Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(8):859-867

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Identification and Quantification flavonoids in three wild edible plants, *Houttuynia cordata, Solanum gilo* and *Solanum kurzii* of North-Eastern region in India, using High Performance Liquid Chromatography with Diode Array Detection

Tapan Seal*, Kausik Chaudhuri and Basundhara Pillai

Plant Chemistry Department, Botanical Survey of India, A. J. C. Bose Indian Botanic Garden, Shibpur, Howrah, INDIA

ABSTRACT

Identification and quantification of flavonoids (aesculin, catechin, rutin ,naringin, myrecetin, coumarin, luteolin, quercetin, naringenin, apigenin and kaempferol) in two different solvent extracts (methanol and 80% aq. ethanol) of three wild edible leaves of viz. Houttuynia cordata, Solanum gilo and Solanum kurzii collected from North-eastern region in India has been performed using a reversed-phase high-performance liquid chromatography with photodiode array detector. The chromatographic separation was carried out on Acclaim C 18 column (5 µm particle size, 250 x 4.6 mm) , Dionex Ultimate 3000 liquid chromatograph and detection was carried out at three different wave lengths (272 , 280 and 310 nm) using a mobile phase of acetonitrile and water with gradient elution . The experimental results showed good amount of catechin in the 80% aq. ethanol extract of H. cordata (6.63±0.046 mg/gm dry extract) and also in the 80% aq. ethanol extract of S. gilo (2.70±0.041 mg/gm dry extract). The high percentage of recovery (97-100%), low coefficient of variation ($R^2 > 0.99$) and low limit of detection (LOD) and limit of quantitation (LOQ) confirm the suitability of the method for simultaneous quantification of flavonoid compounds in the two plants under investigation.

Keywords: Flavonoids; Different solvent extracts; *H. cordata*; *S. gilo*; *S. kurzii*; Gradient HPLC

INTRODUCTION

The flavonoids are a large family of polyphenolic compounds synthesized by plants and structurally derived from the parent substance flavone. Flavonoids present in fruits and leafy vegetables are thought to provide potential and versatile health benefits through radical scavenging and chelating activity. The in-vitro antioxidant activities of the flavonoids are due to their ability to reduce the free radical formation and hence exhibit enormous biological and pharmacological activities and play a major role in optimum protection from oxidative stress caused by the increase in the level of reactive oxygen species in the human organism [1].

Many studies have suggested that flavonoids like rutin, kaempferol, quercetin, apigenin etc. are well- known for its anti-inflammatory, anti-allergic, anti-thrombitic, hepato-protective, anti-spasmodic and anticancer properties [2]. Each different fruits and leafy vegetables are capable to display different extent of antioxidant activities owing to the presence of varied amount of free phenolic and flavonol contents.

The remarkable antioxidant cum nutraceutical properties of phenolics attracted global attention over the past decades. The biological activities like anti-mutagenicity, anti-bacterial action, anti-viral activity, anti-inflammatory traits, apoptotic actions etc. can only be rationalized by detecting and quantitating such compounds [3].

Houttuynia cordata known as 'Jamyr-doh' in Meghalaya state belongs to the family Saururaceae. The whole plant is eaten raw. The leaf juice is taken for the treatment of cholera, dysentery, curing of blood deficiency and purification of blood. The tender young shoots and leaves are eaten raw or cooked as a pot-herb. A decoction of this

plant is used internally in the management of many ailments including cancer, coughs, dysentery, enteritis and fever. Externally, it is used in the treatment of snake bites and skin disorders. The leaves and stems are harvested during the growing season and used fresh in decoctions the leaf juice is antidote and astringent [4].

Solanum gilo Raddi., known as Soh-ngang- heh in Meghalaya state, belongs to the family Solanaceae. The fruits of this plant are cooked as vegetable. It is also bitter to taste [5].

Solanum kurzii Brace known as Soh ngang rit in Meghalaya state belongs to the family Solanaceae. The fruits are eaten raw and also grounded as chutney and as vegetable. It is bitter to taste. The fruit is used as anti-allergy by the Mao Naga tribe of Manipur. The fruit is crushed and juice is applied to the allergic area of the body [6].

The nutritive value and the antioxidant activities of these plants have already been studied by us. The use of the plant in folk medicine and its nutraceutical role provide unambiguous testimony to the fact. Thus, the presences of an appreciable amount of flavonoids in these plants are inferred. The antioxidant activities of the extractive solution represent an important parameter to evaluate the biological property of the plant. Therefore, it is necessary to characterize and quantify the important compounds present in the plant and also to validate the method of separation and identification of active constituents. The extraction of polyphenolic compounds from plant is highly depending on the polarity of the solvent because polar compound is easily extracted using polar solvent. Thus, the solvent used for the extraction of bioactive compounds must be critically chosen because it will influence the quantity and quality of the final extract [7].

The present paper deals with the detection and quantification of nine flavonoids, namely, catechin, naringin, luteolin, quercetin, myricetin, naringenin, kaempferol and apigenin), and two coumarin compounds (aesculin and coumarin) in these two plants under study, using the HPLC with diode array detection in a single run .

