compounds had good scavenging effects on the two free radicals; especially at the concentration of 125 and 62.5 mg/L, the scavenging rate and IC₅₀ values were better than the positive controls Trolox and L-ascorbic acid. In addition, the new compounds exhibited excellent antioxidant activity in vitro. At the same time, from the experimental results, it is very meaningful to synthesize (E) ω -formylcamphene thiazole hydrazone derivatives from (E) ω -formylcamphene thiosemicarbazide.

Acknowledgement: This work is supported by the National Natural Science Foundation (No. 31960295), Jiangxi Province Academic and Technical Leaders Training Program Leading Talents Project (20204BCJ22022), Special Funding for Major Scientific and Technological Research and Development in Jiangxi Province (20203ABC28W016).

Funding Statement: This research was funded by the National Natural Science Foundation (No. 31960295), Jiangxi Province Academic and Technical Leaders Training Program Leading Talents Project (20204BCJ22022), Special Funding for Major Scientific and Technological Research and Development in Jiangxi Province (20203ABC28W016).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study

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DOI: 10.32604/jrm.2022.021073

ARTICLE





Study on Sustained-Release Pesticides Blended with Fosthiazate-Stearic Acid/Expanded Perlite

Huimin Huang¹, Zijun Wu², Zhijian Zhou¹, Qiulin Xu¹, Jie Yan^{1,*} and Qiaoguang Li^{1,*}

¹College of Chemistry and Chemical Engineering, Zhongkai University of Agriculture and Engineering, Guangzhou, 510225, China ²College of Light Industry and Food Science, Zhongkai University of Agriculture and Engineering, Guangzhou, 510225, China ^{*}Corresponding Authors: Jie Yan. Email: yanjie0001@126.com; Qiaoguang Li. Email: liqiaoguang8799@163.com Received: 25 December 2021 Accepted: 01 March 2022

ABSTRACT

The low utilization rate of pesticides makes the migration of pesticides in water and soil, which brings great harm to the ecosystem. The development of pesticide carriers with good drug loading capacity and release control ability is an effective method to realize effective utilization of pesticides and reduce pesticide losses. In this work, fosthiazate-stearic acid/expanded perlite sustained-release particles were successfully prepared by vacuum impregnation using expanded perlite (EP) as carrier, fosthiazate (FOS) as model pesticide and stearic acid (SA) as hydrophobic matrix. The structure and morphology of the samples were studied by BET, FT-IR, TGA, XRD, DSC and SEM. The effects of different mass ratios of FOS to SA on loading capacity and release rate at 24 h were investigated. The sustained release behavior of FOS-SA/EP at different temperatures and pH values was investigated by static dialysis bag method. The results showed that FOS and SA were adsorbed in EP pores by physical interaction. With the mass ratios of FOS to SA decreasing from 7:3 to 3:7, the 24 h release rate of FOS-SA/EP decreased from 18.77% to 8.05%, and the drug loading decreased from 461.32 to 130.99 mg/g. FOS-SA/EP showed obvious temperature response at 25°C, 30°C and 35°C, the cumulative release rate (CRR) of 200 h were 33.38%, 41.50% and 51.17%, respectively. When pH = 5, the CRR of FOS was higher than that of pH = 7, and the CRR of FOS for 200 h were 49.01% and 30.12%, respectively. At different temperatures and pH = 5, the release mechanism of FOS-SA/EP belongs to the Fickian diffusion mechanism; When pH = 7, the diffusion mechanism is dominant, and the dissolution mechanism is complementary.

KEYWORDS

Expanded perlite; fosthiazate; stearic acid; sustained release; release kinetics



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Graphic Abstract



A sustainable-release pesticide

Nomenclature

FOS	fosthiazate
EP	expanded perlite
SA	stearic acid
CRR	cumulative release rate

1 Introduction

Pesticides play an irreplaceable role in agriculture and contribute greatly to the yield and quality of crops [1]. The low utilization rate of pesticides leads to large loss of pesticide active ingredients, as well as soil and water pollution. In recent years, slow and controlled release technology has been widely used in medicine [2], paint [3], cosmetics [4], environmental engineering [5] and other industries and fields. In the field of pesticide dosage form processing, slow and controlled release technology is also an ideal choice to improve the effective utilization rate of pesticides and reduce environmental pollution, and has become an important direction of the development of new pesticide dosage forms [6,7].

Adsorption type sustained release agent is the original drug adsorption in inorganic, organic and other adsorbent carriers, as storage, such as attapulgite [8], bentonite [9], sepiolite [10], diatomite [11], zeolite [12], resin [13], etc. The preparation process of adsorbent sustained-release agent is simple, short time and low cost. Chen et al. [14] used sodium montmorillonite (Na–Mt), zinc nitrate ion and sodium alginate (ALG) to prepare ALG-Zn-Mt, which has high sustained release efficiency and is sensitive to pH. The drug loading of the system for triazolone is 17.75 mg/g. Xu et al. [15] prepared chitosan-grafted polyacrylic acid/attapulgite (CTS-g-PAA/ATP) composite adsorbent using chitosan (CTS), acrylic acid (AA) and attapulgite (ATP) materials. The results showed that the theoretical maximum drug loading of CTS-g-PAA/ATP at 30°C, 35°C and 40°C were 57.8, 54.3 and 51.2 mg/g, respectively. All these studies provide

extensive useful exploration for the preparation and application of sustained release pesticide, however, their loading needs to be further improved.

Therefore, it is necessary to prepare loading materials with large drug loading, good structure and slowrelease performance [16]. The expanded perlite (EP) used in this sustained-release pesticide is formed from the high-temperature transformation of the mineral perlite. Perlite is a natural, common, non-toxic and cheap raw material. EP has the characteristics of lightweight, thermal stability and chemical inertia [17]. Compared with other common synthetic zeolite, nano clay, amorphous silicon and so on, EP has a large load, and has the natural abundance and economic feasibility, so it will hopefully replace other synthetic adsorbents. Furthermore, EP can be used to prepare loading materials, such as sustained-release pesticides, bactericides, antibacterial drugs and so on. Mahkam et al. [18] prepared methacrylic acid (MAA)/perlite composite (APC) via sol-gel route and studied the influence of 3-(trimethoxysilyl) propyl methacrylate ratio on its drug release properties. The results showed that the release rate of 5-aminosalicylic acid in the buffer decreased with the increase of the content of the coupling agent 3-(trimethoxy-methylsilyl-propyl methacrylate) in the composite. Using the cellular structure of EP, Rachel et al. [19] developed a sustained-release biocide system, involving an anchored quaternary ammonium salt (AOAS) embedded in EP substrate. The results showed that the honeycomb cellular microstructure of EP enables the adsorption and accumulation of AQAS monomeric species from methanol solution and acts as a substrate for the formation of oligomeric species after curing, which slowed down the dissolution rate of AQAS, so that AQAS could be released continuously for a long time. Finally, EP can also be used as a soil auxiliary medium for planting tomato, pepper and other crops [20,21]. Therefore, we believe that EP has sufficient potential as a sustained-release pesticide carrier.

Fosthiazate [22] (FOS) is one of the main non-fumigating nematocides against root knot nematodes in vegetables. It is widely used at home and abroad because it is easier to use than fumigating nematocides [23,24]. The method currently used is to apply the FOS to the soil in a single full dose. To achieve the desired control effect, the retention period of nematocides needs to be at least 6–8 weeks. At present, the main dosage form of FOS are granules, followed by emulsion and aqueous emulsion. The effective content of common FOS granules in the market is 10% and the sustained release time is short. Oil and water emulsions have short sustained release time and need to use organic solvents, which will cause secondary pollution to the environment. Therefore, in order to improve the efficacy of pesticides, it is necessary to prepare pesticide preparations with long sustained release period and environmental friendliness.

In this study, in order to improve the load of FOS by EP and prolong the sustained release time of FOS, FOS-SA/EP sustained release agent was prepared using FOS as model pesticide and stearic acid (SA) as hydrophobic matrix by vacuum impregnation method. The structure and morphology of the samples were studied by BET, FT-IR, TGA, XRD, DSC and SEM. The sustained release behavior of the particles at different temperatures and pH values was studied by static dialysis bag method and the responsive release kinetics of FOS to temperature and pH stimulation were studied.

2 Materials and Methods

2.1 Materials

Stearic acid (analytical grade, Tianjin Fuyu Fine Chemicals Co., Ltd., China), methanol (chromatographic pure), fosthiazate (99.7%, Ishihara Industry Co., Ltd., China), pH buffer standard solution (Guangzhou Hewei Pharmaceutical Technology Co., Ltd., China), expanded perlite (Size 5 to 7 mm, Guangzhou Fengzexuan garden materials wholesale).

