Int J Pharm Bio Sci 2012 Oct; 3(4): (P) 478 - 492

Research Article **Pharmacognosy**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

POLYSACCHARIDE FROM THE ROOTS OF *POLYGONUM EQUISETIFORME* SIBTH. & SM.: ISOLATION, CHEMICAL COMPOSITION, ANTIOXIDANT POTENTIAL AND CYTOTOXIC ACTIVITY

TAGHREED A. IBRAHIM [*1,2] AND ATEF A. EL-HELA [3]

[1] Pharmacognosy Department, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia.
[2] Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.
[3] Pharmacognosy Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.

ABSTRACT

A water-soluble polysaccharide (PEP) was isolated and purified from the aqueous extract of *Polygonum equisetiforme* Sibth. & Sm. roots. Structural features of PEP were investigated by a combination of instrumental and chemical analyses, including acid hydrolysis, methylation analysis, IR, GPC, HPLC and GC/MS. The results indicated that PEP has a backbone of (1→4)-linked glucose residues, which occasionally branches at O-6. The branches are mainly composed of (1→4)-linked rhamnose, (1→6)-linked glucose, (1→6)-linked galactose and (1→3)-linked mannose and terminated with arabinose and rhamnose residues. PEP exhibited high ABTS, DPPH, OH free radicals scavenging activities compared to the ascorbic acid reference control. The in vitro cytotoxicity of PEP was dose-dependent with IC_{50} values of 14.67 µg/mL and 22.34 µg/mL against colon and breast cancer cell lines, respectively. These results suggest that PEP may be an effective natural antioxidant compound, with little antitumor activity.

KEYWORDS : Polygonum, Polysaccharide, Gel permeation, Antioxidant, Cytotoxic activities

TAGHREED A. IBRAHIM
Pharmacognosy Department, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia.
Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

INTRODUCTION

Polygonum equisetiforme Sibth. & Sm. of the Polygonaceae family is an annual prostrate herb with small elliptic lanceolate leaves and woody roots that is widely distributed along the Mediterranean coast of Egypt under the local name Qordab [1]. Some species of the Polygonaceae family were reported to have tonic, astringent, anti-septic, anti-malarial, anti-spasmodic, anti-tumour, anti-pneumonia properties, in addition to being used as a fish poison and for insect and snake bites [2,3]. Plants belonging to this family are known to produce a large number of biologically important secondary metabolites, such as flavonoids, anthraquinones, steroids [4,5] and alkaloids [6]. The most characteristic compounds of the Polygonaceae family are polysaccharides, phenylpropane glycosides and stilbenes [7].

Numerous *Polygonum* species are frequently used in traditional medicine [8]. Some species are used in the treatment of cough and diarrhea [9], and several are used as diuretic agents and to treat urinary inflammation [10]. *Polygonum* species are characterised by the presence of drimane sesquiterpenoids, norsesquiterpenoids and sulphated flavonoids [11,12]. Pharmacological investigations of *Polygonum* species revealed that different extracts of the plants possessed antibacterial, analgesic, anti-inflammatory, hypothermia, diuretic and antioxidative properties [13-16].

Polysaccharides are reported to possess a wide range of pharmacological properties, including antioxidant, anti-inflammatory, antitumour, immunomodulatory and antidiabetic activities[17-22]. Polysaccharides from several *Polygonum* species were isolated and characterised [23,24]. Polysaccharides of *Polygonum multiflorum* were shown to have antioxidant activity [25]. No reports on the composition and biological activity of polysaccharides from *Polygonum equisetiforme* Sibth. & Sm. was available in the literature. Therefore, this study was performed to determine the chemical composition, antioxidant properties and cytotoxic activity of polysaccharides isolated from *Polygonum equisetiforme* roots.

MATERIALS AND METHODS

P. equisetiforme Sibth. & Sm. plants were collected from the Garden of the Faculty of Pharmacy, Al-Azhar University, at their flowering stage (April 2010) and were kindly authenticated by Prof. Dr. Abdu Mareay, Assistant Professor of Plant Taxonomy, Faculty of Science, Al-Azhar University, Cairo, Egypt. Voucher specimens were deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy Al-Azhar University, Cairo, Egypt. The collected plant roots were isolated, air-dried, powdered and kept in tightly closed amber coloured glass containers and protected from light at the lowest temperature in refrigerator. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from E. Merck (Darmstadt, Germany). Vitamin C, butylated hydroxytoluene (BHT) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO,USA). Dextrans of different molecular weights were purchased from Pharmacia Co. (Sweden). Other reagents were of analytical grade reagent.

The human cancer cell lines: H 460 (Lung cancer cell line) and HEPG2 (Liver cancer cell line), MCF7 (Breast cancer cell line), HeLa (Cervix cancer cell line), HCT 116 (Colon carcinoma cell line) and U251 (Brain cancer cell line) were provided by National Cancer Institute, Cairo, Egypt.

(i) Extraction, Isolation and Purification of Polysaccharides:

The powdered *P. equisetiforme* root (250 g) was pre-extracted for 48 h in a Soxhlet system with petroleum ether and subsequently for another 48 h with 95% ethanol. The plant residue was extracted three times with hot double-distilled water (90°C, 3 h), and the aqueous extracts were combined, concentrated by rotator evaporator

(Buchi rotavapor R-210/R-215, Germany) and filtered [26]. The extract was deproteinised 5 times using the Sevag reagent [27]; after which, the polysaccharide was free of proteins as determined by scanning the UV Spectra at 260 nm and 280 nm. After removal of the Sevag reagent, the extract was precipitated by adding absolute ethanol (4 times the volume of the aqueous extract), and the mixture was kept overnight at 4°C. The precipitate was collected by centrifugation at 4000 rpm for 20 min, washed successively with petroleum ether, acetone and ethanol. The precipitation procedure was repeated, and then the precipitate was dissolved in water and dialysed against deionised water for 72 h, followed by freeze-drying to yield the crude polysaccharide (14.65%).

Crude polysaccharide (2 g) was subjected to DEAE-cellulose column (2.5 x 80 cm) equilibrated with distilled water and eluted stepwise with 0, 0.1, 0.2, 0.3, 0.4, 0.5 and 1 M NaCl at rate of 0.6 mL/min, the eluate was monitored by the phenol-sulfuric acid method [28]. The collected fractions contained polysaccharides was dialyzed against distilled water for 48 h, and then purified by gel-filtration chromatography on a column of Sephadex G-150 (2.5 x 60 cm). The sample was dissolved in the minimal volume of 0.1 mol/L NaCl solution and added to the column, and then eluted with 0.1 mol/L NaCl solution at a rate of 0.2 mL/min. The main fraction is collected and freeze dried to obtain purified polysaccharide, named PEP (12.34%).

