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Research Article

Antioxidant Behavior of some North East Indian Ethno Medicinal Vegetables and their RP-HPLC Analysis

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Abstract

The antioxidant activities of three ethno-medicinal vegetables viz., *Pogostemon parviflorus*, *Houttuynia cordata* and *Solanum spirale* which are used by the indigenous people of North East India to cure the diseases of stomach, liver etc. were evaluated by spectroscopic methods using 1, 1-diphenyl-2-picrylhydrazyl (DPPH·) radical assay, xanthine/xanthine oxidase assay and hydroxyl radical (OH·) scavenging assay. For these, sequential extraction was done by solvents with increasing polarity viz., hexane, ethyl acetate and methanol. The total phenolic and total flavonoid contents were also measured using standard methods. Results showed that the methanol extracts of all the species showed the highest antioxidant activities and high values for total phenolic and flavonoid contents. A strong correlation was observed between the antioxidant activities and total phenolic and flavonoid contents which indicate that the presences of phenolics are mainly responsible for the antioxidant potential of the plant species. RP-HPLC with UV detection was employed for the identification and quantification of the phenolic antioxidants, present in methanolic extracts. The most abundant phenolic acids were gallic acid (0.08-5.78 mg/g of dry sample) and sinapic acid (0.01-1.07 mg/g of dry sample). Quercetin was the most abundant flavonoid (0.19-3.03 mg/g of dry sample).

Keywords: *Antioxidant, xanthine/xanthine oxidase, hydroxyl radical scavenging, RP-HPLC.*

INTRODUCTION

Antioxidant phytochemicals found in fruits and vegetables play an important role in preventing degenerative diseases such as cancer and cardiovascular diseases as such diseases are associated with oxidative damage, which is a result of oxidative stress. The flavonoids and other phenolic compounds are potential antioxidants among the phytochemicals [1-3]. These can have complementary and overlapping mechanisms of action, including scavenging of oxidative agents, stimulation of the immune system, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism and antibacterial and antiviral effects [4]. Different plants have different compositions of phytochemicals, to different extents. Therefore, sufficient amounts of the appropriate compounds from a variety of dietary sources are recommended for health benefits [5].

North East India is one of the richest repositories of medicinal and aromatic plants in the World. This region is also famous for diverse culture of human races, being the abode of approximately 225 tribes in India, out of 450 in the country. Therefore, the culture and customs of this region have an important role in understanding biodiversity conservation and management issues [6, 7, 8]. Scientific approach for their exploration, conservation and value addition may be the key points for entrepreneurship development by exploiting the indigenous technology knowledge. Research and Development works on medicinal plants get momentum in recent years as some

synthetic drugs are reported to have adverse side effects. However, more research is needed to evaluate their bioactivities or pharmacological effects. The objectives of the present study were to determine the antioxidant activity, total phenolic and total flavonoid content of the different extracts of three ethno-medicinal vegetables of North East India. In addition, a correlation between them was also studied. The plants under study are listed in Table 1, with details.

MATERIALS AND METHODS

Plant materials and chemicals: The plant materials were collected from their natural habitats from nearby areas of Dibrugarh University, Dibrugarh, Assam. One of the plant viz., *Solanum spirale* was collected from Dibong, Arunachal Pradesh. Voucher specimens (*P. parviflorus*: DCH-24, *H. cordata:* DCH-25, *S. spirale*: DCH-26) were deposited at the Herbarium of the Department of Chemistry, Dibrugarh University, Dibrugarh, India. 1, 1-diphenyl-2-picryl hydrazyl (DPPH), (+)-catechin, gallic acid, kaempferol, quercetin, sinnapic acid, protocatechuic acid, 3-hydroxy coumaric acid, gentisic acid, caffeic acid, thiobarbituric acid, ethylenediamine tetra acetic acid (EDTA), xanthine oxidase, nitrobluetetrazolium (NBT) were purchased from Sigma Chemical Co. (MO, USA). Folin-Ciocalteu reagent, hydrogen peroxide, L-ascorbic acid and trichloroacetic acid; methanol, acetonitrile, water a nd acetic acid (HPLC grade) were obtained from Merck Co. (Germany). All other chemicals used were of analytical grade.

Preparation of crude plant extracts: The plant materials were cut into small pieces, shade dried for about a week and then crushed to make coarse powder. The dried powder was extracted with *n*-hexane, ethyl acetate and methanol using a Soxhlet apparatus. The extracts were concentrated under reduced pressure with the help of a rotary vacuum evaporator. The residues were used to carry out radical scavenging assay.

