PLANT SCIENCE TODAY
Vol 12(sp1): 01–09
https://doi.org/10.14719/pst.6903

eISSN 2348-1900

RESEARCH ARTICLE

Anti - inflammatory, antioxidant and antimicrobial activity of *Vitex negundo* and *Hibiscus rosa-sinensis* herbal formulation against oral pathogens

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Received: 25 December 2024; Accepted: 07 June 2025; Available online: Version 1.0: 25 September 2025

Cite this article: Sulochana G, Kaavya S, Rajeshkumar S. Anti - inflammatory, antioxidant and antimicrobial activity of *Vitex negundo* and *Hibiscus rosa-sinensis* herbal formulation against oral pathogens. Plant Science Today. 2025;12(sp1):01–09. https://doi.org/10.14719/pst.6903

Abstract

The aim of the present study was to determine the antimicrobial, anti-inflammatory, antioxidant and cytotoxic activities of *Hibiscus rosa sinensis* and *Vitex negundo*. *Hibiscus rosa-sinensis* Linn. (Family Malvaceae) leaves had been used in ethnomedicine to treat a variety of human diseases including aphrodisiac, hypertension, wound healing, diabetes mellitus and cancer. In traditional medicine, the leaves of *Hibiscus rosa-sinensis* were used to treat dysentery and diarrhoea, to promote the draining of abscesses and as an analgesic. Natural products contained high levels of bioactive compounds that targeted a complex network of proteins involved in a variety of diseases. *Vitex negundo* (*V. negundo*), also known as the "chaste tree," was an ethnobotanical significant plant with powerful medicinal properties. In addition to its anti-diabetic properties, *V. negundo* leaf extract had a beneficial effect on glycoprotein metabolism. Antimicrobial activity was assessed using the agar well diffusion method and MIC assay. Antioxidant activity was evaluated H_2O_2 assay, FRAP assay and DPPH assay. Anti-inflammatory activity was tested using egg albumin denaturation and membrane stabilisation methods. All activities demonstrated very good results for the herbal formulation. Natural products had a high propensity to target specific proteins while causing fewer side effects and lower toxicity and greater effectiveness. Aqueous extract of *V. negundo* and *Hibiscus rosa sinensis* were used as treatment for a variety of diseases and their products have become commercialised, increasing their demand. Overall, the formulation contributed to health protection and showed potential in the management of autoimmune diseases.

Keywords: anti-inflammatory; antimicrobial activity; antioxidant herbal formulation; natural products

Introduction

The vast majority of the world's population used herbal medicines as treatments for various diseases. Polyherbal preparations were formulated from medicinal plants and because they were natural products, they were considered safe. Herbal formulations that gained widespread acceptance as therapeutic agents in India included no tropics, anti-diabetics, hepatoprotective agents and lipid lowering agents. The pharmacological effects of many plants had been studied in various Indian laboratories. However, there were numerous limitations to the safety and efficacy of these preparations (1).

India is one of the nations blessed with a rich heritage of traditional medical systems and biodiversity to supplement the herbal needs of the treatments administered by these systems. Ayurveda, Siddha and Unani were three recognised Indian medical systems that used herbs and minerals in their formulations. India had 15 agro-climatic zones and 4700 plant species, of which 15000 possessed medicinal properties to varying degrees (2).

Hibiscus (Malvaceae) is a genus of herbs, shrubs and trees. Its 250 species were widely distributed in tropical and subtropical regions around the world and were reported to have a variety of medicinal properties. According to research, the plants of the *Hibiscus* genus had the potential to provide biologically active compounds that acted as antioxidants and cardio protective agents (3). As a result, the *Hibiscus* genus was considered a promising natural source for the development of new drugs and offered a cost-effective means of treating cancer and other diseases in the developing world (4). The leaves had been shown to promote hair growth and aid in ulcer healing. Traditional texts agreed that the leaves of *Hibiscus rosa-sinensis* possessed hair growth-promoting and anti-aging properties (5).

Hibiscus leaf extract contained antioxidant compounds that could be used to prevent atherosclerosis in humans through its antihyperlipidemic and anti-LDL oxidation effects. The extract also demonstrated hypoglycaemic activity. Proteins, vitamin C, organic acids (primarily malic acid), flavonoids and anthocyanins were found in leaf extract (6).

Vitex negundo is a member of the (Verbenaceae) family and is commonly known as a five-leaved chaste tree. It was primarily found in tropical to temperate regions, particularly in India, where it had traditionally been used in medicine. It was a large aromatic shrub. The juice from the leaves was used to treat ulcers and joint swelling (7). Secondary metabolites in this plant played an important role in plant-insect interaction and such compounds were found to exhibit hormonal, insecticidal, or anti-feedant activity against insects (8). The presence of alkaloids, steroids, flavonoids, amino acids, phenols, quinones and starch was determined during the phytochemical screening of *V. negundo* (9).

The leaves were primarily used to treat eye diseases, inflammation, leucoderma, toothache, spleen enlargement, skin ulcers, gonorrhoea, rheumatoid arthritis and bronchitis. Additionally, the leaves were used as a vermifuge, lactagogue, tonic, antibacterial, antipyretic and antihistaminic agent. Dried *Vitex negundo* leaves were stored with woollen garments to prevent wool-destroying insects and worms. The indigenous leaves of *V. trifolia* L. were used as a mosquito repellent against *Aedes aegypti* and showed antiparasitic effects. *Vitex* had a significant impact on both male and female reproductive systems. *Vitex* species pharmacological importance included *Vitex agnus-castus, V. rotundifolia, V. negundo* and others (10).

The current study focused on the synthesis of a polyherbal formulation using aqueous extract of *Vitex negundo* and *Hibiscus rosa sinensis.* The antimicrobial efficacy of the herbal formulation was tested against oral pathogens such as *S. aureus, S. mutans, E. faecalis* and *C. albicans* (11, 12). The study aimed to evaluated the antimicrobial, anti-inflammatory, antioxidant and cytotoxic effect of herbal formulation of *V. negundo and Hibiscus rosa sinensis* against oral pathogens.

