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Original Article

HPTLC Fingerprinting and Antioxidant Potential Assessment of Methanolic Extract of *Vitex negundo* Leaves

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ABSTRACT

Background: *Vitex negundo* Linn. is a well- honored medicinal factory known for its different pharmacological properties, largely attributed to its rich phytochemical composition. Glycosidic composites, in particular, contribute significantly to its remedial eventuality, including antioxidant exertion. This study aimed to estimate the phytochemical ingredients and antioxidant exertion of the methanolic excerpt of *Vitex negundo* Linn. leaves, with emphasis on glycosidic enrichment using an optimized birth system. **Materials and Methods:** Methanolic birth of *Vitex negundo* leaves was performed, followed by primary phytochemical webbing. Quantitative estimation of total phenolic content (mg GAE/ g), total flavonoid content (mg shaft/ g), and total tannin content (mg TAE/ g) was carried out using standard spectrophotometric styles. Phytochemical profiling was conducted using Thin Subcaste Chromatography (TLC) and High-Performance Thin- Subcaste Chromatography (HPTLC). An advanced birth protocol involving mild acid hydrolysis, solvent phase partitioning, and chromatographic refinement was employed to widely enrich glycosidic fragments. **Results:** The excerpt showed the presence of flavonoids, phenolics, tannins, glycosides, alkaloids, and saponins, with considerable situations of antioxidant-related composites. TLC and HPTLC biographies verified distinct phytochemical patterns and effective glycoside enrichment. **Conclusion:** *Vitex negundo* leaves retain significant antioxidant eventuality, and the optimized system efficiently amended glycosidic composites of pharmacological significance.

Keywords: Antioxidant, High Performance Liquid Chromatography, Phenolic, Thin Layer chromatography, *Vitex negundo*.

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INTRODUCTION

An extraordinary plant is *Vitex negundo* L. (*Verbenaceae*), frequently referred to as the five/three-leaved chaste tree or nirgundi in India. The Vitex genus consists of approximately 270 recognized species, varying from shrubs to trees flourishing in tropical, subtropical, and temperate climates. *Vitex* is utilized as traditional medicine across Bangladesh, India, China, Indo-China, Indonesia, Nepal, Pakistan, the Philippines, and Sri Lanka.[1]

The significance of nirgundi is documented in De Materia Medica and crucial Ayurvedic scriptures like the Charaka Samhita. *Vitex negundo* has been acknowledged in native healing practices for addressing conditions such as depression, sexually transmitted diseases, malaria, asthma, allergies, wounds, skin disorders, inflammation, pain relief, ulcers, and snakebites. A range of pure compounds, including flavonoids, iridoids, sesquiterpene, diterpenes, lignans and plant steroids have been isolated and identified.[2]

Botanical Description

Vitex negundo is a fragrant, woody deciduous shrub with the capability to develop into a petite tree. Often referred to as the five-leaf chaste tree or monk's pepper, its most remarkable characteristic is a cluster of five-pointed leaves that mirror the form of a palm. This upright tree can attain heights of 2-5 m and showcases slender, quadrilateral branchlets. The leaves are organized in a palmate configuration with five lance-shaped leaflets, sharp, smooth on the upper surface, ranging from 4-10 cm in length, and featuring a hairy underside, tapering to a point at both ends. The terminal leaflet possesses a long petiole, while the lateral leaflets have shorter petioles (Figures 1-11). The flowers, which are bluish-purple, are located in axillary or terminal panicles that can extend up to 30 cm long. The fruit is a succulent globule that darkens when ripe and contains four rounded seeds approximately 4 mm in diameter (Table 1).[3]

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Taxonomic Classification[4]

Table 1: Taxonomic Classification.

Kingdom	Plantae
Subkingdom	Tracheobionta
Super Division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Sub Class	Asteridae
Order	Lamiales
Family	Verbenaceae
Genus	Vitex
Species	negundo

MATERIALS AND METHODS

Plant Material collection

The *Vitex negundo* Linn. leaves were collected from Botanical Garden of Government College of Pharmacy Chhatrapati Sambhajinagar. And Herbarium were Authenticated from the herbarium of the Department of Botany, Dr. Babasaheb Ambedkar Marathwada university, Chhatrapati Sambhajinagar, Maharashtra. Species are *Vitex negundo* Linn. Belong to Family Verbenaceae. standard dried herbarium sheets deposited these valuable voucher specimens at the Department of Botany Dr. Babasaheb Ambedkar Marathwada university, Chhatrapati Sambhajinagar, Maharashtra.

Collected *Vitex negundo* Linn. leaves were washed to remove external contaminants and shade dried (30-60°C) for two to three weeks at room temperature then they powdered by using an electronic blender then it was stored in tightly closed glass containers and kept in the dark at room temperature.

Extraction

Take 100 g of dried *Vitex negundo* Linn. leaves powder was treated with dilute HCl to hydrolyse for 48 hr at room temperature.[5] After two days this hydrolyse powder is extracted with methanol 10:1(mg/L) by Soxhlet extraction method for 17 hr at 45°C. This is the specific process for the extraction of glycoside by modifying the convention Processes by Soxhlet extraction Method.

Isolation

Methanol extract was treated with lead acetate the Flavonoids and tannins were precipitated with lead acetate.[6] And through the filtration processes filtrate moves further. and filtrate again treated with activated charcoal to remove impurity and unwanted pigment to get clear sample solution.[7] After that solvent is evaporated with rotary evaporator and pale-yellow sticky solid sample. Glycoside present in the sample is confirmed by Qualitative and quantitative analysis.