(ii) Determination of the Polysaccharide (PEP)Purification

The purification of the polysaccharides was conducted by a Sephadex G-200 column chromatography and methods of optical rotation [29]. PEP was dissolved in 0.1 mol/L NaCl solution, centrifuged, and then the supernatant was applied to a Sephadex G-200 column (2.5x 90 cm), which was eluted with 0.1 mol/L NaCl solution at a rate of 0.2 mL/min. Polysaccharides were detected by the phenol-sulfuric acid method. Elution curve was drawn by the tube number as abscissa and absorbance as vertical coordinate. In addition, the sample was dissolved in distilled water, and its optical rotation was measured with a WXG-4 polarimeter (Loyal Key Group Shanghai, China) at room temperature.

(iii)IR Analysis of Polysaccharides

The infrared (IR) spectra of PEP (figure 1) was recorded within 4000 cm^{-1} to 400 cm^{-1} using a Varian 1000 Fourier-transform IR (Varian, USA) instrument with KBr pellets.

(iv)Analysis of Monosaccharide Composition

The polysaccharide (10 mg) was hydrolysed with 10 mL of 2 M trifluoroacetic acid (TFA) at 120 ^{o}C for 6 h in a sealed glass tube on the mechanism of acid-catalyzed hydrolysis [30]. Excess acid was removed by co-distillation with methanol after the hydrolysis was completed. Monosaccharides were analysed using high performance liquid chromatography (HPLC). An HP 1050 series equipped with a HP 1050 series pump and HP 1050 series autosampler under computer control (Hewlett Packard, USA) was used with a Lichrospher RP-18 (5 µm) column (250 x 4 mm) and Polygosil 60-5NH_2 (250 x 4.6 mm) Merck, Darmstadt, Germany. The operation conditions were as follows: mobile phase: acetonitrile – water (75: 25 v/ v); detector: RI (HP. 1047A) and flow rate: 2 mL/min. The results are recorded in table 1 and figure 2.

(v) Methylation Analysis

The PEP (20 mg) was methylated thrice according to the method of Hakomori, (1964) [31]. Complete methylation was confirmed by the disappearance of the OH band at 3200–3700 cm^{-1} in the IR spectrum. The methylated products were hydrolyzed, reduced and acetylated [32]. The partially methylated alditol acetates were analyzed by gas chromatography/mass spectrometry (GC/MS). GC/MS was performed on a HP5890 (II) instrument (Hewlett-Packard Component, USA) with an HPS quartz capillary column (25 m x 0.22 mm x 0.2 nm), and at temperatures programmed from 120 to 140 ^{o}C at 1 ^{o}C/min. The methylated sugar

linkages were identified on the basis of relative retention time and fragmentation pattern [33]. The molar ratios of each sugar residue were calibrated using the peak areas and response factor of the flame ionization detector in GC. Results are presented in table 2 and figure 3.

(vi) Determination of Polysaccharide Molecular Weight

The molecular weight of PEP was determined by gel permeation chromatography (GPC) [34] with a Waters HPLC apparatus (Waters 515, Waters Co. Ltd., USA) equipped with an ultra hydrogel column (7.8 × 300 mm). The operation conditions were as follows: mobile phase: 0.2 M phosphate buffer (pH 7.0), flow rate: 0.7 mL/ min, column temperature: room temperature, injection volume: 20 µL, running time: 20 min, and analysed using a Waters 2410 refractive index detector (RID). Dextran standards with different molecular weights (2,500; 4,600; 7,100; 10,000; 21,400; 41,100; 84,400; 133,800; 200,000 Da) were used for establishment of a calibration curve.

(vii) Assays for Antioxidant Activity:

Evaluation of ABTS radical scavenging activity [35,36]:

ABTS radical cations were prepared by mixing ABTS (7 mM, 25 mL in deionised water) with potassium persulfate ($K_2S_2O_8$) (140 mM, 440 µL) and allowing the mixture to stand in the dark at room temperature for 16 h before use. The ABTS radical cation solution was diluted to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C for 30 min. PEP (0.2 mL) at various concentrations (0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 5.0 mg/mL) was mixed separately with 2.0 mL of the diluted ABTS radical cation solution. The reaction was performed at room temperature for 20 min. Then, the absorbance was measured using a UV-visible spectrophotometer at 734 nm. Vitamin C was used as a standard. All the tests were performed in triplicate.

ABTS radical scavenging activity was calculated as follows:

$$\text{ABTS scavenging activity (\%)} = [A_0-(A_s-A_b)]/A_0 \times 100$$

Where A_0 = A734 of ABTS without the sample, A_s = A734 of the sample and ABTS and A_b = A734 of the sample without ABTS. The results are illustrated in table 3 and figure 4.

Evaluation of Hydroxyl radical scavenging activity [37, 38]:

Samples at different concentrations (0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 5.0 mg/mL) were incubated with 2 mM EDTA–Fe (0.5 mL), 3% H_2O_2 (1.0 mL) and 0.360 mg/mL crocin in 4.5 mL sodium phosphate buffer (150 mM, pH 7.4) for 30 min at 37 °C. Hydroxyl radicals were detected by monitoring absorbance at 520 nm. Vitamin C was used as a standard antioxidant. The hydroxyl radical scavenging effect was calculated as follows:

Hydroxyl radical scavenging effect (%) = $[(A_o-A_s) / A_o]\times100$

Where A_o is the A520 with no sample and As is the A520 after addition of the sample. The results are presented in table 3 and figure 5.

***Evaluation of superoxide anion scavenging activity*:**

The superoxide radical is a very harmful species to cellular components as a precursor of more reactive oxygen species. It is known to be produced *in vivo* and can result in the formation of H_2O_2 via a dismutation reaction [39]. In this experiment, measurement of the superoxide anion scavenging activity of PEP was based on the method described by Wang (2008) [40] with some modifications. Tris-HCl buffer (4.5 mL, 50 mmol/L, pH 8.2) and 1.0 mL of the tested samples at various concentrations (0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 5.0 mg/mL) were mixed separately in tubes with lids. The mixture was incubated for 20 min in a water bath at 25°C. Next, 0.4 mL of 25 mmol/L pyrogallol preheated at 25°C was added immediately. After 4 min, the reaction was terminated by adding 0.1 mL HCl solution (8 mol/L), and the mixture was centrifuged at 4000 rpm for 15 min. The absorbance of the sample and the control were determined using a UV spectrophotometer at 325 nm. The curve was made based on the absorbance value. Vitamin C was used as

the positive control. Scavenging activity was calculated using the following equation:

Superoxide anion scavenging activity (%) = $[(A_o-A_s)/A_o] \times 100$

Where A_o is the absorbance without the sample, and A_s is absorbance with the sample at 325 nm. The results are shown in table 3 and figure 6.

