Comparison with Various Parts of *Broussonetia papyrifera* as to the Antinociceptive and Anti-Inflammatory Activities in Rodents

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This study compared the antinociceptive and anti-inflammatory effects of various parts of *Broussonetia papyrifera* (L.) L’Herit. ex Vent. (BP, Moraceae) by chemical-induced pain and inflammation in rodents. All BP parts (1 and 2 g/kg, p.o.) effectively inhibited writhing responses induced by 1% acetic acid. The BP radix, leaf, and fruit effectively inhibited the late-phase licking responses caused by 1% formalin. But only the BP radix and fruit reduced the edema induced by 1% carrageenan at 1–2 h. Furthermore, the BP radix reduced the abdominal Evan’s blue extravasations caused by inflammatory mediators, including serotonin and sodium nitroprusside. Finally, the radix had the highest contents of betulin and betulinic acid among all BP parts. In conclusion, the radix is the better medicinal BP part possessing antinociceptive and anti-inflammatory effects, and its anti-inflammatory effects are partially related to the inhibition of vascular permeability via autocrines and nitric oxide.

**Key words:** *Broussonetia papyrifera*; antinociceptive activity; anti-inflammatory activity; vascular permeability; triterpenoids

The aerial and radix parts of *Broussonetia papyrifera* (L.) L’Herit. ex Vent. (BP, Moraceae) are used in certain therapeutic treatments including pain, edema, and hemorrhage by traditional Chinese physicians. However, the antinociceptive and anti-inflammatory effects of all the BP parts have not been studied. Hence the major aim of the present study was to determine which BP part is apposite by comparing the antinociceptive and anti-inflammatory activities and triterpenoid contents of various BP parts, including radix, fruits, leaves, and stems. First we investigated the antinociceptive and anti-inflammatory activities of various BP parts by an acetic acid-induced writhing test,1) a formalin-induced licking test,2) and a carrageenan-induced paw edema test.3) Then we determined the triterpenoids contents of various BP parts, including betulin, betulinic acid, oleanolic acid, and ursoolic acid, by high performance liquid chromatography and with a photodiode array detector (HPLC-DAD). Finally, we also attempted to clarify the anti-inflammatory mechanism of various BP parts by a skin window test, which measured the vascular permeability by Evan’s blue extravasation caused by certain inflammatory mediators, such as serotonin, histamine, bradykinin, platelet activating factor (PAF), and sodium nitroprusside (SNP).4)

**Materials and Methods**

*Animals.* Male Sprague Dawley rats weighing 200–250 g were used in the study of anti-inflammatory activities and vascular permeability. Male ICR mice weighing 20–25 g were used in testing antinociceptive effects. All animals were handled according to the “Guiding Principles for the Care and Use of Laboratory Animals” of China Medical University. They were housed for at least 1 week before starting the experiment with free access to standard food pellets (designed and supplied by Fwusow Industries, Taichung, Taiwan) and tap water, and kept in a regulated environment (23 ± 1 °C temperature and 60% humidity), under a

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**Abbreviations:** BP, *Broussonetia papyrifera*; PAF, platelet activating factor; SNP, sodium nitroprusside
All the rodents were randomly assigned into several experimental groups, including vehicle-treated group and four test sample-treated groups. Then antinociceptive and anti-inflammatory assays were done using the double-blind method. After behavioral measurement, all animals were killed with carbon dioxide.

Preparation of plant extracts and drug. Plant samples were collected from the Da Ken in Taichung, Taiwan, and deposited in the herbarium of the Graduate Institute of Chinese Pharmaceutical Sciences of China Medical University. Fresh BP radixes, stems, leaves, and fruits were extracted 5 times with 95% ethanol. The resulting extract was combined and concentrated under reduced pressure to obtain ethanolic extracts of BP radix, stem, leaf, and fruit. The yields of BP radix, stem, leaf, and fruit are represented in Table 1. The BP radix, stem, leaf, and fruit (0.6, 1, 2 g/kg) were dissolved in 0.5% carboxymethylcellulose, and were administered orally 60 min prior to injection of the inducer. Acetylsalicylic acid (ASA, 300 mg/kg) and indomethacin (indo, 10 mg/kg) were also prepared as suspension with 0.5% carboxymethylcellulose, and were administered orally 30 min prior to injection of the inducer. Serotonin, histamine, bradykinin, PAF, and SNP (Sigma-Aldrich, St. Louis, MO) were dissolved in normal saline.

Acetic acid-induced abdominal writhing response. Each mouse was given 1% acetic acid (10 ml/kg body weight) intraperitoneally. The mice were placed in individual observation boxes. The symptoms of acetic acid-induced abdominal writhing were similar to those described by Taber et al.\textsuperscript{13} Control animals received vehicle solution in the same experiments. Five min after injection of acetic acid, the number of writhing responses per mouse was counted for 10 min during acetic acid-induced abdominal writhing. Finally, the number of writhing responses permitted us to express the percentage of protection using the following ratio: (control mean – treated mean)/control mean × 100.

