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Research article

Quality control, preparation process optimizing and anti-inflammatory effects of *Premna Puberula* Pamp. Pectin

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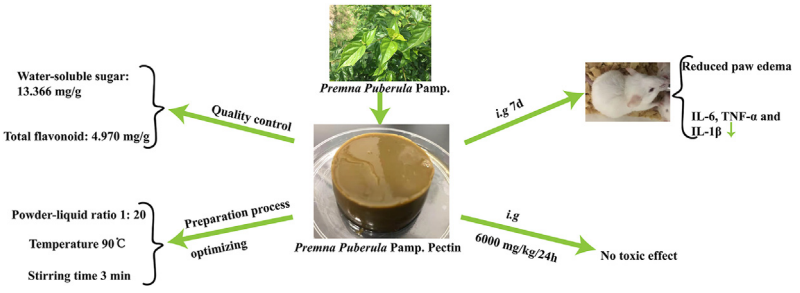
HIGHLIGHTS

- *Premna Puberula* Pamp. Pectin (PP), was a Wudang functional food in China.
- PP's preparation process was optimized by orthogonal test.
- PP had anti-inflammatory effects by reducing IL-6, TNF-α and IL-1β.
- PP was non-toxic to mice at a dose of 6000 mg/kg/24 h.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: *Premna Puberula* Pamp. Pectin (PP) was a Wudang functional food in China. It has the effect of dispelling fire, clearing heat and detoxification in folk medicine. However, little studies have been reported for their preparation, quality control, effects and toxicity.

Methods: The *P. Puberula* leaves were collected from different pharms and seasons. The compounds in PP were identified using UPLC-Q-TOF-MS/MS. UV-VIS spectrophotometry with phenol-sulfuric acid and sodium nitrite aluminum nitrate were conducted for analyzing the water-soluble sugars and total flavonoids, respectively. $L_9(3^4)$ orthogonal experimental method was used to optimize the preparation process of PP. For the pharmacological effects of PP, the swelling right hind paw of ICR mice was modeled using subcutaneous injection of carrageenan gum solution, and the local tissue inflammatory reactions of the model mice were investigated using vernier calipers and HE staining. The serum inflammatory factor expression was detected using ELISA. The acute toxicity experiments were carried out for safety assessment of PP in ICR mice.

Results: Fifty-three compounds were initially identified in PP among which flavonoids were dominant (19 out of 53). The average values of water-soluble sugar content and total flavonoid content of PP were 13.366 and 4.970 mg/g, respectively. The best preparation process of PP was powder-liquid ratio 1: 20, temperature 90 °C, and

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stirring time 3 min. Data showed that PP reduced paw edema and decrease the serum level of IL-6, TNF-α and IL-1β in the model mice. There was no toxic effect of PP on mice at a total dose of 6000 mg/kg/24h.
Conclusion: In summary, by optimizing the preparation process, PP with stable quality can be obtained. PP has anti-inflammatory effects without toxicity.

1. Introduction

Premna puberula Pamp., belonging to the genus *Premna* Linn. of the family Lamiaceae [1, 2], is a functional edible plant and known as "fairy tree" in the Wudang Mountain, Hubei Province of China. Its leaves, known as "fairy leaves", are rich in pectin, protein, cellulose, and vitamins, among which the pectin has been used for the local special food and anti-inflammation in folk medicines [3]. The local folk in Wudang area usually use "fairy leaves" to make "fairy tofu" (*P. Puberula* Pectin, PP) [4], which is a kind of natural pectin-enriched curd with green in color, smooth and slightly bitter in taste.

The previous studies showed that the key factor in the formation of PP is the richness of pectin in the leaves [5]. PP had the function of clearing dampness and heat, and detoxifying and regulating menstruation which can be used for irregular menstruation, rheumarthritis, edema and unidentifiable sores or boils [3, 6, 7]. At present, studies on the pharmacological effects of the plants in the genus *Premna* Linn. have focused on the anti-inflammatory effects. The examples included *Premna microphylla* Turcz., *Premna fulva* Craib., and *Premna integrifolia* Linn [1, 8, 9]. However, little studies have been reported for *P. Puberula*, its chemical compounds, preparation, quality control, effects and toxicity. Therefore, in this study, we investigated the quality, optimized the preparation process, and explored the anti-inflammatory effects as well as safety of PP, aiming to screen natural medicinal candidates through production standardization and pharmacological effects [10].

2. Materials and methods

2.1. Reagents and materials

Food-grade gelatin was purchased from Shangshui County Fuyuan Gelatin Co., Ltd (Zhoukou, Henan Province, China); food-grade agar was purchased from Fujian Shishi Gaoxin Agar Food Co., Ltd. (Shishi, Fujian Province, China); food-grade light calcium carbonate was purchased from Jiangxi Mingyuan High-tech Materials Co., Ltd (Dexing, Jiangxi Province, China); food-grade magnesium chloride was purchased from Qinghai Jiayou Magnesium Industry Co., Ltd. (Geermu, Qinghai Province, China); anhydrous glucose (purity ≥98%) was purchased from Tianjin Kemeou Chemical Reagent Development Center (Tianjin, China); concentrated sulfuric acid was purchased from Luoyang Haohua Chemical Reagent Co., Ltd (Luoyang, Henan Province, China); phenol was purchased from Tianjin Tianli Chemical Reagent Co., Ltd. (Tianjin, China); Rutin (HPLC grade, purity ≥98%) was purchased from Sichuan Weikeqi Biotechnology Co., Ltd (Chengdu, Sichuan Province, China); absolute ethanol was purchased from Shanghai Wokai Biotechnology Co., Ltd. (Shanghai, China); sodium hydroxide was purchased from Tianjin Tianli Chemical Reagent Co., Ltd (Tianjin, China); Sodium nitrite and aluminum nitrate were purchased from Tianjin Hengxing Chemical Reagent Manufacturing Co., Ltd. (Tianjin, China); λ-carrageenan was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Sample and preparation process of Premna Puberula Pamp. Pectin (PP)

The fresh "fairy leaves" were collected from different farms in Shiyan City, Hubei Province of China from June 1, 2019 to October 31, 2021, and were identified as *Premna Puberula* Pamp (Figure 1A). by Prof. Xuanbin Wang. The specimens were Nos. 20190627XXX-20211022XXX according to the number system rule of YYYY/MM/DD/batch number in our lab (Table 5). Then the leaves were dried to constant weight at 60 °C, crushed into powder, and sieved and stored at room temperature in our lab until use.

