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Identification of the chemical composition of the methanolic extract of *Salix tetrasperma* Roxb. using LC-ESI-MS and evaluation its potential as antioxidant agent

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ABSTRACT

Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. A variety of free radical scavenging antioxidants exist within the body which many of them are derived from dietary sources like fruits and vegetables. In the present work, the total phenolic and flavonoid contents of the defatted methanolic extract of Salix tetraspermaRoxb. growing in Egypt and certain fractions (dichloromethane, ethyl acetate, n-butanol and water) derived from it was determined. The ethyl acetate fraction had high phenolic and flavonoid contents (285.48 ± 2.07 mg gallic acid equivalent /g extract and 136.13 ± 2.47 mg rutin equivalent /g extract) respectively. The antioxidant activities of these extracts were evaluated using three tests; 1,1-diphenyl picrylhydrazyl free radical scavenging activity (DPPH) method, total antioxidant capacity (TAC) assay and Azino-bis (3-ethylbenzothiazoline -6-sulfonic acid) (ABTS) assay. The results showed that although all extracts have antioxidant activity but the ethyl acetate fraction is the highest one (SC_{50}= 42.59 ± 0.38 µg/ml for DPPH method; 298.03 ± 6.85 mg ascorbic equivalent/g extract for TAC assay and 925.79± 4.74 mmolTrolox® equivalent / 100 g extract for ABTS assay). The identification of the chemical constituent of the defatted methanolic extract of S. tetrasperma has been performed by HPLC-ESI-MS technique. It was appeared that the chemical constituent of this extract is composed of a mixture of flavonoid and phenolic acid derivatives.

Key words: *Salix tetrasperma*, plant extract, antioxidant activity, phenolic and flavonoid contents, HPLC-ESI-MS.

INTRODUCTION

Since the beginning of civilization, humans have used natural products for healing of different diseases. Plants are biochemical labs that produce inside their cells a variety of complex substances with numerous biological and pharmacological active compounds. Therefore, many plants become the primary source of substance for drug development [1, 2].

An antioxidant is defined as any substance that prevents or delays of the oxidative stress. Antioxidants are of interest to biologists and clinicians because they help to protect the human body against the damages induced by reactive free radicals generated in many diseases such as atherosclerosis, ischemic heart, cancer, Alzheimer and Parkinson diseases [2-7].There are many evidences that many natural products and their derivatives have efficient antioxidant properties and consequently linked to anti-cancer, hypolipidemic, anti-aging and anti-inflammatory activities [3, 5, 7-10]. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace the synthetic antioxidants, which are being restricted due to their side effects[11].

The Genus *Salix* (Family *Salicaceae*) is a very popular herbal species affirmed in the natural habitats and field-cultivated in many countries [1,12]. The phenolic glycosides contained in this species are known for their anti-inflammatory, analgesic, and fever-reducing effects and have been shown to relieve rheumatic disturbances, infections, and headache [13, 14]. The recovery of polyphenols from plant materials is influenced by their solubility in the extraction solvent, the type of solvent, the degree of polymerization of phenols, the interaction of phenols with other plant constituents and the formation of insoluble complexes[15,16-18]. In previous studies, methanol, ethanol, acetone, propanol and ethyl acetate have been used to extract phenols from plants [15,18-20].Therefore, the present study deals with the extraction of the leaves of *Salix tetrasperma*Roxb.with 85% methanol and fractionation of this extract with different organic solvent as well as evaluation of these extracts as an antioxidant agent using three different methods. Also Identification of certain chemical constituents of the defatted methanolic extract of the plant was performed using a sensitive, accurate and specific high performance liquid chromatography (HPLC) coupling with electrospray ionization mass spectrometry.

MATERIALS AND METHODS

1. Chemicals

1,1-diphenyl picrylhydrazyl(DPPH) and 2,2`-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma –Aldrich (SL Louis, USA), potassium persulfate purchased from Biobasic Co. (Canada), aluminum chloride, sodium carbonate, sodium phosphate, ammonium molybedate, sodium hydroxide, sodium nitrite, ascorbic acid and gallic acid and the Folin-Ciocalteu's reagent (FCR) were purchased from Merck Chemical Company (Germany). All solvents are analytical grade and other chemicals were purchased from the Egyptian Company.

2. Plant materials

Fresh leaves of *Salix tetrasperma*were collected from Sharkia Governorate, Egypt. The plant was identified by Prof. Dr. WafaaAmer, Professor of plant taxonomy, Faculty of Science, Cairo University. Voucher specimens of the plant were kept in the Medicinal Chemistry department, Theodor Bilharz Research Institute. The plant was dried in shade, finely powdered with an electric mill, and kept for the extraction process.

3. Extraction and fractionation process

600 grams of fine powdered leaves of *Salix tetrasperma* were extracted with 85% methanol at room temperature for three times and the methanolic extract was filtrated and concentrated to dryness under reduced pressure using rotatory evaporator. The methanolic extract was defatted with petroleum ether. The aqueous defatted methanolic extract was subjected to fractionation using dichloromethane, ethyl acetate and *n*-butanol respectively. Three fractions were concentrated to dryness using rotatory evaporator. The defatted methanol extract and the three fractions derived from it were kept away from moisture in closed vials.

4. Total phenolic content

The total phenolic content in the tested extracts was measured by using Folin- Ciocalteu's reagent (FCR). This method depends on the reduction of FCR by phenols in each plant extract to a mixture of blue oxides. The experiments were performed according to the reported method byParajule*et al.*, 2012 [21].Briefly, a mixture of 100 µl of each tested extract (1 mg/ml) was mixed with 900 µl distilled water, 1 ml of FCR and 1 ml of Na_2CO_3 (10 %). The mixture was shaken and concentrated. After 1 hour, the absorbance was measured at 760 nm against a blank which contain all reagents without the sample at the same condition. Gallic acid was used as standard. All experiments were carried out in triplicates. The total phenolic content is expressed as the number of equivalents of gallic acid (GAE) and calculated by the following formula:

$X=A/A_o$

Where X is the total phenolic content, mg/mg plant extract in GAE, A is the absorption of the plant extract solution; A_o is the absorption of the standard gallic acid solution.