2.2 HPLC Test Conditions of FOS

The HPLC column was COSMOCORE 2.6 μ m C18 (4.6 mm × 100 mm, 5 μ m) and the mobile phase was methanol/water (70:30 v/v). The injection volume was 5 μ L with a flow rate of 1.0 mL/min, and the oven temperature was maintained at 30°C. The UV detection wavelength was 220 nm [25].

2.3 Preparation of Composite FOS-SA/EP

Composite FOS-SA/EP was prepared by vacuum impregnation method [26]. Firstly, molten FOS-SA mixture was prepared with mass ratios of 7:3, 6:4, 5:5, 4:6, 3:7 and 0:10, respectively. Then the mixture and EP were placed in a round-bottom flask at a mass ratios of 10:1 and stirred evenly at 70°C. Third, the round bottom flask is connected to circulating water vacuum pump through the pipeline with a valve. When the vacuum degree of the pump reaches 0.09 MPa, vacuumize continuously for 1 min. Then closed the valve, soaked at 70°C for 1 h. Finally, the sample was thermally filtered out and named FOS-SA/EP-1, FOS-SA/EP-2, FOS-SA/EP-3, FOS-SA/EP-4, FOS-SA/EP-5 and SA/EP.

2.4 Characterization of Composite FOS-SA/EP

BET: The specific surface area of EP was measured by automatic surface area and porosity analyzer (ASAP 2460, Mike Instruments Inc., USA). The sample was degassed in vacuum at 200°C for 8 h, and the N_2 adsorption and desorption isotherms were tested in liquid nitrogen. The BET method was used to calculate the specific surface area of the samples.

FTIR: Fourier transform infrared spectra of FOS, SA, EP and FOS-SA/EP-3 were recorded. Each sample was compressed with KBr powder into a pellet and scanned in the spectral range of $450 \sim 4000 \text{ cm}^{-1}$ (Spectrum 100, Perkin Elmer Inc., USA).

XRD: The structure and crystal form of the samples were measured by X-ray diffractometer (SMARTLAB 9KW, Rigaku, Japan). Cu-target rays were used in the measurement, and the scanning range was $2\theta = 10 \sim 80^{\circ}$, and the scanning speed was 5° /min.

TGA: Thermal stabilities of the samples were determined using a thermo gravimetric analyzer (TGA2, Mettler Toledo, USA), under nitrogen atmosphere of 50 mL/min at a heating rate of 10 °C/min from 40°C to 600°C.

DSC: Thermal analysis of samples was conducted from 20°C to 100°C using a differential scanning calorimeter (Q200, TA Instruments, USA) at a heating rate of 10 °C/min under nitrogen atmosphere of 20 mL/min.

SEM: The FOS-SA/EP sample was placed on a copper sheet, dried using high-power lamp, and the surface of the solid sample was sputter-coated with gold. The images of the samples were acquired by scanning electron microscopy (EVO18, ZEISS, Germany) at an accelerating voltage of 15 kV under nitrogen gas atmosphere, to observe their microstructures.

2.5 Determination of Drug Loading

Accurately weighed 0.500 g of FOS-SA/EP powder into a 100 ml brown volumetric flask and treated in ultrasonic cleaner (JP-020, Skymen, China) for 30 min to release FOS. Removed 1 mL of the solution and diluted it with methanol to 10 mL, and then determined the mass concentration of FOS (mg/L) by HPLC(Waters, USA). The concentration of FOS was calculated using a calibration working curve $A = 7238.4 \text{ C} + 423.66 (R^2 = 0.9999)$, where A is the peak area and C (mg/L) is the injection concentration. Each sample was tested three times and the average value was calculated.

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2.6 Evaluation of Sustained-Release Performance

A certain mass (M_1, g) of drug-loaded EP was weighed and put into a dialysis bag. The bag was placed in brown conical flask containing 100 mL of water or buffer solution. At certain intervals (t), transfered 1.00 mL of the sample solution to a brown volumetric flask and made up to 10 mL with methanol. At the same time, 1.00 mL of the original sustained-release medium was added to the conical flask [27]. The cumulative release rate (M_t/M_{∞} , CRR, %) of FOS was calculated using Eq. (1)

$$\frac{M_i}{M_{\infty}} = \begin{cases} \frac{C_i \times 0.1}{M_1 \times LC} (i=1) \\ \frac{C_i \times 0.1}{M_1 \times LC} + \frac{\sum_{i=1}^{i-1} C_i \times 0.001}{M_1 \times LC} (i=2, 3, 4...) \end{cases}$$
(1)

where, *i* is sampling times; C_i is the concentration of FOS at different time intervals, mg/L; M_1 is the mass of the adsorbed sample, g; *LC* is the drug loading, mg/g.

3 Results and Discussion

3.1 BET Analysis of EP

The N₂ adsorption-desorption isotherms of EP are shown in Fig. 1. It can be seen from the figure that the nitrogen adsorption-desorption isotherm of EP is a typical type IV isotherm with obvious type H3 hysteresis ring, indicating that EP has slit holes formed by the extrusion of laminar particles. This is mainly related to the production and processing method of EP particles. EP is usually a porous material made of perlite under the condition of rapid heating, which vaporizes the bound water and rapidly expands the volume. Most of them are irregular flaky structures. The EP has a specific surface area of 3.4190 m²/g, a pore diameter of 86.358 Å and a pore volume of 0.007381 cm³/g. It is not much different from what is recorded in the literature [28].



Figure 1: N₂ adsorption and desorption isotherms for EP at 77 K

3.2 FT-IR Analysis of Composite FOS-SA/EP

The FT-IR spectra of SA, FOS, EP, FOS-SA and FOS-SA/EP-3 are shown in Fig. 2. The FTIR spectrum of pure SA showed peaks at 2918 and 2849 cm⁻¹ (C–H stretching), 1689 cm⁻¹(C=O stretching), 1467 cm⁻¹

(C–H bending), 1300 and 1100 cm⁻¹ (C–O stretching), and 936, 723, and 685 cm⁻¹ (C–H bending) [29]. The main absorption bands of EP [30] were 3485, 1633, 1024, 789 and 469 cm⁻¹. The peaks at 3485 and 1633 cm⁻¹ were respectively assigned to the stretching and bending modes of the hydroxyl groups which were attached to the surface of EP (mainly Si–OH groups) and the adsorbed water molecules. The peaks positioned at 1024 and 789 cm⁻¹ were ascribed to Si–O stretching vibrations of Si–O–Si and Si–O–Al, respectively. O–Si–O bending vibration was observed at about 469 cm⁻¹. On the FOS spectrum, the characteristic peaks of FOS were 2968 cm⁻¹ (C–H stretching), 1700 cm⁻¹ (C=O stretching), 1250 cm⁻¹ (P=O stretching) and 1020 cm⁻¹ (P-O-C asymmetric stretching), respectively. Compared with FOS-SA, there were no new characteristic peaks appearing in the spectrum of FOS-SA/EP-3, indicating that there is no chemical interaction between FOS-SA and EP. The infrared spectra of FOS-SA/EP-3 showed the characteristic peaks of FOS at 2959, 1700, 1250 and 1020 cm⁻¹, respectively. It can be determined that FOS has been loaded in EP.



Figure 2: FT-IR of FOS, SA, EP and FOS-SA/EP

3.3 TG Analysis of Composite FOS-SA/EP

Figs. 3a and 3b show the TG and DTG curves of FOS, SA and different FOS-SA/EP samples, respectively. It was evident that FOS and SA had one mass loss stage, the decomposition temperature of FOS is 140~250°C, and the mass loss is 66.42%; The decomposition temperature of SA is 160~280°C and the mass loss is 99.6%, indicating that SA can be completely decomposed at this temperature [31]. The thermogravimetric curves of different proportions of FOS-SA/EP showed two stages. The stage I is 140~240°C, mainly due to the decomposition of FOS. The stage II is 240~290°C, mainly the decomposition of SA. As can be seen from the Fig. 3b, the decomposition temperature of FOS-SA/EP system, the decomposition temperature of FOS gradually moved to the right. This indicated that the addition of SA made FOS-SA/EP system have better thermal stability and higher decomposition temperature.


