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Preliminary Phyochemical Analysis and Characterization of Flavonoid Moiety from *Vitex negundo* Leaves Origin in Madhya Pradesh State by HPLC Study

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Abstract

In the present study leaves of an important medicinal plant *Vitex negundo* was subjected to a phytochemical screening, flavonoid content and characterization of flavonoids moiety of *Vitex negundo* leaves extracts. The petroleum ether, chloroform, ethanol, methanol and aqueous extracts were prepared, and further screened for preliminary phytochemical test. The quantification of quercetin was investigated in ethanol extract by HPLC study. Preliminary phytochemical revealed the presence of flavonoids and phenolic compounds in ethanol, methanol and aqueous extracts of leaves of *Vitex negundo*. The ethanol, methanol and aqueous extracts investigated for antioxidant activity, and ethanol extract indicates the presence of highest quantity of flavonoids and polyphenol compared to other extracts. The HPLC chromatogram confirmed the flavonoids moiety was quercetin in ethanol extract, and the yield of quercetin was 42.18 ng/ml.

Introduction

The medicinal plants play significant role in the suppression of dietary and pathogen related ailments of various diseases. The therapeutic activities of plants are due to presence of secondary metabolites. The bioactive and antioxidant potentials of the plants are attributed to the presence of polyphenols, flavonoids, lignins, alkaloids, terpenoids, carotenoids, vitamins, and so forth. These phytoconstituents display different biological activities such as anti-oxidant, anti-inflammatory, antispasmodics, emetics, anti-cancer etc. Similarly, phenolic compounds play important role in antioxidant activity and inhibit the progression of different diseases[1-3].

Madhya Pradesh state of India is blessed with plenty of wild plants which have very significant role in the daily life of rural people. It was also observed that some people of the subject areas use them for the eradication of various diseases. *Vitex negundo* has an important social and economic significance in the Madhya Pradesh. The beneficial properties of *Vitex negundo* seem to be due to its richness in antioxidant constituents.

Vitex negundo possesses numerous biological activities proved by many experimental studies. It represents a class of herbal drug with very strong conceptual or traditional base as well as strong experimental base for its use. Researchers reported various pharmacological activities namely anticancer, analgesic, anti-inflammatory, bronchial relaxant, antibacterial, antifungal, larvicidal, antihelmentic, antioxidant, insecticidal/pesticidal activities etc for *Vitex negundo*[4,5].

Plant is bitter, acrid, astringent, cephalic, stomachic, antiseptic, alterant, thermogenic, depurative, rejuvenating, ophthalmic, anti-gonorrhoeic, anti-inflammatory, antipyretic and useful in bronchitis, asthma and enlargement of spleen. Roots are tonic, febrifuge, anti-rheumatic, diuretic, expectorant and are useful as a demulcent in dysentery, in cephalalgia, otalgia, colic, uropathy, wound and ulcers. Bark is useful in odontalgia, verminosis and ophthalmopathy. Leaves are aromatic, bitter, acrid, astringent, anodyne, anti-inflammatory, antipyretic or febrifuge, tranquillizer, bronchial smooth muscle relaxant, anti-arthritic, antihelmintic and vermifuge. Flowers are cool, astringent, carminative, hepatoprotective, digestive, febrifuge,

vermifuge and are useful in haemorrhages and cardiac disorders. Fruit is nervine, cephalic, aphrodisiac, emmenagogue and vermifuge[6,7].

The merit of the traditional use of *Vitex negundo* has also been supported by the isolation and identification of several possible active chemical constituents, mainly flavonoids, iridoids, terpenoids; fatty acids have been isolated from different parts leaves and twigs, bark, seeds and roots. Among the chemical constituent, flavonoids are the major. Leave and twig were reported to contain the known flavonoids such as casticin, orientin, isoorientin, luteolin, lutecin-7-O-glucoside, corymbosin, gardenins A and B, 3-O-desmethylartemetin, 5-O-desmethylnobiletin and 30, 40, 5, 50, 6,7,8-heptamethyoxy flavone. Besides, many glycosidic iridoids, alkaloids and terpenoids have also been isolated[6,8].

