Seasonal dynamics of the phytochemical constituents and bioactivities of extracts from *Stenoloma chusanum* (L.) Ching

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Abstract

*Stenoloma chusanum* is a traditional Chinese medicinal herb with a very high total flavonoid content (TFC). The seasonal dynamics of the TFC and total phenolic content (TPC) in *S. chusanum*, as well as antioxidant activity, were investigated. The TFC and TPC showed clear seasonal dynamics, reaching their maxima (24.63 ± 1.34% and 9.58 ± 0.41%, respectively) in February. The TFC and TPC in the aerial parts of the plant were much higher than those in the subterranean parts; however, the antioxidant activities of the extracts from the subterranean parts were slightly higher than those from the aerial parts. Moreover, the extracts exhibited higher inhibition against tyrosinase than against arbutin (the positive control). The extract from *S. chusanum* collected in February was associated with the highest proliferation and apoptosis of K562 cells. The phytochemicals in the extract were analyzed using LC-MS, and were found to comprise of 12 flavonoids, five alkaloids, one sesquiterpenoid and one phenypropanoid. In conclusion, *S. chusanum* exhibits multiple bioactivities; these results could contribute to the therapeutic application of the plants in indigenous medicine.

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1. Introduction

The 2015 Nobel Prize for Physiology and Medicine was awarded to scientists studying natural products and neglected diseases. One of the recipients was the Chinese scientist Youyou Tu, who was awarded the prize for discovering the novel antimalarial drug artemisinin from a traditional Chinese medicine. An interesting result has emerged in pharmaceutical development since the late 1990s, namely a return to nature as a source of potential drugs (Lanzotti, 2014; Xiao, 2015; 2016a).

Herbal folk medicine commonly makes use of ferns (Wang and He, 2006; Gao et al., 2003) and it is estimated that about 300 species are available for use in medicine in China (Ching, 1978). Our previous research has demonstrated that several species of fern exhibit a range of bioactivities including antioxidant, acetylcholinesterase inhibition and anti-tumor activities (Cao et al., 2013a, 2014). *Stenoloma chusanum* (L.) Ching, a member of the family Lindsaeaceae, is widely distributed in the south of China (Ching, 1959). In traditional Chinese medicine, its prescription, either alone or in combination with other ingredients, is considered effective in cancer treatments, detoxification and hemostasis, resulting in its reputation as an “all-purpose antidote” (The National Assembly Group of Chinese Herbal Medicine, 1983; Jiangsu New Medical College (ed.), 1977). Previous research has indicated its high flavonoid content, strong antioxidant potential (Xia et al., 2014) and antibacterial activity (Wu and Zhang, 2008). The bioactivities may be related to the presence of flavonoids and phenolic compounds (Xiao et al., 2016; Xiao, 2016b; Cao et al., 2016), which probably fluctuate seasonally (Xie et al., 2015). The objectives of the current work were to investigate the seasonal dynamics of total flavonoid and phenolic contents of *S. Chusanum*, and to evaluate the biological potential of ethanol extracts based on the chemical components identified.

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2. Materials and methods

2.1. Chemicals and materials

Rutin, Folin-Ciocalteu reagent, gallic acid, tyrosinase, acridine orange, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), 2,2′-azinobis-(3-ethylbenothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were all purchased from Sigma Co. (MO, USA). 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (TPTZ) and arbutin were purchased from TCI (Tokyo, Japan). PRMI-1640 medium, fetal bovine serum (FBS) and phosphate buffer saline (PBS) were obtained from Gibco (NY, USA). FITC annexin V apoptosis detection kits were purchased from BD Bioscience (USA). The acetonitrile was HPLC grade and all other chemicals used were of analytical grade. Ultra-pure water was used for any aqueous solutions.