5. Total flavonoid content

The total flavonoid content in the tested extracts was determined using aluminum chloride assay according to the reported procedure by Barku*et al.*, 2013with little modification[22]. 250 µl of the tested extract in methanol (1 mg/ml) was added to 1.3 ml distilled water then add 75 µl of sodium nitrate (5%) followed by incubation period 5 mints. After which mixed with 150 µl of solution $AlCl_3$(10 %) in methanol and the mixture was allowed to stand for 6 minutes at room temperature. 0.5 ml 1 molar sodium hydroxide was finally added and the mixture diluted with 275 µl distilled water. After 15 min, the absorption was measured at 510 nm against the blank using a UV/Vis

spectrophotometer. The blank consisted of reagent solution without any extract. All experiments were performed three times. Total flavonoid content was expressed as mg rutin equivalent (RE)/g extract.

6. DPPH radical scavenging activity

The ability of the defatted methanol extract of *S. tetrasperma* and three fractions derived from it were measured according to the procedure described by Santos *et al.*, 2010 [18] with small modification. 2 ml of each extract at different concentrations was mixed with 2 ml of DPPH (0.1 mM of DPPH in methanol). The mixture was maintained in the dark at room temperature for 20 min. The absorbance at 517 nm was measured using UV/Vis spectrophotometer and compared with a control (only solvent and DPPH without extract). Ascorbic acid was used as a reference compound.

The antioxidant activity was expressed as a percent of inhibition of DPPH radical and calculated from the equation
DPPH scavenging activity (%) = (Absorbance of control- Absorbance of extract/Absorbance of control) × 100

The SC_{50} (concentration of sample required to scavenge 50% of DPPH radicals) values were determined.

7. Total antioxidant capacity

The total antioxidant capacity of the tested extracts was evaluated by the phosphomolybdenum method as described byPrieto *et al*., 1999 [23]. The assay is based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of a green phosphate/ Mo(V) complex. An aliquot of 0.3 ml of each extract (200 μg/ml) solution was mixed with 3 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were sealed then incubated at 95 °C. Thereafter, the tubes were left to cool at room temperature and then the observance of the mixture was measured at 695 nm against the blank using a UV/Vis spectrophotometer. The blank consisted of 3 ml of the reagent solution and the appropriate volume of the solvent and it was incubated under the same conditions. The experiment was repeated for three times and ascorbic acid was used as standard. The total antioxidant activity of the tested extract was expressed as the number of equivalents of ascorbic acid (AAE).

8. ABTS assay

The antioxidant capacity assay was performed using a UV-VIS recording spectrophotometer by the improved $ABTS^{\cdot+}$ (2, 2`-azino-bis (3-ethylbenzothiazoline- 6`-sulfonic acid) method as described byRe *et al.*, 1999 [24]. $ABTS^{\cdot+}$ radical cation was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate after incubation at room temperature (23 ºC) in dark for 16 h. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.050 at 734 nm. The filtered sample was diluted with methanol to give 20–80% inhibition of the blank absorbance with 0.1 mL of sample. $ABTS^{\cdot+}$ solution (1 ml, with absorbance of 0.700 ± 0.050) was added to the tested samples (0.1 ml) and mixed thoroughly. The reaction mixture was allowed to stand at room temperature for 2.5 min and the absorbance was immediately recorded at 734 nm. Trolox® standard solution (final concentration 0–15 µM) in methanol was prepared and assayed at the same conditions. The absorbance of the resulting oxidized solution was compared to that of the calibrated Trolox® standard. Results were expressed in terms of Trolox® equivalent antioxidant capacity (TEAC, mmolTrolox® equivalents per 100 g extract)

10. LC-ESI-MS Analysis

10.1. Preparation of standards mixture and sample solutions

Thirteen standard stock solutions (µg/ml); gallic acid, catechin, caffeic acid, ferulic acid, taxifolin, quercetin-3-*O*-β-D- glucopyranoside (1→6) gallic acid, rutin, quercetin 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-α-L-rhamnopyranoside, myrcetin, kaempferol 3-O-α-L- rhamnopyranoside, quercetin, and apigenin were prepared in HPLC grade solvent mixture of $CH_3CN/MeOH/H_2O$ (1:1:2; v/v/v) and filtered using membrane disc filter (0.45 µm). A mixed stock solution containing 13 standards was prepared for qualitative analysis. For defatted methanol extract of *Salix tetrasperma*, solution (5 mg/ml) was prepared in an HPLC grade solvent mixture of $CH_3CN/MeOH/H_2O$ (1:1:2; v/v/v) and filtered using membrane disc filter (0.45 µm).

10.2. LC-ESI-MS Conditions

LC-ESI-MS analysis system consists of HPLC (Waters Alliance 2695) and mass spectrometry (Waters 3100). The mobile phases were prepared daily by filtering through 0.45 μm membrane disc filter and degassed by sonication before use. The mobile phase for gradient elution consists of two solvents: solvent A (0.1% formic acid (FA) in H_2O) and solvent B (0.1% FA in $CH_3CN/MeOH$ (1: 1; v/v). The linear gradient profile was as follows: 95% A (5 min), 95-90% A (10 min), 90-50% A (55 min), 50-95% A (65 min), and 95% A (70 min). The injection volume was 10 μL. The flow rate (0.6 ml/min) was split 1: 1 before the MS interface. The negative ion mode parameters were as follows: source temperature 150 °C, desolvation temperature 350 °C, cone gas flow 50 L/h, cone voltage 50 eV, capillary voltage 3 kV, and desolvation gas flow 600 L/h. Spectra were recorded in the ESI negative mode between

m/*z* 50-1000. The peaks and spectra were processed using the Maslynx 4.1 software. Unknown peak was tentatively identified by comparing its retention time and mass spectrum with literatures. Known peak was identified by comparing its retention time (R_t) and mass spectrum with a known standard.

