Screening for Bioactive Compounds from *Adiantum capillus–veneris* L.

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Summary: The aim of the present study was to assess the biological properties of *A. capillus-veneris* L. and identified the functional compounds in plant extracts. The antimicrobial activities were determined with broth dilution method using 4 Gram-positive bacteria, 2 Gram-negative bacteria and 1 fungus. The antioxidant activities were studied by employing various *in vitro* antioxidant assays such as DPPH$^{\bullet}$ and ABTS scavenging and Fe^{3+}–Fe^{2+} transformation method. MTT colori metric analysis was used to measure the cytocide rate on SGC-7901 cell line. The content of total phenolics, tannins and flavones were measured by Folin-Ciocalteu reagent and aluminum nitrate methods, respectively. Phytochemical compounds were analyzed by HPLC-DAD-MS/MS technique. EtOAc fraction exhibited broad spectrum antimicrobial activities against all tested microorganism especially against *Candida albicans* with the MIC of 0.95 μg/mL. Similarly, this fraction also exhibited the strongest antioxidant potential among tested samples. The preliminary phytochemical investigation showed that the EtOAc fraction possessed a highest total flavones, total phenolics contents (21.65 and 42.11% respectively) and seven major compounds of the EtOAc fraction were characterized by HPLC-DAD-MS/MS techniques. 3-*p*-coumaroylquinic acid and kaempferol 3-*O*-glucoside were the main compounds. The results indicate the possibility that the observed bioactivities are partly contributed by phenolic acids and flavonoids especially by the compounds of 3-*p*-coumaroylquinic acid and kaempferol 3-*O*-glucoside.

Keywords: *Adiantum capillus–veneris*; antimicrobial; antioxidant; cytotoxicity; HPLC-DAD-MS/MS

Introduction

Adiantum capillus-veneris L. is a widely distributed plant species that has been extensively used in traditional Chinese medicine (TCM). Its beneficial effects are observed against dermatitis and cystitis, cough, toothache dental abscesses, gastritis, respiratory problems and tumors of spleen, liver and other viscera [1-4].

In the literatures, many health benefits associated to occurrence of different classes of bioactive secondary metabolites have been reported. These classes include, among others, flavonoids and polyphenols which possessed antimicrobial, antioxidant and cytotoxic activities [5, 6]. *A. capillus–veneris* L. is an important natural source of compounds from both classes. Because of the high level content of flavonoids and phenols presented in it, the biological properties attributed to this species, including anti-inflammatory, anti-infective and anti-tumors may originate from the these components [3] and the probable functional mechanism were antimicrobial and antioxidant effects [3, 7].

There were some researches for *A. capillus–veneris* L. on its chemical compositions and biological activities of total extract. However, the correlation between chemical compositions and pharmacological action, i.e. active fractions or active ingredients of this species are still unexplored. The aim of this study is to assess biological properties of *Adiantum capillus-veneris*. A HPLC-DAD-MS/MS approach is used for the rapid tentative identification of functional compounds in plant extracts.

Results and Discussion

Antimicrobial Activity

The crude extract of *A. capillus-veneris* L. was examined for their antimicrobial (Table-1) properties. The most susceptible microorganisms were found to be *Staphylococcus aureus* and *Candida albicans* (both IC_{50} were 15.63 μg/mL). *Candida albicans* strains were selected to evaluate the MIC of fractions due to its strong pathogenicity. The EtOAc fraction gave the lowest MIC values (0.95 μg/mL) followed by n-BuOH fraction (2.0 μg/mL). The profile based on the antimicrobial activity of EtOAc fraction correlated to that based on the amount of total phenolics (Table-2). By reference to some literatures [8], it is highly likely that the total phenolics in this plant may be responsible for the potent antimicrobial activity.

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Table-1: MIC values of crude extract and different fractions of *Adiantum capillus-veneris* L.

samples	MIC (μg/mL)						
	Sa	Se	Ef	Bs	Ec	Pa	Ca
Crude extract	**15.63**	**31.2**	**31.25**	**31.25**	**31.25**	**62.50**	**15.63**
Petroleum ether							**>31.25**
EtOAc							**0.95**
n-BuOH							**2.00**
hydromethanol							**3.70**

Sa: *Staphylococcus aureus*; Se: *Staphylococcus epidermidis*; Ef: *Enterococcus faecaelis*; Bs: *Beta-hemolytic streptococcus*; Ec: *Escherichia coli*; Pa: *Pseudomonas aeruginosa*; Ca: *Candida albicans*; MIC: minimum inhibitory concentration.

Antioxidant Activity in vitro

The reduction of carcinogenesis and inhibition of pathogenic bacterial growth is often associated with the termination of free radical propagation in biological systems [9]. Since the antioxidants can interrupt the free radical chain reaction of oxidation by the formation of stable forms of free radicals, prohibited initiate or propagate further oxidation [10]. In order to investigate the mechanism of the antimicrobial and cytotoxicity activity of *A. capillus-veneris* L., series of antioxidant assays were carried out.

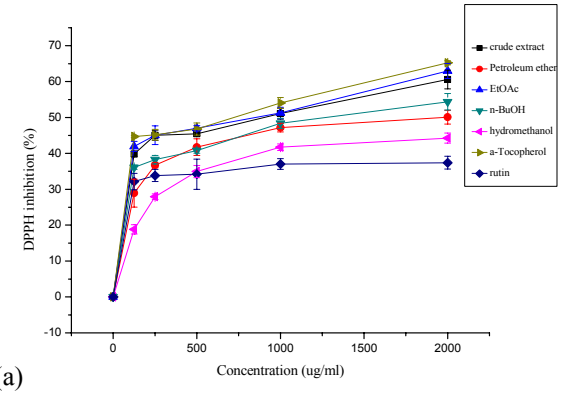
Assays based on the use of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals are among the most popular spectrophotometric methods for determination of the antioxidant capacity of samples [11]. These two assays evaluate the ability of antioxidants to scavenge free radicals [12]. As shown in Fig. 1, EtOAc faction exhibited the most effective free radical scavenging activity (852.5 μg/mL and 28.21 μg/mL respectively).