2.2. Preparation of plant extracts

Whole plants of *Stenoloma chusanum* (L.) Ching were collected from Wenzhou, China every month throughout the year, and identified by Prof. Jianguo Cao at the College of Life & Environmental Sciences of Shanghai Normal University. The materials were divided into aerial and subterranean parts. Fresh plants were air dried at room temperature, ground to powder and passed through a 40-mesh sieve. One gram of the powdered sample was soaked in 25 mL 50% ethanol (v/v) at 80°C for 1.5 h and then an ultrasound-assisted extraction was performed for 20 min, using a Kunshan ultrasound generation system (Kunshan, China). This extraction process was repeated twice for each sample. The extracts were passed through filter paper and the liquid collected. The mixture was allowed to cool for 20 min and concentrated using a rotary evaporator until a residue was dissolved in PRMI-1640 medium and filtered through a 0.22 μm membrane (Millipore, USA) before testing. For other experiments, the residue was suspended in methanol and filtered through a 0.45 μm membrane (Millipore, USA).

2.3. Determination of total flavonoid content (TFC)

The TFC in the extracts was measured as described in our previous paper, Zhang et al. (2012).

2.4. Determination of total phenolic content (TPC)

The TPC was determined with Folin-Ciocalteu reagent according to the method described by Singleton and Rossi (1965).

2.5. LC/DAD-ESI/MS analysis

The phytochemical profile of the extract was determined using a LC-DAD-ESI/MS system as described in our previous papers (Cao et al., 2013a, 2015).

2.6. Antioxidant activity assay

We had previously measured the DPPH, free radical scavenging activity of the extracts from *S. chusanum*, see Xia et al. (2014).

2.6.1. ABTS assay

The ABTS assay of the extracts from *S. chusanum* was performed according to our previous work (http://www.sciencedirect.com/science/article/pii/S092666901530073XXia et al., 2014).

2.6.2. FRAP assay

The FRAP assay of the extracts from *S. chusanum* was carried out according to our previous work (http://www.sciencedirect.com/science/article/pii/S092666901530073X, Cao et al., 2013b).

2.7. Tyrosinase inhibitory assay

The tyrosinase inhibitory potentials of the extracts from *S. chusanum* were determined as described by Zengin et al. (2015). L-3,4-dihydroxyxyphenylalanine (L-DOPA, Sigma) and tyrosinase from mushrooms were employed as the substrate. Briefly, 0.2 mL of extract samples with variable concentrations were added to 0.2 mL of tyrosinase solution (100 U/mL, in PB). After incubation for 90 min in the dark, 0.5 mL of L-DOPA solution (0.03%, in PB) was added and the reaction was stopped by the excess addition of sodium dodecyl sulfate (SDS, 4%) after 5 min, after which the absorbance at 450 nm was measured. Arbutin was used as a positive control. All the determinations were performed in triplicate and found to be reproducible within acceptable experimental errors.

2.8. Antitumor activity in vitro

2.8.1. Cell culture

The human chronic myeloid leukemic cell line K562 was maintained in PRMI 1640 medium supplemented with heat-inactivated FBS (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified 5% CO₂ incubator at 37°C. The medium was changed every day.

2.8.2. MTT assay

The cell cytotoxicity was determined using the MTT assay as reported by El Euch et al. (2015).

2.8.3. Fluorescence microscopy observation

In order to determine whether the extract induced cell apoptosis, an acridine orange stain assay was performed. Cells treated with different concentrations of the extract or blank medium were stained with AO and examined under a fluorescence microscope after treatment for 48 h.

2.8.4. Flow cytometry assay

After incubation with the extracts for 48 h, K562 cells were suspended with both propidium iodide and annexin V(FITC)-conjugated to a Beckman Coulter C250 flow cytometer (CA, USA), allowing FL1 (AV) and FL3 (PI) bivariate analysis. Data from 20,000 cells/sample were collected and then analyzed using FlowJo 7.6 software.

2.9. Statistical analysis

The values are presented as mean ± SD (standard deviation) of triplicate measurements; they were examined statistically using analysis of variance (ANOVA). A level of *p* < 0.05 was considered to indicate statistical significance.