EXPERIMENTAL SECTION

Plant material

The roots of *Houttuynia cordata,* fruits of *Solanum gilo* and *Solanum kurzii* were collected from the local market of Meghalaya state of India. It was duly authenticated and a voucher specimen was kept at the Department of Plant Chemistry of Botanical Survey of India under the Registry No. BSITS 3, BSITS 8 and BSITS 9 for future reference. The plant part was shed-dried, made coarse powder and stored in an air-tight container for extraction.

Chemicals

The standard flavonoid and coumarin compounds like (aesculin, catechin, narigin, rutin, myricetin, luteolin, quercetin, naringenin, apigenin and kaempferol) were purchased from Sigma Chemical Co. (St. Louis , MO , USA) and the HPLC-grade solvents such as chloroform, methanol, water and acetic acid were purchased from Merck (Germany).

HPLC equipment

HPLC analyses were performed with Dionex Ultimate 3000 liquid chromatograph (Germany) with four solvent delivery system quaternary pump (LPG 3400 SD) including a diode array detector (DAD 3000) with 5 cm flow cell, a manual sample injection valve equipped with a 20 μl loop and Chromeleon 6.8 system manager as data processor. The separation was achieved by a reversed-phase Acclaim ^{TM} 120 C18 column (5 μm particle size, i.d. 4.6 x 250 mm).

Preparation of standard solutions

The stock solution of concentration 1mg / ml was prepared by dissolving 1 mg each standard separately in 0.5 ml HPLC-grade methanol followed by sonication for 10 minutes and the resulting volume was made up to 1 ml with the same solvent. The working solutions of the sample under investigation were prepared by further dilution of the standard solution with the mobile phase solvent system. The standard and working solutions were filtered through 0.45 μm PVDF-syringe filter and the mobile phase was degassed before the injection of the solutions.

Preparation of plant extracts

One gm of each coarsely powdered leaf was extracted using 5 ml methanol with constant stirring for 24 hours at the ambient temperature. The extract so prepared was filtered and the plant residue so left was macerated with the same volume of fresh solvent, stirred and filtered. The process was repeated thrice and the extracts were combined. The extracts were finally filtered through 0.45μm PVDF membrane and the volume was made up to 10 ml using the same solvent & stored. The same processes were followed for the preparation of sample extract with 80% aq. ethanol.

Chromatographic analysis of flavonoids

The mobile phase contains water (Solvent A) and acetonitrile (Solvent B), the flow rate was adjusted to 0.7 ml/min, the column was thermostatically controlled at 28^0 C and the injection volume was kept at 20 µl. A gradient elution was performed by varying the proportion of solvent B to solvent A. The gradient elusion was changed from 13 % to 40% B in a linear fashion for duration of 67 min and allowed to run for another 2 min, before the injection of another sample. Total analysis time per sample was 69 min.

HPLC Chromatograms were detected using a photo diode array UV detector at three different wavelengths (272, 280 and 310 nm) according to absorption maxima of analysed compounds. Each compound was identified by its retention time and by spiking with standards under the same conditions.

The quantification of the sample was done by the measurement of the integrated peak area and the content was calculated using the calibration curve by plotting peak area against concentration of the respective standard sample. The data were reported with convergence limit in triplicate.

Validation of the method

According to the USP and ICH guidelines, there are various parameters to validate the reproducibility of the method viz. the effectiveness, the limit of detection (LOD), the limit of quantitation (LOQ), the linearity, the precision and the accuracy.

The effectiveness of the HPLC method was detected with the standard solutions of flavonoids. Generally, methanol of diverse composition is used as eluent but solvents like acetonitrile, acetic acid, formic acid are also reported in the literature. In this study, different proportion of acetonitrile and water was used to achieve the best resolution.

To ascertain the linearity, the stock solution of the standard (1 mg/ml) was diluted to six different concentrations (5, 10, 20, 30, 40, 60 µg/ml) which were fed individually in triplicate to the HPLC system and the calibration curve so obtained by plotting peak area versus concentration for each sample where the square of the correlation coefficient $R^2 > 0.99$ is indicative of the measure of linearity.

The accuracy of the method was determined by application of the standard addition method. The methanol extract of *H. cordata S. gilo* and *S.kurzii* were spiked with two known concentration of calibration solutions (20 µg/ml and 40 µg/ml). The amounts of flavonoids and coumarin compounds present in the investigated plants were previously determined. For each standard compound, the percentage of recovery was calculated as follows

$$\text{Recovery (\%)} = (\text{amount found - amount contained})/\text{amount added} \times 100$$

The high recovery rate in the range of 96 – 103% for the samples is indicative of efficacy & consistency.

Limit of detection and limit of quantification were calculated using the following formula LOD = 3.3 (σ)/S and LOQ = 10 (σ)/S, where (σ) = standard deviation of response (peak area) and S = slope of the calibration curve.

