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SHORT COMMUNICATION

Standardization and HPTLC Fingerprint Studies on the Ethanol Extracts of *Vitex negundo* Leaves and *Opuntia dillenii* Cladode

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Abstract

Background: In traditional Indian system of medicine *Vitex negundo* Linn is referred as *Sarvaroganivarani* - the remedy for all diseases. The cladodes of *Opuntia dillenii* possess anti-inflammatory, potent hypotensive and antihyperglycemic effects. Aim: This study aimed to perform comparative standardization and HPTLC fingerprinting on ethanolic leaf extracts of *Vitex negundo* and cladodes of *Opuntia dillenii*. Methods: Extracts were prepared using cold maceration. Physicochemical studies of powdered crude drugs, preliminary phytochemical analysis, heavy metal analysis for arsenic, lead, cadmium and mercury, test for aflatoxin B1, G1, B2, G2 and microbial analysis were carried out in both ethanolic plant extracts. The chromatographic fingerprints of ethanolic extracts of plants were recorded using HPTLC and the solvent system used was chloroform and methanol in the ratio 9:1. Results: The outcomes of preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, phenols, glycosides and steroids in both the plant extracts. Proteins, saponins and tannins were absent. Analysis of heavy metals and aflatoxins were Below Quantification Limits (BQL). Microbes *E. coli*, *Salmonella, Staphylococcus aureus*, and *Pseudomonas aeruginosa*, were absent. Total aerobic bacterial count and yeast mould count were well within the acceptable limits. The results obtained from HPTLC fingerprint lane analysis showed 12 and13 bands at wavelengths 254nm and 366nm respectively. Conclusion: The present study offers valuable information and act as a tool that can applied in standardization of ethanolic plant extracts of *Vitex negundo* and *Opuntia dillenii.*

Keywords: Aflatoxin, Cladode, Heavy Metals, HPTLC, *Opuntia dillenii*, *Vitex negundo*

1. Introduction

Vitex negundo Linn (*Verbenaceae*), called *Nirgundi* in Sanskrit is a significant medicinal plant that has a range of phytoconstituents with considerable pharmacological properties. The plant was first described in *Charaka samhita* which is the oldest and most authentic text of Ayurveda. It is used in the treatment of cephalgia, otalgia, arthritis, inflammation, colic, rheumatism, skin diseases, urinary disorders and wounds, and ulcers. *Nirgundi* oil is used to treat sinuses, wounds, ulcers, and gangrenous wounds. Bark and leaves are thought to be beneficial for scorpionsting[1]. 15 bioactive compounds, including flavanone, agnuside, casticin, herbacetin rhamnoside, kaempferol, luteolin-7-glucoside, negundoside, p-hydroxybenzoic acid, protocatechuic acid, quinic acid, vitexin, and vitexin A, were found in an ethanolic leaf extract of *V. negundo* by LC-MS/MS analysis[2]. In the traditional system of Indian medicine *Vitex negundo* Linn is referred to as *Sarvaroganivarani* - the remedy for all diseases[3].

Opuntia dillenii additionally referred to as the prickly pear belongs to the family Cactaceae[4]. *Opuntia*

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dillenii fruit has been utilized as a traditional medication to cure gonorrhea and manage spasmodic cough.

The leaves have been used to treat the inflammation of the eye and as well as to all heat and inflammation[5]. Flowers of the *Opuntia dillenii* plant have antiviral, antioxidant, analgesic, and antibacterial properties. This plant's fresh fruit lowers blood glucose levels, and its freeze-dried form of aqueous fruit extract possesses anti-inflammatory qualities[6,7]. *Opuntia dillenii* cladodes have strong hypotensive, anti-inflammatory, and anti-hyperglycemic properties[8-10]. The *Kani* tribes of southern India's Tirunelveli hills consider *Opuntia* species as spiritual. *Opuntia dillenii* is used to cure colds, headaches, coughs, fever and manage toxic animal stings[11]. *O. dillenii* mucilage was reported to have a 6.2% swelling index. Additionally, it includes neutral sugars involving arabinose, rhamnose, and galactose and has anti-obesity qualities[12]. Research studies on *Vitex negundo* Linn and *Opuntia dillenii* on different plant parts and their extracts were reported to possess significant pharmacological activities with multiple beneficial effects on humans. The assessment of their quality and identification is essential for the safe use of herbal medicines. This work is focused on standardization and HPTLC profiling of ethanolic plant extracts of leaves of *Vitex negundo* Linn and cladodes of *Opuntia dillenii*.

