

RSM assisted phytochemical profiling of *Vitex negundo* and molecular docking studies of biological active compounds with target proteins

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Abstract

Vitex negundo (Family-Verbenaceae) is a remarkable medicinal plant with numerous therapeutic applications. In this study, phytochemical screening was performed in *Vitex negundo* leaf extract, and extraction optimization was performed by applying response surface methodology (RSM). Methanolic extract and microwave-assisted extraction (MAE) were found to be appropriate for obtaining the maximum yield of bioactive compounds. In single-factor analysis, solvent concentration, microwave power, and time of extraction variations were analyzed to calculate the amount of TPC, TFC, and TAC in the extract. The quantity of TPC, TFC, and TAC were fairly obtained from 60%–95% (v/v) methanol concentration, 540–900 W microwave power, and 60–90 s extraction time. Furthermore, we also employed the response surface methodology (RSM) for optimizing microwave-assisted extraction of *Vitex negundo* leaves by considering three key parameters: solvent concentration, extraction temperature, and extraction duration. Condition optimization for the extraction were obtained at 95% (v/v) methanol, 720W, and 90s with TPC (227.6 mg gallic acid equivalent (GAE)/g sample), TFC (978.2 mg RE/g sample), and TAC (82.13 mg ascorbic acid equivalents/g sample). The characterization of methanol extract of *Vitex negundo* was done by FTIR and GC–MS to identify the various active groups and types of bioactive constituent's presence in the extract. Subsequently, the compounds were evaluated for their bioactivity through molecular docking interactions with target proteins *in silico*. Furthermore, the antibacterial activity of the optimized extract was studied against two Gram +ve (*Bacillus sphaericus*, *Pectobacterium*) and two Gram-ve (*Escherichia coli*, *Pseudomonas aeruginosa*) bacteria, and the inhibitory effect was observed in all four tested bacteria. The highest antibacterial activity was shown against *B. sphaericus* (19 ± 0.087) and *E. coli* (19 ± 0.064) followed by *Pectobacterium* (18 ± 0.068) and *Pseudomonas* (13 ± 0.093). The hemolytic activity of the optimized leaf extract (at a dose of 20 µg/ml) shows a minimum hemolytic activity, i.e., 1.33 ± 0.06 %. It may be concluded from the study that *Vitex negundo* is potential and safe source of natural antioxidants that can be utilized in green therapeutics.

Highlights

1. Microwave-assisted RSM-based extraction optimization of *Vitex negundo* for making therapeutically sound herbal formulations.
2. Optimized extract indicated antioxidant and anti-inflammatory properties.
3. A molecular docking study also validates the possible role of *Vitex negundo* bioactive compounds in various diseases.
4. Antibacterial and hemolytic analysis suggested the role of *Vitex negundo* in green therapeutics.

1. Introduction

Plant secondary metabolites are organic molecules that perform defence function in plants and protect them under various biotic and abiotic stress conditions (A. Hussein & A. El-Anssary, 2019; Yang et al., 2018). According to their chemical nature and activity, secondary metabolites are of different types, such as phenols, flavanoids, and terpenoids (Jan et al., 2021). Polyphenols are natural antioxidants and possess several positive health impacts. These are thought to reduce the risk of chronic diseases, slow the aging process, support a healthy immune system, and enhance general well-being. In addition, these are being used in the prevention and treatment of various chronic illnesses such as cancer, cardiovascular diseases, diabetes, hypertension, asthma, and several infections (Cory et al., 2018; Rudrapal et al., 2022). Since ancient times, medicinal plants have been used in several herbal formulations because they are a rich source of bioactive components (Boy et al., 2018). *Vitex negundo* (VN), an important ethnobotanical plant belonging to (Family-Verbenaceae), is found widely in Asia, including India, North America, Europe, and the West Indies (Kamal et al., 2022). Several bioactive substances have been extracted from different parts, viz roots, leaves, and shoots, in the form of lignans, iridoids, terpenes, volatile oils, flavonoids, and steroids. These bioactive substances show several medicinal properties such as anti-inflammatory, antioxidant, antidiabetic, anticancer, and antimicrobial properties (Prasathkumar et al., 2021). There are different *Vitex* species present globally that produce various phytochemicals depending on their chemical makeup. VN has also been recognized in the manipulation of biological processes such as apoptosis, cell cycle, sperm motility, polycystic ovary syndrome, and the menstrual cycle (Gill et al., 2018).

The quality and safety of herbal formulations made using the extracted phytochemical are still being researched. The ultimate quality of any herbal preparation is very much affected by method of extraction; therefore, it should be reliable, economic, efficient, and also time saving. Several research findings were studied to determine the impact of the extraction procedure on the superiority and amount of phytochemicals. Microwave-assisted extraction (MAE) has gained attention because of its efficiency and time saving property. (Idris & Mohd Nadzir, 2021). It is a contemporary approach with numerous benefits compared with traditional methods of extraction. It reduces solvent quantity, time, cost and also energy consumption (Al Mamoori & Al Janabi, 2018; Reddy et al., 2020). Efficiency of extraction protocol defines by optimization of several factors viz polarity of the solvent, extraction time, and the extraction method applied. Now a days MAE has been frequently applied to extract bioactive substances including polyphenols, quinones, terpenes, and alkaloids from various sources. (Patra et al., 2022; Pavlić et al., 2019). MAE process provides good amount of yield of the desired chemicals in the extract and also the quality is retained. (Georgiopoulou et al., 2023). Software called Response Surface Methodology (RSM) is employed to analyze and improve data using mathematical and statistical models. It is frequently used in the optimization process when many factors and their interactions affect the response (Reji & Kumar, 2023).

Antibacterial potential against several bacteria have been reported in several medicinal plants (Elisha et al., 2017; Othman et al., 2019) due to presence of diverse secondary metabolites. Plants contain a range of organic chemicals that are quite effective in treating bacterial illnesses (Khameneh et al., 2019; Talib & Mahasneh, 2010).

This study focused to establish an optimized phytochemical extraction protocol from *Vitex negundo* leaves that will help in making efficient herbal formulation. For this, microwave-assisted extraction (MAE) was performed. RSM was executed to obtaining the best conditions for the extraction of bioactive components in the plant extract. Additionally, the optimized extract of *Vitex negundo* leaf extract was analysed for their antibacterial potential and hemolytic activity. GC-MS analysis was executed to recognize the bioactive components responsible for antibacterial and antioxidant activity of VN leaf extract, and FTIR analysis was done to determine the various functionally active groups involved in pharmacological activities. Molecular docking analysis of the identified bioactive components was performed with target proteins to evaluate their antioxidant and anti-inflammatory potential. Our investigation may help

to follow an efficient extraction protocol and facilitate the preparation of a good herbal formulation from *Vitex negundo*. This may be used in green therapeutics to naturally cure several ailments.

2. Experimental procedure

2.1. Collection and preparation of sample

Vitex negundo was procured from the Central Institute of Medicinal and Aromatic Plants (CIMAP, CSIR), Lucknow. After procurement it was sustained at Gautam Buddha University, Greater Noida. Plant samples were identified by the National Herbarium of Cultivated Plants (NHCP), Plant Exploration and Germplasm Collection division, ICAR-NBPGR, PUSA Campus, New Delhi. Fresh leaves of *Vitex negundo* were taken from the GBU campus herbal garden before flowering and rinsed for 10 min in running tap water to remove dirt and repeat the same procedure twice with double distilled water. The washed leaves were dried in an open environment at 22-28 degrees Celsius for 5 days to eliminate leftover moisture. The dried leaves were then homogenized with an electronic grinder to make powder, which was stored in an airtight jar for future use (Kotowaroo et al., 2006).

2.2. Phytochemical extraction and screening

Vitex negundo leaf extract was prepared using various solvents (methanol, ethanol, chloroform, n-hexane & distilled water) and by applying two methods of extraction: room temperature extraction (RTE) and microwave-assisted extraction (MAE). 5 grams of samples were taken in a ratio of 1:20 (sample: solvent w/v) for extraction using solvents (methanol, ethanol, chloroform, n-hexane, and distilled water 100ml.) and kept in a shaking incubator at 120 rpm for 24 h at 37⁰ C in RTE and for MAE at 540 W for 60 s. Mixtures were then centrifuged at 7,000 rpm for 8 min, followed by filtration. The extracts were dried completely and stored at 4°C for further analysis (Nuchdang et al., 2022).

Table 1: Methodology for qualitative screening of phytoconstituents of the VN leaf extract

S. No.	Phytochemical analysis	Name of the Test	Method for phytochemical extraction
1	Alkaloid	Mayer's test (Jha et al., 2012)	500 µL of each extract+few drops of Mayer's reagent (mercury chloride + potassium iodide). Red precipitate- alkaloids present
2	Carbohydrates	Benedict's test (Hernández-López et al., 2020)	1mL of Benedict reagent + 500 µL of extract + 5 min water bath at 80°C. Red precipitates- Carbohydrate present
3	Saponins	Froth test (Gul et al., 2017)	500 µL of the extract + 2.5 ml of D.W.for making dilution and agitated for 5 min. Stable foam layer- Saponins presence
4	Steroids	Aqueous sodium hydroxide test (Shaikh & Patil, 2020)	Chloroform + 500 µL extracts+ sulfuric acid (4-5 drops).Red precipitate- Steroids present
5	Glycosides	Keller-Killiani test (Tingson & Politano, 2021)	500 µL of extract + 1 mL of DDW+2 drops of 1 N NaOH. Yellow precipitate-glycoside presence
6	Tannins	Ferric chloride test (Nathaniel et al., 2019)	500 µL extracts + 1 mL of 5% FeCl ₃ .The presence of tannins indicates a blue– black precipitate.
7	Terpenoids	Salkowski's Test (Sudha et al., 2021)	500 µL of extract + 4 drops of chloroform + 5 drops of strong sulfuric acid – layer formation. Terpenoids are present when the color is reddish-brown.
8	Flavonoids	Lead acetate test (Abdullahi et al., 2020)	2-4 drops of chloroform + 500 µL of extract+ few drops of sulfuric acid + layer formation Terpenoids are present.
9	Phenols	Ferric chloride test (Gupta et al., 2019)	500 µL of extract + 2 drops of a 5% FeCl ₃ solution + Bluish-black color shows the existence of phenolic chemicals.
10	Amino acids	Ninhydrin Test (Nathaniel et al., 2019)	2 drops of 5% ninhydrin + 500 µL of extracts and heated at 100°C for 5 min. The emergence of a blue– green tint confirms amino acids presence.

Phytochemical extracted from the leaves of *V. negundo* was screened by both qualitative and quantitative methods.