The precision refers to the degree of proximity of the results expressible as % relative standard deviation (RSD) of the retention time and the peak area. The repeatability of the retention time and peak areas (Pa) were checked by injecting the mixed standard solutions at two concentration levels (20 µg/ml and 40 µg/ml) into the HPLC system. The RSD of retention time and peak areas were calculated for five replicate determinations.

RESULTS AND DISCUSSION

Validation of HPLC method

A typical HPLC chromatogram of the all standard mixture of flavonoid and coumarin recorded at 272 nm is presented in fig. 1. As shown in the chromatogram, all investigated

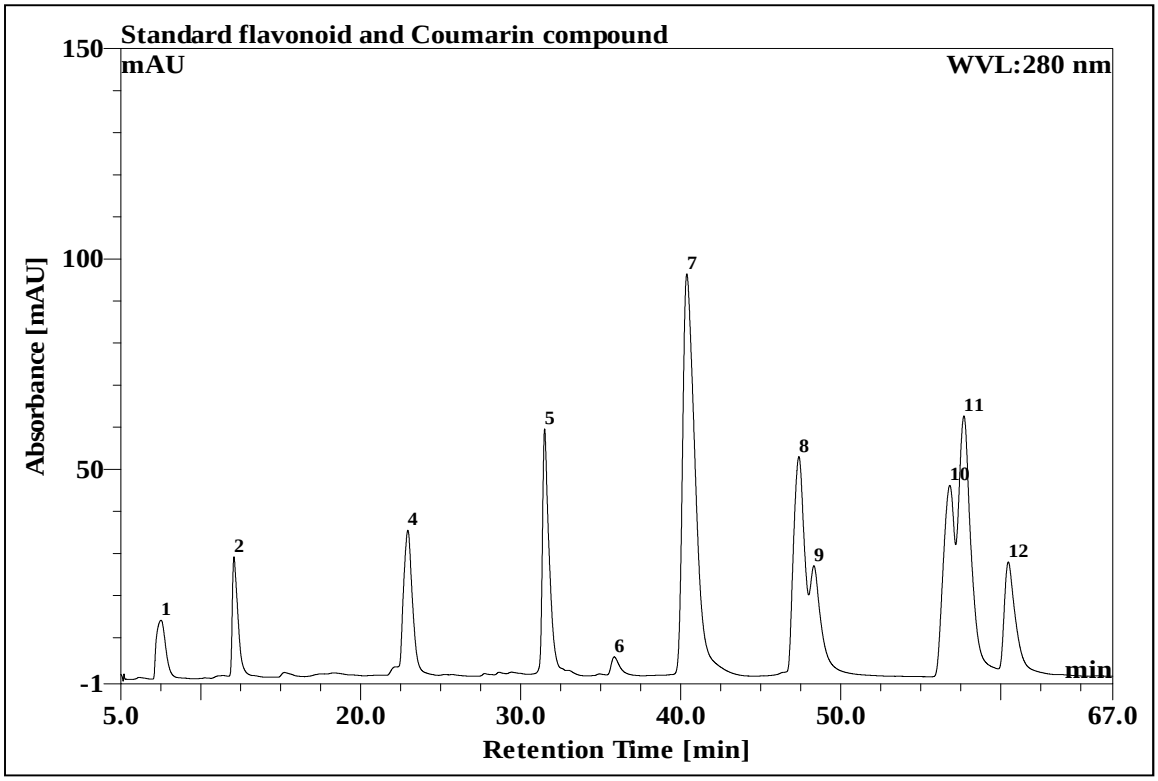

Fig.1. HPLC Chromatogram of standard flavonoids and coumarin compounds
1. *Aesculin* 2. *Catechin* 4. *Rutin* 5. *Naringin* 6. *Myricetin* 7. *Coumarin* 8. *Luteolin* 9. *Quercetin* 10. *Naringenin* 11.*Apigenin* 12.*Kaempferol*

compounds had responses at 280 nm, where they were successfully separated. The constituents under investigation were also identified by the recorded absorption spectra, which were comparable for two different solvent extracts of *H. cordata*, *S. gilo* and *S. kurzii* and standard substances. The regression coefficient together with LOD and LOQ values, are shown in Table 1. The high value of $R^2 > 0.9906$ in the range of analyzed concentrations at 280 nm is indicative of responsive linearity.

