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Study on Antibacterial Effects of Extracts from *Pinus massoniana* Lamb. Needles

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Abstract [**Objectives**] To study the antibacterial effects of extracts from *Pinus massoniana* Lamb. needles. [**Methods**] In this experiment, the components from *Pinus massoniana* Lamb. needles were extracted by systematic solvent extraction method, and the antibacterial activity against four common bacteria, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Aspergillus flavus* and the antibacterial active component were examined for by punch method. [**Results**] Different solvent extraction rate was different, the rates of petroleum ether, chloroform, ethyl acetate, n-butanol, water extracts were 4.2%, 16.7%, 17.4%, 21.1%, 40.6%. All extracts showed inhibitory effect against test bacteria. It was observed that the inhibition of G^+ was stronger than G^-, and the extracts had the best antibacterial activity to *Staphylococcus aureus* while the weakest to *Aspergillus flavus*. The antibacterial activity of the components decreased in the order: ethyl acetate extract > n-butanol extract > chloroform extract > petroleum ether extract > aqueous phase. The extracts were stable under ultraviolet radiation (UV) light and long term storage. The antibacterial activity of the extracts was weaker with the increase of pH value when the pH value ≤8. [**Conclusions**] It is inferred that the antibacterial components in the extract of *Pinus massoniana* needles are widely distributed, and the components with medium polarity or above are the main antibacterial components.

Key words *Pinus massoniana* Lamb., Pine needle extract, Antibacterial activity

1 Introduction

Ecological environment and food safety have increasingly become the focus of public attention, and the research on antibacterial efficacy of plant extracts has also become an academic hot spot. According to historical documents, it has been known since ancient times that there are antibacterial substances in plants that can resist microorganisms or other diseases in China[1]. According to relevant statistics, there are nearly 500 000 plant species in the world, and only about 10% of them have been studied for chemical composition[2]. At present, there are about 1 400 plant species with bactericidal activity[3]. Research results at home and abroad show that plants with natural antibacterial properties mainly include spices, Chinese herbal medicines, fruits and vegetables, *etc.* Over the years, scholars at home and abroad have conducted extensive research on the bacteriostatic effect of Chinese herbal medicines[4], and found that many Chinese herbal medicines have significant inhibitory effects on common pathogenic bacteria[5], and their bacteriostatic spectrum is extensive[6-7].

In recent years, with the rapid development of various disciplines and the cross-integration of disciplines, remarkable progress has been made in the research of bacteriostasis from plants. The research contents cover the general survey of bacteriostatic active plants, the analysis of bacteriostatic active ingredients, the research of bacteriostatic mechanism, and the research of bacteriostatic spectrum and application effect[8-11]. Although there are still some gaps and shortcomings in the research and development of plant-derived bacteriostatic substances in China, the abundant bacteriostatic and bactericidal plant resources in China provide a unique basis for related research. Therefore, the research of plant-derived bacteriostatic substances has broad development prospects.

Pinus massoniana Lamb., an evergreen tree belonging to the *Pinus* genus in the Pinaceae family[12-13], and there are the richest resources in the subtropical regions of China with widespread distribution[14-17]. *P. massoniana* Lamb. needles are the leaf tissue of *P. massoniana* Lamb.[18]. Pine needles taste bitter and are warm in nature. Modern medical research has confirmed that pine needles are rich in a variety of volatile oils, vitamins, soluble sugars, amino acids, proteins, organic acids, mineral elements and other components[19], and have many curative effects such as improving eyesight and tranquilizing the nerves, dispelling wind and promoting blood circulation, detoxifying and relieving itching[20]. Scholars at home and abroad have pointed out that the tannin components in pine needles have the effects of preventing cardiovascular diseases, inhibiting lipid peroxidation, anti-aging and anti-cancer[21-22].

At present, the research on the antibacterial effect of *P. massoniana* Lamb. needles mainly focuses on its water extract and alcohol extract[23-24], while there are few reports on the comparative studies on the antibacterial effects of various components in the extract. In this study, the inhibitory activity of systematic

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solvent extract of *P. massoniana* Lamb. needles on four common bacteria was compared and analyzed by decoction method and systematic separation method, aiming at providing a scientific basis for its application in the field of food preservation.

2 Materials and methods

2.1 Materials *P. massoniana* Lamb. needles were collected in Shikeng Village, Nahuo Town, Dianbai County, Maoming City, Guangdong Province, washed and dried.