2. Materials and Methods

2.1 Plant Material

The fresh leaves of *Vitex negundo* L were gathered from the natural habitant Thiruporur to Chengalpattu highway roadside near Chennai, Tamil Nadu and the cladodes of *Opuntia dillenii* were collected from their natural habitat at Singaperumal koil to Hanumanthapuram main road side during the first week of April 2022. Prof. P. Jayaraman, director of the Plant Anatomy Research Centre (PARC), West Tambaram, Chennai 600045, verified the authenticity of the herbs. For *Vitex negundo* L., the authentication number is PARC/2022/4833, and for *Opuntia dillenii*, it is PARC /2022/4832).

2.2 Preparation of Plant Material

To eliminate dirt and undesirable particles before drying, the collected leaves were washed and dried with water. The plant sections were allowed to dry for 60 days at room temperature (27 ± 1 °C) in the shade. After being removed from undesirable particles, the cladodes were left to dry for 90 days at room temperature (27 ± 1 °C). Using a mechanical grinder, the cladodes and leaves were ground into a course, dry powder. The mixture was then put through a 60 # sieve and kept in an airtight container.

2.3 Physicochemical Studies

Using the procedure outlined in WHO guidelines, several physicochemical parameters, including Loss on Drying (LOD), ash values (total ash, acid in soluble ash), and extractive values (by cold extraction) on the leaves of *Vitex negundo* (VN) and cladodes of *Opuntia dillenii* (OD) powdered crude drug, were determined.

2.4 Cold Maceration Extraction

4g of coarsely powdered leaves of VN and 4g of coarsely powdered cladode of OD were placed in separate glass stoppered maceration jars. The menstruum (ethanol) was 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 mixtures were pressed and strained[13]. The extracts were collected and evaporated to dryness. These ethanol Extracts of *Vitex negundo* (EVN) and ethanolic Extracts of cladodes of *Opuntia dillenii* (EOD) were stored separately and used for further studies.

2.5 Preliminary Phytochemical Screening

Phytochemical examinations of VN and OD were performed to identify the phytoconstituents present[14-16].

2.6 Heavy Metal Analysis

Heavy metals determination in EVN and EOD was done using atomic absorption spectroscopy (Shimadzu). Standard operating parameters for arsenic, lead, cadmium, and mercury at wavelengths of 193.7nm, 283.3nm, 228.8nm, and 253.7nm were used. According to Official Method 999.10, air acetylene was utilized as fuel and as radiation sources in hollow cathode lamps. Atomic absorption spectrophotometry; lead, cadmium, zinc, copper, and iron in food. 1999 saw the first atomic action. NMK-AOAC technique.

2.7 Test for Aflatoxin

The HPLC method approved by the AOAC® Official Methods Program was used to identify aflatoxin in EVN and EOD. Official method 991.31 is applicable for determining aflatoxin B1, B2, G1, and G2 in maize, peanuts, and peanut butter using both fluorometry and HPLC analysis[17].

2.8 Microbial Analysis

The microbial analysis was carried out both in EVN and EOD. The total aerobic bacterial count was determined as per the procedure given in IS 5402:2012[18]. The yeast and mould count were determined as per the procedure given in IS 5403:1999[19]. The enumeration of coliforms by colony count technique was performed as per the procedure given in IS 5401: 2012[20]. The detection of *Salmonella* species was performed in accordance with IS 5887 (Part 3): 1999 procedure[21]. The presence of *Pseudomonas aeruginosa* was determined in accordance with IS 5887 (Part 2):1976 procedure and *Staphylococcus aureus* were determined as per the procedure outlined in IS 5887 (Part 2): 1976[22].