11. Statistical analysis

All data were expressed as mean ± SD and the SC_{50} values were calculated using the SPSS 13.0 program.

RESULTS AND DISCUSSION

1. Antioxidant activity, Total Phenolic and Flavonoid contents

Solvent polarity plays a key role in extraction of plant process and increasing phenolic solubility. The aqueous methanol is very common solvent for extraction of the phenolic compounds from plant materials. In the present study, the defatted 80% methanolic extract of *Salix tetrasperma* was prepared and the total phenolic and flavonoid content of this extract was determined. The results in table 1 showed that the methanolic extract of *S. tetrasperma*give high yield of total phenolic (145.09 ± 3.92 mg gallic acid equivalent/g plant extract) and flavonoids (98.86 ± 0.31 mg rutin equivalent/g plant extract). On the other hand, the defatted methanolic extract of the plant showed a considerable antioxidant activity with three tests used in this study. The results in table 2 revealed that the methanol extract react positively with DPPH test (SC_{50}= 88.46 ± 0.58 µg/ml) where this reaction depend on the ability of this extract as free radical scavenging agent. Also, this extract led to high reduction of Mo(IV) to Mo (V) and the subsequent formation of green phosphate/Mo compounds which gave maximum absorption at 695 nm. This indicated that the methanol extract has high total antioxidant activity (203.83 ± 7.34 mg ascorbic acid equivalent/g extract). The methanolic extract exhibited high ABTS activity which indicating of its potential antioxidant activity (597.87± 7.37 mmolTrolox® equivalent/100 g extract).

Owing to the high antioxidant of the methanolic extract of *Salix tetrasperma*, this extract was fractionated using certain organic solvents such as dichloromethane, ethyl acetate and *n*-butanol. The total phenolic and flavonoids of these fractions were determined. The results in table 1 exhibited that although the four fractions have remarkable phenolic and flavonoid contents but the ethyl acetate fraction had high contents (285.48 ± 2.07 mg gallic acid equivalent /g plant extract and 136.13 ± 2.47 mg rutin equivalent/g plant extract) respectively. Evaluation of these fractions as antioxidant agents using the three tests as shown in table 2 exhibited that the ethyl acetate fraction has high activity (SC_{50}= 42.59 ± 0.38 µg/ml; 298.03 ± 6.85 mg ascorbic acid equivalent/g extract; 925.79 ± 4.74 mmolTrolox® equivalent/100 g extract) respectively whereas the residual aqueous fraction was much smaller. These results are in agreement with the previous reports on the other plant extracts [14, 25]. Also the results indicated that phenolic and flavonoid contents are the major contributors to the antioxidant activity of the plant *S. tetrasperma*extracts (there is a strong correlation between the antioxidant activity of this plant and its total phenolic and flavonoid contents). These results are also in agreement with the previous results [10,14, 15].It was appeared that the phenolic and flavonoid of *S. tetrasperma*could be suggested as a potential natural source of antioxidants appropriate for utilization in nutritional and pharmaceutical fields. Therefore, the identification of the polyphenols of the plant under investigation is required and very important.

Table 1.Total phenolic and flavonoid contents of the 85% defatted methanolic extract of *Salix tetrasperma* and its derived fractions

Plant extract	Total phenols (mg gallic acid equivalent/g plant extract)	Total flavonoids (mg rutin equivalent/g plant extract)
85% Defatted MeOH ext.	145.09 ± 3.92	98.86 ± 0.31
CH_2Cl_2 fraction	47.75 ± 1.24	23.52 ± 3.92
EtOAc fraction	285.48 ± 2.07	136.13 ± 2.47
BuOH fraction	216.46 ± 2.82	65.22 ± 3.76
H_2O fraction	37.62 ± 2.57	11.47 ± 1.23

Values are expressed as mean of triplicate determinations ± standard deviation.

Table 2.DPPH scavenging activity, total antioxidant capacity and ABTS assay of the 85% defatted methanolic extract of *Salix tetrasperma* and its derived fractions

Plant extract	DPPH free radical scavenging activity SC_{50} [µg/ml]	Total antioxidant capacity [mg ascorbic acid equivalent / g extract]	ABTS assay [mmolTrolox® equivalent /100 g extract]
85% Defatted MeOHext.	88.46 ± 0.58	203.83 ± 7.34	597.87 ± 7.37
CH_2Cl_2 fraction	150.78 ± 0.59	123.17 ± 2.61	503.36 ± 10.53
EtOAc fraction	42.59 ± 0.38	298.03 ± 6.85	925.79 ± 4.74
BuOH fraction	72.97 ± 0.35	260.63 ± 4.91	742.09 ± 3.06
H_2O fraction	274.77 ± 2.33	82.33 ± 4.30	261.47 ± 9.58
Ascorbic acid	8.06 ± 0.70	--	--

Values of SC_{50}, total antioxidant capacity and ABTS assay are expressed as mean of triplicate determinations ± standard deviation.

2. LC-ESI-MS analysis of methanolic extract of *S. tetrasperma*

Due to the fact that plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, their separation and identification still remains a big challenge. Liquid chromatography coupled with mass spectrometry (LC/MS) is a powerful and new technique for identification of the complex botanical extracts [26, 27].It provides information for structural elucidation of the components of these extracts. Therefore, in the present work, the defatted methanolic extract of *S. tetrasperma* was submitted to HPLC connected with MS spectrometry in negative ion mode. The identification of the major components of this extract was carried out through their retention times, molecular weights (MW), calculated *m/z*, major fragments which produced under the ionization conditions, as well as by comparison of these data with the standards (Fig. 1) and the previously reported data in the literatures.