2.2 Test strains *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Aspergillus flavus* were strains preserved in the microbiology laboratory of Lingnan Normal University.

2.3 Main experimental reagents and instruments Sodium hydroxide, provided by Chengdu Kelong Chemical Reagent Factory; petroleum ether, produced by Guangdong Guanghua Technology Co., Ltd.; trichloromethane, provided by Hengyang Kaixin Chemical Reagent Co., Ltd.; disodium hydrogen phosphate, citric acid, and ethyl acetate, all provided by Tianjin Guangfu Fine Chemical Research Institute; ethanol, sodium chloride, glucose, n-butanol, supplied by Tianjin Damao Chemical Reagent Factory. All the above drugs were of analytical grade.

Peptone and beef extract, biological reagents, provided by Beijing Aoboxing Biotechnology Co., Ltd.; agar, chemically pure, provided by Lianjiang Taixing Marine Biotechnology Co., Ltd.; YXQ-LS-75SII vertical pressure steam sterilizer, produced by Shanghai Boyi Industrial Co., Ltd. Medical Equipment Factory; GFL-230 electric blast drying oven, manufactured by Tianjin Labotery Instrument Equipment Co., Ltd.; SPL-250 biochemical incubator, provided by Tianjin Labotery Instrument Equipment Co., Ltd.; PHS-3C precision acidity meter, produced by Shanghai Tianda Instrument Co., Ltd.; YRE-5299 rotary evaporator, manufactured by Gongyi Yuhua Instrument Co., Ltd.; THZ-100 constant temperature culture shaker, provided by Shanghai Yiheng Scientific Instrument Co., Ltd.

2.4 Culture medium and bacterial suspension Beef extract peptone medium[25] was dissolved by heating, then packaged, and sterilized by high pressure steam at 121 ℃ for 30 min. The dosage of agar in the semi-solid medium was half of that of the solid medium, and the rest components were the same; agar was not added to the liquid medium. After the test strains were activated, the strains were picked and placed in liquid culture medium. The bacteria were cultured in a constant temperature shaker at 37 ℃ for 24 h, and the fungi were cultured at 28 ℃ for 48 h to prepare a bacterial suspension.

2.5 Experimental methods

2.5.1 Preparation and separation of *P. massoniana* Lamb. needle extract. (i) Preparation of *P. massoniana* Lamb. needle extract. Fresh *P. massoniana* Lamb. needles were taken, washed, air-dried, and cut into approximately 2 cm long sections using scissors. 300 g of *P. massoniana* Lamb. needles were accurately weighed and placed in a porcelain pot. Enough distilled water was added to immerse the pine needles. After soaking for 30 min, they were boiled over high heat, then slightly boiled over low heat. After continuous decocting for 1.5 h, it was filtered to obtain a decoction. The residue was decocted for the second and third time according to the above steps for 60 and 40 min respectively. The three decoctions were combined and the extract was concentrated to 300 mL using a rotary evaporator, so as to obtain the *P. massoniana* Lamb. needle extract stock solution (Y), which was stored at 4 ℃ for subsequent use.

(ii) Separation of *P. massoniana* Lamb. needle extract. 150 mL of the stock solution was taken, and 30 mL of petroleum ether, chloroform, ethyl acetate and n-butanol were used for extraction and separation. The extraction was repeated four times for each solvent, and the extracts of each solvent were combined separately. The remaining solution after n-butanol extraction was taken as an aqueous phase (W). Each solvent extract was concentrated under reduced pressure to recover the respective solvent. After systematic separation, *P. massoniana* Lamb. needle extract was divided into five components: petroleum ether phase (A), chloroform phase (B), ethyl acetate phase (C), n-butanol phase (D) and aqueous phase (W). The above five components were dried and used for later use, and the yield of each extract was calculated.

Extract yield = (Mass of extract, g)/Dry weight of extract, g) × 100%.

2.5.2 Assessment of antibacterial activity of *P. massoniana* Lamb. needle extract. Following the method described by Xing Huizi *et al.*[26], the sterilized semi-solid medium was cooled to approximately 50 – 60 ℃, and then 3 mL of the test bacteria suspension was added, mixed evenly and immediately poured into a Petri dish filled with solid medium. After the culture medium solidified, a 100 μL gun tip was used to punch holes in the semi-solid culture medium, 4 wells were evenly set up in each culture dish, and 30 μL of sample solution was injected into each well; the same amount of sterile water was injected into the well used as blank control and marked as CK.