To prepare PP, the dried leaf powder was added into water with a certain proportion, mixed with the coagulant (Figure 1B) (a recipe containing calcium carbonate) in water bath at 24, 50 or 90 °C, and well stirred and filtered with two-layer gauze. The filtrates were set at 4 °C until the pectins were formed [5, 11] (Figure 1C).

2.3. Compound identification of PP through UPLC-Q-TOF-MS/MS

To ensure the quality of PP, we detected and analyzed the chemical compounds in *P. Puberula* leaves using UPLC-Q-TOF-MS/MS (Waters

Table 1. Mobile phase gradient for LC-MS/MS.

Time (min)	Flow Rate (mL/min)	Mobile phase (B %)
0	0.3	5
5		10
15		20
25		30
35		40
45		70
50		95

Note: B %, 0.1% formic acid aqueous solution in phase A and acetonitrile in phase B.

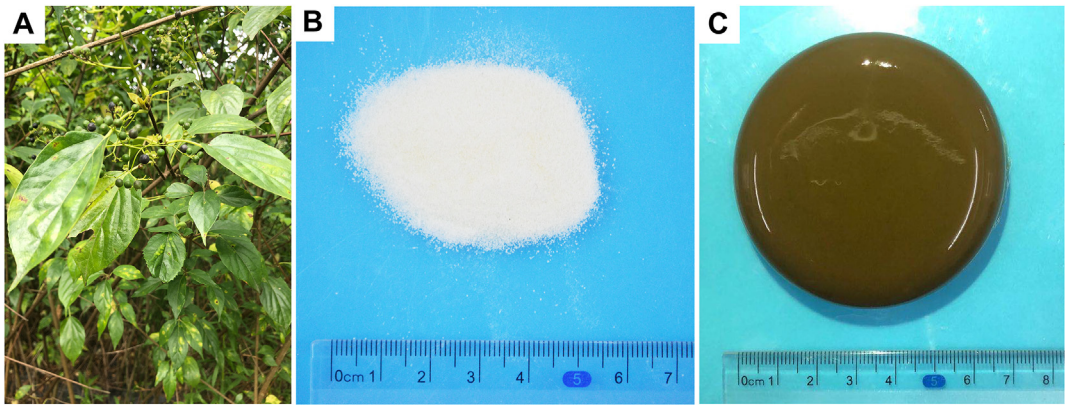

Figure 1. Plant of *Premna Puberula* Pamp. and the pectin (PP). A. Plant. B. The coagulant. C. The pectin (known as "fairy toufu").

Table 2. Mass parameters (Sciex Triple TOF 4600 LC-MS/MS).

MS	Parameter Value	MS/MS	Parameter Value
TOF mass range	50 ~ 1700	MS/MS mass range	50 ~ 1250
Ion Source Gas 1 (psi)	50	Declustering Potential (V)	100
Ion Source Gas 2 (psi)	50	Collision Energy (eV)	±40
Curtain Gas (psi)	35	Collision Energy Spread (eV)	20
Ion Spray Voltage Floating (V)	-4500/5000	Ion Release Delay (ms)	30
Ion Source Temperature (°C)	500	Ion Release Width (ms)	15
Declustering Potential (V)	100		
Collision Energy (eV)	10		

Table 3. The factors and levels of the Orthogonal experiment method (OEM) for *P. Puberula* Pectin (PP).

Level	Powder-liquid ratio (g/mL)	Temperature (°C)	Stirring time (min)
1	1 : 20	24	1
2	1 : 30	50	2
3	1 : 40	90	3

Corporation, Milford, MA, USA) firstly [12]. The *P. Puberula* leaves were extracted three times with 95% ethanol. The extracts were filtered and mixed. The ethanol was volatilized using rotary evaporator. The extracts were concentrated and freeze-dried into 0.0705 g per gram of the dried *P. Puberula* leaves and stored at -4 °C until use.

To conduct LS-MS/MS analysis, the *P. Puberula* leaf extracts were added into 2 mL of methanol, and sonicated to be dissolved completely. The solution was centrifuged in 12000 rpm for 5 min. The supernatants were filtered for LC/MS/MS determination. The Chromatographic column was Waters ACQUITY UPLC HSS T3 (2.1 × 100 mm, 1.8 μm) with the column temperature 30 °C. The injection volume was 2 μL. The detection wavelength range was 190–400 nm with the flow rate 0.3 mL/min. The mobile phase was set 0.1% formic acid aqueous solution for phase A and acetonitrile for phase B. The gradients were shown in Table 1. The mass spectrometry detection mode was ESI-Negative/Positive ion mode (Table 2).

The data acquisition software was Analyst TF 1.7.1 and the data processing software was Peakview 1.2. The compound identification was prioritized by matching the mass spectrometry data of the Natural Products HR-MS/MS Spectral Library 1.1 database (AB Sciex Pte. Ltd., USA). The compounds were initially screened based on the information of each peak score, and further confirmed based on each chromatographic profile. The compounds were further confirmed based on the primary and secondary information of the peaks. The Compounds that were not included in the database were identified based on literature reports, mass spectrometry cleavage patterns, and etc.

