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Active components, antimicrobial and antioxidant activities of extracts from *Melicope patulinervia*

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**Melicope patulinervia** (Merr. and Chun) Huang is the rare and endangered plant in the Hainan Province, China. In the purpose of exploring the value of its exploitation and utilization, this study was conceived to evaluate the antimicrobial, antioxidant activities and the active ingredients of this plant. Spectrophotometry was the basis for the determinations of total phenol and total flavonoids. Meanwhile, the antioxidant activities of the different parts, different solvents extracts of *M. patulinervia* were measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) methods. Seven plant pathogenic fungi species were used to assay for antimicrobial activity of the extracts of this plant. The results showed that 70% methanol extracts of the leaves had higher level of total flavonoids, with a content of 41.3194±0.071 mg·g⁻¹. But it was strange that the contents of total phenol of the extracts of the plant were significantly less than that of total flavonoids. The antioxidant activities of the different parts, different solvents extracts of *M. patulinervia* were all weak. The leaf extract showed antimicrobial activity against 4 of the plant of pathogenic fungi species, especially Oxytetracycline hydrochloride.

**Key words:** *Melicope patulinervia* (Merr. and Chun) Huang, active ingredients, antimicrobial activity, antioxidant activity.

**INTRODUCTION**

It is well known that free radicals formed in living cells play an important role in the origin of life and biological evolution (McCord, 2000; Lander, 1997; Zheng et al., 2000). However, it has been found that those reactive species also play a cardinal role in oxidative damage to cellular constituents which leads to cell injury and death. This has been associated with pathogenesis of various chronic diseases, for example, carcinomas, coronary heart disease and many other health problems related to advancing age. Natural antioxidants, especially phenolic and flavonoids, have biological activity as well as safety, therefore, most studies are on antioxidants of plant origin. *Melicope patulinervia* (Merr. and Chun) Huang belongs to *Melicope* genus, Rutaceae. The genus includes 50 species, among which 2 species grow in China. One is grown in Taiwan, called *M. triohylla* (Lam.) Merr. The other in Hainan, which is just the plant we study in this paper (Editorial board, 2004). *M. patulinervia* is a shrub or tree with papery leaf blade, attractive bluish white flowers and ellipsoid blue-black seeds. It is the rare and endangered plant.

The literature survey indicates that there is no paper study on active components, antimicrobial and antioxidant activities of extracts of *M. patulinervia*. The present study was aimed to examine the contents of total phenolic and flavonoids, and phytochemical analysis of the different parts, different solvents extracts of *M. patulinervia* were screened for antimicrobial, antioxidant activities using standard methods. The purpose of this paper was to supply reference for the exploitation and
utilization of *M. patulinervia*.

**MATERIALS AND METHODS**

**Plant materials**

*M. patulinervia* (Merr. and Chun) Huang was collected in Sanya, Hainan Province, China, which was identified by Huang Shi Man, Professor of phytotaxonomy at Hainan University. The type specimens were kept in our laboratory. The plants were air dried at room temperature to constant weight. The dried plant materials were grinded and sieved with 40-mesh sieve, then stored in the fridge for use.

**Instruments and reagents**

Infinite M 200 Universal Microplate Spectrophotometer (Swiss Tican company, Swiss) was used to measure the absorbance (reducing power and 1,1-diphenyl-2-picryl-hydrayl (DPPH) assays) and UV-2102 PCS uv-vis spectrophotometer (Shanghai Unica Company Ltd, China) was used to measure the absorbance of NaNO$_2$-Al(NO$_3$)$_3$ chromogenic and Folin-Ciocalteu methods.

2,4,6-Tris-(2-pyridyl)-s-triazine (TPTZ), DPPH, 6-Hydroxy-2,5,7,8-tetra methychroman-2-carboxylic (Trolox) are all purchased from Sigma (USA). Standards of quercetin and gallic acid were purchased from China Pharmaceutical and Biological Products Testing Station (The batch numbers were 10080-200306 and 110831-200302, respectively). Nutrient agar (Hangzhou Microbial Reagent Co.Ltd, China), glucose and all the other chemicals used including the solvents were of analytical grade.

