https://africanjournalofbiomedicalresearch.com/index.php/AJBR
Afr. J. Biomed. Res. Vol. 27 (September 2024); 432-443

Research Article

HPTLC and FTIR Studies on Iridoid Glycoside (Negundoside) Isolated from Ethanol Leaf Extracts of *Vitex Negundo* Linn.

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ABSTRACT
In traditional Indian system of medicine *Vitex negundo* Linn is referred as *Sarvaroganivaran*i - the remedy for all diseases. It is reported to possess wide range of medicinal uses and the ethanolic leaf extracts reported to contain flavanone, agnuside, casticin, herbacetin rhamnoside, kaempferol, luteolin-7- glucoside, negundoside, p-hydroxybenzoic acid, protocatechuic acid, quinic acid, vitedoin A, and vitexin. Investigation shows that negundoside is a potent phytopharmaceutical that acts in a novel way in inhibiting liver toxicity by interfering in the key events that are the main causative factors leading to liver dysfunction. This study aimed to isolate the phytoconstituent negundoside from ethanolic leaf extracts of *Vitex negundo* and identify the same by HPTLC fingerprinting and FTIR methods. The ethanol leaf extracts of *Vitex negundo* was subjected to column chromatography in a silica gel (60-120 mesh) glass column loaded onto the column of 46 × 2 cm and eluted with petroleum ether followed by cyclohexane, chloroform, ethyl acetate, methanol with increasing polarity. It resulted in six different fractions (A, B, C, D, E, F). The HPTLC chromatographic fingerprints for all fractions were recorded at 254 nm and 366 nm using the solvent system chloroform, methanol and acetic acid in the ratio 7:3:1. The results obtained for each fraction were compared with the HPTLC fingerprints and Rf values of reference standard negundoside developed under the same conditions. The lane graph and HPTLC fingerprint of isolated fraction E reported to have similar Rf value as compared with reference standard negundoside with varying displayed volumes. The FTIR spectrum of fraction E also showed peak wavelength values similar to that and reference standard negundoside. The present study offers valuable information and acts as a tool that can be used to identify negundoside from the ethanolic leaf extracts of *Vitex negundo.*

Keywords: Column Chromatography, Fourier Transform Infrared Spectroscopy (FTIR), High Performance Thin Layer Chromatography (HPTLC), Negundoside, *Vitex negundo.*

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Receiving date 15/08/2024 Acceptance date 17/09/2024

DOI: https://doi.org/10.53555/AJBR.v27i3.2027

1 INTRODUCTION

Vitex negundo Linn (Verbenaceae), known as *Nirgundi* in Sanskrit is an important medicinal plant with a variety of phytoconstituents having significant pharmacological activities. The plant was first described in Charaka Samhita, which is an oldest and most authentic text of Ayurveda. It is used in the treatment pf cephalgia, otalgia, arthritis, inflammation, colic, rheumatism, skin diseases, urinary disorders and wounds, ulcers. *Nirgundi* oil is used to treat sinuses, wounds, ulcers and gangrenous wounds. Leaves and bark are considered useful in scorpion sting. [1] Ethanolic leaf extract of *Vitex negundo* by LC-MS/MS analysis revealed the

presence of 15 bioactive molecules including flavanone, agnuside, casticin, herbacetin rhamnoside, kaempferol, luteolin-7- glucoside, negundoside, p-hydroxybenzoic acid, protocatechuic acid, quinic acid, vitedoin A, and vitexin. [2]. In traditional system of Indian medicine *Vitex negundo* Linn is referred as *Sarvaroganivarani* - the remedy for all diseases. [3] Research studies on *Vitex negundo* Linn on different plant parts and their extracts reported to possess significant pharmacological activities with multiple beneficial effects to humans [4]. Negundoside is reported to possess anticancer activity both in vitro and in silico studies [5]. Negundoside reported to exert a protective effect on CYP2E1-dependent CCl4 toxicity via inhibition of lipid peroxidation, followed by an improved intracellular calcium homeostasis and inhibition of $Ca^{(2+)}$-dependent proteases [6]. This study is aimed to isolate the iridoid glycoside negundoside by column chromatography from ethanolic leaf extracts of *Vitex negundo* and identify the same by HPTLC fingerprinting profile and FTIR spectra by comparing it with marker reference standard negundoside. The assessment and identification of phytoconstituent is essential for safe use of herbal medicines.

2 METHODS AND MATERIALS

2.1 Chemicals and iridoid glycoside standard

Petroleum ether, cyclohexane, chloroform, ethyl acetate, methanol, silica gel. All the chemicals used were of analytical grade and iridoid glycoside reference standard negundoside was purchased from Sigma-Aldrich Chemicals Private Limited, Bangalore, India.

2.2 Plant material

The fresh leaves of *Vitex negundo* L were collected from their natural habitat at Thiruporur to Chengal- pattu highway roadside near Chennai, Tamil Nadu during the first week of April 2022. The herb was authenticated by Prof P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), West Tambaram, Chennai 600045 and the authentication number is PARC/2022/4833 for *Vitex negundo* L.