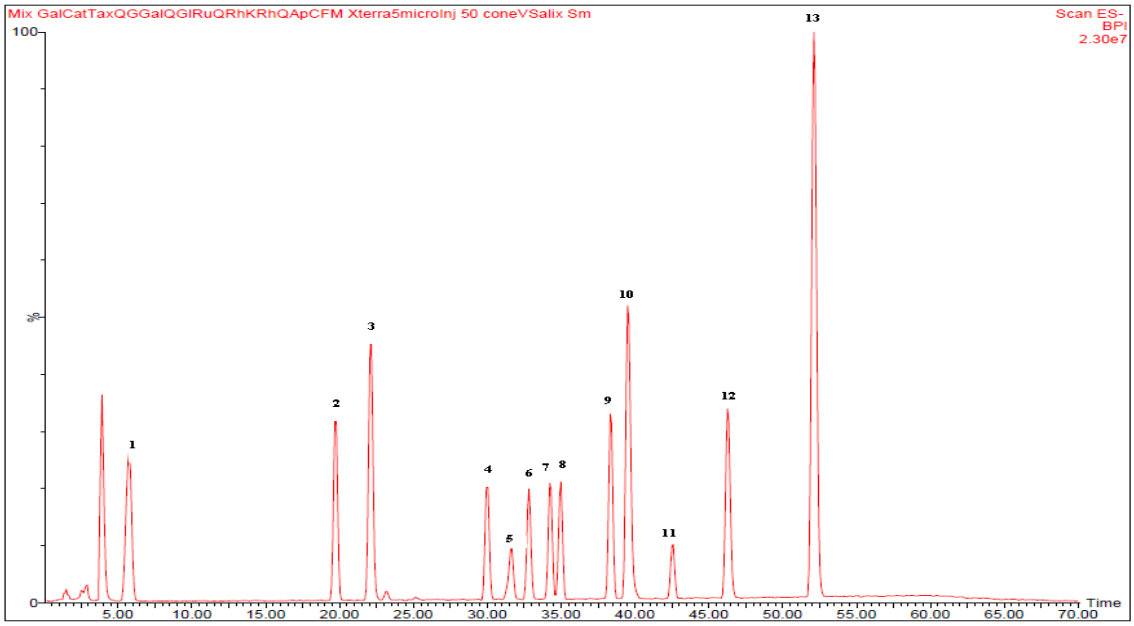


Figure1. HPLC-ESI-MS chromatogram of 13 phenolic compound standards; gallic acid (1), catechin (2), caffeic acid (3), ferulic acid (4), taxifolin (5), quercetin-3-*O*-β-D- glucpyranoside (1→6) gallic acid (6), rutin (7), quercetin 3-O-β-D-glucpyranoside (8), quercetin 3-*O*-α-L-rhamnopyranoside (9), myrcetin (10), kaempferol 3-*O*-α-L- rhamnopyranoside (11), quercetin (12), and apigenin (13)

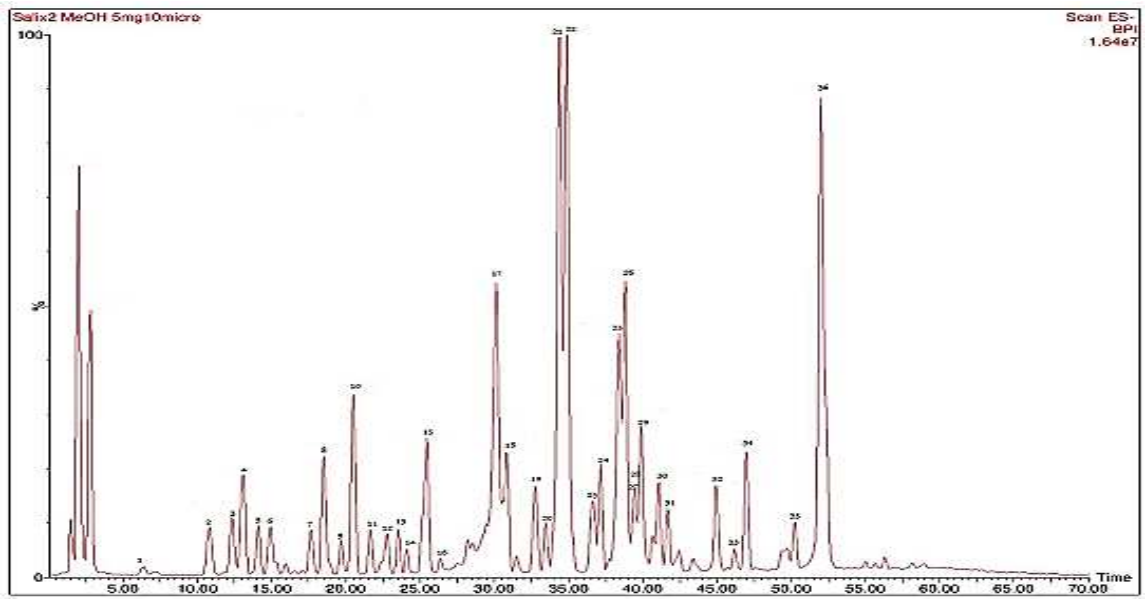


Figure 2.HPLC-ESI-MS chromatogram of *Salix tetrasperma*85% defatted MeOH extract

The results of HPLC-ESI-MS identification of the components of the 85% defatted methanolic extract of *S. tetrapermaa*re represented in Fig. 2 & 3 and table 3. From these results, it was appeared that the tentative identified compounds are a mixture of flavonoid and phenolic acid derivatives.

Table 3.Tentative identification of some compounds in the 85% defatted methanolic extract of *Salix tetrasperma* by LC-ESI (-ve)-MS.