2.4. Quality control of PP using ultraviolet-visible (UV-VIS) analysis

Given PP as a functional food for its pectin and anti-inflammatory folk use, the contents of the water-soluble sugars and the total flavonoids were selected for quality control of PP using UV-VIS spectrophotometry (Shimadzu Suzhou Instruments Mfg. Co., Ltd, Suzhou, China) with phenol-sulfuric acid [13] and sodium nitrite aluminum nitrate [14], respectively. The internal references were anhydrous glucose for the water-soluble sugars and rutin for the total flavonoids. The average contents of the water-soluble sugars and total flavonoids were taken in consideration for the reference of quality control of PP.

2.5. Orthogonal experiment method (OEM) for optimizing the preparation process of PP

As various factors affect the preparation of PP, such as powder-liquid ratio, temperature, and stirring time. To optimize the preparation process of PP, the $L_9(3^4)$ OEM was designed using leaf specimen 20200710002 in this study [15]. The powder-liquid ratio, temperature and stirring time were used as the investigating factors to screen the best preparation process conditions (Table 3). In this study, the comprehensive score (Equation 1) of the water-soluble sugars and total flavonoid content were determined as quality control markers, and the different weight coefficients were assigned according to the importance of each indicator to the preparation process, and the comprehensive score formula was below:

$$\text{Comprehensive score} = (\text{water-soluble sugar content} * 0.5 + \text{total flavonoid content} * 0.5) * 100\% \text{ [16]} \quad (1)$$

The *P*-value of the statistical analysis by the multi-indicator experimental formula method was used as the index of investigation, and the size of the extreme difference R-value was used to judge the priority of the role of each factor, and the best preparation process was obtained by the comprehensive preparation process ANOVA. The three-repeated experiments were conducted according to the optimized protocol [16].

2.6. Animals

The male and female ICR mice (19–21 g) were provided by Hubei University of Medicine (SCXK (E) 2019–0008). All animals were housed in a special pathogen-free grade (SPF) room with controlled ambient temperature (23 ± 2 °C) and humidity (55 ± 5%), 12 h dark/light cycles, and free access to food and water. The animals were habituated to the environment for 7 days prior to experiments. All animal experiments conformed to the British Home Office Regulations (Animal Scientific Procedures Act 1986) for the care and use of animals. This study was reviewed and approved by the Laboratory Animal Welfare Ethics Review Committee of Hubei University of Medicine (No. 2020-Shi 034 and No. 2022-Shi 001). The mice were sacrificed using CO_2 inhalation [17].

2.7. Effects of PP on carrageenan-induced mice paw edema

To investigate the anti-inflammatory of PP, the sixty ICR male mice were randomly divided into 6 groups (10 mice per group): Control group, Model group, Positive group (diclofenac sodium, 15 mg/kg), and PP groups (500, 1000 and 2000 mg/kg). Each group were administered via gavage. The Control and Model groups were given equal volume of normal saline. On the 7th day, all groups except the Control group were injected subcutaneously with 30 μL of 1% carrageenan at the middle toe of the right hind paw after 30 min administration of PP or the positive drug. The toe thickness of mice was measured using vernier calipers at 5 h. The paw edema rate (Equation 2) was calculated as below:

$$\text{Paw edema rate} = (\text{toe thickness after administration} - \text{toe thickness before administration})/\text{toe thickness before administration} * 100\% \quad (2)$$

The anti-inflammatory effect of the drug was evaluated by comparing the degree of paw edema in mice [18].

The swelling toes were sectioned, fixed with 10% formaldehyde for 72 h, and decalcified with 10% nitric acid for 24 h. The hematoxylin-eosin (HE) staining was used for observing histological changes through the microscope (Olympus Corporation, Tokyo, Japan).

2.8. Effects of PP on inflammatory factors IL-1β, IL-6 and TNF-α in mouse serum using enzyme-linked immunosorbent assay (ELISA)

To investigate the effects of PP on the inflammatory factors, the mouse blood was sampled [17]. The blood was centrifuged and the

Table 4. Fifty-three compounds were identified from *Premna puberula* Pamp. via UPLC–Q–TOF-MS combined with the database of Natural Products HR-MS/MS Spectral Library 1.0.