Materials and Methods

Preparation of plant extract

The herbs *Vitex negundo and Hibiscus rosa-sinensis* leaf were collected from the SIMATS campus in Velappanchavadi, India. To remove dust particles from the leaves, the leaves were cut into small pieces and washed twice or three times with distilled water. They were the slightly dried at room temperature. Individually, 10 g of leaves were weighed and boiled in 100 mL of distilled water for 15 min at 50 - 60 °C. After boiling, the plant extract were filtered through muslin cloth and the filtered extract were concentrated at 3 - 5 mL using a heating mantle. The 5 mL of *Vitex negundo* and *Hibiscus rosa-sinensis* were dried using hot air oven at 50 °C. The dried powder was collected and mixed using distilled water and stored in refrigerator 4 °C before being used in biomedical applications.

Antimicrobial activity

The agar well diffusion method was used to test the antimicrobial activity of synthesised plant extract against oral pathogens such as *E. faecalis, S. mutans, S. aureus* and *C. albicans*. Each strain's fresh overnight culture were uniformly swabbed onto individual plates containing sterilised and cooled Mueller Hinton agar. Wells of 9 mm were bored into agar plates. The herbal extracts were then loaded into the wells at various concentrations (25, 50 and 100 µL). The plates were incubated at 37 °C for 24 hr. Commercial antibiotics (ampicillin) were used as a control. After incubation, the zone of incubation formed around the well were measured to evaluate antimicrobial activity.

MIC assay

To determine the MIC of *V. negundo* and *Hibiscus rosa sinensis* (aqueous extract) against oral pathogens, Mueller Hinton broth was prepared and sterilized to provide a standardized growth medium. Subsequently, 6 mL of broth was dispensed into three test tubes, followed by the addition of an overnight bacterial suspension, with concentrations ranging from 5×10^5 CFU/mL. Various concentrations of *V. negundo* and *Hibiscus rosa sinensis* (6.25, 12.5, 25, 50 and 100 µg/mL) were then added to the test tube. Standard antibiotics - amoxycillin, flucanzole) were included as positive control, while a test tube containing only the microbial culture served as negative control. The test tubes were incubated at 37 °C for 24 hr to assess the inhibitory effects of the extract on bacterial growth. To quantify the inhibitory effect, the percentage of dead cells was calculated at regular intervals by measuring the absorbance at 600 nm. This allowed for the determination of the MIC defined as the lowest concentration of plant extract that inhibited visible bacterial growth. By systematically evaluating the impact of aqueous extracts of *V. negundo* and *Hibiscus rosa sinensis* on *S. mutans, S. aureus, C. albicans* and *E. faecalis* growth over time, this approach provided valuable insights into their antimicrobial efficacy and potential as alternative therapeutic agents against oral pathogens.

Antioxidant activity

DPPH method

The antioxidant activity of a biogenic synthesised *Vitex negundo* and *Hibiscus rosa-sinensis* (aqueous extract) herbal formulation was tested using the DPPH assay. The reaction mixture consisted of with 1 mL of 0.1 mM DPPH in methanol and 450 µl of 50 mM Tris HCl buffer (pH 7.4). the mixture was incubated in the dark at room temperature for 30 min. After incubation, the absorbance was measured at 517 nm was used to calculate the reduction in DPPH free radicals. BHT was used as a control. The following equation was used to calculate the percentage of inhibition:

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

Hydroxyl radical scavenging assay

The assay was carried out in accordance with the Halliwell method with minor modifications (13). All solutions were made from scratch. 1.0 mL of the reaction mixture contained 100 µL of 28 mM 2 -deoxy-2-ribose (dissolved in phosphate buffer, pH 7.4), 500 µL of various concentrations of *Vitex negundo* and *Hibiscus rosa-sinensis* (aqueous extract) (10 to 50 µg/mL), 200 µL of 200 M $FeCl_3$ and 1.04 mM EDTA (1:1 v/v), 100 mL H_2O_2 (1.0 mM) and 100 µL ascorbic acid (1.0 mM). The TBA reaction was used to determine the extent of deoxyribose degradation after an hour of incubation at 37 °C. Compare the absorbance at 532 nm to the blank solution. A positive control was included, which was vitamin E.

FRAP assay

This technique worked by decreasing the ability of Fe^{3+} to Fe^{2+} ions in the sample. Ferric-tripyridyltriazine complex was reduced to the ferrous form in the presence of low pH, TPTZ (2,4,6-Tri (2-

pyridyl)-s-triazine) and the formulations indicated the blue color which had absorbed at 593 nm. A total of 0.7 mL of aqueous extract and 2.3 mL of FRAP reagents were combined with five different concertation of *V. negundo* and *Hibiscus rosa sinensis* aqueous extract (10 - 50 µg/mL). The mixed solutions were then left to incubate for 30 min at room temperature in the dark. The ascorbic acid used in the standard was substituted with double-distillation water for the control. Absorbance at 593 nm was measured using UV visible spectrophotometer after incubation. A increased in absorbance values indicated an increase in the reducing capacity of the extract.

Anti-inflammatory activity

Bovine serum albumin denaturation assay

The anti-inflammatory activity of *Vitex negundo and Hibiscus rosa -sinensis* (aqueous extract) was tested according to the method proposed by Muzushima and Kabayashi with specific alterations (14). A volume of 0.05 mL of *Vitex negundo* and *Hibiscus rosa-sinensis* aqueous extract of various concentrations (10, 20, 30, 40, & 50 µg/mL) was added to 0.45 mL 1 % aqueous bovine serum albumin solution.) The pH of the mixture was adjusted to 6.3 using small volume of 1 N hydrochloric acid. These samples were incubated at room temperature for 20 min and then heated at 55 °C in a water bath for 30 min. The samples were cooled and the absorbance was measured spectrophotometrically at 660 nm. Diclofenac Sodium was used as the standard and. DMSO served as a control. The percentage of protein denaturation was calculated using following equation,

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Egg albumin denaturation assay