2.3 Preparation of plant material

The leaves collected were washed and rinsed with water to remove dirt and unwanted particles prior to the drying process. The plant parts were dried under shade for 60 days at room temperature (27 ± 1°C). The leaves were pulverized into a coarse dry powder (Figure 1) with a mechanical grinder and passed through 60 # sieve and stored in airtight container.

Figure 1. Drying of leaves of *Vitex negundo*

2.4 Preparation of ethanol plant extract of *Vitex negundo*

50 g of coarsely powdered leaves of VN was placed in separate glass stoppered maceration jars. The menstruum (ethanol) were poured on to the top of each maceration jar, until it completely covered the drug material. The containers were closed and kept for three days with occasional shaking. After 3 days, the mixture was pressed and strained. [7]. The extracts were collected and evaporated to dryness. The ethanol extracts of *Vitex negundo* (EVN) was stored separately and used for further studies.

2.5 Column Chromatography

The ethanolic extracts of *Vitex negundo* (EVN) was subjected to Silica gel (60-120 mesh) column chromatography for the isolation of phytoconstituents. The column was rinsed well with acetone and completely dried before packing. A piece of glass wool was placed at the bottom of the column using a glass rod. Above the glass wool added sea sand upto 1 cm height. About 20 g of EVN was mixed with 100 g of silica gel and loaded onto the vertical column of 46 × 2 cm and eluted with petroleum ether followed by cyclohexane, chloroform, ethyl acetate and methanol with increasing polarity.

2.6 TLC of fractions

The fractions were collected and subjected to TLC (20 × 20 cm aluminium sheets coated with silica gel 60 F254) to detect the presence of phytoconstituents. The Rf values of each spot was calculated. Fractions exhibiting same Rf values were polled together and concentrated to dryness using rotary evaporator. The fractions obtained from TLC were analyzed for the presence of negundoside using HPTLC and FTIR methods.

2.7 High Performance Thin Layer Chromatography (HPTLC)

The fractions obtained from ethanol plant extracts of the leaves of *Vitex negundo* L by column chro- matography were subjected to HPTLC fingerprint analysis following standard methods. The fractions and reference standard negundoside were applied on to the TLC aluminium plate precoated with silica gel 60 F254 using Camag's ATS4 applicator. 10 µL of sample was applied on to the plate with 6 mm band width fitted with a micro syringe using CAMAG HPTLC Linomat IV sample applicator. The plate was developed using mobile phase chloroform: methanol: acetic acid (7:3:1 ratio) upto 85 mm in a twin trough CAMAG glass chamber previously saturated with mobile phase for 20 minutes at 25°C. After development, the plate was air dried and photo documented using Camag's TLC Visualizer under 254 nm and 366 nm and their chromatogram finger print profiles were recorded. Just TLC software version 4.6.1 used for lane analysis of extracts.

2.8 Fourier Transform Infrared Spectroscopy (FTIR)

The HPTLC finger print and Rf values of fractions, which is compatible with that of reference standard of negundoside, was

utilized for FTIR studies. 10 mg of compatible fraction and 10 mg of reference standard negundoside were encapsulated separately in 100 mg of KBr pellet to prepare translucent discs. The test fraction and reference standard loaded in FTIR spectroscope (SHIMADZU, IR, Affinity 1, Japan) with scan range of 500 to 4000 cm^{-1} [8] and a resolution of 4 cm^{-1}. Happ-Genzel function of apodization was selected to achieve a good balance between ripple size and resolution. The spectral data of test fraction were compared with a reference standard to identify the relationship between the functional groups existing between them.

3 RESULTS

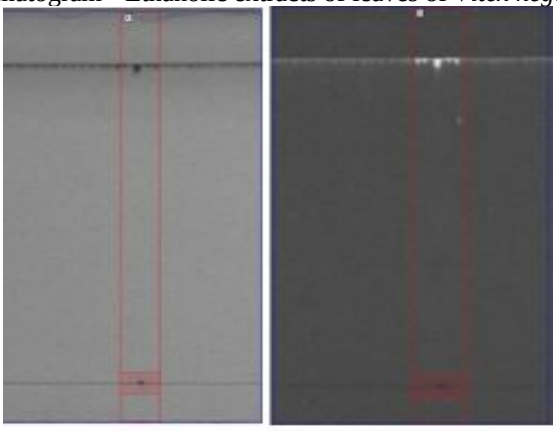
The column chromatography resulted in six fractions from the ethanol plant extracts of the leaves of *Vitex negundo* L using solvents cyclohexane, chloroform, ethyl acetate and methanol with increasing polarity. cyclohexane: chloroform in the ratio 9:1 gave fraction A. In the ratio 7:3 of cyclohexane: chloroform resulted in fraction B and in the ratio 6:4 gave fraction C. Fraction D resulted using the solvent cyclohexane and chloroform in the ratio 4:6. In the ratio of 2: 8 of chloroform and ethyl acetate fraction gave fraction E. ethyl acetate and methanol (8:2) gave fraction F. All the collected fractions were subjected to TLC coated with silica gel 60 F254. TLC with mobile phase of benzene: chloroform (8:2) gave Rf value of 0.4 and 0.6 for fractions A and D with dark black colour spot. TLC mobile phase of chloroform: ethyl alcohol (7:3) as the developing solvent system for sample E, F detected as yellow spots. The fractions were tested for the presence of phytoconstituent negundoside using HPTLC and FTIR studies. The yields of the fractions obtained are given in (Table 1).