For the measurements of the reductive ability of samples, the Fe^{3+}–Fe^{2+} transformation was investigated using the method proposed by Yildirim et al [13]. Still, Fig. 1 showed the EtOAc fraction had the most effective reducing power (EC_{50}=495.3 μg/mL) which indicates its electron donor properties. It was confirmed that the electron donating capacity, reflecting the reducing power of bioactive compounds, is associated with antioxidant activity [14].

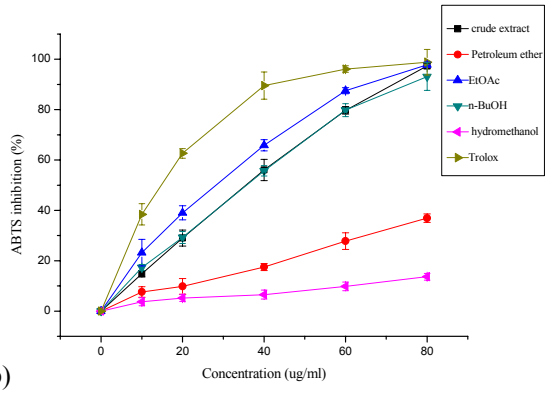
In Table-2, a positive correlation was observed between the antioxidant activity and the amount of flavonoids of the samples in our study. This correlation was also confirmed by other literatures [15, 16]. So it can be inferred the total flavonoids presented in this plant may be responsible for potential antioxidant activity. Furthermore, the antioxidation may be contributed to the antimicrobial and cytotoxicity activity.

Cytotoxicity Activity

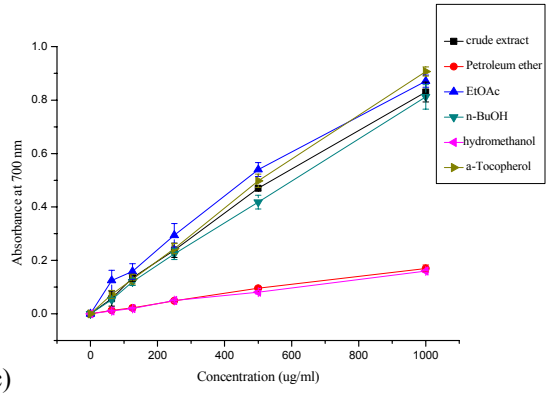
EtOAc fraction of *A. capillus-veneris* L. was evaluated for the inhibitory activity against the growth of human gastric carcinoma SGC-7901 cells *in vitro*. 5-fluorouracil (5-FU) was used as positive control. As illustrated in Table-2 and Fig. 2, EtOAc fraction showed certain inhibitory activity against this cell line with dose dependent pattern.



(a)



(b)



(c)

Fig. 1: free radical scavenging activity and reducing power of different successive fractions and crude extract of *Adiantum capillus-veneris* L. and compared with α-tocopherol Trolox and rutin. All the data are reported as the means ± SD for three measurements. (a) DPPH free radical scavenging activity ($DPPH^{\bullet}$: 1,1-diphenyl-2-picryl-hydrazyl free radical). (b) ABTS radical scavenging activity ($ABTS^{\bullet+}$: 2.2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)).(c) Reducing power (Fe^{3+}–Fe^{2+} transformations).

Table-2: Antioxidant activity, cytotoxicity and total amount of phenolics, tannins and flavonoids of crude extract and different fractions of *Adiantum capillus-veneris* L.

samples	Total phenolics as gallic acid equivalents (mg/g of samples)	tannins as gallic acid equivalents (mg/g of samples)	Flavonoids as rutin equivalents (mg/g samples)	DPPH assay SC_{50} (mg/mL)[a]	ABTS assay SC_{50} (mg/mL)[a]	Reducing power EC_{50} (mg/mL)[a]	Cytotoxicity IC_{50} (mg/mL)[a]
Crude extract	**175.4±0.03**	**104.9±0.02**	**161.2±0.09**	**888.42±91.87**	**35.56±5.17**	**557.14±34.21**	
Petroleum ether	**13.2±0.01**	**0.7±0.03**	**24.7±0.06**	**1965.7±128.28**	**>80.00**	**>1000.00**	
EtOAc	**421.1±0.02**	**168.3±0.02**	**216.5±0.02**	**856.5±67.82**	**28.21±2.16**	**452.00±13.20**	**126.53±16.62**
n-BuOH	**334.7±0.07**	**190.7±0.08**	**120.2±0.02**	**1274.1±69.14**	**35.70±1.16**	**595.03±5.30**	
hydromethanol	**113.9±0.04**	**18.3±0.04**	**24.8±0.03**	**>2000**	**>80.00**	**>1000.00**	
Trolox [b]					**14.80±1.27**		
Rutin [b]				**>2000**			
a-Tocopherol [b]				**719.86±25.76**		**513.75±29.17**	
5-FU [b]							**8.71±0.30**

[a] values were expressed as mean ± SD (n=3)
[b] positive controls

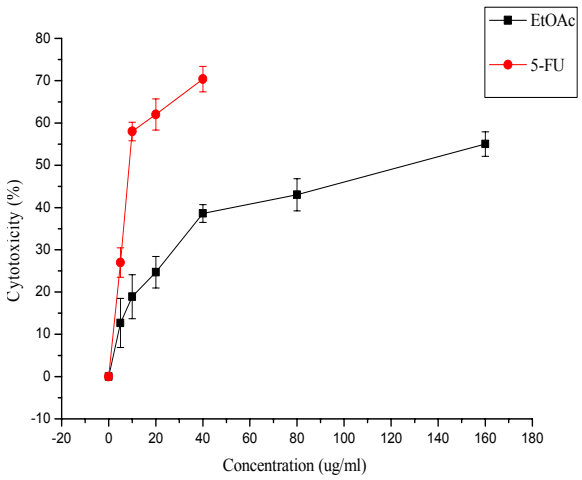


Fig. 2: Growth inhibition curves of EtOAc fraction of *Adiantum capillus-veneris* L. on the SGC-7901 cell line for 48 h in vitro. 5-fluorouracil (5-FU) was used as positive control. All the data are reported as the means ± SD for three measurements.

