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Characterization of Volatile and Polar Compounds of Jiaogulan Tea [*Gynostemma pentaphyllum* (Thunb.) Makino] by Hyphenated Analytical Techniques

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Jiaogulan [G*ynostemma pentaphyllum* (Thunb.) Makino] is a Chinese medicinal plant from southern Asia that has rapidly gained popularity and interest for its health-promotive and therapeutic properties. The volatile composition of jiaogulan tea was analyzed by using headspace-solid phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS). A total of 29 volatile components were detected in the jiaogulan tea, with major compounds being benzaldehyde (15.3 %), 1,8-cineole (8.7 %), 6-methyl-5-hepten-2-one (7.8 %), (*Z*)-3-hexenal (7.6 %) and α-ionone (6.3 %). The chemical characterization of methanolic extract and 5 % infusion from the *G. pentaphyllum* leaves was determined by using LC-ESI-MS/MS systems. Chlorogenic acids, flavonoids and gypenosides were determined. The compounds quercetin 3-*O*-hexoside, isorhamnetin rutinoside and kaempferol dimethyl ether were identified in the extracts for the first time. Phenolic content was determined by gallic acid equivalents and antioxidant activity of the extracts was evaluated by DPPH, β-carotene/linoleic acid and ABTS radical scavenging assays. Both extracts showed weak antioxidant activity.

Keywords: Gypenosides, Gynosaponins, Volatiles, HS-SPME, LC-ESI-MS/MS, Antioxidant activity.

INTRODUCTION

Gynostemma pentaphyllum (Thunb.) Makino or jiaogulan, is a Chinese medicinal herb of the family Cucurbitaceae, growing wild in southern China, Japan, India and Korea [1]. Other names for *G. pentaphyllum* include five-leaf ginseng, poor man's ginseng, southern ginseng, immortality herb (English) *amachazuru* (Japanese) and *dungkulcha* (Korean) [2]. Commonly used as an herbal tea in Southern Asia, *G. pentaphyllum* has been used as food (longxu), tea (jiaogulan tea) and botanical supplements (jiaogulan extracts) and has become increasingly popular as an adaptogen, helping the body to adapt to certain stresses and promoting homeostasis and as an antioxidant, helping the body to fight against the effects of free radicals [3,4]. In addition, *G. pentaphyllum* has been found to have significant bioactivity in treatment of a wide range of diseases, including various types of cancer [5,6] and type 2 diabetes [7].

Although much interest has focused on the biological activities of saponins, there is also interest in the health-promoting activities of flavonoids (*e.g.*, quercetin and rutin) produced by *G. pentaphyllum* [8,9]. In addition, polysaccharides from *G. pentaphyllum*, beyond serving as structural components and energy sources have been investigated for their chemistry and health-promoting properties [10-12]. Gypenosides, dammarane (triterpenoid) saponins are considered to be the major bioactive compounds in jiaogulan and tend to vary by provenance, season, taste and genotype [13]. Aktan *et al.* [14] proposed that the mechanism underlying the therapeutic effects of gypenosides in numerous diseases (such as inflammation and atherosclerosis) involves the suppression of nitric oxide in murine macrophages. No human toxicity has been found with *G. pentaphyllum* in multiple studies [15,16]. Nevertheless, *G. pentaphyllum* is reported to have insect antifeedant properties against the tobacco cutworm, *Spodoptera litura* Fab. [17] and molluscicidal properties against the freshwater snail *Oncomelania hupensis* Gredler [18].

Jiaogulan tea has been used by local Chinese people in the mountainous regions of Southern China to increase endurance, strength and to relieve fatigue and for treating common colds and other infectious diseases [3]. However, only limited information has been available on the volatile composition of jiaogulantea. In the present study, the volatile compounds of jiaogulan tea were analyzed by HS-SPME/GC-MS (headspace-

solid phase microextraction) and phytochemicals in infusions and methanol extracts were determined by LC-ESI-MS/MS.

