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Screening and Characterization of Vitexin from *Vitex negundo* by LCMS, pHPLC and ^{13}CNMR Analysis

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Abstract: Complementary herbal medicines are currently required to overcome the drastic side effects of chemical based Disease-modifying antirheumatic drugs (DMARDS) and Non-steroidal anti-inflammatory drugs (NSAIDS). Herbal based drugs may include herbal preparations, purified compounds, vitamins and their products. *Vitex negundo* commonly called 'Nochi' in Tamil is an annual edible medicinal plant predominantly used as mosquito repellent. Traditionally leaves and root of *V. negundo are* used to treat cough, asthma, colitis, dysentery, diarrhoea, flatulence, fever, vomiting, and rheumatism. *V. negundo* is also found to possess analgesic, anti-oxidant, anti-inflammatory, anti-arthritic, anti-hyper pigmentation, immuno-stimulant, hepato-protective, anti-androgenic, pesticidal, anti-cancerous and anti-metastatic activity. *V. negundo* contains ample bioactive leads that might be pharmacologically beneficial. Therefore, the scientific validation of this folkloric herbal practice is needed. The present study aimed to assess the free radical scavenging role of methanolic extract of leaf and root of *Vitex negundo*. The study also focused on screening, isolation and characterization of bioactive compound Vitexin. Vitexin is a pharmacologically beneficial flavonoid found to be in the root extract of *V. negundo*. The roots of *V. negundo* were subjected to *in vitro* qualitative and quantitative phytochemical screening and the results showed that both the leaf and root extract may possess effective free radical scavenging role. Studies on root extract revealed the presence of abundant flavonoids in comparison with leaf extract of *V. negundo*. The root extract was further subjected to preparative Thin Layer Chromatography (pTLC), Liquid Chromatography Mass Spectrophotometry (LCMS), and preparative High performance Liquid Chromatography (pHPLC) for the screening and isolation of flavonoids. The structure of Vitexin was confirmed by ^{13}C NMR characterization analysis on comparison with the literaure. The present work may develop simple and reliable method for the purification of bioactive flavonoids from the methanolic root extract of the medicinal plant *V. negundo* which may possesses abundant biomedical importance against inflammatory diseases.

Keywords: Preparative Thin Layer Chromatography, Liquid Chromatography Mass Spectrophotometry, Preparative High performance Liquid Chromatography, ^{13}CNMR, Vitexin, *Vitex negundo*

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Introduction

Imbalance between formation and neutralization of oxidants result in oxidative stress and release free radicals and reactive oxygen species. Those radicals are the important cause for various disorders in humans. They cause oxidative damage to macromolecules and may cause many chronic inflammatory diseases, cancer, and also degenerative diseases in humans. There are certain biological enzymes that have the ability to scavenge those free radicals and fight against them (Gangwar *et al*., 2014). Traditionally many herbal formulations from plants have been used to treat various diseases. It is necessary to check the pharmacologically effective bioactive compounds from the herbs to evaluate the scavenging tendency. *V. negundo* is ethano medically bioactive plant used in Indian medicines from ancient times. It is known that *V. negundo* extracts are rich in phenols, flavonoids, glycosides and many therapeutic compounds (Bano *et al*., 2015). The present study aimed to screen and isolate a particular flavonoid that can act as remedy for various chronic diseases. According to literature, vitexin is a flavone glycoside which has a potential to scavenge free radicals and reactive oxygen species (Aslam, 2015). This study aimed to evaluate the total flavonoids constituents from the different extracts of *V.negundo* to evident that there is fingerprint for the presence of vitexin. Assessment of vitexin qualitatively and quantitatively with various chromatographic spectroscopic analysis is needed to scientifically validate the pharmaco-beneficiary effects of folk chloric plant. This may pave the way for studying interaction of vitexin from *V. negundo* in various signalling pathways of chronic inflammatory diseases.

Materials and Methods

Collection of Plant:

Leaves and root of *V. negundo* was collected in and around South Chennai, Tamil Nadu, and India and was authenticated by Prof. P. Jayaraman, Senior Taxonomist, Plant Anatomy Research Centre, Tambaram and Chennai, India (PARC/2019/4198 and PARC/2019/4199).

Extraction:

Extract was prepared by taking 5 g of plant leaf and root powder and subjecting to successive (aqueous, acetone and methanol) extraction by hot continuous method using a Soxhlet extractor for 24 h. Extract was filtered using Whatman No.1 filter paper and the filtrate was diluted with methanol (HPLC grade) to obtain final concentration of 20 µg/ml.