Qualitative screening: To determine the presence of active phytochemicals such as alkaloids, flavonoids, tannins, carbohydrates, phenolic compounds, terpenoids, glycosides, steroids, amino acids, fixed oils, and fats in extracts, preliminary qualitative phytochemical screening was performed as per standard

methods described by Trease & Evans (1989; Santhi & Sengottuvel, 2016). For **quantitative screening**, Total phenolic content (TPC), Total antioxidant capacity(TAC) and total flavonoid content (TFC) were analyzed.

TPC: of the prepared extracts was determined by the Folin–Ciocalteu colorimetric method described by Singleton, V. and Rossi, J. (1965) (Dini et al., 2020). TPC was calculated in gthe sample by making a standard curve using 10, 20,40, 80 and 100 µg/ml of gallic acid. 50µl of different concentration of gallic acid and 500 µL of water were added with 100 µl of folin reagent and kept at RT for 5 min in dark conditions. After 5 min, 300 µl of 20% sodium carbonate was mixed and the again it was incubated for 1 h at RT in dark condition. After incubation, the sample OD was taken at 750 nm. TPC were denoted as mg gallic acid equivalent (mg GAE/g) of the extracted sample.

TFC: was calculated by using the aluminum chloride colorimetric method (Mahboubi et al., 2013). The total amount of flavonoid in the extract was determined by a calibration curve prepared by adding different concentrations of rutin (20, 40, 60, 80, 100, 120, 140 and 160 µg/mL) from a stock solution of 1000 µg/ml. 0.5 ml of Plant extract was incubated with 100µl of 20% AlCl₃ solution for 5 min. Following this, 100 µl of potassium acetate was added and the volume was makeup to 5 ml by distilled water and incubated for 45 min providing dark condition. After incubation, the absorbance was measured at 415 nm. TFC was expressed as rutin equivalent per gram (mg of RE/g of extract).

TAC: was determined using the method described by Prieto P et al. (Christodoulou et al., 2022). TAC was examined using a calibration curve of ascorbic acid 5, 10, 20, 30, 40 and 50 µg/mL. A total of 50 µL of extract and 500 µL of reagent solution (a mixture of 50 ml of concentrated 98 % H₂SO₄, 0.2298 gm sodium phosphate, and 0.41 gm of ammonium molybdate) were added to each test tube. Test tubes were incubated at 90°C for 45 min in a water bath to complete the reaction and absorbance of samples was taken at 695 nm. The TAC was measured as the AA(ascorbic acid) equivalent per gram (mg of AA/g of extract).

2.3. BBD experimental strategy

2.3.1. Single factor optimization

The different variables (Concentration of methanol, M (20–95%, v/v), time of extraction(T, (30–100 s), and microwave power ,P(360–900 W)—were taken on the basis of single-factor analysis results on the TPC, TFC, and TAC, to standardized all variables using the BBD design to have maximum extraction efficiency.

2.3.2. Optimization of Extraction Variables Using the BBD Method

To investigate the effect of factors on responses, a Box– Behnken design (BBD) with five central-point replicates was applied using Design Expert software (Version 10, Stat-Ease Inc., Minneapolis, MN, USA) based on single-factor analysis. Table 2 lists different independent variables: CM, P, T and their values. For a total of three responses, TPC, TFC, and TAC, a total of 17 experimental runs were generated which comprise four central points. Second order polynomial equation was used obtain the yield TPC, TFC, and TAC (Table 3). Three-dimensional plots were constructed and studied to find the effects of the different variable on TPC, TFC, and TAC. Variables were considered to be significant on the basis of *p*-values ≤ 0.05.

Table 2: Different variables used for applying the Box–Behnken design for extraction optimization.

Variable	Tag	Levels	
		-1	1
Concentration of methanol(%)	CM	60	95
Microwave power (W)	P	540	900
Time of extraction (s)	T	60	90

Table 3: Different parameters used in Box–Behnken experimental design and analysis of the responses of TPC, TFC, and TAC

Run	Types of Variables			Response		
	CM (% v/v)	P (W)	T (sec)	TPC (mg GAE/g)	TFC (mg Rutin/g)	TAC (mg AA/g)
1	77.5	720	75	105	511.1	80.5
2	95	720	60	168.8	754.4	86.74
3	60	900	75	159.6	633	102.34
4	77.5	700	60	133.2	500.1	89.21
5	95	720	75	105	511.1	80.5
6	60	900	75	187.3	656.4	87.22
7	60	720	90	193.7	511.1	96.8
8	77.5	900	60	149.4	978.2	89.3
9	77.5	540	60	132.28	710.7	70.1
10	77.5	700	75	105	511.1	89.21
11	95	540	75	165.38	613.4	81.4
12	77.5	720	75	105	511.1	80.5
13	95	720	90	227.6	978.2	110.34
14	77.5	900	90	167	710.7	69.3
15	77.5	540	90	149.8	647.8	97.6
16	77.5	720	75	105	511.5	80.5
17	60	540	75	140	511.5	101.2

2.4. GC-MS analysis

GC-MS analysis of the RSM optimized extract was carried out in 5977C GC/MSD GC-MS instrument (Agilent tech) fitted with a fused new Hydro Inert source, an HES InertPlus EI column, an inert extractor EI source, and a JetClean self-cleaning ion source interfaced with a GC detector. Software Platform MassHunter OpenLab CDS, hydrogen gas was used as the carrier gas. Ratio (m/z) 0.6–1091, scan speed $\leq 20,000$ Da/s, software platform Mass hunter, open lab CDS. The contents of phytochemicals present in the test samples were identified based on a comparison of their retention time (min), peak area, peak height, and mass spectral patterns with those in the spectral database of authentic compounds stored in the National Institute of Standards and Technology (NIST) (Linstrom, 1997) (Updated: January, 2023).

2.5. Fourier transform infrared spectroscopy

FT-IR was performed to find out the probable functionally active groups and the resolution in the spectral area ($4000\text{--}400\text{ cm}^{-1}$) was used. The dried VN leaf extract powder (10 mg) was mixed with 100 mg of KBr salt pellet using a mortar and pestle and compressed into a thin pellet (Satapute et al., 2019).

2.6. Molecular Docking

2.6.1. ADMET characteristics prediction

Compounds from methanolic leaf extract of *Vitex negundo* were examined for ADME characteristics using the Swiss Institute of Bioinformatics (SIB) SwissADME (Daina et al., 2017). The probable parameters help in monitoring whether the compounds adhere to the drug-like properties established by Lipinski's rule of five and its modifications. A molecule will be considered as a drug like candidate from the listed phytochemicals if that complies with not more than one violation of these criteria, besides having molar refractivity between 40 and 130, has a greater probability of being orally bioavailable (Benet et al., 2016; Jangam et al., 2019). The toxicity of the screened phytochemicals was investigated using ProTox-II (Banerjee et al., 2018). This software computed computational toxicity for chemical and molecular targets using predictive modeling by classifying toxicity prediction systems into two major groups: organ toxicity and analyze various toxicological parameters such as carcinogenicity, immunotoxicity and cytotoxicity).

2.6.2. Molecular Docking Simulations

All GC-MS recognized phytocompounds were analysed through molecular docking to study their supposed role as antioxidant and anti-inflammatory. The structures of these phytocompounds were obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) (S. Kim et al., 2023) as an SDF file. The SDF file was converted into the PDBQT format using OpenBabel (Yoshikawa & Hutchison, 2019). The three-dimensional structures of target proteins were obtained from the RCSB PDB Database (<https://rcsb.org/>) (Burley et al., 2023). To find out the protein-ligand interactions of bioactive compounds of Vitex with sodium dismutase (SOD1) (PDB ID: 5YUL), tumor necrosis factor-alpha (TNF-a) (PDB ID: 7JRA), glutathione reductase (GSR) (PDB ID: 2AAQ),

glutathione peroxidase (GPX7) (PDB ID: 2P31), catalase (CAT) (PDB ID: 7P8W), prostaglandin G/S synthase 2 with cyclooxygenase activity (PTGS2) (PDB ID: 51FA), and interferon-gamma (INF- γ) (PDB ID: 3BES) structure- based molecular docking was done. All target proteins were prepared by adding missing residues, polar hydrogens, and Kollman charges using AutoDockTools 1.5.7 (Bitencourt-Ferreira et al., 2019). The binding regions and active site residues were identified by literature search and database exploration (see Table). The receptor grid for each target protein was generated using AutoDockTools 1.5.7 (Bitencourt-Ferreira et al., 2019). AutoDock Vina was used to predict the local and global minimum conformations for nine different binding modes of the ligand (Eberhardt et al., 2021). The exhaustiveness of the conformational search was set at 100 to obtain consistent docking. Interactions between receptor and ligand was visualized by using BIOVIA Discovery Studio (Biovia Dassault Systemes, 2017).

2.7. Antibacterial Activity Evaluation

To evaluate the antibacterial potential of *Vitex negundo* optimized extract was analyzed by zone of inhibition method, Minimum inhibitory concentration (MIC), and Maximum lethal concentration (MLC). Two Gram-positive bacteria (*Escherichia coli* MTCC443 and *Bacillus sphaericus* MTCC7542) and two Gram-negative bacteria (*Pseudomonas aeruginosa* MTCC 2474 and *Pectobacterium carotovorum* MTCC 1428) were collected from MTCC, Chandigarh. Each strain was set to a concentration of 10^8 cells/ml. with the help of 0.5 McFarland standards.

2.7.1. Disc diffusion method

In this experiment susceptibility testing of all bacteria were done by the disc diffusion method recommended by Bauer et al. (1966). The bacterial suspension was diluted with a sterile MHA to 10^8 CFU/ml. 1000 μ L of was placed on the Mueller–Hinton agar surface and the inoculum was allowed to dry completely. 50 μ L of sample solution was placed individually on the surface of the MHA. Neomycin and methanol were used as a positive control and negative control respectively. Above stated protocol was applied for all four bacteria, and the plates were incubated at 37°C overnight. The plates were checked for the inhibition zone and was measured using a ruler (Karimi et al., 2018).

2.7.2. MIC and MLC

MIC was performed on the surface of the MHA broth according to Jain et al. 2020. The MICs of the extracts were used in concentrations 1.25, 2.5, 5, and 10 % with 10 μ L bacteria containing 10^8 CFU/ml and incubated overnight at 37°C. Kanamycin (10mg/ml) and methanol (50 μ L) was used as a positive and negative control respectively. Bacterial growth was determined using a plate reader at 600 nm. The lowest concentration was observed as the MIC value while the MLCs of the extract were determined at the lowest concentration of the extract at which no bacterial growth in the media was observed. Results were determined by calculating the MBC/MIC ratio which state that MBC/MIC ratio is <2- the extract has bactericidal activity, otherwise it is bacteriostatic. If MBC/MIC>16- ineffective extract.