**Microbial cultures**

Seven strains of plant pathogenic fungus were used as test microorganisms. The plant pathogenic fungus included *Penicillium* sp, *Oxytetracycline* hydrochloride, *Fusarium graminearum*, *Botrytis cinerea*, Northern Leaf Blight of Corn, *Lecannosticta acicola*, *Rhizoctonia solani*. All microorganisms were obtained from the Research Institute of Forest Protection Zhejiang A and F University.

**Preparation of the extracts**

Dried and pulverized sample powders (1 g) were extracted for 3 times (20 min each time) with 15 ml of Petroleum ether, EtOAc, CHCl$_3$, EtOH, MeOH, respectively, using ultrasonic wave at room temperature. Extracted 1 g of dried and pulverized stems, leaves and the whole parts of *M. patulinervia* with 75% methanol by ultrasound 3 times (20 min each time), respectively.

All the extracts were made into dry powder through filtering and pressure reducing concentration, then were determined capacity by 75% methanol solution to 50 ml (with 50 ml flask volumetric), respectively. The final dried samples were stored in labeled sterile bottles and kept at 4°C.

**Determination of total flavonoids**

The total flavonoid content of *M. patulinervia* extract was determined using a spectrometric method with minor modifications (Yang et al., 2003). Draw standard curve for determination of the content of quercetin and extracts separately, finding that there was a strong absorption at 510 nm. Consequently, 510 nm was chosen as detection wavelength. The extract samples (1 ml) was added sequentially with 0.5 ml of 5% NaNO$_2$ solution, then was shaken. After 5 min, 0.5 ml of 10% Al(NO$_3$)$_3$ solution were added to the mixture solution. After mixing and standing for 5 min, 4 ml of 4% NaOH were added and put some more 75% methanol to make exact 10 ml (with 10 ml flask volumetric). After reacting for 15 min, the absorbance of the mixture was read at 510 nm using a spectrophotometer. A standard curve was prepared at 0, 55, 110, 165, 220 and 275 µg ml$^{-1}$ of quercetin dissolved in 75% methanol. The amount of total flavonoids was expressed as quercetin equivalent (µg QE·ml$^{-1}$). The absorbance was used as the Y-axis and the concentration of quercetin as the X-axis by drawing standard curve. The regression equation was $y = 12.93x + 0.01$, $R^2 = 0.9988$.

**Determination of total phenols**

The total phenol content of plant extracts was determined using Folin-Ciocaltel assay method with minor modification (Wolfe et al., 2003). Draw standard curve for determination of the content of gallic acid and extracts separately, finding that there was a strong absorption at 700 nm. Consequently, 700 nm was chosen as detection wavelength. The extracted sample (1 ml) was added distilled water to 5 ml, then was mixed with 0.5 ml of Folin-Ciocaltel reagent and incubated at 25°C for 5 min. The mixture reacted with 1 ml of 10% Na$_2$CO$_3$ solution and left for 1 h at 25°C. A standard curve was prepared at 0, 0.48, 0.96, 1.44, 1.96 and 2.4 µg ml$^{-1}$ of quercetin dissolved in 75% methanol. The amount of total phenols was expressed as gallic acid equivalent (µg GAE·ml$^{-1}$). The absorbance was used as the Y-axis and the concentration of as gallic acid as the X-axis by drawing standard curve. The regression equation was $y = 466.3x - 0.006$, $R^2 = 0.9997$.