The anti-inflammatory activity was determined using a slightly modified version of egg albumin denaturation method (15). The test samples consisted of 0.2 mL of fresh egg albumin, 2.8 mL of phosphate-buffered saline (PBS, pH 6.4) and 0.6 mL of different concentrations of the *Vitex negundo* and *Hibiscus rosa-sinensis* (aqueous extract) dissolved in 0.2 % DMSO (v/v) resulting in final concentrations of 1.670, 0.835, 0.4175, 0.209 and 0.104 mg/mL in the total reaction solution. The samples were incubated at 37 °C for 10 min before and then heated in a water bath at 70 °C for an additional 20 min. The mixture was cooled and the absorbance was measured at 660 nm. A negative control consists of 0.6 mL of 0.2 % DMSO (v/v), 0.2 mL of fresh egg albumin and 2.8 mL of PBS were used as negative controls. As a control, sodium diclofenac was used. The test was repeated in triplicate. The percentage inhibition of protein denaturation (anti-inflammatory activity) was calculated as follows:

$$\% \text{ inhibition} = (As/Ac-1) \times 100$$

Where, As = absorbance of sample, Ac = absorbance of control. The concentration at which 50 % protein denaturation (IC_{50}) was calculated from log -dose graph.

Membrane stabilisation assay

A membrane stabilisation technique was used to test the in vitro anti-inflammatory activity of extract using human red blood cells (HRBC) (16). Anti-inflammatory activity was expressed as a percentage inhibition of RBC lysis. HRBC membrane act similarly to the lysosome membrane. It stabilises the lysosome membrane by using the extract. The haemoglobin content in the suspension was estimated using a spectrophotometer in 560 nm. Blood was collected from healthy human volunteers with recent use of NSAIDs within the previous two weeks of the experiment, which was a primary exclusion criterion. To prevent clotting, Na-Oxalate was used. All blood samples were stored at 4 °C for 24 hr prior to experiment. Supernatant was removed by centrifugation for 5 min at 2500 rpm. The resulting pellet was washed three times sterile saline solution (0.9 % w/v NaCl) with centrifugation after each wash of 5 min at 2500 rpm. The packed cell volume was measured after each time. A 40 % suspension (v/v) was prepared by mixing the packed red blood cells with phosphate-buffered saline (PBS, 10 mM pH 7.4). The PBS solution was composed of NaH_2Po_4. $2H_2O$ (0.26 g): Na_2HPO_4 (1.15 g): NaCl (9 g), dissolved in 1 L of distilled water and used as cellular reconstitution.

Heat induced haemolysis

In a separate set of centrifuge tubes, 5 mL of isotonic buffer containing blood was mixed with 10 µL, 20 µL, 30 µL, 40 µL and 50 µL of *Vitex negundo* and *Hibiscus rosa-sinensis* aqueous extracts. The tubes were immersed in a boiling water bath at 56 °C for 30 minutes. After cooling, the samples were centrifuged at 2500 rpm for 5 min. The supernatant was collected and absorbance was measured at 560 nm. Diclofenac sodium was used as standard dose and DMSO used as the control.

Cytotoxic effect

Two grams of iodine free salt were measured and dissolved in 200 mL of distilled water. Approximately 10-12 mL of the prepared saline water was dispersed into each well of a 6-well ELISA plates. Ten newly hatched Brine Shrimp (*Artemia salina*) larvae (nauplii) were transferred into six wells. Aqueous extract of *V. negundo* and *Hibiscus rosa-sinensis* were synthesised and added to each well at varying concentrations of 5, 10, 20, 40 and 80 µL. One well was designed as the standard sample, which contained live nauplii but no extracts of *V. negundo* and *Hibiscus rosa-sinensis.* After samples were left alone for 24 hr. After the incubation periods, the number of live nauplii in each well was recorded and the data were graphed to show mortality trends across different concertation. The percentage mortality was calculated using the formula:

$$\frac{\text{Total number of dead nauplii}}{\text{Total number of dead nauplii} + \text{Total number of live nauplii}} \times 100$$

Biocompatibility study

Cytotoxicity evaluation by MTT assay

3T3-L1 mouse fibroblast cells, sourced from the National Centre for Cell Science (NCCS), Pune, were cultured in 25 cm^2 vented flasks under controlled conditions of 37 °C and 5 % CO_2 within a humidified incubator. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Life Technologies, USA) supplemented with 10 % fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 1 % penicillin-streptomycin (Life Technologies, USA). Once the cells reached approximately 70-80 % confluence, they were harvested and seeded into 96-well

plates at a density of 3 × 10^3 cells per well. These cultures were incubated for 24-48 hr to allow for adhesion and the establishment of a confluent monolayer. A stock solution of the aqueous extract of *V. negundo* and *Hibiscus rosa-sinensis* (100 mg/mL, w/v) was prepared using dimethyl sulfoxide (DMSO) and diluted in DMEM to achieve final concentrations ranging from 5 to 100 µg/mL. Untreated cells served as the control group. Following the adherence phase, the fibroblasts were treated with different concentrations of the test solution and incubated for 24 hr. After incubation, the treatment media were removed and 50 µL of MTT solution (5 mg/mL) was added to each well. The plate was then incubated at 37 °C for 2 hr to allow formazan crystal formation. Subsequently, 150 µL of DMSO was added to each well to dissolve the formazan crystals and absorbance was measured at 490 nm using a TECAN microplate reader. Additionally, to assess cytotoxic effects, morphological changes in fibroblasts were examined under a phase-contrast microscope and images were captured to document any structural alterations in the treated cells.

Results

Preparation of Herbal extract

Vitex negundo and *Hibiscus rosa-sinensis* aqueous extracts appeared in dark brown colour. The boiled extracts were used for the biomedical applications (Fig. 1). Fresh leaves of *Vitex negundo* and *Hibiscus rosa-sinensis* were edible and have been used in many medicinal and cosmetic products.