2.8. Hemolytic activity

The leaf extract of *Vitex negundo* was tested for hemolytic activity against normal mouse erythrocytes. Three milliliters of mouse blood were mixed with 5 ml of 1 $\times$ Phosphate-Buffer Saline (PBS) and the mixture was centrifugation at 3000 rpm for 5 min. The obtained pellets were dissolved in 5 ml of PBS and from this 180 μ L of was then added to 20 μ L, 40 μ L, 60 μ L, and 80 μ L of plant extract dissolved in dimethyl sulfoxide (DMSO). After incubation at 37°C for 30 min, it was centrifuged for 5 min at 13,000 rpm, and refrigerated. Free haemoglobin in the supernatant was detected using a spectrophotometer at 576 nm. The negative control (DMSO) and positive controls (1% Triton X-10)were taken, where A_t = absorbance of plant extract, A_n = absorbance of negative control, and A_s = absorbance of positive control (Habib et al., 2020).

$$\%Hemolysis = (A_t - A_n)/A_s \times 100$$

2.9. Statistical Analysis

Variability among different factors was analyzed by applying single factor analysis ANNOVA (One way analysis of variance). The best values of all the response variables were projected by their optimal values by applying BBD by Design Expert 10 software. All the experiments were performed in triplicates and results are presented as mean $\pm$ SE.

3. Results And Discussion

3.1. Phytochemical extraction and screening

Plant phytochemicals, mainly polyphenols, are thought to account for the majority of the antioxidant activity of plant extracts (Hano & Tungmunthum, 2020; Tungmunthum et al., 2022). *Vitex negundo* is an important medicinal plant, and its phytochemicals have many therapeutic applications. It will be extremely useful to find an optimized method of phytochemical extraction from its leaves. Therefore, it would be used in various pharmaceutical and medicinal applications. The selection of the solvent and the conditions of the extractions are the main factors for the good-quality extraction of bioactive compounds.

Table 4: Presence of different constituents in the room temperature extraction and microwave-assisted extraction methods

Constituents	Methanol		Ethanol		n-Hexane		Chloroform		Distilled water	
	RTE	MAE	RTE	MAE	RTE	MAE	RTE	MAE	RTE	MAE
Alkaloid	++	+++	-	++	++	+++	-	++	++	+
Carbohydrate	+	++	+	++	++	+	+++	+++	++	+++
Saponin	-	-	-	-	+++	+++	++	+	-	-
Steroid	+++	+++	+	+++	++	++	++	++	+	+++
Glycoside	++	+++	++	++	+++	+++	+	+++	++	++
Tannin	+++	+++	++	+++	-	-	++	-	-	-
Terpenoid	++	+++	+	+	++	+++	++	+++	++	++
Flavonoid	++	++	-	-	++	++	+	++	++	+++
Phenol	+++	++	++	++	-	-	-	-	-	-
Amino-acid	++	+++	-	+++	-	-	-	-	++	+++

(+ sign shows presence, - sign shows absence)

Vitex negundo leaf extract was prepared in different solvents, and screening was performed by applying different methods. Initial phytochemical screening suggested many compounds such as alkaloids, carbohydrates, steroids, glycoside, tannin, terpenoids, flavonoids, phenol, and amino acids are present in the extract. (Table 4.) Based on qualitative results, further analysis of the TPC, TFC and TAC of the crude leaf extracts of *Vitex negundo* were performed.

TPC, TFC, and TAC were estimated and expressed as gallic acid eq/gm, rutin eq/gm, and ascorbic acid equivalents (AAE) per gram leaf extract, respectively. The highest amount of total phenol was obtained using the MAE method (23.59 mg GAE/gm), followed by RTE (18.26 mg GAE/gm.). In the case of TFC, it was observed that the MAE and RTE methods generated the total flavonoid content (45.23 mg rutin/gm) and (34.52 mg rutin/gm), respectively. Total antioxidant activity was obtained in the MAE method (18.77 mg AA/gm) that was followed by the RTE method (18.35 mg AA/gm) (Fig. 1)

Medicinal plants are a rich source of polyphenols that are function as antioxidants. This antioxidant activity works against reactive oxygen species and is effective against various diseases (Huyut et al., 2017; Rudrapal et al., 2022) because of the presence of hydroxyl groups, which play an important role in their scavenging ability. Flavonoids are also polyphenolic compounds that contain many phenolic groups and work as antioxidants and anti-inflammatory agents in chronic diseases (S. Kumar & Pandey, 2013; Shahidi & Ambigaipalan, 2015). They play an active role in the quenching of free radicals because of their redox properties (Panche et al., 2016). The total antioxidant capacity of the plant is assumed to be due to the presence of polyphenols. Most of the bioactivities of plant crude extracts are due to the high flavonoids and phenols that are being used in the medicine industry for their antioxidative properties.

3.2. BBD experimental design

3.2.1. Single-factor analysis

As we observed that methanol and microwave-assisted extraction gave appropriate results in qualitative and quantitative analyses, the extraction procedure of phytochemicals from *Vitex negundo* was further optimized using response surface methodology. Single-factor analysis was performed and the effect of CM, T, and P on the accumulation of TPC and TFC in the extract and the relative change in TAC were also studied. In the single-factor analysis, *Vitex negundo* leaf extract was studied at five different methanol concentrations (20, 40, 60, 80, and 95% v/v) while maintaining other parameters (60 sec. and 900 watts) constant. Total phenolic content increases as we increase CM from 20–95% (v/v). and also TFC yield followed the same pattern (Fig.2A). The TAC showed better scavenging activity at 95% (v/v) CM. In previous studies, methanol concentration in water has also been reported to affect the TPC in the extract (Aryal et al., 2019). According to the obtained data, a range between 60 % and 95% (v/v) methanol was selected for RSM, and 95% (v/v) was used in the subsequent experiments.

Effects of methanol concentrations: Methanol offers various advantages over other solvents, including higher extraction ability in the above-mentioned compound extract, faster extraction, slighter toxicity, and lesser cost, and it is the most often used solvent in phytochemical extraction. Several studies have suggested that when we use methanol as extraction solvent the yield of low molecular weight polyphenols are high whereas in aqueous acetone extraction yield of higher-molecular-weight flavanols were obtained. Our results also showed that methanol is a suitable solvent for polyphenol extraction.

Effects of Extraction Time: The effect of various time intervals on the extraction efficiency was studied, and extraction was carried out at 30, 60, 90, and 120 s under extraction conditions of 900 W and 95% (v/v) methanol. Fig. 2b shows that the TPC and TFC yields were highest in the 90s compared with other time durations. Antioxidant activity also followed the same pattern as TPC and TFC. TPC and TFC yields increased when time was increased from 30 s to 90 s but slightly decreased at 120 s, while the highest activity for antioxidants was recorded at 90 s. Therefore, antioxidant capacity was also observed low by exceeding time more than 100 s which further reduces the total phenolic content and total flavonoids A longer extraction time degrades the antioxidant from the extract. After a certain time limit, phenols and flavonoids decompose and their content in the final extract reduced. (Michiels et al., 2012). The optimized time range was selected as 60–90 s for further optimization.

Effects of microwave power. Microwave power significantly affect the efficiency of extraction procedures. The efficiency of phytochemical extraction depends on the volumetric heating of plant cells (Flórez et al., 2015). After optimization of two variables 95% methanol(v/v) and 60 s, now the effect of P at

different watts 360, 540, 720, and 900 was evaluated. In Fig. 2c it is clearly observed that as the P increased from 360 to 540 W, the TPC sharply increased from 18.5 to 33.8 (mg GAE/g) and TFC also changes from 12.5 to 22.8 (mg QE/g). As we proceed further to increase P to 720 W, TPC and flavonoid levels increased slightly to 33.4 (mg GAE/g) and 23.4 (mg QE/g). By increasing P to 900W, the TPC and TFC were reduced. It shows that the P generate thermal effect on the extraction process. Our results are similar with another study given by Le et al. (2019) which stated that specifically, heating effect of microwave results in decomposition of phenols and flavonoids. Therefore, 540–900 W was selected for applying RSM for proper optimization of the extraction method.

3.2.2. BBD Method optimization

3.2.2.1. Fitting model

RSM was executed to find the best settings for the optimization of the extraction procedure. Numerous reports are documented on the RSM which highlights the effects of different factors such as solvent concentration, particle size, liquid-solid ratio, and extraction time on the antioxidant component yields of some plant extracts (Huang et al., 2020; Pelalak et al., 2021; Sankaran et al., 2023). To determine the quadratic model of the experiments Analysis of variance (ANOVA) was to check the significance of the model based on their F-value and p-value (Rheem et al., 2017). Model stability was determined by comparing the predicted and adjusted values of R² based on their difference, If the difference is it ensures the model reliability. A p-value should be less than 0.05, if it is higher than 0.05 then it was not significant. Table 5 presents responses for each model presenting its significance. All p-values were obtained less than 0.05. High significance was shown for TPC (F-value = 16.83; p < 0.005), followed by TFC (F-value = 12.31; p < 0.0035) and antioxidant capacity (F-value = 8.71; p < 0.004). Each response exhibited the lack of fit value for each response that determine the model was effective. All responses showed R² and adjusted R² of 1.0, which were pretty similar. The outcomes shows that the statistical models were good.

Table 5: Experimental model showing value of Analysis of variance (ANOVA)

Model	TPC	TFC	Total antioxidant capacity
F-value	16.83	12.31	8.71
p-value	0.006	0.0037	0.0047
R ²	0.955	0.9129	0.9181
Adjusted R ²	0.911	0.899	0.8992
Lack of fit	325.97	283.12	91.36

3.2.2.2. Conditions for optimal extraction

TPC from *Vitex negundo* leaf extract using the MAE method were obtained in a range of 105 to 227.6 mg GAE/g with a mean of 147.00 mg GAE/g. Experimental run -13, 95% (v/v) CM, 720 W P, and T of 90 s showed best suited conditions as the highest yield of TPC (227.6 mg GAE/g) was observed. The p-value of CM was highly significant (p < 0.1), followed by T and P (Table 6). It was also confirmed by the quadratic model, CM (A2) and T (C2) were significant (p < 0.0001). Interaction studies among variables (AB, AC and BC) posed a significant impact on TPC (p < 0.05).

Table 6: Quadratic model fitting for TPC, TFC, and TAC of the extract obtained by the microwave-assisted method.

