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A preliminary study on the anti-inflammatory and analgesic effects of two extracts of pine pollen on anoxia and fatigue resistance

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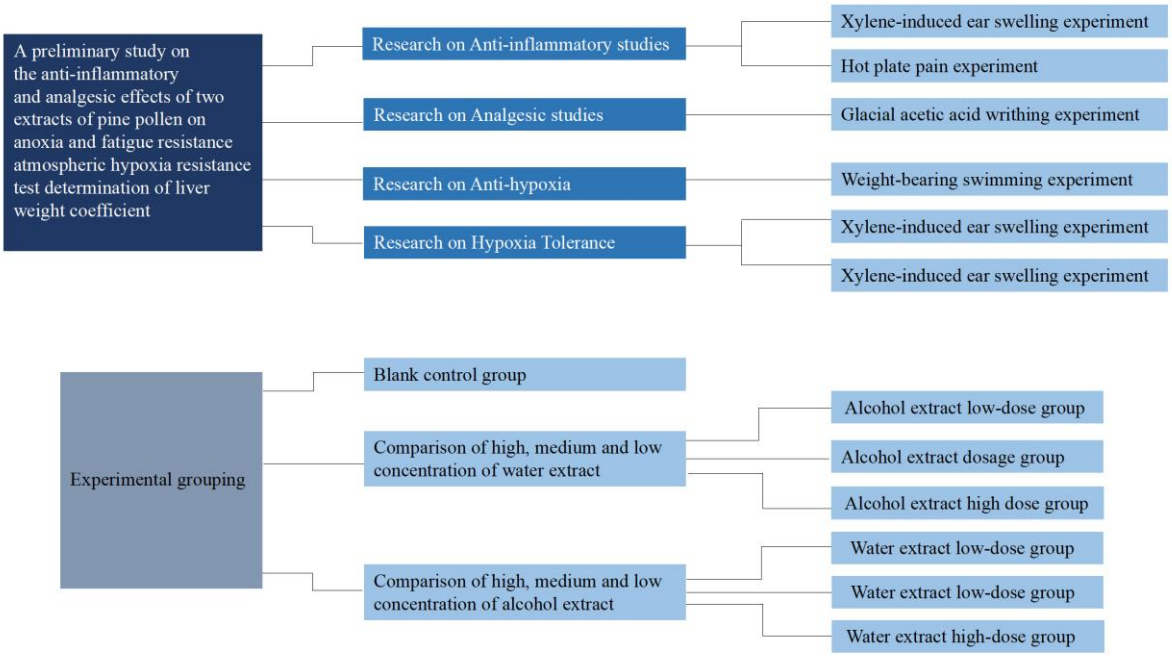
Highlights

This study on pine pollen is of great significance. This study shows that pine pollen has good application value in the field of anti-aging, which is worth further study. For example: scavenging excessive free radicals in the body can delay aging and improve disease resistance, anti-inflammatory and analgesic can be used for skin beauty, skin disease treatment and so on.



Abstract

Background: To study the anti-inflammatory, analgesic, fatigue resistant and antihypoxia effects of ethanol extract and water extract of pine pollen. **Methods:** Two different extracts of pine pollen were prepared into there different concentrations, that is 1.5 mg·mL^{-1}, 4.5 mg·mL^{-1} and 7.5 mg·mL^{-1} respectively. The extract were studied by xylene-induced ear swelling, acetic acid distortion test and hot plate test. The antihypoxia and antifatigue effects were explored by weight-bearing swimming experiment, routine pressure hypoxia tolerance experiment and liver weight coefficient comparison. **Results:** Through the study of the four pharmacological effects of ethanol extract and water extract, we found that the anti-inflammatory, analgesic, antithyposia and antifatigue effects of ethanol extract were better than that of water extract. Moreover, the experimental effects significantly improved with the increase of the concentration, and the effect of alcohol increased dose group was accurate ($P < 0.05$). **Conclusion:** Pine pollen has excellent effects of anti-inflammatory, analgesic, antihypoxia and anti-fatigue. Besides, with the increase of drug concentration, effects tend to be more obvious with positive correlation.