3. Results

3.1. The seasonal dynamics of TFC and TPC

Table 1 presents the TFC and the TPC values for *S. chusanum*. TFC ranged from 4.31 ± 0.56% to 24.63 ± 1.34%, while TPC ranged from 2.57 ± 0.12% to 9.58 ± 0.41%. The annual average values of TPC and TFC in the aerial parts of the plants were much higher than that in subterranean parts. The TFCs and TPCs in both aerial and subterranean parts exhibited similar seasonal trends (Fig. 1). The TFCs in both parts were higher in winter and spring (from December to...
August has weak DPPH free radical scavenging potential. Radical at various concentrations (10⁻⁸ to 10⁻⁴ M) from S. chusanum showed slightly higher DPPH free radical scavenging potential than the aerial parts; the samples collected in August, however, showed slightly higher DPPH free radical scavenging potential than those from August, and the subterranean parts exhibited the greatest potential.

All the investigated samples showed dose-dependent reducing power (Fig. 2I–L). The extracts from the aerial parts were less effective in this respect than those from the subterranean parts, with the exception of the sample collected in February. The samples collected in May and August exhibited similar capacities in both parts.

3.3. Tyrosinase inhibitory activity

The results pertaining to tyrosinase inhibitory capacity of the extracts from S. chusanum (collected in February) are presented in Fig. 4. Generally, the inhibitory ratios of the two parts were found to be dose-dependent, and the aerial part showed higher inhibition. The IC₅₀ values of the extracts from aerial, subterranean parts and arbutin (the positive control) were 107.25 ± 2.82, 137.48 ± 2.67 and 190.39 ± 3.22 μg/mL, respectively, indicating the excellent inhibitory capacity of the extracts from S. chusanum against tyrosinase.

3.4. Anti-tumor effect on K562 cells

The cytotoxicity of the extracts from S. chusanum on K562 cells is shown in Fig. 5. The inhibitory effect of the extracts from the aerial part on cell proliferation was greater than that of the extracts from the subterranean part. The IC₅₀ value for the extracts from the aerial part was found to be 707.11 ± 5.43 μg/mL. However, the IC₅₀ value for the extracts from the subterranean parts could not be calculated because the cytotoxicity was too low.

In order to ascertain whether the cytotoxicity towards K562 cells was mediated through apoptosis, flow cytometry and fluorescence microscopy were used to examine the treated cells. The results of the flow cytometry analysis are presented in Fig. 6 and Table 2. Apoptosis and the necrotic rate of K562 cells were determined by flow cytometry using PI and AV staining. PI detects dead cells and AV, which detects externalized phosphatidylserine residues, is a marker of apoptotic cells. The graph is divided into four parts, Q1–Q4, which represent primary necrosis (AV⁻/PI⁺), late apoptosis/secondary necrosis (AV⁻/PI⁻), early apoptosis (AV⁺/PI⁻) and normal cells (AV⁻/PI⁻) respectively. The original concentration of the extract from the aerial part (2.35 μg/mL) was adjusted according to its IC₅₀ value in the MTT assay. On the basis of its apoptotic and necrotic rate (Table 4), we can see that the extract from S. chusanum was able to induce both necrosis and apoptosis of K562 cells, and the percentage of cell apoptosis responded in a dose-dependent manner (from 6.86% to 32.92%) (P < 0.05).

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Moreover, Fig. 7 shows the appearance of stained K562 cells under fluorescence microscopy after treatment with the extracts of the aerial part of *S. chusanum* collected in February. The concentration of the added extract (235, 471 and 707 μg/mL) was adjusted according to its IC50 value in the MTT assay. It is clear that the number of K562 cells emitting orange-yellow fluorescence reduced with increasing concentration of the extracts from *S. chusanum*. Furthermore, typical features of apoptotic cells, such as cytoplasmic membrane blebbing and nuclear contraction, were visible under fluorescence microscopy after treatment with the extracts from *S. chusanum* (Fig. 8).