Qualitative phytochemical analysis:

Leaf extracts and root extracts of *V.negundo* were subjected to qualitative phytochemical screening (Harborne, 1998; Kokate, 2001)

Quantitative phytochemical analysis:

The total phenolic content (TPC), flavonoids content (TFC) and total glycoside content (TGC) were assessed by standard protocols using quercetin as standard (Elolemy *et al*., 1994; Mcdonald *et al*., 2001; Chang *et al*., 2002)

Free radical Scavenging Activity:

The radical scavenging activity of leaf and root extracts of *V. negundo* were determined by 2,2-diphenyl-1-picryl-hydrazyl hydrate (DPPH) radical scavenging assay and Ferric ion Reducing Antioxidant Power (FRAP) assay, The results were compared with quercetin equivalents (Poongodi *et al*., 2012; Mohanasundaram *et al*., 2019).

Screening of Total by LCMS flavonoids:

The dried methanolic root extracts were re-dissolved in methanol and subjected to LCMS analysis under Shimadzu LCMS 2020 model with ESI positive and negative mode. The preferred scan range was 50-2000 m/z. The system was equipped with column Zorbax Eclipse C18 250X4.6 mm, 5 µ. LCMS program was carried out with mobile phase acetonitrile and 0.1% formic acid upon linear gradient elution. 20 µl was injected at the flow rate of 0.3 ml/min (Zhang *et al*., 2018)

Identification of total flavonoids by pTLC:

The dried methanolic root extracts of *V. negundo* were subjected to thin layer chromatography. Silica gel was used as the stationary phase and the mobile phase used was diethyl ether: acetone: acetic acid: water at the ratio 6:2:2:1. Ethylene glycol in ethanol was used for visualization. The bands obtained were carefully eluted and extracted with methanol. The resulting extracts were allowed to evaporate and residue thus obtained was subjected to further analysis (Leena and Annam, 2013)

Separation of total flavonoids by HPLC:

HPLC analysis was performed on 1260 infinity series HPLC (Agilent, USA) equipped with PDA at Zorbax Eclipse plus C18 column 4.6X250 mm, 5 µ. 20 µl of sample dissolved in methanol and injected and recorded at Wave length of 330, 254 and 210 nm. Mobile phase used were 0.1% acetic acid and HPLC grade methanol at a flow rate of 1 ml/min and gradient elution was performed (Roy *et al.*, 2013)

Purification of Vitexin by pHPLC:

The vitexin fraction was further purified by pHPLC. The purification and separation were performed with PDA at Zorbax Eclipse plus C18 column using acetic acid 0.05% and HPLC grade methanol was used as the mobile phase in an isocratic mode at a flow-rate of 3 ml/min.

Characterization of Vitexin by UV-VIS spectroscopy:

The fraction collected from the pHPLC was further scanned in UV-VIS spectroscopy using Thermospectronicunicam UV spectrophotometer 540. The wavelength of the detection corresponded to the local maxima (peaks), it was selected after recording the absorption spectra at wavelengths of 200-800 nm. From a vitexin stock solution (100 µg/ml) prepared in methanol:water (1:1,v/v), different standard solutions were prepared with the varying concentration : 5, 10, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0 and 50.0 µg/ml. Standard calibration was plotted with spectrophotometric reading at 340 nm.

Characterization of Vitexin by ^{13}C NMR:

The ^{13}C NMR spectra were acquired in CDCL3 as solvent on a Bruker 400-MHz NMR spectrometer (El-Menya, Egypt) at 400 MHz. Standard pulse sequence and parameters were used to obtain one-dimensional ^{13}C NMR and chemical shifts were measured in ppm. The Topspin software was used for this analysis.

Statistical analysis:

The results were analysed and the data are statistically represented as Mean ± SD using SPSS Statistics tool with significant value of $p<0.05$.

Results and Discussion

Qualitative phytochemical analysis:

The aqueous, acetone and methanol extracts showed difference in extraction of phytochemicals. The percentage yield of acetone extract of leaf and methanol extract of root of *V. negundo* was calculated and found that the yield of leaf extract was higher in leaf (93 %), compared with root extract (81%). The qualitative analysis of the leaf (aqueous and acetone) and root (aqueous and methanol) extracts confirms the presence of carbohydrates, fatty acids, proteins, saponins, tannins, carotenoids, polyphenols, flavonoids, alkaloids and glycosides in variable quantities (Table 1). On comparing the results the methanolic extract of root was found to contain more flavonoids and glycosides. This showed that the methanolic root extract might favour the isolation of vitexin since vitexin is a flavone glucoside. The results obtained are in conformity with findings of Ullah *et al*. (2012)

Quantitative phytochemical analysis:

The total phenolic content and total flavonoid content of methanolic root extract of *V.negundo* was found to be higher than leaf extract. Phenols and flavonoids of biogenetic origin have proven as excellent antioxidants as well as boost the activity of antioxidant enzymes and protects against oxidative stress. Hence, the extract might possess anti-inflammatory, anti-allergic, anti-tumour, antibacterial, anti-fungal, and anti-thrombotic