		TPC		TFC		Total Antioxidant				
Parameter	df	Estimated Coefficients		Prob p-vale	Estimated Coefficients		Prob p-value	Estimated Coefficients		Prob p-value
Linear										
A	1	75.00		0.0070	75.00		0.0082	-8.66		0.0012
B	1	23.79		0.0525	23.79		0.2850	-0.2675		0.0756
C	1	93.04		0.0021	93.04		0.0027	-0.8425		0.2490
Interaction										
AB	1	-19.63		0.0225	-19.63		0.2211	1.17		0.0310
AC	1	-8.48		0.0432	-8.48		0.0790	1.26		0.0169
BC	1	25.00		0.0973	25.00		0.0181	-11.88		0.0014
Quadratics										
A ²	1	113.27		0.0001	113.27		0.0052	11.12		0.0018
B ²	1	-20.80		0.0477	-20.80		0.4866	-0.3260		0.0899
C ²	1	123.25		0.2408	123.25		0.0082	-0.3410		0.2849

When we increased methanol concentration from 60 % to 95% (v/v), the yield of TPC in the extract also enhanced. Figure 3(a-c) shows the interactions among variables. In quadratic interaction, methanol concentration showed significance with extraction time but it was non-significance ($p > 0.05$) with microwave power. It could also be confirmed by other studies that increase in polarity also enhance the phenolics extraction. (Kaczorová et al., 2021; Shi et al., 2022). At 60%(v/v), the yield of TPC is low and it shows increasing pattern as polarity increased. Maximum TPC was obtained at 90%(v/v). In case of microwave power, TPC reached its maximum at 720 W and slightly decreased as microwave power increased (Figure 2b). It may justify as the thermal degradation of phytochemicals at higher microwave power is due to generation of heat. It could be too strong for plant cells, causing the breakdown of phytochemicals (Chamutpong et al., 2021; Ismail-Suhaimy et al., 2021). The extraction time also influenced the yield of TPC (Figure 2c). Generally, it is observed that the quantity of analytes in a sample is improved by increasing the extraction time. It was observed that 90 s of irradiation time in this study was better for obtaining a good yield of TPC. These results were in agreement with other studies in which increasing extraction time also increased the yield of TPC (Moyo & Tavengwa, 2023; Tomasi et al., 2023).

Figure 3(d-e) shows the interactions among variables, in which the quadratic and interactions of methanol concentration and microwave power were significant, but it was nonsignificant in the case of extraction time (p value $> .005$) on the yield of flavonoids. TFC was increased as microwave power reached from 360 to 900 W. As heating increased to 900 W, there was a slight decrease in TFC. A power of 720 W was found to be suitable for the maximum yield of TFC. An increase in the rate of heating tends to degrade the compounds over prolonged exposure to microwave power (Ma et al., 2009).

TAC response was also studied with the help of the model and it was observed that the TAC of *Vitex negundo* leaf extract was obtained in the range of 69.3 % to 106.43% with a mean of 87.16%. Experimental run no. 4 under extraction conditions of 60% (v/v) CM, 720 Watt P, and T of 60 s was found suitable for obtaining the highest antioxidant yield (106.43%) (Fig.3g-i). Analysis of variance study showed that only methanol(A) is showing significant linear effects ($p < 0.0020$) as compared to (B) and (C). The antioxidant activity increased with increasing CM, indicating that it was more effective at a lower P and lower T. Antioxidant activity results correlate with total TFC and TPC in the extract. We found the same optimal conditions in terms of CM, T, and P as in TFC and TPC. This may be because the antioxidant potential of any plant extract is correlated with the presence of total phenols and flavonoids.

Our results similar to other findings which discuss the role of polarity of the solvent which increases the phenolic compounds solubility and in turn increases the antioxidant activities. Antioxidant activity increases and then decreases with an increase in P and prolonged T. The extract with high antioxidant activity was correlated with high TPC and TFC values, indicating a correlation between antioxidant activity and polyphenol content. (Rani et al., 2018).

3.3. FTIR analysis

Flavonoids are hydroxylated phenolic compounds that possess antioxidant properties and have the potential to treat diseases caused by free radicals. The infrared spectra of *Vitex negundo* were recorded by Perkin Elmer Spectrum IR version 10.6.0 Fourier transform infrared spectroscopy (FTIR) and run in the IR range of 400-4000 cm^{-1} as shown in figure 5. The band at 638.73 cm^{-1} indicated C=C bending of alkenes. Bands identified at 1032.81 cm^{-1} and 1055.32 cm^{-1} represent C-N stretching of amine and C-F stretching of fluoro compounds, respectively. The band at 1381.75 cm^{-1} showed O-H bending of alcohols and phenols and C-F stretching of fluoro compounds. C-H bending of alkanes, C=N stretching imine/oxime, or C=O stretching conjugated ketone or alkenes were also observed at 1636.24 cm^{-1} .

The band at 2929.02 cm^{-1} represents intramolecular bonded alcohol OH stretching and at 3437 cm^{-1} shows the presence of carboxylic acid with an O-H group. The stretching and bending vibrations at the abovementioned wavenumber represent the presence of amino acids, nitrates, alkenes, and OH groups.

The OH functional group have significant role in antioxidant and antibacterial activities. As in a previous study, they showed that the antibacterial effect of curcumin analogs was very much dependent on the aromatic hydroxyl group. Hydroxycurcumin with an additional aromatic hydroxyl group on the curcumin scaffold showed antibacterial activity against all pathogens tested and remained effective even against ampicillin-resistant *Enterobacter cloacae* (M. K. Kim et al., 2012).

Polyhydroxylated natural phenolic compounds, especially those with low molecular weights, are also characterized by their ability to eliminate free radicals as they act as strong antioxidants (Ali Al-Mamary & Moussa, 2021).

3.4. Gas chromatography –mass spectrometry analysis

The optimization of extraction was done by applying RSM, and GC-MS analysis was carried out for the identification of bioactive compounds in *Vitex negundo* methanolic leaf extract. The GC– MS profile of the methanolic extract of *Vitex negundo* leaves is shown in Fig. 5. The key constituents recognized in the extract are described in Table 7. Compounds in the extract were identified on the basis of their retention times. Interpretation of the mass spectrum was conducted National Institute of Standards and Technology (NSIT) database. GC-MS chromatogram of the methanolic extract of *Vitex negundo* recorded 9 peaks which shows different types of bioactive compounds present in the extract. The major components in *Vitex negundo* extract were 2-propanon-1-hydroxyacetone, 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester, 2,7-dioxatricyclo[4.4.0.0(3,8)]dec-4-ene,benzofuran, phenol, phen-1,4-diol, 2,3-dimethyl-5-trifluoromethyl, 1H-naphtho[2,1-b]pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-, [3R-(3a,4aβ,6aa,10aβ,10ba)]-, 5-hydroxy-methyl furfural, and 5- (7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-enal.

Identified compounds have been reported as antioxidant, antimicrobial, and anti-inflammatory by previous studies. These compounds have different chemical natures, such as phenolic compounds, esters, alkanes, aldehydes, alkenes, and ketones. All these bioactive compounds could be responsible for the antioxidant, antibacterial, and anti-inflammatory properties of *Vitex negundo* leaf extract. Compound 1,2-benzenedicarboxylic acid has been reported as an anti-inflammatory, antiproliferative, and strong antioxidant agent. 1,2-benzene dicarboxylic acid, bis (2-methyl propyl) ester (BDCE fraction). The BDCE fraction was evaluated for its antiproliferative potential against human osteosarcoma MG-63, human neuroblastoma IMR-32, and human lung carcinoma A549 cell lines (A. Kumar et al., 2022). The role of 5-hydroxyfurfural has also been studied as an anti-inflammatory in lung diseases. In this study, it was concluded that the 5-HMF attenuated lipopolysaccharide (LPS) induced ALI has protective effect on endotoxin induced acute lung injury in mice mitigating alveolar destruction. (Zhang et al., 2021). In another study,%-hydroxyfurfural was isolated from Cola hispid, and it was reported as a potential lead for novel drug development for the management of oral cancer and inhibition of a multidrug-resistant strain of *S. aureus* (Onoja, 2021). Several other medicinal plants have similar phytoconstituents, as obtained in our study, in their methanolic and ethanolic extracts that are responsible for antibacterial, anti-inflammatory, and antioxidant potential (Ochieng Nyalo et al., 2022). Cyclic compounds are unsaturated, so they are responsible for their antioxidant potential (AlMousa et al., 2022; Naz et al., 2020).

Table 7: Biologically active plant components recognized in the methanol extracts of *Vitex negundo* leaves using gas chromatography– mass spectrometry.

Peak No.	Retention time (RT)	Name of compound	Molecular formula	Molecular weight (g/mol)	Structure of the compound	Mass peak fragments
1	15.02	2-propane-1-hydroxyacetone	C ₃ H ₆ O ₂	74.08	CC(=O)CO	15,18,31,43,57,68
2.	17.4	1,2-Benzenedicarboxylic acid and bis(2-methyl propyl) ester	C ₁₆ H ₂₂ O ₄	278.34	CC(C)COC(=O)C1=CC=CC=C1C(=O)OCC(C)C	57,65,76,93,104,121,132,149, 167,189,205,223
3.	20.4	Benzofuran	C ₈ H ₆ O	118.10	C1=CC=C2C(=C1)C=CO2	29,39,51,6374,90
4.	21.74	2,7-Dioxatricyclo[4.4.0.0(3,8)]dec-4-ene	C ₈ H ₈ O ₂	138.15	C1=CC(=CC(=C1)C=O)CO	27,39,44,53,6681,94,109,120
5.	23.93	Phenol	C ₆ H ₆ O	94.11	C1=CC=C(C=C1)O	27,39,55,66,74
6.	23.77	Phen-1,4-diol, 2,3-dimethyl-5-trifluoromethyl-	C ₉ H ₉ F ₃ O ₂	206.16	COC1=CC=CC(=C1)C(C(F)(F)F)O	43,57,68,77,83,91,101,123,141149, 165,175,190
7.	26.54	1H-Naphtho[2,1-b]pyran, 3-ethenyldodecahydro-3,4a,7,7,10a-pentamethyl-, [3R-(3a,4aβ,6aa,10aβ,10ba)]-	C ₂₀ H ₃₂ O	290.47	CCC1(CCC2C1(CCC3C2CC=C4C3CCCC4)C)O	29,43,55,67,81,95,109,123,137,149163, 177,192205,220,230,245,257,275
8.	28.77	5-hydroxy-methyl furfural	C ₆ H ₆ O ₃	126.11	C1=C(OC(=C1)C=O)CO	29,41,53.69,81,97,109
9.	28.91	5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-enal	C ₂₀ H ₃₄ O	290.5	CC(=CCCC(=CCCC(=CCCC(=CCO)C)C)C)C	29,42,5569,81,95107,121,135,149, 161,177,191205,217,231245,273

3.5. Molecular Docking Simulations

3.5.1. ADMET Analysis

The pharmacokinetic efficiency and oral bioavailability of 9 compounds from *Vitex negundo* Linn. were assessed using Lipinski's Rule of Five and Veber's Rule. The data for each compound were retrieved from the SwissADME webserver. All compounds showed good oral bioavailability (Table 8). 2 of 9 compounds showed exactly one violation of Lipinski's Rule of Five (Bolenol and geranyl geraniol). No compounds showed any violations of Veber's rule. Based on this, we suggest that all the extracted phytoconstituents have good oral bioavailability and drug likeliness.