Evaluation of DPPH radical scavenging activity [41-43]**:**

The DPPH radical scavenging assay is a widely used method to evaluate the free radical scavenging activity of natural compounds. Briefly, 3 mL of sample (0.01 to 5.0 mg/mL) were added to 1mL of 0.1mM methanol solution of DPPH. Vitamin C was used as the reference material. The absorbance at 517 nm was measured after keeping the solution at room temperature for 30 min. The DPPH radical scavenging activity was calculated as follows:

DPPH scavenging activity (%) = $[A_o-(A-A_b)]/A_o \times 100$

Where A_o = A517 of DPPH without the sample, A = A517 of the sample and DPPH, and A_b = A517 of the sample without DPPH. The results are illustrated in table 3 and figure 7.

Evaluation of reducing power [44,45]**:**

BHT was used as the reference material. PEP and BHT were used at different concentrations (0.01 to 5.0 mg/mL). The sample (1.0 mL) was mixed with phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL,1%) and incubated at 50°C for 20 min. Then, the reaction was terminated by adding 2.5 mL trichloroacetic acid solution (0.1%), and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 6 mmol/L), and the absorbance was measured spectrophotometrically at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. A curve was established showing the relationship between PEP concentration and the absorbance at 700 nm (Figure 8).

(viii) Evaluation of Cytotoxic Activity

The potential cytotoxicity of the polysaccharide isolated from the roots of *Polygonum equisetiforme* was tested *in vitro* [46] against six human cell lines: U251 (brain cancer cell line), MCF7 (breast cancer cell line), H460 (lung cancer cell line), HeLa (cervical cancer cell line), HCT 116 (colon cancer cell line) and HEPG2 (liver cancer cell line).

The potential cytotoxicity of PEP was evaluated using the sulphurhodamine B assay (SRB), in which the cells were plated in a 96-well plate (10^4 cells/well) for 24 hours before treatment with PEP to allow attachment of cells to the wall of the plate. Different concentrations of PEP in DMSO (1, 2.5, 5 and 10 µg/mL) were added to the cell monolayer. DMSO was used as negative control and doxorubicin (Sigma-Aldrich Co.) as positive control, and preparing six concentrations of doxorubicin from 64 to 2 µg/mL using two fold serial dilution in DMSO. Triplicate wells were incubated with the samples, negative and positive control, for 48 hours at 37 ºC in an atmosphere of 5% CO_2. The cells were then fixed, washed and stained with sulphurhodamine B stain. Excess stain was washed with 1% glacial acetic acid and attached stain was recovered with Tris-EDTA buffer. The colour intensity was measured in an ELISA reader at 564 nm. Percent cell survival was calculated compared to the untreated cell (negative control) that was assumed as 100% viable. The relationship between the surviving fraction and the drug concentration is plotted to obtain the survival curve for each cancer cell line after treatment with the specified test sample and positive control. The curves were fitted using a linear equation and the IC_{50} was calculated. The results are recorded in table 4.

(ix)Statistical Analysis

All antioxidant and cytotoxic activity assays were replicated three times. All data are shown as the mean ± SD. Statistical comparisons of data were performed using an ANOVA and t-test by the SPSS 18.0 system considering P-value<0.05 significant.

RESULTS AND DISCUSSION

1. Chemical and spectral analysis of the polysaccharide

The purified polysaccharide PEP is a fine aqueous polysaccharide obtained from *P. equisetiforme* roots (yield = 12.34% calculated for dry wt) by boiling water extraction, ethanol precipitation, deproteinisation and DEAE-cellulose column chromatography. It was further purified by gel chromatography on Sephadex G-150 column. The purification of PEP was identified on both Sephadex G-200 column and WXG-4 optical polarimeter at room temperature. The results showed only one symmetrical peak on gel-filtration chromatography and the same optical rotation in different low concentrations of ethanol, clearly demonstrating that PEP is single composition. The IR absorption spectrum of PEP (figure 1) shows a broad intense peak at 3600-3200 cm^{-1}, which is characteristic of hydroxyl groups, and a weak C-H band at approximately 2930 cm^{-1}. The relatively strong absorption peak at approximately 1600-1650 cm^{-1} is characteristic of the C=O group; the peaks at 950-1200 cm^{-1} suggest the presence of C-O-C and C-O-H bonds [47], and the absorption at 870 cm^{-1} was due to mannose.

HPLC identification was performed by comparing the retention times and relative retention times of the individual sugars to those of authentic samples. The concentration was determined by measuring the peak area. As shown in table 1, PEP is composed of galactose, glucose, rhamnose, mannose and arabinose. Their percentage are 5.6%, 42.3%, 33%, 8.6% and 0.8%, respectively, as determined by HPLC analysis (figure 2).

Figure 1

IR spectrum of the polysaccharide isolated from the roots of Polygonum equisetiforme.