EXPERIMENTAL

A female plant of *G. pentaphyllum* was grown in a 56.7 L container in a controlled-environment greenhouse at the South Mississippi Branch Experiment Station (SMBES) in Poplarville, Mississippi, USA. Voucher specimen #693 was deposited at the SMBES for future reference. Aerial parts were harvested from the plant in May, July and December and air-dried for three weeks inside an air-conditioned building (25 °C maximum). Dried plant material was consolidated and packed loosely into cardboard boxes to avoid crushing and stored in the same building until subsequent processing.

Extraction procedure: 1 g of the air dried plant material was macerated with MeOH 3 times over 8 h. 1 g of plant material was infused with 20 mL of boiling water, then filtered and lyophilized. The methanolic extract and the infusion were used for LC-ESI-MS/MS analysis. The infusion for SPME was prepared at a 10 % concentration.

Headspace-solid phase microextraction: A manual SPME device (Supelco, Bellafonte, PA, USA) with a fiber-precoated 65-μm-thick layer of polydimethylsiloxane/divinylbenzene (PDMS/DVB-blue) was used for infusion. The vial containing the plant extract was sealed with parafilm. The fiber was pushed through the film layer for exposure to the headspace of the extract for 15 min at room temperature. The fiber was then immediately inserted into the injection port of the GC-MS for desorption of the adsorbed volatile compounds for analysis.

Analysis of volatile compounds: The volatiles were analyzed by GC-MS using an Agilent 5975 GC-MSD system. An Innowax FSC column (60 m × 0.25 mm, 0.25 mm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min and kept constant at 220 °C for 10 min and then programmed to 240 °C at a rate of 1 °C/min. The injector temperature was set to 250 °C. Mass spectra were recorded at 70 eV. Mass range was *m/z* 35 to 450. Identification of the volatile components was carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes by comparison with fragmentation patterns in mass spectra with those stored on commercial libraries [19,20], an in-house library 'Baser Library of Essential Oil Constituents' built up by genuine compounds and components of known oils) and MS literature data [21,22]. Relative percentage amounts of the separated compounds were calculated from TIC chromatograms. The volatile compounds identified are listed in Table-1.

LC-ESI-MS/MS analysis: Experiments were performed with a Shimadzu 20A HPLC system coupled to an Applied Biosystems 3200 Q-Trap LC-MS/MS instrument equipped with an ESI ion source used in the negative ionization mode. Separations were performed on an ODS 150 × 4.6 mm i.d., 3 μm particle sizes, octadecyl silica gel analytical column operating at 40 °C at a flow rate of 0.7 mL/min. The mobile phase was used acetonitrile:water:formic acid (10:89:1) (solvent A) and acetonitrile:water:formic acid (89:10:1) (solvent B). The composition of B was increased from 15 to 40 % in 15 min, increased to 45 % in 3 min and held for 12 min and increased to 75 % in 5 min; then the composition of B was increased to 100 % in 10 min. Chromatograms were recorded at 280 nm.

TABLE-1
VOLATILE COMPOSITION OF
JIAOGULAN (*Gynostemma pentaphyllum*) TEA

RRI[a]	Compound[b]	Content (%)[c]	Identification method
1213	1,8-Cineole	8.7	RRI, MS
1225	(Z)-3-Hexenal	7.6	MS
1247	6-Methyl-2-heptanone	2.1	MS
1280	*p*-Cymene	0.3	RRI, MS
1328	2,2,6-Trimethylcyclohexanone	0.6	MS
1348	6-Methyl-5-hepten-2-one	7.8	MS
1400	Nonanal	1.6	MS
1600	Isophorone	2.1	MS
1445	Filifolone	2.1	MS
1496	2-Ethyl hexanol	1.3	MS
1497	α-Copaene	2.1	MS
1541	Benzaldehyde	15.3	RRI, MS
1553	Linalool	0.4	RRI, MS
1602	6-Methyl-3,5-heptadien-2-one	2.8	MS
1638	β-Cyclocitral	1.1	MS
1658	Benzene acetaldehyde	4.6	MS
1703	6-Oxoisophorone	0.4	MS
1719	Borneol	1.0	RRI, MS
1740	α-Muurolene	0.3	MS
1766	1-Decanol	0.8	RRI, MS
1865	Isopiperitenone	2.4	MS
1871	α-Ionone	6.3	MS
1896	Benzylalcohol	0.2	RRI, MS
1958	(E)-β-Ionone	1.4	MS
1983	Piperitenone oxide	0.4	RRI, MS
2009	*trans*-β-Ionone-5,6-epoxide	0.9	MS
2131	Hexahydrofarnesyl acetone	2.1	MS
2239	Carvacrol	1.9	RRI, MS
2380	Dihydroactinidiolide	0.4	MS
	Total	78.6	