Preliminary Phytochemical test of Methanolic extract of *Vitex negundo* Linn.

Alkaloids

Mayer's test

Two drops of Mayer's reagent are added along the sides of test tube in too few amounts of plant extract. The presence of alkaloids is indicated by a white creamy precipitate.[8]

Wagner's test

A few drops of Wagner's reagent are added to a few amount of plant extract and a reddish brown precipitate depicts the presence of alkaloids.[9]

Dragendroff's test

The addition of few drops of Dragendroff's reagent into the extract gives red precipitate if alkaloids are present in the sample.

Hager's test

A small amount of Hager's reagent is added to the extract. The formation of yellow precipitate indicates the presence of alkaloids.[10]

Glycosides

Keller-Killani Test

2 mL of plant extract were treated with 2 mL glacial acetic acid containing a drop of $FeCl_3$. A brown coloured ring or brown-violet under a brown greenish layer indicated the presence of cardiac glycosides.[11]

Borntrager's test

To 2 mL of filtered hydrolysate, 3 mL of chloroform is added and shaken, chloroform layer is separated and 10% ammonia solution is added to it. Pink colour indicates presence of glycosides.[12]

Legal's test

50 mg of extract is dissolved in pyridine; sodium nitroprusside solution is added and made alkaline using 10% NaOH. Presence of glycoside is indicated by pink colour.[12]

Test for flavonoids

1. **Ferric chloride test:** 2 mL test solution was taken and added few drops of ferric chloride solution indicate blackish red colour presence of flavonoid.[13]
2. **Lead acetate solution test:** 2 mL test solution treated with few drops of 10% lead acetate solution formation of yellow precipitate indicate the presence of flavonoids.[13]

Test for Saponins

Foam test

1 mL solution of extract was diluted with distilled water to 20 mL and shaken in a graduated cylinder for 15 min. Development of stable foam suggests the presence of saponins. b) 1 mL extract was treated with 1% lead acetate solution. Formation of white precipitates indicates the presence of saponins.[14]

Test for phenols and tannins

Ferric chloride test

2 mL of 5% solution of $FeCl_3$ were added to 1 mL crude extracts. A black or blue-green colour indicated the presence of tannins and phenols.[11]

Test for terpenoids

5 mL of each extract was mixed in 2 mL of chloroform. 3 mL of concentrated H_2SO_4 was then added to form a layer. A reddishbrown precipitate colouration at the interface formed indicated the presence of terpenoids.[15]

Solkowski test

0.5 mL test solution was taken and added 2 mL chloroform and 1 mL conc. sulphuric acid then colour turned into red brown at the interface indicates presence of terpenoids.[13]

Test for Steroids

Salkowski's test

A red color produced in the lower chloroform layer when 2 mL of organic extract was dissolved in 2 mL of chloroform and 2 mL concentrated sulphuric acid was added in it, indicates the presence of steroids.

Liebermann Burchard test

Development of a greenish color when 2 mL of the organic extract was dissolved in 2 mL of chloroform and treated with concentrated sulphuric acid and acetic acid indicates the presence of steroids.[16]

QUANTITATIVE ANALYSIS

Determination of

Total Phenolic Content

To quantify the total phenolic content, 1.0 mL of either the sample extract or a standard gallic acid solution was transferred to a test tube. Then, 5.0 mL of pre-diluted Folin-Ciocalteu reagent was added to the tube. After allowing the mixture to incubate at room temperature for 5 min, 4.0 mL of a 0.7 M sodium carbonate solution was introduced. The contents of the tube were vortexed thoroughly and left to react in the dark at room temperature for 60 min. Once the reaction was complete, the absorbance of the resultant blue solution was measured at 765 nm, with a methanol blank used as the absorbance reference.[17]

To facilitate quantification, a calibration curve was generated using gallic acid standards within a typical concentration range of 20-100 µg/mL. The corresponding absorbance values were plotted against these concentrations, enabling the creation of a linear regression equation. Using this equation, the total phenolic content of the plant extract was calculated and expressed as milligrams of gallic acid equivalents per gram of dried extract (mg GAE/g).

$$T = \frac{(C \times V)}{M}$$

Were, T=Total content of phenolic compounds, milligram per gram dry weight of plant extract, in GAE; C=the concentration of Gallic Acid established from the calibration curve, milligram per millilitre; V=the volume of extract, millilitre; M=the weight of methanolic plant extract, gram.

Determination of Total Flavonoid Content (TFC)

The overall flavonoid concentration of the methanolic extract from the leaves of *Vitex negundo* Linn. was assessed using the aluminium chloride colorimetric technique. To create a 10% aluminium chloride solution, 10 g of $AlCl_3$ was dissolved in 100 mL of methanol, and a 1 M potassium acetate solution was made by dissolving 9.8 g of CH_3COOK in 100 mL of distilled water. Rutin served as the standard reference compound, with standard solutions prepared in the range of 20-100 µg/mL by diluting a 1000 µg/mL stock solution in methanol. For the assay, 1.0 mL of either the standard or sample solution was combined with 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate, and 2.8 mL of methanol, achieving a total volume of 4.0 mL. The mixture was incubated at room temperature for 30 min, and the absorbance was recorded at 415 nm against a reagent blank.[18,19] The TFC was calculated from a rutin calibration curve and expressed as milligrams of rutin equivalent per gram of dry extract (mg RE/g), using the formula:

$$TFC = \frac{(C \times V)}{M}$$

Where **C** is the concentration of flavonoids obtained from the standard curve (mg/mL), **V** is the volume of extract used (mL), and **M** is the mass of the extract (g).

