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Identification and quantification flavonoids in two wild edible plants, *Viburnum foetidum* and *Perilla ocimoides* of North-Eastern region in India, using high performance liquid chromatography with diode array detection

Tapan Seal and Kausik Chaudhuri

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 two wild edible leaves of *viz. Viburnum foetidum* and *Perilla ocimoides*, 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 high amount of myricetin (36.19±0.020 mg/gm dry extract) and luteolin (30.242±0.033 mg/gm dry extract) present in the 80% aq. ethanol extract of *P. ocimoides* and a good amount of naringin (18.304±0.022 mg/gm dry extract) is detected in the methanol extract of *V. foetidum*. 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, *V. foetidum, P. ocimoides,* 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 amazing 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].
Viburnum foetidum Wall, known as Soh lang in Meghalaya state, belongs to the family Caprifoliaceae. It is common in Khasi Hills of Meghalaya and in Assam of India. The plant is astringent, juice of the leaves used internally in menorrhagia and in post-partum haemorrhage. It yields essential oil and crystalline alkaloid. The fruits of this plant are edible [4].
Perilla ocimoides Linn., known as Nei in Meghalaya state, belongs to the family Labiatae. The leaves, stems and seeds of this plant considered as diaphoretic and cephalic in China and Indo-China. In Meghalaya state the seeds are roasted, crushed or pounded with salt and eaten as

chutney. The seed contains a fixed oil similar in taste, odour and drying qualities to our common linseed oil. In Manchuria, this oil is used for edible purposes [5].

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 [6].

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.

Materials and Methods

Plant material

The fruits of *Viburnum foetidum* and seeds of *Perilla ocimoides* 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 2 and BSITS 6 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, naringin, 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.

Extraction of plant samples using two solvents of different polarity

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 fruits extract of *V. foetidum* and seeds extract of *P. ocimoides* 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

Recovery (%) = (amount found - amount contained)/amount added × 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 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 both for fruits extract of *V. foetidum* and seeds extract *P. ocimoides* 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.

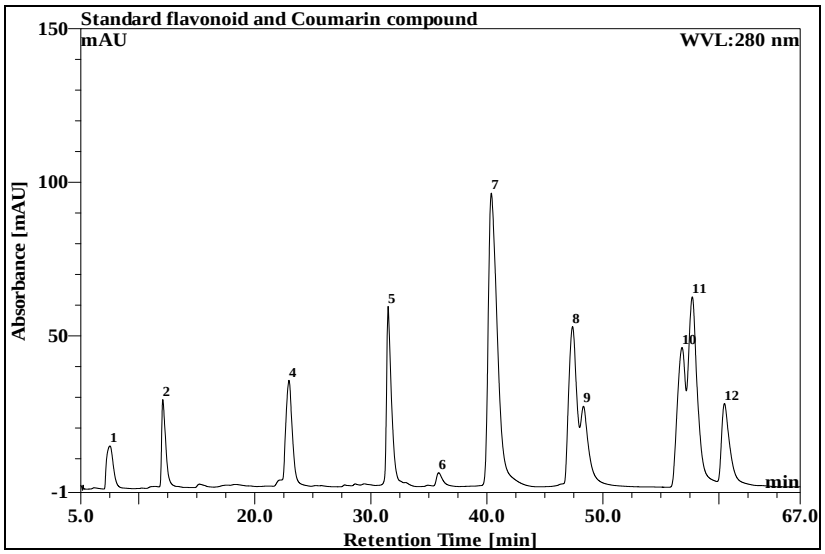

Fig 1: HPLC Chromatogram of standard flavonoids and coumarin compounds

1. Aesculin 2. *Catechin* 4. *Rutin* 5. *Naringin* 6. *Myricetin* 7. *Coumarin* 8. *Luteolin* 9. *Quercetin* 10. *Naringenin* 11. *Apigenin* 12. *Kaempferol*

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 the plant

The HPLC chromatogram of methanol extract of the fruits of *V. foetidum* showed the presence of naringin, coumarin, luteolin and naringenin in varying amount whereas the 80 % aq. ethanol extract of this plant contained naringin and coumarin as presented in fig. 2 and fig.3.