**1,1-Diphenyl-2-picryl-hydrayl (DPPH) radical scavenging activity** (Wettasinghe et al., 2000; Xiong et al., 2006)

The DPPH radical scavenging ability in the different extracts of *M. patulinervia* was evaluated by the spectrophotometric method. Each sample was diluted with 75% methanol to obtain 6 different concentrations (1, 2, 4, 8, 12 and 16 mg·ml$^{-1}$) and each diluted sample (20 µl) was mixed with 200 µl of DPPH solution. After 30 min of reaction, the increase in absorbance at 530 nm was recorded (the reaction took place in enzyme label plate). The scavenging activity was calculated by the following formula:

$$\text{DPPH scavenging activity (%) = } [1-(AP-AC)/A_{max}] \times 100$$

Where, AP equals the absorbance of the plant extract with DPPH; AC equals the absorbance of the plant extract with 75% methanol and Amax equals that of 75% methanol with DPPH. The antioxidant activity was expressed as IC$_{50}$ which was defined as the final concentration of the tested sample required for the inhibition of the formation of DPPH radical by 50%. A standard curve was prepared at 0, 7.35, 14.7, 22.05, 29.4, 44.1 and 58.8 µg·ml$^{-1}$ of Trolox dissolved in 75% methanol. The absorbance was used as the Y-axis and the concentration of Trolox as the X-axis by drawing standard curve. The regression equation was $y = 7.261x + 0.535$, $R^2 = 0.9979$.

**Total antioxidant activity (ferric reducing antioxidant property (FRAP) assay)**

A modified method of Benzie and Strain (Benzie et al., 1996) was
adopted for the FRAP assay. The stock solutions included 300 ml acetate buffer (3.1 g C₆H₅NaO₂·3H₂O and 16 ml C₆H₂O₇·3H₂O), pH 3.6, 0.01 M TPTZ solution in 0.04 M HCl, and 0.02 M FeCl₃·6H₂O solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ and 2.5 ml FeCl₃·6H₂O. The temperature of the solution was raised to 37°C before using. Plant extracts (20 µl) were allowed to react with 200 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 595 nm. A standard curve was prepared at 0, 7.35, 14.7, 22.05, 29.4, 44.1 and 58.8 µg/ml of Trolox dissolved in 75% methanol. The absorbability was used as the Y-axis and the concentration of Trolox as the X-axis by drawing standard curve. The regression equation was $y = 7.768x + 0.340$, $R^2 = 0.9987$.

The antioxidant activity was expressed as Trolox equivalent antioxidant capacities (TEAC) which was defined as µg ml⁻¹.

### Preliminary research on antifungal activity

Antifungal activity (Liu et al., 2008): Potato dextrose agar (PDA) medium was used to antifungal experiments, which was prepared by dissolving 200 g potato, 20 g glucose and 20 g gelatin in 1000 ml distilled water (pH 7.0 to 7.2). All plant pathogenic fungi species were cultured in PDA nutrient agar medium at 28°C for 2 to 4 days. The antibacterial activity was tested by the confrontation method and according to the diameter of antibacterial ring to evaluate the antimicrobial capacity of the extracts. Put the Oxford cup onto the center of the plate which had 15 ml PDA in it. Put 4 pieces of the plant pathogenic fungi species (diameter 6 mm) onto 4 different positions of the plate, which were at circumference with Oxford cup. The Fungi were incubated at 28°C for 2 to 4 days. After incubation period, the inhibition zone obtained around the Oxford cup was measured. A negative control, involving the presence of microorganisms without test material (blank) was used. The experiments were performed in triplicate.

### RESULTS AND DISCUSSION

#### Total phenolic, flavonoids contents

Results obtained in the present study revealed that the amount of phenol and flavonoids in the methanol extracts of the leaves of *M. patulinervia* was the largest by ultrasonic extraction. The leaves obviously had higher levels of total phenol and flavonoids than the stems and all plants. The extracts of leaves were rich in flavonoids, but not phenol. Remarkably, the contents of total phenol of the plants were significantly less than that of total flavonoids (Tables 1 and 2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Content of TP (mg·g⁻¹)</th>
<th>Content of TF (mg·g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>0.1888±0.031</td>
<td>1.1462±0.054</td>
</tr>
<tr>
<td>EtOAc</td>
<td>0.2041±0.027</td>
<td>2.1852±0.037</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>0.2298±0.015</td>
<td>2.2409±0.034</td>
</tr>
<tr>
<td>EtOH</td>
<td>0.2815±0.036</td>
<td>2.0128±0.031</td>
</tr>
<tr>
<td>MeOH</td>
<td>0.3721±0.035</td>
<td>5.5437±0.082</td>
</tr>
</tbody>
</table>

Values are expressed as means ± standard deviation (n = 3).