Peak No.	R_t	MW	[M-H]	MS fragments	Tentative identified compounds
85% defatted methanolic extract					
1	6.34	320	319	179,163,119	Coumaroyl-1,5-quinolactone
2	10.85	316	315	169,153,109	Protocatechuic acid hexoside
3	12.35	494	493	331,313,169,109	Gallic acid dihexoside
4	13.10	618	617	571,331, 285, 165,123	Gallic acid hexose derivative
5	14.19	610	609	447,315,163,152	Isorhamnetin-hexoside-pentoside
6	14.94	354	353	191, 179	3-caffeoyl quinic acid
7	17.61	444	443	337,191,163	Coumaroylquinic acid derivatives
8	18.53	338	337	191,163,119	3-Coumaroyl quinic acid
9	19.70	326	325	195,185,163, 119	Coumaroyl-hexoside
10	20.54	354	353	191	5-caffeoyl quinic acid
11	21.71	354	353	191,179, 173, 135	4-caffeoyl quinic acid
12	22.79	338	337	191,173,163,119	Coumaroylquinic acid isomer
13	23.54	388	387	207,173,119	Unknown
14	24.13	416	415	269,161,62	Apigenin-*O*-deoxyhexose isomer
15	25.46	338	337	191, 173,163,119	Coumaroylquinic acid isomer
16	26.38	594	593	537,489,327,195,179	Unknown
17	30.14	848	847	469, 423, 317, 273,162	Unknown
18	30.81	480	479	317,155	Myricetin-*O*-hexoside
19	32.73	440	439	233, 145,62	Unknown
20	33.48	464	463	317,147	Myrictin-*O*-deoxy hexose
21	34.31	610	609	463,447,301,179	Rutin[1]
22	34.90	464	463	301,179,151	Quercetin-3-*O*-β-D-glucopyranoside[1]
23	36.57	434	433	301,245,187,113	Qurecetin-*O*-pentoside
24	37.15	448	447	284,233,175,145	Luteolin-*O*-hexoside
25	38.32	448	447	285, 163,145	Kaempferol-*O*-hexoside
26	38.82	478	477	314,234,161,113	Isorhmentin-*O*-hexoside
27	39.41	432	431	269,175,163,145	Apigenin-*O*-hexoside isomer
28	39.49	432	431	269,175,163,145	Apigenin-*O*-hexoside isomer
29	39.91	476	475	423, 299,273,145	Unknown
30	41.08	562	561	435, 423,313,273,285	Unknown
31	41.66	562	561	431,307, 285,163,145	Kaempferol-*O*-deoxyhexose–pentoside
32	44.92	470	469	423,316,248	Unknown
33	46.17	302	301	273,179,151	Quercetin[1]
34	46.92	416	415	285,163,145	Kaempferol-*O*-pentoside
35	50.26	528	527	415,317,248,145	Unknown
36	52.10	528	527	405,269,161,137,121	Apigenin derivative
Standard compounds					
1	5.76	170	169	125, 97, 69	Gallic acid
2	19.71	290	289	244, 221, 150, 136, 123	Catechin
3	22.13	180	170	135, 107, 69	Caffeic acid
4	29.98	194	193	178, 134	Ferulic acid
5	31.64	304	303	284, 274, 217, 179, 151	Taxifolin
6	32.81	616	615	463, 313, 301, 271, 169, 151, 147	Quercetin-3-*O*-β-D-glucopyranoside(1→6) gallic acid
7	34.23	610	609	463, 301, 179, 151, 147	Rutin
8	34.99	464	463	300, 271, 254, 179, 151	Quercetin 3-*O*-β-D-glucopyranoside
9	38.33	448	447	300, 270, 179, 151	Quercetin 3-*O*-α-L-rhamnopyranoside
10	39.49	318	317	179, 151	Myrcetin
11	42.50	432	431	248, 254, 227, 198, 147	Kaempferol 3-*O*-α-L- rhamnopyranoside
12	46.26	302	301	179, 151	Quercetin
13	52.10	270	269	225, 199, 159, 151, 117	Apigenin

[1]*Compounds identified by comparison with standards.*

2.1. Phenolic acid derivatives

Phenolic acid derivatives were tentative identified which belonging to hydroxybenzoic acid derivatives (protocatechuic and gallic acids) and hydroxycinnamic acid derivatives (*p*-coumaric and caffeic acids). These acids are found in their conjugated forms usually linked to sugars as shown in table 3.

2. 1(a). Hydroxybenzoic acid derivatives

The mass spectrum of compound **2** (R_t= 10.85) gave deprotonated molecule $[M-H]^-$ at *m/z*= 315 and base peak at *m/z*= 153 $[M-H - 162]^-$ due to lose of hexose sugar as well as other peak at *m/z*= 109 $[M-H- 44]^-$ related to the liberation of CO_2. This reflected the presence of protocatechuic acid hexoside[28].Compound **3** and **4** (R_t = 12.35

and 13.10 respectively), were tentative identified as gallic acid derivatives through appearing the characteristic peak ion at *m/z*= 331 for galloyl hexose. Compound **3** showed molecular ion [M-H]$^-$ at *m/z*= 493 and two fragment ions at *m/z*= 331 [M-H- 162]$^-$ and *m/z*=169 [M-H-2×162]$^-$ due to loss of two hexoside moieties, Therefore compound **3** was elucidated as gallic acid dihexoside. Whereas compound **4** yield molecular ions at *m/z* = 617 and 331 for gallic acid hexoside derivative[28].

