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A COMPREHENSIVE COMPARATIVE STUDY OF FIVE MEDICINAL *VITEX* SPECIES USING PHYSICOCHEMICAL, PHYTOCHEMICAL, HPTLC AND FTIR ANALYSES

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ABSTRACT :

Background: This study aims to perform a comparative analytical evaluation of five **Vitex** species—*Vitex agnus-castus*, *V. cannabifolia*, *V. negundo*, *V. ovata*, and *V. trifolia*—to identify their similarities and differences. While *V. negundo* is the accepted source of *Nirgundi*, a key Ayurvedic herb for inflammation management, other species are also used under this name. The findings will aid in assessing their therapeutic equivalence and support the rational use of *Vitex* species in Ayurvedic formulations. **Materials and Methods:** Leaves of five *Vitex* species were collected, authenticated, and subjected to physicochemical analysis (loss on drying, ash value, extractive value), and qualitative phytochemical screening of aqueous and methanolic extracts was done as per API standards. HPTLC was performed using the method given by the HPTLC association. FTIR analysis was carried out using UATR. **Results:** Physicochemical analysis showed variations in loss on drying, ash, and extractive values among *Vitex* species compared to *V. negundo*. Phytochemical screening confirmed the presence of alkaloids, flavonoids, and phenolics in all Vitex genus species. HPTLC provided distinct fingerprints with shared bands, while FTIR revealed characteristic hydroxyl, carbonyl, and aromatic groups, highlighting chemical similarities and differences. **Conclusion:** This study shows similarity in the physicochemical and phytochemical parameters. Also, HPTLC and FTIR fingerprinting show similar results, imparting the similarity in chemical profiles of all five species. Thus, these findings support sustainable use of alternative species, reducing reliance on *Vitex negundo* and promoting resource conservation in Ayurvedic formulations.

KEYWORDS: Analytical evaluation, Ayurvedic formulations, FTIR analysis, HPTLC profiling, *Vitex* species.

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1. INTRODUCTION

The genus *Vitex*, belonging to the family Lamiaceae, comprises 205 taxonomically recognized species, predominantly found in tropical and subtropical regions worldwide. Commonly referred to as chaste tree or monk's pepper, 51 species within this genus are renowned for their medicinal values.[1] In India, *Vitex* species are distributed across diverse ecological regions. *Vitex trifoliolata Linn*. is predominantly found in coastal regions and lowland areas[2],while*Vitex ovata* Thunb. is adapted to sandy terrains and is commonly seen in the Northeast and the Western Ghats[3]. *Vitex agnus-castus* Linn., though native to Africa and Europe, has established itself in localized regions of Karnataka, Kerala, Rajasthan, and Tamil Nadu. *Vitex cannabifolia* Siebold & Zucc., on the other hand, is restricted to the moist forests and riverbanks of Northeast India. Among these, *Vitex negundo* Linn. is widely distributed throughout the country.[4] In the traditional systems of Ayurveda and Unani medicine, the leaves of *Vitex negundo* are extensively utilized for managing rheumatism and inflammatory joint conditions.[5] Commercially, *V. negundo* is incorporated into a range of products such as Acne-n-Pimple Cream, Joint Care B Cream, Muscle and Joint Rub, Pilex tablets and cream, Rumalaya gel and tablets.[6]

The Ayurvedic Pharmacopeia of India (API) mentions *Vitex negundo* as the botanical source of *Nirgundi,* a classical Ayurvedic herb, which is described to have properties like *Shophahara* (anti-inflammatory), *Kushthahara* (useful in skin diseases), and *Shoolahara* (analgesic).[7] Despite this, other *Vitex* species are often utilized as *Nirgundi* depending on regional availability. This unintentional substitution of *Vitex negundo* with other species of *Vitex* highlights the necessity of generating evidence regarding their similarities and differences to ensure their rational therapeutic use. Such data also help reduce the burden on a single species and underscore the need for sustainable exploration and utilization of alternative species to balance ecological and pharmacological demands.

Ethnobotanical studies have also documented the therapeutic use of several *Vitex* species in India, such as *Vitex agnus-castus* (analgesic, anti-anxiety, fungicide, anti-helminthic, antiseptic, digestive and carminative[8]), *Vitex cannabifolia* (antiseptic and analgesic[9]), *Vitex negundo* (analgesic, anti-inflammatory, anti-rheumatic, vermifuge, ophthalmic, and stomachic[10]), *Vitex ovata* (expectorant, analgesic, anti-inflammatory and antimicrobial[11]), and *Vitex trifolia*(Anti-inflammatory, anti-rheumatic, insecticidal, anodyne and anti-helminthic[12]). These ethnomedicinal uses align with the pharmacological activities described in Ayurveda literature[13], suggesting a potential alternative to be used as *Nirgundi*.