All six fraction were subjected to HPTLC analysis. The HPTLC chromatogram under short UV λ = 254 nm and long UV λ = 366 nm and lane graphs were recorded for all six fractions. Similarly, the HPTLC chromatogram for reference standard negundoside was recorded under the same conditions.

Table 1. Experimental yield of ethanol plant extracts of the leaves of *Vitex negundo* L

S. No.	Solvent system	Name of the fraction	Yield of fraction(g)
1.	Cyclohexane: Chloroform (9:1)	A	1.25
2.	Cyclohexane: Chloroform (7:3)	B	1.02
3.	Cyclohexane: Chloroform (6:4)	C	1.10
4.	Cyclohexane: Chloroform (4:6)	D	0.85
5.	Chloroform: Ethyl acetate (2:8)	E	2.20
6.	Ethyl acetate: Methanol (8:2)	F	0.95

Figure 2. HPTLC chromatogram - Ethanolic extracts of leaves of *Vitex negundo* (EVN) Fraction A

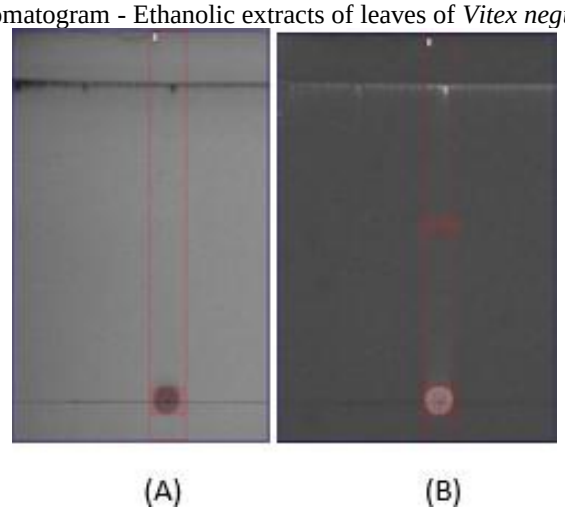


(A) Under short UV λ = 254 nm, (B) Under long UV λ = 366 nm

Figure 3. HPTLC - Lane profile graph of EVN Fraction A at UV λ 254 nm

Figure 4. HPTLC - Lane profile graph of EVN Fraction A at UV λ 366 nm

Figure 5. HPTLC chromatogram - Ethanolic extracts of leaves of *Vitex negundo* (EVN) Fraction B



(A) Under short UV λ = 254 nm, (B) Under long UV λ = 366 nm

Figure 6. HPTLC - Lane profile graph of EVN Fraction B at UV λ 254 nm

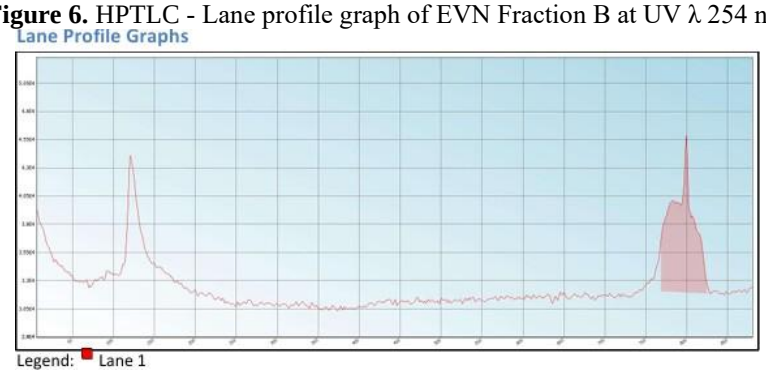
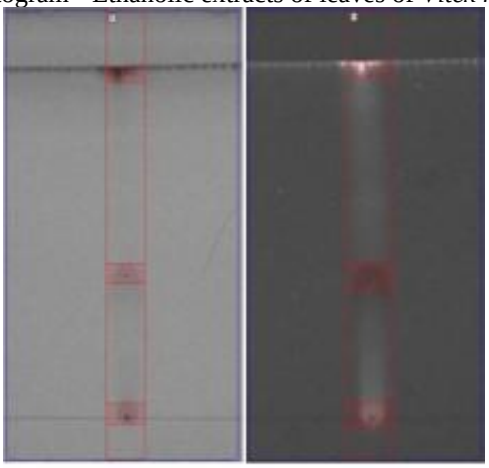


Figure 7. HPTLC - Lane profile graph of EVN Fraction B at UV λ 366 nm

Figure 8. HPTLC chromatogram - Ethanolic extracts of leaves of *Vitex negundo* (EVN) Fraction C