Phytochemical Screening

The phytochemical results of the samples are given in Table-3. All samples contained phenols and flavonoids except petroleum ether fraction. Interestingly, petroleum ether fraction did not exhibit obvious antimicrobial and antioxidant activity compared with other fractions. The results provided strong evidence of an association between phenols and flavonoids constituents and their biological activities.

Total Phenolics, Tannins and Flavonoids

Table-2 showed the total phenolic, casein-absorbed tannin contents and total flavonoids of the all samples (extract and fractions). The content of the phenolics and flavonoids in EtOAc fraction was the highest (421.1 mg/g GAE and 216.5 mg/g RE, respectively), whereas the amount of the casein-absorbed tannins was highest in n-BuOH fraction (190.7 mg/g GAE), followed by EtOAc fraction (168.3 mg/g).

Table-3: Phytochemical constituents of crude extract and different fractions of *Adiantum capillus-veneris* L.

sample	phytocompounds										
	Ph	**Ta**	**F**	**Ant**	**Tr**	**St**	**Sa**	**An**	**Oa**	**SPG**	**APP**
Crude extract	+	+	+	-	+	+	+	+	+	+	+
petroleum ether	-	-	-	-	-	+	-	-	-	-	-
EtOAc	+	+	+	-	+	+	+	+	+	+	-
n-BuOH	+	+	+	-	+	-	+	-	+	+	-
hydromethanol	+	+	-	-	-	-	+	-	-	+	+

Ph, Phenols; Ta, Tannins; F, flavonoids; Ant, Anthocyanidins; Tr, Triterpenoids; St, Sterols; Sa, Saponins; An, Anthraquinones; Oa, Organic acid; SPG, Saccharides/ polysaccharides/ glycoside; APP, Amino acid/ polypeptide/ protein

HPLC-DAD-MS/MS Analysis for Components Identification

Because EtOAc fraction exhibited the strongest antimicrobial, antioxidant and cytotoxic activities, it was significant to analysis its chemical composition. Since the technical of hyphenated LC-DAD-MS/MS instrumentation is an effective technique for a rapid mixtures screening and structure elucidation [17, 18], it was employed for chemical analysis.

In order to obtain chromatograms with good separation and strong total ion current (TIC), low-gradient slope and CH_3OH/H_2O-HCOOH (100:1, v/v) was found to be the optimal mobile phase in both HPLC and MS analyses. The addition of HCOOH had substantial effect on the selectivity and efficiency. Four detection wavelengths of 220, 260, 280 and 310 nm were chosen to record chromatograms. The 280 nm wavelength was chosen for a sufficiently large number of detectable peaks and smooth baseline obtained on the chromatographic profiles. The chromatograms of the EtOAc fraction were presented in Fig. 3.

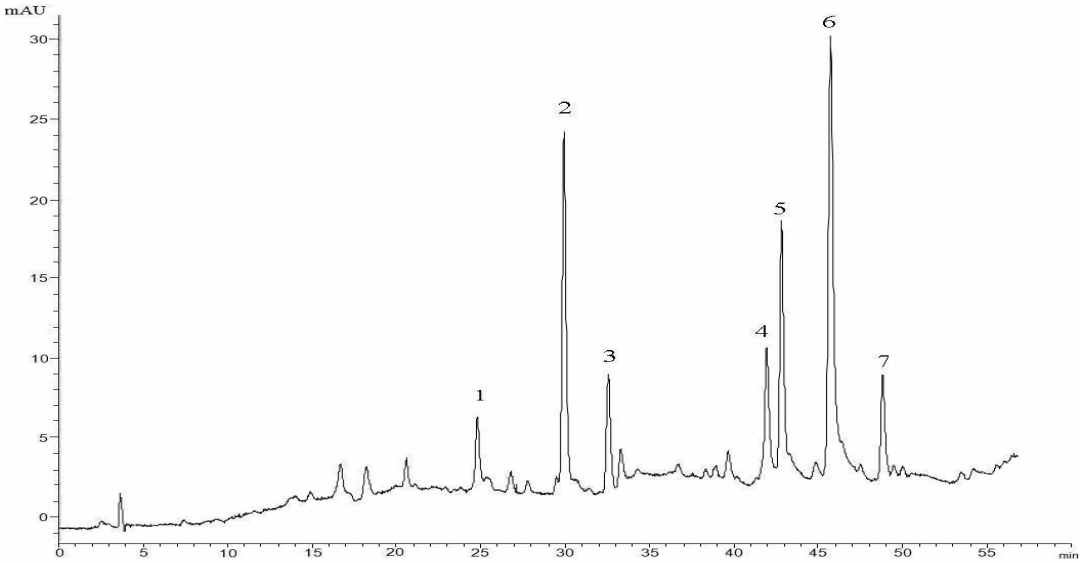


Fig. 3: Chromatographic profiles acquired by HPLC-DAD with the Agilent TC-C_{18} column at 280 nm of the EtOAc fraction of *Adiantum capillus-veneris* L. Peak 1=5-*O*-caffeoylquinic acid; 2=3-*p*-coumaroylquinic acid; 3=4-*p*-coumaroylquinic acid; 4=quercetin 3-*O*-glucoside; 5=epicatechin 7-*O*-rutinoside; 6= kaempferol 3-*O*-glucoside; 7= hydroxycinnamic derivative.