2. 1(b). Cinnamic acid derivatives

Nine Compounds **(1, 6-12 and 15)** are hydroxycinnamic acid derivatives. Three of them (**6**, **10** and **11**) had R_t = 14.94, 20.54 and 21.71 respectively, were tentative annotated as caffeoylquinic acid isomers (isomers of chlorogenic acid) on the basis of presence a permanent peak at *m/z* = 353 in their spectrums. Compound **6** was 3-*O*-caffeoyl quinic acid where, this compound yielded two peaks at *m/z*= 191 (100 %) and 179 (80 %). Compound **10** was identified as 5-*O*-caffeoyl quinic acid on a basis of clearing a base peak at *m/z*= 191 (100 %), while compound **11** gave two characteristic ions *m/z*=179 (100%) and 173 (85%) for 4- *O*- caffeoylquinic acid [26, 29].Five compounds (**1**, **7, 8, 12** and **15** with R_t = 6.34, 17.61, 18.53, 22.79 and 25.46 respectively) were tentative identified as coumaroylquinic acid derivatives according to presence of the characteristic peak at *m/z*= 337 in their spectra beside other fragment ions at *m/z*= 191,173,163 and 119 [26, 28,30]**.** Compound **1** showed base peak *m/z*= 319 [M-H]$^-$ and other fragments at *m/z*= 163 [M-H-156]$^-$ which means the loss of quinolactone unit, which indicated compound **1** is coumaroyl-1,5-quinolactone isomer. Compound **7** showed *m/z*= 443 [M-H]$^-$ and other fragments at *m/z*= 191 (40%) and 163 (75%) so compound **7** is *p*-coumaroylquinic acid derivatives and compound **8** showed *m/z* = 337 [M-H]$^-$ and base peak at *m/z*= 163(100 %) and 119 indicating the presence of 3-*O*-*p*-coumaroyl quinic acid while compounds **12** and **15** exhibited deprotonated molecule at *m/z* = 337 and a base peaks at *m/z*= 173 (100%) and 163 (25%) reflecting the presence of two isomer of 4-*O*-*p*-coumaroyl quinic acid **(**26,31**).** Compound **9** (R_t = 19.70) gave a deprotonated molecules [M-H]$^-$ at *m/z*= 325 and base peak at *m/z*= 163 (100%) [M-H-162]$^-$ and another peak at *m/z*= 119. Therefore compounds **9** being proposed as *p*-coumaroylhexoside[26, 28, 29].

2.2. Flavonoid derivatives

Compounds **5** and **26** (R_t = 14.19 and 38.82 respectively) were tentative identified as isorhamnetin glucoside derivatives. Compound **5** gave a deprotonated molecule [M-H]$^-$ at *m/z*= 609 and other fragment at *m/z*= 447 [M-H-162]$^-$ indicate the liberation of hexose unit. There is another fragment at m/z= 315 [M-H-162-132]$^-$due to the loss of another pentose unit. Therefore, compound **5** was tentative identified as isorhamnetin-*O*-hexoside-pentoside. Compound **26** yield [M-H]$^-$ at *m/z* = 477 beside a characteristic peak at *m/z* = 314 [M-H-162]$^-$ due to losing of hexoside unit so compound **26** tentative identified as isorhamnetin-*O*-hexoside respectively [26,28].

Each of compounds **18** and **20** (R_t =30.81 and 33.48 respectively) which gave a characteristic myricetin ion peak at *m/z*= 317 in their structures indicating the two compounds are myricetin derivatives. Compound **18** gave a deprotonated molecule at *m/z*= 479 [M-H]$^-$and other fragment ion at *m/z*= 317 [M-H-162]$^-$ which reflected that compound **18** is myricetin-*O*-hexoside**[**28**,**31]. On other hand compound **20** yield a deprotonated molecule at *m/z*= 463 [M-H]$^-$ and other fragment at *m/z*= 317 [M-H- 146]$^-$ which indicated the liberation of deoxyhexose unit. This indicated that compound **20** is myricetin-*O*-deoxyhexose[26, 28].

Compound **24** (R_t = 37.15) showed deprotonated ion at m/z= 447 [M-H]$^-$ and other fragment at *m/z*= 284 [M-H-163]$^-$ which indicated the liberation of hexose unit. So, compound **24** identified as luteolin -*O*-hexoside[32,33].

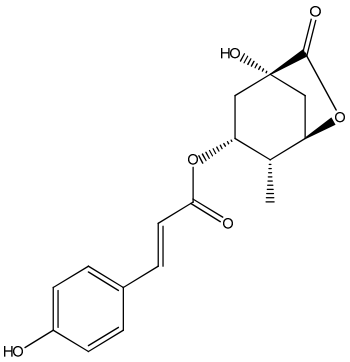
Compounds **25**, **31** and **34** (R_t =38.32, 41.66 and 46.92) were tentatively identified as kaempferol derivatives. Compound **25** gave a deprotonated molecule at *m/z* = 447 [M-H]$^-$and other fragment ion at *m/z*= 285 [M-H-162]$^-$ indicating that compound **25** could be identified as kaempferol-O-hexoside. Compound **31** showed deprotonated molecule [M-H]$^-$ at *m/z*= 561 and two fragment ions at *m/z*= 431 [M-H-130]$^-$ and 285 [M-H -132-146] due to loss of pentose and deoxyhexose unit so compound **31** kaempferol-*O*-deoxyhexose-pentoside[26,28]**.** Compound **34** yield a deprotonated molecule [M-H]$^-$ at *m/z*= 415 and fragment ion at *m/z*= 285 [M-2H-130]$^-$ for losing of pentose unit. Therefore, compound **34** is kaempferol-*O*- pentose [26,28].

Four compounds **14**, **27**, **28** and **36** (R_t = 24.13, 39.41, 39.49 and 52.10 respectively) were assigned to apigenin derivatives, compound **14** yield deprotonated molecule at *m/z*= 415 [M-H]$^-$ and fragment at *m/z*= 269 [M-H-146]$^-$ for losing deoxyhexose unit. Therefore, compound **14** is apigenin-*O*-deoxyhexose isomer. Compounds **27** and **28** gave the same deprotonated molecule at *m/z*= 431[M-H]$^-$ and a peak at *m/z*=269 [M-H-162]$^-$ due to loss of hexose unit beside the characteristic fragment ions of apigenin. Therefore, the two compounds may be apigenin-*O*-hexoside isomers. Compound **36** showed deprotonated molecule at *m/z*=527 [M-H]$^-$ and a peak at *m/z*=269 which characteristic for apigenin, so compound **36** is apigenin derivative [26,34].