The toxicity assessment (Table 9) suggested 2 out of 7 compounds (Benzofuran and Phenol) belonging to Class III were toxic if swallowed, whereas 3 compounds (3-formylbenzyl alcohol, Trifluoro methoxyphenyl ethanol, and bolenol) predicted to be Class IV were harmful if swallowed. 3 compounds (hydroxyacetone, 5-hydroxymethoxy furfural, and geranyl geraniol) were predicted to be in Class V. These compounds having predicted LD50 values above 2000 were grouped as "maybe harmful if swallowed". Di-isobutyl phthalate was the only compound to be predicted in Class VI and can be considered non-toxic. Di-isobutyl phthalate and bolenol were also identified to be active in the estrogen receptor alpha-mediated toxicological pathway. Bolenol was also predicted to be highly active in androgen receptor (AR) and androgen receptor-ligand binding domain (AR-LBD) mediated toxicological pathways, along with immunotoxicity. Bolenol was mildly active in ER-ligand binding domain (ER-LBD) mediated toxicology. In addition, benzofuran was predicted to be highly active in carcinogenicity, whereas trifluoro methoxyphenyl ethanol was predicted to be highly active in immunotoxicity. Moreover, dibutyl phthalate, trifluoro methoxyphenyl ethanol, and 5-hydroxymethoxy furfural were predicted to be mildly active in carcinogenesis. Based on these results, we consider di-isobutyl phthalate, 5-hydroxymethoxy furfural, geranyl geraniol, and hydroxy acetone to have low toxicity. Thus, these phytocompounds are potential drug-like candidates.

3.5.2. Molecular Docking Simulations

GC-MS analysis revealed that *Vitex negundo* extracts contained 9 compounds that are biologically active. These all nine were analyzed for their activities against antioxidant and anti-inflammatory target proteins. Docking studies performed for phytocompounds using AutoDock Vina to explain the binding affinities to the selected target proteins. Binding energies and non-bonding interactions were studied to predict the binding affinity and conformation of every phytocompound with each target protein. Lowest binding energy interaction was determined among various conformations to suggest best binding conformation. All four phytocompounds (5-hydroxy methoxy furfural, propane-2-hydroxyacetone (hydroxy acetone), 5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-enal (geranyl geraniol), and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester (di-isobutyl phthalate)) filtered out based on ADME and toxicity analysis showed lower binding affinities (i.e., high inhibitory potential) as well as good non-covalent interactions. Among these four compounds, geranyl geraniol showed the highest binding energy (ranging between -5.2 to -9.2 kcal/mol), followed by di-isobutyl phthalate (ranging between -4.7 to -7.4 kcal/mol), 5-hydroxy methoxy furfural (ranging between -4.0 to -5.4 kcal/mol), and 2-propane-1-hydroxy acetone (ranging between -3.0 to -4.1 kcal/mol) (Table-10).

Table 8: ADME characteristics identified using SwissADME

Compound Name	Hydroxyacetone	Diisobutyl phthalate	Benzofuran	3-formylbenzyl alcohol	Phenol	Trifluoromethoxy phenylethanol	Bolenol	5-hydroxymethyl furfural	Geranyl geraniol
Heavy atoms	5	20	9	10	7	14	21	9	21
Aromatic heavy atoms	0	6	9	6	6	6	0	5	0
Rotatable bonds	1	8	0	2	0	3	1	2	10
H-bond acceptors	2	4	1	2	1	5	1	3	1
H-bond donors	1	0	0	1	1	1	1	1	1
MR	17.9	77.8	36.21	37.96	28.46	44.06	90.26	30.22	97.52
TPSA	37.3	52.6	13.14	37.3	20.23	29.46	20.23	50.44	20.23
iLOGP	0.9	3.31	1.9	1.43	1.24	2.07	3.72	0.91	4.75
Lipinski #violations	0	0	0	0	0	0	1	0	1
Veber #violations	0	0	0	0	0	0	0	0	0

The two-dimensional interaction between the ligands and targets suggested that protein– ligand interactions are stabilized by conventional hydrogen bonds, pi-alkyl, pi-sigma, and pi-pi interactions. Based on this, we state that all of these compounds have a high potential to act as antioxidant and anti-inflammatory agents. The in silico ADMET studies suggested that 5-hydroxy methoxy furfural, 2-propane-1hydroxyacetone (hydroxyacetone), geranyl geraniol, and di-isobutyl phthalate have good orally bioactive drug-like characteristics. The molecular docking simulation studies indicate that all of these compounds have a high potential to act as antioxidant and anti-inflammatory agents because of their low binding energies and multiple non-covalent interactions. However, to better understand the structural consequences of these potential inhibitors, molecular dynamics simulation studies and experimental designs are further required.

Table 9: Toxicity analysis using ProTox-II

Compound	Hydroxyacetone	Diisobutyl phthalate	Benzofuran	3-formylbenzyl alcohol	Phenol	Trifluoromethoxy phenylethanol	Bolenol	5-hydroxymethyl furfural	Grenyl geraniol
Predicted LD50	2200	10000	196	1630	270	800	667	2500	5000
Predicted Toxicity Class	5	6	3	4	3	4	4	5	5
Avg Similarity	100	100	64.32	96.43	100	64.27	100	100	100
Prediction Accuracy	100	100	68.07	72.9	100	68.07	100	100	100
Hepatotoxicity	-	--	-	-	-	-ve	-	-	-
Carcinogenicity	--	--	+	-	-	++	-	++	-
Immunotoxicity	-	-	-	-	-	+	+	-	-
Mutagenicity	-	-	-	-	-	-	-	-	-
Cytotoxicity	-	-	-	-	-	-	-	-	-
AhR	-	-	-	-	-	-	-	-	-
AR	-	-	-	-	-	-	++	-	-
AR-LBD	-	-	-	-	-	-	++	-	-
Aromatase	-	-	-	-	-	-	--	-	-
ER	-	-	-	-	-	-	++	-	-
ER-LBD	-	-	-	-	-	-	++	-	-
PPAR-Gamma	-	-	-	-	-	-	-	-	-
nrf2/ARE	-	-	-	-	-	-	-	-	-
HSE	-	-	-	-	-	-	-	-	-
MMP	-	-	-	-	-	-	++	-	-
TP53	-	-	-	-	-	-	-	-	-
ATAD5	-	-	-	-	-	-	-	-	-

-: inactive, +: Active, ++:

Table 10: Binding energies of nine phytocompounds against tested target proteins

S. No.	Targets	SOD1 (5YUL)	TNF- α (7JRA)	GSR (2AAQ)	GPX7 (2P31)	CAT (7P8W)	PTGS2 (5F1A)	INF- γ (3BES)
	Compound Name	Binding affinity (kcal/mol)						
1.	2-propane-1-hydroxyacetone	-3.3	-3.7	-3.6	-3.3	-3.8	-4.1	-3.0
2.	1,2-Benzenedicarboxylic acid and bis(2-methylpropyl) ester	-5.1	-5.2	-6.6	-4.7	-7.3	7.4	-5.6
3.	Benzofuran	-4.4	-5.1	-5.3	-4.2	-6.4	-6.1	-4.9
4.	2,7-Dioxatricyclo[4.4.0.0(3,8)]dec-4-ene	-5.1	-5.5	-5.5	-4.4	-6.3	-6.2	-4.8
5.	Phenol	-4	-4.8	-4.5	-3.6	-5.2	-5.7	-4.2
6.	Phen-1,4-diol, 2,3-dimethyl-5-trifluoromethyl-	-5.3	-6.1	-6.5	-4.8	-7.2	7.8	-5.9
7.	1H-Naphtho[2,1-b]pyran, 3-ethenyl-dodecahydro-3,4a,7,7,10a-pentamethyl-, [3R-(3 α ,4 α ,6 α ,10 α ,10b α)]-	-6.4	-7.1	-7.6	-6.4	-8.2	-8.2	-7.3
8.	5-hydroxymethoxyfurfural	-4.5	-5.3	-4.9	-4.0	-5.4	-5.4	-4.2
9.	5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-pent-2-enal	-5.4	-5.2	-6.2	-5.8	-9.2	-6.6	-6.2

3.6. Antibacterial activity

Vitex negundo leaf extract was investigated to evaluate its antibacterial activity against two strains of Gram-positive bacteria (*Escherichia coli* MTCC443 and *Bacillus sphaericus* MTCC7542) and two gram-negative bacteria (*Pseudomonas aeruginosa* MTCC 2474 and *Pectobacterium carotovorum* MTCC 1428) using the disk diffusion method. The zone of inhibition by plant extracts is illustrated in figure 6. The results revealed that *Vitex negundo* plant extract was potentially effective in suppressing bacterial growth with variable potency. The highest zone of inhibition was against *B. sphaericus* (19 $\pm$ 0.87mm) and *E. coli* (19 $\pm$ 0.64mm) at a concentration of 10 mg/ml.

3.6.1. MIC and MLC of plant extract

To see the bacteriocidal and bacteriostatic effect of plant extract was evaluated by MIC and MBC by the microtiter broth dilution method. The concentration effect of the plant extract is reported in Table 11. The inhibitory effect of *Vitex negundo* started at 5%, whereas at a concentration of 10%, bacterial growth was completely suppressed. The MIC and MBC of the were equal in the case of *E. coli* with a mean concentration of 1250 μ g/ml. the MIC and MBC for *B. sphaericus* and *P. carotovorum* were 2500 μ g/ml and 5000 μ g/ml, respectively. As mentioned in table 6, the methanol extract of *Vitex negundo* had the maximum and equal MIC and MBC against *Bacillus sphaericus* and *P. carotovorum*. However, in the case of *E. coli*, the MIC and MBC values were minimal. From the above findings, it might be concluded that the *Vitex negundo* leaf extract works well on *E. coli* and a broad spectrum of bacteria. The MIC is not the same as MBC against the bacteria, which means that the leaf extract shows a bacteriostatic effect, not a bacteriocidal effect. The same can be validated by calculating the MBC/MIC ratio, and in the case of all bacteria, this ratio is 2. If the MBC/MIC ratio is less than 2, then the extract shows a bacteriocidal effect; otherwise, it shows a bacteriostatic effect. In the present study, the MBC/MIC ratio was 2, and from this, it could be concluded that the leaf extract showed a bacteriostatic effect.