Antimicrobial activity

The antimicrobial activity of the aqueous extracts *Vitex negundo* and *Hibiscus rosa-sinensis* was evaluated using agar well diffusion method against *E. faecalis, S. aureus, S. mutans* and *C. albicans* (Fig. 2). The maximum zone of inhibition was noted in *E. faecalis* in three different concertation in (25, 50 & 100 µL) with inhibitory zones of 10 mm, 11 mm and 12 mm. For *S. aureus* for a consistent zone of inhibition zones of 9 mm was recorded across all concertation matching the standard inhibition zone of 9 mm. Similarly, *S. mutans* and *C. albicans* showed the inhibitory zone of 9 mm and the standard control showed significantly larger zone of 43 mm and 32 mm, respectively (Fig. 2).

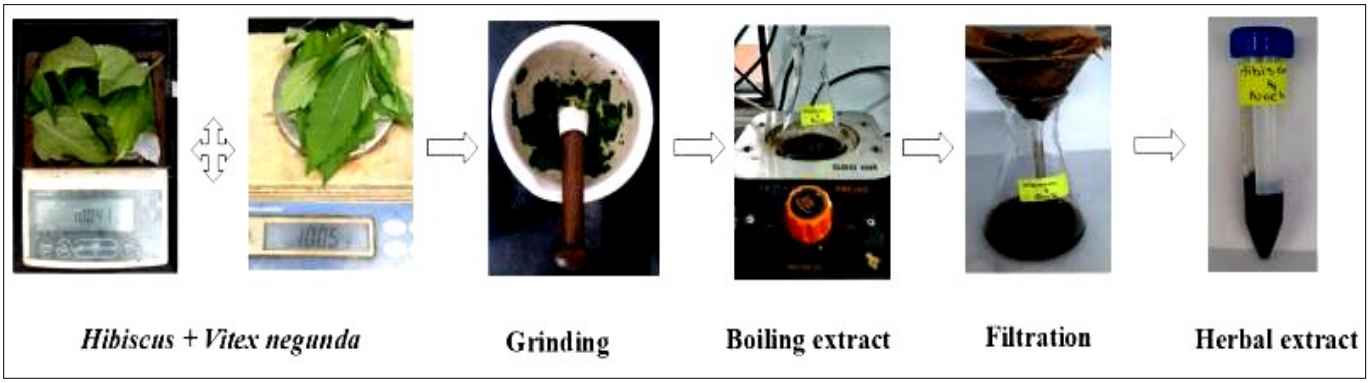

Fig. 1. Preparation of *Vitex negundo* and *Hibiscus rosa-sinensis* aqueous extract.

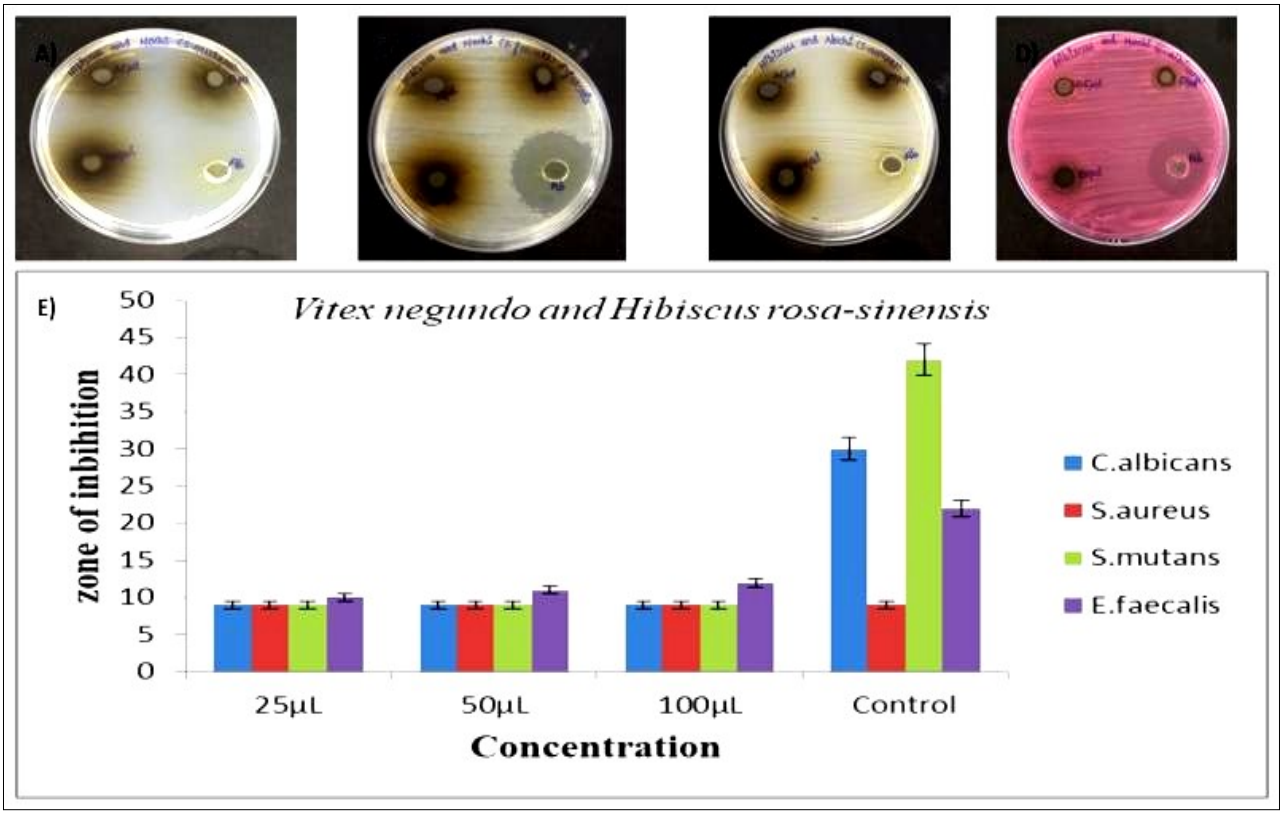

Fig. 2. Antimicrobial activity of *V. negundo* and *Hibiscus rosa sinensis* aqueous leaf extracts against oral pathogens. A) *S. mutans;* B*) E. faecalis; C) S. aureus;* D) *C. albicans;* and E) Graphical representation of the zone of inhibition for each pathogen.