(A) Under short UV λ = 254 nm, (B) Under long UV λ = 366 nm

Figure 9. HPTLC - Lane profile graph of EVN Fraction C at UV λ 254 nm

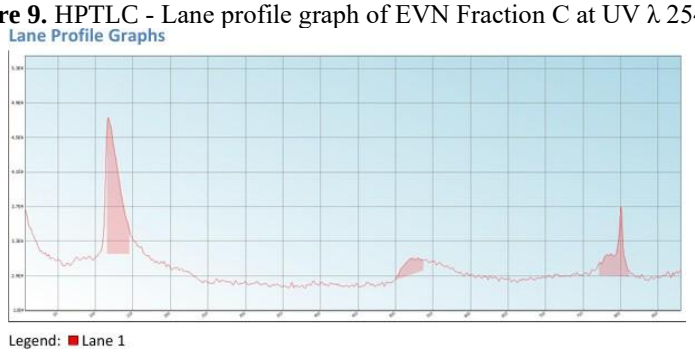


Figure 10. HPTLC - Lane profile graph of EVN Fraction C at UV λ 366 nm

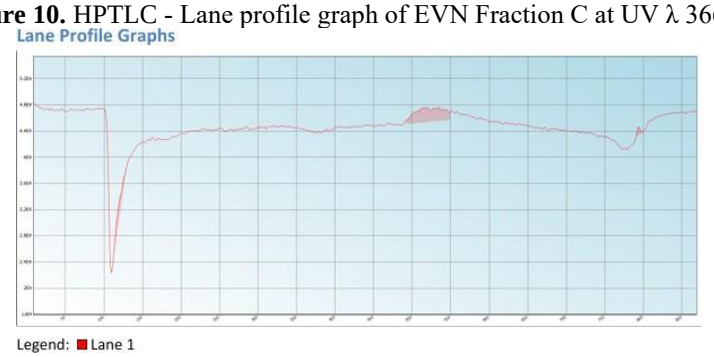
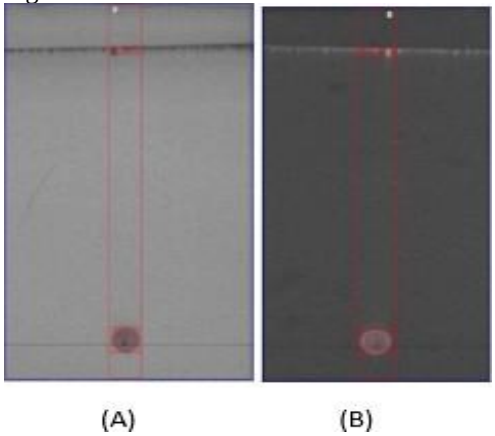


Figure 11. HPTLC chromatogram - Ethanolic extracts of leaves of *Vitex negundo* (EVN) Fraction D

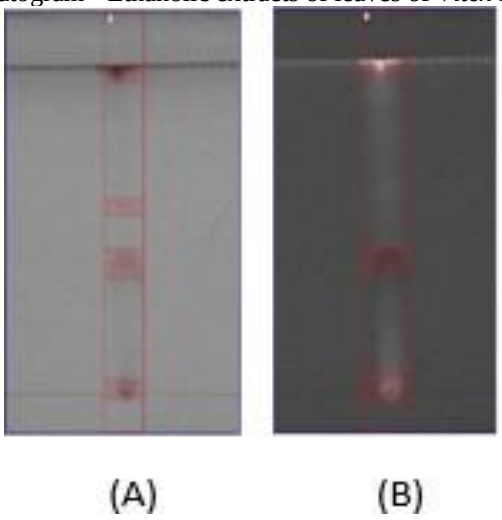


(A) Under short UV λ = 254 nm, (B) Under long UV λ = 366 nm

Figure 12. HPTLC - Lane profile graph of EVN Fraction D at UV λ 254 nm

Figure 13. HPTLC - Lane profile graph of EVN Fraction D at UV λ 366 nm

Figure 14. HPTLC chromatogram - Ethanolic extracts of leaves of *Vitex negundo* (EVN) Fraction E