In the present study, we investigated preliminary phytochemical screening, flavonoids content and characterization of active constituent of *Vitex negundo* extracts.

2 Material and Methods

2.1 Plant material

Fresh plant parts (leaves) of *Vitex negundo* were collected randomly from Botanical garden of Govt. Motilal Vigyan Mahavidyalaya, Bhopal, Madhya Pradesh, India. The taxonomic identity of the plant was confirmed by prof. Dr. Madhuri Modak, Department of Botany, Govt. Motilal Vigyan Mahavidyalaya Bhopal, India. The preparation of various extracts was made from the shade dried and powdered leaves of *Vitex negundo* L.

2.2 Preparation of extract

The powdered leaves of *Vitex negundo* about 1 Kilogram were packed in soxhlet apparatus and extracted with petroleum ether, chloroform, ethanol, methanol and distilled water separately, until the completion of the extraction. The extract was filtered while hot, and the resultant extract was distilled in vacuum under reduced pressure in order to remove the solvent completely, and later dried in a desiccator.

2.3 Preliminary Phytochemical analysis

Preliminary phytochemical screening was performed to identify secondary metabolites (phytoconstituent) in extracts[9].

2.4 Total polyphenol content

Total polyphenol content was determined using colorimetric method. 1.0 ml of the prepared extract was oxidized using 2.5 ml of Folin- Ciocalteu reagent, and 2.0 ml of sodium carbonate solution (75 g/l) was then added to the reaction mixture. The absorbance readings were taken at 760 nm after incubation at room temperature for 2 h. The amount was calculated using the gallic acid calibration curve. The results were expressed as gallic acid equivalent (GAE) mg per 100 ml of the sample (extract).

2.5 Total flavonol content

Flavones and flavonols contents were analyzed by the colorimetric method. 9.8 ml of the prepared extract was mixed with a 10% solution of aluminum chloride (200 µl). After 30 min, absorption was measured at a 425 nm wavelength. The amount was calculated using quercetin calibration curve. The results were expressed as the quercetin equivalent (QE) mg per 100 ml of the sample[10,11].

2.6 HPLC study of extract

2.6.1 Standard solutions and sample preparation

Standard stock solutions of quercetin were prepared at a concentration of 2 mg/mL in 80% methanol. The standard solutions of quercetin were serially diluted with 80% methanol to obtain calibration standard solutions at concentrations of 20, 40, 60, 80 and 100 ng/ml.

The ethanol extracts was dissolved in 80% methanol at a concentration of 250 mg/mL and were centrifuged at 13,200 rpm for 5 min. The supernatant was collected and filtered through a 0.45-µm polytetrafluoroethylene (PTFE) syringe filter.

2.6.2 Instrumentation and chromatographic conditions

The analyses were carried out using an HPLC system (Waters, USA) consisting of a solvent delivery pump (Model LC-10 ADvp), a variable wavelength UV/VIS detector (Model SPD 10 AVP), a manual injection valve (Rheodyne®, USA) with a 20 µL loop, and degasser (DGU 14A). Data collection and analyses were performed using CLASS-VPTM System Software. A gradient elution was performed on a Thermo Scientific Hypersil C18 column (250 x 4.0 mm i.d., 5 µm particle size) (Thermo Scientific, USA). The mobile phase composed of Acetonitrile: Methanol (50:50v/v) and was isocratically eluted at a flow rate of 1 mL min^{-1}. All solutions were degassed and filtered through a 0.45 µm pore size filter (Millipore, USA). A small sample volume of 20 µL was used for each sample run, being injected into the HPLC system. The chromatogram was monitored with UV detection at a wavelength of 258 nm.

A thermospectronic model of Labindia 3000 + UV/VIS Spectrophotometer with 1cm. matched quartz cells was used for determination of λmax. The HPLC system (Waters) consisted of a pump, a U.V.Visible detector, a Thermo C18 (250 X 4.6 mm, 5µm) column, a Data Ace software.