Table 11: MIC and MBC in (μ g/ml) *Vitex negundo* plant extract against bacteria

Microorganisms	<i>Vitex negundo</i> leaf extract		MBC/MIC
	MIC (μ g/ml)	MBC (μ g/ml)	
<i>Bacillus sphaericus</i>	2500 $\pm$ 0.02	5000 $\pm$ 0.03	2
<i>Escherichia coli</i>	1250 $\pm$ 0.03	2500 $\pm$ 0.06	2
<i>Pectobacterium</i>	2500 $\pm$ 0.04	5000 $\pm$ 0.03	2

Previously, research has been conducted on *Psidium guajava*, *Salvia officinalis*, *Ziziphusspina Christi*, *Morusalba* L., and *Olea europaea* L leaf extracts to evaluate the antimicrobial effects on several Gram- positive and Gram-negative bacteria. They found that some leaf extracts showed bacteriocidal and bacteriostatic effects against microbes (Hemeg et al., 2020). In another study, the antibacterial effect of *Sida rhombifolia* was evaluated against *E. coli*, *S. aureus*, *S. typhi*, *Citrobacter*, and *K. pneumoniae*, and in this study, the extract showed both bacteriostatic and bacteriocidal effects (Debalke et al., 2018). Therefore, the present study results are in line with the previous study.

4. Conclusion

The significance of natural herbal formulations extends beyond their therapeutic efficacy. Sustainability is a key consideration in an era where environmental concerns are paramount. This multifaceted approach not only addresses specific health concerns but also promotes overall wellness. Secondary

metabolites in plants have revealed a good source of bioactive compounds with remarkable antioxidant and antibacterial potential. Our investigation has shed light on phytochemical extraction optimization, screening, and the profound potential of *Vitex negundo* methanolic extract. The optimization of the extraction process in medicinal plants is a pivotal step for making therapeutically sound herbal formulations. Through systematic experimentation and analysis, our study successfully identified optimal extraction conditions for microwave-assisted extraction of *Vitex negundo* leaf extract through RSM. Furthermore, GC-MS profiling and FTIR analysis confirmed the presence of bioactive compounds that are having specific biological activities and potential therapeutic effects. The results of the present investigation indicate the antioxidant, antibacterial, and hemolytic properties of the methanolic extract of *Vitex negundo*. Molecular docking study helps us to predict that out of nine compounds, four have promising binding affinity toward the binding sites of target proteins of oxidative and inflammatory conditions. In the future, these compounds can be validated by other simulation methods of protein model. Experimental studies on animal models also assist to confirm its status as a novel compound. Thus, the knowledge of the current work not only advances our understanding of traditional medicinal practices but also propels us toward the development of effective, safe, and standardized herbal remedies with broad applications in the healthcare industry.

Declarations

Author contribution statements

Eti Sharma: Conceptualization, Validation, Original Draft., Nisha Gaur: Data Curation, Writing - review & editing., Sandeep Singh: Performed designed experiments, Kartavya Mathur: Bioinformatics data analysis, Krishan Kumar: GC-MS and FTIR data analysis, Nitin Wahi: Second draft Editing, Gunjan Garg: Final editing & overall guidance

Conflict of Interest

The authors affirm that they have not any competing financial interests and also no personal relationships that could influence the work described in this paper.

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Figures

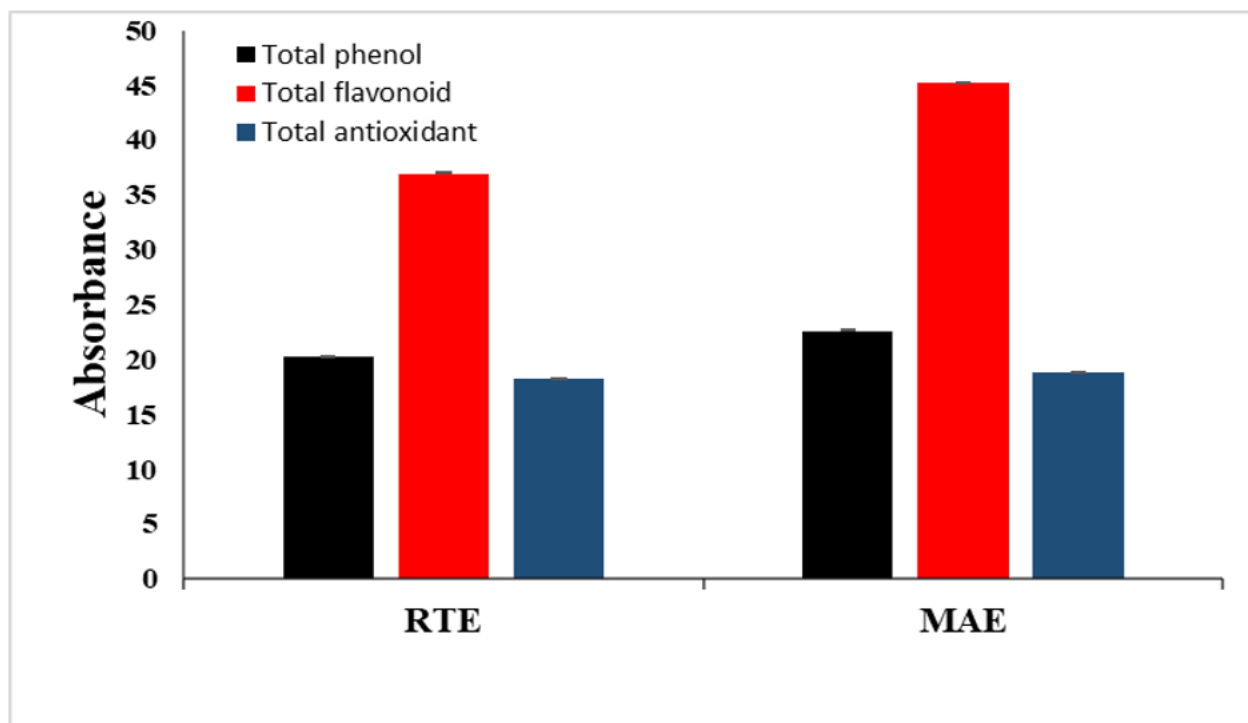


Figure 1

Estimation of TPC, TFC, and TAC in vitex negundo leaf extract using different extraction methods

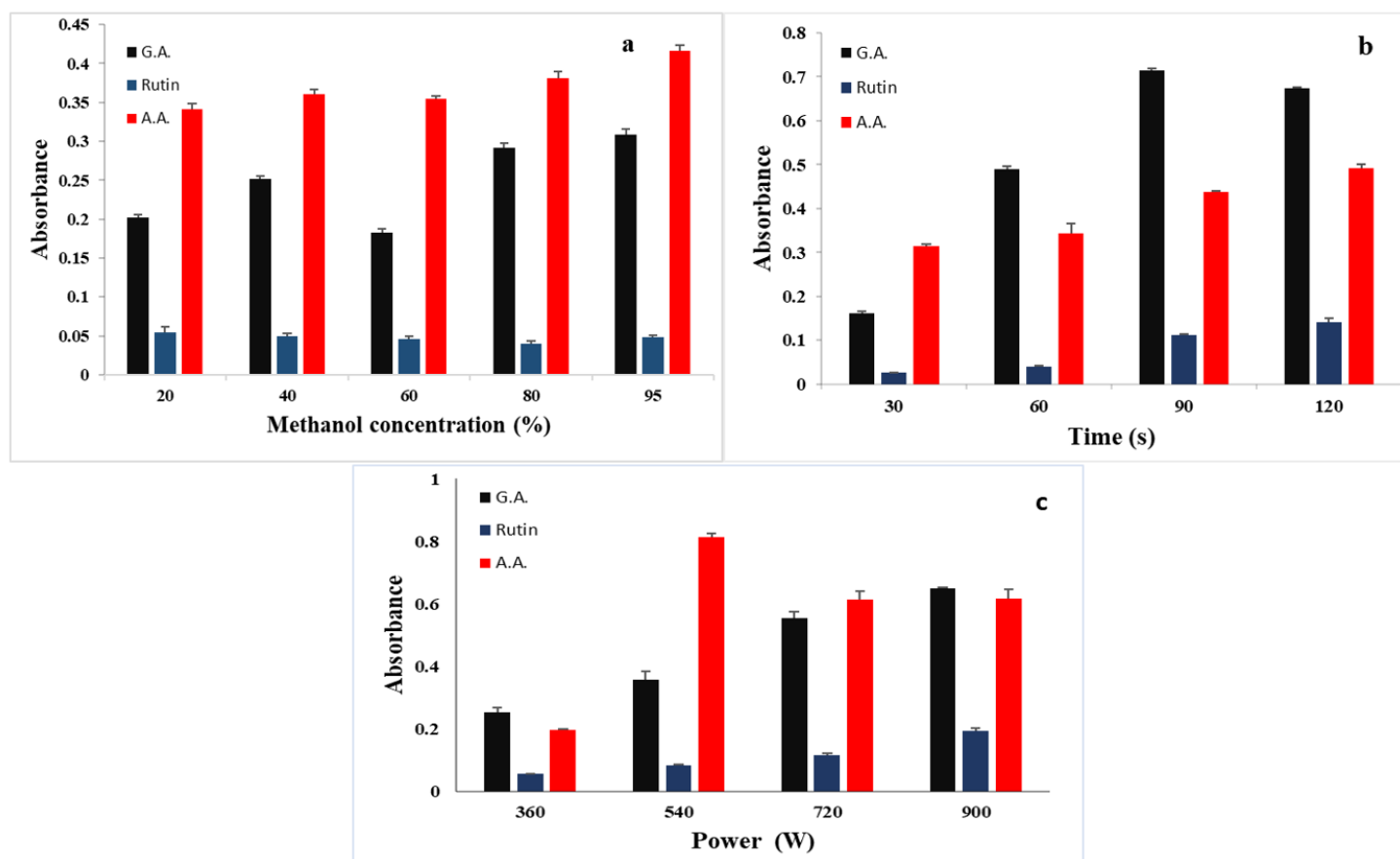


Figure 2

Effects of (a) methanol concentrations, (b) extraction time, and (c) microwave power on the TPC, TFC, and TAC of VN leaf extract