The bacteria were cultured in a constant temperature environment of 37 ℃ for 24 h, and the fungi were cultured in an environment of 28 ℃ for 48 h. The diameter of the inhibition zone was measured by the cross method[27-28].

2.5.3 Influence of *Pinus massoniana* Lamb. needle extract concentration on antibacterial effect. The *P. massoniana* Lamb. needle extract was prepared according to the concentrations listed in Table 1, and the influence of different concentrations of *P. massoniana* Lamb. needle extract on the antibacterial effect was studied using the punch method according to the above experimental steps.

2.5.4 Stability analysis of antibacterial active ingredients of *P. massoniana* Lamb. needles. (i) Effect of storage time on the stability of antibacterial active ingredients of *P. massoniana* Lamb. needles. The extract of *P. massoniana* Lamb. needles was placed at room temperature, and its antibacterial activity was measured by the punch method every 10 d. Four test bacte-

ria were used as indicator bacteria, and the stock solution (Y) was used for the experiment.

(ii) Effect of medium pH on the stability of antibacterial active ingredients of *P. massoniana* Lamb. needles. The pH of C and D extracts of *P. massoniana* Lamb. needles was adjusted to 3, 4, 5, 6, 7 and 8 with citric acid and disodium hydrogen phosphate, and the aqueous solution with corresponding pH was used as the control. Three kinds of bacteria were used as indicator bacteria to measure and compare the antibacterial effects.

Table 1 Preparation of *Pinus massoniana* Lamb. needle extract with different concentrations

Liquid	100%	80%	60%	40%	20%	CK
Extract stock solution (Y)	2.5	2.0	1.5	1.0	0.5	0
Sterile water	0	0.5	1.0	1.5	2.0	2.5

(iii) Effect of ultraviolet irradiation on the stability of antibacterial active ingredients of *P. massoniana* Lamb. needles. According to the method of Liu Wenduo *et al.* [29], the D extract was placed on an ultra-clean workbench, and the ultraviolet lamp was turned on for irradiation for 10, 20, 30, 40, 50 and 60 min. The untreated sample solution was used as the control, *S. aureus* and *E. coli* were used as indicator bacteria, and the diameter of the inhibition zone was measured to determine the effect of ultraviolet irradiation on the stability of the antibacterial active ingredients of *P. massoniana* Lamb. needles.

3 Results and analysis

3.1 Yield of systemic solvent extract of *P. massoniana* Lamb. needle The extraction yield of *P. massoniana* Lamb. needle extract by petroleum ether, chloroform, ethyl acetate, N-butanol, and water was 4.2%, 16.7%, 17.4%, 21.1%, and 40.6%, respectively. The components extracted from petroleum ether A were the least, and the extraction yield was only 4.2%, showing dark green oiliness; the extract of n-butanol D was the most, and the yield was 21.1%, showing orange-yellow color; the extract of chloroform B and ethyl acetate C was green, and their extraction yields were similar, reaching 16.7% and 17.4%, respectively; the substance dissolved from W was the most, reaching 40.6%. It can be seen that different solvent polarity led to different extract yields. Most of the extracts of *P. massoniana* Lamb. needles were highly polar components, and there were few weakly polar components.

3.2 Antibacterial activity of each component of *P. massoniana* Lamb. needle extract As can be seen from Fig. 1 and Table 2, the inhibitory activity of each component of *P. massoniana* Lamb. needle extract against the test bacteria was different. C had the best antibacterial effect, because it may contain flavonoids, tannins, lignans, organic acids and other substances; the antibacterial effects of B and D were equivalent, but the antibacterial effect of D was better; A also had certain antibacterial activity; the antibacterial activity of W was very low.

E. coli is a G^- bacterium, *S. aureus* and *B. subtilis* are G^+ bacteria, and *A. flavus* is a fungus. From the experimental results, it can be seen that the extracts of *P. massoniana* Lamb. needles with different polar solvents had inhibitory effects on the test bacteria, indicating that *P. massoniana* Lamb. needles had broad-spectrum antibacterial effect, and the antibacterial components were widely distributed. However, different solvent extracts had different inhibitory activity on different bacteria, and the antibacterial effects of the extracts in each phase showed the strongest inhibition on *S. aureus*.