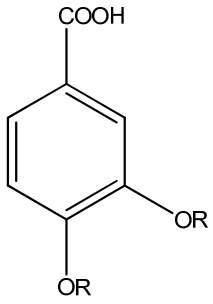
Compounds **21, 22,** and **33** with R_t = 34.31, 34.90 and 46.17 respectively were ascertained identified as rutin, quercetin 3-*O*-β-D-glucopyranoside and quercetin by comparing their mass spectra and $R_t$with standards. Compound **23** has *m/z*= 433 $[M-H]^-$ and other fragment at *m/z*= 301 $[M-H-132]^-$ which revealed that loss of pentose unit so compound **23** is tentative identified as quercetin-*O*-pentoside**[**26,32,35].

CONCLUSION

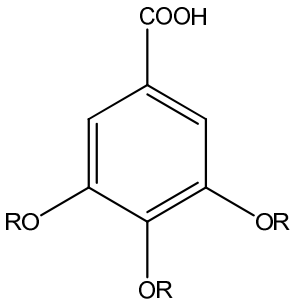
The present study showed that the methanolic extract of *Salix tetrasperma*and its derived fractions had antioxidant activity and high phenolic content especially EtOAc fraction. These extracts could be suggested as a potential natural source of antioxidants appropriate for utilization in nutritional and pharmaceutical fields. The characterization process using HPLC-ESI-MS indicated that the methanolic extract contains flavonoid and phenolic acid derivatives.



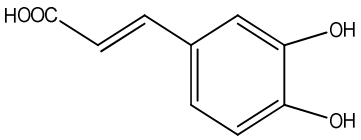
(1) Coumaroyl-1, 5-quinolactone



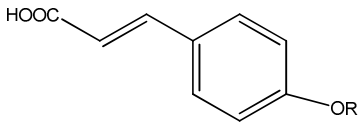
2R= H; Protocatechuic acid
(2) 1R= Hexose, 1R= H



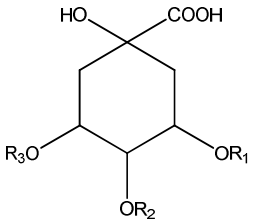
3R= H; Gallic acid
(3) 2R= Hexose, 1R= H or
1R= Hexose-O-Hexose, 2R=H



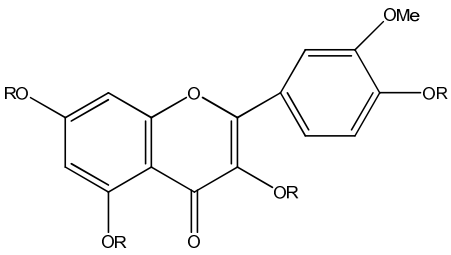
Caffeic acid (CA)



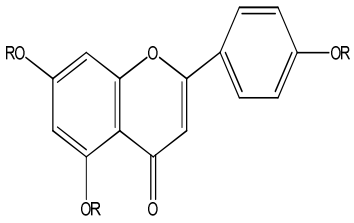
R=H; P-Coumaric acid (pCoA)
(9) R= Hexose



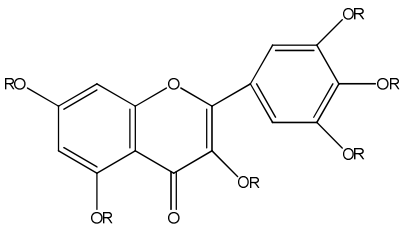
R_1, R_2, R_3 = H Quince acid
(6) R_2, R_3 = H, R_1- CA
(8) R_2, R_3 = H, R_1= PCoA
(10) R_1, R_2 = H, R_3= CA
(11) R_1, R_3 = H, R_2= CA
(12) 2R= H, 1R PCoA
(15) 2R= H, 1R= PCoA



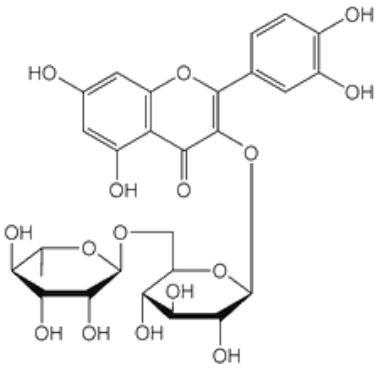
4R= H; Isorhmentin
(5)1R= Hexose, 1R- Pentose, 2R= H or 1R= Hexose-O-Pentose, 3R=H
(26) 1R= Hexose, 3R= H



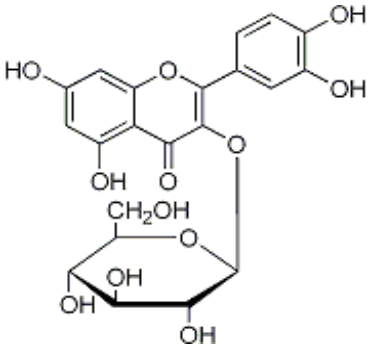
3R= H; Apigenin
(14) 2R = H, 1R= Deoxy- hexose
(27) 2R = H, 1R= Hexose
(28) 2R = H, 1R= Hexose



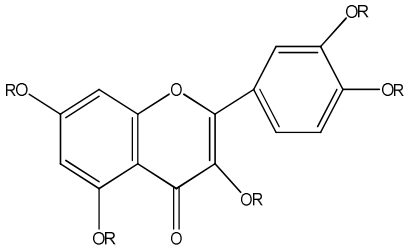
6 R = H, Myricetin
(18) 1 R = Hexose, 5 R = H
(20) 1 R = Deoxyhexose, 5 R = H



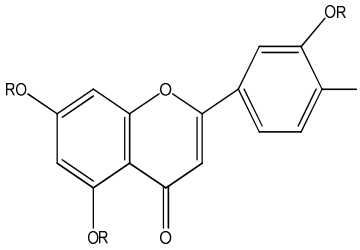
(21)Rutin



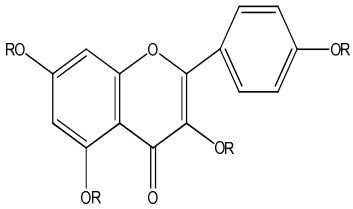
(22) Quercetin-3-*O*-β-D- glucopyranoside