Analytical studies are essential in herbal drug research for ensuring the quality, identity, and purity of plant materials. These analyses assess the physical and chemical properties of herbs. Physicochemical parameters, such as ash value and moisture content, confirm the authenticity of crude drugs, while phytochemical analysis identifies bioactive constituents. Advanced techniques like High-Performance Thin Layer

Chromatography (HPTLC) and Fourier Transform Infrared Spectroscopy (FTIR) provide additional authentication. HPTLC offers a chemical fingerprint by detecting major active compounds, while FTIR identifies functional groups. These methods collectively ensure the safety, efficacy, and standardization of herbal medicines.

Hence, this study aims to conduct an analytical, qualitative phytochemical comparison using HPTLC and FTIR analysis of five species of *Vitex*, to generate preliminary evidence about their similarities and differences. This data will contribute to a better understanding of the scope for interchangeable use of closely related species in Ayurvedic formulations.

Objectives

To evaluate and compare the physicochemical and phytochemical parameters of five plants of the Vitex genus and create a fingerprint of the same using HPTLC and FTIR techniques.

2. MATERIALS AND METHODS

Collection of plant material and authentication:

Leaves of five species of *Vitex—Vitex agnus-castus* (VAC)*, Vitex cannabifolia*(VC)*, Vitex negundo* (VN)*, Vitex ovata* (VO) and *Vitex trifolia* (VT) were collected from the herbal garden of All India Institute of Ayurveda, Sarita Vihar, New Delhi in the month of June 2023, washed and shade dried. The samples were authenticated from BGIR-BSI, Noida and voucher specimens preserved (BSI/BGIR/TECH./2024/177 and BSI/BGIR-2021/Id/05) at the institute. (Annexure 1)

Physicochemical and Phytochemical Analysis:

The shaded dried powder of the herb was used for physiochemical and phytochemical analysis. Physicochemical analysis including loss on drying, ash value, extractive value and qualitative phytochemical analysis, was done as per standard guidelines mentioned in API and the observations were then compared to reference values given in API[14],[15],[16].

HPTLC analysis: (done as per method given by HPTLC association)

HPTLC was performed on the stationary phase—Merck HPTLC Silica gel 60 F254, 20×10cm HPTLC plates with ethyl acetate: glacial acetic acid: water [16:2:1 (v/v)] as the mobile phase. Methanolic extracts of five samples of genus Vitex (1:10) were subjected to HPTLC plates by CAMAG-Linomat 5 band applicators. CAMAG TLC Scanner 4 was used to densitometrically, to quantify the bands using WIN CATS software. The scanner operating parameters were: (Mode: absorption; slit dimension: 6×0.45 mm; scanning rate: 20 mm/s and monochromator bandwidth: 20 nm at an optimized wavelength of 254, 366 nm and in the visible range). After derivatizing with derivatizing reagent—vanillin sulfuric acid (2.0 mL), plate was heated at 100°C for 3 min. and again scanned and observed at 540 nm[17].

Table-1- Details of sample application for HPTLC

Track	Vial ID	Description	Volume	Type
1	1	*V. agnus castus*	3.0 µL	Sample
2	1	*V. agnus castus*	5.0 µL	Sample
3	1	*V. agnus castus*	7.0 µL	Sample
4	2	*V. cannabifolia*	3.0 µL	Sample
5	2	*V. cannabifolia*	5.0 µL	Sample
6	2	*V. cannabifolia*	7.0 µL	Sample
7	3	*V. negundo*	3.0 µL	Sample
8	3	*V. negundo*	5.0 µL	Sample

9	3	*V. negundo*	7.0 µL	Sample
10	4	*V. ovata*	3.0 µL	Sample
11	4	*V. ovata*	5.0 µL	Sample
12	4	*V. ovata*	7.0 µL	Sample
13	5	*V. trifolia*	3.0 µL	Sample
14	5	*V. trifolia*	5.0 µL	Sample
15	5	*V. trifolia*	7.0 µL	Sample

FTIR analysis-

The sample was carefully positioned onto the diamond crystal surface of the Universal Attenuated Total Reflectance (UATR) accessory, ensuring a smooth and even contact for optimal results. Gentle pressure was applied using the UATR clamp, maintaining consistent contact without exerting excessive force to prevent damage to the ATR crystal. Next, the Fourier Transform Infrared (FTIR) spectrometer was set up with a spectral range typically spanning 4000-400 cm^$^{-1}$ and a resolution of about 4 cm^$^{-1}$. To ensure a robust signal-to-noise ratio, the appropriate number of scans was selected.