Table 1: Preliminary qualitative phytochemical analysis of *Vitex negundo*

S. No.	Phytochemicals	Acetone (leaf)	Methanol (root)	Aqueous (both)
1	Carbohydrates	++	+++	++
2	Fatty acids	+++	+	++
3	Proteins	++	++	+++
4	Amino acids	++	++	+++
5	Saponins	+	+	+
6	Tannins	++	-	+
7	Carotenoids	-	-	-
8	Polyphenols	++	+++	+
9	Flavonoids	++	+++	+
10	Alkaloids	++	++	+++
11	Glycosides	+++	++	+

+ Mild; ++ Moderate +++ More – Absent

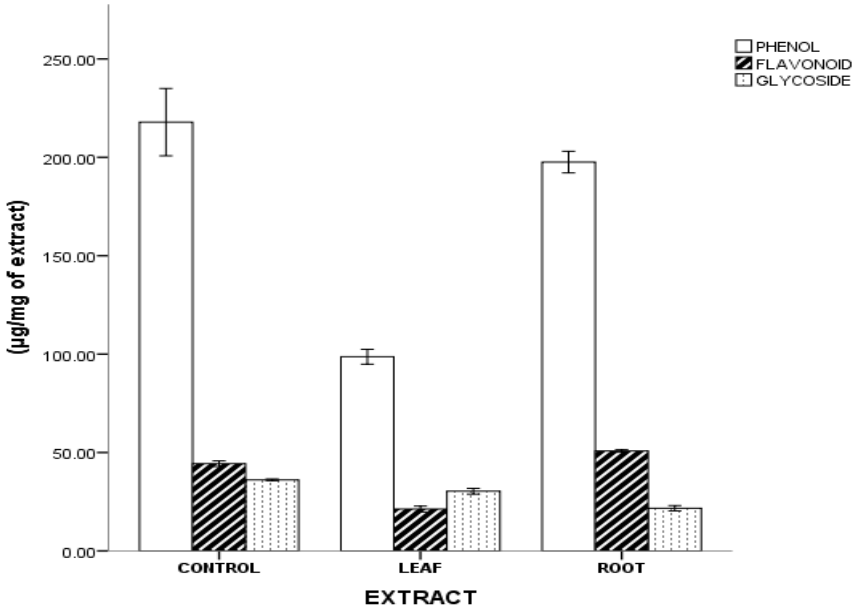

Fig. 1: Total phenolic, flavonoid and glycoside content of *V. negundo.* IC_{50} values are calculated and Data are presented as Mean ± S.D ($p<0.05$).

activity. Leaf extract (acetone) of *V.negundo* was found to contain more glycoside (Fig. 1) comparing with root extract. The presence of glycosides ensures that the extract might boost up the pharmacokinetic properties and also enhance the activity of phenols and flavonoids (Xiao *et al*., 2016).

Radical Scavenging activity:

Table 2 shows that the free radical scavenging activity of root extract of *V. negundo* is comparably higher than leaf extract. The results showed that the bioactive compounds present in the root extract scavenge more DPPH radicals formed and induce the decolourisation of resultant mixture (Kedare and Singh, 2011). The ability of root extract to reduce more ferric to ferrous ion showed the antioxidant potential of bioactive compounds in the extract. The presence of phytochemicals especially phenols, flavonoids and glycosides in estimated amount in the root extract of *V. negundo* evident the radical scavenging

Table 2: Radical scavenging activity of *V. negundo*

S. No.	Extracts	DPPH (IC50 µg/ml of extract) Mean ± SD	FRAP (IC50 µg/ml of extract) Mean ± SD
1	Root	35.36±0.50	20.29±0.61
2	Leaf	79.29±0.59	53.43±0.47

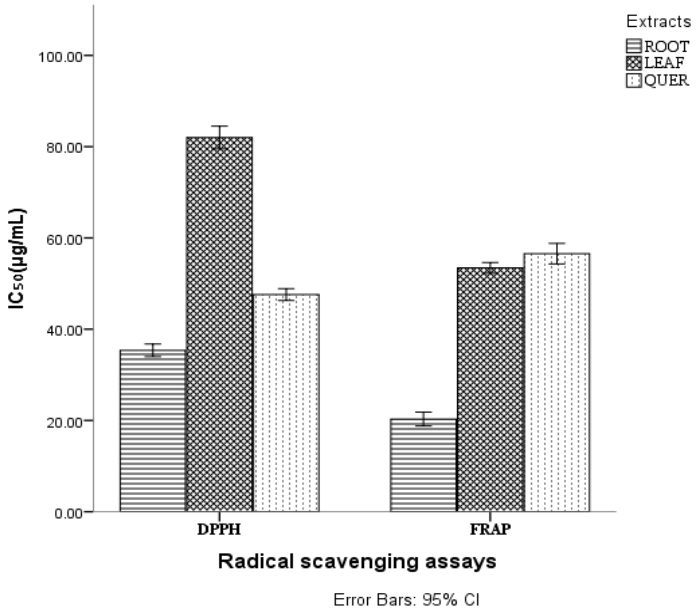

Fig. 2: Radical Scavenging activity of *V. negundo* (IC_{50} values of DPPH and FRAP assay results are presented as Mean ± S.D ($p<0.05$)

potential and ensures the ability to fight against disease and might possess varying health benefits (Fig. 2). Hence it is necessary to screen and isolate the biologically active pharmakon for more pharmacological benefits.