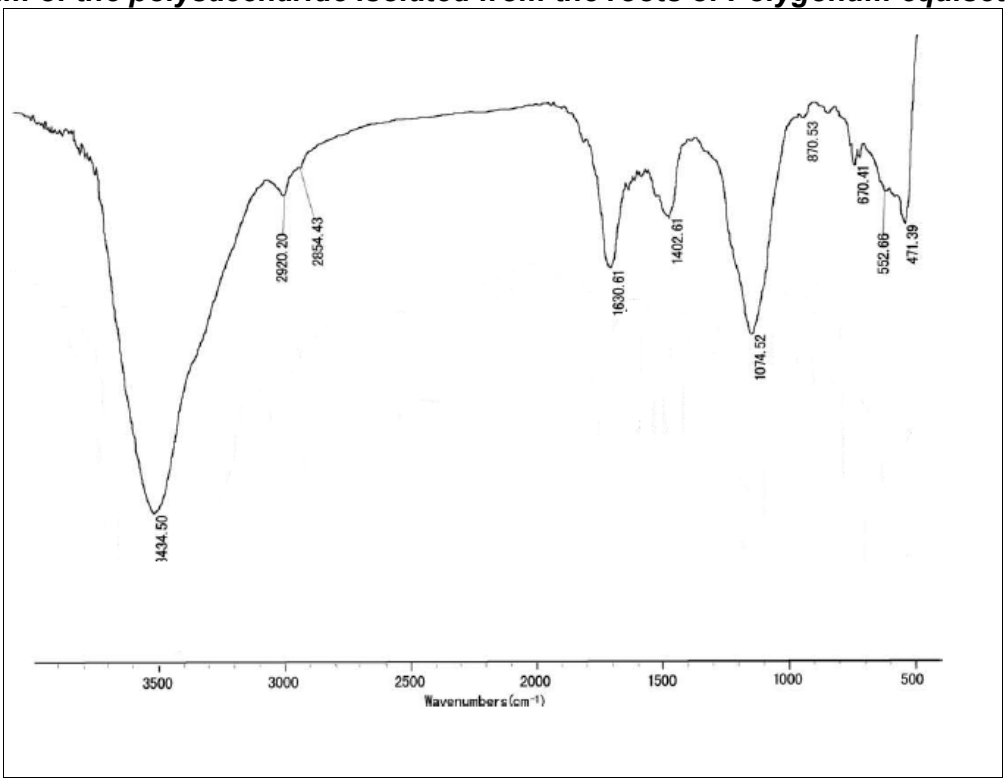
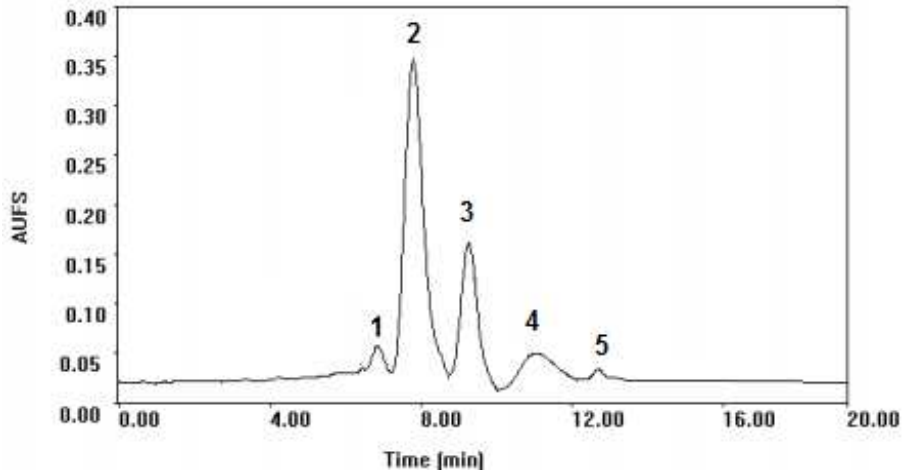


Table 1
HPLC analysis of monosaccharide content in the polysaccharide extracted from the roots of Polygonum equisetiforme.

No.	Compound	R_t (min)	RR_t	Concentration (%)
1	Galactose	7.222	0.917	5.6
2	Glucose	7.876	1.000	42.3
3	Rhamnose	9.321	1.183	33
4	Mannose	10.213	1.297	8.6
5	Arabinose	12.711	1.614	0.8

R_t: Retention time (min) ***RR_t: Relative retention time***

Figure 2
HPLC chromatogram of monosaccharides derived by acid hydrolysis of PEP



2. Methylation analysis of polysaccharides

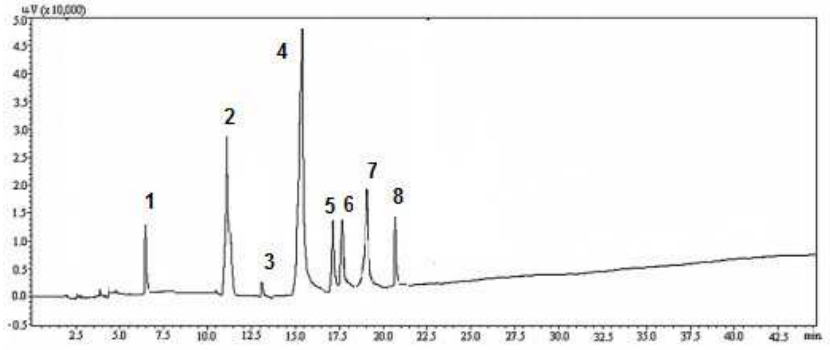
Products of PEP after methylation and hydrolysis were converted into alditol acetates for GC/MS analysis. The combination of the fragmentation pattern and retention time of PEP revealed the presence of eight main components, as shown in table 2, figure 3 with their methylation analysis. The results of acid hydrolysis and methylation linkage analysis of PEP indicated that (1 → 4)-linked glucose was the major component of the backbone structure and the branch residue was (1 → 4, 6)- linked glucose. Residues of branch structure were (1 → 4)-linked rhamnose, (1 → 6)- linked glucose, (1 → 6)- linked galactose and (1 → 3)- linked mannose. Residues of terminal sugars were 1 → linked arabinose and 1 → linked rhamnose.

Table 2
The results of methylation analysis of PPE

No.	Retention time (min.)	Methylated sugar	Linkage	Molar ratio
1	6.46	2,3,4-Me_3-Rha	1-linkage (end group)	0.671
2	11.48	2,3-Me_2-Rha	1,4-linkage	1.324
3	12.98	2,3,5-Me_3-Ara	1-linkage (end group)	0.114
4	15.58	2,3,6-Me_3-Glu	1,4-linkage	2.005
5	17.29	2,3,4-Me_3-Glu	1,6-linkage	0.673
6	17.85	2,3,4-Me_3-Gal	1,6-linkage	0.676
7	19.04	2,3-Me_2-Glu	1,4,6-linkage	1.000
8	20.75	2,4,6-Me_3-Man	1,3-linkage	0.673

Ara: Arabinose, Gal: Galactose, Glu: Glucose, Man: Mannose, Rha: Rhamnose

Figure 3
The gas chromatogram of the products derived by methylation of PEP



3. Determination of the molecular weight of the polysaccharide

Using gel filtration chromatography, the molecular weight (MW) of PEP was estimated to be 79.09 kDa based on a calibration curve of standard dextrans with known molecular weights.

4. Polysaccharide antioxidant activity

The polysaccharide isolated from the roots of *Polygonum equisetiforme* showed a dose-dependent, radical scavenging activity (Table 3). The radical scavenging activity was enhanced with increased concentration. The ABTS, hydroxyl radical and DPPH scavenging activity of the polysaccharide were 96.45%, 79.02% and 83.47%, respectively, at a concentration of 1 mg/mL. In contrast, low free radical scavenging activity was detected for the superoxide anion (44.04%) at the same concentration. This result indicated that the polysaccharide isolated from the roots of *Polygonum equisetiforme* had strong ABTS and DPPH free radical scavenging activity.

Table 3
Free radical scavenging activity of the polysaccharide extracted from the roots of Polygonum equisetiforme.