(A) Under short UV λ = 254 nm, (B) Under long UV λ = 366 nm

Figure 15. HPTLC - Lane profile graph of EVN Fraction E at UV λ 254 nm

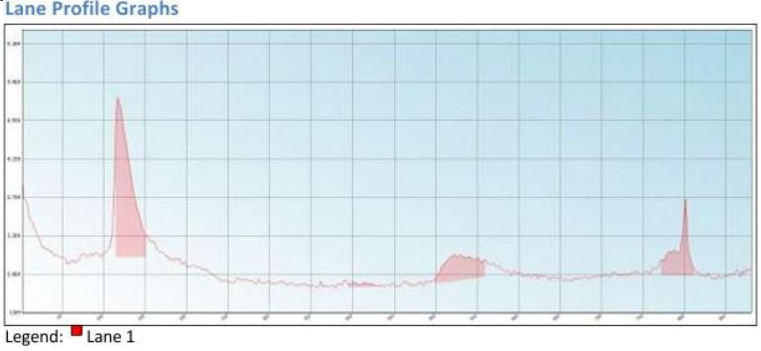


Figure 16. HPTLC - Lane profile graph of EVN Fraction E at UV λ 366 nm

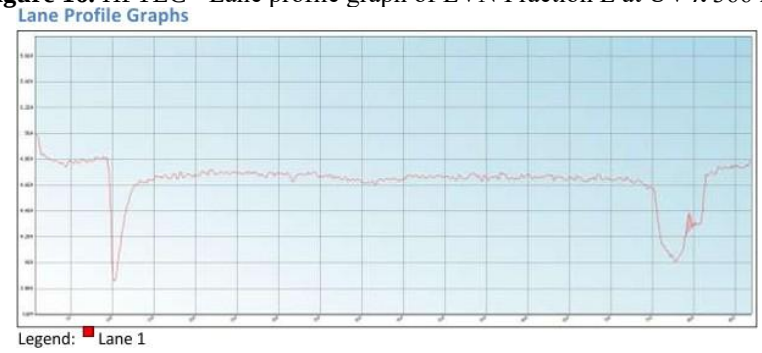
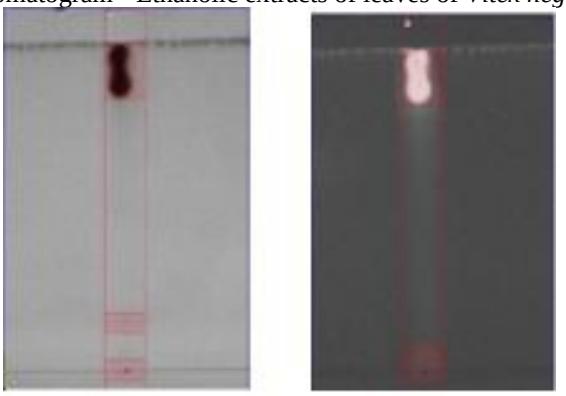


Figure 17. HPTLC chromatogram - Ethanolic extracts of leaves of *Vitex negundo* (EVN) Fraction F



(A) Under short UV λ = 254 nm, (B) Under long UV λ = 366 nm

Figure 18. HPTLC - Lane profile graph of EVN Fraction F at UV λ 254 nm

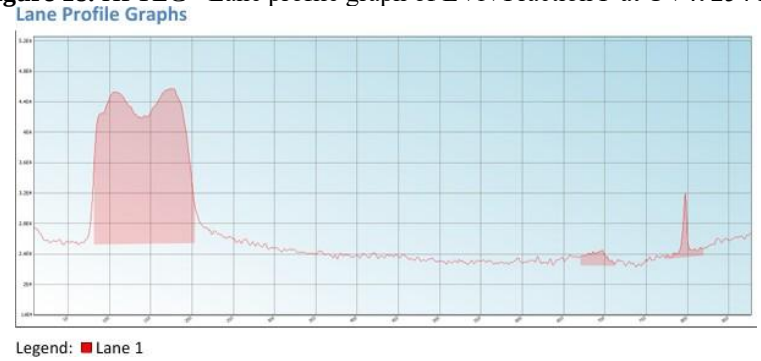


Figure 19. HPTLC - Lane profile graph of EVN Fraction F at UV λ 366 nm

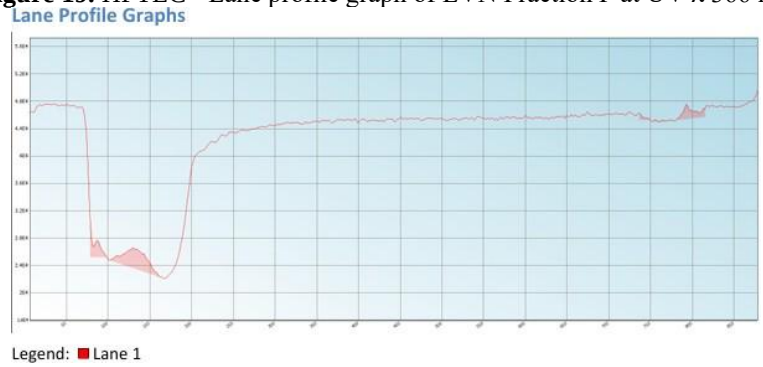
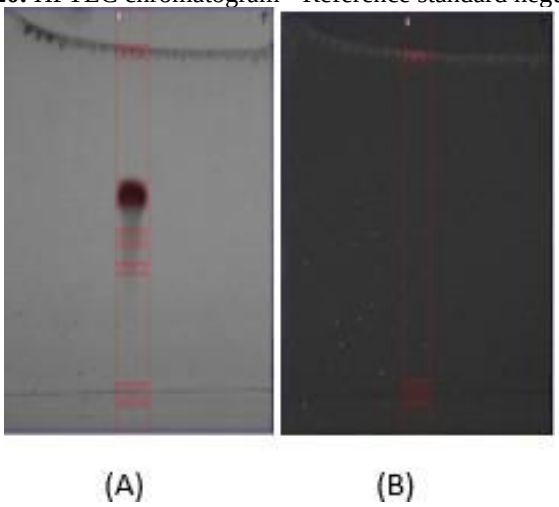