Screening of Flavonoids by LCMS:

The quantifications of flavonoids was performed using ion transitions at m/z. In the present study the methanolic root extract of *V. negundo* showed 27 different peaks at 254 nm at 311.1m/z. Analysis of plant based herbal products are highly facilitated by LCMS detection techniques. Mass spectrometry provides higher selectivity and sensitivity to lower most levels of detection. The glycosylated flavonoids can be easily identified by LCMS (Table 3, Fig. 3). The spectral data analysis of the root extract represents the presence of vitexin in *V. negundo* that is correlated with the data for screening of vitexin from *Scorzonera austriaca* species (Zhang *et al*., 2018)

Purification of Vitexin by pTLC:

In pTLC analysis the light yellow coloured compound was visualized and the RF value calculated as 0.87 cm and compared with quercetin standard (Fig. 4). Analysis was carried out with modifications in the procedure (Zhu *et al.,* 2015). The eluted compound results positive for flavonoids, Ammonia test and it was soluble in methanol and water. The resultant was subjected to structural conformational analysis (Leena and Annam, 2013)

Isolation of Vitexin by HPLC:

The HPLC analysis of the purified residue showed

Table 3: LCMS analysis of methanolic root extract of *V. negundo*

Detector A Channel 2 254nm

Peak#	Ret. Time	Area	Height	Height%	Area%
1	9.234	753030	14394	0.598	0.595
2	14.651	1595401	16055	0.666	1.261
3	17.536	1136176	21190	0.880	0.898
4	18.817	36902	3163	0.131	0.029
5	20.579	28519	1940	0.081	0.023
6	21.042	169478	6468	0.269	0.134
7	21.682	40094	1685	0.070	0.032
8	22.193	121427	6518	0.271	0.096
9	22.691	169755	8337	0.346	0.134
10	24.156	484929	17247	0.716	0.383
11	24.797	3139110	45628	1.894	2.481
12	26.587	12191755	327870	13.610	9.637
13	27.565	3305155	136469	5.665	2.612
14	27.873	3616397	113491	4.711	2.858
15	28.910	3037314	113461	4.710	2.401
16	29.261	4059905	145907	6.057	3.209
17	30.021	36238871	563567	23.395	28.644
18	32.484	25366460	247124	10.259	20.050
19	34.532	2326525	95659	3.971	1.839
20	35.350	3824799	138966	5.769	3.023
21	35.666	10612832	170097	7.061	8.389
22	37.327	5596073	75831	3.148	4.423
23	39.072	1021412	36957	1.534	0.807
24	40.063	6229805	76175	3.162	4.924
25	42.057	330236	9821	0.408	0.261
26	43.166	116264	5050	0.210	0.092
27	44.506	967405	9895	0.411	0.765
Total		126516030	2408965	100.000	100.000

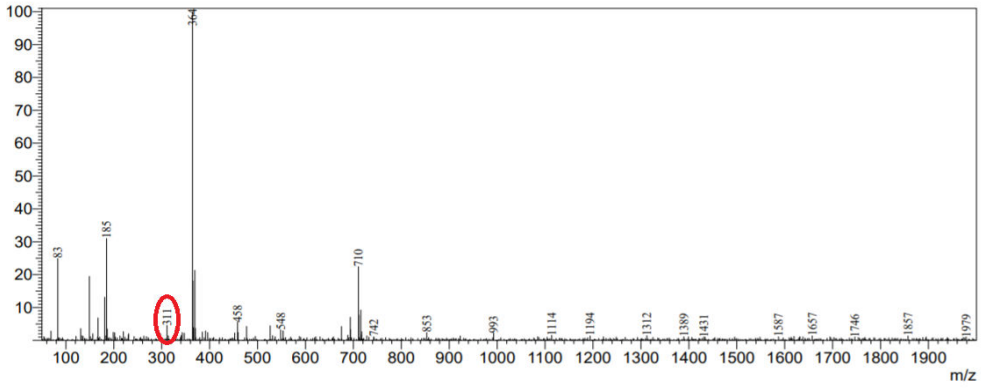

Fig. 3: LCMS analysis of Methanolic root extract of *V. negundo.*

the presence of 83 different peaks. There are many peaks identified from 2 to 50 min of the retention time. Vitexin was identified at 254 nm 5.590 retention time with an area of 0.46%. (Table 4, Fig. 5). Flavonoid compounds isolated were compared with literature and observed that the isolated compound was Vitexin (Roy *et al*., 2013). Flavonoids undergo decomposition at high temperature. HPLC can be performed at room temperature hence, this technique can validate and isolate flavonoids easily (Mittal and Ali, 2013). This serves as an evident that the isolated compound contains vitexin in the root extract of *V. negundo.*