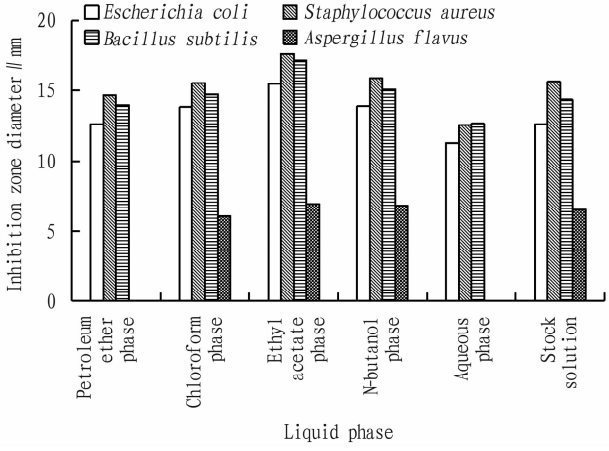

Fig. 1 Effect of systematic solvent extract of *Pinus massoniana* Lamb. needle on the inhibition zone diameter of test bacteria

3.3 Relationship between the concentration of *P. massoniana* Lamb. needle extract and antibacterial effect It can be seen from Fig. 2 and Fig. 3 that the greater the concentration of *P. massoniana* Lamb. needle extract, the better the antibacterial effect. At the same time, *P. massoniana* Lamb. needle extract showed the strongest inhibitory effect on *S. aureus*, and the results showed that the bacteriostasis against G^+ bacteria was stronger.

3.4 Stability of antibacterial active ingredients of *P. massoniana* Lamb. needles

3.4.1 Effect of storage time on the stability of antibacterial active ingredients of *P. massoniana* Lamb. needles. As can be seen from Fig. 4, the antibacterial activity of *P. massoniana* Lamb. needle extract showed a slight decreasing trend after being placed for different time. It may be because the components in the extract interacted with light and oxygen at room temperature, the antibacterial active ingredients changed, and the content of some effective antibacterial active ingredients decreased, resulting in the reduction of the antibacterial effect of the extract.

Table 2 Effect of systematic solvent extract of *Pinus massoniana* Lamb. needle on the diameter of inhibition zone of test bacteria

Strains	Inhibition zone diameter//mm						
	A	B	C	D	*W*	*Y*	*CK*
Escherichia coli	12.63 ±0.32	13.80 ±0.27	15.47 ±0.15	13.88 ±0.08	11.28 ±0.54	12.60 ±0.02	–
Staphylococcus aureus	14.65 ±0.21	15.52 ±0.09	17.62 ±0.51	15.88 ±0.34	12.57 ±0.01	15.60 ±0.13	–
Bacillus subtilis	13.93 ±0.17	14.72 ±0.26	17.13 ±0.52	15.07 ±0.02	12.64 ±0.43	14.32 ±0.37	–
Aspergillus flavus	–	6.07 ±0.11	6.93 ±0.09	6.82 ±0.31	–	6.52 ±0.09	–

NOTE " – " indicates that there is no inhibition zone.

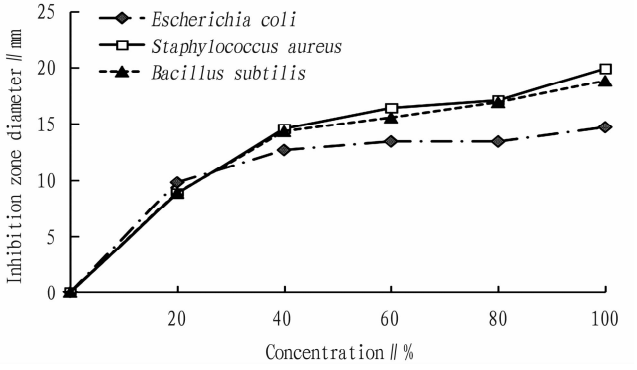

Fig. 2 Effect of different concentrations of *Pinus massoniana* Lamb. needle extracts on the inhibition zone diameter of the test bacteria