Figure 3: (a) TGA and (b) DTG curves of FOS, SA and different FOS-SA/EP samples

3.4 XRD Analysis of Composite FOS-SA/EP

The XRD patterns of EP, SA, SA/EP and different FOS-SA/EP samples are shown in Fig. 4. In the XRD pattern of SA, the characteristic peaks appear at 21.64° and 24° [32]. EP had no obvious and sharp diffraction peaks, indicating that it was an amorphous material with an amorphous structure [28]. Due to the relatively low diffraction intensity of EP, it was not observed in the XRD pattern of the composite, but only two characteristic peaks of SA were observed. In addition, no new diffraction peaks were found in the XRD patterns of the composites, indicating that no new substances were formed after mixing SA and EP, SA and FOS, and FOS-SA and EP, which further proved that there was no chemical reaction between them. In the FOS-SA/EP composite, diffraction peaks with different intensities are displayed. In addition, with the increase of SA content, the peak intensity of the FOS-SA/EP samples became stronger, which means that the crystallinity of the sample became higher [31].



Figure 4: XRD patterns of EP, SA, SA/EP and different FOS-SA/EP samples

3.5 DSC Analysis of Composite FOS-SA/EP

Fig. 5 shows the DSC curves of SA and different FOS-SA/EP samples. The melting temperature of single component and composite system was further studied. At the same heating rate, the melting peak of SA appeared at 59.06°C. The melting temperatures of FOS-SA/EP-1, FOS-SA/EP-2, FOS-SA/EP-3, FOS-SA/EP-4 and FOS-SA/EP-5 were 44.19°C, 46.52°C, 49.44°C, 51.74°C and 53.06°C, respectively. The results in Table 1 show that with the addition of SA, the T_m and ΔH_m of the samples increased gradually. There was no melting peak of FOS in DSC. With the increase of FOS content, the crystallinity and melting point of FOS-SA/EP system decreased. This is because the addition of FOS destroys the crystallization process of SA, destroys the regularity of SA molecular structure and decreases the melting point. With the increase of crystallinity, the molecules in the FOS-SA/EP system were more closely aligned and the permeability resistance was stronger.



Figure 5: DSC curves of FOS, SA and different FOS-SA/EP samples

Samples	T _m /°C	$\Delta H_m/(J/g)$	X _C /%
FOS-SA/EP-1	44.19	30.72	14.61
FOS-SA/EP-2	46.52	50.79	24.54
FOS-SA/EP-3	49.44	57.18	27.62
FOS-SA/EP-4	51.74	56.52	27.30
FOS-SA/EP-5	53.06	75.32	36.39
SA	59.06	150.38	76.65

Table 1: DSC determination results of FOS-SA/EP

Note: T_m : the dissolution peak temperature; ΔH_m : melting enthalpy; X_C : the crystallinity.

3.6 SEM Analysis of Composite FOS-SA/EP

Figs. 6a–6e show the SEM images of EP, SA/EP and FOS-SA/EP-3. Fig. 6a describes the SEM micrographs of EP. EP does not show a specific shape or crystal structure, but looked like a thin sheet. The interior of EP material had a well-defined polygonal honeycomb structure (sometimes referred to as "popcorn" structure) surrounded by a relatively smooth outer surface (sometimes referred to as "eggshell") [17]. These characteristics were considered to be caused by the high internal pressure

generated by the water vapor in the original perlite minerals at the EP transition temperature (850°C). The porous structure of EP made it a good material for absorbing molten SA and FOS-SA.

Fig. 6b shows the section of the SA/EP particles. It can be observed from Fig. 6b that the edge of EP became smooth, the honeycomb structure was not obvious, and the pores became smaller, indicating that SA was uniformly distributed in the pores of EP. The surface of SA/EP is shown in Fig. 6c. It can be seen from Fig. 6c that the surface of EP loaded with SA became smooth and flat, indicated that SA was uniformly coated on the surface of EP, and the holes on the surface of EP were closed.

Fig. 6d reveals the section morphology of FOS-SA/EP-3 particles. The cross section of FOS-SA/EP-3 was similar to Fig. 6b, the edges were smooth, and the sheet structure was not obvious, which indicating that FOS-SA has been loaded in the pores of EP. Fig. 6e shows the surface of the FOS-SA/EP-3 particles. The surface of FOS-SA/EP-3 was relatively smooth, the honeycomb structure was not visible, and no pores appeared. This means that water molecules need to dissolve the FOS on the surface and generate holes before they can enter the interior of the EP to dissolve the internal drug and take it out. Therefore, FOS-SA/EP-3 has a certain sustained release ability.



Figure 6: SEM images of (a) EP, (b) Sectional view of SA/EP, (c) Surface map of SA/EP, (d) Sectional view of FOS-SA/EP-3, (e) Surface map of FOS-SA/EP-3

3.7 Effect of Mass Ratios of FOS to SA on Drug Loading and 24 h Release Rate

Fig. 7 examines the drug loading and 24 h release rate of FOS in FOS-SA/EP at different ratios of FOS to SA. It was speculated that the addition of hydrophobic inclusions can act as additional physical barriers to slow down drug release and delay water infiltration and drug diffusion [33]. As expected, with the mass ratios of FOS to SA decreasing from 7:3 to 3:7, the 24 h release rate of FOS decreased from 18.77% to 8.05%, and the absorption capacity of FOS decreased from 461.32 to 130.99 mg/g. The results showed that the 24 h release rate and drug loading of FOS in FOS-SA/EP gradually decreased with the increase of SA. One of the reasons for this results may be that SA plays a hydrophobic role in FOS-SA/EP, and the increase of SA will reduce the contact area between water molecules and effective drugs, thus slowing down the drug release rate and playing a slow-release role. Another reason may be that the internal space of EP is limited. As the amount of SA increases, the drug loading will decrease, resulting in slow release rate. Fig. 6 shows that the drug loading and 24 h release of different FOS-SA ratios change regularly. In

practical production and application, different FOS-SA ratios can be selected according to the demand. FOS-SA/EP-3 (FOS:SA = 5:5) was selected for the experiment below.



Figure 7: Drug loading and 24 h release rate of different FOS-SA/EP samples

3.8 Effect of Temperature on Sustained Release Performance

Fig. 8 shows the drug release curves of FOS-SA/EP at different temperatures. The drug release rate was $35^{\circ}C > 30^{\circ}C > 25^{\circ}C$. With the increase of temperature, the release rate of FOS-SA/EP increased, and the sustained release effect became worse. The increase of temperature, on the one hand, increases the solubility of FOS, and on the other hand, accelerates the diffusion rate of FOS, leading to easier release of drugs into sustained-release media. The release rate was fast in the first 24 h, and then gradually slowed down, and the CCR were 17.04%, 22.94% and 30.57% at 25°C, 30°C and 35°C for 24 h, respectively. After 200 h of continuous release, the CCR were 33.38%, 41.50% and 51.17%, respectively. Since then, the release is very slow. FOS-SA is loaded inside EP through physical capillary action without chemical reaction. Theoretically, FOS will eventually be released completely from EP.



Figure 8: Effect of temperatures on sustained release performance of FOS

3.9 Effect of pH Value on Sustained Release Performance

According to the literature [34], the half-life of FOS in soil with pH > 7 is only 14.1~20.7 d, which was easy to decompose in alkaline environment. Therefore, the paper only carried out experiments in neutral and acidic environment. As can be seen from Fig. 9, the release rate was pH = 5 > pH = 7. After 200 h, the CRR were 49.01% and 30.12%, respectively. Under acidic conditions, the drug release rate is relatively fast, indicating that the FOS-SA/EP sustained-release system was acid-responsive.



Figure 9: Effect of pH on sustained release performance of FOS

3.10 Kinetic Studies

The release mechanisms of FOS from FOS-SA/EP at 25°C, 30°C and 35°C were studied. The Zero-order, First-order, Higuchi and Korsmeyer-Peppas models [35] were used to fit the release curve data, and the results were shown in Table 2 and Fig. 10. When the temperatures were 25°C, 30°C and 35°C, the sustained release data were consistent with the Korsmeyer-Peppas model, and the regression coefficients (R²) were 0.9958, 0.9901 and 0.9751, respectively. In the Korsmeyer-Peppas model, *n* was the release index. When $n \le 0.45$, drug release corresponds to Fickian diffusion mechanism [36]. When the temperature of the sustained-release medium is 25°C, 30°C and 35°C, its release index *n* is 0.3070, 0.3055 and 0.2932, respectively. All satisfy $n \le 0.45$, indicating that the drug release is carried out by the Fickian mechanism, which means the release process slower and longer [37]. Based on the above results, the release process of FOS in particles can be divided into two stages: (1) In the initial stage, sudden release. When the water-soluble medium moistens the particles, the free drug (FOS) in the outer layer is rapidly dissolved. (2) After the outer FOS dissolves in water, holes are generated on the particle surface, and water enters the inside of the particle. The bound FOS molecules are dissolved and migrated. Due to the long diffusion path, the release is slow [38].