2.9 High Performance Thin Layer Chromatography

The ethanol plant extracts of the leaves of *Vitex negundo* L and Ethanol Plant Extracts (EVN) of cladodes of *Opuntia dillenii* (EVD) were subjected to HPTLC fingerprint analysis following standard methods. Using Camag's ATS4 applicator, the plant extracts were applied to the TLC aluminum plate that had been precoated with silica gel $60F_{254}$. With a Camag HPTLC Linomat IV sample applicator equipped with a micro syringe and a 6mm bandwidth, 15µL of sample was put to the plate. Utilizing a 9:1 ratio of methanol and chloroform as the mobile phase, the plate was produced up to 85mm in a twin trough Camag glass chamber that had been saturated with mobile phase for 20min. at 25°C. After development, the plate was allowed to air dry before being exposed under 254 and 366 nm using Camag's TLC Visualizer, which also recorded the chromatogram fingerprint profiles. The only software used for extract lane analysis is TLC version 4.6.1.

Table1. Physicochemical analysis of crude powders of VN leaf and OD cladode

S.no	Parameters	VN	OD
1	Loss on drying at 105°C	7.4%	6.8%
2	Total ash value (%w/w)	8	15.8
3	Acid in-soluble ash value(%w/w)	0.84	0.57
4	Alcohol soluble extractive value(%w/w)	17.5	20.5
5	Water soluble extractive value(%w/w)	21.4	24.8

3. Results and Discussion

Physical-chemical characteristics, like drying at 105°C, total and acid-insoluble ash values, and water-soluble and alcohol-soluble extractive values, were measured. Results for VN and OD are given in (Table 1). Loss on drying is the measure of moisture content present in the given sample and it directly promotes fungal growth and deterioration of the drug sample. It was found that drying at 105 °C resulted in losses in VN and OD of 7.4 and 6.8% w/w, respectively. The total ash value of the plant material shows the presence of earthy and mineral components in addition to the plant material. The total ash value for the VN and OD content, according to analytical data, was 8.0 and 15.8% w/w, respectively. The plant samples had acid-insoluble matter contents of 0.84 and 0.57% w/w, respectively. For VN and OD, the extractive values that were soluble in alcohol were 17.5% and 20.5% w/w, respectively. For both VN and OD, the water-soluble extractive value indicated the presence of inorganic compounds, sugar, and acids at 21.4 and 24.8% w/w. The quantitative values obtained for VN were within the acceptable limits provided by Pharmacopoeia Commission[23]. The quantitative parameters for OD are not established in API. The total ash value, water-soluble and alcohol-soluble extractive values of OD were found to be higher than that of VN.

Preliminary phytochemical analysis of EVN and EOD indicated the presence of alkaloids, flavonoids, phenols, glycosides, and steroids in both plant extracts. Proteins, saponins and tannins were absent in EVN and EOD. Results of preliminary phytochemical analysis of EVN and EOD are given in (Table 2).

Analysis of heavy metals (arsenic, cadmium, lead, mercury) and Aflatoxins (B1, G1, B2, G2) in EVN and EOD were Below Quantification Limits (BQL). The heavy metal analysis of As, Pb, Cd, and Hg on EVN and EOD are furnished in (Table 3). Studies on the presence

Table 2. Preliminary phytochemicals analysis of EVN and EOD

S.no	Phytochemical constituents	EVN	EOD
1	Alkaloids	+	+
2	Flavonoids	+	+
3	Phenols	+	+
4	Proteins	-	-
5	Reducing sugar	+	+
6	Glycosides	+	+
7	Saponins	-	-
8	Terpenoids	+	-
9	Steroids	+	+
10	Tannins	-	-

+Present. -Absent

Table 3. Test for heavy metals in EVN and EOD

S. no.	Para meters	Units	EVN	EOD
1	Arsenic	ppm	BQL[LOQ:0.05]	BQL[LOQ:0.05]
2	Lead	ppm	BQL[LOQ:0.01]	BQL[LOQ:0.01]
3	Cadmium	ppm	BQL[LOQ:0.01]	BQL[LOQ:0.01]
4	Mercury	Ppm	BQL[LOQ:0.01]	BQL[LOQ:0.01]

BQL-Below Quantification Limit, LOQ-Limit of Quantification

of aflatoxin B1, G1, B2, and G2 in EVN and EOD are summarized in (Table 4).