Determination of Total Tannin Content

The total tannin level of the methanolic extract obtained from the leaves of *Vitex negundo* Linn. was assessed using the ferric chloride-potassium ferrocyanide colorimetric approach. A 0.1 M solution of ferric chloride was prepared in 0.1 N hydrochloric acid, alongside a 0.008 M solution of potassium ferrocyanide dissolved in distilled water. Tannic acid (1 mg/mL in methanol) served as the reference standard, and serial dilutions were carried out to achieve concentrations ranging from 10 to 50 µg/mL. In

the assessment, 5.0 mL of either the standard or sample solution was combined with 3.0 mL of ferric chloride solution and 3.0 mL of potassium ferrocyanide solution. The resultant mixture was allowed to incubate for 10 min at ambient temperature, after which the absorbance was recorded at 720 nm using a UV-visible spectrophotometer (Schimadzu UV-vis 1700). A calibration curve was generated by plotting absorbance against the concentrations of tannic acid.[20]

Thin Layer Chromatography

Preparative TLC is the technique for segregating the active components based on their R_f values. This method is also suitable for extracting specific constituents. Utilizing this technique, the purity can be achieved to the desired level. It additionally serves as a method to separate milligrams/grams of the sample.[21]

High Performance Thin Layer Chromatography (HPTLC)

High-Performance Thin Layer Chromatography (HPTLC) represents an advanced instrumental technique leveraging the complete potential of thin layer chromatography. The benefits of automation, scanning, thorough optimization, selective detection principles, minimal sample preparation, and hyphenation, among others, empower it as a formidable analytical tool for obtaining chromatographic data from intricate mixtures of inorganic, organic, and biomolecular substances. HPTLC serves as an invaluable asset for trustworthy identification.[22]

In vitro Antioxidant Activity

In vitro antioxidant activity refers to the ability of a compound or extract to neutralize free radicals or prevent oxidative damage outside of a living organism, typically in a controlled laboratory environment. This activity is measured using various chemical assays to evaluate a substance's potential to act as an antioxidant.[23]

RESULTS

Table 2: Result of Preliminary Phytochemical Screening.

Sl. No.	Phytochemical	Test	Result
1.	Alkaloids	Wagner's test	+
		Hager's Test	+
		Mayer's test	-
		Dragendroff's Test	-
2.	Tannins	Ferric chloride test	+
		Lead acetate test	+
3.	Flavonoids	Ferric chloride test	+
		Shinoda Test	+
4.	Glycoside	Borntrager's test	+
		Legal test	+
		Keller-Kiliani Test	+
5.	Terpenoids	Vanillin test	+
		Salkowski Test	+
6.	Steroids	Liebermann-Burchard Test	+
7.	Saponins	Foam Test	+
8.	Protein	Biuret Test	-
9.	Carbohydrate	Molisch's test	+

Result of Quantitative Estimation

Gallic acid (10-100 ppm), rutin (200-1000 ppm), and tannic acid (10-60 ppm) calibration curves showed strong linearity with consistent absorbance values collected in triplicate. The methanolic extract's total phenolic, flavonoid, and tannin content were quantitatively estimated using these standard curves. Determined the quantitative estimation of total phenolic content, total flavonoid content and total tannin content of Methanolic extract of *Vitex negundo leaves* shown in Tables 3-6.

Thin-layer Chromatography study of Extract

Thin-layer chromatography study reveals that all extracts contain more than one constituent. the result is given below in tabular forms.

High-Performance Thin Layer Chromatography (HPTLC) Profiling

The High-Performance Thin Layer Chromatography (HPTLC) (CAMAG HPTLC) analysis of the methanolic extract of the plant unveiled several unique bands when exposed to UV light (254 nm and 366 nm), and following derivatization with anisaldehyde-sulfuric acid reagent, observed at 540 nm. The chromatogram, created using the mobile phase combination of Ethyl acetate: Glacial acetic acid: Water (80:10:05), displayed distinct spots, signifying the existence of various phytochemical components. Notable R_f values identified in the plant extract were around 0.11, 0.20, 0.31, 0.39, 0.52, and 0.80. The band at R_f ~0.31-0.33 appeared to be significant and pronounced in both UV and derivatized states, indicating it as a primary component in the extract.

Interpretation of Each Chromatogram

Each graph represents a 3D densitometric scan of HPTLC tracks, showing Absorbance Units (AU) on the Y-axis, R_f values on the X-axis, and sample tracks (lanes) on the Z-axis.

3D Chromatogram at 254 nm before Derivatization

Major Peaks at R_f ~0.33 and ~0.34 The R_f 0.33 peak is the most prominent and sharp in most tracks, indicating a major compound present in sample tracks.