No.	Category	Molecular formula	Molecular weight	Compounds	T_R (min)	MS/MS fragments
1	Flavonoid	$C_9H_8O_4$	180.04	Caffeic acid	8.673	135.0445; 89.0385
2	Flavonoid	$C_{16}H_{12}O_5$	284.07	Genkwanin	36.478	283.0588; 268.0363; 240.0425; 171.0464
3	Flavonoid	$C_{15}H_{10}O_6$	286.05	Luteolin	23.73	285.0404; 199.0402; 175.0418; 151.0045; 133.0295
4	Flavonoid	$C_{16}H_{12}O_6$	300.06	Diosmetin	28.505	299.0562; 284.0323; 256.0366; 227.0357; 151.0027
5	Flavonoid	$C_{15}H_{10}O_7$	302.04	Quercetin	23.675	301.0339; 273.0388; 178.9981; 151.0034; 121.0290
6	Flavonoid	$C_{17}H_{14}O_6$	314.08	Pectolinargenin	37.871	313.0705; 298.0485; 283.0223; 255.0291
7	Flavonoid	$C_{16}H_{12}O_7$	316.06	Rhamnetin	32.43	315.0476; 300.0293; 193.0147; 165.0189; 149.9942
8	Flavonoid	$C_{18}H_{16}O_7$	344.09	Eupatilin	39.394	343.0796; 328.0567; 313.0327; 298.0105; 285.0385
9	Flavonoid	$C_{19}H_{18}O_7$	358.11	3'-hydroxy-5,6,7,4'-tetramethoxyflavone	42.429	359.1125; 343.0812; 329.0659; 315.0853; 298.0829
10	Flavonoid	$C_{19}H_{18}O_8$	374.1	Vitexicarpin	39.049	373.0942; 358.0682; 343.0458; 315.0548; 300.0265
11	Flavonoid	$C_{20}H_{20}O_7$	372.12	Isosinensetin or isomer	34.168	373.1293; 357.0973; 343.0821; 339.0868; 312.0998
12	Flavonoid	$C_{20}H_{20}O_7$	372.12	Sinensetin or isomer	33.118	373.1295; 357.0976; 343.0822; 329.1028; 312.0999
13	Flavonoid	$C_{21}H_{20}O_{11}$	448.1	Luteoloside	15.965	447.0936; 285.0399; 133.0330
14	Flavonoid	$C_{24}H_{28}O_{13}$	524.15	6'-O-Caffeoylcatalpol	11.41	523.1432; 323.0742; 179.0339; 161.0240; 133.0285
15	Flavonoid	$C_{27}H_{30}O_{14}$	578.16	Rhoifolin	17.605	579.1655; 433.1069; 271.0602
16	Flavonoid	$C_{27}H_{30}O_{15}$	594.16	Luteolin 7-O-neohesperidoside	15.494	593.1526; 447.0897; 285.0383; 175.0412
17	Flavonoid	$C_{28}H_{32}O_{15}$	608.17	Diosmin	18.69	609.1815; 463.1211; 301.0697; 286.0460
18	Flavonoid	$C_{30}H_{26}O_{14}$	610.13	Luteolin-7- O-(6"-O-E-caffeoyl)-β-D-glucopyranoside	21.827	611.1446; 287.0553; 163.0396
19	Flavonoid	$C_{28}H_{32}O_{16}$	624.17	Diosmetin 7-O-gentiobioside	16.927	625.1717; 463.1212; 301.0693; 286.0460
20	Fatty acid	$C_{18}H_{30}O_3$	294.22	13-Hydroxyoctadeca-9,11,15-trienoic acid isomer	43.45	/
21	Fatty acid	$C_{18}H_{30}O_3$	294.22	13-Hydroxyoctadeca-9,11,15-trienoic acid	43.155	293.2107; 275.2024; 235.1701; 171.1029; 121.1016
22	Fatty acid	$C_{18}H_{32}O_3$	296.24	9-hydroxy-10E,12Z-octadecadienoic acid	44.82	295.2281; 277.2190; 195.1376; 171.1012
23	Fatty acid	$C_{18}H_{32}O_5$	328.22	9,12,13-Trihydroxy-10,15-octadecadienoic acid	28.077	327.2165; 291.1973; 239.1295; 229.1444; 211.1333
24	Fatty acid	$C_{18}H_{34}O_5$	330.24	Pinellic acid	30.329	329.2326; 314.0411; 299.0182; 271.0238; 243.0267
25	Fatty acid	$C_{18}H_{28}O_9$	388.17	Tuberonic acid glucoside	9.826	387.1629; 207.0990; 113.0252; 59.0143
26	Heterocyclics	$C_{30}H_{48}O_5$	488.35	Tormentic acid	42.627	487.3443
27	Heterocyclics	$C_{19}H_{34}O_{10}$	422.22	1-Octen-3-yl primeveroside	20.042	421.2050; 289.1608; 161.0402
28	Heterocyclics	$C_{30}H_{48}O_4$	472.36	Sumaresinolic acid	45.501	437.3391; 409.3439; 203.1769; 123.1173
29	Heterocyclics	$C_{30}H_{48}O_4$	472.36	Corosolic acid	45.25	473.3553; 437.3419; 409.3455; 203.1814; 189.1639
30	Heterocyclics	$C_{30}H_{46}O_5$	486.33	Actinidic Acid	39.727	485.3271; 467.3058; 441.3337; 423.3214
31	Heterocyclics	$C_{30}H_{48}O_5$	488.35	Arjunolic acid	40.496	487.3405; 469.3250; 423.3221
32	Heterocyclics	$C_{25}H_{44}O_{15}$	584.27	1-Ethenylhexyl-O-α-L-arabinopyranosyl-(1→6)-O-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside	17.692	583.2584; 421.2134; 289.1647
33	Phenolic acids	$C_{15}H_{18}O_9$	342.1	Glucocaffeic acid	6.51	281.0671; 221.0451; 179.0358; 161.0246; 135.0452
34	Phenolic acids	$C_{15}H_{18}O_8$	326.1	1-O-p-coumaroyl-β-D-glucose	6.75	325.0889; 179.0345; 161.0238; 135.0447
35	Phenolic acids	$C_{15}H_{18}O_8$	326.1	6-O-p-coumaroyl-D-glucose	7.64	325.0919; 221.0439; 203.0357; 179.0333; 161.0233
36	Phenolic acids	$C_{15}H_{18}O_9$	342.1	6-O-caffeoyl-β-D-glucose	7.44	341.0861; 281.0633; 179.0346; 161.0240; 135.0450

(*continued on next page*)

Table 4 (*continued*)