Purification of Vitexin by pHPLC:

The HPLC separation of total flavonoids yielded resolved peaks from acetic acid and methanol elution. Purification of target compound vitexin was achieved by fractions collected at the specific peak isolated 5.590 retention time. The eluted fraction of purified vitexin was further confirmed

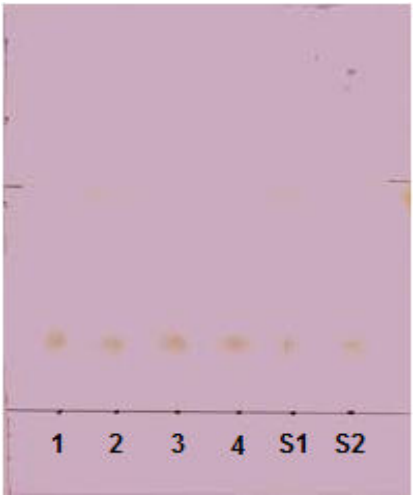

Fig. 4: pTLC chromatogram of Methanolic root extract of *V. negundo.* 1-4 – Methanol Extract; S1 and S2 – Quercetin Standard.

Table 4: Different peaks obtained from HPLC analysis of *V. negundo*

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.647	BV	0.2212	882.94092	51.70803	3.1029
2	3.048	VV	0.2515	330.94138	18.42911	1.1630
3	3.653	VB	0.4940	479.25400	14.04860	1.6842
4	5.590	BV	0.3776	132.75798	5.13541	0.4665
5	6.411	VV	0.2802	184.46587	10.33856	0.6483
6	6.949	VB	0.3860	439.49231	17.43938	1.5445
7	7.938	BV	0.3108	2517.53711	121.97203	8.8473
8	8.698	VB	0.2454	132.96214	8.45792	0.4673
9	9.341	BV	0.2449	241.71086	14.62243	0.8494
10	9.878	VV	0.2588	220.77972	12.57522	0.7759
11	10.435	VB	0.4014	398.77203	13.70935	1.4014
12	11.879	BB	0.5287	118.35446	3.01038	0.4159
13	12.972	BV	0.4176	5698.95947	196.93587	20.0276
14	14.233	VV	0.6392	1845.17151	42.94906	6.4844
15	15.166	VB	0.5590	932.78601	24.56800	3.2780
16	18.966	BV	0.8369	1079.80042	16.77746	3.7947
17	19.850	VB	0.7931	1499.59546	27.80877	5.2700
18	22.917	BB	0.6872	685.83429	14.35950	2.4102
19	25.309	BV	0.5459	355.15274	9.54986	1.2481