The screening, identification and further characterizing of the components in EtOAc fraction were performed firstly by HPLC–MS in both the positive and negative ion modes. Their TIC chromatograms are shown in Fig. 4. The results showed that ESI in positive mode was particularly sensitive to all chemical components of EtOAc fraction in *A. capillus-veneris* L. The data of DAD and MS/MS were then checked to identify and characterize the chemical constitution.

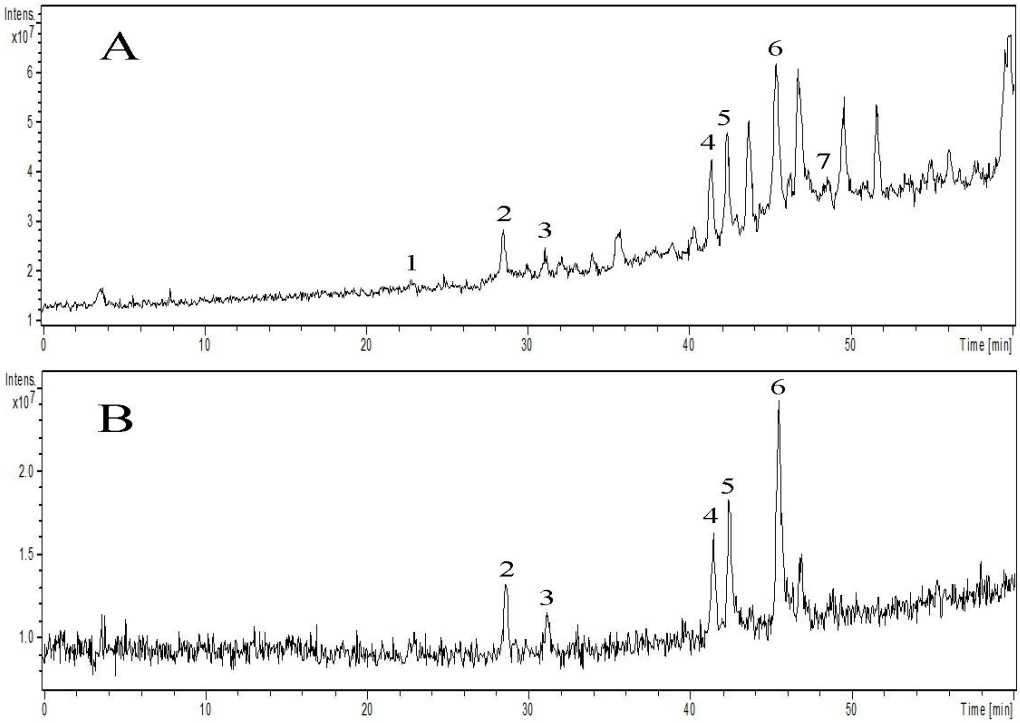


Fig. 4: Electrospray ionization mass spectrometry (ESI/MS) analysis of the EtOAc fraction of *Adiantum capillus-veneris* L. was performed by both positive (A) and negative (B) ionization modes.

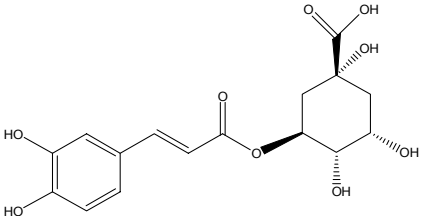
Compounds **4** and **6** were unambiguously identified by comparing with the authentic compounds. For unknown constituents, the structures were characterized based on their MS data, literature reports, retention times, and UV-vis spectra. MS, MS/MS and UV data are summarized in Table-4. The structures of the major components are depicted in Fig. 5.

Table-4: Characterization of compounds in the EtOAc fraction of *Adiantum capillus-veneris* L. by HPLC-DAD-MS/MS.

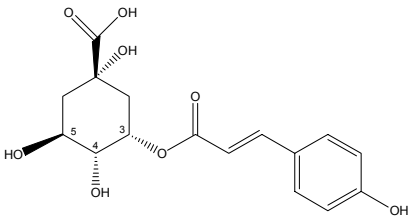
Peak No.	Retention Time (min)	Compound	$\lambda_{max\ (nm)}$	$[M+Na]^+$	$[M-H]^-$	Fragments ions m/z
1	**22.78**	**5-*O*-caffeoylquinic acid**	**326.8**	**377**		**$[M+Na-H_2O]^+$: 359 $[M+Na-HCOOH]^+$: 331 $[caffeoyl+Na]^+$: 185 $[qunic\ acid+Na]^+$: 215**
2	**28.39**	**3-*p*-coumaroylquinic acid**	**313**	**361**	**337**	**$[M+Na-H2O]^+$: 343 $[M+Na-HCOOH]^+$: 315 $[qunic\ acid+Na]^+$: 215 $[qunic\ acid+Na-H_2O]^+$: 197 $[qunic\ acid+Na-H-COO]^+$: 169 $[hydroxycinnamic\ acid]^+$: 147**
3	**30.96**	**4-*p*-coumaroylquinic acid**	**306**	**361**	**337**	**$[M+Na-H2O]^+$: 343 $[M+Na-HCOOH]^+$: 315 $[qunic\ acid+Na]^+$: 215 $[qunic\ acid+Na-H_2O]^+$: 197 $[qunic\ acid+Na-H-COO]^+$: 169 $[hydroxycinnamic\ acid]^+$: 147**
4[a]	**41.20**	**quercetin 3-*O*-glucoside**	**255.7, 353.4**	**487**	**463**	**$[M+Na-Glu]^+$: 325 $[Glu+Na]^+$: 185**
5	**42.17**	**epicatechin 7-*O*-rutinoside**	**265.2, 348.3**	**621**	**597**	**$[M+Na-H_2O]^+$: 603 $[M+Na-H_2O-Rha]^+$: 457 $[M+Na-H_2O-Rha-Glu]^+$: 295**
6[a]	**45.21**	**kaempferol 3-*O*-glucoside**	**285**	**471**	**447**	**$[M+Na-Glu]^+$: 309 $[Glu+Na]^+$: 185**
7	**48.43**	**hydroxycinnamic derivative**	**291.2, 309.1**	**373**	**349**	**$[M+Na-$ hydroxycinnamic acid$]^+$: 227**

Glu, glucose; Rha, rhamnose.