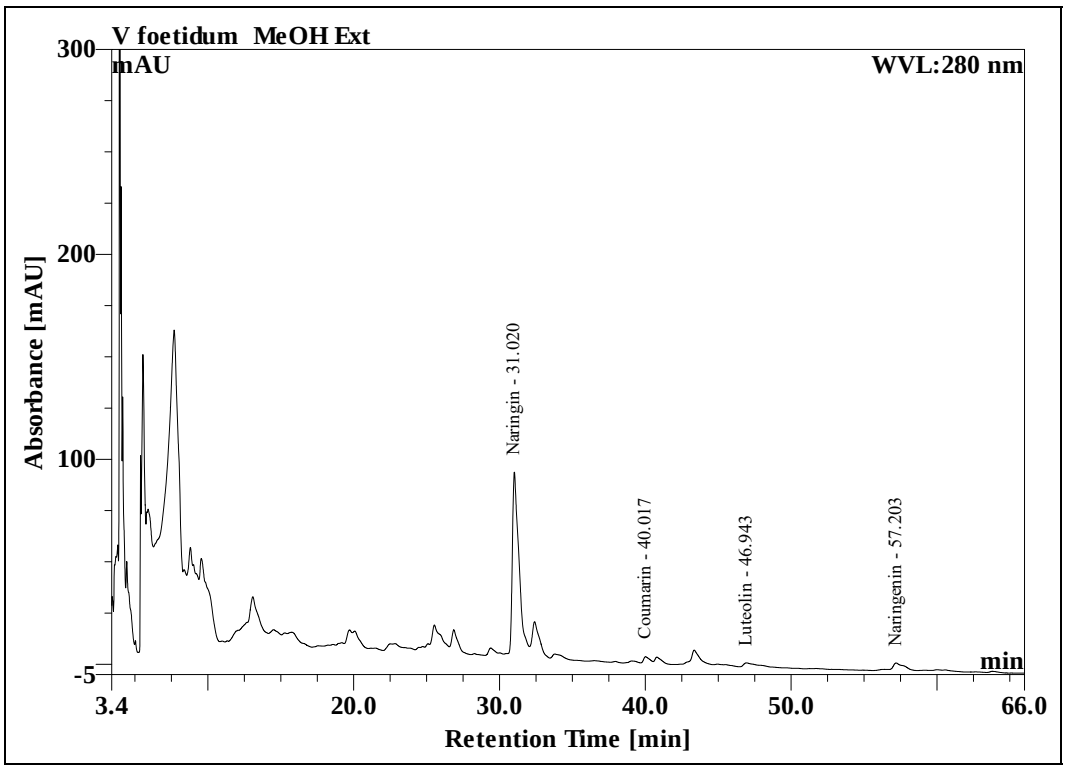

Fig 2: HPLC chromatogram of the methanol extract of *V. foetidum*

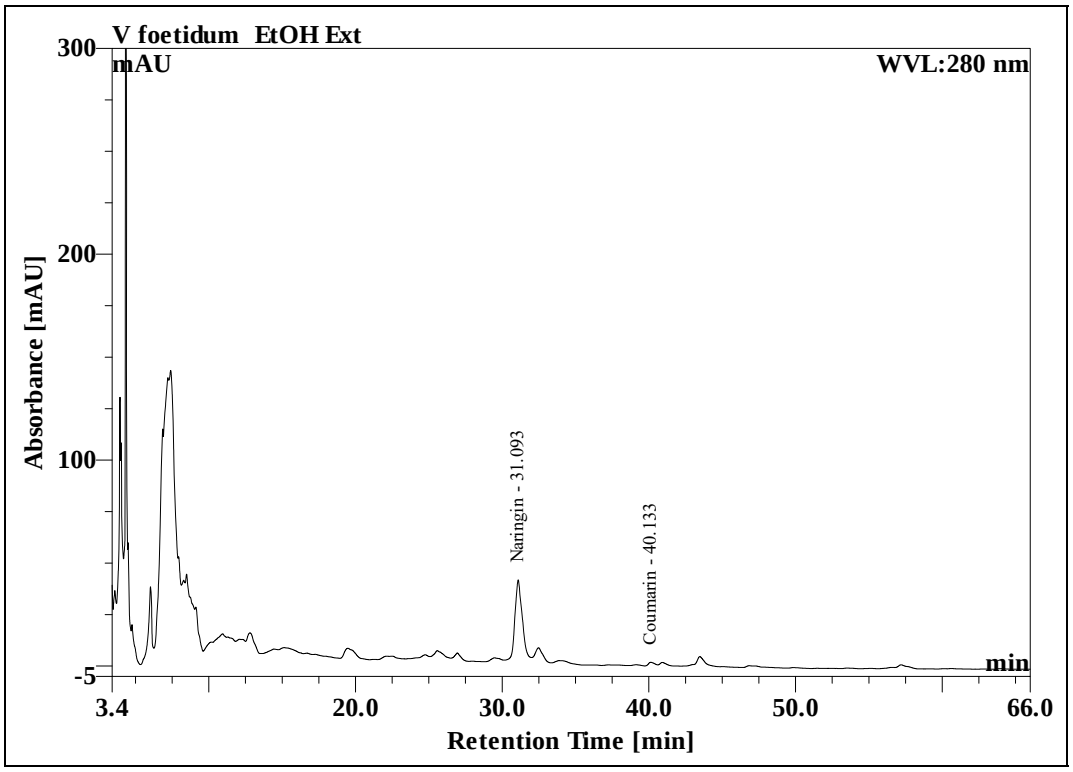

Fig 3: HPLC chromatogram of the 80% aq. ethanol extract of *V. foetidum*

The methanol extract of the seeds of *P. ocimoides* revealed the presence of high amount of luteolin along with moderate amount of apigenin. A higher amount of luteolin and remarkable amount of myricetin were detected in the 80% aq. ethanol extract of the seeds of this plant as depicted in the HPLC chromatogram in fig. 4 and fig. 5.