No.	Category	Molecular formula	Molecular weight	Compounds	T_R (min)	MS/MS fragments
37	Phenolic acids	$C_{17}H_{22}O_{10}$	386.12	1-O-sinapoyl-β-D-glucose	10.005	385.1145; 161.0247; 133.0295
38	Phenolic acids	$C_{20}H_{28}O_{13}$	476.15	Primeverin	7.09	475.1502; 251.0615; 179.0379; 161.0254; 133.0309
39	Phenolic acids	$C_{24}H_{24}O_{12}$	504.13	1,6-Dicaffeoyl glucose	19.252	503.1198; 341.0872; 281.0665; 251.0569; 179.0364
40	Phenolic acids	$C_{27}H_{34}O_{12}$	550.21	Tracheloside	20.042	549.1938; 387.1660; 161.0257; 133.0304; 89.0360
41	Phenolic acids	$C_{27}H_{30}O_{15}$	594.16	Vicenin-2	11.271	593.1537; 503.1260; 473.1145; 413.0892; 383.0786
42	Phenolic acids	$C_{27}H_{30}O_{16}$	610.15	Luteolin 7-O-β-D-gentiobioside	13.728	609.1491; 447.0872; 285.0395
43	Phenolic acids	$C_{30}H_{38}O_{16}$	654.22	Scropolioside F	13.626	653.2049; 491.1556; 377.1214; 163.0394; 145.0293
44	Phenolic acids	$C_{30}H_{38}O_{16}$	654.22	Scropolioside F or isomer	16.604	653.2106; 491.1601; 377.1260; 309.1012; 163.0410
45	Phenolic acids	$C_{30}H_{38}O_{17}$	670.21	Callicoside E/F or isomer	11.573	669.2040; 507.1513; 325.0883; 307.0815; 161.0248
46	Phenolic acids	$C_{30}H_{38}O_{17}$	670.21	Callicoside E/F or isomer	14.168	669.2056; 325.0949; 161.0243
47	Phenolic acids	$C_{30}H_{38}O_{17}$	670.21	Callicoside E/F or isomer	12.102	669.2089; 325.0975; 307.0834; 161.0250; 135.0469
48	Phenolic acids	$C_{31}H_{40}O_{17}$	684.23	Scropolioside G	14.487	683.2198; 521.1711; 407.1346; 193.0505; 175.0399
49	Phenyl ethanoid glycosides	$C_{23}H_{26}O_{10}$	462.15	6'-O-coumaroyl-1'-O-[2-(3,4-dihydroxyphenyl)ethyl]-beta-D-glucopyranoside	18.852	461.1432; 315.1072; 161.0239; 145.0291
50	Phenyl ethanoid glycosides	$C_{23}H_{26}O_{10}$	462.15	(4-Hydroxyphenethyl)-6-O-(E)-caffeoyl-β-D-glucopyranoside	18.448	461.1460; 281.0665; 179.0337; 161.0239
51	Phenyl ethanoid glycosides	$C_{23}H_{26}O_{11}$	478.15	Desrhamnosylverbascoside	16.42	477.1405; 315.1090; 161.0242; 133.0291
52	Phenyl ethanoid glycosides	$C_{29}H_{36}O_{15}$	624.21	Acteoside	16.251	623.2002; 461.1674; 161.0252; 133.0281
53	Phenyl ethanoid glycosides	$C_{29}H_{36}O_{16}$	640.2	β-Hydroxyacteoside	14.949	639.1936; 477.1603; 315.1044; 161.0240

Note: T_R: retention time in UPLC.

Table 5. Contents of the water-soluble sugars and total flavonoids in different batches of PP.

Places	Leaf specimen (YYYY/MM/DD/XXX)	Sample archive number	Water-soluble sugars (mg/g)	Total flavonoids (mg/g)
Danjiangkou City	20190627001	PP-1	10.367	4.986
	20190804001	PP-2	12.528	3.584
Xianggong Village	20200710002	PP-3	19.052	5.433
	20200912002	PP-4	17.444	3.271
	20201029002	PP-5	12.388	2.512
	20210518002	PP-6	17.087	3.862
	20210708002	PP-7	11.944	4.956
	20210806002	PP-8	14.081	5.120
	20210920002	PP-9	9.501	11.196
	20211022002	PP-10	15.121	1.454
Dachuan County	20210518003	PP-11	14.134	4.621
	20210708003	PP-12	9.674	3.706
	20210806003	PP-13	9.968	6.590
	20210908003	PP-14	12.592	5.027
Yuezhu Village	20210908004	PP-15	14.612	8.231
Average			13.366	4.970

supernatant was aspirated for experiment. The contents of IL-1β, IL-6 and TNF-α were determined according to the ELISA kit instructions (Shanghai Jianglai industrial Limited By Share Ltd, Shanghai, China).

2.9. Safety assessment of PP through the maximum dosing method

Ten males and Ten females of ICR mice were administered by gavage at a maximum dose of 6 g/kg in three times within 24 h. The mice were observed for 14 d. The number of toxic reactions and deaths were recorded daily [19].

2.10. Statistical analysis

Statistical analysis was performed with SPSS 22.0 software. The measurement data were expressed as $\overline{X}\pm SD$, and One-way ANOVA was used for comparison between groups.

3. Results

3.1. Flavonoids were the dominant compounds in P. Puberula

Fifty-three compounds were initially identified in PP among which flavonoids were dominant (19 out of 53), indicating the potential role of flavonoids in the effects of PP. The other compounds were fatty acids, heterocyclics, phenolic acids, and phenyl ethanoid glycosides (Table 4).

3.2. Average contents of the water-soluble sugars and total flavonoids in PP

PP was prepared as Figure 1C. The standard curves for the water-soluble sugars and total flavonoids were obtained (Figure 2). The data showed that the average values of water-soluble sugar content and total

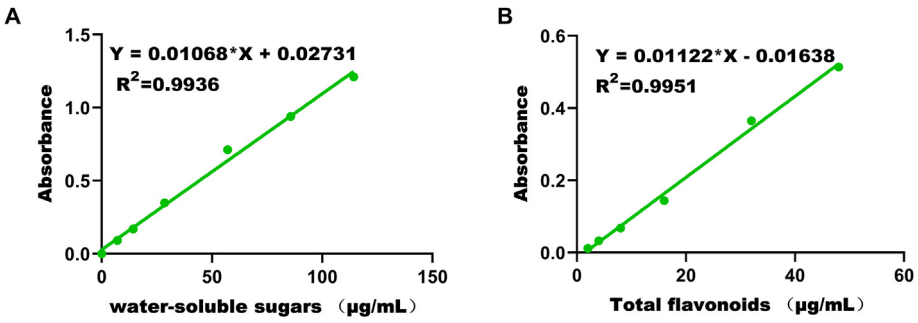

Figure 2. Standard curves for the quality control markers. A: Water-soluble sugars; B: Total flavonoids.

Table 6. Results of OEM.