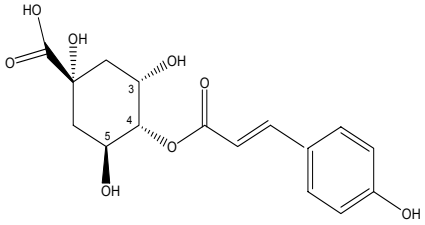
[a] Compared with authentic compounds.



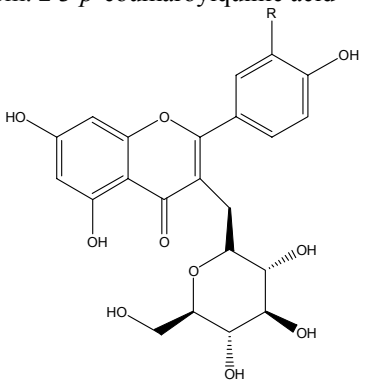
Com. 1 5-*O*-caffeoylquinic acid



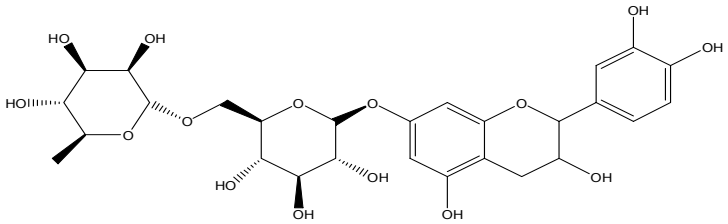
Com. 2 3-*p*-coumaroylquinic acid



Com. 3 4-*p*-coumaroylquinic acid



Com. 4 R=OH, quercetin 3-*O*-glucoside
Com. 6 R=H, kaempferol 3-*O*-glucoside



Com. 5 Epicatechin-7-*O*-rutinoside

Fig. 5: Chemical structures of the major components were identified in the EtOAc fraction of *Adiantum capillus-veneris* L.

Compound **1** presented a λ_{max} at 326.8, attributable to the caffeoyl group. The positive ESI-MS spectrum exhibited a signal at *m/z* 377 ($[M+Na]^+$), 355 ($[M+H]^+$) and a fragment at *m/z* 215 and 185. This suggested a very high tendency of the quinic acid unit and caffeoyl unit. By compared with literature [19], compound **1** was tentatively identified as 5-*O*-caffeoylquinic acid.

Compounds **2** and **3** were a pair of isomers. Both of them exhibited the same $[M+Na]^+$ ion at *m/z* 361 and $[M-H]^-$ ion at 337 and the same MS^2 ion (at *m/z* 343, 315, 215, 197, 169 and 147) at positive mode in ECI MS^2. The occurrence of the ion at *m/z* 215 and 147 allowed identifying the presence of the quinic acid unit and hydroxycinnamic acid unit. But different UV-vis spectra (λ_{max} at 313 and 306 nm respectively) and different retention times (28.5 and 30.9 min respectively) were observed. With reference to relevant literature [20], compounds **2** and **3** were plausibly identified as 3-*p*-coumaroylquinic acid and 4-*p*-coumaroylquinic acid, respectively.

Compounds **4**, **5** and **6** were all flavonoids of glycosylated derivatives of three flavonols, i.e. quercetin (255.7 and 353.4 nm for 3-glycosides), flavanol (285.3 nm) and kaempferol (265.2 and 348.3 nm for 3-glycosides) [21]. The presence of $[M+Na]^+$ signals in the positive ESI-MS spectra of compounds **4**, **5** and **6** together with the $[M-H]^-$ ions in the negative ESI-MS spectra allowed the determination of the molecular weights (464, 598 and 448 respectively). Compounds **4** and **6** presented the same ions of $[glucose+Na]^+$ and $[M+Na-162]^+$, which suggested the quercetin and kaempferol group connected with the glucosyl group. Compared to the characteristic fragmentation of authentic compounds, compounds **4** and **6** were identified as quercetin 3-*O*-glucoside and kaempferol 3-*O*-glucoside, respectively. The MS^2 ions of compound **5** at the m/z 603, 457, 279 that originated from the successive losses of rhamnose and glucose group, indicated it was flavanol group connected with the rutinose group. By referring to the literature data [22], it was tentatively identified as Epicatechin-7-*O*-rutinoside.

Compound **7** presented a λ_{max} at 291.2 and 309.1, attributable to the hydroxycinnamic acid chromophore. The positive ESI-MS spectrum exhibited a signal at m/z 373 $[M+Na]^+$ and the negative ion ESI-MS spectrum showed ions at m/z 349 $[M-1]^-$. The MS^2 ion at m/z 227 showed a high tendency to lose hydroxycinnamic acid. Therefore compound **7** may be regarded as a hydroxycinnamic derivative.

Experimental

Plant Material

The whole herb of *A. capillus–veneris* L. were collected in September 2008 from Dali, Yunnan, China and were kindly identified by Dr. Jianping Wang from Tongji Medical Center of Huanzhong University of Science and Technology, Wuhan, China. A voucher specimen (AC0810) was deposited in the key laboratory of natural medicinal chemistry and resources evaluation of Tongji Medical Center of Huanzhong University of Science and Technology. The plants were dried in shade, finely powdered with electric precision cracker for future use.