3.5. Identification phytochemicals in various parts via LC-MS

The extracts from *S. chusanum* collected in February with high TFC and TPC were analyzed by LC-DAD-ESI/MS. As listed in Tables 3 and 4, each compound was identified according to its ion spectrum and UV absorbance spectrum based on references (Megdiche-Ksouri et al., 2015; Toth et al., 2015; Wijayanto et al., 2015; Karakose et al., 2015; Xie et al., 2015). Twelve flavonoids (flavones, isoflavones and flavonols), five alkaloids (isoquinolines, amides, indoles and carbolines), one sesquiterpenoid and one phenypropanoid in the extracts were identified. The aerial parts
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4. Discussion

Flavonoids occur commonly in plants and are frequently components of the human diet. Some of them have been found to possess antioxidant, antitumoral, anti-inflammatory and antibacterial characteristics (Chen et al., 2005; Terao et al., 1994; Alvesalo et al., 2006; Subarnas and Wagner, 2007). S. chusanum is used as a traditional medicine, and the flavonoids it contains have been considered to exhibit bioactivity (Wu et al., 2010). However, the total flavonoid content reported by different researchers has varied from 2.27% to 34.42% (Xia et al., 2014; Zhang et al., 2008; Wu et al., 2010). The present investigation has demonstrated that although the flavonoid content in the aerial parts of the plant are clearly higher than that in the subterranean parts, both exhibit excellent antioxidant capacity. Indeed, the IC50 value indicates that the antioxidant ability of the subterranean part is relatively higher than that of the aerial part. Perhaps the flavonoids in the subterranean parts exhibit some crucial capacity for scavenging. The high antioxidant ability of S. Chusanum may be attributed to structural elements leading flavonoids to control its antioxidative behavior, potentially including the following features: 1) occurrence and arrangement of multiple hydroxyl groups; 2) presence of additional substituents such as double bonds or conjugation; and 3) glycosylation (Monica et al., 2011; Heim et al., 2002). For example, the B-ring hydroxyl configuration is the most significant: a 3’-4’-catechol structure in the B-ring strongly enhances antioxidant activity (Dugas et al., 2000; Ratty and Das, 1988).

Quercetin-3-rhamnoside and luteolin-7-O-glucoside with the 3’-4’-catechol structure were found in our work and may play a role in antioxidant effect. Other flavonoids like kaempferol in S. Chusanum may also contribute to this activity as a result of its 5-OH structure (Ratty and Das, 1988). In contrast, aglycones are more potent antioxidants than their corresponding glycosides (Lai and Chen, 2002; loku et al., 1995). Luteolin and quercetin aglycones significantly exceed the 3-, 4- and 7-O-glucosides with respect to antioxidant activity. We found that the antioxidant capacity of the extract from the aerial part of the fern, although higher in flavonoids/phenolics, was inferior to the subterranean part. This may be attributed to the larger number of glycoside moieties in the flavonoid compounds from the aerial parts.

Tyrosinase, known as a key enzyme in melanin biosynthesis, is involved in determining the color of mammalian skin and hair. Melanin overproduction may cause hyperpigmentation diseases (Xue et al., 2008). Plant polyphenols are effective natural tyrosinase inhibitors (Chang, 2009; Kubo et al., 2000). The extracts from S. Chusanum exhibited high inhibition of tyrosinase. The inhibitory ability of the extract (from plants collected in February) in both parts was obviously superior to the positive control (arbutin) and, based on IC50 values, the aerial part was the most effective. It has been reported that the flavonoids kaempferol and luteolin-7-O-glucoside, both found in S. Chusanum, exhibit tyrosinase inhibitory capacity.

Another important facet of this work pertains to the cytotoxicity and apoptosis of the S. Chusanum extract (February) on K562 cells. Our result demonstrated that the S. Chusanum extract of both plant parts was harmful to K562 cells, and the sample from the aerial parts was more effective at inhibiting the growth of cells. In the cell apoptosis study (flow cytometry), the extract of the aerial part was found to induce both necrosis and apoptosis of K562 cells. Furthermore, some typical apoptotic features were captured: cytoplasmic membrane blebbing and nuclear contraction.