Number	Powder-liquid ratio (g/mL)	Temperature (°C)	Stirring time (min)	Blank	water-soluble sugars (mg/g)	Total flavonoids (mg/g)	Comprehensive score
1	1 : 20	24	1	1	12.512	2.304	7.408
2	1 : 20	50	2	2	15.956	4.618	10.287
3	1 : 20	90	3	3	18.584	5.533	12.058
4	1 : 30	24	2	3	9.862	0.503	5.182
5	1 : 30	50	3	1	16.703	1.243	8.973
6	1 : 30	90	1	2	16.980	2.554	9.767
7	1 : 40	24	3	2	9.197	0.801	4.999
8	1 : 40	50	1	3	9.701	1.189	5.445
9	1 : 40	90	2	1	10.531	3.197	6.864
K1	9.918	5.863	7.540	7.748			
K2	7.974	8.235	7.444	8.351			
K3	5.769	9.563	8.677	7.562			
R	4.149	3.700	1.233	0.789			

Table 7. Results of analysis of variance.

Source	Sum of squares	Degrees of freedom	Mean Square	F value	*P* value	Significance
Powder-liquid ratio	25.847	2	12.924	25.323	0.038	*
temperature	21.080	2	10.541	20.654	0.046	*
stirring time	2.820	2	1.410	2.763	0.266	
blank	1.021	2	0.511			

$F_{0.05}$ (2, 2) = 19.00.

Table 8. Validation test results in triple individual experiments (n = 3).

Number	water-soluble sugars (mg/g)	RSD (%)	total flavonoids (mg/g)	RSD (%)
1	19.066	1.23	5.521	2.20
2	18.610		5.741	
3	18.914		5.521	

flavonoid content of the samples were 13.366 (9.674–19.052) and 4.970 (1.454–11.196) mg/g, respectively (Table 5).

3.3. The process of PP preparation was optimized with OEM

The ANOVA showed that the order of influencing the preparation effect was powder-liquid ratio > temperature > stirring time, while the powder-liquid ratio was 1:20 > 1:30 > 1:40, temperature 90 > 50>24, and stirring time 3 > 2>1 (Table 6). The factors powder-liquid ratio and temperature had a significant effect, while stirring time had no significant difference. Combined with the actual production operation, the best preparation process was determined as powder-liquid ratio 1:20, temperature 90 °C and stirring time 3 min (Table 7). The RSD was 1.23% for the water-soluble sugars and 4.40% for the total flavonoids, indicating the preparation process is stable and reliable (Table 8).

3.4. PP prevented the hind paw edema in carrageenan-induced mice

The rate of paw edema was significantly decreased in PP-treated groups compared with the model group, indicating that PP attenuated the hind paw edema in carrageenan-induced mice ($P < 0.05$, $P < 0.01$ or $P < 0.001$) (Table 9).

3.5. PP alleviated the neutrophil infiltration of paw edema in carrageenan-induced mice

HE staining showed that the toe tissue of mice in the model group had a large amount of neutrophil infiltration compared with the control group, while those in the medium (1000 mg/kg) and high dose (2000 mg/kg) of PP were significantly reduced (Figure 3).

Table 9. Effects of PP on carrageenan gum-induced paw edema in mice (n = 10).

Time (h)	Size of paw (mm)					
	Control group	Model group	PP-500 mg/kg	PP-1000 mg/kg	PP-2000 mg/kg	DS
0	2.04 ± 0.16	1.99 ± 0.06	2.00 ± 0.08	1.97 ± 0.11	2.00 ± 0.05	1.92 ± 0.10
0.5	2.07 ± 0.13	2.67 ± 0.27###	2.66 ± 0.33	2.60 ± 0.22	2.63 ± 0.31	2.55 ± 0.27
1.0	2.13 ± 0.11	2.75 ± 0.30###	2.70 ± 0.45	2.54 ± 0.12	2.58 ± 0.27	2.56 ± 0.22
2.0	2.08 ± 0.13	2.75 ± 0.18###	2.61 ± 0.24	2.72 ± 0.20	2.53 ± 0.17	2.44 ± 0.17**
3.0	2.05 ± 0.11	2.89 ± 0.47##	2.64 ± 0.43	2.59 ± 0.26	2.52 ± 0.12	2.31 ± 0.26**
4.0	2.10 ± 0.09	3.06 ± 0.37###	2.65 ± 0.46*	2.50 ± 0.22***	2.52 ± 0.08***	2.20 ± 0.18***
5.0	2.06 ± 0.07	3.26 ± 0.37###	2.79 ± 0.36*	2.51 ± 0.24***	2.47 ± 0.16***	2.03 ± 0.21***
the paw edema rat	0.98%	63.82%	39.50%	27.41%	23.50%	5.73%

Notes: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ *vs.* model; ##$P < 0.01$ and ###$P < 0.001$ *vs.* control. DS: diclofenac sodium; PP: *P. Puberula* Pectin.

3.6. PP inhibited inflammatory factors in the serum of carrageenan-induced mice

The levels of IL-1β, IL-6 and TNF-α were significantly higher in the model group compared with the control group ($P < 0.001$), while those of the high-dose and medium-dose administration groups were significant decrease compared with the model group ($P < 0.001$ or $P < 0.05$), indicating that PP inhibited inflammatory factors IL-1β (Figure 4A), IL-6 (Figure 4B) and TNF-α (Figure 4C) in carrageenan-induced mice.

3.7. PP showed no toxicity for mice at the dose of 6000 mg/kg

The dose of 6000 mg/kg of PP was non-toxic to mice during the 14-day observation period. There was no animal mortality and the animals did not exhibit any of the typical signs associated with toxicity, such as spasms, convulsions, and diarrhea. This indicated that PP was safety for mice as a functional food.

4. Discussion

PP is a functional food and known as "fairy tofu" in Wudang Mountain area in China. However, up to date, its quality control, preparation process optimizing, pharmacological effects and safety of PP have yet been reported.