MIC – Minimum Inhibitory Concentration

MIC values of *V. negundo* and *Hibiscus rosa sinensis* aqueous extracts were determined against oral pathogens over a 5 hr period using the broth dilution method in a 96-well microliter plate (Fig. 3). Both extracts exhibited consistently low MIC values (100 µg/mL) against *E. faecalis* and *S. aureus* at all time points indicating strong antibacterial potential. In contrast, the distilled water control showed higher MIC values, indicating inefficacy. These results confirmed the potent and sustained antibacterial efficacy of *V. negundo* and *Hibiscus rosa sinensis* aqueous extracts against *E. faecalis* and *S. aureus* across concentrations and incubation times.

Antioxidant activity

The antioxidant activity of *V. negundo* and *Hibiscus rosa sinensis* aqueous leaf extracts, were evaluated using the DPPH and H_2O_2 assay. In the DPPH assay, the extract demonstrated a percentage inhibition ranging from 63.38 % to 90. 47 % which was comparable to the standard ascorbic acid which showed inhibition between 66.25 - 93.15 % (Fig. 4A). Similar, H_2O_2 scavenging assay as 49.7 - 85.8 % while the standard vitamin C showed inhibition ranging from 51.1 to 89.9 % (Fig. 4B). The FRAP assay showed the percentage inhibition in the range 67.72 % to 88.04 % at concentration between 10-50 µL and this was compared with ascorbic acid which showed inhibition ranging 72.98 % to 90.24 % at the same concentration (Fig. 4C). The *V .negundo* and *Hibiscus rosa sinensis* aqueous leaf extracts demonstrated excellent antioxidant properties. The standards used for comparison in the H_2O_2, DPPH and FRAP assay differed slightly. The result showed that as the concentration of herbal extract increased, the percentage of inhibition also increased proportionally.

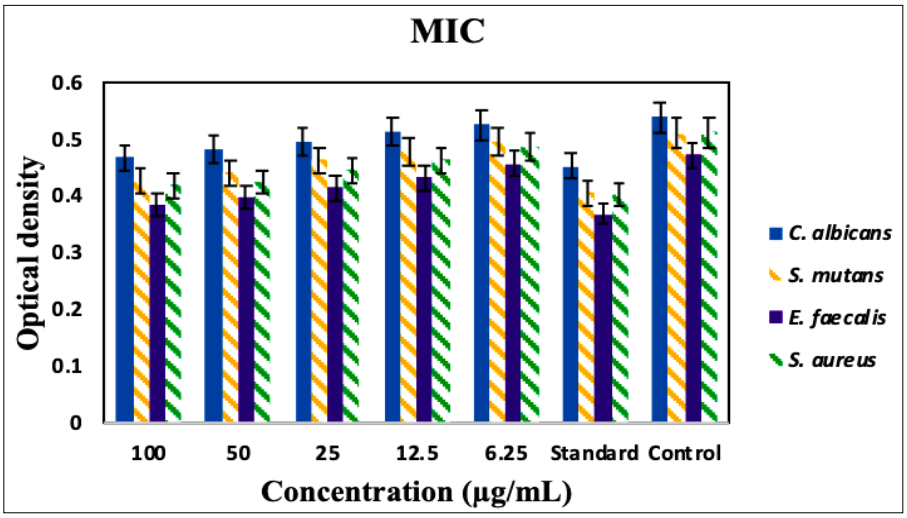

Fig. 3. Minimum inhibitory concentration (MIC) of *V. negundo and Hibiscus rosa sinensis* aqueous leaf extracts against oral pathogens.