Key words: Pine pollen, Alcohol extract, Water extract, Anti-inflammatory effect, Analgesic effect, Antihypoxia, Fatigue resistance

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***Competing interests*:**

The authors declare that they have no conflict of interest.

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Background

Pine pollen is a single pollen of pines, such as Chinese pine and masson pine, which are the natural products for our traditional food and medicine [1–2]. In the ancient Chinese medicine record *Shen Nong Ben Cao Jing* (*Shengnong's classic of Materia Medica*, unknown author, written in the Han Dynasty) and other ancient medical works of China, the pine pollen has multi health functions, such as supplementing blood and removing internal moisture, promoting muscle growth and stanching bleeding [3]. Owing to its sweet taste, warm nature, non-toxic, rich nutrition, balanced composition and other characteristics, the pine flower has outstanding edible and medicinal value in daily life [4–6].

Many scholars have confirmed pine pollen is rich in protein, unsaturated fatty acid, phytosterol, flavonoids and other active substances. Based on its characteristics, the anti-virus and immune regulation have been widely used in food, health products, medical supplies and other fields [7–9]. Additionally, the sporopollenin in the cell wall is acid and alkali resistant, temperature and pressure-resistant, possesses with stable physical and chemical properties for digestive system enzymes and gastric acid as its cell characteristics. Therefore, the extraction of nutrients from pine pollen is complicated, which extremely limits the development and utilization of pine pollen. As a result, it is essential to pretreat pine pollen, such as breaking walls and soaking [10–11].

Currently, our national researches on the pharmacological effects of pine pollen are mainly on liver protection, lipid reduction and immune regulation [12–13]. It still lacks of comprehensive research on anti-inflammatory, analgesic, antihypoxia and fatigue resistance. This study chiefly discusses the anti-inflammatory and analgesic effects of ethanol extract and the pure water extract of pine pollen.

Materials and methods

Experimental animals

Three hundred and fifty SPF Kunming mice, the ratio of male to female is 2 : 3, body weight (20 ± 2) g, week age unknown, which provided by the Experimental Animal Center of Youjiang Medical College of Nationalities, Baise City, Guangxi Province, license: No. SCXK GUI 2012-0003.

Drug identification

The dried pollen of *Pinus massoniana Lamb*. was identified by Professor Huang Suoyi, the director of the Scientific Experiment Center of Youjiang Medical College for Nationalities.

Instruments and consumables

Rb-200 intelligent hot plate instrument (Chengdu Taimeng Software Limited Company); TWCL-T5000 magnetic stirrer (Shanghai Biaohe Instrument Limited Company); BLSB-5 low temperature coolant circulating pump (Zhengzhou Changcheng Technology Industry and Trade Limited Company); JSC electronic balance CN-BH (Fuzhou Kedi Electronic Technology Limited Company); FA1204B electronic balance (Shanghai Jingke Tianmei Instrument Limited Company); JMF-320G multi-stage flasher (Henan Zhijing Biotechnology Limited Company); TH-RP-10 real laboratory special ultra-pure water machine (Shandong Tenghai Analytical Instrument Limited Company); clean experimental rat maintenance feed (Jiangsu Synergetic Pharmaceutical Bioengineering Limited Company).

Animal grouping and administration

The experiment was divided into 7 experimental groups by random number table method and 5% picric acid mark, namely blank control group, water extract low, medium and high dose groups, alcohol extract low, medium and high dose groups. Additionally, in the hot plate experiment, all the female mice were selected, and the other experimental groups were divided into cages according to the ratio of male and female 1 : 1 and randomly assigned to feed, with 10 in each group. Pure water and ethanol extract were respectively prepared into 1.5 $mg \cdot mL^{-1}$, 4.5 $mg \cdot mL^{-1}$ and 7.5 $mg \cdot mL^{-1}$ solution, corresponding to the low, medium and large experimental groups of pure water and ethanol extracts.