2.6.3 Chromatographic conditions

The chromatographic analysis was performed at ambient temperature on a RP-C18 analytical column with a mobile phase composed of Acetonitrile: Methanol (50:50, v/v) and was isocratically eluted at a flow rate of 1 mL min^{-1}. A small sample volume of 20 µL was used for each sample run, being injected into the HPLC system. The chromatogram was monitored with UV detection at a wavelength of 258 nm.

2.6.4 RP-HPLC Method

HPLC method was developed using a Thermo C-18 column with 250 x 4.6 mm i.d. and 5-µm particle size column. Mobile phase selected for this method contained Methanol and Acetonitrile (50:50) was taken in same proportion was filtered through 0.2-micron membrane filter. Flow rate employed was 1.0 ml/min. Detection of eluent was carried out at 258 nm[12-14].

3 Results and Discussions

3.1 Phytochemical study

The evaluation was done to define the nature of chemical constituents present in leaves extracts. The phytoconstituent may be beneficial indicator of both efficacy and potential toxicity of plants. Preliminary phytochemical investigations of the extracts of leaves of *Vitex negundo* revealed the presence of flavonoids, tannins, phenolic compounds, alkaloids, glycosides, carbohydrates, fats and oil. The details are presented in table 1.

From the result of phytochemical screening, the petroleum ether extract of leaves of *Vitex negundo* demonstrated the presence of fats and oils. The chloroform extracts consisting of alkaloids. The Alkaloids, glycosides, flavonoids, tannins and polyphenol were found in ethanol extracts of leaves of *Vitex negundo*, and similar constituents were present in methanol extract except alkaloids. Similarly glycosides, carbohydrates, tannins and polyphenol were present in aqueous extracts of leaves of *Vitex negundo*. The maximum phytocostituents were observed in ethanol and methanol extracts of *Vitex negundo*. The ethanol and methanol extracts of *Vitex negundo* were selected for further *in vitro* antioxidant activity evaluation as this extract revealed the presence of flavonoids and phenolic compounds.

Table 1: Phytochemicals present in extract of *Vitex negundo* leaves

Phytoconstituent		Petroleum ether extract	Chloroform extract	Ethanol extract	Methanol extract	Aqueous extract
Alkaloids	Dragendorff's test	-	+	+	-	-
	Hager's test	-	-	+	-	-
	Legal's test	-	-	+	+	-
Glycosides	Keller killiani test	-	-	+	++	+
	Saponin glycoside	-	-	+	+	+
	Coumarin glycoside	-	-	-	-	-
Carbohydrates	Molish test	-	-	-	-	++
	Benedict's test	-	-	-	-	-
	Tannic acid test for starch	-	-	-	-	-
Tannins and Phenolic compound	5%$Fecl_3$ solution	-	-	++	+	+
	Lead acetate solution	-	-	++	+	+
	Bromine water	-	-	+	+	+
	Acetic acid solution	-	-	-	-	-
	Dilute potassium permanganate solution	-	-	+	-	+
Flavonoids	Shinoda test	-	-	++	++	-
Steroid test	Liebermann burchard test	-	-	-	-	-
	Liebermann's reaction	-	-	-	-	-
Protein	Biuret test	-	-	-	-	-
	Ninhydrin test	-	-	-	-	-
Fat and oil test	Solubility Test	++	-	-	-	-
	Filter paper staining	+	-	-	-	-

+ = Detected, ++ = Strongly detected, - = Not detected

3.2 Total phenolic content of Vitex negundo

The concentration of total phenolic content in ethanol, methanol and aqueous extract of *Vitex negundo* was evaluated. The standard curve of gallic acid was calculated and plotted in distilled water for determining absorption data. The linear equation of gallic acid was found to be y = 0.0394 x +0.0115 (Fig 1).