Free radical scavenging activity: DPPH Assay: The scavenging activity of DPPH by the different plant extracts was determined by a slightly modified spectrophotometric method of Brand-Williams[9]. A solution of DPPH in methanol was prepared daily, before UV measurement. From this stock solution, for each measurement, 0.2 mL of DPPH solution was made up to 3 mL by adding methanol to make a test solution (0.07 mM). The absorbance of the test solution was recorded at 517 nm. Antioxidant activity of the plant extract was studied by adding 0.1 mL of the stock solution of the extract to a test solution of DPPH. The solution was shaken well and incubated in the dark for 30 minutes at room temperature. The decrease in absorbance was measured at 517 nm and was compared with a control of methanol in a Hitachi UV-visible spectrophotometer. The percentage inhibition of the radicals due to the antioxidant properties of the plant extract was calculated using the formula:

$$\text{Inhibition (\%)} = (A_{control} - A_{sample})/A_{control} \times 100 \quad \text{---- (1)}$$

Superoxide anion radical scavenging activity: NBT/XO assay: The method of Kirby and Schmidt was used [2, 10]. Reagents were prepared in 50 mM KH2PO4-KOH buffer, pH 7.6. The reaction mixture contained 20 µL 15 mM Na2EDTA (pH 7.6), 30 µL of 3 mM xanthine, 1 mL of 0.6 mM NBT, 500 µL of xanthine oxidase solution (1 U in 10 mL buffer) and 100 µL of plant extracts in buffer (a diluted sonicated solution of 10 mg /250 mL buffer) or 150 µL of KH2PO4-KOH buffer (as control). The reaction was initiated by the addition of xanthine oxidase at 37°C and superoxide anion radical ($O_2^{\bullet-}$) production was evaluated spectrophotometrically by monitoring the reduction of NBT to nitro blue diformazan at 560 nm. Results were expressed as a percentage of inhibition of the superoxide radical calculated by equation (1).

Hydroxyl radical ($OH^{\bullet}$) scavenging activity: Hydroxyl radicals were measured by the deoxyribose method [11, 12]. Hydroxyl radicals generated by ferric-ascorbate-EDTA-H_2O_2, which attacks on deoxyribose to form products called thiobarbituric acid reactive substances (TBARS), which upon heating with TBA at low pH yield pink chromogen. The hydroxyl scavenger, when added, competes with deoxyribose for hydroxyl radicals and decreases TBARS formation and pink chromogen. The reaction mixture containing 10mM deoxyribose, 10mM ferric chloride, 1mM EDTA, 1mM ascorbic acid and 10mM H_2O_2 in 50mM phosphate buffer pH 7.4 was added 100 µl of plant extracts. After incubating for 30 min at 37°C, the reaction was added to 1ml of 10% trichloroacetic acid and 1ml of 1% TBA to yield a final volume 3ml. The reaction mixture was kept in a boiling water bath for 30 min., cooled and the absorbance was measured at 532 nm. Scavenging action was expressed as percentage of inhibition of hydroxyl radical calculated as in Equation (1).

Determination of total phenolic content (TPC): The amounts of total phenolics in the plant extracts were determined by the Folin-Ciocalteu reagent (FCR)

method [2, 13, 14]. To 1.5 mL of a dilute extract of each plant, 5 mL of 10% diluted FCR and 4 mL of aq. Na_2CO_3 (7.5%) were added and the resulting mixture was allowed to stand for 30 minutes, and the total phenolics were determined using an UV-Visible spectrophotometer at 760 nm. The average of triplicate measurements was used to calculate the phenolic content as mg gallic acid equivalents (GAE) / g dry weight of the plant extract. Gallic acid was used for the preparation of standard curve.

Determination of total flavonoid content (TFC): TFC was determined by the aluminum chloride colorimetric method [2, 14, 15]. The calibration curve was prepared by preparing catechin solutions at different concentrations (20-100 mg/L). A 1 mL aliquot of appropriately diluted sample or standard solutions of catechins (20, 40, 60, 80 and 100 mg/mL) was added to a 10 mL volumetric flask containing 4 mL double distilled water. At zero time, 0.3 mL 5% $NaNO_2$ was added to the flask. After 5 min, 0.3 mL 10% $AlCl_3$ was added. At 6 min, 2 mL of 1M NaOH was added to the mixture followed by addition of 2.4 mL distilled water. Absorbance of the resulting pink mixture was determined at 510 nm versus the prepared water blank. The amounts of flavonoids in the plant extracts were expressed in mg catechin equivalents/g dry weight of the plant extract.