For the qualitative and quantitative analysis for the leaves of *P. Puberula*, some components have been detected in the previous studies, including water-soluble sugars, protein, starch, pectin and total flavonoids [20]. However, as a pectin, quality of PP depends on its acidic heteropolysaccharides comprising of D-galacturonic Acids (D-Gal-A) linked with α-1,4-glycosidic bond. The degraded products of the later are soluble sugar. Thus the soluble sugars was selected for quality analysis for PP [21]. On the other hand, regarding a functional food, the active ingredients play a critical role in anti-inflammatory effects of PP. In this study, 19 flavonoids were the major components in PP (Table 4). Since the flavonoid active ingredients of the genus *Premna* Linn. have anti-inflammatory effects [22], the total flavonoids were analyzed as the quality control markers as well (Tables 5 and 6, Figure 2). The results showed that the average contents of the water-soluble sugars and total flavonoids in different batches of PP were 13.366 mg/g and 4.970 mg/g, respectively (Table 5), which may be reference for the quality standard for PP in the future. Notably, the previous study showed that the total flavonoid content in the leaves of *P. Puberula* differed from season to season [23]. In our study, the total flavonoid content of PP varied with different leaf specimen. This may be related to the soil environment of different origins, and especially, the leaves harvested in different seasons. Moreover, the total flavonoid content data for the quality control and the orthogonal test were 2.512–11.196 (Table 5) and 0.503–5.553 (Table 6), respectively. This difference may result from that, the data for OEM were sampled only from the leaf specimen NO. 20200710002 and conducted the different processing, while the data for the quality control were sampled from the all-specimen batches and conducted the optimized process.

As the best preparation processing, OEM is a design method to study multiple factors and levels. It can find the best processing in the least experimental times, so that the preparation via OEM is more effective, time saving and economical [24], while the response surface method (RSM) is a simple and feasible to give a visible result for the best processing. In this study, $L_9(3^4)$ OEM was exploited, and the results revealed that the best preparation process was the powder-liquid ratio of 1:20, temperature of 90 °C and stirring time of 3 min. However, OEM has some limitation that it doesn't cover all conditions and may only present the best condition at certain range, while RSM has some shortages that it needs a large number of tests to screen the best optimized range [25]. It should be better to integrate the two methods to gain the best optimized process in the future studies [26].

For the pharmacological effects of PP, few literatures have been reported though the genus *Premna* Linn. has been studied to have significant anti-inflammatory and immunomodulatory effects [27]. The total flavonoids of *Premna fulva* Craib. (The congeneric plant of *Premna*

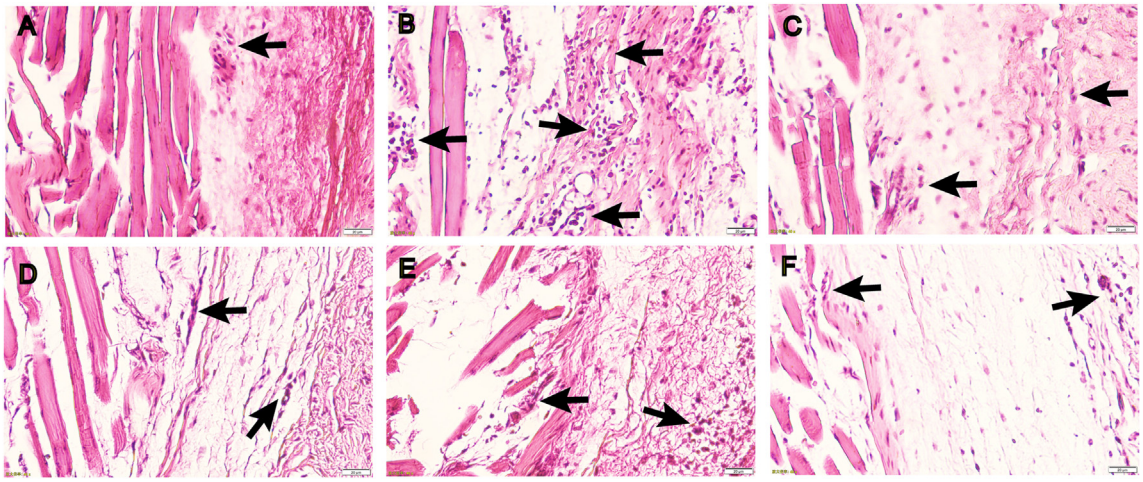

Figure 3. PP alleviated the neutrophil infiltration (black arrow) of paw edema in carrageenan-induced mice using HE staining. A. Control group; B. Model group; C. Low-dose of PP (500 mg/kg); D. Medium-dose group (1000 mg/kg); E. High-dose (2000 mg/kg); F. Positive group (diclofenac sodium).