Leukemia is a complex disease affecting all blood cell lineages, it contained more flavonoids than that the subterranean parts, which was consistent with their total flavonoid contents. Moreover, these two parts contained similar compounds, namely flavones (apigenin–7–O–β–D–glucoside), isoflavones (scadenone), flavonols (quercetin–3–rhamnoside) and phenypropanoids (chlorogenic acid). Polyphenols in the extracts included phenypropanoids (chlorogenic acid), flavones (luteolin–7–O–glucoside and apigenin–8–C–glucoside) and flavonols (quercetin–3–O–rhamnoside, kaempferol and kaempferol–3–O–rutinoside).
affects millions of people worldwide each year, especially children and adolescents, and is responsible for almost one-third of all cancer deaths (The Leukemia & Lymphoma Society, 2012). Many peer-reviewed publications have reported anticancer activities of polyphenols (Lamoral-Theys et al., 2010; Wolter et al., 2001), exhibiting anti-proliferative and apoptotic properties. In this work, we consider that the high phenolic/flavonoid content explains the extracts’ cytotoxic and apoptosis activity. Furthermore, two compounds, apigenin and kaempferol, that we detected in S. Chusanum, are reported to exhibit anti-proliferative and apoptotic properties in leukemia cells (Chen et al., 2005; Mahbub et al., 2013). It would be valuable to isolate pure polyphenol/flavonoid compounds with antitumor activity in the future.

5. Conclusions

Our results indicate that the extracts from S. Chusanum possess considerable amounts of flavonoids/phenolics and the contents exhibit seasonal dynamics. Furthermore, a number of bioactivities were found, including antioxidant, anti-tyrosinase, cytotoxic and apoptotic potentials. Some pure flavonoid in S. Chusanum appears to be responsible for these effects. The extract from the aerial part of the fern exhibited high anti-tyrosinase, cytotoxic and apoptotic activities but marginally lower antioxidant activity than that from the subterranean part. It is possible that S. Chusanum could be exploited as a natural antioxidant and anti-tyrosinase, as well as an anti-tumor agent, but the exact active ingredients remain unidentified. Further studies of S. Chusanum focusing on isolating the active constituents are needed to allow the development of specific medical treatments.

Table 2
Apoptosis and necrotic rate of K562 cells detected by cytomter with PI and AV staining methods.

<table>
<thead>
<tr>
<th></th>
<th>Primary necrosis (%)</th>
<th>Late apoptosis (%)</th>
<th>Early apoptosis (%)</th>
<th>Total apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.90 ± 0.91</td>
<td>1.97 ± 0.24</td>
<td>0.97 ± 0.02</td>
<td>2.94</td>
</tr>
<tr>
<td>235 µg/mL</td>
<td>3.90 ± 0.16</td>
<td>2.55 ± 0.9</td>
<td>4.31 ± 0.25</td>
<td>6.86</td>
</tr>
<tr>
<td>471 µg/mL</td>
<td>20.97 ± 4.8</td>
<td>17.53 ± 3.30</td>
<td>8.64 ± 0.49</td>
<td>26.17</td>
</tr>
<tr>
<td>707 µg/mL</td>
<td>34.00 ± 0.8</td>
<td>36.87 ± 1.5</td>
<td>2.05 ± 0.07</td>
<td>38.92</td>
</tr>
</tbody>
</table>

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Fig. 7. K562 cells stained with AO and viewed with fluorescence microscopy. (A, B and C) Treatments with various concentrations (235, 471 and 707 μg/mL) of the extracts from S. chusanum. (D) Control cells.

Fig. 8. Characteristic features of typical apoptotic cells found in treatments of the extracts from S. chusanum. (A) Normal cell; (B) nuclear contraction; (C) cytoplasmic membrane blebbing.

Table 3
Compounds identified in the extracts from the aerial part of S. chusanum.

<table>
<thead>
<tr>
<th>No</th>
<th>tR(min)</th>
<th>m/z</th>
<th>MW</th>
<th>UV λmax (nm)</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>32.436</td>
<td>593[M- H]+; 595[M+H]+</td>
<td>594</td>
<td>200/265/340</td>
<td>Quercetin-3-O-rhamnoside</td>
</tr>
<tr>
<td>7</td>
<td>34.429</td>
<td>431[M- H]+; 433[M+H]+</td>
<td>432</td>
<td>200/270/345</td>
<td>Apigenin-7-O-glucoside</td>
</tr>
<tr>
<td>14</td>
<td>56.767</td>
<td>269[M- H]+; 271[M+H]+</td>
<td>270</td>
<td>265/345</td>
<td>Apigenin</td>
</tr>
</tbody>
</table>

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