RP-HPLC analysis: HPLC analysis was accomplished on a Shimadzu Prominence reverse phase (RP)-HPLC instrument equipped with a manual injector, a programmable wavelength photodiode array (PDA) UV detector (200-400 nm) and a modified silica gel (C18) column [16]. The flow rate was 1 mL/min and the injection volume was 20 µL. The mobile phase was a binary solvent system consisting of (A) water: acetonitrile (91:9) with 2% acetic acid, and (B) acetonitrile: water (80:20) with 2% acetic acid. The freshly prepared infusions from methanolic plant extracts were filtered through a 0.2 µm Nylon 66 membrane filter and 20 µL of each plant extracts were injected. The compounds were identified by comparing with a standard of each identified compound using the retention time and the absorbance spectrum profile. Quantification of the compounds was based on peak area, obtained with valley-to-valley integration using standard compounds. For this 1 mg of each standard compounds and extracts were diluted to 10 mL in methanol.

Statistical analysis: All analyses were performed in triplicate. The data were recorded as means ± standard deviations (SD). Correlation coefficients (*R*) to determine the relationship between two variables (between radical scavenging activities and TPC and TFC) were calculated using MS Excel Software (CORREL statistical function).

RESULTS AND DISCUSSIONS

Free radical scavenging activity: DPPH Assay

The n-hexane, ethyl acetate and methanol extracts of all three plants investigated were found to reduce DPPH within 30 minutes (Table 2). The methanol extracts of all three species showed the highest DPPH radical scavenging activity (P. parviflorus -91.06%, H. cordata -79.83% and S. spirale – 64.14%) at a concentration of ~ 4 mg dry extract/mL of solvent. These percentages can be considered as full absorption inhibition of DPPH, because after completion of the reaction, the final solution always possesses some color and, therefore, its absorption inhibition compared with colorless methanol solution cannot reach 100%. The ethyl acetate and n- hexane extracts were considerably less effective radical scavengers than the methanol extracts, with the ethyl acetate extracts being more active than the n-hexane extracts. The results provided in Table 2 demonstrate that the highest antiradical activity was exhibited by the methanol extract of P. parviflorus.

Superoxide anion radical scavenging activity: NBT/XO assay:

The NBT/XO superoxide radical scavenging assay relies on the competition between test substrate and nitro blue tetrazolium chloride (NBT) for superoxide anion generated by the xanthine/xanthine oxidase system. The decrease in absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture[2].

The results in Table 3 show that of the extracts tested, the methanol extracts of all three plants had the highest superoxide anion radical scavenging potential in comparison with their n-hexane and ethyl acetate extracts, although the ethyl acetate extracts were also good superoxide anion inhibitors. Of the three medicinal plants, P. parviflorus had the best superoxide anion radical scavenging activity, followed by H. cordata and S. spirale.

Hydroxyl radical ($OH^{\bullet}$) scavenging activity:

The ability of three vegetables to scavenge hydroxyl radicals was measured by studying competition between deoxyribose and plant extracts for hydroxyl radical generated from ferric-ascorbate-EDTA-H_2O_2 system. Hydroxyl radicals attack deoxyribose starting a set of reactions which eventually results in

thiobarbituric acid reactive substance (TBARS), which upon heating with thiobarbituric acid yield a pink chromogen. When a tested extract scavenges hydroxyl radicals, the TBARS formation is decreased. It was observed that the methanol extracts of all three species had the maximum of hydroxyl radical scavenging activity. Among the three medicinal plants, S. spirale showed the highest hydroxyl radical scavenging activity, followed by P. parviflorus and H. cordata (Table 4).

Determination of total phenolic content (TPC):

Phenolics constitute one of the major groups of compounds acting as primary antioxidants. Hence, it was reasonable to determine their total amount in the selected plant extracts. The phenolic compounds were estimated under basic conditions using the Folin-Ciocalteu reagent (FCR) method; the results are expressed in terms of gallic acid equivalents (GAE) (Table 5).

The results showed high total phenolic contents (TPC), with gallic acid equivalents from 31.95 ± 0.03 and 120.64 ± 0.01 mg/g of extract. The highest values were obtained for the methanol extracts of each of the three species. As the antiradical activity was also found to be highest in the methanol extracts, a direct correlation between the antiradical activity and the TPC of these medicinal vegetables has been observed.