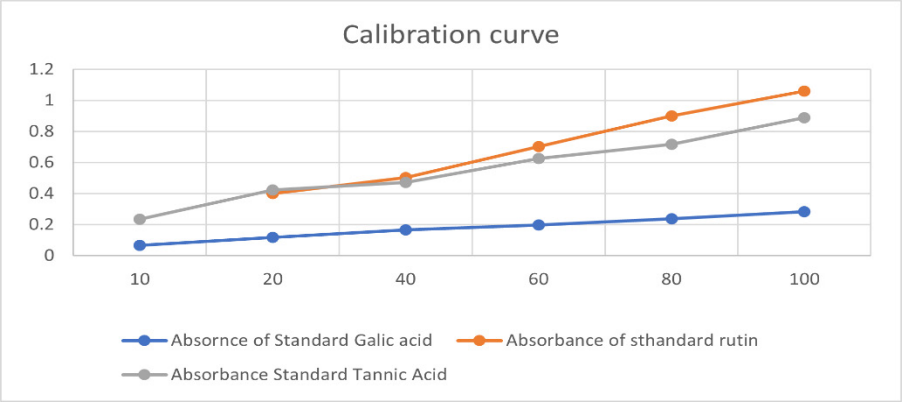


Figure 1: Calibration curve of Gallic acid, Rutin, Tannic acid.

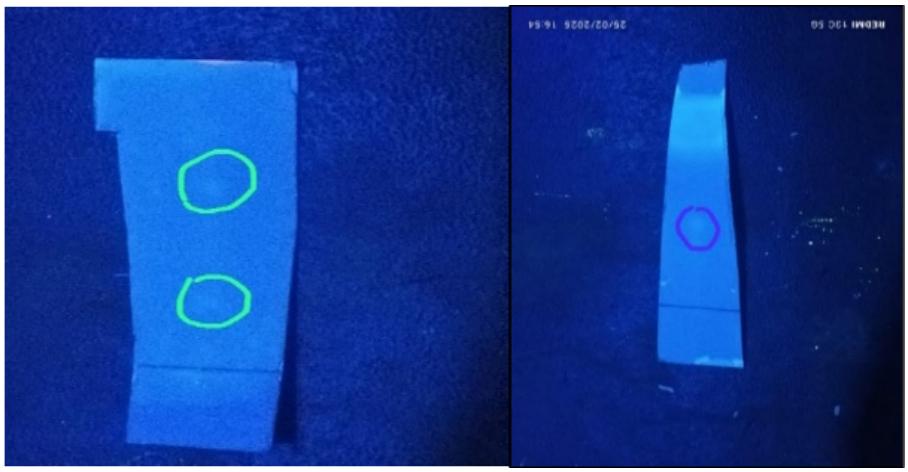


Figure 2: TLC under Uv Cabinet (R/340/OC).

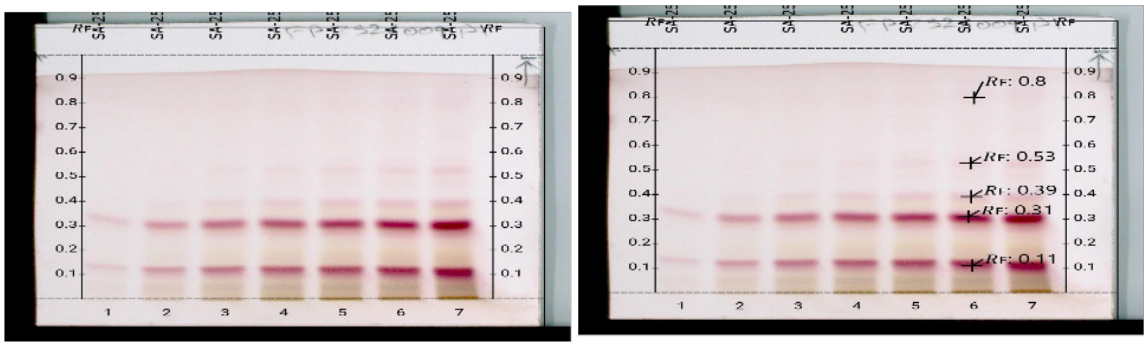
Table 3: Total phenolic content and total flavonoid content, and total tannin content of the methanolic extract of *Vitex negundo* Linn. leaves.

Sample	Total Phenolic content GAE/g dry weight	Total Flavonoid Content Rutin Equivalent (RE)/g extract	Total Tannin Content
Vitex negundo Linn. leaves extract	2.818 mg GAE/g extract	9.078 mg RE/g extract	3.64 µg/mL Tannic Acid Equivalent
			% Total Tannin Content: 36.4%

Table 4: TLC Result of extract.