Fitting formula	Fitting equation	R ²
$M_{t'}/M_{\infty} = k_{I}t$	$M_t/M_\infty = 0.0014 t + 0.1149;25^{\circ}\mathrm{C}$	0.8070
	$M_t/M_\infty = 0.0017 t + 0.1477;30^{\circ}\mathrm{C}$	0.7856
	$M_t / M_\infty = 0.0021 t + 0.1951;35^{\circ} \text{C}$	0.7443
$M_t/M_\infty = k_1[1 - exp(-k_2t)]$	$M_t/M_{\infty} = 0.2735[1 - \exp(-0.0538 t)]; 25^{\circ}C$	0.8596
	$M_t/M_\infty = 0.3482[1 - \exp(-0.0564 t)];30^{\circ}\text{C}$	0.8755
	$M_t/M_{\infty} = 0.4389[1 - \exp(-0.0659 t)];35^{\circ}\text{C}$	0.8875
	Fitting formula $M_t/M_{\infty} = k_1 t$ $M_t/M_{\infty} = k_1 [1 - exp(-k_2 t)]$	Fitting formulaFitting equation $M_t/M_{\infty} = k_I t$ $M_t/M_{\infty} = 0.0014 \ t + 0.1149;25^{\circ}C$ $M_t/M_{\infty} = 0.0017 \ t + 0.1477;30^{\circ}C$ $M_t/M_{\infty} = 0.0021 \ t + 0.1951;35^{\circ}C$ $M_t/M_{\infty} = k_I [1 - exp(-k_2 t)]$ $M_t/M_{\infty} = 0.3482 [1 - exp(-0.0538 \ t)];25^{\circ}C$ $M_t/M_{\infty} = 0.3482 [1 - exp(-0.0564 \ t)];30^{\circ}C$ $M_t/M_{\infty} = 0.4389 [1 - exp(-0.0659 \ t)];35^{\circ}C$

Table 2: Release kinetic model fitting results of FOS-SA/EP-3 at different temperatures

(Continued)

Table 2 (continued)			
Kinetic model	Fitting formula	Fitting equation	R^2
Higuchi	$M_t/M_\infty = k_I t^{1/2}$	$M_t/M_\infty = 0.0213 t^{1/2} + 0.0577;25^{\circ}\text{C}$	0.9573
		$M_t/M_\infty = 0.0273 t^{1/2} + 0.0712;30^{\circ}\mathrm{C}$	0.9466
		$M_t/M_\infty = 0.0343 t^{1/2} + 0.0973;35^{\circ}\text{C}$	0.920
Korsmeyer-Peppas	$M_t/M_\infty = k_I t^n$	$M_t/M_\infty = 0.0657 t^{0.3070}; 25^{\circ}C$	0.9960
		$M_t/M_\infty = 0.0846 t^{0.3055};30^{\circ}\mathrm{C}$	0.9908
		$M_t/M_\infty = 0.1145 t^{0.2932};35^{\circ}\mathrm{C}$	0.9758

Note: In the fitting equation, M_t/M_{∞} represents CRR (%), t refers to release time (h), k is defined as drug dissolution constants, and n in the Korsmeyer–Peppas equation is the release index.



Figure 10: Model fitting of data of sustained release profiles at varying temperatures

Kinetic model	Fitting formula	Fitting equation	R^2
Zero-order	$M_t/M_\infty = k_I t$	$M_t/M_\infty = 0.0020 t + 0.1524; \text{ pH} = 5$	0.8238
		$M_t/M_\infty = 0.0013 t + 0.0670; pH = 7$	0.9178
First-order	$M_t/M_\infty = k_1[1 - exp(-k_2 t)]$	$M_t/M_{\infty} = 0.4244[1 - \exp(-0.0271 t)]; \text{ pH} = 5$	0.8901
		$M_t/M_{\infty} = 0.3050[1 - \exp(-0.0136 t)]; \text{ pH} = 7$	0.9178
Higuchi	$M_t / M_\infty = k_I t^{1/2}$	$M_t/M_\infty = 0.0317 t^{1/2} + 0.0552; \text{ pH} = 5$	0.9671
		$M_t/M_\infty = 0.0207 t^{1/2} + 0.0080; \text{ pH} = 7$	0.9834
Korsmeyer-Peppas	$M_t/M_\infty = k_I t^n$	$M_t/M_\infty = 0.0745 t^{0.3508}$; pH = 5	0.9888
		$M_t/M_{\odot} = 0.0239 t^{0.4770}$; pH = 7	0.9823

Table 3: Release kinetic model fitting results of FOS-SA/EP-3 at varying pH values



Figure 11: Model fitting of data of sustained release profiles at varying pH values

In order to further explore the drug release behavior of FOS-SA/EP under different pH conditions, zeroorder, first-order, Higuchi and Korsmeyer-Peppas kinetic models were fitted for drug release data of its drug delivery system. The results are shown in Table 3 and Fig. 11. The above-mentioned FOS-SA/EP release curve at pH=5 has the highest degree of fit with the Korsmeyer-Peppas kinetic model, with R² being 0.9888 and *n* being 0.3508. Since $n \le 0.45$, indicating that the drug release in FOS-SA/EP at pH=5 is performed by Fickian mechanism. The drug release curves at pH=7 fit well with Higuchi and Korsmeyer-Peppas kinetic models, R² being 0.9834 and 0.9823, and n=0.4770. Since 0.45 < n < 0.89, drug release corresponds to non-Fickian diffusion mechanism [36], it indicated that the diffusion mechanism is dominant and dissolution mechanism is secondary under the pH=7 condition.

4 Conclusion

The results showed that the sustained-release particles with different drug loading and sustained-release performance could be obtained by changing the mass ratios of FOS to SA, and the sustained-release rate and drug loading of FOS-SA/EP decreased with the increase of SA ratio. The release rate of FOS-SA/EP was $35^{\circ}C > 30^{\circ}C > 25^{\circ}C$, pH = 5 > pH = 7, which indicates that FOS-SA/EP is a temperature-responsive and acid-responsive sustained-release system. The release curves of FOS-SA/EP at different temperatures and pH = 5 conformed to the Korsmeyer-Peppas kinetic model, and the drug release conformed to the Fickian diffusion mechanism. The fitting results showed that FOS-SA/EP was dominated by diffusion mechanism and supplemented by corrosion mechanism in the environment of pH = 7. In general, in this study, FOS-SA/EP sustained-release particles were prepared by vacuum impregnation method with EP as the FOS sustained-release carrier and SA as the hydrophobic matrix. The method is simple, solvent-free, good release performance and environmentally friendly, which provides an effective and promising method for EP as the pesticide sustained-release carrier.

Funding Statement: This work was supported by the Guangdong Provincial Science and Technology Project (No. 2015B020215012), State Key Laboratory of Woody Oil Resource Utilization, Co-Built by Provincial and Ministry of China (No. GZKF202108) and National Natural Science Foundation of China (32101475).