Determination of total flavonoid content (TFC):

Many flavonoids are found to possess effective radical scavenging activity because of their phenolic hydroxyl groups[2,8]. The total flavonoid content (TFC) was determined by an aluminum chloride method using catechin as a reference compound.

The TFC's of the three medicinal vegetables are given in Table 6. The content of flavonoids (mg/g), in catechin equivalents (CE), varied from 6.52 ± 0.03 to 108.55 ± 0.03. Of the three extracts of each vegetable, the methanol extracts had the highest flavonoid contents, with that of P. parviflorus being the highest (108.55 ± 0.03).

Correlation between radical scavenging activity and the total phenolic and flavonoid contents:

The free radical scavenging activities of the three medicinal vegetables determined by different methods were correlated to their TPC and TFC, as shown in Table 7. From the results of the radical scavenging assays (DPPH, $O_2^{\bullet-}$ and $^{\bullet}OH$), a strong correlation was found between them (Table 7). In case of H. cordata, the correlation was not so significant between DPPH vs $O_2^{\bullet-}$ and DPPH vs $^{\bullet}OH$ (0.728 and 0.883 respectively).

The free radical scavenging activities were also correlated to their total phenolic content. DPPH$^{\bullet}$, $O_2^{\bullet-}$ and $^{\bullet}OH$ assay shows significant corrrelationship with the total phenolic content which signifies the direct relationship between the total phenolic compound present in the plant extracts with their free radical scavenging activities. Similarly strong positive correlations were also found for free radical scavenging activities with the total flavonoid content of the plant extract (Table 7**)**.

RP-HPLC analysis:

The methanol extracts of all the three medicinal vegetables having maximum antiradical activity as well as they contained considerable amount of TPC and TFC. Moreover, the TPC, TFC and the antioxidant activities of the plant species are positively correlated (Table 7). This signifies that the phenolic compounds contributed to the antioxidant activities of the plant extracts. So, HPLC was carried out on the plant extracts to identify the phenolic compounds responsible for antioxidant activity. The methanol extracts of all three species were analyzed by RP-HPLC, and some phenolic acids and flavonoids were identified by comparison with authentic compounds (Table 8 and Figure 1-3).

CONCLUSION

The methanol extracts of the three medicinal vegetables showed high antiradical activity in DPPH, $O_2^{\bullet-}$ and $^{\bullet}OH$ assays. The methanol extracts were also found to be rich in TPC and TFC. The results of this study clearly show the presence of substantial amount of phenolic and flavonoid components in the three plant species, which can be considered as good sources for medicinal and food application. A strong correlation was observed between DPPH, $O_2^{\bullet-}$ and $^{\bullet}OH$ assays. RP-HPLC analysis of the extracts of the three plants showed the presence of different phenolic compounds. These findings indicate that the polyphenolic rich extracts of these plant species have a significant potential to allow future exploitation as a natural antioxidant and dietary source for the mitigation of oxidative stress-induced liver injury.

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Table 1
Plants investigated

Sl No	Plant names	Parts used/ Form of use	Method/purpose of use
1	*Pogostemon parviflorus* Benth. [Lamiacea]	Leaf and root	Leaves are taken as medicinal vegetables in cooked form and considered good for liver problems
2	*Houttuyinia cordata* Thunb. [Saururaceae]	Leaf	Leaves are taken as medicinal vegetables in cooked form and good for liver functions
3	*Solanum spirale* Roxb [Solanaceae]	Leaves, berries and root	Used as vegetables considering good for liver and stomach disorders and high blood pressure

Table 2
Inhibition (%) of DPPH by three vegetables extracts

Plants	DPPH inhibition (%) (mean± SD)		
	n-Hexane	EtOAc	MeOH
P. parviflorus	64.83 ± 0.041	82.71 ± 0.037	91.06 ± 0.037
H.cordata	26.76 ± 0.042	60.13 ± 0.021	79.83 ± 0.016
S. spirale	42.25 ± 0.037	51.92 ± 0.054	64.14 ± 0.044

[mean ± Standard deviation (SD) of triplicate measurements].