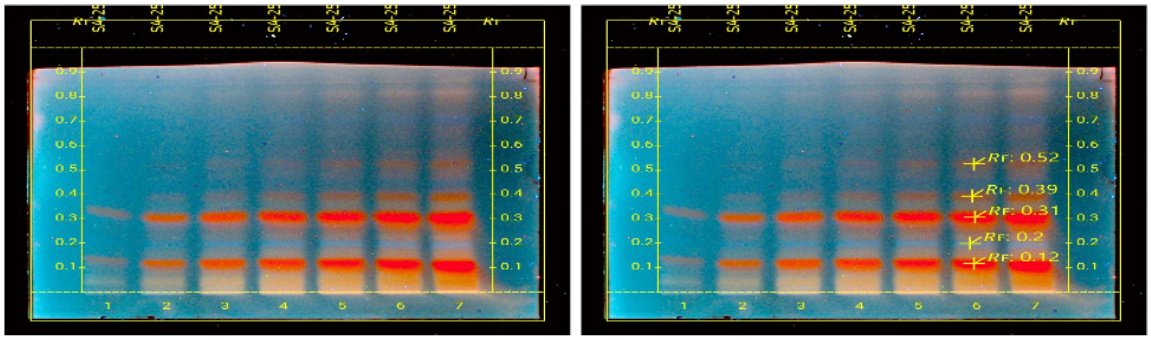
Sl. No.	Name	Solvent System	Visualizing agent	Number of Spot	R_f value
1.	Methanol Extract	N-Butanol: Glacial acetic acid: Water (4:3:5)	U.V cabinet chamber	2	Lower Spot: R_f=0.36
					Upper Spot: R_f=0.60
2.	Methanol Extract	Ethyl acetate:Formic acid:Glacial acetic acid: Water (10:1.1:1.1:2.6)	U.V cabinet chamber	1	R_f=0.53

Plate Image under R white after derivatization with ASR, with and without Rf



Track Details: Track 1-7: *Vitex negundo* herbal extract in increasing volume : 0.5 µl,2 µl,4 µl,6 µl,8 µl,10 µl and 15 µl.

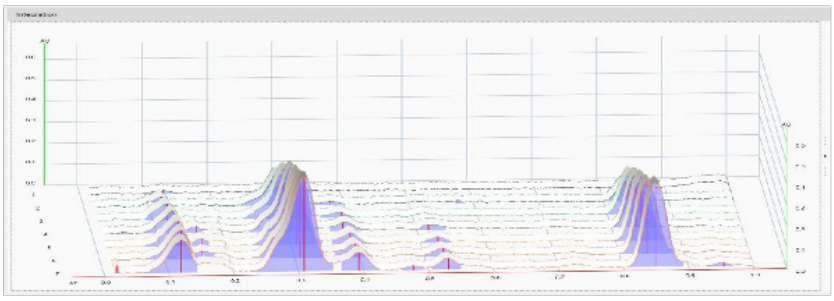
Plate Image under R white after derivatization with ASR, with and without Rf



Track Details: Track 1-7: *Vitex negundo* herbal extract in increasing volume : 0.5 µl,2 µl,4 µl,6 µl,8 µl,10 µl and 15 µl

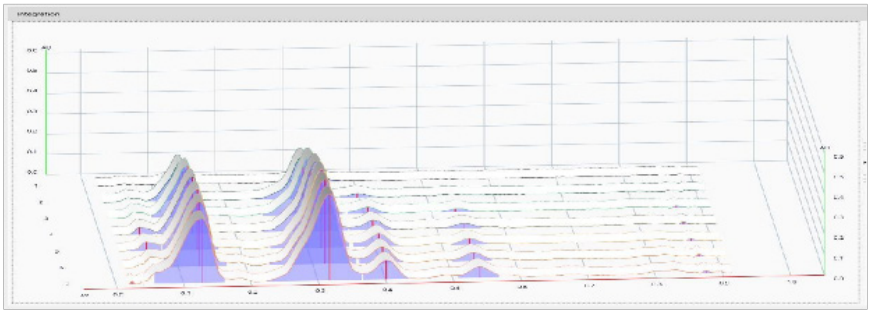
Figure 3: HPTLC (CAMAG HPTLC) Fingerprinting of Methanolic sample (*Vitex negundo* Linn.) after reagent spray.

3D Chromatogram at 254 nm before derivatization



Track Details: Track 1-7. *Vitex negundo* herbal extract in increasing volume . 0.5 µl,2 µl,4 µl,6 µl,8 µl,10 µl and 15 µl.

3D Chromatogram at 580 nm after derivatization with ASR



Track Details: Track 1-7: *Vitex negundo* herbal extract in increasing volume : 0.5 µl,2 µl,4 µl,6 µl,8 µl,10 µl and 15 µl.

Figure 4: 3D Chromatogram at 254 nm and 580 nm before and after derivatization.

Table 5: HPTLC Profiling of the methanolic extract of *Vitex negundo* Linn. leaves.

Sl. No.	Mobile Phase	Compound	R_f Value	Remarks
1.	Ethyl acetate: Glacial acetic acid: Water (80:10:05)	Swertiamarine	0.33	Anisaldehyde-sulfuric acid
2.	Ethyl acetate: Glacial acetic acid: Water (80:10:05)	Negundoside	0.31	Visible at 254 nm

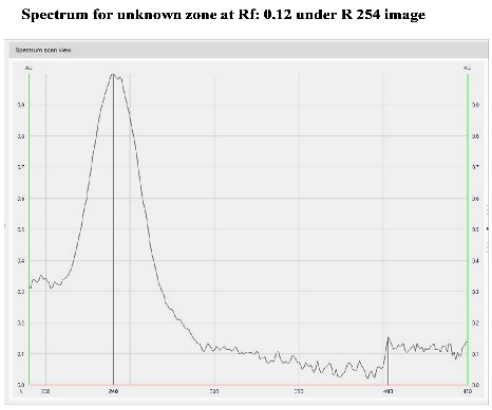


Figure 5: Spectra for unknown zone at R_f 0.12 under R 254.