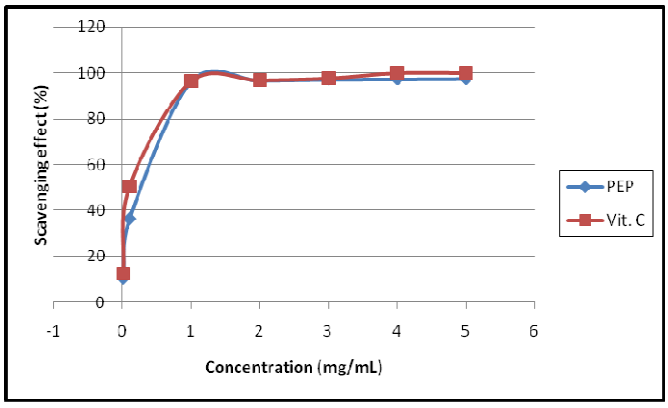
Concentration (mg/mL)	ABTS		Hydroxyl radical		Superoxide Radical		DPPH	
	Scavenging effects (%)	IC_{50}	Scavenging effects (%)	IC_{50}	Scavenging effects (%)	IC_{50}	Scavenging effects (%)	IC_{50}
0.01	10.55 ± 0.34		8.76 ± 0.34		10.34 ± 0.95		9.41 ± 0.74	
0.1	36.54 ± 0.53		31.88 ± 0.52		32.62 ± 0.42		31.17 ± 0.36	
1.0	96.45 ± 0.47		79.02 ± 0.86		44.04 ± 0.34		83.47 ± 0.46	
2.0	96.82 ± 0.38	0.299	79.56 ± 0.32	1.018	45.12 ± 0.41	-	84.52 ± 0.87	0.836
3.0	97.27 ± 0.22		81.07 ± 0.41		45.42 ± 0.25		85.31 ± 0.53	
4.0	97.38 ± 0.34		81.10 ± 0.25		45.55 ± 0.56		86.22 ± 0.73	
5.0	97.54 ± 0.57		81.10 ± 0.52		45.55 ± 0.43		87.15 ± 0.42	

Values are Mean ± SD (n=3).

ABTS radical scavenging activity

The ABTS radical assay can be used in both organic and aqueous solvent systems [48]. Therefore, it is often used to evaluate the total antioxidant power of both single compounds and complex mixtures from various plants [49,50]. In this experiment, the ABTS scavenging ability of the polysaccharide isolated from *P. equisetiforme* was measured using vitamin C as a standard. The scavenging activities of PEP and vitamin C correlated well with increasing concentrations. At the low dose (0.01 mg/mL), the scavenging activities of both samples were poor: 10.55% for PEP and 12.45% for vitamin C. In contrast, PEP exhibited high scavenging power at higher doses (1.0 mg/mL to 5.0 mg/mL), which were comparable to that of vitamin C: 96.45% at 1.0 mg/mL, 97.27% at 3 mg/mL and 97.54% at 5 mg/mL. From figure 4, an IC_{50} of 0.299 mg/mL was calculated. These results indicated that PEP had strong scavenging activity against ABTS radicals and suggest it as a potential novel antioxidant.

Figure 4
The scavenging activity of PEP on ABTS.



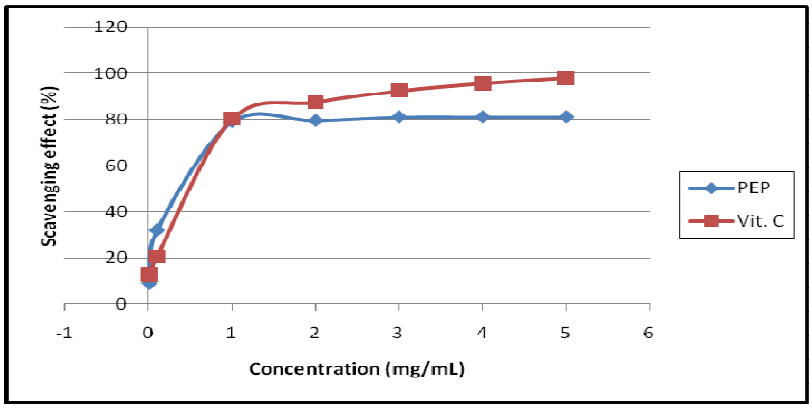
The results are presented as the means ± standard deviations (n = 3).
Differences are considered to be statistically significant if P < 0.05 relative to the control.

Hydroxyl radical scavenging activity

In cells, the hydroxyl radical can easily cross cell membranes at specific sites and react with most biomolecules to cause tissue damage and cell death. Thus, removing hydroxyl radicals is very important for the

protection of living systems [51]. The vitamin C standard showed high radical scavenging activity (97.9%) at 5.0 mg/mL. PEP exhibited strong scavenging activity (81.07%) at 3.0 mg/mL. The increase in the hydroxyl radical scavenging activity was insignificant at concentrations greater than 3.0 mg/mL. From figure 5, an IC_{50} of 1.018 mg/mL could be calculated. Therefore, PEP has an appreciable scavenging activity against hydroxyl radicals.

Figure 5
The scavenging activity of PEP on hydroxyl radicals.

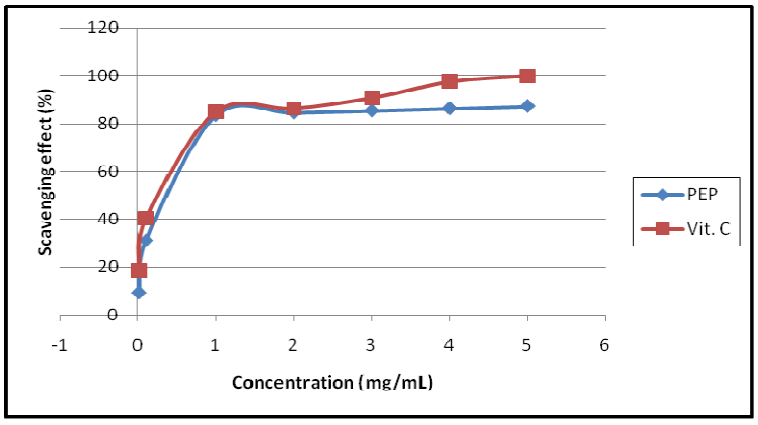


The results are presented as the means ± standard deviations (n = 3).
Differences are considered to be statistically significant if P < 0.05 relative to the control.

Superoxide radical scavenging activity

The superoxide anion radical is also known as the initial radical and plays an important role in the formation of other reactive oxygen species, such as hydrogen peroxide or singlet oxygen in living systems [52]. (The vitamin C standard had a high radical scavenging activity (87.12 to 89.46%) at the higher doses (3.0 mg/mL to 5.0 mg/mL). PEP, however, demonstrated very low radical scavenging activity at every concentration. Therefore, the polysaccharide PEP has no significant effects on superoxide free radical scavenging as shown in table 3 and figure 6.