The body weight of the experimental animals was measured one by one after 1 week of adaptive feeding. To ensure the accuracy of the intragastric dose and the preciseness of experimental operation, the body mass was tested every three days during the experiment. In the experiment, each group was given the corresponding solution according to the body weight of 20 $mL \cdot kg^{-1}$, the dose was 20 $g \cdot kg^{-1}$. The model control group was normal saline group, and 0.9% normal saline was equally allocated. Strickly follow the principle of 24-hour intragastric and ethical requirements of animal experiments.

Drug extraction

Referring to the ethanol extraction method of literature [14–15], weigh 2 kg pine pollen and extract it three per-time with ten times volume of ethanol by ultrasound, combine the filtrate for flash concentration and freeze-drying to obtain the drug-dried powder. Prepare 1 $g \cdot mL^{-1}$ solution with pure water, take 2 kg pine pollen, decoct for 1 h with 10 times volume of water for the first time, next, 1 h

with 8 times volume of water , for the third time, add 6 times of the amount of water to boil for 1 hour, combine with 3 times of the extract to filter, flash and condense to a water decoction with a crude drug content of 1 g·mL^{-1}. After natural cooling, the two extracts placed in a 4 ℃ refrigerator for cold storage.

Experimental study on the anti-inflammatory effect of two extracts of pine pollen

Firstly, we selected 35 male and 35 female mice, then divided into groups, dosing and intragestric administration were carried out according to "2.1". Anti-inflammation test requires intragastric administration for 14 days. On the 14th day, 30 minutes later after administering, 0.04 mL xylene was applied on both sides of the right ear of mice to cause inflammation, while the left ear was not treated. Thirty minutes later, the mice were killed by cervical dislocation, and the ear pieces were taken from the same part with a 6 mm diameter perforator. The weight of the earpieces was weighed up, the swelling degree was indicated by the mass difference between the left and right ear pieces and calculated the swelling inhibition rate.

Swelling inhibition rate (%) = (mean value of model group − mean value of administration group)/mean value of model group × 100%.

Experimental study on the analgesic effect of two extracts of pine pollen

Analgesic effect of glacial acetic acid on writhing response in mice. Seventy mice were selected. The ratio of male to female, grouping, administration and body weight was exactly the same as "2.3", gavage for 14 days. Thirty minutes after the last administration, 20 mL/kg of 0.5% glacial acetic acid solution was injected intraperitoneally. The first writhing time and the number of writhing reactions were recorded within 30 minutes after the injection, then we calculated the writhing inhibition rate. The calculation formula is as below.

Writhing inhibition rate (%) = (mean number of writhing in the control group − mean number of writhing in the administration group)/mean number of writhing in the control group × 100% [17].

Analgesic effect of the hot plate on pain response in mice. Seventy female mice were divided into groups, administrated and their body mass was determined in the same way as the acetic acid writhing test. Before the experiment and 1 hour after the last administration, put mice on the hot plate instrument, temperature set at (55 ± 0.5) ℃ and the timing was immediately recorded. We recorded the time since them touch the instrument to lick their hind feet and the value as the pain threshold for the mice, the increase rate of the pain threshold was calculated as follows.

The improvement rate of pain threshold (%) = (mean pain threshold after medication − mean pain threshold before medication)/mean pain threshold before medication × 100%.

Experimental study on the anti-hypoxia effect of two extracts from pine pollen

Seventy mice were subjected to hypoxia tolerance test under normal pressure, the ratio of male and female, group and administration were the same as "2.3", 21 days after gavage. On the 21st day, 30 minutes after gavage, put the inhibited mice into a 50 mL wide mouthed bottle and smear the bottle mouth with vase line and covered it tightly, record and observe the respiratory stop time of mice, which is the time of hypoxia tolerance under normal pressure [18]. The mice were killed by cervical dislocation. The liver of the mice was stripped completely and weighed. The liver weight of each mouse was recorded. Liver weight coefficient is calculated as follows.