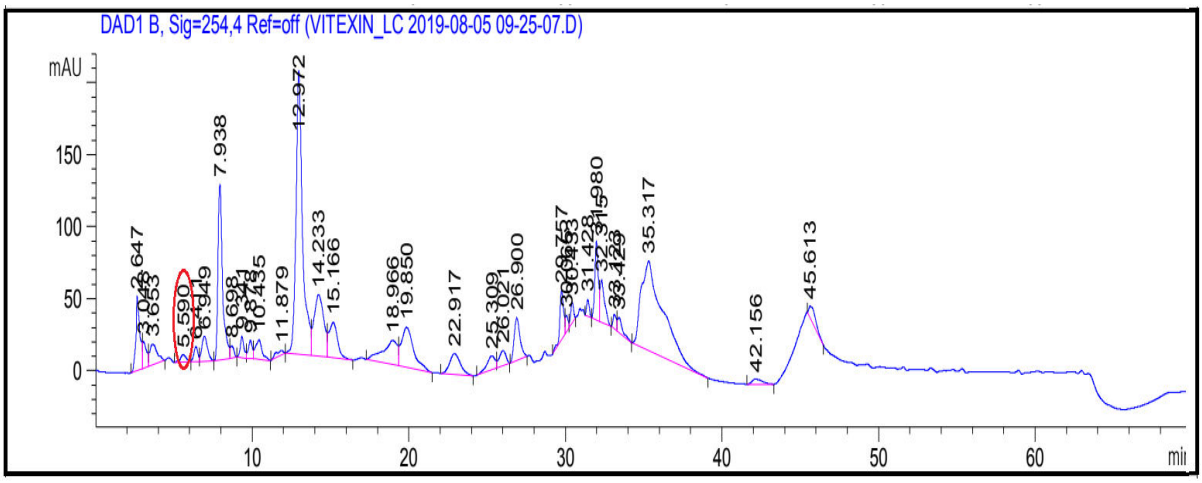

Fig. 5: HPLC chromatogram of Methanolic root extract of *V. negundo.*

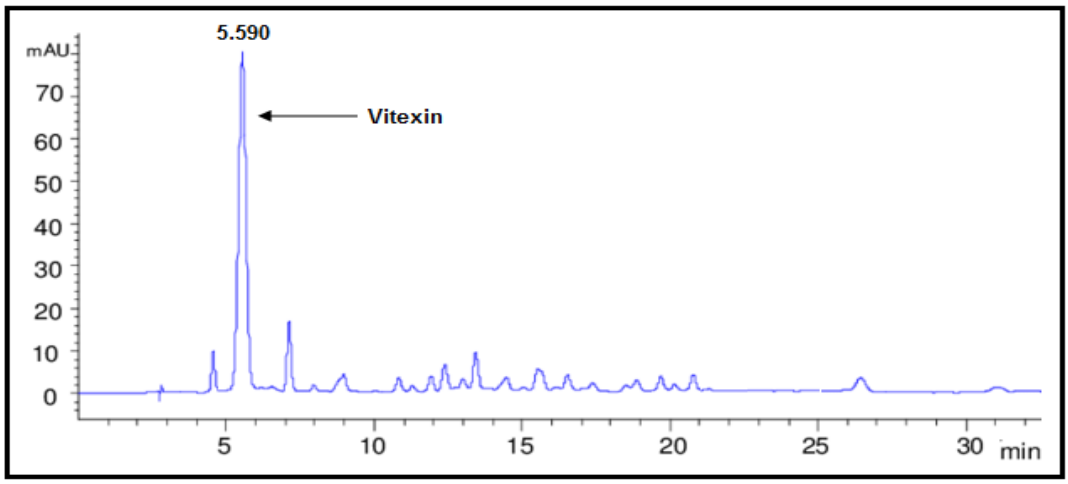

Fig. 6: pHPLC chromatogram of Vitexin from *V. negundo.*

by the HPLC analysis (Fig. 6). Raghu and Agrawal (2016) reported that the reverse phase HPLC method was used to purifiy the phytochemical vitexin from the medicinal plant *Justicia gendarussa* and considered as a simple, sensitive, precise and reproducible method by HPLC for the quantification of vitexin which correlates with our present findings.

Characterization of Vitexin by UV-VIS spectroscopy:

The UV-VIS spectroscopy analysis of the fraction collected from the HPLC was done to identify the wavelength of the purified vitexin. The vitexin sample was scanned under UV-VIS spectrophotometer from 200-800 nm. The scanning result showed absorption maxima at 340 nm which was also compared with the standard flavonoids quercetin (Fig. 7). The reproducibility of the compound was tested with the different concentrations of vitexin and its absorption at 340 nm showed good linearity, accuracy and reproducibility (Fig. 8). Similar simple and rapid spectral validation method was also carried out by Shuayprom *et al.* (2016) from *Passiflora foetida* which correlates with our findings. The present method provided reliable practical method for routine analysis and could be used for pharmaceutical quality control of raw materials for regulatory purposes. The per cent recovery for