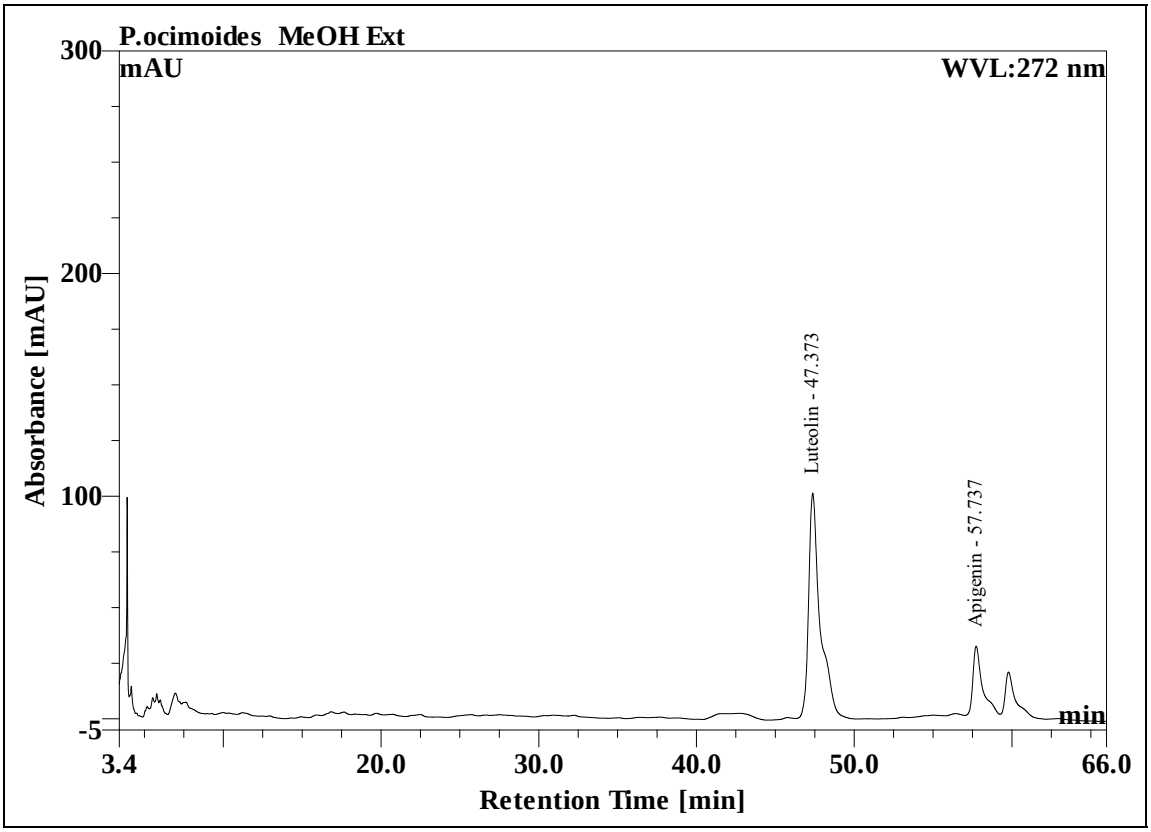

Fig 4: HPLC chromatogram of the methanol extract of *P. ocimoides*

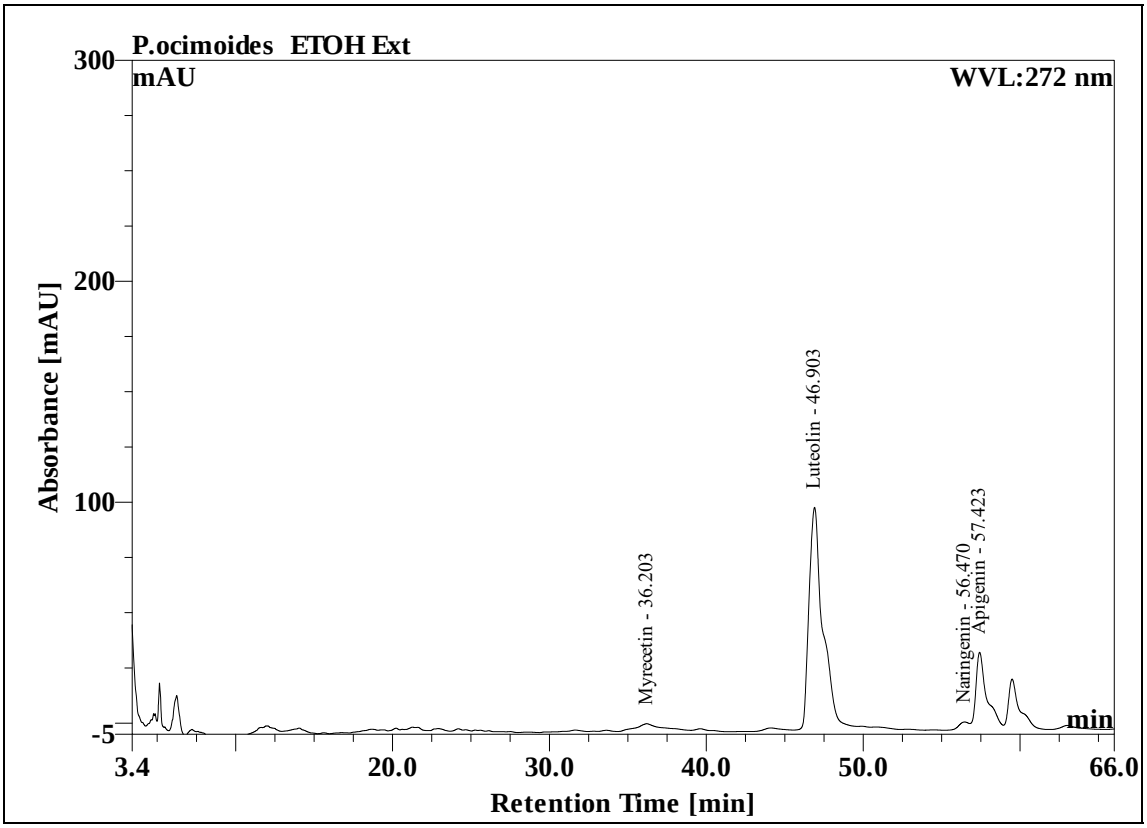

Fig 5: HPLC chromatogram of the 80 % aq. ethanol extract of *P. ocimoides*

Quantification of flavonoids and coumarin compounds in two different solvent extracts of the plant

The present study indicated the occurrence of large amount of naringin and minor amount of naringenin in the methanol extract of fruits of *V. foetidum* as shown in table 2. A good amount of naringin and naringenin were also detected in the 80% aq. ethanol extract of *V. foetidum* and *P. ocimoides*.