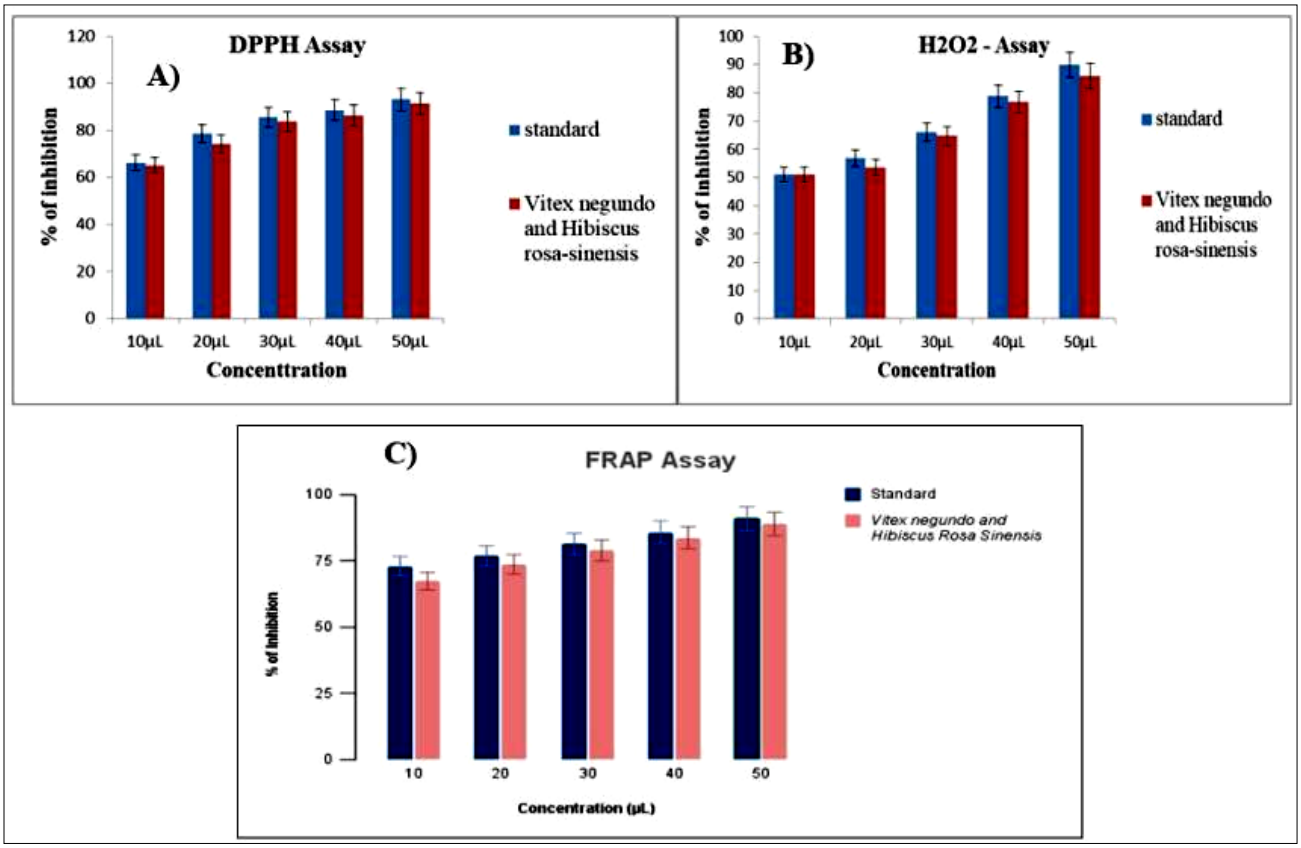

Fig. 4. Graphical representation of the antioxidant activity of *V. negundo* and *Hibiscus rosa sinensis* aqueous leaf extracts. A) DPPH; B) H_2O_2 assay and C) FRAP assay.

Anti-inflammatory activity

The anti-inflammatory activity of the herbal formulation consisting of aqueous extracts of *V. negundo* and *Hibiscus rosa sinensis,* was evaluated using egg albumin denaturation assay, bovine serum albumin denaturation assay and the membrane stabilisation assay.

Bovine Serum Albumin (BSA) denaturation assay

At 10 µL the extract showed 46 % inhibition, compared to the standard at 43 %. At 20 µL the extract showed 58 %, while the standard 55 %. At 30 µL inhibition was 63.4 % for the extracts and 68 % for standard. At 40 µL inhibition was 72.3 % for extract and 74 % for standard and at 50 µL inhibition was 79.6 % extracts and 79 % for standard (Fig. 5A). The IC_{50} values extracts and standard (Diclofenac sodium), in the assay were 65.46 ± 25.76 µL and 68.43 ± 29 µL, respectively.

Egg Albumin denaturation assay

At 10 µL is the extract showed 53 % inhibition and standard 54 %. At 20 µL 64.3 % inhibition by extract and the standard 62 %. At 30 µL extracts showed 71.4 % and standard 65 %. At 40 µL 72 % (extracts) 70 % (standard) whereas at 50 µL it was 76 % for extracts 79 % for standard (Fig. 5B). The IC_{50} values aqueous extracts and standard (Diclofenac sodium), in the assay were 68.58 ± 26.63 µL and 69.06 ± 29.91 µL, respectively.

Membrane stabilisation assay

At 10 µL, 51.6 % inhibition by extracts and 55 % by the standard. At 20 µL it was 60.3 % (extracts) and 65 % (standard). At 30 µL it was 69.4 % and 74 % and at 40 µL 71.5 % for extracts and 79 % for standard. At 50 µL 74 % for extracts and 85 % for standard (Fig. 5C). The IC_{50} values of aqueous extracts, in the assays, was reported as 70.27 ± 17.83 µL.

Overall, the anti-inflammatory properties of the herbal combination of *V. negundo* and *Hibiscus rosa sinensis* aqueous extracts were found to be comparable to those of the standard drug diclofenac sodium at all concentrations (10 - 50µL.) The result indicated that the herbal formulation exhibited significant anti-inflammatory activity.

Cytotoxic effect

The cytotoxic effect of herbal formulation was determined using the brine shrimp lethality experiment. For percentage mortality calculations, the control group remained stable ad showed no lethality. At a concentration of 40 µL aqueous extracts of *Vitex negundo* and *Hibiscus rosa-sinensis* exhibited 80 % mortality. At 80 µL the mortality rate reached 100 % indicating a strong cytotoxicity effect on higher concentrations (Fig. 6).

MTT assay

The cytotoxic effects of *Vitex negundo* and *Hibiscus rosa-sinensis* (aqueous extracts) on mouse fibroblast (3T3-L1) cells were evaluated