Figure 6
The scavenging activity of PEP on superoxide radicals.

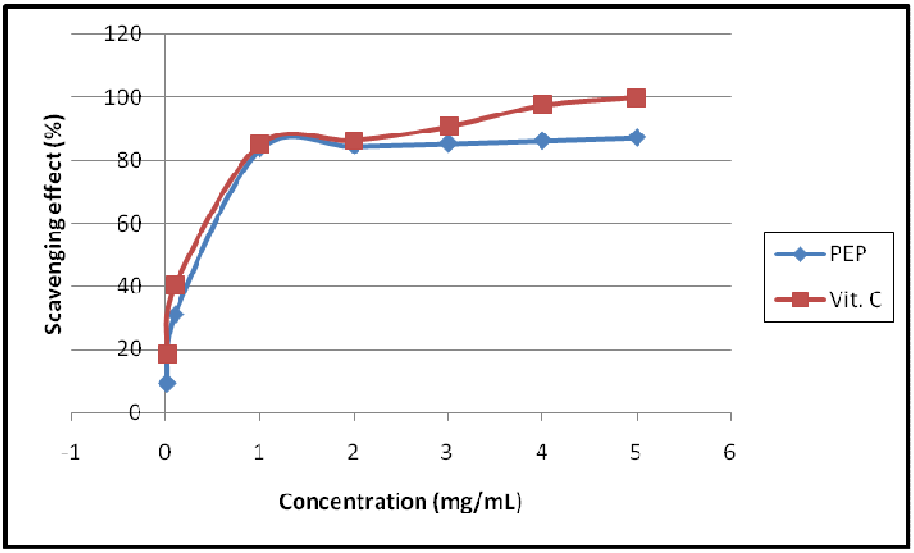


The results are presented as the means ± standard deviations (n = 3).
Differences are considered to be statistically significant if P < 0.05 relative to the control.

DPPH radical scavenging activity

DPPH is frequently used as a substrate to evaluate antioxidant activity. DPPH is a stable free radical with a maximum absorption at 517 nm in methanol [53]. The DPPH radical scavenging activity of antioxidants was determined to be a result of their proton-donating properties. In a DPPH test, antioxidants were able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine [54]. Based on this principle, the antioxidant activity of a substance can be expressed as its ability to scavenge DPPH free radicals. The results indicate that both vitamin C and PEP showed a dose-dependent DPPH radical scavenging activity. Furthermore, the scavenging activities of both increased very significantly with increasing concentrations. At 1.0 mg/mL, the scavenging activity increased insignificantly. At 5.0 mg/mL, vitamin C exhibited very high scavenging activity (99.77%), and PEP also had strong scavenging activity on DPPH radicals (87.15%). From figure 7, an IC_{50} of 0.836 mg/mL could be calculated. Therefore, the polysaccharide PEP has strong antioxidant activity against DPPH radicals at higher concentrations as reported in table 3 and figure 7.

Figure 7
The scavenging activity of PEP on DPPH.

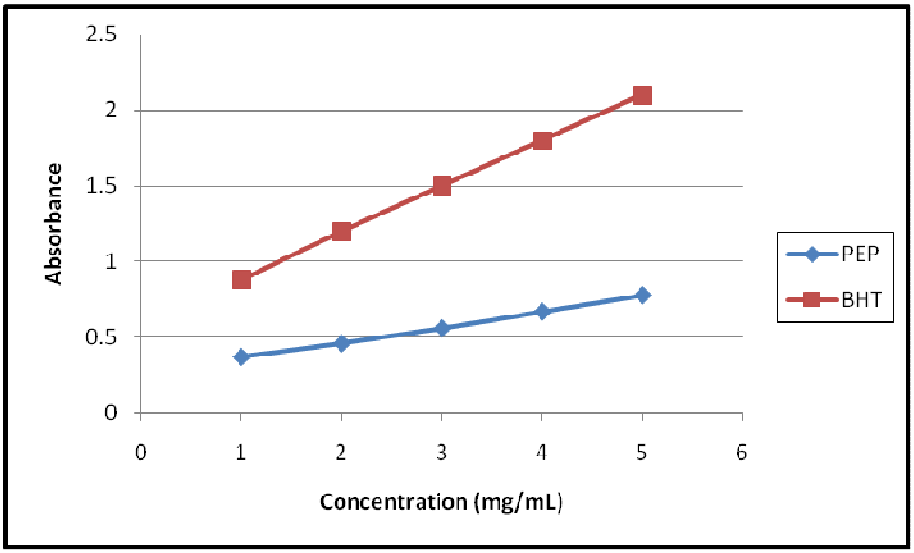


The results are presented as the means ± standard deviations (n = 3).
Differences are considered to be statistically significant if P < 0.05 relative to the control.

Analysis of reducing power

It has been demonstrated that there is a direct correlation between antioxidant activity and reducing power [55]. The reductive power of PEP, Fe^{3+}-Fe^{2+} transformation in the presence of different concentrations of the test compound were investigated using BHT as a reference material. The reductive properties of PEP and the reference material are shown in figure 8. PEP and BHT showed a concentration-dependent reducing capability. At the high doses (1.0 to 5.0 mg/mL), BHT exhibited higher absorbance (2.103), which indicated that BHT has greater reducing power. PEP had a lower absorbance than BHT at every concentration. At the high dose (5.0 mg/mL), the absorbance of PEP was 0.78, indicating that PEP had weak reducing capacity, relative to the reference standard. These results suggested that PEP does not have significant reducing properties.

Figure 8
Reducing power of PEP on Fe^{3+}-Fe^{2+} transformation.



The results are presented as the means ± standard deviations (n = 3).
Differences are considered to be statistically significant if P < 0.05 relative to the control.

5. Cytotoxic activity

As shown in table 4, colon (HCT116) and breast (MCF7) cancer cell lines were the most sensitive of all human cell lines examined to the polysaccharide followed by the HeLa cervical carcinoma cell line. As shown in table 4, the response was dose-dependent with regression coefficients (R^2) of 0.9838 and 0.9579 for the colon and breast cancer cell lines, respectively. IC_{50} values of 14.67 µg/mL and 22.34 µg/mL were calculated for the colon and breast cancer cell lines, respectively. IC_{50} values of positive control doxorubicin were calculated; 2.122 µg/mL and 2.531 µg/mL for the colon and breast cancer cell lines, respectively. The cytotoxic activities of PEP were calculated in comparison to positive control doxorubicin to be 11.33% and 14.46% against colon and cancer cell lines, respectively.

Table 4
Cytotoxic activity of PEP on different human cell lines.