Microbial studies" to detect the presence of total aerobic bacterial count, yeast and mould count, *E. coli* spp., *Salmonella* spp., *Pseudomonas aeruginosa,* and *Staphylococcus aureus* in EVN and EOD are provided in (Table 5). Microbes *E. coli, Salmonella, Staphylococcus aureus*, and *Pseudomonas aeruginosa*, were absent both in EVN and EOD. Total aerobic bacterial count and yeast mould count were found to be 127 CFU/g and 143 CFU/g in EOD, and ≤10 for EVN, indicating all well within the acceptable limits. The standardization of leaves of *Vitex negundo* (VN) and cladodes of *Opuntia dillenii* (OD) powdered crude drug and their ethanolic extracts indicates the medicinal herb selected for the present study complies with the guidelines and safety standards for herbal medicines mandated by API and WHO.

HPTLC chromatograms of EVN and EOD at wavelengths 254 nm and 366nm are furnished in (Figure 1). The fingerprint lane profile graph at 254 nm

Table 4. Test for aflatoxins in EVN and EOD

S. no.	Parameters	Units	EVN	EOD
1	AflatoxinB1	µg/kg	BQL[LOQ:0.1]	BQL[LOQ:0.1]
2	AflatoxinG1	µg/kg	BQL[LOQ:0.1]	BQL[LOQ:0.1]
3	AflatoxinB2	µg/kg	BQL[LOQ:0.1]	BQL[LOQ:0.1]
4	AflatoxinG2	µg/kg	BQL[LOQ:0.1]	BQL[LOQ:0.1]

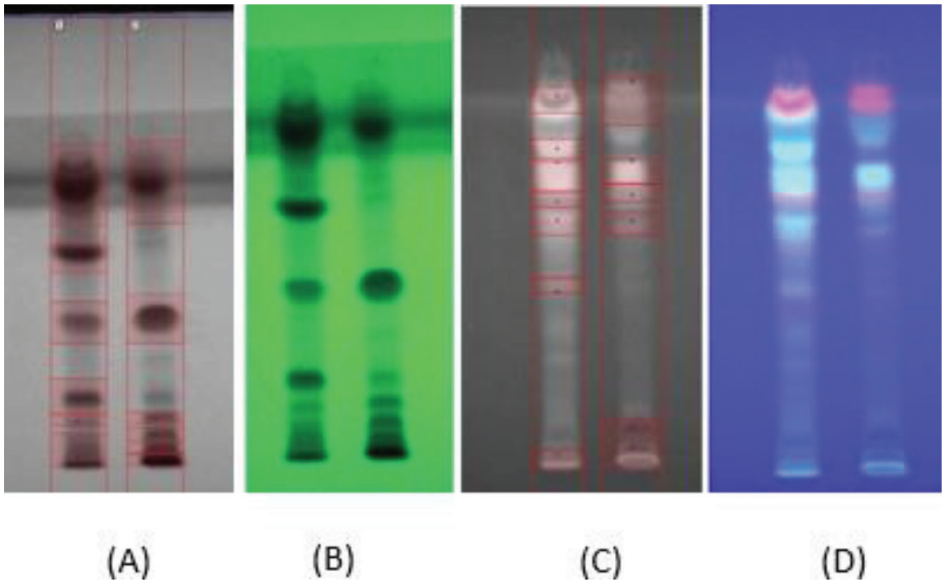
BQL-Below Quantification Limit, LOQ-Limit of Quantification