Conflicts of Interest: We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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DOI: 10.32604/jrm.2022.020204

ARTICLE





Determining the *in vitro* Anti-Aging Effect of the Characteristic Components from *Eucommia ulmoides*

Xuesong Wang^{1,#}, Zhihong Wang^{1,2,#}, Qiuling Yang¹, Sheng Peng³ and Mijun Peng^{1,*}

¹Guangdong Provincial Key Laboratory of Chemical Measurement and Emergency Test Technology, Guangdong Provincial Engineering Research Center for Ambient Mass Spectrometry, Institute of Analysis, Guangdong Academy of Sciences (China National Analytical Center, Guangzhou), Guangzhou, 510070, China

²Guangdong Provincial Key Laboratory of Silviculture, Protection and Utilization, Guangdong Academy of Forestry, Guangzhou, 510520, China

³National & Local United Engineering Laboratory of Integrative Utilization Technology of *Eucommia ulmoides* Jishou University, Jishou, 427000, China

*Corresponding Author: Mijun Peng. Email: pengmj163@163.com

[#]These authors contributed equally to this work

Received: 10 November 2021 Accepted: 27 December 2021

ABSTRACT

To evaluate the potential anti-aging ability of Eucommia ulmoides, four characteristic components (chlorogenic acid, geniposidic acid, aucubin, quercetin) were selected to assess their effects on H₂O₂-induced oxidative damage model of human umbilical vein endothelial cell (HUVEC). Oxidative damage indexes, inflammatory factors, cell cycle, cell apoptosis, cell senescence, and their related proteins were analyzed by methyl thiazolyl tetrazolium (MTT) assay, enzyme-linked immunosorbent assay (ELISA), propidium iodide (PI) staining, annexin V-FITC/PI double staining, SA β -galactosidase staining, and western blotting (WB). The results showed that H₂O₂-induced cell growth inhibition rate decreased as supplementation with characteristic components when compared to H₂O₂ group. Meanwhile, the contents of antioxidant indexes (reactive oxygen species, lactate dehydrogenase, molondialdehyde, superoxide dismutase, glutathione), inflammatory factors (nuclear factor kappa-B, intercellular cell adhesion molecule-1, vascular cell adhesion protein 1), and functional factors (NO, Endothelin-1) in characteristic components treated groups improved if comparison with H_2O_2 group, suggesting the characteristic components of E. ulmoides could alleviate H2O2-induced oxidative damage. Moreover, cell cycle, cell apoptosis, cell senescence, and their related proteins under characteristic components treatment exhibited a better effect than under H₂O₂ treatment, implying the characteristic components could participate in anti-aging via multiple pathways. These results manifested that the characteristic components of E. ulmoides posses the capacity of anti-aging, which provided a basis for investigating the anti-aging ability of E. ulmoides itself.

KEYWORDS

Anti-aging; Eucommia ulmoides; characteristic components; oxidative damage; cell senescence

1 Introduction

Free radical theory of aging, is a popular suggested hallmarks of aging [1], implicates the gradual accumulation of oxidative cellular damage as a fundamental driver of cellular aging [2]. Plenty of factors



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are responsible for free radical excessive production, such as genome instability, proteostasis failure, and environmental influence [3]. These factors can cause abnormal energy supply and metabolite production, which results in free oxygen radicals *in vivo* elevation. Reactive oxygen species (ROS) is one of the most important free oxygen radicals and can lead to numerous oxidative damages, such as carbonylation, oxidized methionine, glycation, aggregation of proteins, and DNA damage. Therefore, ROS extensively generation contributes to aging and age-related diseases (ARDs) occur, e.g., Alzheimer's disease, cancer, atherosclerosis, and metabolic diseases [4].

In recent years, the molecular mechanisms relevant to age-related progression through external natural products intervention have attracted much attention. For instance, natural products can modulate cellular longevity through histone post-translational modification and can induce the up-regulation of autophagy [5]. Meanwhile, kinds of natural products have been reported to slow anti-aging progress and can extend lifespan of organisms, such as resveratrol, astaxanthin, and gallic acid [6–8]. To date, more than 300 compounds or clinical drugs are announced with anti-aging activity. Among them, 185 compounds and 55 complex or extracts belong to natural products [3]. In addition, some of these compounds receive popular interest and present anti-aging activities in multiple aging models under vigorous investigation, such as chlorogenic acid from *Eucommia ulmoides* reveals anti-aging activity in *Caenorhabditis elegans* aging model [9].

Eucommia ulmoides Oliv. is the unique medicinal plant of China, and a total of 138 kinds of natural active compounds are separated, purified, and identified from the barks and leafs [10], such as chlorogenic acid, geniposidic acid, and aucubin. Based on the rich material foundation, *E. ulmoides* has multiple pharmacological activities, such as anti-inflammatory and anti-hyperlipidemia [11,12]. Chlorogenic acid (CA) is the most important characteristic component of *E. ulmoides*. It is reported that CA can protect mice against Cd-induced hepatorenal injury [13]. Moreover, research shows that the serum levels of Immunoglobulin A (Ig A), immunoglobulin G(Ig G), and immunoglobulin M (Ig M) increase under the function of *E. ulmoides* treatment, which contributes to immunity enhancement in animals [14]. Except CA, there are other characteristic components with high contents in *E. ulmoides*, e.g., geniposidic acid (GPA), aucubin (AU), and quercetin (QU). These components possess the abilities of free radical scavenging, anti-inflammatory, and anti anti-cancer [15–17]. However, although literatures about the four characteristic components reveal good to excellent biological functions, there is no systematic study about their effect on anti-aging, especially for GPA and AU.

In this study, the potential anti-aging activities of the four characteristic components from *E. ulmoides* were investigated. The antioxidant indexes, inflammatory factors, cell cycle, cell apoptosis, cell senescence, and their related proteins were analyzed. We hypothesis that the four characteristic components of *E. ulmoides* can alleviate H_2O_2 -induced cell senescence based on their pharmacological activities. Our study will provide a theoretical foundation for evaluating the anti-aging ability of *E. ulmoides* itself, which contributes to the actually application of *E. ulmoides* in the field of functional foods and medicals.

2 Materials and Methods

2.1 Cell Material and Treatment

Human umbilical vein endothelial cell (HUVEC) was provided by Jiangsu KeyGEN BioTECH Co., Ltd., Nanjing, China, and incubated in F-12K medium (90%) with adding fetal bovine serum (FBS, 10%). The culture condition was under saturation humidity, 37°C, and 5% CO₂. The oxidative damage model of HUVEC was established by culturing 70% HUVEC *in vitro* containing 100 μ M H₂O₂. Four major characteristic components of *E. ulmoides*, including chlorogenic acid (CA), geniposidic acid (GPA), aucubin (AU), and quercetin (QU), were adopted to access their potential anti-aging abilities. A negative control was prepared without adding H₂O₂ and characteristic components. Therefore, six groups were generated and divided into Control group, H₂O₂ group, H₂O₂ + CA group, H₂O₂ + GPA group, $H_2O_2 + AU$ group, and $H_2O_2 + QU$ group. Meanwhile, the groups inclusion H_2O_2 were denoted as H_2O_2 treated groups. The HUVEC of each group was treated for 24 h and these treated cells were used for antiaging evaluation unless otherwise specified.

2.2 Methyl Thiazolyl Tetrazolium (MTT) Cell Proliferation Assay

The cell suspension (100 μ L) was added to each well (96-well plates) at a cell density of 5 × 10⁴/mL and incubated at 37°C, 5% CO₂ for 24 h. Meanwhile, medium alone (100 μ L) was prepared as negative control and incubated at the same condition. After incubation, 5 mg/mL MTT solution (20 μ L) was added to each well and incubated for another 4 h. When incubation completed, the medium of each well was subsequently removed. DMSO (150 μ L) was added to each well and mixed thoroughly with the pipette. The optical density (OD) value of each well was recorded at 490 nm.

2.3 Intracellular ROS/Anti-Oxidation Indexes Detection

The intracellular ROS level was assayed with 2,7-dichlorodi-hydrofluorescein diacetate (DCFH-DA) fluorescent probe. The treated HUVEC of each group was washed with phosphate buffered saline (PBS) and collected at 1000 rpm for 5 min. The cell density was adjusted to 1×10^6 /mL. Then, the cells were resuspended in DCFH-DA and incubated at 37°C for 20 min with vibrating every five minutes. Loaded cells were washed three times by serum-free cell culture medium. The fluorescence intensity of each group was determined by using a fluorescence microscope with an excitation wavelength of 488 nm and emission wavelength of 530 nm. The intracellular anti-oxidation related indexes were determined by using relevant detection kit, including lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) (Jiangsu KeyGEN BioTECH Co., Ltd., Nanjing, China). The pertinent details of detection was followed the instruction of each kit.

2.4 Functional and Inflammatory Factors Detection by Enzyme-Linked Immunosorbent Assay (ELISA)

Two functional factors (NO, ET-1) and three inflammatory factors (NF- κ B, ICAM-1, VCAM-1) of the treated HUVEC from each group were determined by using relevant detection kits. The NO content was assayed by chemical method and the ET-1, NF- κ B, ICAM-1, and VCAM-1 contents were monitored by ELISA. All of the kits were purchased from Jiangsu KeyGEN BioTECH Co., Ltd, Nanjing, China. The pertinent details of detection was followed the instruction of each kit.

2.5 Cell Cycle Detection by Propidium Iodide (PI) Staining

The most commonly used dye for cell cycle analysis was propidium iodide (PI) staining. After digested with 0.25% trypsin and washed twice with PBS, cells were harvested at a density of 5×10^{5} /mL. The prepared single cell suspension was fixed with 70% ethanol at 4°C for 2 h. Then, the cell suspension was mixed with a solution containing 100 µL RNase A (37°C, 30 min) and 400 µL PI, and incubated in the dark at 4°C for 30 min. The red fluorescence of each group was recorded at 488 nm.