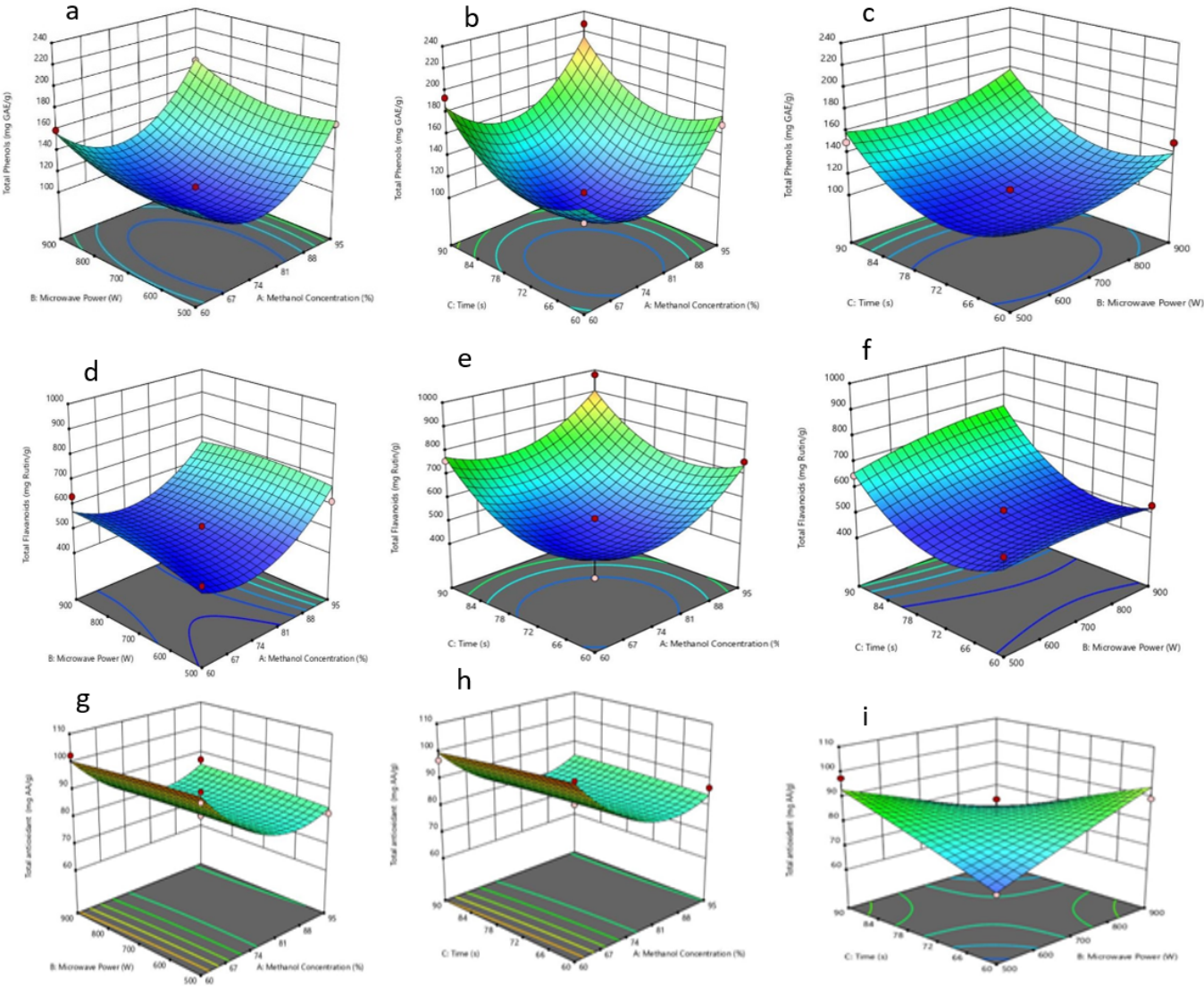


Figure 3

3D plots showing the interaction effect on (a-c)TPC, (d-f)TFC, and (g-i)TAC, as a function of methanol concentration, time, and microwave power