Table 5. Microbial analysis of EVN and EOD

S. no.	Parameters	Units	EVN	EOD
1	Total aerobic bacterial count	CFU/g	≤10	127
2	Yeast and Mould count	CFU/g	≤10	143
3	*E. coli* spp.	per g	Absent	Absent
4	*Salmonella* spp.	per25g	Absent	Absent
5	*Pseudomonas aeruginosa*	per g	Absent	Absent
6	*Staphylococcus aureus*	per g	Absent	Absent

CFU/g: colony-forming units per gram

and366 nm for EOD and EVN are given in (Figures 2 and 3). HPTLC peak observations for EOD and EVN at 254nm are provided in (Tables 6 and 7) and HPTLC peak observations for EOD and EVN at 366 nm are shown in (Tables 8 and 9). HPTLC fingerprinting is useful in phytochemical profiling and quantification of compounds present in plant samples[24]. HPTLC fingerprint lane analysis for EOD and EVN showed twelve bands at 254nm. At 366 nm EOD and EVN showed thirteen bands. Lane 1 for EOD revealed 6 spots with Rf values of 0.787, 0.601, 0.404, 0.189, 0.128, 0.031 at 254 nm and 7 spots with R_f values of 0.829, 0.711, 0.68, 0.592, 0.55, 0.392, 0.028 at 366 nm. Lane 2 for EVN showed 6 spots with R_f values 0.802, 0.415, 0.138, 0.09, 0.051, 0.018 at 254 nm and 6 spots with R_f values 0.862, 0.683, 0.608, 0.546, 0.089, 0.047 at 366 nm. The displayed volume at 254 nm and 366 nm was found to be higher in EOD. Comparing Rf values of Lane 1 (EOD) and 2 (EVN) at 254 nm revealed one phytoconstituent with similar R_f values at 0.787 and 0.802. The comparison of Rf values of Lane1and 2 at 366nm expressed a similar band with Rf values 0.68 and 0.683. The comparative studies of R_f value indicate that both EVN and EOD have one phytoconstituent in common. The maximum area



(A)Under short UV λ=254nm, (B) Under fluorescence light UV λ =254nm, (C) Under long UV λ = 366 nm, (D) Under fluorescence light long UV λ = 366 nm

Figure 1. HPTLC chromatogram-Ethanolic extracts of leaves of *Vitex negundo* (EVN) and ethanolic extracts of cladodes of *Opuntia dillenii (*EOD).

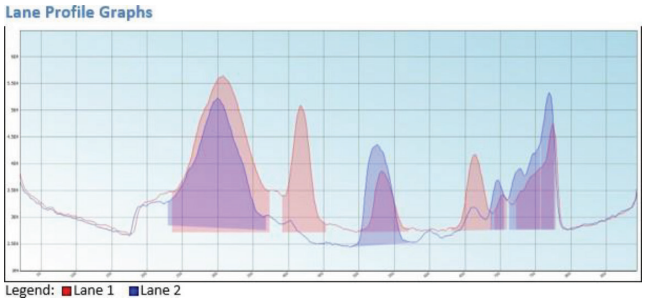


Figure 2. HPTLC-Lane profile graph of EVN and EOD at UV λ 254nm.

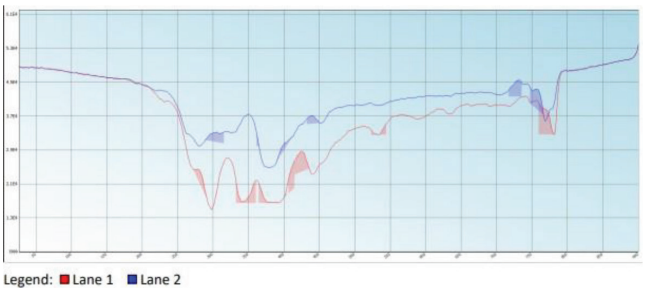


Figure 3. HPTLC-Lane profile graph of EVN and EOD at UV λ 366nm.