Figure 20. HPTLC chromatogram - Reference standard negundoside



(A) Under short UV λ = 254 nm, (B) Under long UV λ = 366 nm

Figure 21. HPTLC - Lane profile graph of reference standard negundoside at UV λ 254 nm

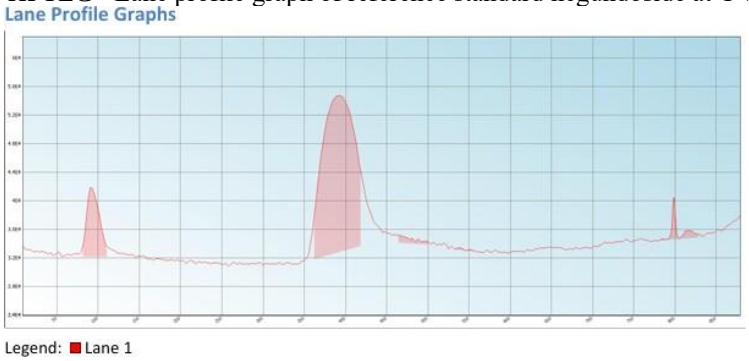


Figure 22. HPTLC - Lane profile graph of reference standard negundoside at UV λ 366 nm

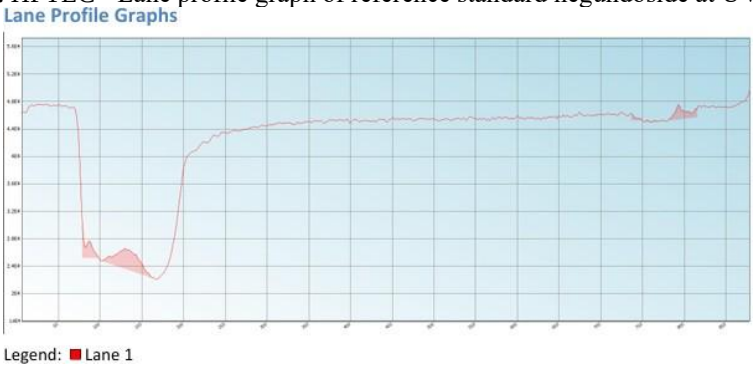


Table 2. HPTLC - Lane observations for EVN fractions and reference standard negundoside at UV λ 254 nm

Lane ID	Width	Bands	Volume	Displayed Volume	Notes
1.	64	1	4597056	45.97	Fraction A
1.	77	1	29912267	299.12	Fraction B
1.	76	3	33784736	337.85	Fraction C
1.	64	2	37005504	370.05	Fraction D
1.	77	4	45001110	450.01	Fraction E
1.	89	3	201733541	2017.33	Fraction F
1.	104	5	127225072	1272.26	Reference

Table 3. HPTLC - Lane observations for EVN fractions and reference standard negundoside at UV λ 366 nm

Lane ID	Width	Bands	Volume	Displayed Volume	Notes
1.	85	1	2748900	27.49	Fraction A
1.	69	2	540339	5.4	Fraction B
1.	81	3	10183482	101.84	Fraction C
1.	72	2	0	0	Fraction D
1.	94	3	10539374	105.39	Fraction E
1.	93	2	16721121	167.21	Fraction F
1.	83	2	1450093	14.5	Reference

Table 4. HPTLC - Band Peak observations of EVN fractions and reference standard negundoside at UV λ 254 nm

Lane ID	Band ID	Rf	Area	Volume	Displayed Volume	Notes
1.	1	0.038	2944	4597056	45.97	Fraction A
1.	1	0.044	4312	29912267	299.12	Fraction B
1.	1	0.968	2280	21712592	217.13	Fraction C
1.	2	0.411	2888	3728484	37.28	Fraction C
1.	3	0.032	3040	8343660	83.44	Fraction C
1.	1	0.966	1152	9239296	92.39	Fraction D
1.	2	0.045	3840	27766208	277.66	Fraction D
1.	1	0.979	2849	26102230	261.02	Fraction E
1.	2	0.577	2541	832986	8.33	Fraction E
1.	3	0.401	4697	9473079	94.73	Fraction E
1.	4	0.03	3080	8592815	85.93	Fraction E
1.	1	0.906	10858	190410783	1904.1	Fraction F
1.	2	0.177	3827	5136724	51.37	Fraction F
1.	3	0.031	4183	6186034	61.86	Fraction F
1.	1	0.981	3016	17881864	178.82	Reference standard
1.	2	0.585	5824	101909912	1019.1	Reference standard
1.	3	0.476	3744	2371824	23.72	Reference standard
1.	4	0.388	2600	466960	4.67	Reference standard
1.	5	0.035	4888	4594512	45.95	Reference standard