Concentration (µg/mL)	Mean of Surviving Fraction ± SD					
	Brain cancer cell line (U251)	Breast cancer cell line (MCF7)	Lung cancer cell line (H460)	Cervix cancer cell line (HELA)	Colon cancer cell line (HCT 116)	Liver cancer cell line (HEPG2)
0.000*	1.000 ± 0.01	1.000 ± 0.01	1.000 ± 0.01	1.000 ± 0.01	1.000 ± 0.01	1.000 ± 0.01
1.000	1.000 ± 0.06	0.976 ± 0.04	1.000 ± 0.02	0.974 ± 0.04	1.000 ± 0.04	1.000 ± 0.07
2.500	0.936 ± 0.01	0.920 ± 0.04	0.988 ± 0.04	0.931 ± 0.02	0.900 ± 0.06	0.978 ± 0.04
5.000	0.882 ± 0.02	0.862 ± 0.05	0.964 ± 0.04	0.877 ± 0.05	0.841 ± 0.03	0.953 ± 0.01
10.000	0.863 ± 0.03	0.795 ± 0.01	0.892 ± 0.01	0.831 ± 0.08	0.662 ± 0.06	0.895 ± 0.04

**** Negative control*** ***Values are Mean ± SD (n=3).***

CONCLUSIONS

This study provides strong evidence for the antioxidant properties of a polysaccharide isolated from the roots of *Polygonum equisetiforme* that could be considered a valuable medicinal plant species. Results indicate that PEP's scavenging activity is mediated through several mechanisms, including hydrogen donating activity and inhibition of hydroxyl ion production. This could be potentially useful for the treatment of

major free radical-induced degenerative diseases including brain dysfunction, inflammation, liver disorders and cancer. PEP showed a little cytotoxic effect against colon and breast cancer cell lines compared to standard doxorubicin. Additional *in vivo* studies are still needed to confirm these results.

ACKNOWLEDGEMENTS

This research project was supported by a grant from the Research Centre of the Female Centre for Scientific and Medical Colleges of King Saud University.

REFERENCES

1. Täckholm V, Students' flora of Egypt. Beirut: Cairo University, Cooperative Printing Co. (1974).
2. Belkin M and Fitzgerald DB, Distribution of antineoplastic activity in plants. J. Pharmaceut. Sci. 55 (3): 139–148, (1966).
3. Shimada H, Semipurified fractions from *Rumex hymeno-sepalus* Shoyakugaku Zasshi. J. Pharamceut. Sci. 55 (3): 49–57, (1966).
4. Baraka DM, Ecological and Phytochemical Study on one Species of Polygonaceae. M.Sc. Thesis, Fac. Sci. Zagazig University (Benha) Egypt, p. 171, (1985).
5. Marjorie MC, Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12 (4): 564–582, (1999).
6. Iwu MW, Duncan AR and Okunji CO, New antimicrobials of plant origin. In: Janick, A.S. (Ed.), Perspectives on New Crops and New Uses. ASHS Press, Alexandria, VA, p. 457–462, (1999).
7. Marc EB, Nelly A, Annick DD and Dupont F, Plants used as remedies antirheumatic and antineuralgic in the traditional medicine in Lebanon. J Ethnopharmacol. 120: 315-334, (2008).
8. Rab J, Népi növényismeret a Gyergyói-medencében. Pallas- Akadémia Könyvkiadó: Csíkszereda, p. 236, (2001).
9. Cakilcioglu U and Turkoglu I, An ethnobotanical survey of medicinal plants in Sivrice (Elaziğ-Turkey). J Ethnopharmacol. 132: 165-175, (2010).
10. Hegnauer R, Chemotaxonomie der Pfanzen Volume 9. Birkhäuser Verlag: Basel, Boston, Berlin, p. 268-284, (1990).
11. Fukuyama Y, Sato T, Asakawa Y and Takemoto T, A potent cytotoxic warburganal and related drimane-type sesquiterpenoids from *Polygonumhydropiper*. Phytochemistry. 21(12): 2895-2898, (1982).
12. Haraguchi H, Ohmi I, Sakai S, Fukuda A, Toihara Y, Fujimoto T, Okamura N and Yagi A, Effect of *Polygonum hydropiper* sulfated flavonoids on lens aldose reductase enzymes. J Nat Prod. 59: 443-445, (1996).
13. Li, Y and Gong, Y, The research progress on the chemical component and the pharmacology of *Polygonum capitatum* Ham ex D. Don. Journal of Guizhou University (Natural Sciences). 24: 205-207, (2007).
14. Liu M, Luo C, Zhang Y and Qiu D, Analgesic, anti-inflammatory, and diuretic effects of *Polygonum capitatum* and *Toddalia asiatica*. Guizhou Medical Journal. 31: 370-371, (2007).
15. Liu Z, Qi J, Zhu D and Yu B, Chemical constituents from *Polygonum capitatum* and their antioxidation activities in vitro. Zhong Yao Cai (Journal of Chinese Medicinal Materials). 31: 995-998, (2008).
16. Ren G, Chang F, Lu S, Zhong H and Zhang G, Pharmacological studies of *Polygonum capitatum* Buch Ham. Ex D Don. Zhongguo Zhongyao Za Zhi (China Journal of Chinese Materia Medica). 20: 107, (1995).