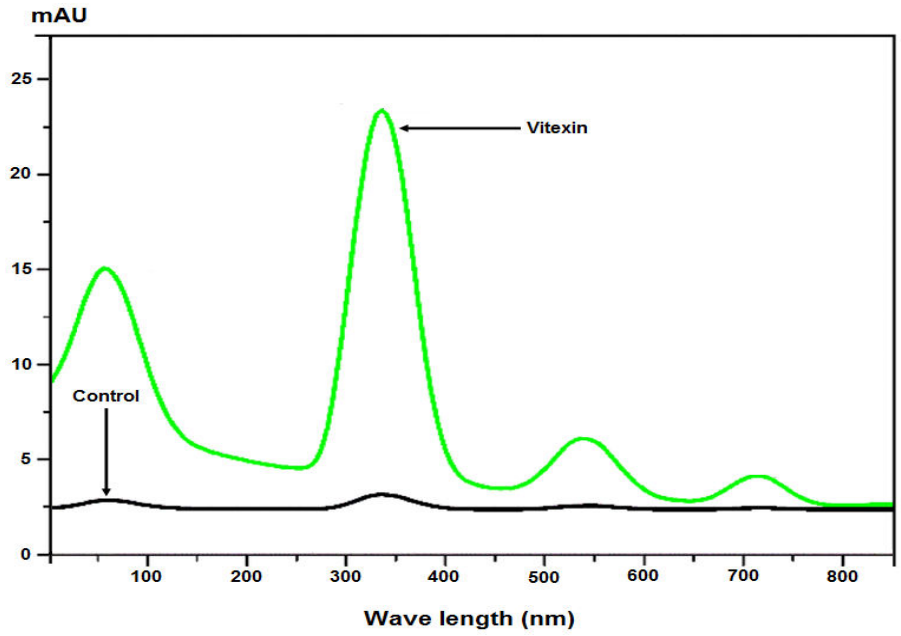

Fig. 7: UV-VIS spectrum of Vitexin from *V. negundo.*

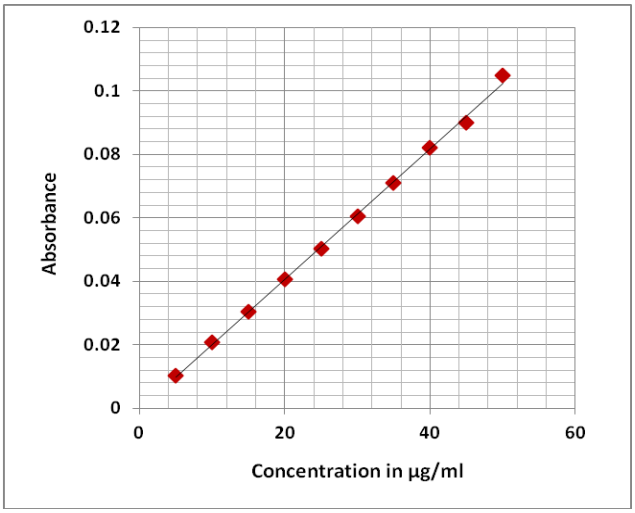

Fig. 8: Linearity plot of Vitexin from *V. negundo.*

the method proposed here is comparable with previous methods explained by Pongpan *et al*. (2007)

Characterization of Vitexin by ^{13}CNMR:

The ^{13}C NMR spectra are presented in Figure 9 and Table 5. ^{13}C NMR shifts relative to ring carbon was identified. Based on the carbon shift, the result on NMR study was compared with the standards and the carbon skeleton structure of the compound was predicted and confirmed. According to the results, ^{13}C NMR spectrum showed the carbon shifts obtained for aglycone carbons at the positions C2(164.22ppm), C3(102.43 ppm), C4(182.24 ppm), C5(160.98 ppm), C6(98.10 ppm), C7(162.33 ppm), C8(104.61 ppm), C9(156.00 ppm), C10(104.46 ppm), C1'(121.60 ppm), C2'(129.12 ppm), C3'(115.78 ppm), C4'(161.14 ppm), C5'(117.78 ppm), C6'(129.12 ppm) and for glycone carbons at the positions C1"(71.9 ppm), C2"(82.1 ppm), C3"(78.6 ppm), C4"(70.5 ppm), C5"(81.6 ppm), C6"(61.3 ppm). All the carbon positions obtained by the NMR assay were compared with the standard flavonoid and the structure was confirmed as vitexin (Fig. 10). The overall results of NMR analysis confirmed that the

Table 5: ^{13}C NMR chemical shift of Vitexin from *V. negundo*

Aglycone C position	Type of Carbon	^{13}C Shift (ppm)	Aglycone C position	Type of Carbon	^{13}C Shift (ppm)	Glycone C position	Type of Carbon	^{13}C Shift (ppm)
C2	>C—	164.51	C1’	>C—	121.81	C1”	>C—	71.02
C3	>CH	102.37	C2’	>CH	128.98	C2”	>CH—OH	82.24
C4	>C=O	180.24	C3’	>CH	115.24	C3”	>CH—OH	79.21
C5	>C—OH	159.95	C4’	>C—OH	160.70	C4”	>CH—OH	69.80
C6	>CH	98.59	C5’	>C—	114.98	C5”	>C—	80.77
C7	>C—OH	162.26	C6’	>C—	128.78	C6”	>CH—OH	60.84
C8	>C—	104.47						
C9	>C—	156.13						
C10	>C—	103.91						