[a]Relative retention indices calculated against *n*-alkanes.
[b]Identification method based on the relative retention indices (RRI) of authentic compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.
[c]Calculated from TIC data.

Quantification of total phenolics in the extracts: Total phenolics were estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/1 g extract [23].

ABTS radical scavenging assay: Determination of the ABTS radical ($ABTS^{\bullet+}$) scavenging was carried out as described by Papandreou *et al.* [24]. The results are expressed as the Trolox equivalent antioxidant capacity (TEAC, mmol/L Trolox). Vitamin E was used as positive control.

1,1-Diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging activity: The potential DPPH radical scavenging activity of the extracts was determined according to Kumarasamy *et al.* [25]. After 30 min UV absorbance was recorded at 517 nm.

Determination of inhibition of β-carotene/linoleic acid co-oxidation: Antioxidant activity of water and methanolic extracts of *G. pentaphyllum* was determined according to the β-carotene bleaching method [26]. Absorbance was measured

on a spectrophotometer at 470 nm. The samples were then subjected to thermal autoxidation by keeping them in a constant temperature water bath at 50 °C for 2 h for the acceleration of the oxidation. The rate of bleaching of β-carotene was monitored by taking the absorbance at 15 min intervals. Antioxidative activity (AA) was calculated according to Oomah *et al.* [26] as follows:

$$AA\ (\%) = \left(1 - \frac{(Abs^{0}_{sample} - Abs^{105}_{sample})}{(Abs^{0}_{control} - Abs^{105}_{control})}\right) \times 100$$

RESULTS AND DISCUSSION

A total of 29 constituents, representing 78.6 % of the total composition, were identified in the jiaogulan tea. Benzaldehyde (15.3 %), 1,8-cineole (8.7 %), 6-methyl-5-hepten-2-one (7.8 %), (*Z*)-3-hexenal (7.6 %) and α-ionone (6.3 %) were the dominant volatile components in the jiaogulan tea (Table-1). The chemical profile of methanolic extract and 5 % infusion from the *G. pentaphyllum* leaves was determined by LC-MS/MS systems. Based on the LC-ESI-MS/MS analysis, 32 peaks were determined and listed as four major groups: chlorogenic acid derivatives (1, 3, 6), flavonoids (4, 7-12, 19, 22, 23 and 27), gypenosides (21, 24, 26, 27, 29) and unknown molecules (2, 5, 13-20, 25, 31 and 32) (Table-2). In the present study, peaks 9, 12 and 23 were identified in *G. pentaphyllum* for the first time.

Peak 1 was identified as quinic acid with a deprotonated molecular ion at *m/z* 191 and product ions at *m/z* 155, 129 and 211. Based on Clifford *et al.* [27,28], peaks 3 and 6 were identified as 5-dihydro caffeoylquinic acid and 4-caffeoylquinic acid, respectively.

Peak 4 was shown to be a pseudo molecular ion $[M-H]^-$ at *m/z* 593 and yielded to *m/z* 473 $[(M-H)-120]^-$, 503 $[(M-H)-90]^-$, 383 $[(M-H)-120-90]^-$ and 353 $[(M-H)-120-120]^-$. The fragmentation characteristic for *C*-glycoside flavonoids showed characteristic loss of sugar parts as 90 and 120 amu. The fragmentation pattern of peak 4 was consistent with two sugar units. Thus, the aglycon part of peak 4 must be 324 amu lower than the molecular ion peak, which can be the aglycon part's molecular weight of 269 amu (apigenin). Based on this data, peak 4 was identified as apigenin 6,8-*C*-di-hexoside (vicenin 2).