5 R = H, Quercetin
(23) 1 R = Pentose, 4 R = H
(33) 5 R =H



4 R = H; Luteolin
(24) 1 R = Hexose, 3 R=H



4 R = H; Kaempferol
(25) 1 R = Hexose, 3 R = H
(31) 1 R = pentose, 1 R = Deoxyhexose, 2 R = H or
1 R = Deoxyhexose- *O*- pentose, 3 R = H
(34) 1 R = Pentose, 3 R = H

Figure 3: Chemical structures of some compounds identified and tentatively identified

REFERENCES

[1] M T Khayya l, M AEl-Ghazaly, D M Abdallah, S N Okpanyi, O Kelber, D Weiser. *Arzneimittelforschung*, **2005**, 55(11): 677- 687.
[2] A Hemati, M Azarnia, A H Angaji. *J Sci Res*, **2010**, 5(3): 174-176.
[3] J M Mates, C Perez-Gomez, I Nunez de Castro. *Clin.Biochem*, **1999**, 32(8): 595-603.
[4] E Souri, G Amin, HFarsam, H Jalalizadeh, S Barezi. *J of Pharm Res*, **2008**, 7 (2): 149-154.
[5] O I Aruoma. *Nutri. Res*, **2003**, 523-524: 9-20.
[6] C B Cochrane, P K Nair, S J Melnick, A P Resek, C Ramachandran. *Anticancer Res*, **2008**, 28 (2A): 965-971.
[7] M H Rhee, H J Park, J Y Cho. *J Med Plants Res*, **2009**, 3(8): 548-555.
[8] L K Han, M Sunmiyoshi, J Zhang, M X Liu, X F Zhang, Y N Zheng,H Okuda, Y Kimura. *Phytother Res*,**2003**, 17(10): 1188-1194.
[9] S Sultana, M Saleem. *J Ethanopharmacol*, **2004**, 91: 267-276.
[10]M S Alam, G Kaur, Z Jabbar, K Javed, M Athar. *Phytothe. Res*, **2006**,20 (6):479-483.
[11] I M C Brighente, M Dias, L G Verdi, G Pizzolatti. *Pharm Biol*, **2007**, 45 (2): 156-161.
[12] U Gawlik-Dziki, D Sugier, D Dziki, P Piotr Sugie. *Sci World J*, **2014**, 1-10.
[13] K Krauze-Baranowska, E Szumowicz. *PostepyFitoterapia*, **2004**, 5 (2): 77–86.
[14] S Jan, M R Khan, U Rashid, J Bokhari. *Osong Public Health Res Perspect*, **2013**, 4 (5): 246-254.
[15] D O Kim, S W Jeong, C Y Lee. *Food Chem*, **2003**, 81(3): 321-326.
[16] X Li, Z Liu, X F Zhang, L J Wang, Y N Zheng, C C Yuan, G Z Sun. 2008,*Molecules*, 13(8): 1530-1537.
[17] S A Santos, P C Pinto, A J Silvestre, C PNeto. *Industrial Crops Prod*, **2010**, 31(3): 521-526.
[18] M M El-Sayed, S A Abo-Sedra, H A El-Nahas, M M Abdel-Gawad, M A Maher, S A El-Sayed, S A Waffa, E AEzzat. *Aust J Basic and ApplSci*, **2011**,5(9): 344-352.
[19] K S Kumer, K Ganesan, P V Rao. *Food Chem*, **2008**, 107 (1): 289-295.
[20] A Sonboli, M Mojarrad, S N Ebrahimi, SEnayat. *Iranian J Pharm Res*, **2010**, 9: 293-296.
[21] S Parajule, T P Nirmala, P Shraddha, JNirmala. *JHAS*, **2012**, 2(1): 27-31.
[22] V Y Barku, Y Opoku-Boahen, E Owusu-Ansh, E F Mensah. *Asian J Plant Sci Res*, **2013**, 3(1): 69-74.
[23] P Prieto, M Pineda, M Aguilar. *Anal Biochem*, **1999**: 269, 337-240.
[24] R Re, N Pellegrini, AProteggente, A Pannala, M Yang, C Rice-Evans. *Free Radical Biol Med*, **1999**, 26: 1231-1237.

[25] N Saeed, M Khan, M Shabbi. *Alternat Med*, **2012**, 12:221-233.
[26] A Plazonic, F Bucar, Z Males, AMrnar, B Nigovic, N Kujundzic. *Molecules*, **2009,** 14: 2466-2490.
[27] A Zhang, L Wan, C Wu, Y Fang, G Han, H Li, Z Zhang, H Wang. *Molecules*, **2013**, 18: 14241-14257.
[28] I M Abu-Reidah, M S Ali-Shtayeh, R M Jamous, D AArraez-Roman, A Segura-Carretero. *Food Chem*,**2015**, 166: 179-191.
[29] J Sun, F Liang, Y Bin, P Li, C Duan. *Molecules*, **2007**: 12, 670-693.
[30] N P Rodrigues, N Bragagnolo. *J Food Compos Anal*, **2013**, 32: 105-115.
[31] L LSaldanha, W Vilegas, A L Dojjedal. *Molecules*, **2013**, 18: 8402-8416.
[32] M B Hossain, D K Rai, N P Brunton. *Agric Food Chem*,**2010**, 58: 10576 -10581.
[33] O R Pereira, A Peres, A M Silva, M R Domongues, Cardoso S M. *Food Res Int*,**2013**, 54(2), 1773-1780.
[34] J Oszmianski, J Kolniak-Ostek, A Wojdylo. *Agric. Food Chem*, **2012**, 61:176-184.
[35] L Z Lin, J M Harnly. *Food Chem*, **2010**, 120: 319-326.