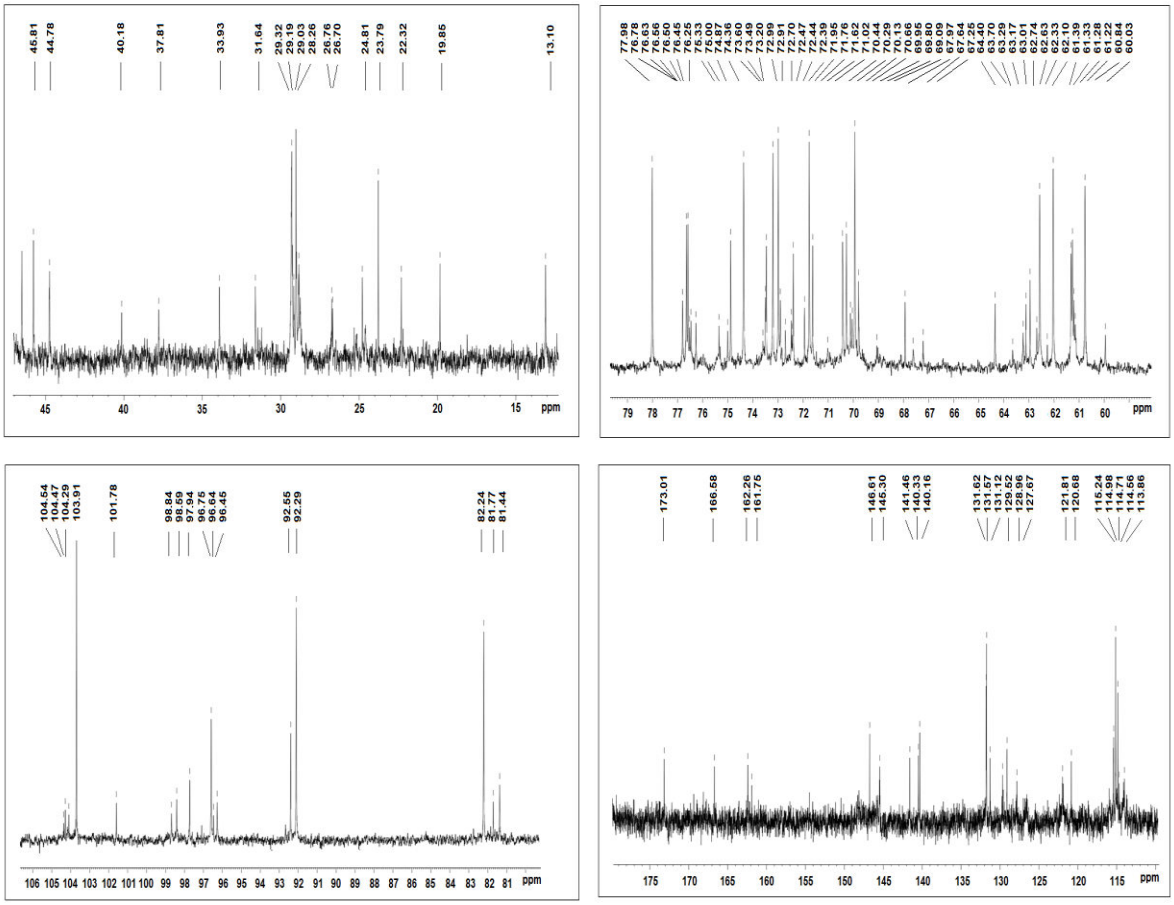

Fig. 9: ^{13}C NMR spectrum of Vitexin from *V. negundo.*

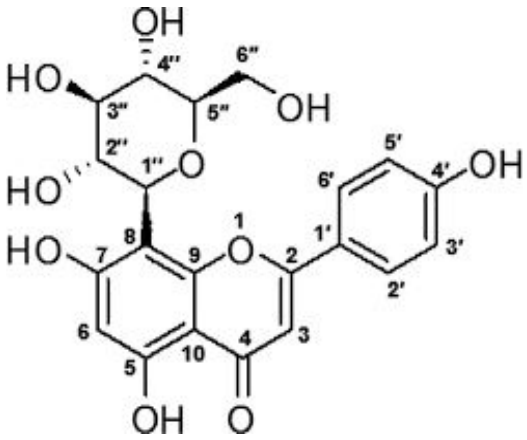

Fig. 10: Structure of Vitexin.

purified compound from *V. negundo* analysed by LCMS, pTLC, and HPLC was Vitexin. The ^{13}CNMR results also further proved that the overall compound possess 21 carbon atoms and 1st position is occupied by oxygen atom, the aglycon ring structure of vitexin contains 14 carbon atoms (C2-C10; C1'-C6') and the glycone ring structure of vitexin contains 6 carbon atoms (C1"-C6"). Sharma *et al*. (2014) reported that the phytochemicals Orientin and Vitexin were isolated from the bark of *V. negundo* and their structures were confirmed by IR, ^{1}HNMR, ^{13}C NMR, MS studies were also correlated with the present findings. Another report by Thenmozhi and Subasini (2014) identified the structure of vitexin from *Vitex pinnata* leaves which also supported the current investigation.

Conclusion

The current study proved that the traditional herb *V. negundo* has ample amount of antioxidants and rich phytochemicals to scavenge the free radicals. Hence, it is a potent herb to fight oxidative stress. This study validated that the traditional application of herb for various diseases may be due to the presence of flavonoids, phenols and glycosides. The present study standardized a method for the screening, isolating and structural conformation of pharmakon vitexin from the root sample of *V. negundo*. Studying the interaction of the purified vitexin in *in vitro* and *in vivo* methods may find more pharmaco economic values in the treatment of chronic inflammatory diseases.

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