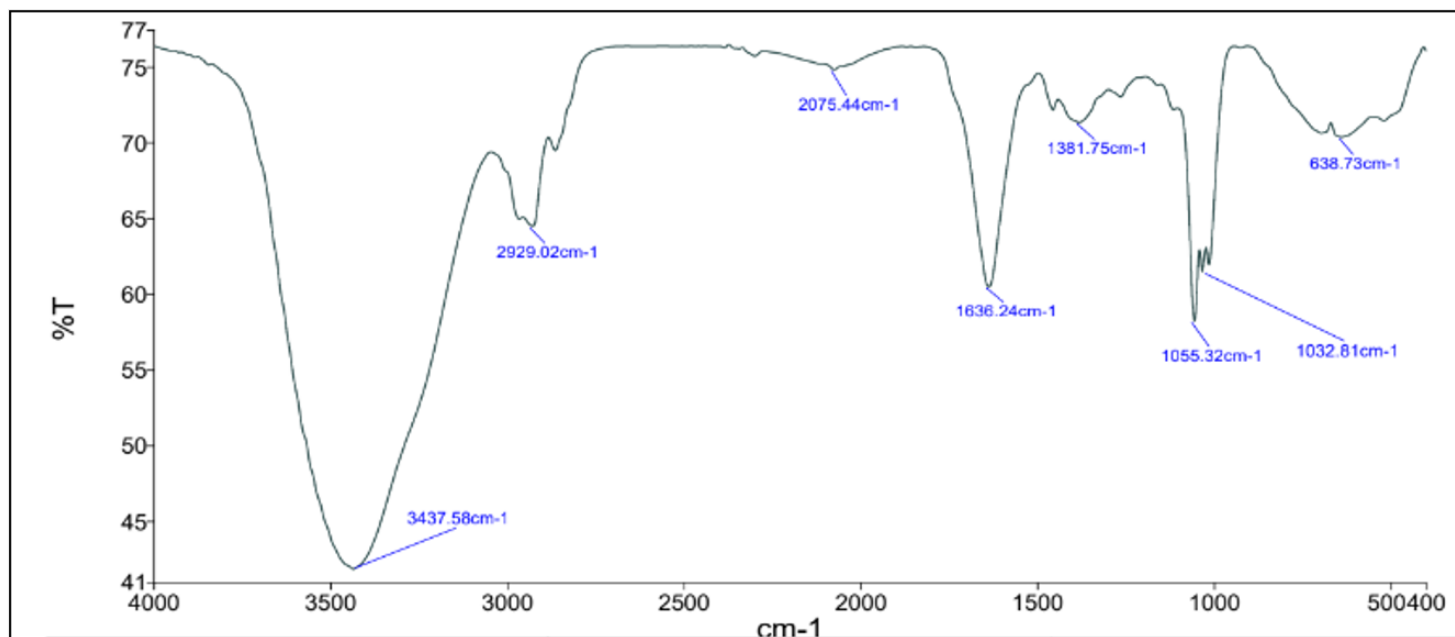


Figure 4

FTIR spectrum of the leaf extract of *Vitex negundo*

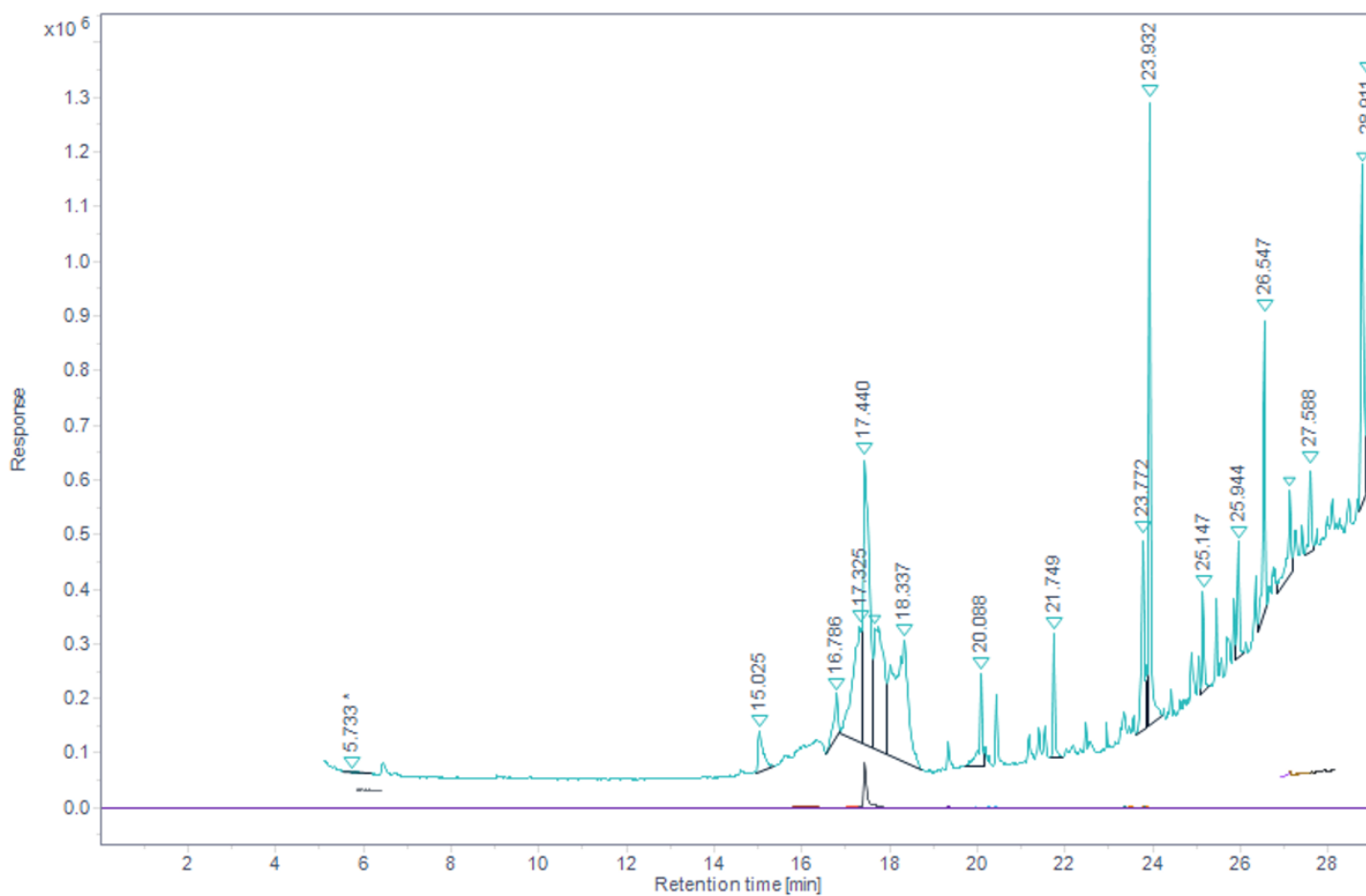


Figure 5

GC- MS chromatogram of *Vitex negundo* leaf extract

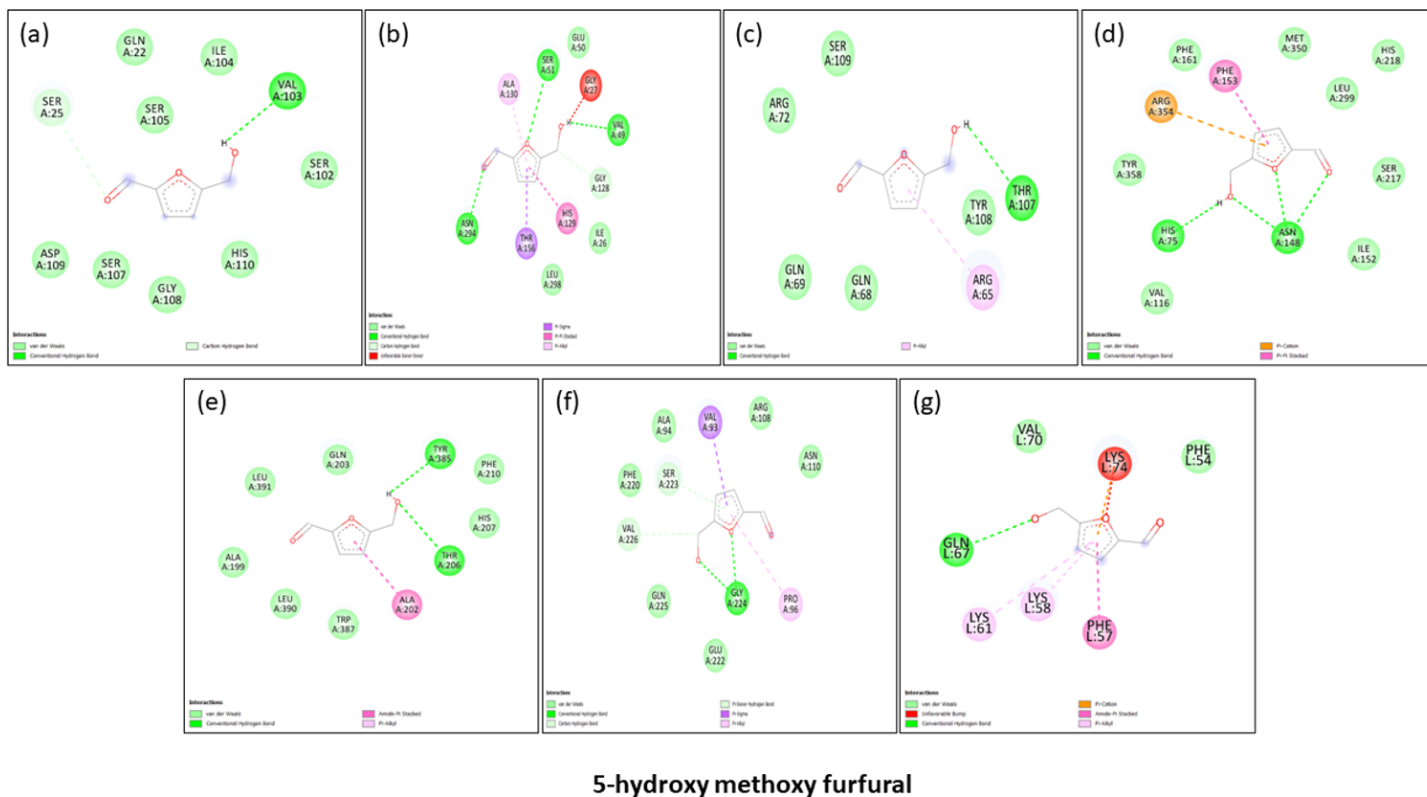


Figure 6

2D interactions of 5-hydroxymethoxy furfural with (a) sodium dismutase (SOD1), (b) glutathione reductase (GSR), (c) glutathione peroxidase (GPX7), (d) catalase, (e) prostaglandin G/S synthase (PTGS2), (f) tumor necrosis factor-alpha (TNFA), and (g) interferon-gamma (IFNG).

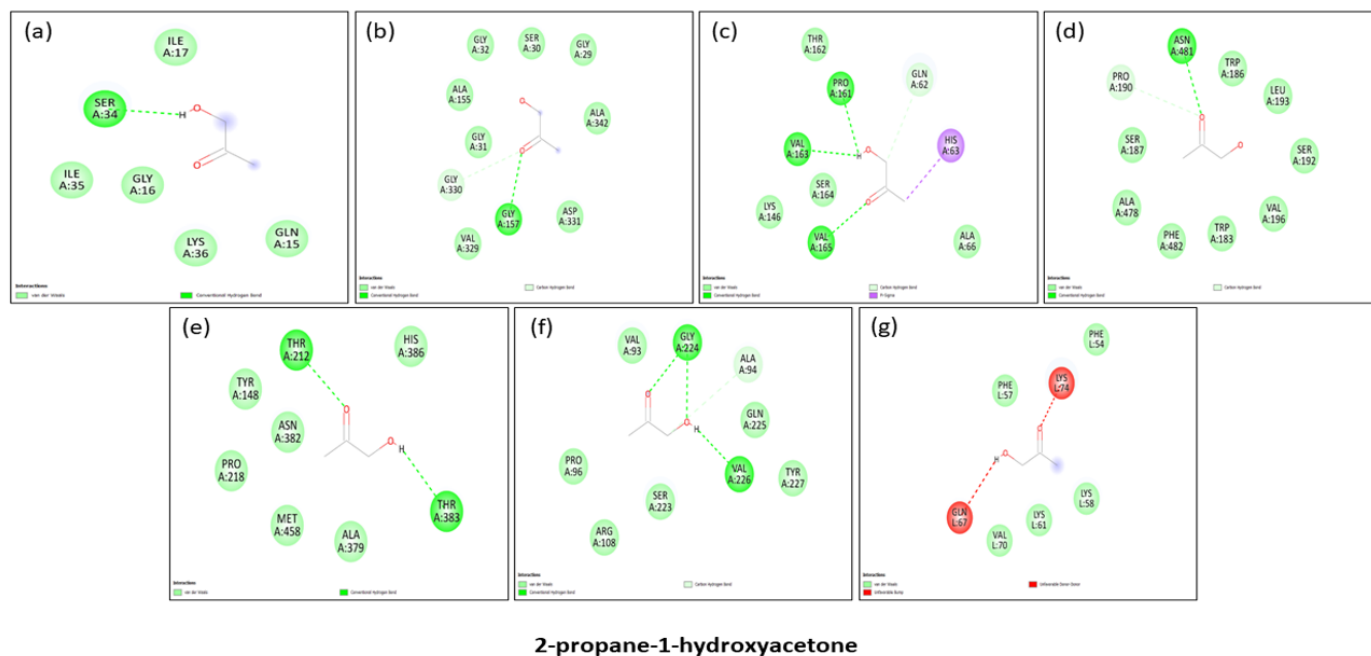


Figure 7

2D-interactions of 2-propane-1-hydroxyacetone with (a) sodium dismutase (SOD1); (b) glutathione reductase (GSR); (c) glutathione peroxidase (GPX7); (d) catalase (e) prostaglandin G/S synthase (PTGS2); (f) tumor necrosis factor-alpha (TNFA); and (g) interferon-gamma (IFNG).

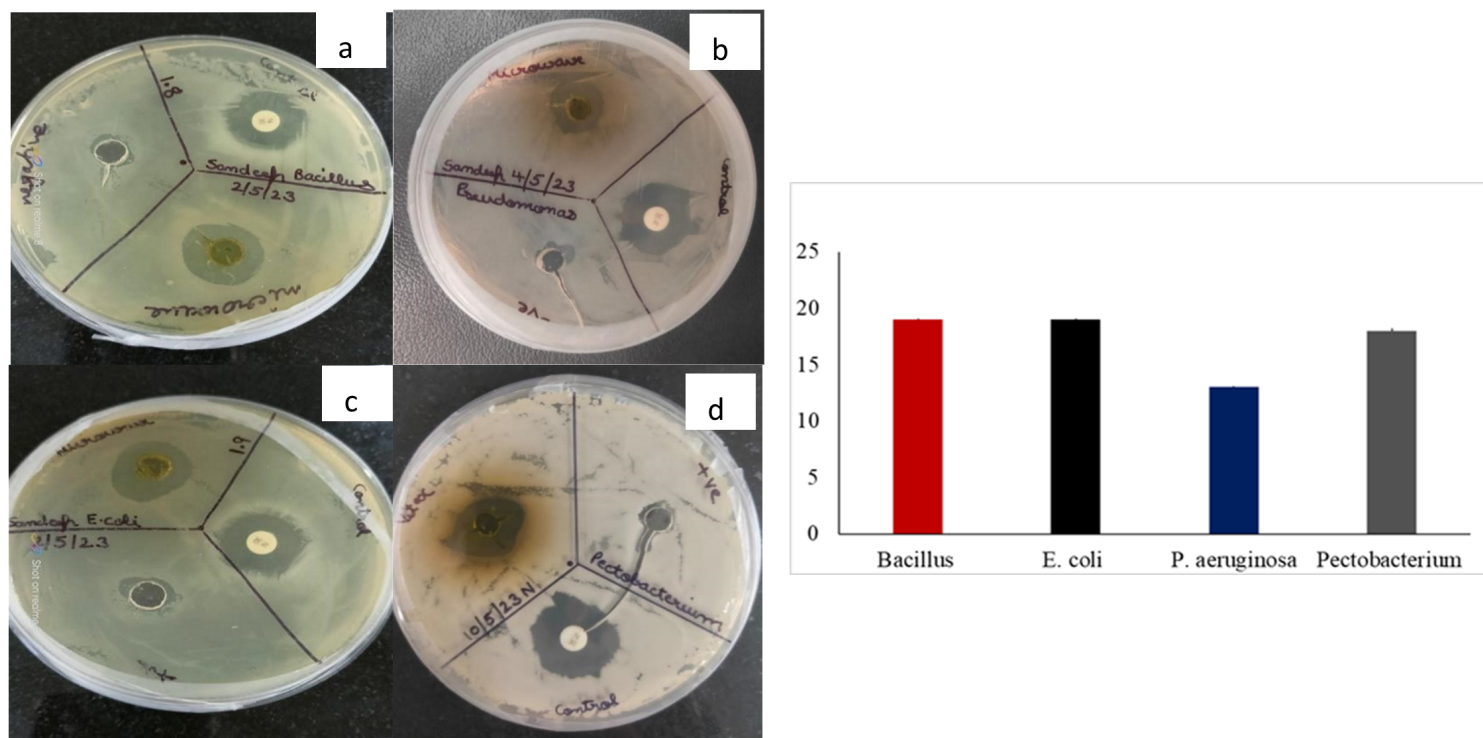


Figure 10

Zone of inhibition test of *Vitex negundo* against (a) *B. sphaericus*, (b) *P. aeruginosa* (c) *E. coli*, and (d) *P. carotovorum*

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GA.png](#)