Table 1. Retention time and parameters of calibration curve, precision and repeatability, LOD, LOQ and percent recovery study of standard flavonoids and coumarin compounds for HPLC method validation

Name of the Standard	Detected at wavelength λ nm	Retention time	RSD (%) of the retention time	RSD (%) of the peak area at conc 20 µg/ml	RSD (%) of the peak area at conc 40 µg/ml	Regression Coefficient R^2	LOD µg/ml	LOQ µg/ml	Percentage of recovery (%)
Aesculin	280	7.61	0.625	0.137	0.033	0.9996	0.091	0.276	98.71
Catechin	280	12.08	0.048	0.043	0.039	0.9820	0.028	0.084	97.26
Rutin	280	22.89	0.131	0.186	0.158	0.9750	0.112	0.339	100.47
Naringin	280	31.50	0.018	0.103	0.101	0.9990	0.065	0.198	97.08
Myricetin	280	35.99	0.181	3.293	0.256	0.9969	0.576	1.747	98.15
Coumarin	280	40.35	0.052	0.055	0.075	0.9998	0.035	0.107	98.01
Luteolin	280	47.35	0.044	0.078	0.078	0.9970	0.054	0.163	99.20
Quercetin	280	48.31	0.036	1.293	0.059	0.9967	0.097	0.295	98.23
Naringenin	280	56.75	0.067	0.151	0.059	0.9962	0.098	0.296	98.77
Apigenin	280	57.66	0.044	0.118	0.041	0.9581	0.069	0.210	97.87
Kaempferol	280	60.49	0.010	0.326	0.094	0.9947	0.146	0.441	97.41

Note : RSD Relative standard deviation, LOD Limit of detection, LOQ limit of quantification

The repeatability of the retention time for all the standard samples and that for the peak areas two standards *viz*., 20 µg/ml and 40 µg/ml was found to be below one percent. The significant high rate of recovery of the standard phenolics and the flavonoids worth's mention. It follows that the method under consideration is characterized by precision, accuracy, meticulousness and could be used for the qualitative as also quantitative assay of flavonoids in the different extracts of these two plants under investigation.

Identification of different flavonoids and coumarin compounds in two different extracts of H.cordata, S. gilo and S. kurzii

The HPLC chromatogram of methanol extract of the roots of *H. cordata* showed the presence of rutin whereas the 80 % aq.ethanol extract of this plant found to contain good amount of catechin along with small amount naringin and kaempferol as presented in fig. 2 and fig.3.