Table 3
Inhibition (%) of superoxide radical by three vegetables extracts

Plants	$O_2^{\bullet-}$ inhibition (%) (mean± SD)		
	n-Hexane	EtOAc	MeOH
P. parviflorus	27.34± 0.042	49.25± 0.026	70.67± 0.037
H.cordata	28.36± 0.044	50.04± 0.024	65.01± 0.026
S. spirale	26.35± 0.041	48.18± 0.071	63.55± 0.054

[mean ± Standard deviation (SD) of triplicate measurements].

Table 4
Inhibition (%) of hydroxyl radical by three vegetables extracts

Plants	$^{\bullet}OH$ inhibition (%) (mean± SD)		
	n-Hexane	EtOAc	MeOH
P. parviflorus	37.48± 0.024	63.46± 0.099	76.08± 0.024
H.cordata	38.38± 0.024	64.26± 0.016	73.11± 0.016
S. spirale	39.56± 0.041	65.37± 0.021	79.89± 0.004

[mean ± Standard deviation (SD) of triplicate measurements].

Table 5
Total phenolic contents of the three vegetable extracts (GAE: mg/g ofextract).

Plants	Total phenolic content (mean± SD)		
	n-Hexane	EtOAc	MeOH
P. parviflorus	52.53 ± 0.03	78.44 ± 0.04	120.64 ± 0.01
H.cordata	38.84 ± 0.03	46.76 ± 0.04	70.61 ± 0.01
S. spirale	31.95 ± 0.03	36.93 ± 0.03	45.95 ± 0.01

[mean ± Standard deviation (SD) of triplicate measurements].

Table 6
Total flavonoid contents of the three vegetable extracts (CE: mg/g of extract).

Plants	Total flavonoid content (mean± SD)		
	n-Hexane	EtOAc	MeOH
P. parviflorus	14.27 ± 0.03	89.52 ± 0.02	108.55 ± 0.03
H.cordata	6.52 ± 0.03	19.84 ± 0.025	25.75 ± 0.03
S. spirale	8.31 ± 0.03	17.41 ± 0.01	31.95 ± 0.01

[mean ± Standard deviation (SD) of triplicate measurements].

Table 7
Pearson's Correlation (R) between AOAs, TPC and TFC

Plants	DPPH vs. $O_2^{\bullet -}$	DPPH vs $^{\bullet}OH$	$O_2^{\bullet -}$ vs. $^{\bullet}OH$	DPPH vs. TPC	$O_2^{\bullet -}$ vs. TPC	$^{\bullet}OH$ vs. TPC	DPPH vs. TFC	$O_2^{\bullet -}$ vs. TFC	$^{\bullet}OH$ vs. TFC
P. parviflorus	0.980	0.999	0.982	0.941	0.989	0.945	0.922	0.948	0.991
H. cordata	0.809	0.992	0.728	0.883	0.958	0.887	0.999	0.977	0.999
S. spirale	0.986	0.974	0.998	0.995	0.965	0.948	0.998	0.973	0.958

Table 8
Phenolic constituents of the methanol extracts of the three plant extracts.

Compounds	Retention Time	Quantity of the identified compounds (mg/g)		
		P. parviflorus	*H. cordata*	*S. spirale*
Gallic Acid	4.686	5.78	0.08	0.17
Protocatechuic Acid	7.536	0.09	--	0.01
Catechin	12.886	--	--	0.09
Gentisic Acid	14.482	--	--	0.67
Caffeic Acid	17.306	2.87	--	--
m- Coumaric Acid	24.764	0.12	0.18	0.26
Sinapic Acid	25.209	1.07	0.01	0.58
Quercetin	30.216	3.03	1.09	0.19
Kaempferol	33.710	4.30	--	--