Peaks 7 and 8 co-eluted and showed the same molecular ions at *m/z* 609, which were determined to be quercetin rhamno hexoside and rutin (quercetin-3-*O*-rutinoside), respectively.

TABLE-2
ASSIGNMENT OF PEAKS FROM THE AQUEOUS AND METHANOL EXTRACTS OF AERIAL PARTS OF JIAOGULAN (*Gynostemma pentaphyllum*)

Peak	TR[a]	$[M-H]^-$	Fragments	Identification[b]	I[c]	M[d]
1	5.2	191	155, 129, 111	Quinic acid	+	+
2	9.4	455	–	Unknown	–	+
3	10.8	355	209, 191	5-dihydrocaffeoylquinic acid	+	
4	11.0	593	503, 473, 397, 383, 353, 325, 297	Vicenin 2	+	+
5	12.7	431	385, 223, 205, 161, 153	Unknown	+	+
6	14.4	353	191, 173, 179, 155, 111	4-caffeoylquinic acid	+	-
7	15.9	609	300, 301	Quercetin-rhamno-hexoside	+	+
8	15.9	609	343, 300, 301, 271, 255, 179, 151	Rutin	+	+
9	17.4	463	299, 271, 255, 243, 179, 151	Quercetin 3-*O*-hexoside	+	+
10	17.6	593	357, 327, 284, 255, 227, 151	Kaempferol-rhamno-hexoside	+	+
11	18.3	593	327, 284, 255, 227	Kaempferol rutinoside	+	+
12	18.8	623	315, 300	Isorhamnetin rutinoside	+	–
13	19.4	403	371, 343, 327, 315, 205, 151	Unknown	–	+
14	19.9	691	667, 631, 619, 355, 335, 317	Unknown	+	+
15	21.2	313	295, 285, 255, 159, 151, 148	Unknown	+	+
16	22.3	607	583, 561, 299, 284, 255	Unknown	+	+
17	22.6	675	599, 557, 391, 319	Unknown	+	–
18	24.1	675	643, 599, 557, 391, 355, 319	Unknown	+	–
19	24.9	637	329, 314, 299, 285, 271	Ombuoside	+	+
20	25.1	387	355, 323, 295, 221, 189, 151	Unknown	+	+
21	27.1	1093	–	Gypenoside XXII, Gypenoside LVI, Gypenoside LXII, Gypenoside LXVII, Gypenoside LXX, Gypenoside LXXI	+	+
22	28.2	301	273, 179, 151	Quercetin	–	+
23	28.2	313	298, 283, 255, 227	Kaempferol dimethyl ether	–	+
24	29.9	1123	1077	Gypenoside XLII, Gypenoside XLVII	+	–
25	30.5	327	309, 291, 229, 211, 283, 251	Unknown	+	+
26	32.4	1107	1061	Gypenoside XXXIV [Gypenoside XXXVI + HCOOH-H]	–	+
27	32.5	285	257, 151	Kaempferol	+	+
28	33.3	961	915	Gypenoside XLIV, Gypenoside XLVI	+	+
29	33.7	315	300, 283, 271	Isorhamnetin	–	+
30	34.1	961	915	Gypenoside XLIV, Gypenoside XLVI	+	
31	34.3	387	343, 327, 297, 283, 238	Unknown	+	+
32	34.5	287	269, 155	Unknown	+	+

[a]Retention time; [b]Identification was performed by comparison to standards or previously reported in *G. pentaphyllum* or mass fragmentation reported in plant samples; [c]Infusion; [d]Methanolic extract.

Both compounds gave the characteristic ions at *m/z* 300 and 301 (quercetin) due to the loss of a rutinose and rhamnose + hexose moiety. Because of the cleavage of $^{0,2}X_0$, the rutinose part produced the ion at *m/z* 343. Quercetin rhamno hexoside (peak 7) and rutin (peak 8) were previously determined in *G. pentaphyllum* with the same eluting order [29,30].