The spectrometer was then activated to collect the FTIR spectrum, and stable readings were obtained, with constant monitoring of the background spectrum to correct for any environmental variations. Once the data collection was complete, the recorded spectrum was analysed to identify characteristic peaks and bands. These features were compared against known reference libraries for qualitative and quantitative analysis, providing insights into the chemical composition of the sample.[18]

Fig. 1: Five species of genus *Vitex*A- *V. agnus castus*, B- *V. cannabifolia,*C- *V. negundo*, D- *V. ovata*, E- *V. trifolia*

3. RESULTS AND DISCUSSION

Physico-chemical analysis:

Table 2 - Comparative physicochemical analysis of five species of *Vitex* genus

Parameter	VAC	VC	VN	VO	VT	VN (API)
Loss on drying	0.05%	0.11%	0.07%	0.13%	0.07%	-
Alcohol soluble extractive	17.63%	16.34%	18.93%	17.92%	18.27%	Not less than 10%
Aqueous soluble extractive	26.44%	22.2%	26.96%	24.71%	26.44%	Not less than 20%
Ash value	7.50%	7.28%	6.90%	6.33%	7.58%	Not more than 8%

**Vitex agnus-castus*(VAC), *Vitex cannabifolia*(VC), *Vitex negundo* (VN), *Vitex ovata* (VO), *Vitex trifolia*(VT), API (Ayurvedic Pharmacopeia of India)

The physicochemical evaluation of the plant samples (VAC, VC, VN, VO, VT) revealed variations within acceptable limits when compared to the Ayurvedic Pharmacopoeia of India (API) standards for VN. All samples had low moisture content, indicated by loss on drying values ranging from 0.05% to 0.13%, well within ideal limits. Alcohol-soluble extractive values ranged from 16.34% to 18.93%, exceeding the minimum API requirement of not less than 10%, showing good solubility in alcohol across all samples. Aqueous-soluble extractive values were also high (22.2% to 26.96%), all above the minimum standard of 20%, indicating efficient extraction in water. The total ash content ranged from 6.33% to 7.58%, remaining below the maximum limit of 8% set by the API, suggesting low inorganic residue (Table no 2). Overall, all plant samples, including VN, met the quality criteria, indicating their suitability for pharmacological or therapeutic use.

Phyto-chemical analysis:

Table-3- Comparative Phyto-chemical analysis of Methanolic and Aqueous extract of leaves of five species of genus *Vitex*

Phytochemicals	VAC		VC		VN		VO		VT	
	MeOH	Aq	MeOH	Aq	MeOH	Aq	MeOH	Aq	MeOH	Aq
Alkaloids	+	+	+	+	+	+	+	+	+	+
Tannins	-	-	-	-	-	+	-	-	-	-
Flavonoids	+	+	+	+	+	+	+	+	+	+
Proteins	-	-	-	-	-	-	-	-	-	-
Saponins	-	+	-	+	-	+	-	+	-	+

**Vitex agnus-castus*(VAC), *Vitex cannabifolia*(VC), *Vitex negundo* (VN), *Vitex ovata* (VO), *Vitex trifolia*(VT), Methanolic extract (MeOH), Aqueous extract (Aq)

*(+ indicates presence of phytochemical and – indicates their absence)

The phytochemical analysis of VAC, VC, VN, VO, and VT extracts using methanol and aqueous solvents revealed notable trends. Alkaloids and flavonoids were consistently present in all extracts, regardless of the plant or solvent, indicating their ubiquitous distribution and solvent-independent solubility. Proteins were absent across all samples and solvents, suggesting they are either not present or below detectable levels. Saponins were detected only in the aqueous extracts of all five samples, implying they are water-soluble and not extractable with methanol. Tannins were uniquely found in the aqueous extract of VN, suggesting a plant-specific and solvent-dependent occurrence. Overall, aqueous extraction was more effective for isolating saponins and tannins, while both solvents were equally effective for alkaloids and flavonoids (Table no 3).