Formalin-induced licking response. This method is modified from Shibata et al.\textsuperscript{21} Each mouse was placed in the observation chamber on a transparent acrylic plate floor for 5 min prior to formalin injection. Beneath the floor, a large mirror was inclined at a 45° angle in order to allow clear observation of the animal’s paws. The animals were administered 25 μl of 1% formalin into the right subplantar. Then each animal was returned to the chamber where two distinct periods of the intensive licking response was observed. The first period (early phase) was recorded 0–5 min after injection of formalin, and the second period (late phase) was recorded 10–35 min after injection. The time spent in licking response of the injected paw was measured as the indicator of pain response.

Anti-inflammatory study in rats. Anti-inflammatory activity was determined in the rats by measuring the mean increase in hind paw volume after subplantar injection of inflammatory agents such as carrageenan.\textsuperscript{3} The animals were injected with 0.1 ml of 1% carrageenan in the right hind foot under plantar aponeurosis. The inflammation was quantitated in terms of ml using a plethysmometer (7150 Ugo Basile, Comerio, VA), which recorded small differences in water level caused by volume displacement. Before treatment, the average volume of the back paws of each animal was determined (Vo) by three measurements that did not differ by more than 4% (preciseness of the apparatus). Then 1, 2, 3, and 4 h after injection of the inflammatory agents, the average volume of the back paws of each animal was determined (Vt) by three measurements that did not differ by more than 4%. The percentage of edema at each record was calculated by comparing the average volume of the back paws of each animal (Vt) after injection of the inflammatory agents with the average volume of the back paws of each animal (Vo) before treatment. Percentages of inhibition were obtained for each group using the following ratio: [(Vt/Vo)control − (Vt/Vo)treatment]/(Vt/Vo)control × 100.

Microvascular permeability test in rats. Microvascular permeability was determined in the rats by measuring the absorbance change in the abdominal Evans’ blue extravasations after intradermal injection of inflammatory mediators such as serotonin, histamine, bradykinin, PAF, and SNP. Sixty min after sample treatment, the rats were anesthetized with 30 mg/kg of pentobarbital, and then their abdominal hairs were shaved, and six 2-cm diameter circles were marked in the abdominal skin. After intravenous injection of 25 mg/kg of Evans’ blue, the animals were injected with 50 μl saline, serotonin (1 nM), histamine (20 μM), bradykinin (20 nM), PAF (40 nM), and SNP (100 nM) into the central area of the six circles in the abdominal skin. After 1 h all the rats were sacrificed, and the stained skin of the injected site was excised. These stained skins were infiltrated with 750 μl of sodium sulfate and 1.75 ml of acetone overnight to extract the abdominal Evans’ blue extravasations. The infiltrated solutions were centrifuged at 2,000 × g for 20 min, the supernatant was collected, and the absorbance was measured at 620 nm.\textsuperscript{6,7} The

### Table 1. The Yield and Triterpenoid Contents of Various BP Parts Extracted with 95% Ethanol

<table>
<thead>
<tr>
<th>Groups</th>
<th>Yield (g/Kg)</th>
<th>Betulin (μg/g)</th>
<th>Betulinic acid (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP radix</td>
<td>43.24</td>
<td>631.91 ± 19.87</td>
<td>1476.58 ± 45.77</td>
</tr>
<tr>
<td>BP stem</td>
<td>30.94</td>
<td>159.73 ± 9.41</td>
<td>3.44 ± 2.52</td>
</tr>
<tr>
<td>BP leaf</td>
<td>102.13</td>
<td>—</td>
<td>570.97 ± 6.30</td>
</tr>
<tr>
<td>BP fruit</td>
<td>68.55</td>
<td>—</td>
<td>444.90 ± 7.10</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SEM. N = 3.
alteration of vascular permeability was obtained for each group using the following ratio: 
\[ \frac{A_{\text{induced}} - A_{\text{saline}}}{A_{\text{saline}}} \times 100 \]
where \( A_{\text{induced}} \) is the absorbance of Evan’s blue extravasation in circle treated with inflammatory factor, and \( A_{\text{saline}} \) is the absorbance of Evan’s blue extravasation in circle treated with saline.