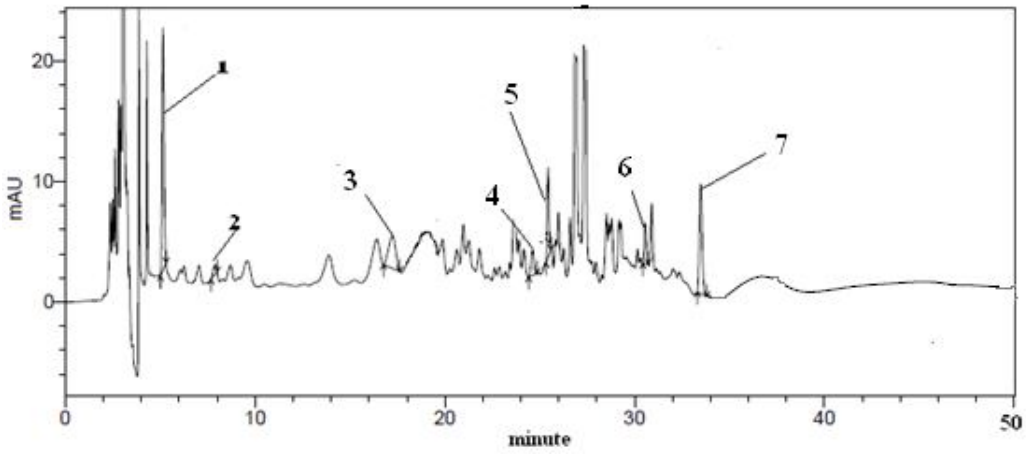

Figure 1: HPLC chromatograms of *Pogostemon parvifloras*

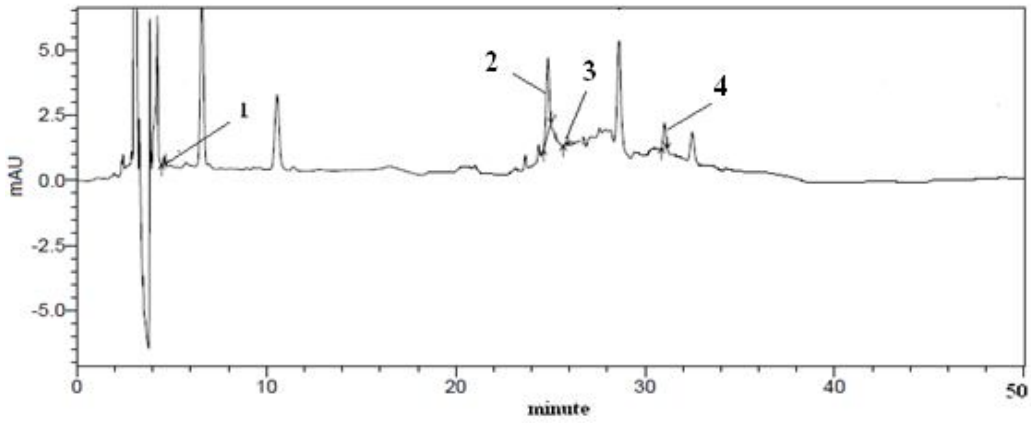

Figure 2: HPLC chromatograms of *Houttuynia cordata*

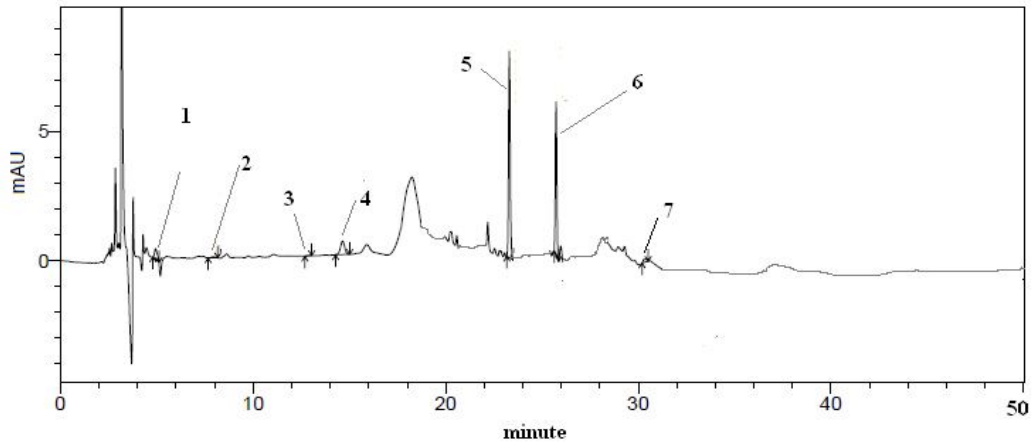

Figure 3: HPLC chromatograms of *Solanum spirale*

In **Figure 1:** (1) Gallic Acid, (2) Protocatechuic Acid, (3) Caffeic Acid, (4) m-Coumaric Acid, (5) Sinapic Acid, (6) Quercetin and (7) Kaempferol; **Figure 2:** (1) Gallic Acid, (2) m-Coumaric Acid, (3) Sinapic

Acid, and (4) Quercetin; and **Figure 3:** (1) Gallic Acid, (2) Protocatechuic Acid, (3) Catechin, (4) Gentisic Acid, (5) m-Coumaric Acid, (6) Sinapic Acid, and (7) Quercetin.

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