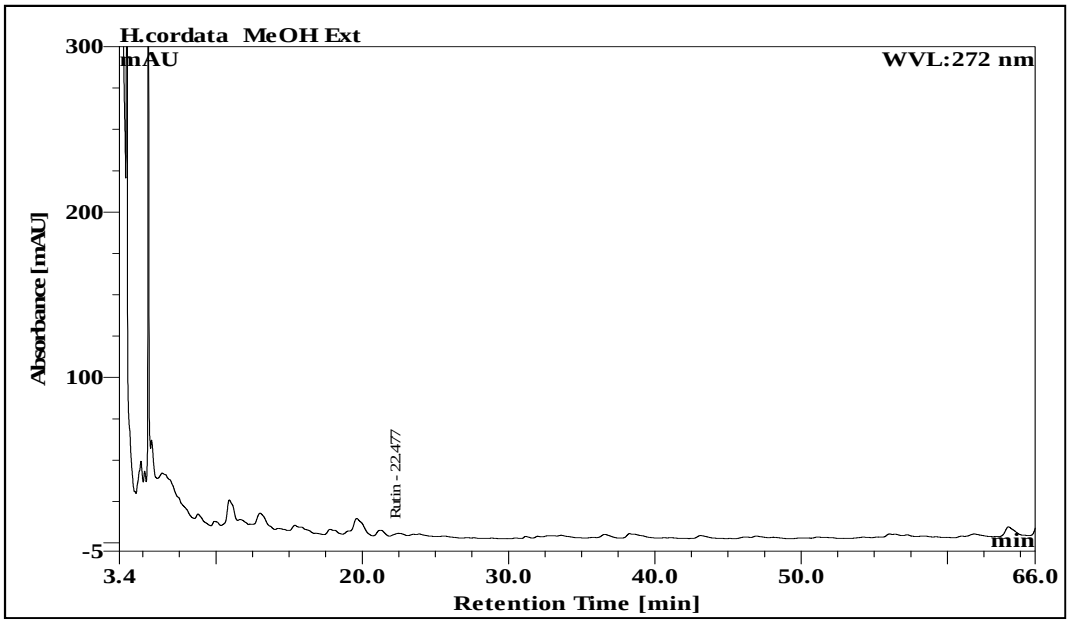

Fig.2. HPLC chromatogram of the methanol extract of *H. cordata*

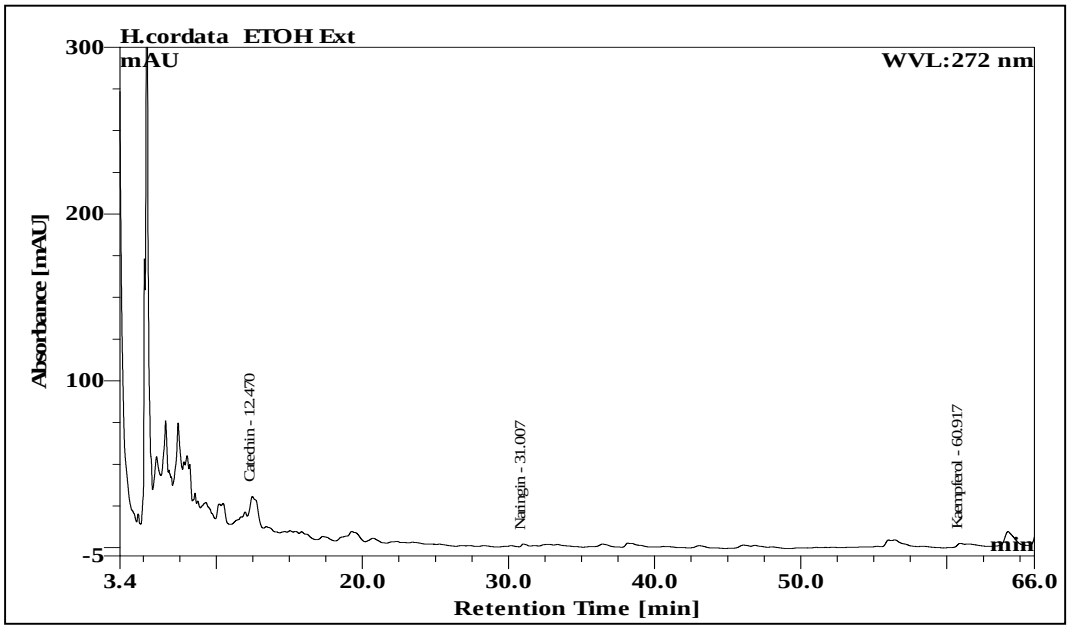

Fig.3. HPLC chromatogram of the 80 % aq.ethanol extract of *H. cordata*

The methanol extract of the fruits of *S. gilo* revealed the presence of naringenin whereas catechin, rutin and naringenin were detected in the 80% aq. ethanol extract of the fruits of this plant as depicted in the HPLC chromatogram in fig. 4 and fig. 5.