of the HPTLC chromatogram peak at 254 nm for lane1 (EOD) is 34.32% contributed by Band 1 with Rf value 0.787. The maximum area of the HPTLC chromatogram peak at 254 nm for Lane 2 (EVN) is 45.72% contributed by Band1 with R_f value 0.802. The maximum area of the HPTLC chromatogram peak at 366 nm for Lane1 (EOD) is 22.94% contributed by Band 3 with a R_f value 0.68. The maximum area of the HPTLC chromatogram peak at 366nm for Lane 2 (EVN) is 31.73% contributed by Band 1 with R_f value 0.862. The present study indicates that EOD and EVN have one phytoconstituent in common in addition to the phytoconstituents. Further studies using marker compounds will provide insights into the nature of phytoconstituents present. The development of a chromatogram employing solvent system chloroform: methanol (9:1) and the R_f values obtained offers valuable information and acts as a tool that can applied in the standardization of plant extracts of EOD and EVN.

Table 6. HPTLC-Lane observations for EVN and EOD at UV λ 254 nm

Lane ID	Width	Bands	Volume	Displayed Volume	Notes
1	77	6	361095273	3610.93	EOD
2	79	6	280314989	2803.18	EVN

Table 7. HPTLC-Band Peak observations for EVN and EOD at UV λ 254nm

Lane ID	Band ID	R_f	Area	Volume	Displayed Volume	Notes
1	1	0.787	10626	193212789	1932.1	EOD
1	2	0.601	4774	56682164	566.82	EOD
1	3	0.404	5390	25729627	257.3	EOD
1	4	0.189	4466	29612583	296.13	EOD
1	5	0.128	1386	7437738	74.38	EOD
1	6	0.032	4312	48420372	484.2	EOD
2	1	0.802	10981	139087400	1390.9	EVN
2	2	0.415	6557	56812218	568.12	EVN
2	3	0.138	1501	11422531	114.23	EVN
2	4	0.09	1817	17822163	178.22	EVN
2	5	0.051	1659	22032863	220.33	EVN
2	6	0.018	1501	33137814	331.38	EVN

Table 8. HPTLC-Lane observations for EVN and EOD at UV λ 366 nm

Lane ID	Width	Bands	Volume	Displayed Volume	Notes
1	73	7	25218434	252.17	EOD
2	85	6	16519750	165.2	EVN

Table 9. HPTLC-Band Peak observations for EVN and EOD at UV λ 366nm

Lane ID	Band ID	Rf	Area	Volume	Displayed Volume	Notes
1	1	0.829	2774	2332204	23.32	EOD
1	2	0.711	2044	4169395	41.69	EOD
1	3	0.68	2847	2345490	23.45	EOD
1	4	0.592	1533	6967485	69.67	EOD
1	5	0.55	2701	398653	3.99	EOD
1	6	0.392	1533	1059011	10.59	EOD
1	7	0.028	1752	7946196	79.46	EOD
2	1	0.862	5610	2412810	24.13	EVN
2	2	0.683	2720	343145	3.43	EVN
2	3	0.608	2125	1305940	13.06	EVN
2	4	0.546	2720	2033795	20.34	EVN
2	5	0.089	1530	4615840	46.16	EVN
2	6	0.047	2975	5808220	58.08	EVN

4. Conclusion

Present studies on ethanol leaf extracts of *Vitex negundo* (EVN) and ethanolic extracts of cladodes of *Opuntia dillenii* (EOD) will help in establishing standards for the quality of the plant material as per pharmacopoeial requirements. The Below Quantification Limit (BQL) reported for aflatoxins and heavy metals in the present study emphasizes the importance of safety standards set forth for the safe use of herbals. One important contribution of our work is the reporting of HPTLC fingerprints of EVN and EOD and these fingerprint peak profiles can be considered valuable information for the authentication and standardization of plant extracts. The occurrence of one band in common with similar Rf values of 0.68 and 0.683 reported for EOD and EVN is an interesting area to focus upon to identify the phytoconstituent using suitable marker compounds already reported in these plants like negundoside, vitexin and quercetin.

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