2.6 Cell Apoptosis Detection by Annexin V-FITC/PI Double Staining

The cell apoptosis rate of the treated HUVEC from each group was determined by using annexin V-FITC/PI double staining detection kit (Jiangsu KeyGEN BioTECH Co., Ltd., Nanjing, China). After digested with 0.25% trypsin and washed twice with PBS, cells were harvested at a density of 5×10^{5} /mL. The prepared single cell suspension was mixed with 500 µL Binding Buffer, 5 µL Annexin V-FITC, and 5 µL PI completely. Then, the cell suspension was incubated in the dark for 15 min. The cell apoptosis rate was detected on a flow cytometer (FCM).

2.7 Cell Senescence Detection by SA β -galactosidase Staining

The treated HUVEC of each group was inoculated in a 6-well plate and washed with PBS. A working solution of β -galactosidase (1 mL) stain was then added, mixed well, and incubated at 37°C for 15 min. After incubation, the β -galactosidase stain solution was removed and washed with PBS for three times every three minutes. Then, the PBS was draw out and 1 mL β -galactosidase stain solution was added to each well. The senescence of cells in each group was observed under microscope.

2.8 Western Blotting (WB) Analysis

Ten inflammatory, apoptosis/senescence, and DNA-repair process related proteins were selected for western blotting (WB) analysis. The detail information of the ten proteins is shown in Table S1. The total protein of the treated HUVEC from each group was isolated by using Whole Cell Lysis Assay kit (Jiangsu KeyGEN BioTECH Co., Ltd., Nanjing, China). Protein quantitation for the isolated whole protein was carried out by using BCA Protein Quantitation Assay kit (Jiangsu KeyGEN BioTECH Co., Ltd., Nanjing, China). Then, the total protein (30 µg) of each group was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was prepared by SDS-PAGE Prepare kit (Jiangsu KeyGEN BioTECH Co., Ltd., Nanjing, China). The band of target protein was transferred to a nitrocellulose (NC) membrane at 200 mA for 2 h and subsequently sealed in 5% nonfat-dried milk for another 2 h. Then, the NC membrane was saturated in a plate inclusion primary antibody at 4°C overnight. The next day, the NC membrane was washed three times (10 min/time) with Tris Buffered Saline Tween (TBST) and saturated in a 5% nonfat-dried milk inclusion secondary antibody (Goat Anti-Rabbit Ig G-HRP) for 2 h. After incubation, NC membrane was washed three times (10 min/time) with TBST and detected by electrochemiluminescence (ECL). The imaging of selected proteins were photographed by BOX Chemi XR5 (SYNGENE, Cambridge, United Kingdom) and the relative expression of target protein was analyzed by Gel-Pro32 software.

2.9 Statistical Analysis

All data were analyzed by using Microsoft Office and SPSS 19.0, and were expressed as the mean \pm SD. One-way analysis of variance (ANOVA) and the least significant difference (LSD) tests were performed to identify significant differences among the tested groups. Tukey test was used to correct multiple comparisons.

3 Results and Discussions

3.1 Inhibition Rate of HUVEC

The inhibition rates of the four characteristic components against HUVEC are shown in Fig. 1A. At the threshold of inhibition rate < 10%, the selected optimum concentrations of CA, GPA, AU, and QU were 0.78, 3.13, 50.00, and 6.13 μ M, respectively. Meanwhile, the inhibition rate of H₂O₂ against HUVEC was greater than 50% (Fig. 1B), suggesting the HUVEC under 100 μ M H₂O₂ treatment was the desirable oxidative damage model for anti-aging analysis. Significant decrease in inhibition rate was observed when H₂O₂ supplementation with characteristic component treatment (p < 0.05, Fig. 1B). However, the inhibition rates of HUVEC revealed no significant difference among the four characteristic components added groups (p > 0.05).

3.2 Contents of ROS/ROS-Related Indexes

Cell injury is tightly related to oxidative stress, which induces amount of ROS generation and cause oxidative damage as well as cell dysfunction [18]. As a strong oxidant, H_2O_2 can induce lipid peroxidation and promote ROS production [19]. In our study, the ROS contents in all H_2O_2 treated groups were higher than that of in control group (Fig. 2, Fig. S1). Compared to H_2O_2 group, the characteristic component added groups revealed lower ROS contents, especially in GPA and AU added groups, indicating the characteristic components of *E. ulmoides* could reduce the H_2O_2 -induced oxidative damage.



Figure 1: The inhibition rate (%) of HUVEC. (A) Inhibition rate of HUVEC under four characteristic components treatment; (B) Inhibition rate of HUVEC of the six tested groups. CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively



Figure 2: The intracellular ROS content among the six tested groups. CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively

It is reported that LDH activity and MDA content can reflect the effect of H_2O_2 on cell membrane integrity and the degree of lipid peroxidation [20]. Besides, intracellular SOD activity and GSH content can positively take part in cell damage response via scavenging free radical [21]. Hence, these four ROSrelated indexes are explored. The MDA exhibited the similar profile with ROS and its content was significantly higher in H_2O_2 group, followed by CA, AU, and QU added groups, and was significantly lower in control and GPA added group (p < 0.05, Table 1). The GSH content and SOD activity in GPA and AU added groups were significantly higher than in other H_2O_2 treated groups, but were significantly lower than in control group (p < 0.05, Table 1). On the contrary, the energy-related LDH activity in GPA and AU added groups were significantly lower than that of in other H_2O_2 treated groups, but were significantly higher than that of in control group (p < 0.05, Table 1). According to these results, it was easily to summary that the characteristic components of *E. ulmoides* could enhance the antioxidant ability of cells and had advantages in maintaining cell function as normal, especially in GPA and AU added groups.

3.3 Contents of Functional and Inflammatory Factors

Because the H_2O_2 -induced oxidative damages inevitably occur, the consequent inflammatory response in HUVEC is therefore explored. Three typical inflammatory factors were investigated in our study, including NF- κ B, ICAM-1, and VCAM-1 (Table 2). The contents of NF- κ B, ICAM-1, and VCAM-1 increased in H₂O₂ group and significantly decreased in characteristic components added groups (p < 0.05). Apart from the effect of QU on ICAM-1, the overall effects of supplementation with GPA and AU on NF- κ B, ICAM-1, and VCAM-1 contents were better than CA and QU. Previous researches have indicated the three inflammatory factors are well associated with HUVEC inflammation and can promote the migration and adhesion of white blood cell (WBC) to HUVEC [22]. Among these factors, NF-kB dominates the key position as its activation is ROS-dependent [23] and many ARDs are coordinated by increasing NF- κ B levels [24]. For instance, ICAM-1 and VCAM-1 are tightly involvement with NF- κ B based on their expressions are regulated by NF- κ B activation in HUVEC [25]. Thus, our results revealed that the characteristic components of E. ulmoides could alleviate H₂O₂-induced inflammation.

 Table 1: The intracellular anti-oxidation indexes contents among the six tested groups

Group	Control	H_2O_2	$H_2O_2 + CA$	$H_2O_2 + GPA$	$H_2O_2 + AU$	$H_2O_2 + QU$	
LDH (U/L)	$22.66 \pm 1.00 \ e$	$58.39\pm1.00\ a$	$39.43 \pm 1.64 \ c$	$33.77 \pm 1.00 \ d$	$32.03 \pm 1.31 \ d$	$45.32\pm1.00\ b$	
MDA (nmol)	$7.42\pm0.42\ c$	24.96 ± 1.70 a	$13.33 \pm 0.45 \ b$	$8.32\pm0.30\ c$	$13.96\pm0.48\ b$	$13.70\pm0.36\ b$	
GSH (gGSH/L)	$437.94 \pm 9.11 \ a$	$154.22 \pm 8.70 \ e$	$271.24 \pm 27.87 \ c$	$376.05 \pm 7.15 \ b$	$378.13 \pm 1.96 \ b$	$236.65 \pm 17.45 \ d$	
SOD (U/ml)	$59.95\pm0.70~a$	$25.89 \pm 2.24 \ d$	$27.76\pm2.58~cd$	$39.62\pm1.46\ b$	$36.59\pm1.28\ b$	$29.56\pm1.40\ c$	
Note: CA GPA ALL and OLL represent chlorogenic acid, geninosidic acid, aucubin, and guercetin, respectively.							