**Determination of triterpenoids by HPLC-DAD.** All ethanolic extracts of BP parts were separately dissolved in methanol and then filtered with a 0.22-μm filter. The HPLC system used in the measurement consisted of a Shimadzu VP series LC-10ATvp pump, a SCL-10AVP system controller, a SPD-M10AVP photodiode array detector (DAD), and an SIL-10AF autosampler. Data were monitored by the Shimadzu Class-VP™ chromatography data system. A Supelco Discovery® C18 (150 × 4.6 mm, 5 μm) column (Sigma-Aldrich, St. Louis, MO) was used. Standard solutions of ursolic acid,oleanolic acid, betulinic acid, and betulin (Extran/Chimie, Genay Cedex, France) at a concentration of 1 mg/ml were prepared in methanol and stored in a refrigerator. Working solutions at 50–250 μg/ml were prepared freshly every day by dilution of standard solution with methanol. The mobile phase for triterpenoids was a mixture of methanol and water (80:20, v/v) and contained 0.05% phosphoric acid. The flow rate was 1 ml/min, and the detection wavelength was 210 nm. All chromatographic operations were carried out at ambient temperature. The chromatographic peaks of the four triterpenoids were confirmed by comparing their retention times and UV spectra.

**Statistical analysis.** All data obtained during the antinociceptive, anti-inflammatory, and microvascular permeability tests were expressed as mean ± standard errors (SEM), and analyzed by ANOVA one-way analysis of variance, followed by Dunnett’s test. When the probability (P) was less than 0.05, the difference was considered to be significant.

**Results and Discussion**

**Antinociceptive activity of all BP parts in mice**

To compare the antinociceptive activities of all BP parts, we elicited the acetic acid-induced abdominal writhing response and the formalin-induced licking response in mice. The formalin-induced licking response is a very useful model for studying pain and assessing antinociceptive drugs. There are obvious differential properties in the early and late phases of formalin-induced licking responses in that the early phase is caused by central nerve fiber activation while the late phase is dependent on functional changes in the peripheral nerves.\(^{21}\) Administering all BP parts at 1 and 2 g/kg orally inhibited the acetic acid-induced writhing response in a dose-dependent manner (Table 2). Three BP parts, the radix, leaf, and fruit, administered orally at 2 g/kg shortened the late-phase but not early-phase licking time induced by formalin (Fig. 1). There were similar inhibition percentages caused by all BP parts at 2 g/kg as form the acetic acid-induced writhing response and the late phase of the formalin-induced licking response. The results for the all positive acetylsalicylic acid control (300 mg/kg) and for indomethacin (10 mg/kg) were similar to our earlier report.\(^{5}\) These also decreased the acetic acid-induced writhing response and inhibited the late phase of the formalin-induced licking response.

We found that BP possesses antinociceptive effects, and the effects of BP radix and fruit were slightly better than those of BP stem and leaf in the two pain models used. The antinociceptive activities of BP radix and fruit were mainly via peripheral nervous systems, as demonstrated from their inhibition effects on the acetic acid-induced writhing response and the late phase of the formalin-induced licking response in mice. Base on the mechanism of the formalin-induced licking response described by Shibata \textit{et al.}, the early phase of the formalin-induced licking response is related to bradykinin and substance P, and the late phase is related to bradykinin, autocinines, and prostaglandin.\(^{21}\) On the other hand, we found that there was no difference in the \( IC_{50} \) of the four BP parts as to the inhibition of cyclooxygenase-2 activity (data not shown). Hence the antinociceptive effects of BP radix and fruit might be mainly related to bradykinin and autocinines.

**Anti-inflammatory activity of all BP parts in rats**

Due to the better inhibitory activities of all BP parts on inflammatory algesia (late phase) caused by the formalin-induced licking response, we assessed the anti-inflammatory activity of all BP parts against carrageenan-induced edema formation in the rats. Subplantar injection of carrageenan induced paw edema, which reached maximal edema about 3h after carra-
geenan administration. Only BP radix and fruit at 2 g/kg decreased carrageenan-induced edema from 1 to 2 h after carrageenan administration. The BP stem and leaf did not. The results for the positive control indomethacin (10 mg/kg), were similar to our earlier study,5) which also effectively inhibited carrageenan-induced edema formation throughout the measurement intervals in the rats (Fig. 2). Consistently with the antinociceptive results, BP radix and fruit possessed better anti-inflammatory effects than the other BP parts. Moreover, the mechanism of carrageenan-induced edema usually separates into three phases. The first phase (1.5 h after carrageenan treatment) is related to autocrines and PAF. The second phase (from 1.5 h to 2.5 h after carrageenan treatment) is related to kinins. The third phase (2.5 h after carrageenan treatment) is related to prostaglandins and leukotriens.8–11) Based on the our above results, the antinociceptive and anti-inflammatory mechanisms of BP might be related mainly to kinins and autocrines, because BP radix and fruit mainly inhibited the late-phase of the formalin-induced licking response and the first two phase of carrageenan-induced paw edema.