Peak 9 was shown to be a pseudo molecular ion at *m/z* 463 that produced an aglycone anion at *m/z* 300 ($Y_0 - H^-$) and low intense aglycon ion at *m/z* 301 (Y_0). This fragmentation behaviour indicated that peak 9 is a quercetin 3-*O*-hexoside [31]. To our best of knowledge, this is the first report of quercetin 3-*O*-hexoside in *G. pentaphyllum* extracts.

Peaks 10 and 11 were shown to be the same pseudo molecular ion $[M-H]^-$ at *m/z* 593 and shared the same base peak $[M-H]^-$ at *m/z* 284, which has an aglycon anion of kaempferol. Peaks 10 and 11 were identified as kaempferol rhamno hexoside and kaempferol rutinoside, which was previously reported from *G. pentaphyllum* [9,30].

Peak 12 was shown to be a molecular ion $[M-H]^-$ at *m/z* 623 that yielded product ions at *m/z* 315 (base peak) and *m/z* 300. The fragment ion at *m/z* 308 indicates rhamnose and glucose co-loss. The absence of other fragmentation between the molecular ion and base peak ion indicated a rutinose moiety. Wu *et al.* [32] previously reported isorhamnetin from *G. pentaphyllum*, which gave the same aglycon fragmentation with aglycon of peak 12. Therefore, peak 12 was assigned as isorhamnetin rutinoside and has not been previously reported in *G. pentaphyllum* extract.

Peak 19 presented a molecular ion $[M-H]^-$ at *m/z* 637 and base peak ion at *m/z* 329 that was associated with loss of the methyl units at *m/z* 314 and *m/z* 288. This compound was identified as ombuoside (7,4'-di-*O*-methylquercetin-3-*O*-β-rutinoside) and was reported previously from *G. pentaphyllum* [33].

Peaks 22, 27 and 29 were identified as quercetin, kaempferol and isorhamnetin, respectively, after comparison with our home library of standard compounds and based on previously published papers on *G. pentaphyllum* [8,32].

Peak 23 was shown to be a molecular ion at *m/z* 313 $[M-H]^-$, which is 28 amu higher than kaempferol. Based on the similarity of mass spectrum patterns of dimethoxy flavonoids (like ermanin, cirsimaritin and pilloin), peak 23 was identified as kaempferol dimethyl ether, with this compound being reported for the first time in this present study.

Peaks 21, 24, 26, 28, 30 were identified as dammarane-type saponins, which were identified as gypenoside derivatives based on previously published reports [34,35]. It is not possible to obtain further fragmentation of these group compounds because triterpenic structures exhibit low fragmentation behaviour.

Peak 2 was shown to be a pseudo molecular ion at *m/z* 455 and yielded no other product ions. 20R-21,24-cyclo-3β,25-dihydroxyldammar-23(24)-en-21-one is a dammarane-type saponin determined previously in *G. pentaphyllum* based only its molecular weight [9,36]. However, the poor ionization and unmatched retention time with other triterpenic compounds led us to identify this peak 2 as unknown.

Peak 5 was shown to be a molecular ion at *m/z* 431 $[M-H]^-$ and gave fragmentation similar to roseoside and sinapoyl-β-D-glucose [37,38]. However, this peak cannot be identified as roseoside and sinapoyl-β-D-glucose without having the full mass fragmentation pattern. Thus, we identified peak 5 as unknown.

Peak 14 was shown to be a molecular ion $[M-H]^-$ at *m/z* 691 and several fragments noted in Table-2 that suggested this compound to be myricetin-3-*O*-(6"-galloyl) glycoside derivative [39]. Due to absences of myricetin as an aglycon in *G. pentaphyllum* extracts, it is not possible to completely identify peak 14.

Peak 16 was fully matched with diosmetin rutinoside (diosmin) with molecular ion $[M-H]^-$ at *m/z* 607 and other fragments given in Table-2. Due to absence of diosmetin as an aglycon in the *G. pentaphyllum* extracts, this compound cannot be indicated as diosmin. Zhao *et al.* [30] also determined the compound as unknown in *G. pentaphyllum*.

Peak 32 showed molecular ion $[M-H]^-$ at *m/z* 287 and further fragmentation was observed at *m/z* 269 and 155, suggesting this compound to be dihydrokaempferol. This compound was identified as unknown because of lack of literature data for a tentative identification.