#### 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity

Figures 1 and 2 showed the dose-response curve of DPPH radical scavenging activity of the extracts of different solvents and parts. The DPPH radical scavenging activity of different solvents that showed in

#### Total ferric reducing antioxidant power (FRAP)

The Figures 5, 6, 7 and 8, and Table 4 showed the reducing power of total antioxidant power of the extracts of different solvents and parts. The larger amount of TEAC, the higher total antioxidant power it had. The reducing power of different solvents showed in Figures 5 and 7 was MeOH>EtOH>CHCl₃>EtOAc>Petroleum ether. From Table 3, Figures 2 and 4, it was observed that the extract of the leaves had higher activity than that of the stem and all plants. The $IC_{50}$ of the extract of leaves was 1.633±0.035 mg·ml⁻¹, while that of the stem was 18.560±0.023 mg·ml⁻¹.

#### Antimicrobial activity of the extracts of different part

Table 5 showed the antimicrobial activity of the extracts of different parts. It was observed that among the 7 plant
Table 3. The regression equation of the scavenging effects on DPPH free radicals in all different extracts.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Regression equation</th>
<th>$R^2$</th>
<th>$IC_{50}$ (mg · ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>$y = 0.007x + 0.232$</td>
<td>0.927</td>
<td>38.29±0.044</td>
</tr>
<tr>
<td>EtOAC</td>
<td>$y = 0.01x + 0.275$</td>
<td>0.984</td>
<td>22.50±0.009</td>
</tr>
<tr>
<td>CHCl$_3$</td>
<td>$y = 0.014x + 0.266$</td>
<td>0.995</td>
<td>16.71±0.010</td>
</tr>
<tr>
<td>EtOH</td>
<td>$y = 0.012x + 0.307$</td>
<td>0.994</td>
<td>16.08±0.020</td>
</tr>
<tr>
<td>MeOH</td>
<td>$y = 0.015x + 0.342$</td>
<td>0.958</td>
<td>10.53±0.025</td>
</tr>
<tr>
<td>All plant</td>
<td>$y = 0.022x + 0.408$</td>
<td>0.958</td>
<td>4.182±0.031</td>
</tr>
<tr>
<td>Leave</td>
<td>$y = 0.030x + 0.451$</td>
<td>0.966</td>
<td>1.633±0.035</td>
</tr>
<tr>
<td>Stem</td>
<td>$y = 0.009x + 0.333$</td>
<td>0.884</td>
<td>18.56±0.023</td>
</tr>
</tbody>
</table>

Values are expressed as means ± standard deviation (n = 3).

Table 4. TEAC values of different extracts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>The whole plant</th>
<th>Stem</th>
<th>Leaf</th>
<th>Petroleum ether</th>
<th>Methanol</th>
<th>Ethanol</th>
<th>Etyl acetate</th>
<th>Dichloromethane</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEAC (µg·g$^{-1}$)</td>
<td>110.64±0.028</td>
<td>81.68±0.032</td>
<td>188.19±0.022</td>
<td>16.41±0.070</td>
<td>61.34±0.009</td>
<td>41.11±0.031</td>
<td>32.67±0.023</td>
<td>35.12±0.019</td>
</tr>
</tbody>
</table>

Values are expressed as means ± standard deviation (n = 3).

Table 5. Antimicrobial activity of the extracts of different part.

<table>
<thead>
<tr>
<th>Tested pathogen</th>
<th>Diameter of inhibitory zone/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf</td>
</tr>
<tr>
<td>Oxytetracycline hydrochloride</td>
<td>30.19 (+++)</td>
</tr>
<tr>
<td>F. graminearum</td>
<td>20.36 (+++)</td>
</tr>
<tr>
<td>B. cinere</td>
<td>25.01 (+++)</td>
</tr>
<tr>
<td>Northern Leaf Blight of Corn</td>
<td>18.01 (+++)</td>
</tr>
<tr>
<td>L. acicola</td>
<td>-</td>
</tr>
<tr>
<td>Penicillium sp.</td>
<td>-</td>
</tr>
<tr>
<td>R. solani</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: All values are means of three replicates. "+++" means diaphanous, "++" means clear, "+" means eyeable, "-" means no.