**Effects of BP radix and fruit on the microvascular permeability in rats**

It is evidence that inflammation cascade and edema formation caused by lipopolysaccharide or lipoteicholic acid were mediated many inflammatory factors including autocrines, kinins, prostaglandins, which lead to a dilation of arterioles and venules and to an increased vascular permeability.6,7) Hence, to further clarify the anti-inflammatory mechanism of BP radix and fruit at the dosage used in the carrageenan-induced edema experiment, we performed plasma leakage induced by autocrines, bradykinin, PAF, and SNP by Evan’s blue extravasation in rats. The abdominal Evan’s blue extravasation in the intradermally marked circle with saline was represented as 100%. The increased percentages of abdominal Evan’s blue extravasation in the intradermally marked circle with inflammatory mediators is shown in Fig. 3. BP radix at the dosage used in the carrageenan-induced edema experiment decreased the percentage of abdominal Evan’s blue extravasation caused by serotonin and SNP. However, BP fruit at the dosage used in the carrageenan-induced edema experiment only decreased the percentage of abdominal Evan’s blue extravasation caused by PAF. Therefore, the antinociceptive and anti-inflammatory effects of BP radix on the formalin-induced licking response and carrageenan-induced paw edema might be related to modulation of the inflammatory mediators, including serotonin and nitric oxide, but the antinociceptive and anti-inflammatory effects of BP fruit might be related to modulation of PAF.
According to pharmacological and phytochemical reports on Moraceae plants, they possess antinociceptive and anti-inflammatory potency and enrich certain triterpenoid constituents such as betulinic acid, ursolic acid, and oleanolic acid.\textsuperscript{12–16} The above triterpenoids possess antinociceptive and anti-inflammatory activity in rodents.\textsuperscript{17–19} Hence, we further measured the triter-

**Fig. 2.** Effects of Various BP Parts (1 and 2 g/kg) and Indomethacin (indo, 10 mg/kg) on the Carrageenan-Induced Paw Edema in Rats. Each value is represented as mean ± SEM (N = 7–10). *P < 0.05, **P < 0.01, ***P < 0.001 as compared with the VEH group.

**Fig. 3.** Effects of BP Radix and Fruit (2 g/kg) on Vascular Permeability Increased by Serotonin, Histamine, BK, PAF, and SNP in Rats. Each value is represented as mean ± SEM (N = 6). *P < 0.05 as compared with the VEH group.
penoid contents of all BP parts by HPLC-DAD, although no report indicated that BP contained these triterpenoids. The HPLC chromatography is shown in Fig. 4. The calibration curves for ursolic acid, oleanolic acid, betulinic acid, and betulin were drawn in a concentration range of 50–250 μg/ml. The correlation coefficients of the calibration plots were equal to 0.994–0.999, indicating good linearity in the four cases. The R.S.D. values

Fig. 4. HPLC Chromatographic Profiles at 210 nm of (A) Standard, (B) BP Radix, and (C) BP Fruit.
for intra-day variability at three times on the same day at 150 μg/ml were 0.90% for ursolic acid, 0.96% for oleanolic acid, 1.11% for betulinic acid, and 1.09% for betulin. The R.S.D. values for inter-day variability at three times on three days at 150 μg/ml were 1.24% for ursolic acid, 1.51% for oleanolic acid, 2.24% for betulinic acid, and 0.87% for betulin.

The results indicate that betulinic acid existed in all the BP parts, but that only the BP radix and stem contained betulin. We found that BP radix contained the highest contents of betulinic acid and betulin, but there was a lack of oleanolic acid and ursolic acid in all the BP parts (Table 1). Based on our above results and the inhibiting potency of betulinic acid on phospholipase A<sub>2</sub> and nitric oxide<sup>18,20</sup>, we suggest that betulinic acid and betulin are two active constituents of BP radix, because the anti-inflammatory potency is consistent with the phytochemical content. However, the contents of betulin and betulinic acid at the dosage used of BP radix in carrageenan-induced edema were lower than the dosage used in other studies<sup>21</sup>. Moreover, other researchers have indicated that certain flavonoids such as kzinol B, broussochalcone A, and papyriflavanol A isolated from BP possess inhibiting activity on lipoxygenase, but not on cyclooxygenase or inducible nitric oxide synthase<sup>22,23</sup>. The present study also found there was no difference in the IC<sub>50</sub> of the four BP parts on the inhibition of cyclooxygenase-2 activity (data not shown). Therefore, flavonoids are other active constituents of BP require more measurement by HPLC-DAD and animal study in the future.

In conclusion, BP has antinociceptive and anti-inflammatory activities in rodents, and the radix and fruit possess better antinociceptive and anti-inflammatory potency than the other parts. Betulinic acid and betulin might be two of active constituents in BP radix as to antinociceptive and anti-inflammatory activity. Hence its anti-inflammatory mechanism might be related to modulation of the inflammatory mediators including serotonin and nitric oxide, partially via its one active ingredient, betulinic acid, which inhibited paw edema caused by carrageenan and serotonin.<sup>18</sup>

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References