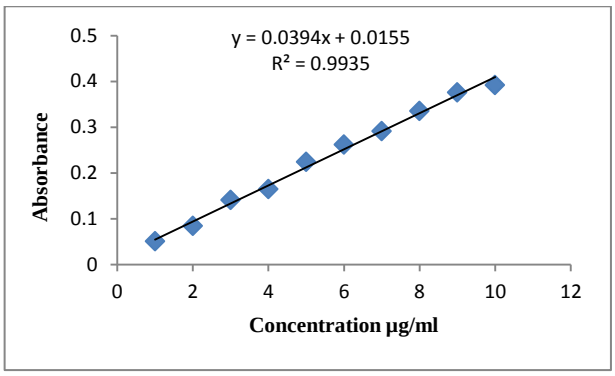

Fig 1: Calibration curve of gallic acid in distilled water

The findings of total phenolic content in extracts are displayed in table 2. The total phenolic content in extracts, expressed as gallic acid equivalents. Total phenolic content of ethanol, methanol and aqueous extract of *Vitex negundo* were 82.13±0.79, 43.65±0.58 and 25.19±0.47 GAE mg/gm, respectively. The order of total phenol content for ethanol, methanol and aqueous extract of *Vitex negundo* were ethanol extract > methanol extract > aqueous extract. The ethanol demonstrated higher quantity of total polyphenol content compared to other extracts.

Table 2: Determination of total polyphenol content in extract of *Vitex negundo* leaves

Extract	Total polyphenol content (GAE mg/gm)
Ethanol	82.13±0.79
Methanol	43.65±0.58
Aqueous	25.19±0.47

Data expressed as gallic acid equivalent (GAE) mg per gm of the extract, Values are mean ± SEM of triplicate determinations

3.3 Total flavonol content of Vitex negundo

The concentration of flavonoids in ethanol, methanol and aqueous extract of *Vitex negundo* by using aluminum chloride. The content of flavonoids was expressed in terms of quercetin equivalents. The standard curve of quercetin was calculated and plotted in distilled water for determining absorption data. The linear equation of quercetin was found to be y = 0.0381 x +0.0116 (Fig 2). The outcomes of total flavonol content in extracts are displayed in table 3. Total flavonol content of ethanol, methanol and aqueous extract of *Vitex negundo* were 73.62±0.27, 39.15±0.64 and 21.84±0.49 QE mg/gm, respectively. The order of total flavonol content for ethanol, methanol and aqueous extract of *Vitex negundo* were ethanol extract > methanol extract > aqueous extract. The ethanol demonstrated higher quantity of total flavonol content compared to other extracts.

The flavonoids are considered to produce potent therapeutic effects against different diseases such as cancer, liver cirrhosis, diabetes, inflammation etc. The flavonoids control the disease by their scavenging properties of reactive oxygen species. The therapeutic property of *Vitex negundo* suggests the presence of antioxidant component in reference to flavonoids. Further the therapeutic efficacy of *Vitex negundo* depend on the quantity of flavonoids. The ethanol extract showed maximum flavonoids and polyphenol content, and it was selected to investigate the nature and quantity of flavonoid component present in the ethanol extract.

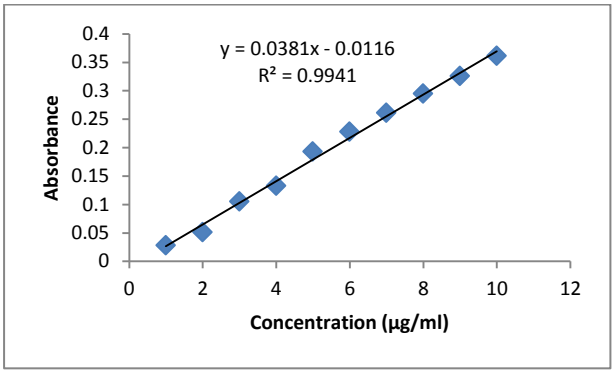

Fig 2: Calibration curve of quercetin in distilled water for quantification of total flavonol content

Table 3: Determination of total flavonol content in extract of *Vitex negundo* leaves

Extract	Total flavonol content (QE mg/gm)
Ethanol	73.62±0.27
Methanol	39.15±0.64
Aqueous	21.84±0.49