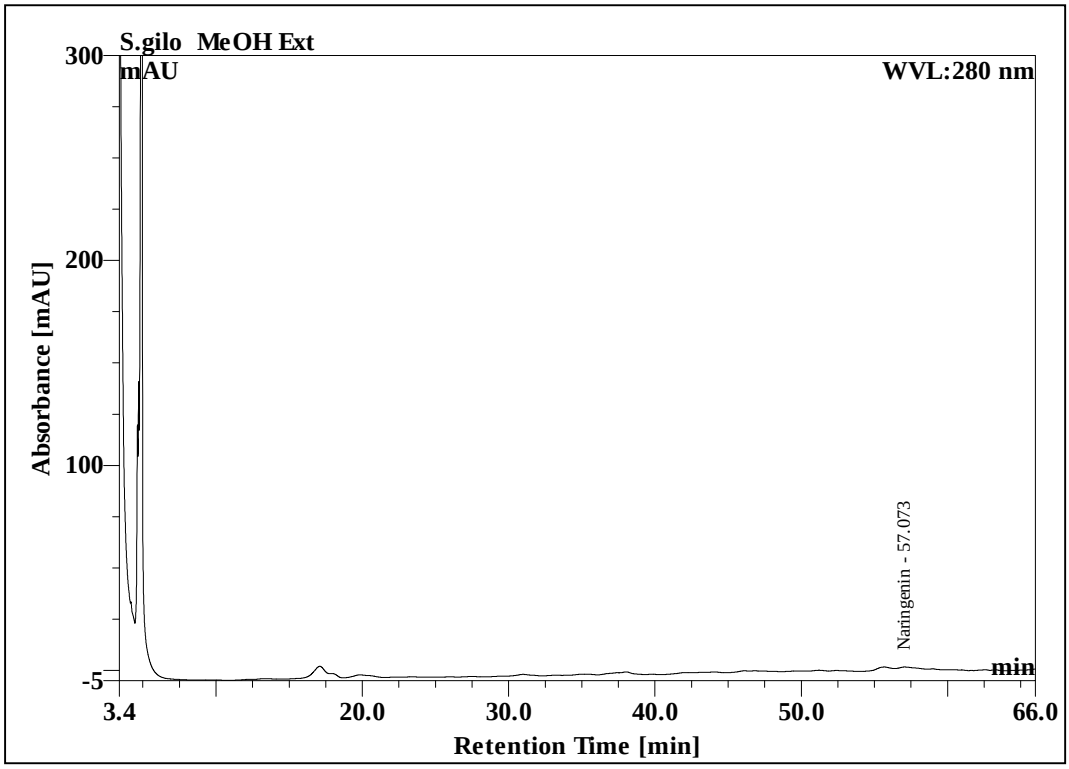

Fig. 4. HPLC chromatogram of the methanol extract of *S. gilo*

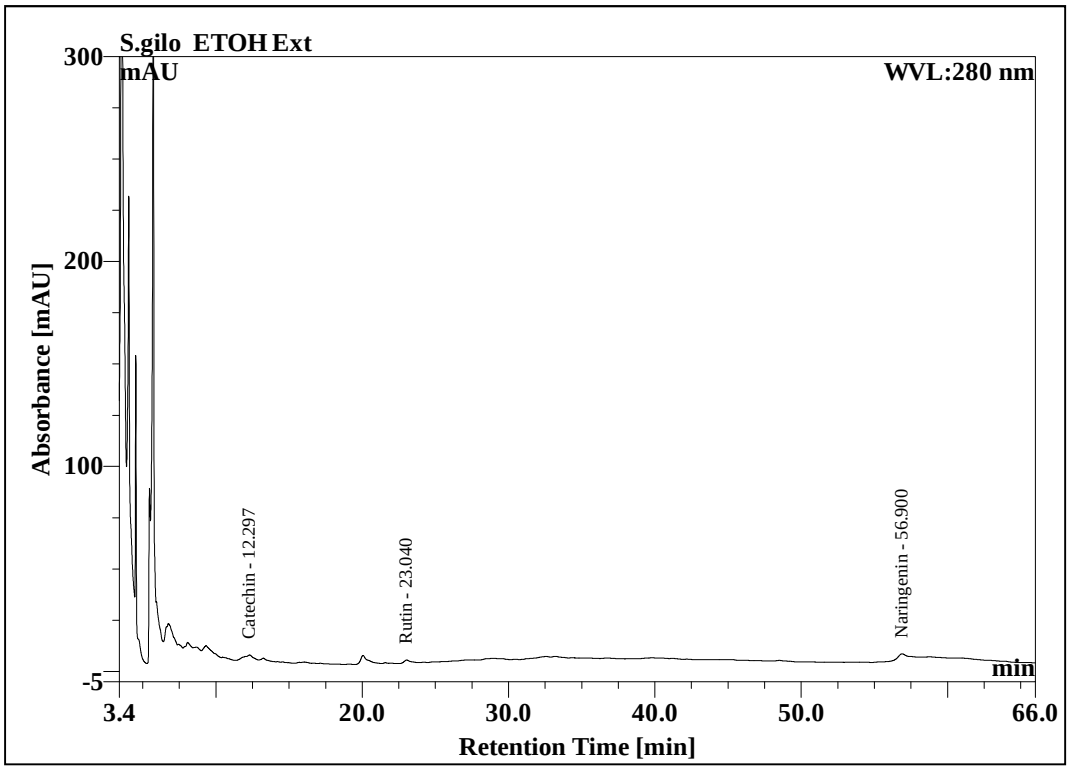

Fig. 5. HPLC chromatogram of the 80 % aq. ethanol extract of *S. gilo*

The HPLC chromatogram also showed the presence of naringenin in both the methanol and 80 % aq.ethanol extract of the fruits of *S. kurzii* in minor amount as presented in fig. 6 and fig.7.