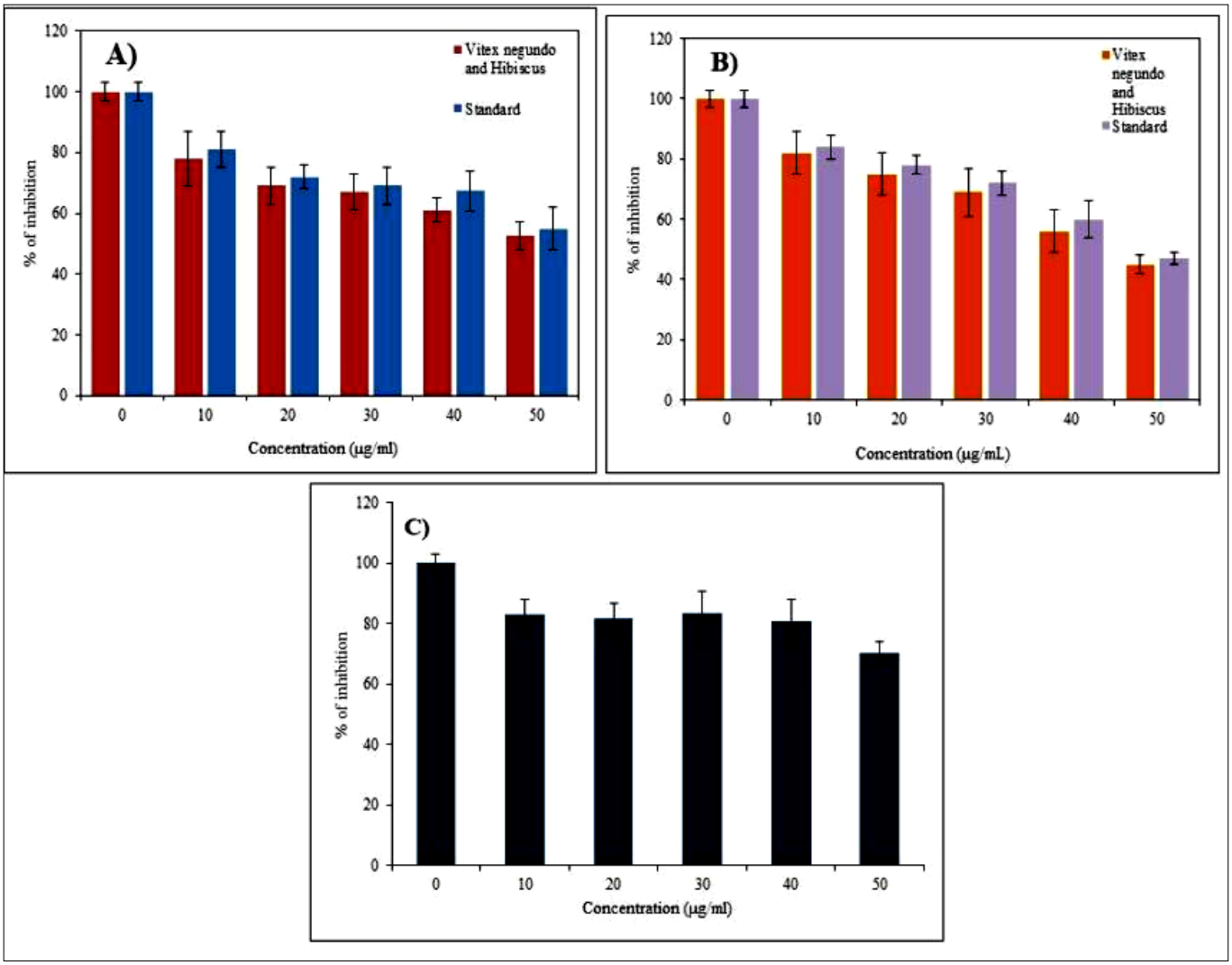

Fig. 5. Graphical representation, of the anti-inflammatory activity of *V. negundo* and *Hibiscus rosa sinensis* aqueous leaf extracts. A) BSA assay; B) EA assay and C) Membrane stabilisation assay.

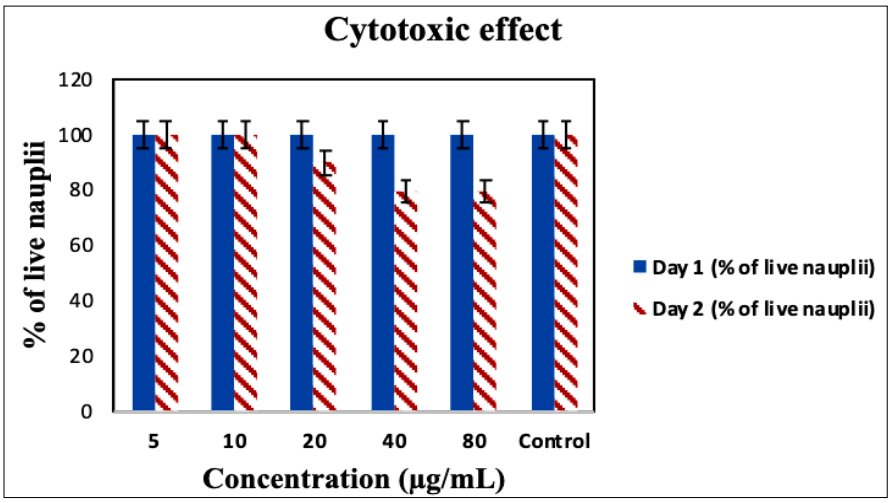

Fig. 6. Graphical representation, of the cytotoxic effect of *V. negundo* and *Hibiscus rosa sinensis* aqueous leaf extracts using Brine Shrimp Lethality assay.

by MTT assay. Cells were treated with extract concertation ranging from 5 to 100 µg/ml for 24 hr and cell viability was evaluated (Fig. 7). Data were shown as means ± SD ($n = 3$). Statistical significance was noted compared to the control group ($p < 0.05$). The results indicated in a concentration-dependent decline in cell viability, suggesting a mild cytotoxic effect at higher concentrations. At 5 µg/mL, cell viability remained high at 96.66 ± 0.49 %, showing minimal toxicity. As the concentration increased, a gradual reduction in viability was observed: 94.41 ± 0.20 % at 10 µg/mL and 91.70 ± 0.21 % at 40 µg/mL, still indicating good biocompatibility. At higher concentrations, a more noticeable decline was observed, with 88.94 ± 0.20 % at 80 µg/mL and 86.73 ± 0.22 % at 100 µg/mL, suggesting a mild cytotoxic effect at elevated doses. However, cell viability remained above 80 %, across all concentrations, indicating that aqueous extracts maintained good compatibility with fibroblast cells, particularly at lower doses. Overall, these findings suggest that the *Vitex negundo* and *Hibiscus rosa-sinensis* (aqueous extract) exhibited favorable biocompatibility, with only a moderate reduction in cell viability observed at higher concentrations.

Discussions

India is rich in natural medicinal herbs. many which are traditionally used in treating various inactions. In particular, herbal formulation have been recognized for their antibacterial properties against oral pathogens such as *E. faecalis,* which is regarded as the main culprit among the oral pathogenic microorganisms. The objective of the study was to prepare a herbal formulation and evaluate its cytotoxic, anti-inflammatory, antimicrobial and antimicrobial efficacy against oral infections (17).