Liver weight coefficient = liver wet weight (g)/weight (g) × 100%.

Experimental study on the fatigue resistance of two extracts from pine pollen

Seventy mice were selected for the swimming experiment. The proportion of male and female, grouping and administration were the same as "2.3", gavage for 21 days. Thirty minutes after the last gavage, each group of mice was loaded with iron wire at the tail (7% of the mice body weight), and 2 mice were placed in plastic barrels with a water depth of 28 cm (more than the tail length, body length of the mouse is appropriate), the water temperature control at (30 ± 2) ℃ [19]. Stir the water surface to make the mice swim continuously, record time from entering the water to the head of mice unable to float out of the water for 6 seconds, which is the exhausted swimming time of the mice. Take out the mice after recording the time.

Data processing and method statistics

The experimental data of each group were expressed as x ± s, the statistical software SPSS 17.0 was used for statistical analysis. The comparison between the two experimental groups was performed by t-test of variance analysis, and multigroup difference analyzed by univariate ANOVA method. The test level was $\alpha = 0.05$.

Results and analysis

Effects of two extracts of pine pollen on the anti-inflammatory effect of xylene induced acute inflammation in mice

In the acute anti-inflammatory experiment, compared with the blank control group, the experimental group showed various degrees of swelling inhibition, and

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the swelling inhibition rate increased significantly with the raise of the concentration, the two were positively correlated. Swelling inhibition rates of mice in the three concentration groups of alcohol extract were 56.67%, 69.17% and 73.75% respectively, the effect of alcohol extract group was the best ($P < 0.05$), see Table 1 for details.

Comparative study on the analgesic effect of two extracts of pine pollen on mice

Effect of two extracts of pine pollen on analgesic effect of writhing mice with glacial acetic acid. The two extracts of pineal flowers had effects on the first writhing time, the number of writhing in 30 minutes and the inhibition rate of writhing induced by glacial acetic acid. Moreover, the inhibitory effect of alcohol extract on writhing of mice was obvious, the inhibitory rates of writhing were 20.34%, 57.67% and 61.64% individually. The alcohol increased dose group had an excellent inhibitory effect on the writhing of mice induced by acetic acid ($P < 0.05$) (Table 2).

Effects of two extracts of pine pollen on pain response of mice induced by hot plate. The effect of alcohol high dose group on pain inhibition of mice caused by hot plate was significant ($P < 0.05$), and the increased rate of pain threshold was 61.54%. There were significant differences in the time and the rate of pain threshold improvement after licking (Table 3).

Effects of two extracts of pine pollen on anoxia in mice

Compared with the blank control group, the death time of the experimental group was obviously prolonged. Among them, the effect of hypoxia tolerance was the best in the alcohol group ($P < 0.01$), and the liver weight coefficient was rather higher ($P < 0.05$), as shown in Table 4.

The effect of two extracts of pine pollen on fatigue test in mice

Compared with (157.087 ± 27.63) min in the blank control group, the exhaustive swimming time of mice in the three concentration groups of alcohol extraction was (297.90 ± 19.65), (660.35 ± 28.15), (783.24 ± 39.88) min respectively. There was a statistical difference in the alcohol high dose group ($P < 0.05$) (Table 5).

Table 1 Effects of two pine pollen extracts on ear swelling caused by xylene in mice ($\bar{x}$ ± s, n = 10)

Group	Dose/g·kg^{-1}	Left ear/mg	Right ear/mg	Swelling degree/mg	Swelling inhibition rate/%
Blank control group	-	6.913 ± 0.026	5.418 ± 0.019	2.780 ± 0.225	-
Water low dose group	20	6.481 ± 0.201 [a]	5.196 ± 0.129 [a]	1.084 ± 0.147 [a]	41.67
Water dose group	20	7.385 ± 0.324	6.226 ± 0.290	1.409 ± 0.011	47.73
High dose water group	20	6.382 ± 0.197 [a]	5.526 ± 0.118	1.134 ± 0.016 [a]	58.17
Alcohol low dose group	20	7.864 ± 0.206 [a]	6.761 ± 0.138	1.283 ± 0.120 [a]	56.67
Middle alcohol dose group	20	8.083 ± 0.045	7.448 ± 0.445 [a]	0.963 ± 0.999	69.17
Alcohol high dose group	20	6.290 ± 0.182 [a]	5.496 ± 0.105	0.931 ± 0.117 [a]	73.75

Compared with the blank control group, [a] $P < 0.05$, with statistical difference.
-, not mention.