Figure 1. The scavenging rate of the extracts of different solvent on DPPH$^•$ with different concentrations.

Figure 2. The scavenging rate of the extracts of different parts on DPPH$^•$ with different concentrations.
pathogenic fungi species, the extracts of different parts almost had none antifungal activity against L. acicola, Penicillium sp and R. solani. On the other hand, the antifungal activity of the plant against Oxytetracycline hydrochloride, F. graminearum, B. cinerea, Northern Leaf Blight of Corn was very significant, especially Oxytetracycline hydrochloride. The extract of the leaves had higher activity than that of the stems and the whole plants. At a concentration of 20 mg·ml⁻¹, the activity of the diameters of inhibitory zone of the plant against Oxytetracycline hydrochloride of the leaves was 30.19 mm, while at the same concentration, that of the stem was 19.23 mm.

A great deal of research has been conducted to plant resources of Hainan Island in my lab. All of these plants have strong antioxidant, antimicrobial activities. M. patulinervia is considered as ornamental plant, not as medicine, in China. But compounds isolated from this plant have many kinds of important pharmacology activities. Some plants of Melicope genus are used as folk medicine in other countries. For example, Melicope

Figure 3. The IC₅₀ values of the different solvent extracts on DPPH⁻.

Figure 4. The IC₅₀ values of the different parts extracts on DPPH⁻.

Figure 5. The reducing power of extracts of the different solvents with different concentrations.

Figure 6. The reducing power of the extracts with different concentrations.
borbonica has been traditionally used for wound healing, blood cleansing and as a sudorific in the Réunion Island. Currently, it is used for its aromatic properties and against rheumatism (Ting et al., 1989; Su et al., 1998; Simoes et al., 1990; Chen et al., 2008). Therefore, it was valuable to explore the exploitation and utilization of *M. patulinervia*.

In this paper, the antimicrobial, antioxidant activities and total phenolic, flavonoids contents of the extracts of *M. patulinervia* (Merr. and Chun) Huang were reported for the first time. The experimental results demonstrated that the amount of flavonoids in the methanol extracts of the leaves of *M. patulinervia* was larger, which was up to 41.3194±0.071 mg g⁻¹. The level of flavonoids in the methanol extracts of leaves was considerable. On the other hand, that of stems was very low. It was strange that the contents of total phenol of the plants were significantly less than that of total flavonoids. Retrieving literatures, it was found that the substituting group of flavonoids contained in this plant was almost methoxy group, not hydroxyl group (Tsann et al., 1998; Williams et al., 2004; Chung et al., 2008; Richard et al., 1996). It may be the reason that led to this reversal.

Compared with the higher level of flavonoids in *M. patulinervia*, the weaker antioxidant activity it had was speculated that the special structure of flavonoids may be just the reason. For the weak antioxidant activities, the extracts of this plant could not suitable for serving as free radical inhibitors or scavengers.

Preliminary research on antimicrobial activity showed that the activity of the plant against Oxytetracycline hydrochloride, *F. graminearum*, *B. cinerea*, Northern Leaf Blight of Corn was very significant, especially Oxytetracycline hydrochloride. And the activity of the extract of leaves was obvious higher than stem and the whole plant. The study showed that the extracts of this plant, especially leaves, could serve as pesticides for specific species.

**Conclusion**

The 70% methanol extracts of the leaves of *M. patulinervia* had higher level of total flavonoids, with a content of 41.3194±0.071 mg g⁻¹. Because of the special structure of flavonoids (the substituting group of flavonoids contained in this plant was almost methoxy group, not hydroxyl group), the antioxidant activities of the different parts, different solvents extracts of *M. patulinervia* were all weak. The leaf extract showed antimicrobial activity against 4 of the plant of pathogenic fungi species, especially Oxytetracycline hydrochloride.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