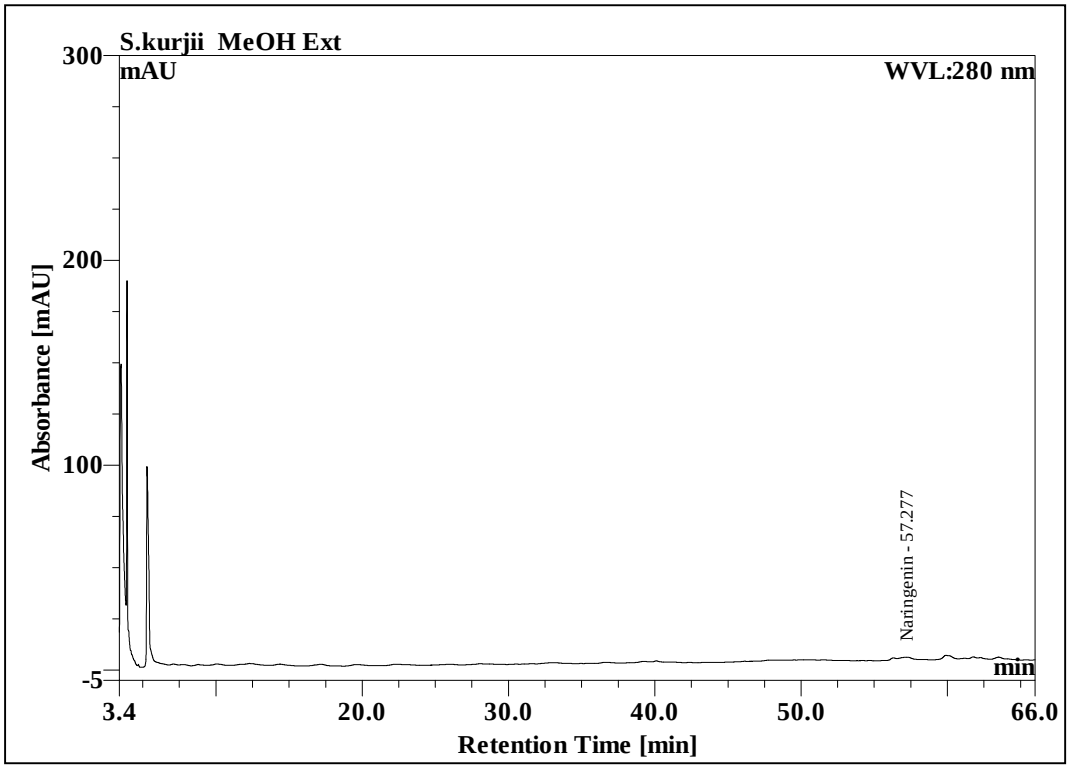

Fig. 6. HPLC chromatogram of the methanol extract of *S. kurzii*

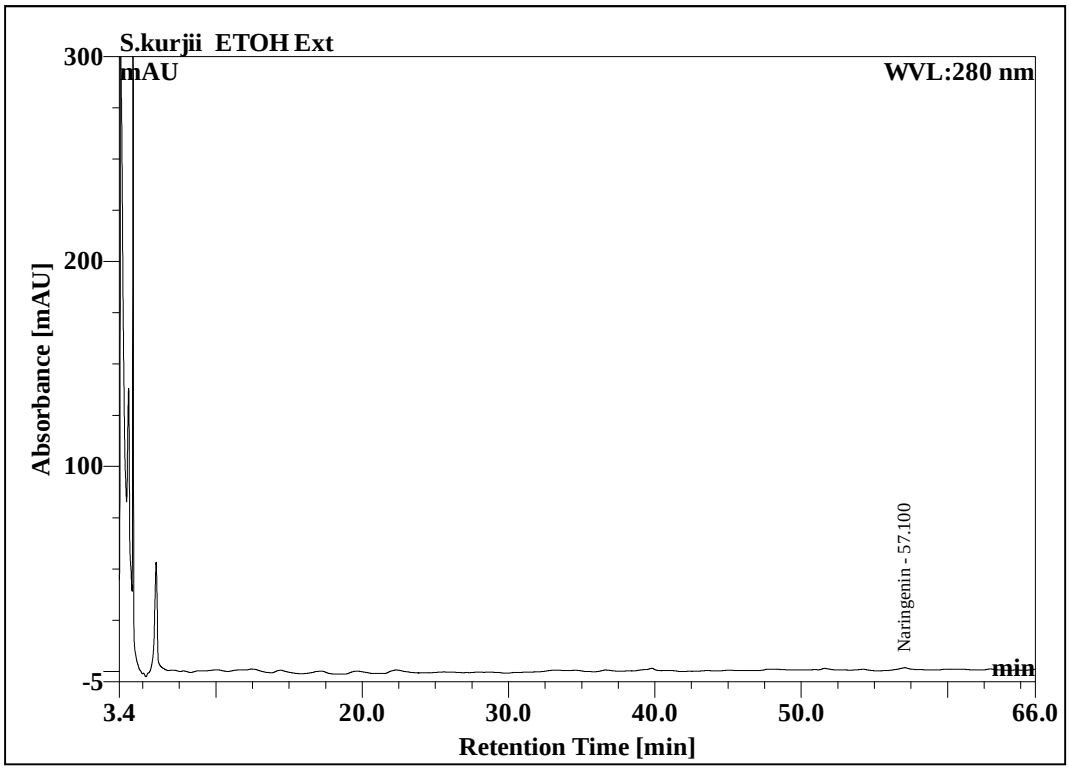

Fig. 7. HPLC chromatogram of the 80 % aq. ethanol extract of *S. kurzii*

Quantification of flavonoids and coumarin compounds in two different solvent extracts of H. cordata, S. gilo and S. kurzii

The present study indicated the occurrence of large amount of catechin (6.632±0.046 mg/gm dry extract) and minor amount of naringin (0.172±0.004 mg/gm dry extract) in the 80 % aq. ethanol extract of *H. cordata* as shown in table 2. A very small amount of rutin (0.227±0.004 mg/gm dry extract) was also detected in the methanol extract of

this plant. The HPLC analysis also revealed the presence of varying amounts of naringenin in the methanol and 80 % aq. ethanol extract of *S.gilo* and *S. kurzii*. A moderate amount of catechin (2.70±0.041 mg/gm dry extract) and small amount of rutin were also detected in the 80 % aq. ethanol extract of *S. gilo* as shown in table 2.

Table 2. Quantification of flavonoids and coumarin compounds in two different extract of *H. cordata, S.gilo* and *S. kurzii*

Flavonoids	Amount of flavonoids and coumarin compounds (mg/gm dry extract) in two different extracts of *H. cordata, S. gilo* and *S. kurzii*					
	H.cordata		*S. gilo*		*S. kurzii*	
	Methanol	80 % aq. ethanol	Methanol	80% aq. ethanol	Methanol	80 % aq. ethanol
Aesculin	ND	ND	ND	ND	ND	ND
Catechin	ND	6.632±0.046	ND	2.70±0.041	ND	ND
Rutin	0.227±0.004	ND	ND	0.445±0.020	ND	ND
Naringin	ND	0.172±0.004	ND	ND	ND	ND
Myricetin	ND	ND	ND	ND	ND	ND
Coumarin	ND	ND	ND	ND	ND	ND
Luteolin	ND	ND	ND	ND	ND	ND
Quercetin	ND	ND	ND	ND	ND	ND
Naringenin	ND	ND	1.04±0.009	1.002±0.015	0.224±0.004	0.284 ±0.004
Apigenin	ND	ND	ND	ND	ND	ND
Kaempferol	ND	ND			ND	ND