Table 2 Effects of two extracts of pine pollen on writhing reaction induced by acetic acid in mice ($\bar{x}$ ± s, n = 10)

Group	Dose/g·kg^{-1}	First twisting time/s	Thirty number of twists in minutes	Torsion inhibition rate/%
Blank control group	-	249.61 ± 53.36	64.77 ± 5.17	-
Water low dose group	20	255.26 ± 39.55	51.42 ± 13.07	8.47
Water dose group	20	269.14 ± 62.08	57.28 ± 4.63	13.41
High dose water group	20	288.61 ± 58.98	37.00 ± 3.65	39.55
Alcohol low dose group	20	292.11 ± 68.12 [a]	53.37 ± 3.77	20.34
Middle alcohol dose group	20	326.84 ± 70.62	17.58 ± 1.68	57.69
Alcohol high dose group	20	343.52 ± 90.36 [a]	8.833 ± 1.21	61.64

Compared with the blank control group, [a] $P < 0.05$, with statistical difference.
-, not mention.

Table 3 Effects of two extracts of pine pollen on the pain response induced by hot plate in mice ($\bar{x}$ ± s, n = 10)

Group	Dose/g·kg^{-1}	Before administration/s	Fourteen days after administration/s	Increase rate of pain threshold/%
Blank control group	-	249.61 ± 53.36	64.77 ± 5.17	-
Water low dose group	20	255.26 ± 39.55	51.42 ± 13.07	8.47
Water dose group	20	269.14 ± 62.08	57.28 ± 4.63	13.41
High dose water group	20	288.61 ± 58.98	37.00 ± 3.65 [a]	39.55
Alcohol low dose group	20	292.11 ± 68.12 [a]	53.37 ± 3.77	20.34
Middle alcohol dose group	20	326.84 ± 70.62 [a]	17.58 ± 1.68 [a]	57.69
Alcohol high dose group	20	343.52 ± 90.36 [a]	8.833 ± 1.21 [a]	61.64

Compared with the blank control group, [a] $P < 0.05$, with statistical difference.
-, not mention.

Table 4 Effects of two extracts of pine flower on hypoxia tolerance in mice ($\bar{x}$ ± s, n = 10)

Group	Number	Dose/g·kg^{-1}	Survival time/S	Liver weight coefficient
Blank control group	10	-	446.50 ± 30.96	0.047 ± 0.009
Water low dose group	10	20	555.08 ± 34.04	0.052 ± 0.006
Water dose group	10	20	498.30 ± 40.19	0.051 ± 0.004
High dose water group	10	20	491.22 ± 24.05	0.055 ± 0.005
Alcohol low dose group	10	20	454.24 ± 18.33	0.053 ± 0.008
Middle alcohol dose group	10	20	497.655 ± 23.12	0.056 ± 0.007
Alcohol high dose group	10	20	505.371 ± 33.11	0.059 ± 0.003

Compared with the blank control group, [a] $P < 0.05$, with statistical difference.
-, not mention.

Table 5 Effects of two extracts of pine pollen on exhausted swimming time in mice ($\bar{x}$ ± s, n = 10)

Group	Number	Dose/g·kg^{-1}	Exhausted swimming time/s
Blank control group	10	-	157.087 ± 27.63
Water low dose group	10	20	171.04 ± 14.12
Water dose group	10	20	180.438 ± 9.12
High dose water group	10	20	226.445 ± 23.70 [a]
Alcohol low dose group	10	20	297.90 ± 19.65 [a]
Middle alcohol dose group	10	20	660.35 ± 28.15 [a]
Alcohol high dose group	10	20	783.24 ± 39.88 [a]

Compared with the blank control group, [a] $P < 0.05$, with statistical difference.
-, not mention.