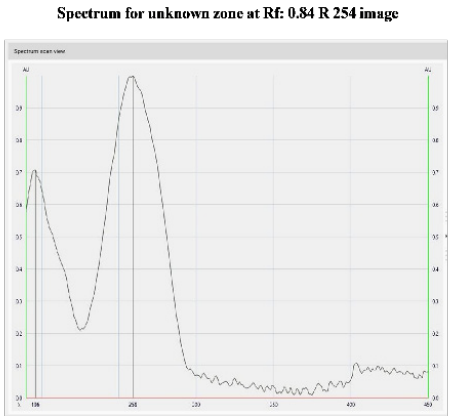


Figure 7: Spectra for unknown zone at R_f 0.84 under R 254.

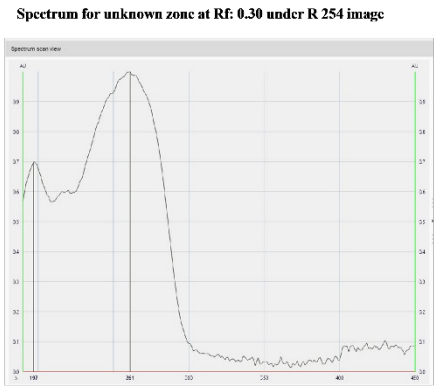


Figure 6: Spectra for unknown zone at R_f 0.30 under R 254.

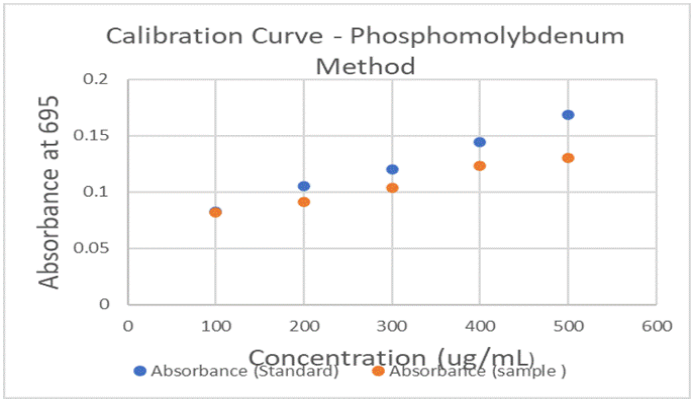


Figure 8: Absorbance at 695 nm of *Vitex negundo* Linn. leaves extract.

Two major peaks at R_f ~0.18 and ~0.33

The values R_f 0.33 and R_f 0.31 are uniform across both chromatograms, reinforcing the hypothesis that these signals represent the target compound (most likely Swertiamarin and Negundoside). HPTLC densitometric assessment unveiled a significant peak at 0.33 in the methanolic extract of the sample, thereby validating the presence of swertiamarin. Additional peaks identified at R_f 0.18 and 0.78 may align with other iridoid glycosides or phytochemicals. The consistency of peaks throughout various tracks signifies the reproducibility and trustworthiness of the method.

Detection of Phytoconstituents by the following spectra

Multiple peaks were detected at distinct R_f values (e.g., 0.11, 0.20, 0.39, 0.52, and 0.80) under UV detection at 254 nm and 366 nm. phytoconstituents present in the extract, warranting further investigation for characterization and structural elucidation.

In vitro Antioxidant Activity

Total Antioxidant Capacity by Phosphomolybdenum Assay

The Total Antioxidant Capacity (TAC) of the extract was evaluated using the phosphomolybdenum technique, with

ascorbic acid serving as the reference benchmark. The assay quantifies the conversion of Mo (VI) to Mo (V) by the antioxidant substances found in the sample *Vitex negundo* . The findings were conveyed in terms of Ascorbic Acid Equivalent (AAE) per mL of the extract. The phosphomolybdenum assay was used to assess the methanolic extract of *Vitex negundo* leaves' overall antioxidant activity. The sample's absorbance showed a concentration-dependent antioxidant effect, rising from 0.0819 ± 0.0021 at 100 µg/mL to 0.130±0.00252 at 500 µg/mL. The greatest activity was noted at 500 µg/mL, and the corresponding total antioxidant capacity values rose from 10.48 to 34.66 µg AAE/mL. At a concentration of 500 µg/mL, the sample achieved a peak TAC value of 34.66 µg AAE/mL, signifying notable antioxidant capabilities. This gradual rise in TAC values with increasing concentrations indicates the existence of bioactive phytochemicals that can convert molybdenum (VI) to molybdenum (V), a defining characteristic of antioxidant effectiveness.

Reducing Power Assay (Potassium Ferricyanide Method)

The (Schimadzu UV-vis 1700) extract's absorbance gradually increased from 0.275±0.0036 at 100 µg/mL to 0.391±0.0036 at 500 µg/mL in the reducing power assay, indicating a dose-dependent rise in reducing ability, but less than that of normal ascorbic acid. The methanolic extract of *V. negundo* Linn. Demonstrated a gradual increase in reducing capability based on dosage, as illustrated in. At a concentration of 100 µg/mL, the absorbance measured 0.275±0.0036, progressively rising to 0.391±0.0036 when reaching 500 µg/mL. In comparison to the standard (ascorbic acid), the extract showed a moderate level of antioxidant strength.