Table 2: The functional and inflammatory factors contents among the six tested groups

Group	Control	H_2O_2	$H_2O_2 + CA$	$H_2O_2 + GPA$	$H_2O_2 + AU$	$H_2O_2 + QU$
NO (uM/L)	32.37 ± 1.99 a	$14.47 \pm 1.83 \ d$	$16.56\pm0.45~cd$	$24.80\pm2.02\ b$	$19.45 \pm 2.07 \ c$	$27.81 \pm 1.24 \ b$
ET-1 (pg/mL)	182.00 ± 7.59 c	254.95 ± 12.38 a	$220.44\pm6.77~b$	$223.61\pm6.78\ b$	$221.15\pm5.90\ b$	$214.88\pm7.90\ b$
NF-κB (ng/ml)	$10.21 \pm 1.61 \ d$	35.17 ± 2.67 a	$20.30\pm2.22\ bc$	$21.39\pm1.93\ bc$	$19.49\pm0.64\ c$	$23.62 \pm 1.52 \ b$
ICAM-1 (ng/ml)	$8.44\pm0.55~e$	26.51 ± 1.09 a	$14.62\pm0.75~b$	$12.68\pm0.81~\text{c}$	$13.82\pm0.83\ bc$	$11.03 \pm 0.93 \ d$
VCAM-1	$163.52 \pm 13.78 \text{ d}$	342.46 ± 12.44 a	$307.33\pm9.54\ b$	282.79 ± 8.55 c	$265.23 \pm 14.08 \ c$	$314.25 \pm 15.53 \text{ b}$
(pg/ml)						

Note: CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively.

Together with the inflammatory factors, the proteins related to NF- κ B activation were also analyzed, including p65, P-Rb, and P-ERK (Table 3, Fig. 3, Table S2). Functioned as the subunit of NF- κ B, p65 comprises a powerful transcriptional activation domain [26]. Simultaneously, P-Rb and P-ERK positively participate in regulation the expression of NF- κ B activation [27,28]. Interestingly, no significant difference was observed for the expressions of p65 among H_2O_2 treated groups (p > 0.05). However, the relative expressions of P-Rb and P-ERK were induced significantly up-regulation under H_2O_2 treatment (p < 0.05) and would decrease as supplementation with characteristic components, especially in GPA and AU added groups. Based on above-mentioned points, we thus speculated that the characteristic components of E. ulmoides contributed to inflammation remission.

Except the inflammatory factors, the HUVEC secretion related vascular active factors are studied in tandem, including NO and ET-1, which participate in physiological and pathological reactions of vasoconstriction and vasodilatation [29]. As shown in Table 2, NO content significantly decreased in H₂O₂ group, whereas the content increased in characteristic components added groups, especially in GPA and QU added groups (p < 0.05), which was in accordance with E. ulmoides extract could activate NO production in Dahl salt sensitive rats [30]. Opposite of NO content, up-regulation of ET-1 content was observed in H₂O₂ group and the content would significantly decrease as supplementation with characteristic components (p < 0.05). Consequently, we obtained that the characteristic components of E. ulmoides could reduce H₂O₂-induced inflammation and keep the normal cellular function of HUVEC.

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Group	р	Control	H ₂ O ₂	$H_2O_2 + CA$	$H_2O_2 + GPA$	$H_2O_2 + AU$	$H_2O_2 + QU$
DNA-repair	p16	$0.06\pm0.01\ d$	$0.61\pm0.03~a$	$0.41\pm0.02\ b$	$0.35\pm0.04\ c$	$0.33\pm0.03\ c$	$0.44\pm0.03~b$
process	p21	$0.14\pm0.03\ c$	$0.42\pm0.04~a$	$0.39\pm0.06\ a$	$0.25\pm0.04\ b$	$0.24\pm0.06\ b$	$0.36\pm0.03~a$
	p53	$0.04\pm0.01\ e$	$0.28\pm0.02~a$	$0.16\pm0.01\ c$	$0.11 \pm 0.01 \ d$	$0.12\pm0.01\ d$	$0.20\pm0.01\ b$
Inflammatory	p65	$0.05\pm0.01\ b$	$0.22\pm0.02~a$	$0.24\pm0.02~a$	$0.25\pm0.02\ a$	$0.25\pm0.04\ a$	$0.24\pm0.02~a$
	P-Rb	$0.06\pm0.01\ d$	$0.25\pm0.02~a$	$0.16\pm0.02\ b$	$0.11 \pm 0.01 \ c$	$0.12\pm0.01\ c$	$0.18\pm0.02\ b$
	P-ERK	$0.45\pm0.02\ c$	$0.75\pm0.04\ a$	$0.71\pm0.05~a$	$0.59\pm0.01\ b$	$0.60\pm0.02\ b$	$0.55\pm0.05\ b$
Apoptosis/	Bax	$0.08\pm0.01\ c$	$0.28\pm0.02\ a$	$0.29\pm0.02\ a$	$0.23\pm0.01\ b$	$0.28\pm0.02\ a$	$0.28\pm0.02~a$
Senescence	Sirt1	$0.20\pm0.01\ a$	$0.02\pm0.01\ d$	$0.04\pm0.01\ c$	$0.07\pm0.01\ b$	$0.05\pm0.01\ c$	$0.04\pm0.01~c$
	Bcl-2	$0.10\pm0.01\ a$	$0.02 \pm 0.002 \ cd$	$0.04\pm0.01\ bc$	$0.04\pm0.003\ b$	$0.04\pm0.002\ b$	$0.02\pm0.01\ d$
	Survivin	$0.26\pm0.02~a$	$0.14\pm0.01~bc$	$0.16\pm0.02\ b$	$0.14\pm0.01\ bc$	$0.14\pm0.02\ bc$	$0.11\pm0.01~c$

Table 3: The relative expression of inflammatory, apoptosis/senescence, and DNA-repair process related proteins among the six tested groups

Note: CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively.



Figure 3: Effects of H_2O_2 and characteristic components on the expression levels of selected proteins. CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively

3.4 Cell Cycle

Previous research has certified that H_2O_2 -induced DNA-damage can lead to cell cycle arrest [31]. Since natural active components possess the ability of radical scavenging, these components reveal excellent protective effects against H₂O₂-induced DNA-damage, such as phenolics oleuropein and hydroxytyrosol from olive oil [32]. In this study, the cell cycle under H₂O₂ as well as under characteristic component treatments was determined (Fig. 4, Fig. S2). The proportion of G1 phase in H_2O_2 group was higher than that of in control group, accompanied with lower proportion of S phase, indicating that HUVEC got arrested in the G1 phase under H_2O_2 treatment. Although the proportion of G1 phase in characteristic components added groups decreased to the same level with control group, the proportion of S phase revealed obviously difference. Compared to control group, the proportion of S phase was higher in GPA and AU added groups, but was lower in CA and QU added groups, implying GPA and AU could facilitate HUVEC progress through to the S phase (stage of DNA synthesis) to avoid the H₂O₂-induced DNA-damage. DNA-damage response (DDR) is responsible for the coordination of DNA-damage cell cycle-checkpoint activation with DNA-repair processes. The DDR is a kinase-based signaling network that controls various effector proteins to achieve the coordination of the DNA-repair process, including p16, p21, and p53 [33]. Hence, the relative expressions of p16, p21, and p53 were investigated (Table 3). The expressions of p16, p21, and p53 significantly increased in H_2O_2 group and significantly decreased as supplementation with characteristic components (p < 0.05), especially in GPA and AU added groups. Therefore, it could infer that the characteristic components of *E. ulmoides* positively participated in the DNA-repair processes and ensued DNA synthesis as normal.