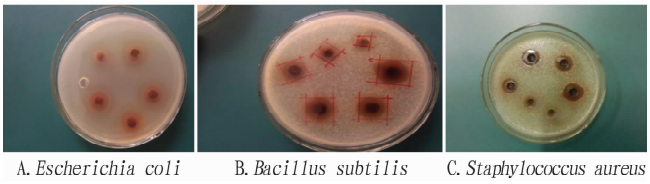

Fig. 3 Antibacterial effect of different concentrations of *P. massoniana* Lamb. needle extracts on the test bacteria

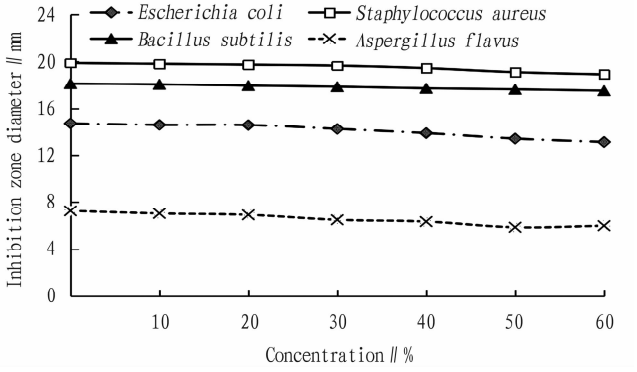

Fig. 4 Effect of storage time on antibacterial activity of ethyl acetate extract of *Pinus massoniana* Lamb. needles

3.4.2 Effect of medium pH on the stability of antibacterial active ingredients of *P. massoniana* Lamb. needles. It can be seen from Fig. 5 and Fig. 6 that when the pH of the medium ≤8, the inhibitory effect of *P. massoniana* Lamb. needle extract on the test bacteria decreased significantly with the increase of the pH of the medium. When the pH of the medium was 8, there was little inhibitory effect on bacteria. Therefore, the pH of the medium had a significant effect on the antibacterial active ingredients of *P. massoniana* Lamb. needle extract, and the antibacterial effect of *P. massoniana* Lamb. needle extract was better under acidic conditions.

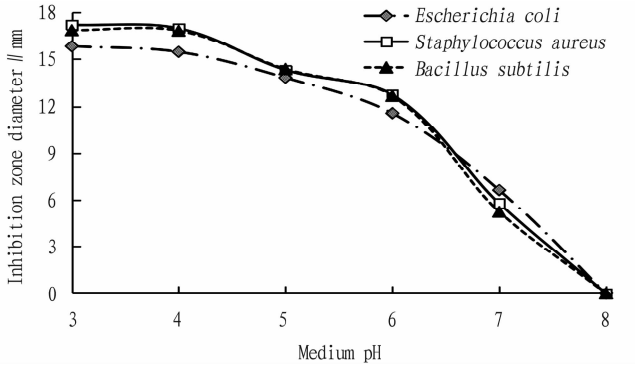

Fig. 5 Effect of medium pH on antibacterial effect of ethyl acetate extract of *Pinus massoniana* Lamb. needles

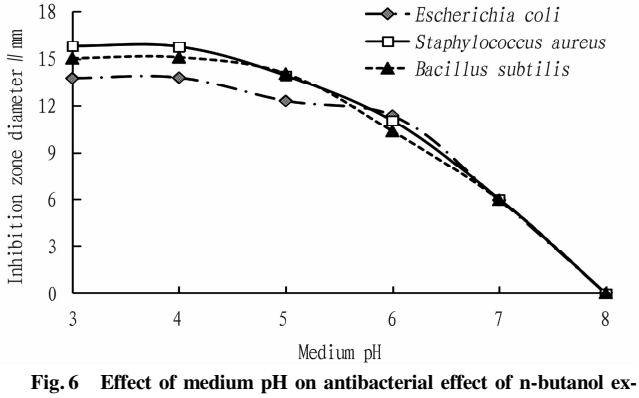

Fig. 6 Effect of medium pH on antibacterial effect of n-butanol extract of *Pinus massoniana* Lamb. needles