17. Ananthi S, Raghavendran HRB, Sunil AG, Gayathri V, Ramakrishnan G and Vasanthi HR, In vitro antioxidant and in vivo anti-inflammatory potential of crude polysaccharide from *Turbinariaornate* (Marine Brown alga). Food and chemical toxicology. 48: 187-192, (2010).
18. Ding X, Zhu F and Gao S, Purification, antitumor and immunomodulatory activity of water-extractable and alkali-extractable polysaccharides from *Solanum nigrum* L. Food Chemistry. 131(2): 677-684, (2010).
19. Ghosh K, Chandra K, Ojha AK, Sarkar S and Islam SS, Structural identification and cytotoxic activity of a polysaccharide from the fruits of *Lagenaria siceraria* (Lau). Carbohydr Res. 344(5):693-8, (2009).
20. Pedrinaci S, Algarra I and Garrido F, Protein-bound polysaccharide (PSK) induces cytotoxic activity in the NKL human natural killer cell line. Int J Clin Lab Res. 29(4):135-40, (1999).
21. Schepetkin IA and Quinn MT, Botanical polysaccharide: Macrophage immunomodulation and therapeutic potential. International Immunopharmacology. 6(3): 317-333, (2006).
22. Shonima GM, Jiji T and Muraleedhara GK, In vitro antioxidant and antitumor activity of polysaccharide isolated from *Ulva fasciata*. Int J Pharm Bio Sci. 3(3): 238 – 246, (2012).
23. Yakovlev AI, Churilov GI and Ginak AI, Polysaccharide composition of *Polygonum aviculare*. Chemistry of Natural Compounds. 21(5): 579-582, (1985).
24. Yunuskhodzhaeva BNA., Abdullabekova VN and Kurbanova AD, Polysaccharide composition of *Polygonum hydropiperis* and *P. Aviculare*. Chemistry of Natural Compounds. 42(5): 600-601, (2006).
25. Aoxue L, Yijun F and Aoshuang L, In vitro free radicals scavenging activities of polysaccharide from *Polygonum Multiflorum* Thunb. Journal of Medicinal Plants Research. 5(6): 966-972, (2011).
26. Luo AX, He XJ, Zhou SD, Fan YJ, He Tao and Chun Z, In vitro antioxidant activities of a water-soluble polysaccharide derived from *Dendrobium nobile* Lindl. extracts. Int. J. Biol. Macromol. 45(4): 359-363, (2009).
27. Navarini L, Gilli R, Gombac V, Abatangelo A, Bosco M and Toffanin R, Polysaccharides from hot water extracts of roasted *Coffea arabica* beans: isolation and characterization. Carbohyd. Polym. 40:71-81, (1999).
28. Dubois M, Gilles K A, Hamilton J K, Rebers P A and Smith F, Colorimetric method for determination of sugars and related substances. Analytical Chemistry. 28(3): 350–356, (1956).
29. Wang ZJ, Liang ZY and Luo DH, Structure investigation of water-soluble polysaccharide HPI isolated from the Hericium Caput-medusa. Chemical Journal of Chinese Universities. 8: 1474–1476, (2004).
30. Erbing B, Jansson PE, Widmalm G and Nimmich W, Structure of the capsular polysaccharide from the *Klebsiella* K8 reference strain-1015. Carbohydrate Research. 273(2): 197-205, (1995).
31. Hakomori S, A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. Journal of Biochemistry. 55: 205–208, (1964).
32. Sweet DP, Shapiro RH and Albersheim P, Quantitative analysis by various GLC Response-factor theories for partially methylated and partially ethylated alditol acetates. Carbohydrate Research. 40(2): 217–225, (1975).
33. Needs PW and Selvendran RR, Avoiding oxidative degradation during sodium hydroxyl dimethyloidine mediated carbohydrate methylation in dimethyl sulfoxide. Carbohydrate Research. 245: 1–10, (1993).
34. Fan YJ, He XJ, Zhou SD, Luo AX, He Tao and Chun Z, Composition, analysis and antioxidant activity of polysaccharide from *Dendrobium denneanum*. Int. J. Biol. Macromol. 45(2): 169-173, (2009).

35. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C, Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26(9-10): 1231-1237, (1999).
36. Zhao XY, Sun HD, Hou AJ, Zhao QS, Wei TT and Xin WJ, Antioxidant properties of two gallotannins isolated from the leaves of *Pistacia weinmannifolia*. Biochimica et Biophysica Acta. 1725: 103-110, (2005).
37. Luo AX, He XJ, Zhou SD, Fan YJ, Luo AS and Chun Z, Purification, composition, analysis and antioxidant activity of the polysaccharides from *Dendrobiumnobile* Lindl. Carbohyd. Polym. 79(4): 1014-1019, (2010).
38. Wang J, Zhang QB, Zhang ZS and Li Z, Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica*. Int. J. Biol. Macromol. 42(2): 127-132, (2008).
39. Li XM, Li XL and Zhou AG, Evaluation of antioxidant activity of the polysaccharides extracted from *Lycium barbarum* fruits in vitro. Eur. Polym. J. 43(2): 488-497, (2007).
40. Wang BS, Li BS, Zeng QX and Liu HX, Antioxidant and free radical scavenging activities of pigments extracted from molasses alcohol wastewater. Food Chem. 107: 1198-1204, (2008).
41. Braca A, De Tommasi N, Di Bari L, Pizza C, Politi M and Morelli I, Antioxidant principles from *Bauhinia terapotensis*. J. Nat. Prod. 64:892-895, (2001).
42. Shimada K, Fujikawa K, Yahara K and Nakamura T, Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J. Agric. Food Chem. 40(6): 945-948, (1992).
43. Sun H and Wang ZB, Effects on exercise endurance capacity and antioxidant properties of *Astragalus membranaceus* polysaccharides (APS). J. Med. Plants Res. 4(10): 982-986, (2010).
44. Raza F, Gholam AG and Mohammad HHK, Antioxidant activity of various extracts of old tea leaves and black tea wastes (*Camellia sinensis* L.). Food Chem. 100: 231-236, (2007).
45. Yen GH and Chen HY, Antioxidant activity of various tea extracts in relation to their antimutagenicity. J. Agric. Food Chem. 43: 27-32, (1995).
46. Skehan P, Storeng R, Scudiero D, Monks A, McMahom JM, Vistica D, Warren J, Bokesch H, Kenney S and Boyd MR, New colorimetric cytotoxicity assay for anticancer-drug screening. J. natl. Cancer Inst. 82: 1107-12, (1990).
47. Barker SA, Bourne EJ, Stacey M and Whiffen DH, Infra-red spectra of carbohydrates. Part I. Some derivatives of D-glucopyranose. Journal of the Chemical Society. 171–176, (1954).
48. Wu LC, Hsu HW, Chen YC, Chiu CC, Lin YI and Ho JA, Antioxidant and antiproliferative activities of red pitaya. Food Chem. 95(2): 319-327, (2006).
49. Huang SS, Huang GJ and Ho YL, Antioxidant and antiproliferative activities of the four *Hydrocotyle* species from Taiwan. Bot. Stud. 49: 311-322, (2008).
50. Katalinic V, Milos M, Kulisic T and Jukic M, Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. Food Chem. 94(4): 550-557, (2006).
51. Yang JX, Guo J and Yuan JF, In vitro antioxidant properties of rutin. LWT. 41(6): 1060-1066, (2008).
52. Stief TW, The physiology and pharmacology of single oxygen. Med. Hypotheses. 60(4): 567-572, (2003).
53. Xu WT, Zhang FF, Luo YB, Ma LY, Kou XH and Huang KL, Antioxidant activity of a water-soluble polysaccharide purified from *Pteridium aquilinum*. Carbohyd. Res. 344(2): 217-222, (2009).
54. Blois MS, Antioxidant determinations by the use of a stable free radical. Nat. 181: 1199-1200, (1958).
55. Yildirim A, Mavi A and Kara AA, Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. J. Agric. Food Chem. 49(8): 4083-4089, (2001).