Discussion and prospect

Pine pollen, as a part of Pinus massoniana, which has the functions of regulating body activity, promoting metabolism, eliminating free radical and enhancing immunity [20–21]. At present, there are two main types of anti-inflammatory drugs in clinical application, one is corticosteroid anti-inflammatory drugs, the other is non-steroidal anti-inflammatory drugs, such as aspirin, celecoxib and other antipyretic, analgesic and anti-inflammatory drugs. Nonsteroidal drugs mainly inhibit the aggregation of white blood cells indirectly by inhibiting the synthesis of prostaglandins, effectively reduce the formation of kallikrein then achieve the purpose of anti-inflammatory by inhibiting the aggregation of platelets. While non-steroidal anti-inflammatory drugs inhibit prostaglandin synthesis to complete the anti-inflammatory purposes. It is easy to stimulate gastrointestinal organs then cause upper gastrointestinal bleeding, accompanied by sleepiness and other neurological symptoms as well as kidney toxicity and other symptoms. It has been found that pine pollen can greatly reduce the production of

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inflammatory cytokines through the mechanism of immune regulation in vivo, so the anti-inflammatory and analgesic effect in vivo is more visible [22]. Hypoxia, like a lousy stimulus that directly damages the body, firstly causes the lack of oxygen supply to all organs of the body, the decrease of the intensity and level of aerobic oxidation in all tissues and organs, the acceleration of anaerobic glycolysis, and the significant reduction of adenosine triphosphate production. Hypoxia further stimulates the brain and heart and other vital organs, and organ cells begin to apoptosis in the large areas due to lack of energy supply. Based on this, the body produces large numbers of free radicals that damage organs and tissues in varying degrees [23]. Qin Wanning [24] confirmed the flavonoids in pine pollen had more robust antioxidant activity than vitamin C, show a strong antioxidant effect. It has been studied that free radicals are harmful groups produced when the body self-regulation ability is damaged, and the increase of skeletal muscle free radicals is an essential cause of body fatigue. pine pollen can reduce the sense of fatigue and improve the active exercise time by its potent free radical scavenging effect [25]. In conclusion, the macroscopical results show that pine pollen has strong ant-hypoxia and anti-fatigue effect.

In this experiment, compared with the blank control group, the experimental group showed anti-inflammatory and analgesic effects and antianoxia and antifatigue active, the difference was statistically significant. After comparing the results of water extract and alcohol extract, we found that under the same concentration, the experimental effect of pine pollen alcohol extract was better than that of water extract, which showed that the affinity of effective components in pine pollen to ethanol was significantly better than that of pure water, which could notablly reduce the formation of inflammatory cytokines and a large number of free radicals in the body caused by the change of external factors. This study is of great significance in applying pine pollen in anti-inflammatory, analgesic, faitigue and hypoxia tolerance. The research shows that pine pollen has a good application value in the field of anti-aging, which is worthy for further study. pine pollen has very high application value in eliminating excessive free radicals, delaying aging and improving disease resistance. It is anti-inflammatory and analgesic effects can be used in skin beauty, skin disease treatment and pregnant women health care.

Pine pollen contains vitamin C, vitamin E, selenium, flavonoids, β-carotene, trace elements selenium and other chemicals, which have anti-inflammatory, antioxidant and free radical scavenging effects. In the study, we demonstrate that the alcohol extract of pine flower powder has more obvious anti-inflammatory and analgesic active, anti-anoxia and anti-fatigue effects than water extract. However, there a son of the findings are still unknown. The possible reasons are that the solubility of effective components in ethanol is higher than that in water, or some substances in pine pollen have new combination and reaction in ethanol. The chemical monomers or parts of the pollen and other substances with the above pharmacological effects need further study.

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