Table 2: Quantification of flavonoids and coumarin compounds in two different extract of *V. foetidum* and *P. ocimoides*

Name of the flavonoids	Amount of flavonoids and coumarin compounds (mg/gm) in two different extracts of *V. foetidum* and *P. ocimoides*			
	V. foetidum		*P. ocimoides*	
	Methanol	**80 % aq. ethanol**	**Methanol**	**80 % aq. ethanol**
Aesculin	ND	ND	ND	ND
Catechin	ND	ND	ND	ND
Rutin	ND	ND	ND	ND
Naringin	18.304±0.022	8.30±0.026	ND	ND
Myricetin	ND	ND	ND	36.19±0.020
Coumarin	0.176±0.003	0.086±0.004	ND	ND
Luteolin	0.426±0.002	ND	3.61±0.026	30.242±0.033
Quercetin	ND	ND	ND	ND
Naringenin	0.779±0.016	ND	ND	1.70±0.038
Apigenin	ND	ND	0.775 ±0.020	7.49±0.035
Kaempferol	ND	ND	ND	ND

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

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 [7].

Myricetin, a natural flavonol, widely available in fruits, vegetables, tea, berries and red wine. It is not only a good antioxidant, but also been shown to be a potent anticarcinogen and antimutagen. The presence of a very good amount of myricetin (36.19±0.020 mg/gm dry extract) in the 80% aq. ethanol extract of the seeds of *P. ocimoides* might be attributed to its strong anti-inflammatory, anti-hyperlipidemia and anti-aldose reductase activities. So the plant rich in myricetin may be useful to the prevention of diabetes mellitus and diabetic complications [8-9].

Several studies have also demonstrated that myricetin exhibits favourable effects on bone health, either by decreasing bone resorption or by increasing osteoblastic activity and bone formation [10].

Luteolin, is another important flavonoid that exists in many types of plants including fruits, vegetables, and medicinal herbs. Plants rich in luteolin have been used in Chinese traditional medicine for the treatment of various diseases such as hypertension, inflammatory disorders, and cancer. Furthermore, recent epidemiological studies have attributed a cancer prevention property to luteolin [11].

A very high amount of luteolin (30.242±0.033 mg/gm dry extract) has been quantified in the 80% aq. ethanol extract of *P. ocimoides* which can contribute to the health benefits of the plant. Because of potent anti-oxidant and radical scavenging activities the plant may be useful for the management of several neurodegenerative diseases.

Apigenin, a naturally occurring plant flavone, abundantly present in common fruits and vegetables is which is known to reduce the risk of cardiac ailments, neurological syndromes, mutagenesis. This bioactive flavonoid shown to possess anti-inflammatory, antioxidant and anticancer properties. Numerous studies suggest that a diet rich in flavones is related to a decreased risk of certain cancers and certain hematological malignancies [12]. It is reported that raw parsley is abundant in this nutraceutical (3.02 mg/gm) and the amount of this component detected in a moderate amount in the 80% aq. ethanol extract of the leaves of *P. ocimoides* (7.49±0.035 mg/gm dry extract) matched well with the harvested vegetables, such as, celery (0.046 mg/gm), cabbage (0.0001 mg/gm), sweet potato leaves (0.0012 mg/gm), peppermint (0.087 mg/gm) etc (Mohammad, 2013). So the 80% aq. ethanol extract of the seeds of *P. ocimoides* containing good amount of apigenin could play an important role in the treatment of cardiovascular and neurological disorders, although more research needs to be conducted in this regard. The fruits of *V. foetidum* was not found to contain apigenin at present study.

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 *V. foetidum* and *P. ocimoides.* 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 naringin, myricetin and luteolin in these plants under study and variation of quantity determined based on the polarity of the solvent taken for extraction process, ensures its unequivocal recommendation for the use in the pharmaceutical and nutraceutical sector.

Acknowledgements

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