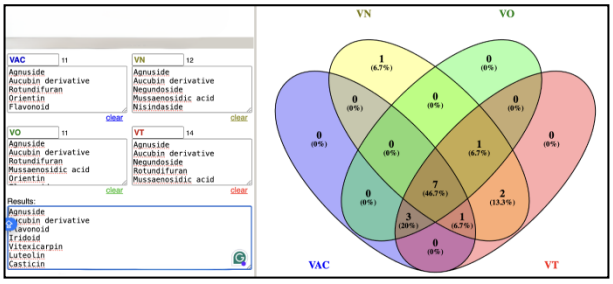

Fig. 2: Venn diagram comparing the phytochemical profiles of four different *Vitex* species:*Vitex agnus-castus*(VAC), *Vitex negundo* (VN), *Vitex ovata* (VO), *Vitex trifolia*(VT) The central overlapping region (shared by all species) shows 7 compounds common to all four *Vitex* species.[19]

The Venn diagram illustrates the distribution of phytochemical constituents among four plant samples: VAC, VN, VO, and VT. A total of seven compounds (46.7%) are common to all four, indicating a significant overlap in their phytochemical profiles. VAC contains three unique compounds (20%), while VT possesses two exclusive compounds (13.3%), highlighting their distinct chemical compositions. Additionally, one compound (6.7%) is shared solely between VN and VAC, and another (6.7%) is common only to VO and VT. VN also has one unique compound (6.7%) not found in the other samples. No compounds are exclusively shared between VAC and VO, VN and VO, VN and VT, or VAC and VT. Overall, while the samples share a core group of common compounds, each exhibits some unique or selectively shared constituents that contribute to their individual phytochemical identities.

HPTLC analysis:

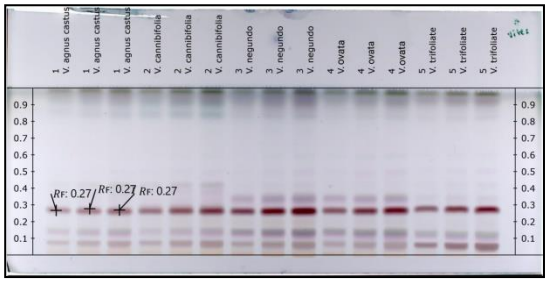

Fig 3: Spectroscopic scan of HPTLC plate developed after derivatisation @540nm

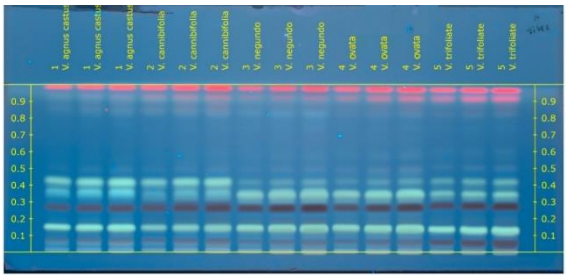

Fig 4: Spectroscopic scan of HPTLC plate under UV light @366nm

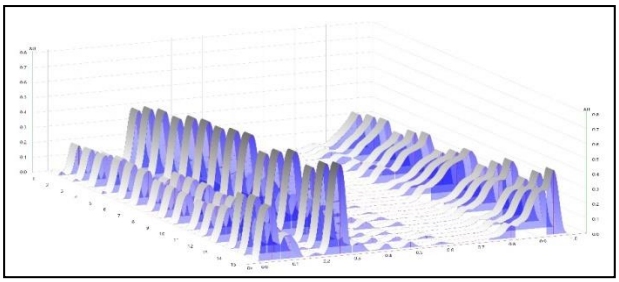

Fig. 5: Densitogram @366nm showing three distinct peaks in all five samples

Table-4: HPTLC analysis of Methanolic extract of five samples of genus *vitex* @540nm

S. No.	Sample	No. of peaks observed
1	*V. agnus castus*	8
2	*V. cannabifolia*	8
3	*V. negundo*	10
4	*V. ovata*	8
5	*V. trifolia*	10

The results from chromatographic analysis show presence of 8 similar peaks in all the five sample, which indicate the presence of similar

phytochemicals and in turn comparable pharmacological activities. Also, *V. negundo* and *V. trifolia* shows presence of two distinct peaks, this can be used as the differentiating parameter from other species.