Data expressed as gallic acid equivalent (GAE) mg per gm of the extract, Values are mean ± SEM of triplicate determinations

3.4 Quantification of quercetin in extract

3.4.1 Linearity of the calibration curve of pure quercetin

Validation data was collected from three analytical runs. The linear regression equation analyte was y = 28.168x + 307.48 (Fig 3). The linear range for quercetin was adequate for this method to be used in the determination of quantity of quercetin present in extract. The HPLC chromatogram of pure quercetin showed R_t value at 3.18 min (Fig 4) on solvent system Acetonitrile: Methanol (50:50, v/v).

3.4.2 Quantification of quercetin in ethanol extracts

The quantification of quercetin in ethanol extract was determined by HPLC. The pure quercetin was used as a standard compound and the quercetin were expressed as ng/ml using the standard curve equation: y = 28.168x + 307.48 (Fig 3).

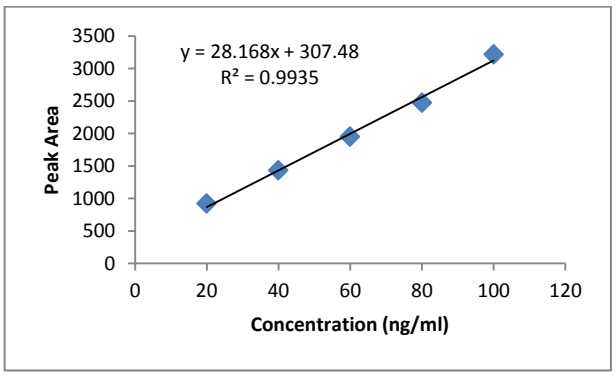

Fig 3: HPLC Calibration curve of quercetin

3.4.5 HPLC Chromatogram

The HPLC analyses were performed on the ethanol extracts of *Vitex negundo* leaves to investigate the quercetin as a chief constituents. The analysis of sample can be completed within 15 minutes. The HPLC chromatogram of ethanol extracts depicted R_t value at 3.20 min (Fig 5), by using solvent system Acetonitrile: Methanol (50:50, v/v), and was similar to R_t of pure quercetin. The peaks which could be identified in the chromatogram were quercetin. They were identified by comparison with the compounds chromatogram reported in literature. The concentration of quercetin present in the ethanol extract was found to be 42.18. Additionally, the established chromatogram can be used as fingerprinting for quercetin present in *Vitex negundo* leaves. This method possesses the advantages of simplicity, rapidity, high sensitivity and good reproducibility and will be applicable to the quality control of *Vitex negundo*.