Hydrogen Peroxide (H_2O_2) Scavenging Assay

The antioxidant capacity of the tested methanolic extract from the leaves of *Vitex negundo* Linn. Was assessed utilizing the Hydrogen Peroxide (H_2O_2) scavenging assay. This technique relies on the capability of the compound under investigation to neutralize H_2O_2, a reactive oxygen species that can inflict cellular harm. Different concentrations of the extract (25-150 µg/mL) were analysed spectrophotometrically (Schimadzu UV-vis 1700) at 230 nm to evaluate their H_2O_2 scavenging effectiveness. With an IC_{50} value of 17.86 µg/mL, the hydrogen peroxide scavenging experiment showed a dose-dependent rise in percentage scavenging activity, reaching 71.65% at 150 µg/mL. The assay's dependability and the extract's notable capacity to scavenge free radicals are demonstrated by the strong linear correlation (R^2=0.9831). A linear regression plot of % scavenging versus concentration gave the following equation:

Table 6: Ascorbic Acid Equivalent (µg/mL) and TAC of the Methanolic extract of *Vitex negundo* Linn. leaves.

Sl. No.	Concentration (ug/mL)	Ascorbic Acid Equivalent (µg/mL)	TAC
1.	100 ug/mL	104.8	10.48
2.	200 ug/mL	152.5	15.25
3.	300 ug/mL	215	21.5
4.	400 ug/mL	311.6	31.16
5.	500 ug/mL	346.6	34.66

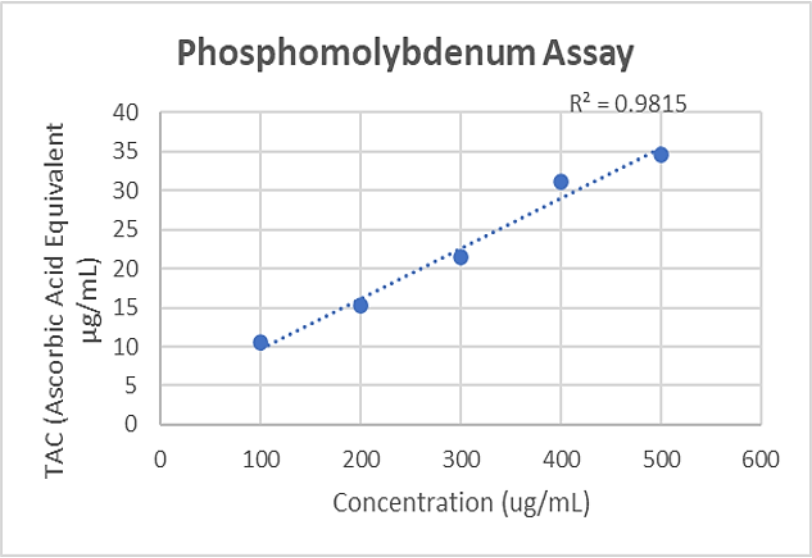


Figure 9: Total Antioxidant Capacity (TAC) of *Vitex negundo* Linn. Sample by phosphomolybdenum method.

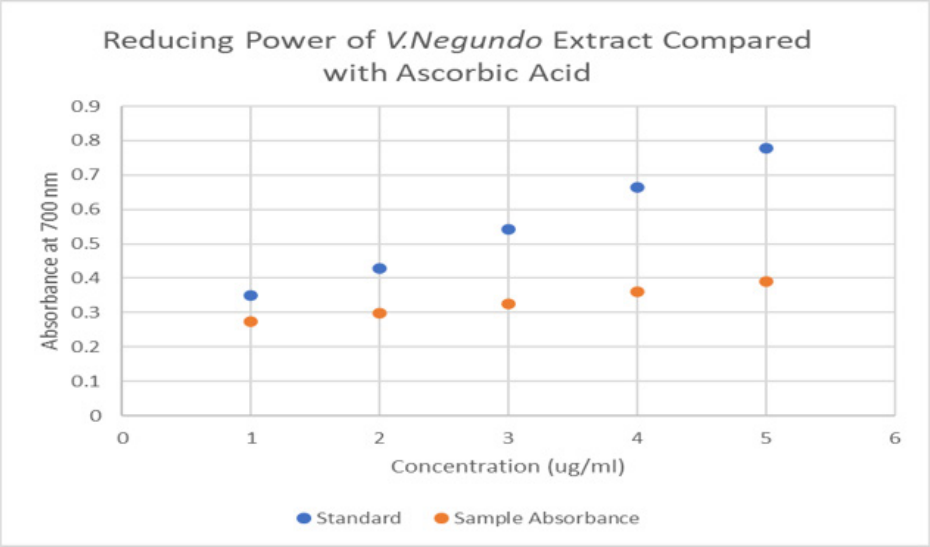


Figure 10: Reducing Power of *V. negundo* Linn. Extract Compared with Ascorbic Acid.