FTIR spectroscopy using UATR:

Table-5 FTIR spectroscopy of Leaf powder of five samples of genus *Vitex*

Name of species	Compound class observed
V. Agnus-castus	Amine salt (2917.11) Fluoro compound (1606.59, 1016.32)
V. cannabifolia	Amine salt (2917.30), Fluoro compound (1604.63, 1017.59)
V. negundo	Amine salt (2916.77, 2848.94), Fluoro compound (1606.90, 1012.53)
V. ovata	Amine salt (2916.84), Fluoro compound (1013.45)
V. trifolia	Amine salt (2916.73, 2848.91), Fluoro compound (1729.94, 1015.24)

4. DISCUSSION

Comparative analytical studies play a crucial role in ensuring accurate taxonomic identification and maintaining the authenticity, purity, and therapeutic efficacy of plant species within a genus.[20] By examining physicochemical and phytochemical characteristics, these studies contribute to the development of robust quality standards for herbal medicines. Such precision is essential in herbal medicine production, providing assurance to consumers, healthcare practitioners, and regulatory authorities.[21]

Physicochemical analysis focuses on the quantitative assessment of physical and chemical properties of plant materials. This includes determining parameters such as moisture content, ash values, extractive values, and pH levels. By comparing these parameters across different plant species within a genus, variations can be recognized that may impact the overall quality and efficacy of herbal medicines. The Ayurvedic Pharmacopoeia of India provides data exclusively on the physicochemical parameters of *Vitex negundo* leaves [22], while the ICMR quality standard database includes data for two species, *Vitex negundo* [23] and *Vitex agnus-castus* [24] leaves. All species exhibit acceptable loss on drying, with *Vitex ovata* showing the highest value (0.13%) and *Vitex agnus-castus* the lowest (0.05%), indicating better stability for the latter. The other physicochemical parameters, namely water-soluble extractive value, alcohol-soluble extractive value, and ash value, fall within the permissible limits specified in the available standard data. Therefore, all the analyzed samples are of good quality and suitable for use. Qualitative Phytochemical analysis involves the identification of bioactive compounds present in plant extracts. Methanolic and aqueous extracts of all five species of *Vitex* have shown the presence of alkaloids and flavonoids. Saponins were present only in the aqueous extract, while other phytochemicals were not found. *Vitex negundo* leaves are rich in flavonoids, a class of polyphenolic compounds recognized for their strong antioxidant and anti-inflammatory effects. [25] Previous studies have reported the presence of flavonoids along with agnuside, aucubin derivatives, and iridoids in all five species. However, vitexicarpin, luteolin, and

casticin are common to all other species except VC [26]. The presence of these similar phytochemicals suggests the possibility of comparable phytochemical activities across the species.

Comparative High-Performance Thin-Layer Chromatography (HPTLC) allows for the simultaneous analysis of multiple plant samples, facilitating the identification and quantification of various chemical constituents. By comparing the chromatographic profiles of plants belonging to the same genus, it becomes easy to recognize subtle differences in the composition of secondary metabolites. Additionally, HPTLC enables the assessment of the consistency and quality of herbal products derived from different plant species within the genus, contributing to the standardization and quality control of herbal medicines. In the present study, HPTLC analysis was performed according to the method provided by the HPTLC Association, designed for the simultaneous estimation of Negundoside (Rf 0.32) and Agnuside (Rf 0.25). Both these phytochemicals are well-documented for their hepatoprotective, anti-inflammatory, and anti-arthritic activities [27]. From the present study, it can be inferred that all five *Vitex* species may contain agnuside in varying proportions, as the HPTLC results showed an Rf value of 0.253 ± 0.02 across all five samples. Furthermore, chromatographic comparison of all the samples revealed the presence of similar peaks, indicating their comparable pharmacological activities. *Vitex negundo* and *Vitex trifolia* exhibited two additional peaks, which may serve as distinguishing features from the other species.

FTIR spectroscopy provides molecular insights into plant extracts by detecting functional group vibrations, generating unique spectral fingerprints. It identifies chemical bonds, elucidates structures, and characterizes phytochemicals, aiding in species differentiation and classification. This study reveals similar functional groups and compound classes across five *Vitex* species, suggesting comparable chemical compositions, which in turn suggests similar pharmacological profiles.

5. CONCLUSION

From this study, it can be concluded that the five species of the genus *Vitex* mentioned above exhibit significant physicochemical and phytochemical similarities. This may serve as a basis for their potential similar pharmacological actions, which can be further validated through clinical studies. Therefore, this study can be considered a pioneering effort in supporting the acceptance of various *Vitex* species as *Nirgundi*.

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