Table 5. HPTLC - Band Peak observations of EVN fractions and reference standard negundoside at UV λ 366 nm

Lane I D	Band ID	Rf	Area	Volume	Displayed Volume	Notes
1.	1	0.049	4080	2748900	27.49	Fraction A
1.	1	0.555	2208	46299	0.46	Fraction B
1.	2	0.011	4416	494040	4.94	Fraction B
1.	1	0.951	2835	2279745	22.8	Fraction C
1.	2	0.408	4617	7903737	79.04	Fraction C
1.	3	0.021	4212	0	0	Fraction C
1.	1	0.986	1728	0	0	Fraction D
1.	2	0.052	4536	0	0	Fraction D
1.	1	1	3760	3123150	31.23	Fraction E
1.	2	0.411	4982	7416224	74.16	Fraction E
1.	3	0.014	5922	0	0	Fraction E
1.	1	0.82	11439	12706125	127.06	Fraction F
1.	2	0.03	7347	4014996	40.15	Fraction F
1.	1	0.971	1660	0	0	Reference standard
1.	2	0.049	3984	1450093	14.5	Reference standard

Figure 23. FTIR spectra of EVN Fraction E

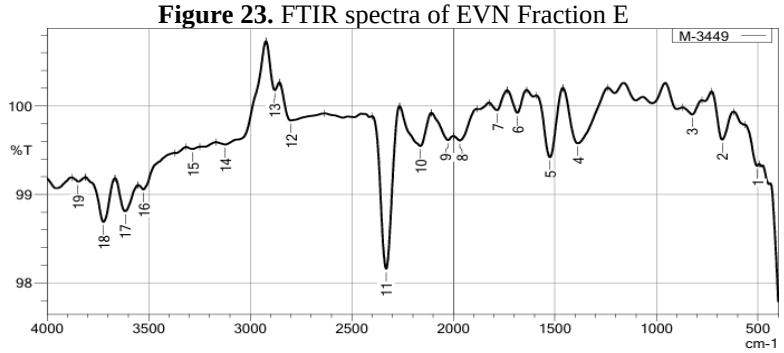


Figure 24. FTIR spectra of reference standard negundoside

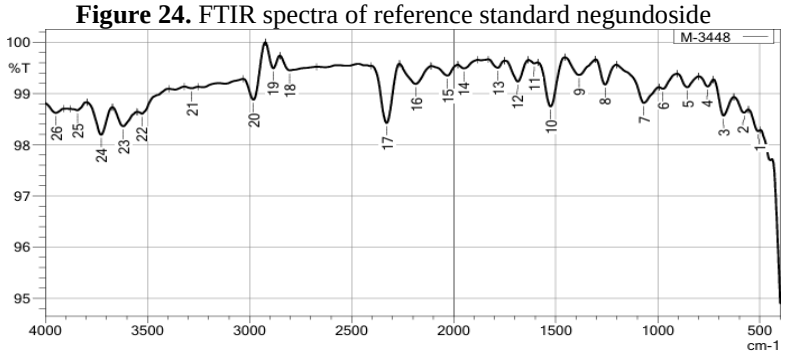
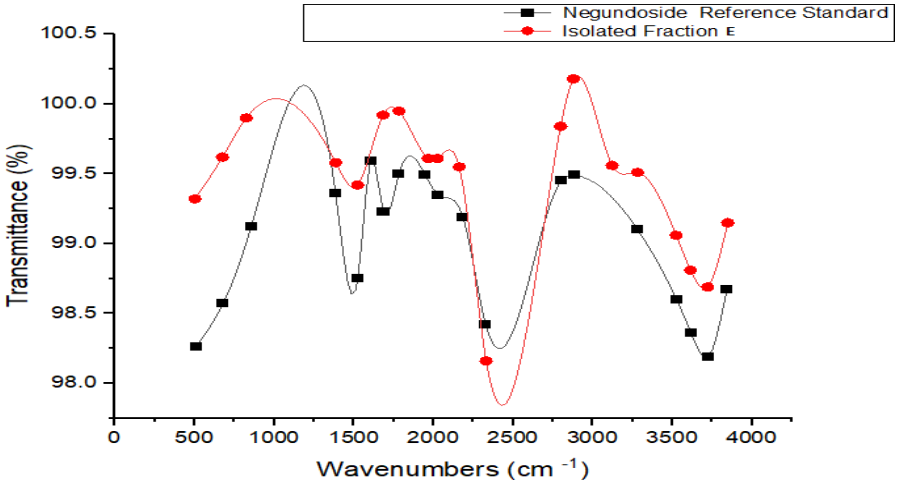


Table 6. Functional group analysis of FTIR spectra of EVN fraction E and reference standard negundoside