Preparation of the Methanol Extract and its Successive Fractions

Fine powdered herbs of the plant (100 g) were refluxed with methanol (1500 ml × 3) at 70 ^oC for 60 min. The solvent was evaporated using a rotatory evaporator (70 ^oC) to produce a crude extract (10 g). Crude extract (9 g) was dissolved in 300 ml distilled water and then partitioned with petroleum ether, ethyl acetate (EtOAc), n-butanol (n-BuOH) successively to give petroleum ether fraction (2.3 g), EtOAc fraction (0.7 g), n-BuOH fraction (3.0 g) and the remaining hydromethanolic soluble fraction (2.9 g). The solvents of those five samples (total extracts and four fractions) were evaporated to get the residues, which were stored at -20 ^oC prior to the biological testing and phytochemical analysis.

Antimicrobial Assay

Microbial Strains and Cultivation

The samples were evaluated against a panel of microorganisms, including four Gram-positive bacteria, (*Staphylococcus aureus, Staphylococcus epidermidis*, *Beta-hemolytic streptococcus*, *Enterococcus faecaelis*), two Gram-negative bacteria, (*Escherichia coli*, *Pseudomonas aeruginosa*) and one fungus (*Candida albicans)*. These microorganisms were procured from Tongji medical college and Tongji hospital, Wuhan, China. All of the microorganisms were pure. Each microbial suspension (100 µl, 0.5-1.0× 106 CFU/ml) was inoculated into 5 ml of broth (MHB/PDB). Bacterial strains were incubated at 37^oC for 12 h and fungus was incubated at 30^oC for 48 h.

MIC Testing

The minimum inhibitory concentration (MIC) of the crude extract of *A. capillus-veneris* L. was determined by using the broth dilution method [23]. The plant extract dissolved in 1% dimethyl sulfoxide (DMSO) was two-fold diluted from 100.0 to 0.2 µg/mL (final volume =1 ml). A volume of 100 µl bacterial or fungus suspensions (0.5-1.0 × 106 CFU/ml) was inoculated into 5 ml sterile tubes which were incubated at 37 ^oC for 24 h for bacteria and at 30 ^oC for 48 h for fungus. The same tests were performed simultaneously for growth control (broth + bacteria/fungus) and sterility control (broth + extracts). The MIC values were calculated as the

highest dilution showing complete inhibition of the tested strains. According to the MIC values, one of the most susceptible pathogenic strains was selected to evaluate the MIC of fractions of this plant.

Antioxidant Activity in vitro

DPPH Assay

The DPPH$^{\bullet}$ (1,1-diphenyl-2-picryl-hydrazyl free radical) assay was performed as described by Shirwaikar et al. [24]. This method was based on the reduction of purple DPPH$^{\bullet}$ to a yellow colored diphenyl picrylhydrazine. The remaining DPPH$^{\bullet}$ which allowed maximum absorption at 517 nm wavelength was measured [25]. About 1.5 ml of various concentrations (0.125-2.000 mg/mL) of each extract was added to 2.5 ml solution of 0.1 mM DPPH$^{\bullet}$ (Sigma-Aldrich, Germany). A mixture of 1.5 ml methanol and 2.5 ml DPPH$^{\bullet}$ served as control. After 20 min of incubation at 37 ^{o}C in the dark, the absorbance was recorded against a blank (2.5 ml methanol and 1.5 ml extract) at 517 nm wavelength. The experiment was performed in triplicates.

Percentage inhibition was obtained from the following equation:

DPPH radical inhibition(%)=($A_{control}$-Asample)/Acontrol×100

The antioxidant activity of the samples was also expressed as IC_{50} (inhibitory concentration), which was defined as the concentration of sample required to inhibit the formation of DPPH radicals by 50%. α-tocopherol (Sigma-Aldrich, Germany) . Rutin (NICPBP, Beijing, China) were used as positive control.

ABTS Assay

ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) formed a relatively stable free radical, which decolorizes in its non-radical form [24]. The ABTS$^{\bullet+}$ scavenging activity was determined according to the method of Re et al [26]. In this method, an antioxidant is added to a pre-formed ABTS radical solution. After a fixed time period, the remaining ABTS$^{\bullet+}$ was quantified spectrophotometrically at 734 nm wavelength. ABTS$^{\bullet+}$ was produced by reacting 7mM ABTS in methanol with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 12 hours. The ABTS$^{\bullet+}$ solution was diluted to give an absorbance of 0.51±0.02 at 734 nm. Then, 1 ml of various concentrations (20-100 μg/mL) of each extract was added to 4 ml ABTS$^{\bullet+}$ solution. The absorbance was recorded at 30 ^{o}C after mixing 6 min. The percentage of radical scavenging was calculated for each concentration with respect to a blank containing no scavenger. The experiment was performed in triplicates.

Percentage inhibition was obtained from the following equation:

ABTS radical inhibition(%)=(Acontrol-Asample)/Acontrol×100

IC_{50} values were calculated. Trolox was used as positive control.

Reducing Power

For the measurements of the reductive ability of such samples, the Fe^{3+}–Fe^{2+} transformation was investigated using the method of Yildirim et al. [13] with slight modification. 2 ml various concentrations (63-500 mg/mL) of each extract was mixed with 2 ml phosphate buffer (0.2 M, PH 6.8) and 2.0 ml 1% (w/v) potassium ferricyanide (K3FeCN6). After that, the mixture was incubated at 50 ^{o}C for 20 min. About 2 ml (10%, w/v) trichloroacetic acids were added to the mixture and centrifuged at 3000 rpm for 10 min. Finally 2.5 ml of the supernatant solution was mixed with 2 ml distilled water and 0.5 ml (0.1%, w/v) $FeCl_3$, and the absorbance was recorded at 700 nm after mixing 10 min. α-tocopherol was used as positive control. The experiment was performed in triplicates. The increased absorbance of the reaction mixture meant the increased reducing power. The EC_{50} (effective concentration) values (μg/mL) were calculated, which indicated the effective concentration at which the absorbance was 0.5 for reducing power.