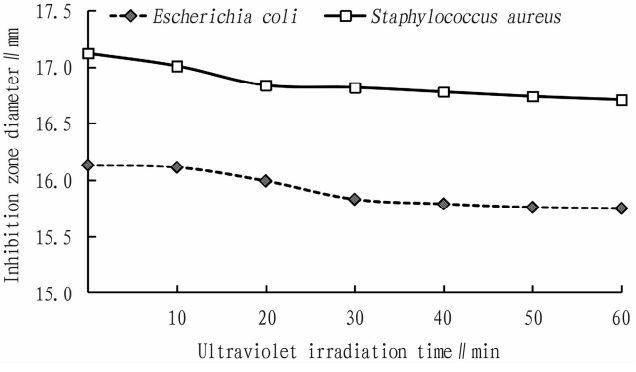

Fig. 7 Effect of ultraviolet light on the antibacterial effect of n-butanol extract of *Pinus massoniana* Lamb. needles

3.4.3 Effect of ultraviolet irradiation on the stability of antibacterial active ingredients of *P. massoniana* Lamb. needles. As can

be seen from Fig. 7, the irradiation treatment with ultraviolet light had little effect on the antibacterial effect of the *P. massoniana* Lamb. needle extract. Compared with the antibacterial effect of the extract without ultraviolet irradiation, the diameter of the inhibition zone of *E. coli* and *S. aureus* after 60 min of ultraviolet irradiation only decreased by 0.38 and 0.41 mm, which indicated that the antibacterial active ingredients in the extract of *P. massoniana* Lamb. needles had good stability to ultraviolet irradiation.

4 Conclusion and discussion

In this study, following the principle of similarity and compatibility, petroleum ether, chloroform, ethyl acetate and n-butanol with gradually increasing polarity were selected as solvents to systematically separate the extract of *P. massoniana* Lamb. needles to obtain the antibacterial active ingredients with different polarity levels, and the antibacterial activity of these ingredients was deeply studied. The results showed that the extraction yields of each solvent were as follows: aqueous phase > n-butanol > ethyl acetate > chloroform > petroleum ether; the antibacterial activity of each component in *P. massoniana* Lamb. needle extract was as follows: ethyl acetate phase > n-butanol phase > chloroform phase > petroleum ether phase > aqueous phase. According to this, it is inferred that the antibacterial components in the extract of *P. massoniana* Lamb. needles are widely distributed, and the components with medium polarity or above are the main antibacterial components. Each phase showed inhibitory effects on the four test bacteria, and the order of antibacterial activity was: *S. aureus* > *B. subtilis* > *E. coli* > *A. flavus*. *S. aureus* and *B. subtilis* are both G^+ bacteria, and *E. coli* is G^- bacteria.

The experimental results showed that the inhibitory effect of *P. massoniana* Lamb. needle extract on G^+ bacteria was stronger than that of G^- bacteria, but the inhibitory effect on fungi was relatively small. The study also showed that after the extract of *P. massoniana* Lamb. needles was left to stand for a period of time, the color changed and the antibacterial activity also decreased. It is speculated that the main reason is that the antibacterial active ingredients react with light, air and other factors, resulting in a decrease in the content of some active ingredients, thus reducing the antibacterial ability. The pH value of the medium had a significant effect on the antibacterial effect of ethyl acetate and n-butanol extracts of *P. massoniana* Lamb. needles. When pH ≤ 8, the antibacterial effect gradually weakened with the increase of pH value. This may be due to changes in the structure of the bacteriostatically active ingredients under alkaline conditions, resulting in loss of bacteriostasis. After UV irradiation, the inhibitory activity of ethyl acetate extract against the test bacteria almost did not change, which indicated that the antibacterial active ingredients in the extract had high stability to UV irradiation.

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projects. Through engineering measures, plant measures, farming measures and temporary measures, soil erosion can be effectively reduced and ecological environment can be protected. However, there are still some shortcomings in the current research on the sustainability of technology promotion and the differences in applicability among different regions.

Future research should further explore soil and water conservation technologies suitable for different topographical and climatic conditions, especially the application effects under complex topography and extreme climatic conditions. In addition, research on soil erosion monitoring and assessment technology should be strengthened, and modern technical means such as unmanned aerial vehicles, remote sensing and big data should be used to improve monitoring accuracy and efficiency. At the same time, it is necessary to promote community participation and government support to form a multi-party collaborative soil and water conservation management model to ensure the effective promotion and application of technology.

In short, the research of soil and water conservation for mountain photovoltaic power generation projects requires multidisciplinary and multi-field collaborative cooperation. Through continuous innovation and practice, it can provide scientific basis and technical support for achieving the win-win goal of ecological environment protection and energy development.

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