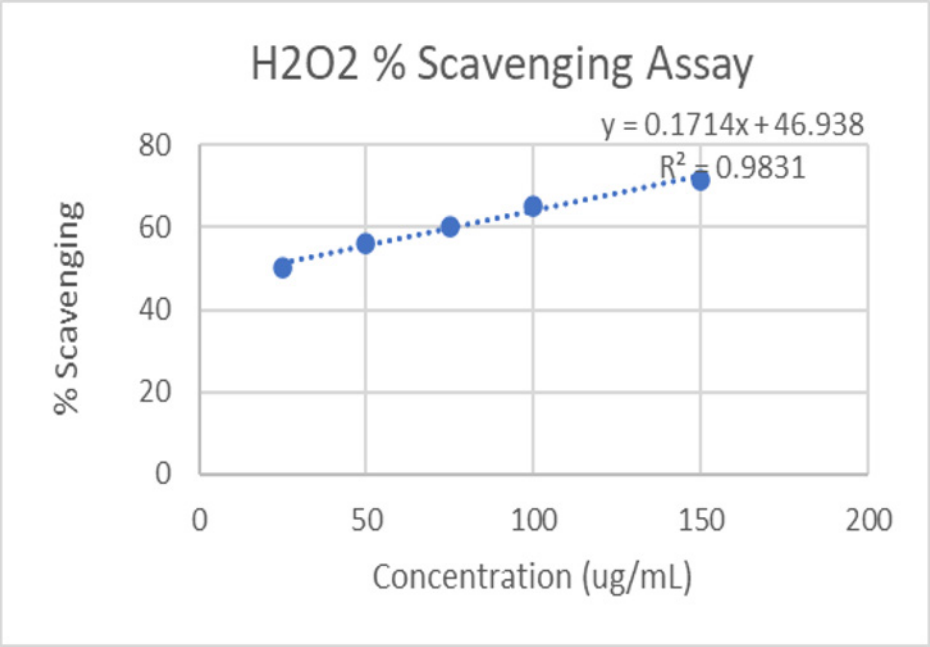


Figure 11: H_2O_2% Scavenging Activity.

y=0.1714x+46.938 (R2=0.9831)

The tested sample exhibited a pronounced concentration-dependent scavenging effect on hydrogen peroxide radicals. The IC_{50} value of 17.86 µg/mL demonstrates a remarkable antioxidant capacity when juxtaposed with various plant-derived extracts, which typically show IC_{50} values ranging from 50 to 200 µg/mL. The elevated R^2 value of 0.9831 signifies an excellent linear correlation, reaffirming the assay's dependability and the dose-response linkage. Although hydrogen peroxide is less reactive than other reactive oxygen species, it can permeate cell membranes and produce highly reactive hydroxyl radicals in conjunction with transition metals. Consequently, a compound's ability to neutralize H_2O_2 is vital for averting oxidative stress and cellular harm.

DISCUSSION

The current research validates that the leaves of *Vitex negundo* Linn. are an abundant source of bioactive phytochemicals, especially glycosides and phenolic compounds, which supports their traditional medicinal applications. The primary phytochemical analysis indicated the presence of glycosides, flavonoids, tannins, terpenoids, steroids, saponins, alkaloids, and carbohydrates. Quantitative assessments revealed significant levels of total phenolics (2.818 mg GAE/g extract), flavonoids

(9.078 mg quercetin/g extract), and tannins (3.64 µg/mL TAE; 36.4), all of which are recognized contributors to antioxidant activity. The extraction and isolation method was optimized to preferentially enhance glycosidic components, with subsequent purification techniques minimizing interference from flavonoids, tannins, and pigments. Thin Layer Chromatography (TLC) and High-Performance Thin Layer Chromatography (HPTLC) profiling exhibited distinct and reproducible chromatographic patterns, with notable bands at R_f 0.33 and R_f 0.39, aligning with previously reported values for iridoid glycosides such as swertiamarin and negundoside. The extract demonstrated considerable antioxidant potential, evidenced by a maximum total antioxidant capacity of 34.66 µg AAE/mL at a concentration of 500 µg/mL, moderate reducing power, and effective hydrogen peroxide scavenging (IC_{50}=17.86 µg/mL). Collectively, these results affirm that the leaves of *Vitex negundo* represent a promising natural source of antioxidant glycosides.

CONCLUSION

The current study illustrates that the leaves of *Vitex negundo* Linn. serve as a significant source of bioactive phytochemicals, especially glycosides and phenolic compounds. Phytochemical screening and quantitative analysis have verified notable levels of phenolics, flavonoids, and tannins, which enhance the observed antioxidant potential. Chromatographic profiling through TLC and HPTLC has yielded a reproducible phytochemical fingerprint and provided initial evidence for the existence of iridoid glycosides, including swertiamarin and negundoside. The methanolic extract demonstrated robust *in vitro* antioxidant activity, as indicated by a high total antioxidant capacity, effective hydrogen peroxide scavenging, and concentration-dependent reducing power. In summary, these results scientifically substantiate the traditional applications of *Vitex negundo* and underscore its potential as a natural source of antioxidant glycosides. Further research involving advanced spectroscopic characterization and *in vivo* assessments is necessary to validate the identified compounds and investigate their therapeutic uses.

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ABBREVIATIONS

GAE: Gallic Acid Equivalents; **RE:** Rutin Equivalent; **TAE:** Tannic Acid Equivalent; **HPTLC:** High-Performance Thin-Layer Chromatography; **TLC:** Thin Layer Chromatography; **TPC:** Total Phenolic Content; **TFC:** Total Flavonoid Content; **TTC:** Total Tannin Content; **UV:** Ultraviolet; **TAC:** Total Antioxidant Capacity; **AAE:** Ascorbic Acid Equivalent; **IC_{50}:** Half maximal inhibitory concentration; **HCl:** Hydrochloric acid; **$AlCl_3$:** Aluminium chloride; **CH_3COOK:** Potassium acetate; **H_2SO_4:** Sulphuric acid; **NaOH:** Sodium hydroxide; **H_2O_2:** Hydrogen Peroxide.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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