Biological Activity

Cell culture

SGC-7901, a kind of human gastric carcinoma cells from the biochemistry laboratory (Huazhong University of science and technology, Wuhan, China) was cultured in RPMI 1640 medium (GIBCO, Shanghai, China) with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/mL streptomycin under an atmosphere of 5% CO_2 at 37 ^{o}C. The numbers of four kinds of cells were maintained at 1×10^6 /ml by daily adjusting cell concentration.

Cytotoxicity Assay

A cytotoxicity effect on SGC-7901 was observed only for EtOAc fraction. MTT [3-(4,5-dimethythiazoyl-2-yl) 2,5-diphenylte-trozo-liumbromide] (sigma) colori metric analysis was used to measure the cytocide rate of EtOAc fraction [27]. Cells in log growth period diluted with 200 μl RPMI 1640 supplemented with 10% fetal calf serum were seeded into 96-well plates at 1×10^4/well. After

culturing for 12 h, different concentrations of the samples (0 (control), 5, 10, 20, 40, 80, 100, 200, 400 μg/mL) were added. 48 h later, 20 μl MTT solutions (5 mg/mL) was put into plates. Then the supernatant was discarded after 4 hours and 150 μl DMSO was added to melt crystal. The values were recorded at 490 nm wavelength. 5-FU was used as positive control.

Cytocide rate was calculated by the following formula:

Cytocide rate (%) = ($OD_{control}$ - OD_{sample})/OD_{sample} × 100

IC_{50}, the EtOAc fraction concentration resulting in 50% cytotoxicity was determined from the graph.

Phytochemical Screening

The screening of the chemical constituents of each sample was carried out employing the chemical methods described by Farnsworth [28].

The Content Determination of Total Phenolics, Tannins and Flavones

The amount of phenolic compounds in the plant samples was determined with the Folin-Ciocalteau method [29] with some modifications. About 1.0 ml sample was resuspended in distilled water (2 mg/mL), and then mixed thoroughly with the Folin-Ciocalteau reagent (FCR, 1 ml), distilled water (10 ml) and 29% Na_2CO_3 solution (12 ml). After a 30 min incubation period at 30 ^oC, the absorbance was recorded against a blank at 730 nm. All determinations were performed in triplicates. The gallic acid was used as a standard for calibration curve. The total content of the phenolic compounds was expressed in mg/g plant samples (crude extract and fractions), in gallic acid equivalents (GAE).

The total content of tannins was determined using FCR [29]. About 20 ml (2 mg/mL) of each sample was mixed with 600 mg casein with interval shaking at 30 ^oC for 1 hour and then filtered. The total casein-absorbed tannins contents were determined also using Folin-Ciocalteau method as described before and expressed as the number of equivalents of gallic acid (GAE).

The amount of flavonoids was determined as previously described [30-32]. Rutin was used as the standard for the calibration curve. About 1.0 ml sample was dissolved in methanol (2 mg/mL) and then mixed with 9 ml methanol and 1 ml 5% $NaNO_2$. 1 ml 5% $Al(NO_3)_3$ and 10 ml 4% NaOH were added to the mixture subsequently after 6 min. The mixture was allowed to stand at 20 ^oC for 15 min. The absorbance of it was measured at 501 nm wavelength. The blank was prepared from the plant samples (1 ml) and diluted to 25 ml in methanol. All determinations were performed in triplicates. The total content of the flavonoids was expressed in mg/g plant samples, in rutin equivalents (RE).

HPLC-DAD-MS/MS Analyses

Standards

Authentic standards of kaempferol 3-*O*-glucoside, quercetin 3-*O*-glucoside were purchased from NICPBP (Beijing, China).

Solvents

HPLC-grade methanol and formic acid were purchased from Kermel Company Inc. (Kermel, China). Distilled water was produced using a water purification system (Heal Force super NW, Hong-Kong).

Sample Preparation

The dry extracts of EtOAc fractions were dissolved in methanol (0.25 mg/mL) and filtered through a 0.45 μm membrane filter (JINTENG, China).

HPLC-DAD-MS/MS Instrumentation and Conditions

HPLC-DAD-MS/MS (liquid chromatography coupled to electrospray tandem mass spectrometry) analysis were carried out using a Agilent 1100 LC/MSD instrument (Agilent, USA) linked simultaneously to both of a diode array detector (DAD) (Agilent 1100, USA) and a Trap-XCT mass spectrometer (Agilent, USA), equipped with a Z-spray electrospray ionization (ESI) source operating in both negative and positive mode. LC/MSD trap software (version 5.2, Agilent, USA) was used to control the instruments, and for data acquisition and processing.

Sample solutions were separated on an Agilent TC-C_{18} column (4.6×250 mm, i.d. 5μm, Serial No. USEGK03151, USA) with Agilent C_{18} guard column (4.6 mm ID × 12.5 mm, Part No. 820888-901), which was maintained at 22 ^oC. A linear gradient elution of (A) MeOH and (B) H_2O–HCOOH (100:1, v/v) was used. The gradient programmer was used according the following profile: 0–60min, 10% A linear increase to 70% A. The solvent flow rate was 1 ml/min. 20 μl of sample solution was injected in each run. The effluent was introduced into a photo diode array detector (PDA detector) (scanning range 190–400 nm, resolution 1.2 nm) and subsequently into an electrospray source (Dry Temp 350 ^oC, Dry Gas 10 l/min, HV capillary

3500 V). The split ratio of HPLC flow between PAD detector and MS detector was 2:1.

Conclusion

In this work, a general procedure to carry out the screening for bioactive compounds is presented and applied to *A. capillus-veneris*. The results were encouraging as EtOAc fraction showed the significant pharmacological activities and the major compounds in this fraction have been elucidated. This methodology looks promising for it allows establishing the correlation of the functional activities and compounds found and can be considered as a powerful tool to find functional interesting compounds from *A. capillus-veneris*. However, the action mechanisms of these compounds and the real bioactive compounds are still unknown and need further investigation.