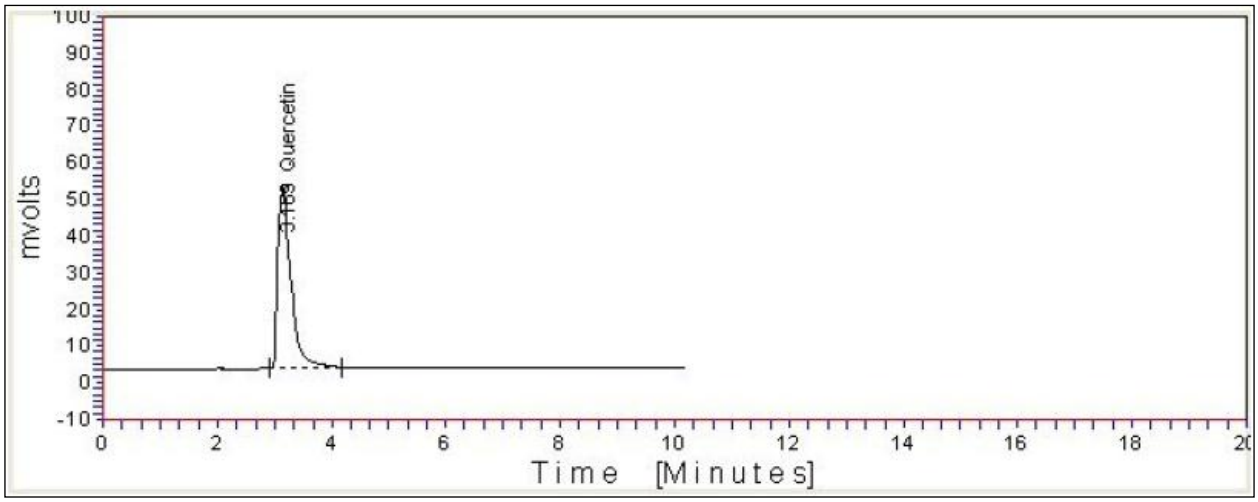

Fig 4: HPLC chromatogram of pure quercetin

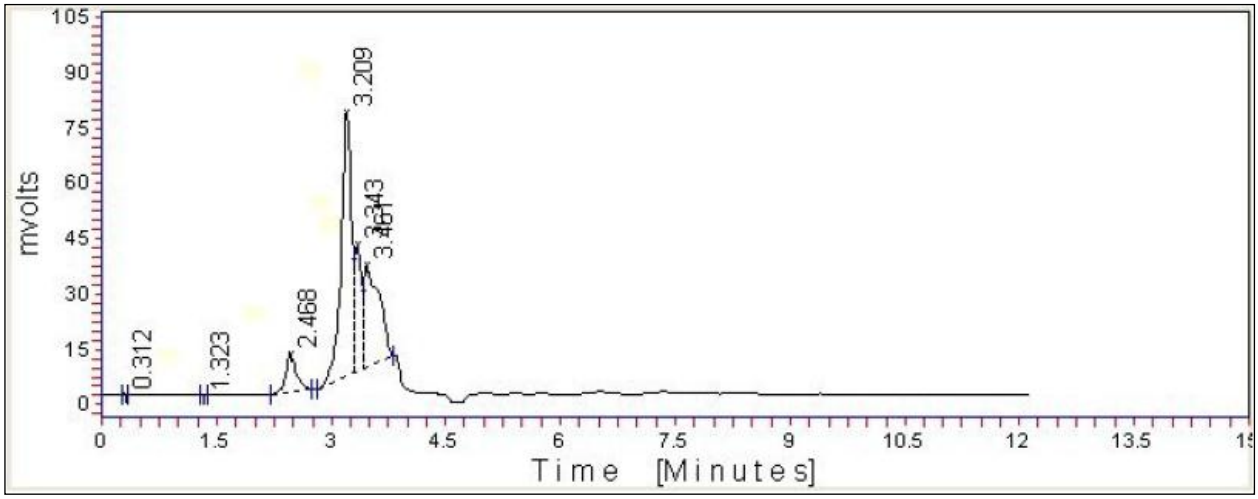

Fig 5: HPLC chromatogram of ethanol extract of *Vitex negundo*

The quercetin biochemical activity is well documented. It is one of the most potent antioxidants among polyphenols. Quercetin has also been demonstrated to display the antiviral, antibacterial, anticarcinogenic, hepatoprotective, anti-arthritic, anxiolytic, anti-amnesic and anti-inflammatory effects. It's

suggested the presence of quercetin in *Vitex negundo* leaves is accountable for the therapeutic efficacy.

4 Conclusion

Vitex negundo reported various therapeutic activities for the treatment of cancer, liver cirrhosis, kidney failure etc due to its antioxidant properties. The quercetin present in *Vitex negundo* contributing its antioxidant activity. The findings of the present study showed that the ethanol and methanol extracts of *Vitex negundo* leaves contained flavonoid moiety. The HPLC chromatogram confirmed the flavonoids moiety was quercetin in ethanol extract. The developed methods herein would prove to be applied to intrinsic quality control of this drug or other medicinal preparations containing quercetin.

5 Conflict of interest

We declare that we have no conflict of interest.

6 Author contributions

FAD and KJ have carried out the research work in the laboratory. BJ and MM compiled and analyzed the data of present work. All authors approved the final manuscript.

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