Figure 4: The cell cycle of HUVEC among the six tested groups. CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively

3.5 Cell Apoptosis Rate

Accompany with cell cycle arrest under H_2O_2 treatment, the cell apoptosis rate will increase [31]. In the present study, the cell apoptosis rate in control group was 6.49% and was apparently lower than that of in H_2O_2 group (40.66%) (Table 4, Fig. S3), indicating that H_2O_2 treatment caused oxidative damage. A slightly reduction in cell apoptosis rate was observed in characteristic component added groups. Among the four characteristic components added groups, GPA added group revealed the best result and its apoptosis rate decreased to 36.64%, followed by CA (37.94%) and AU (37.99%) added groups, and was higher in QU added group (38.94%). Meanwhile, GPA and AU mainly reduced the early apoptosis of HUVEC and their apoptosis rates were 26.09% and 27.51%, respectively, which were lower than in CA (30.90%) and QU (30.07%) added groups. Cell apoptosis is closely related to cell senescence in DNAdamaged cells and there are kinds of proteins function in DDR-associated pathways, such as Sirt1, Bcl-2, Survivin, and Bax. Former study has indicated that Sirt1 is necessary for p53 deacetylation and its loss promotes cell apoptosis [34]. Meanwhile, Bcl-2 acts as anti-apoptotic factors in the mitochondria apoptotic signaling pathways, whereas Bax acts as an pro-apoptotic factor. These two factors can regulate apoptosis via controlling mitochondrial membrane permeability to release apoptosis activators [35,36]. Moreover, Survivin is an inhibitor of apoptosis (IAP) family protein, which is involved in various proteinprotein interactions (PPIs) and thereby regulates cell apoptosis [37]. Therefore, Sirt1, Bcl-2, and Survivin take function in anti-apoptosis, whereas Bax takes function in pro-apoptosis. Accordance with the profile of apoptosis rate alteration, the relative expressions of Sirt1, Bcl-2, and Survivin significantly decreased under H_2O_2 treatment, but the decreasing amplitude would be suppressed as characteristic components supplemented (Table 3). Besides, the relative expression of Bax would be induced significant up-regulation under H_2O_2 treatment and its expression in GPA added group significantly decreased (p < 0.05). These results suggested that the characteristic components of *E. ulmoides* could inhibit cell apoptosis.

3.6 SA β -gal Staining

Apart from the results of cell cycle and cell apoptosis, the senescence-associated SA- β -gal activity analysis is conducted to reflect the senescent cells in culture [38]. Compared to control group, the SA β -gal stained positive cells increased in all H₂O₂ treated groups with squamous cell emerging (Fig. 5), suggesting that H₂O₂ treatment accelerated cell senescence. However, the SA β -gal stained positive cells in characteristic components added groups were lower than that of in H₂O₂ group, indicating the characteristic components of *E. ulmoides* possessed the ability of protecting cells and could alleviate the H_2O_2 -induced cell aging. Consistent with former results, the GPA added group revealed the least stained positive cells among the four characteristic components added groups, followed by AU added group. Moreover, at the threshold of cell inhibition rate < 10%, the optimum concentration of the four characteristic components ranked as CA (0.781 μ M) < GPA (3.125 μ M) < QU (6.125 μ M) < AU (50 μ M). Therefore, the dosage of GPA was kept at a relative low level. Combine the above-mentioned points together, it could get that GPA had a great promise in anti-aging research.

Group	UL (%)	UR (%)	LL (%)	LR (%)	Apoptosis (%)
Control	2.06	2.38	91.45	4.11	6.49
H_2O_2	2.79	7.77	56.55	32.89	40.66
$H_2O_2 + CA$	2.97	7.04	59.09	30.90	37.94
$H_2O_2 + GPA$	3.31	10.55	60.04	26.09	36.64
$H_2O_2 + AU$	3.12	10.48	58.90	27.51	37.99
$H_2O_2 + QU$	3.10	8.87	57.97	30.07	38.94

 Table 4: The cell apoptosis rate among the six tested groups

Notes: CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively. UL, UR, LL, and LR represent upper left, upper right, lower left, and lower right quadrant in the spectrum of Annexin V-FITC/PI double staining, respectively.



Figure 5: The SA β -gal staining of HUVEC among the six tested groups. CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively

4 Conclusion

Comprehensive the above-mentioned points, it can conclude that the four characteristic components of *E. ulmoides* reveal the ability of facilitating DNA synthesis, inhibiting cell apoptosis, and alleviating cell

senescence, and these results may further certify *E. ulmoides* possess the ability of anti-aging. The conclusions from the preliminary data and discussion can be summarized as follows:

- 1. The H₂O₂-induced cell growth inhibition rate as well as the contents of ROS related antioxidant indexes (LDH, MDA, SOD, GSH) improve as supplementation with characteristic components, indicating the four characteristic components of *E. ulmoides* can alleviate H₂O₂-induced oxidative damage.
- 2. Typical inflammatory factors (NF-κB, ICAM-1, VCAM-1) and functional factors (NO, ET-1) are investigated to reflect the impacts of characteristic components on cells. The four selected characteristic components of *E. ulmoides* contribute to inflammation remission and have advantages in maintaining cellular function as normal.
- 3. The cell cycle, cell apoptosis, and cell senescence of H_2O_2 -treated HUVEC ameliorate under characteristic components treatment. Meanwhile, DNA biosynthesis processes are activated by characteristic components, suggesting the characteristic components of *E. ulmoides* facilitate cell division to slow the aging process.
- 4. Among the four characteristic components, GPA reveals the most potential capacity in anti-aging, followed by AU. These two compounds of *E. ulmoides* possess tremendous application foreground in drug field and provide a scientific basis for *E. ulmoides* itself serving as a anti-aging material.

Funding Statement: This study was supported by the National Natural Science Foundation of China (Grant No. 42107020) and the Science and Technology Project of Hunan Province (2020SK2028).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Figure S1: The spectrum of intracellular ROS content among the six tested groups. CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively



Figure S2: The spectrum of PI staining among the six tested groups. CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively



Figure S3: The spectrum of Annexin V-FITC/PI double staining among the six tested groups. CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively

Protein	Molecular weight (kDa)	Dilution rate	Gel concentration for SDS-PAGE
p16	16	1:1000	12%
p21	21	1:1000	12%
p53	53	1:200	10%
p65	65	1:1000	10%
P-Rb	106	1:1000	10%
P-ERK	44	1:1000	10%
Bax	21	1:1000	12%
Sirt1	110	1:20000	10%
Bcl-2	26	1:1000	12%
Survivin	16	1:5000	12%

Table S1: The detail information of the ten selected proteins for western blotting (WB) analysis

	Control	H_2O_2	$H_2O_2 + CA$	$H_2O_2 + GPA$	$H_2O_2 + AU$	$H_2O_2 + QU$
Sirt1	519	62.928	131.28	210.67	135.09	108.57
	504.33	56.33	118.66	231.97	149.67	98.84
	533.67	69.53	143.9	189.37	120.51	118.3
p53	95.589	768.81	461.97	338.26	336.33	549.93
	101.38	827	446.39	302.75	307.17	566.5
	89.8	710.61	477.55	373.77	365.49	533.36
p21	380.4	1161.4	1144.4	750.99	685.29	991.19
	299.81	1039.49	1318.25	626.91	529.91	1075.36
	460.99	1283.31	970.55	875.07	840.67	907.02
p16	150.16	1662.9	1204.8	1044	937.49	1199.8
	162.97	1750.565	1145.004	1151.51	862.293	1106.75
	137.35	1575.235	1264.596	936.49	1012.687	1292.85
GAPDH-1	2650	2733.5	2940.8	3019.1	2803.1	2750
P-Rb	185.32	834.97	554.34	410.51	414.43	657.26
	211.56	897.7135	650.26	373.914	380.982	716.128
	159.08	772.2265	493.69	447.106	447.878	598.392
Bcl-2	345	81.226	128.48	152.93	132.61	87.654
	391.35	73.49	155.99	141.58	124.56	121.87
	298.65	88.97	100.97	164.28	140.66	53.43
Bax	267.28	945.5	1022	866.14	970.84	1034
	307.65	1016.462	1092.29	824.012	1025.178	1121.95
	226.91	874.538	951.71	908.268	916.502	946.05
p65	172.5	748.25	909.81	1015.4	998.49	879.61
	151.94	693.239	806.21	894.598	895.21	820.462
	193.06	803.261	801.79	875.34	754.41	975.262
GAPDH-2	3287.3	3356.95	3527	3726.6	3473.6	3650.4
P-ERK	960.77	1549.6	1550.6	1357.6	1444.012	1301
	915.28	1624.769	1653.43	1353.29	1384.359	1178.06
	1006.26	1474.431	1447.77	1361.91	1342.686	1423.94
Survivin	566.13	294.58	392.39	323.71	330.47	268.46
	607.18	322.246	327.7	355.63	276.007	234.904
	525.08	266.914	313.25	291.79	384.933	302.016
GAPDH-3	2152.5	2052.7	2193.8	2310.7	2299.7	2362.8

Table S2: The fluorescence value of inflammatory, apoptosis/senescence, and DNA-repair process related proteins among the six tested groups. CA, GPA, AU, and QU represent chlorogenic acid, geniposidic acid, aucubin, and quercetin, respectively

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