S.No	Reference standard negundoside wave number cm^{-1}	Fraction E wave number cm^{-1}	Functional groups
1	1385.64	1387.07	CH3 bend
2	1525.63	1524.20	C=C aromatic
3	1687.05	1684.20	C=C aromatic
4	1782.76	1784.19	C=O anhydride
5	1949.90	1968.47	C=C=C stretching
6	2802.71	2799.85	C-H aldehydic
7	2884.13	2879.85	-C-H stretch
8	2981.27	3122.69	carboxylic acid OH stretch
9	3282.68	3282.68	alcohol OH stretch
10	3526.96	3525.53	alcohol OH stretch
11	3618.38	3615.52	alcohol OH stretch

Figure 25. FTIR spectra comparison of fraction E and reference standard negundoside



4 DISCUSSION

Column chromatography of EVN resulted in fractions A, B, C, D, E and F. HPTLC fingerprinting is useful in phytochemical profiling and quantification of compounds present in plant samples [9]. The isolated fractions were subjected to HPTLC fingerprinting, the lane graph and Rf values at UV λ 254 nm and UV λ 366 nm for each fraction was recorded. HPTLC finger print lane analysis of fraction A showed one band with Rf value of 0.038 at UV λ 254 nm and one band with Rf value of 0.049 at UV λ 366 nm (Figure 2). Fraction B at UV λ 254 nm showed one band with Rf value of 0.044 and at UV λ 366 nm showed two bands with Rf values 0.555 and 0.011 (Figure 5). Fraction C showed three bands at Rf values of 0.968, 0.411, 0.032 at UV λ 254 nm and three bands at UV λ 366 nm with Rf values 0.951, 0.408, 0.021 (Figure 8). Fraction D exhibited two bands at UV λ 254 nm and UV λ 366 nm with Rf values 0.966, 0.045, 0.986, 0.052 respectively (Figure 11). Fraction E showed a maximum of four bands at UV λ 254 nm and two bands at UV λ 366 nm (Figure 14). The last fraction F exhibited three bands and two bands at UV λ 254 nm and UV λ 366 nm with Rf values 0.906, 0.177, 0.031 and 0.82, 0.03 (Figure 17). The HPTLC chromatogram for reference standard negundoside at UV λ 254 nm and two bands at UV λ 366 nm are shown in (Figure 20). The lane observations for all fractions of EVN and reference standard negundoside at UV λ 254 nm and UV λ 366 nm are summarized in (Table 2) and (Table 3). Similarly, the peak observations for all fractions of EVN and reference standard negundoside at UV λ 254 nm and UV λ 366 nm are summarized in (Table 4) and (Table 5). The Rf values for fraction E and reference standard exhibited closely related values of 0.979, 0.577, 0.401, 0.03 for fraction E and 0.981, 0.585, 0.388, 0.035 for reference standard at UV λ 254 nm (Figure 14 and Figure 20). The lane graph for fraction E and reference standard showed similar finger prints with varying volumes (Figure 15 and Figure 21).

From HPTLC analysis, fraction E was found to be in close proximity with standard reference negundoside based on their reported Rf values and HPTLC finger print patterns. From outcome of the present HPTLC studies, fraction E was subjected to further FTIR studies and it was compared with IR spectra of reference standard negundoside to compare the similarities in functional group between them. The individual FTIR spectra of fraction E and reference standard are provided in (Figure 23 and Figure 24). The peaks at wave number 1385.64 cm^{-1} and 1387.07 cm^{-1} indicates the presence of functional groups CH3 bend. The presence of C=C aromatic is witnessed at wave numbers 1525.63 cm^{-1} and 1524.20 cm^{-1}. Functional group C=O anhydride is shown at peak values 1782.76 cm^{-1} and 1784.19 cm^{-1}. The peaks at wave number 1949.90 cm^{-1} and 1968.47 cm^{-1} indicates the presence of functional group C=C=C stretching. The functional group carboxylic acid OH stretch is indicated by the peak value wave numbers 2981.27 cm^{-1} and 3122.69 cm^{-1}. The results of functional group analysis are summarized in (Table 6)

The FTIR spectra comparison with fraction E and reference standard negundoside showed similar peak patterns and overlapping of spectra (Figure 25) indicating the isolated fraction E is negundoside.

5 CONCLUSION

The iridoid glycoside negundoside has been reported to be beneficial in treating different ailments and possess remarkable pharmacological effects on humans. The present study has reported the methods of isolation of negundoside from ethanol leaf extracts of *Vitex negundo* (EVN) by silica gel column chromatography using solvents with increasing polarity. The isolation resulted in six fractions. The comparison of HPTLC analysis of six fractions and HPTLC analysis of reference compound negundo-side showed that fraction E exhibited Rf values similar to that of reference marker compound negundoside. The FTIR spectra results for fraction E also exhibited peak wavelengths in accordance with FTIR spectra obtained from reference marker compound negundoside. The overlapping of peak wavelengths between fraction E and reference marker compound validates the isolated fraction is negundoside. The important contribution of our work is the reporting of HPTLC and FTIR spectra data for the identification of an important iridoid glycoside negundoside present in the ethanol leaf extracts of *Vitex negundo* that can be utilized by the research community to establish its identity. The present study also offers an avenue to dwell upon on other five reported isolated fractions for further research studies of pharmacological and phytochemical importance.

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