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Declaration of Interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

References

1. S. Lee, C. Xiao, S. Pei, *Journal of Ethnopharmacology*, **117**, 362 (2008).
2. M. T. Palmese, R. E. Uncini Manganelli, P. E. Tomei, *Fitoterapia*, **72**, 619 (2001).
3. M. Singh, N. Singh, P. B. khare, A. K. S. Rawat, *Journal of Ethnopharmacology*, **115**, 327 (2008).
4. L. J. McGaw, N. Lall, J. J. M. Meyer, J. N. Eloff, *Journal of Ethnopharmacology*, **119**, 482 (2008).
5. R. W. Soeder, *Journal of Botanical Review*, **51**, 442 (1985).
6. T. P. Cushnie, A. J. Lamb, *International Journal of Antimicrobial Agents*, **26**, 343 (2005).
7. F. Pourmorad, S. J. Hosseinimehr, N. Shahabimajd, *African Journal of Biotechnology*, **5**, 1142 (2006).
8. R. Puupponen-Pimiä, L. Nohynek, C. Meier, M. Känhkönen, A. Hopia, K. M. Oksman-Caldentey, *Journal of Applied Microbiology*, **90**, 494 (2001).
9. Q. Y. Zhu, R. M. Hackman, J. L. Ensunsa, *Journal of Agric Food Chemistry*, **50**, 6929 (2002).
10. S. Arabshahi-Delouee, A. Urooj, *Journal of Food Chemistry*, **102**, 1233 (2007).
11. K. Thaipong, U. Boonprakob, K. Crosby, L. Cisneros-Zevallosc, D. H. Byrne, *Journal of Food Composition and Analysis*, **19**, 669 (2006).
12. J. Pérez-Jiménez, F. Saura-Calixto, *Food Research International*, **39**, 791 (2006).
13. A. Yildirim, A. Mavi, A. A. Kara, M. Otkav, O. F. Alqur, V. Bilaloqlu, *Journal of Agriculturaland Food Chemistry*, **48**, 5030 (2000).
14. S. Arabshahi-Delouee, A. Urooj, *Food Chemistry*, **102**, 1233 (2007).
15. R. L. Fabri, M. S. Nogueira, F. G. Braga, E. S. Coimbra and E. Scio, *Bioresource Technology*, **100**, 428 (2009).
16. E. S. S. Abdel-Hameed, *Food Chemistry*, **114**, 1271 (2009).
17. Y. Chen, Y. Yan, M. Y. Xie, S. P. Nie, W. Liu, X. F. Gong and Y. X. Wang, *Journal of Pharmaceutical and Biomedical Analysis*, **47**, 469 (2008).
18. M. Yang, J. Sun, Z. Lu, G. Chen, S. Guan, X. Liu, B. Jiang, M. Ye and D. A. Guo, *Journal of Chromatography* A, **1216**, 2045 (2009).
19. G. M. Weisz, D. R. Kammerer and R. Carle, *Food Chemistry*, **115**, 758 (2009).
20. L. Z. Lin and J. M. Harnly, *Journal of Agricultural and Food Chemistry*, **56**, 10105 (2008).
21. A. Romani, P. Vignolini, L. Isolani, F. Ieri, D. Heimler, *Journal of Agricultural Food Chemistry*, **54**, 1342 (2006).
22. I. Parejoa, E. Capraia, J. Bastida, F. Viladomat, O. Jauregui and C. Codina, *Journal of Ethnopharmacology*, **94**, 175 (2004).
23. NCCLS (National Committee for Clinical Laboratory Standards). Performance standard for antimicrobial susceptibility testing; Ninth Informational Supplement, NCCLS document M100-S9. NCCLS, Wayne, PA, pp 120-126 (2008)
24. A. Shirwaikar, A. Shirwaikar, K. Rajendran and I. S. R. Punitha, *Biological & Pharmaceutical Bulletin*, **29**, 1906 (2006).
25. M. Burits and F. Bucar, *Phytotherapy Research*, **14**, 323 (2000).
26. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, *Free Radical Biology & Medicine*, **26**, 1231 (1999).
27. F. Yin, Y. Shi, W. Zhao, B. Xiao, J. Miao and D. Fan, *World Journal of Gastroenterology*, **6**, 664 (2000).
28. N. R. Farnsworth, *Journal of Pharmaceutical Sciences*, **55**, 225 (1966).
29. Pharmacopoeia Commission of People's Republic of China. Pharmacopoeia of the Peoples Republic of China, vol. 1, Chemical Industry

Press, Beijing, China, appendix 57, 99 (2005).
30. M. I. Nieva Moreno, M. I. Isla, A. R. Sampietro and M. A. Vattuone, *Journal of Ethnopharmacology*, **71**, 109 (2000).
31. I. Hussain, M. A. Khan and J. Ali, *Journal of the Chemical Society of Pakistan*, **33**, 499 (2011).
32. M. K. Khosa, S. A. S. Chatha, M. Nisar, K. M. Zia, Khalil-Ur-Rehman, M. A. Jamal, M. Yousaf, Ali, *Journal of the Chemical Society of Pakistan*, **33**, 421 (2011).