Each value in the table was obtained by calculating the average of 3 experiments and data are presented as Mean ±SEM "ND" denotes not detected

Catechins belong to a category of compounds known as flavanols and are found only in foods and drinks derived from plants. The 80 % aq. ethanol extract of *H. cordata* was found to contain the highest concentration of catechin amounting 6.632±0.046 mg/gm. Appreciable quantities of catechin were also detected in the 80 % aq. ethanol extract (2.70±0.041 mg/gm) whereas the absence of this active constituent in the methanol extracts of the plants under investigation, signifying that polarity of the solvent was the important factor for the isolation of the bioactive constituents from plant. This indicates that catechin found in 80 % aq.ethanol extract of *H. cordata* and *S. gilo* may contribute to its medicinal and antioxidant properties [8].

Rutin is a phenolic compound with glycosidic linkage and is used for the treatment of varicose veins, haemorrhoids, haemorrhagic stroke and mucositis. The rutin content in the 80% aq. ethanol extract of *S. gilo* (0.445±0.020 mg/g) and in the methanol extract of *H. cordata* (0.227±0.004 mg/gm) are comparable to that in the leaves of *Fagopyrum esculentum* (0.12 mg/g) and *Melisa officinalis* (0.30 mg/g) [9].

Naringenin is a flavanone, a type of flavonoid. It is the predominant flavanone in grapefruit. Naringin is a flavanone 7-*O*-glycoside found in grapes and citrus fruits. Both naringin and naringenin are strong antioxidants, however, naringin is less potent compared with naringenin because the sugar moiety in the former causes steric hindrance of the scavenging group. Due to the presence of naringin and its aglycone naringenin in the plants under investigation showed potent anti-inflammatory and antioxidant activities. Several investigations also suggest that both naringin and naringenin supplementations are beneficial for the treatment of obesity, diabetes, hypertension, and metabolic syndrome [10].

CONCLUSION

The reversed-phase HPLC method with diode array detection was developed for the quantitative estimation of flavonoid and coumarin compounds like (aesculin, catechin, narigin, rutin, myricetin, luteolin, quercetin, naringenin, apigenin and kaempferol in the two different solvent extracts of *H. cordata*, *S.gilo* and *S. kurzii.* These flavonoids are extremely common and wide spread in the plant kingdom as their glycosides and useful in treating several diseases. The established HPLC assay showed a well separation of the compounds and also the developed method was linear, sensitive, accurate, meticulous and reproducible. Therefore, the method can be used for the simultaneous determination of flavonoids in different formulations with 'shorter run time' and 'high efficiency'. The presence of significant amount bio-active components like catechin, rutin, naringin and naringenin in these plants under study and variation of quantity determined based on the polarity of the solvent taken for extraction process, ensures its clear recommendation for the use in the pharmaceutical and nutraceutical sector.

Acknowledgements
The Authors owe deep gratitude to Dr. P. Singh, Director, Botanical Survey of India, Kolkata for extending necessary scientific facilities and express sincere indebtedness to Mr. R. Shanpru, Scientist, Botanical Survey of India, North Eastern Circle, Shillong, Meghalaya for identifying the plant specimens.

REFERENCES

[1] M. Olszewska. *Acta Chromatographica*, **2007**, 19, 253-69
[2] SK Maheshkumar; SL Kirti. *Asian Journal of Traditional Medicines*, **2012**, 7, 220-25.
[3] Pirjo Mattila; Jarkko Hellstrom. *Journal of Food Composition and Analysis* , **2007**, 20, 152-160.
[4] RN Chopra; SL Nayar; IC Chopra. Glossary of Indian Medicinal Plants. Seventh reprint, **2006**, 170-241.
[5] T Seal. *Journal of Medicinal Food Plants*, **2010**, 2(2), 6-13.
[6] A A Mao; TM Hynniewta, M Sanjappa, Indian *Journal of Traditional Knowledge*, **2009**, *8*(1), 96-103.
[7] AR Nur Syukriah; MS Liza; Y Harisun; AAM Fadzillah. *International Food Research Journal*, **2014**, 21, 1067-1073.
[8] Tapan Seal. *J App Pharm Sci*, **2016**, 6 (02), 157-166.
[9] B Moghaddasian ; D Eradatmand Asli; A Alaghemand. *Bull. Env. Pharmacol. Life Sci* , **2013**, 2, 35- 38.
[10] MA Alam; N Subhan; MM Rahman; SJ Uddin; HM Reza; SD Sarker. *Advances in Nutrition; An International Review Journal* **2014**, 5, 404–417.