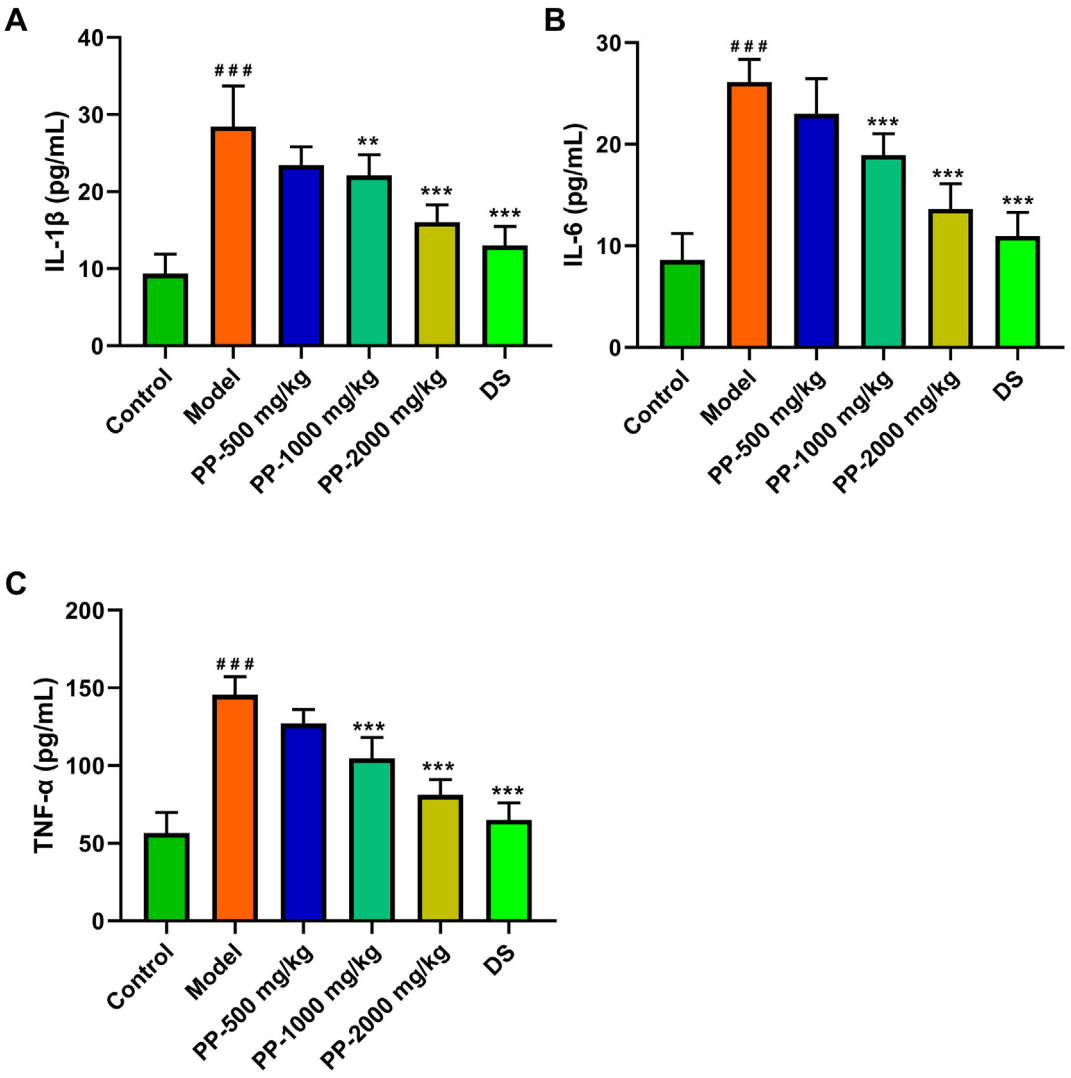

Figure 4. PP inhibited inflammatory factors in the serum of carrageenan-induced mice. A. The serum level of interleukin-1β (IL-1β) in PP-treated mice. B. The serum level of interleukin-6 (IL-6) in PP-treated mice. C. The serum level of tumor necrosis factor (TNF-α) in PP-treated mice. DS: diclofenac sodium. $^{###}$: $P < 0.001$ *vs.* control; ***: $P < 0.001$; **: $P < 0.01$ *vs.* model.

Puberula Pamp.) had significant anti-inflammatory and analgesic effects when administered transdermally in a dose-dependent manner [22]. The petroleum ether extract of the leaves of *Premna integrifolia* Linn (Another congeneric plant of *Premna Puberula* Pamp.) decreased the formation of nitric oxide (NO), reduced pro-inflammatory cytokines (IL-1β, IL-6) and prostaglandin E2 (PGE2), induced anti-inflammatory cytokine (IL-2) and down-regulated COX-2, 5-LOX, TNF-α, IL-1β and iNOS [9]. Furthermore, in an in vivo study, the petroleum ether extracts significantly reduced paw edema in a carrageenan-induced inflammation model in mice [9]. Since flavonoid components have significant anti-inflammatory effects in other plants of the genus *Premna* Linn. according to the literature [22]. On the other hand, nineteen flavonoid components were identified in the alcoholic extracts of the *P. Puberula* leaves in this study. This strongly suggested that the anti-inflammatory effects of PP might be related to total flavonoids and need to be studied in depth. In this study, using the carrageenan gum-induced paw edema model, we found that PP reduced paw edema, The mechanisms may result from PP decreased the levels of IL-1β, IL-6 and TNF-α, and reduced neutrophils in toe tissue, suggesting that PP acts similarly to its congeneric plants and has anti-inflammatory effects, the mechanism of which needs to be studied in depth.

Acute toxicity test is important to examine the safety of food and medicines. The methodology includes median lethal dose (LD_{50}) test [19, 28], limit test [29] and maximum tolerated dose test [30]. As LD_{50} test requires about 30–100 mice, it is always used for high toxic drugs. Whereas limit test and maximum tolerated dose test only requires 10–20 mice with the maximum dose no more than 5 g/kg, and the duration is for 14 days. Thus, limit test and maximum tolerated dose test are often used for low toxic drugs and food. In this study, PP is food and low toxic, so we used the maximum dose at 6 g/kg for each mouse, which is higher than that in maximum tolerated dose test. However, PP did not kill any mice, indicating its safety as a functional food.

5. Conclusion

The main constituents of *Premna puberula* Pamp. are flavonoids. By optimizing the preparation process, stable quality pp with rich nutrition was obtained. PP had anti-inflammatory effects by reducing IL-6, TNF-α and IL-1β. Furthermore, PP was non-toxic to mice at a dose of 6000 mg/kg/24 h. Thus, PP is safe and non-toxic, and has anti-inflammatory effects, which is worthy of further study.

Declarations

Author contribution statement

Kaiqi Liu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yingying Guo, Xiaojing Chen, Jufeng Duan: Performed the experiments; Wrote the paper.

Lin Chen, Bei Li: Analyzed and interpreted the data; Wrote the paper.

Ming Liu: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Minglun Li, Yibin Feng: Conceived and designed the experiments; Wrote the paper.

Hongliang Li: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Xuanbin Wang: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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