Total phenolic content was determined as 22.3 mg GAE (gallic acid equivalents) in 1 g infusion and 32.3 GAE in 1 g MeOH extract. The results are quite similar with previously published data [40]. The potential antioxidant capacities in *G. pentaphyllum* extracts were determined with *in vitro* methods such as DPPH, β-carotene/linoleic acid and ABTS radical scavenging assays. IC_{50} value of the DPPH radical scavenging activity for MeOH extract was determined as 0.94 mg/mL; however, the IC_{50} value of the DPPH radical scavenging activity of the infusion was determined as >5 mg/mL. The IC_{50} value for positive control gallic acid showed as 0.02 mg/mL. Inhibition of β-carotene/linoleic acid co-oxidation of the infusion and MeOH extracts at a concentration of 10 mg/mL were determined as 35.28 % and 42.09 %, respectively. ABTS radical scavenging activity was determined as 1.48 mM TEAC for infusion and 1.43 mM TEAC for MeOH extract at the concentration of 10 mg/mL for both extracts. The positive control gallic acid was determined as 2.3 mM TEAC at 0.1 mg/mL concentration. Due to low total phenolic contents in both extracts, the infusion and MeOH extracts had weak antioxidant activity. *G. pentaphyllum* extracts contain more saponins than flavonoids which could have reduced the antioxidant activity [41].

Volatile compounds in *G. pentaphyllum* have received limited investigation so far. Liu [42] found 45 compounds in the essential oil of wild-collected *G. pentaphyllum* from Qinba Mountain in China, with the major compounds obtained by steam distillation identified as 3-hexen-1-ol (22.0 %), 1-hexanol (14.8 %), linalool (9.9 %), caryophyllene (9.1 %) and hexadecanoic acid (7.2 %) [18]. Niu *et al.* [43] collected *G. pentaphyllum* from five locations in four provinces in China and found the main constituents as aldehydes, ketones, alkanes, alkenes, aromatic hydrocarbons, alcohol and esters using the SPME/GC-MS method. Benzaldehyde (63.2 % and 38.5 %) was the most abundant compound in two samples and linalool was the second highest in one sample [45]. Results from our analysis of methanolic extract and 5 % infusion from the *G. pentaphyllum* leaves using LC-ESI-MS/MS are in agreement

with previously identified compounds [8,9,27-30,33]. Peaks 9, 12 and 23 were identified in *G. pentaphyllum* for the first time. Our extracts from *G. pentaphyllum* were rich in gypenosides (gynosaponins) and such compounds are gaining more attention due to their lipid-lowering effects in animal models and human studies. Saponin-rich Gynostemma tablets have been approved by the Chinese government for lowering blood lipid levels [44]. However, more toxicity studies and development of standardized methods are still needed. Identification of saponin-rich cultivars of *G. pentaphyllum* could also be valuable for development of commercial products with useful biological activities.

Conclusion

The current study was an endeavor to reveal the volatile composition of jiaogulan tea and the chemical profile of infusion and methanol extracts. Teas are popular beverages worldwide, with some used for health benefits. Each tea has its own odor and taste, which depends on the tea's origin, genetics and processing (*e.g.*, fresh or fermented). In the current study, we found that our jiaogulan tea contained volatile components such as aldehydes, alcohols, ketones, terpenes and aromatic compounds (Table-1). In the jiaogulan tea, the most odorous volatiles compounds benzaldehyde (bitter almond) [45], benzene acetaldehyde (hyacinth, lilac) [45], (*Z*)-3-hexenal (herbal apple, green leafy) [45], α-ionone (woody, berry, floral, nutty) [46], β-ionone (woody, floral, rose, violet) [46], 1,8-cineole (camphorous) [47] and 6-methyl-5-hepten-2-one (woody, spicy, cinnamon) [48], together with the polar compounds (chlorogenic acids, flavonoids, gypenosides and unknown molecules) might influence the tea's odor and taste. To better understand the composition of jiaogulan, more studies are needed involving samples from different geographic location and comparisons between wild and cultivated samples.

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