According to this study, the herbal formulation of *Vitex negundo* and *Hibiscus rosa-sinensis* exhibited a higher zone of inhibition compared to both the individual herbal extracts and the standard antibiotic ampicillin (18). In the previous study, a larger zone of inhibition was observed against *Vitex negundo* and *Hibiscus rosa sinensis* against the pathogens *E. faecalis* (24 mm), *S. aureus* (20 mm), *S. mutans* (32 mm) and *C. albicans* (9 mm) (19). Similar work, various control groups- including crude extract, precursor compounds, biosynthesized *Vitex negundo* and *Hibiscus rosa-sinensis* and the standard antifungal - Ketaconazole, were used to assess antifungal efficacy. However, no clear zone of inhibition was observed for *C. albicans* indicating limited antifungal activity under those conditions (20). Although *E. faecalis* was commonly part of the normal intestinal flora, a previous studies have demonstrated its high pathogenic potential, particularly oral infections (21, 22). Compared to other oral infections, *E. faecalis* caused a larger inhibition zone when treated with the herbal formulation, suggesting that *Hibiscus rosa sinensis* and *V. negundo* possess strong antibacterial properties. In MIC, the previous research showed that *A. indica* AgNPs and TSC

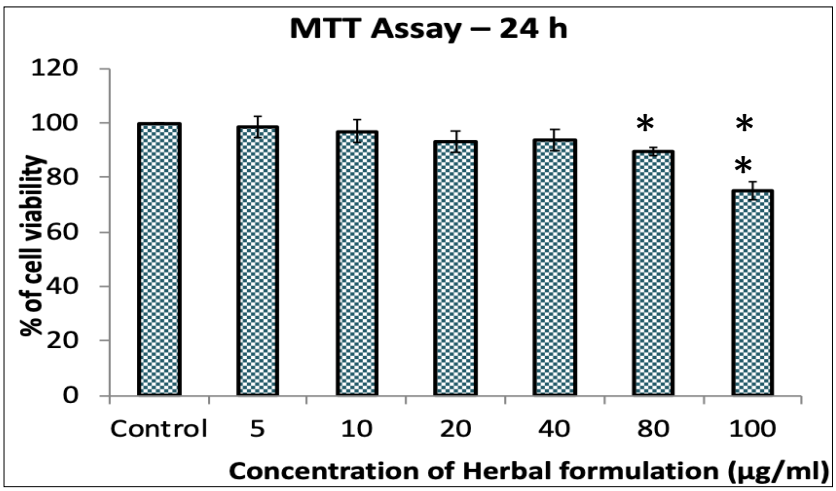

Fig. 7. Graphical representation, of the cytotoxic effect of *V. negundo* and *Hibiscus rosa sinensis* aqueous leaf extracts using the MTT assay on mouse fibroblast (3T3-L1) cell line.

AgNPs inhibited *E. faecalis* at different concentrations over 5 hr (23). Similarly, another MIC based study using serial dilution methods reported that the lowest effective concentration against *E. faecails* was 62.5 µg/mL, confirming its antimicrobial sensitivity at low doses (24).

In similar research, the antioxidant properties of plant - derived dietary components, which play a significant role in disease prevention have attracted considerable attention in recent years. Therefore, the ability to act as an antioxidant has become a key criterion for selecting bioactive components for medicinal use (25). According to findings of current study, *H. rosa* extract possesses a high total antioxidant capacity, which was dependent on reaction time particularly at concentration of 500 µg/ml (26). In previous study, antioxidants offer natural protection against various degenerative diseases and ailments. The herbal formulations of *Hibiscus rosa sinensis* and *V. negundo* were tested for their antioxidant capacity using the DPPH and H_2O_2 assays. For comparison purpose ascorbic acid was used as standard for DPPH while DMSO was the control solvent in the H_2O_2 assay. At various concentrations, the percentage of inhibition for the herbal combination was recorded revealing notable antioxidant activity. During DPPH assay, a visible colour changed from purple to brown was observed, indicating hydrogen donation and high free radical scavenging activity (27). In the FRAP assay, the antioxidant activity of Mn-doped ZnO nanoparticles ranged from 0.79 ± 0.11 to 10.9 ± 0.11 µM/mL. Mn-doped ZnO nano-particles exhibited significantly higher FRAP values compared to undoped ZnO nano-particles (28).

The anti-inflammatory activity of herbal formulation was assayed using bovine serum albumin, egg albumin denaturation assay and membrane stabilization assay. In a similar study reported that clove and cardamom mediated silver nanoparticles have anti-inflammatory activity (29). Earlier a combination of *C. sativus* + *C. macroptera* and glycerol extract demonstrated percentage inhibition 48 %, 54 %, 72 %, 74 % and 76 % at concentrations of 10, 20, 30, 40 and 50 µg/mL respectively using bovine serum albumin denaturation assay (30). *Acorus calamus* DMSO extract on membrane stabilization assay showed a inhibition ranging from 55 % to 86 % (31).

The cytotoxic effect of herbal formulation was evaluated using brine shrimp lethal assay. It was reported that *Pongamia pinnata* extract showed increasing toxicity with concentration, while *Nutmeg oleoresin* caused at least 30 % reduction in live nauplii at both 80 µL and 40 µL indicating significant cytotoxicity (32, 33). In contrast to the herbal formulations of *Vitex negundo* and *Hibiscus rosa-sinensis* demonstrated lower mortality, showing low toxic effect and better compatibility. In similar research using, MTT assay titania nanoparticles (TiO_2 NPs) synthesised with probiotic *Bacillus coagulans,* exhibited high cell viability at four different concentrations in µg/mL of 25, 50, 75 and 100 with up to 93.3 % of viability after 24 hr supporting the safety profile of natural ad nanoparticle based compound (34).

Conclusion

A significant portion of the population pursue the herbal remedies derived from the *V. negundo* and *Hibiscus rosa sinensis* (aqueous extract) for their healthcare purpose. Several bioactive compounds from medicinal plants are either used directly or indirectly for the therapeutic applications. The study confirmed that herbal formulation of *V. negundo* and *Hibiscus rosa sinensis* (aqueous extract) exhibited strong antibacterial, antioxidant, anti-inflammatory and cytotoxic effect particularly against oral pathogens. The formulation also showed low toxicity as evidence by high cell viability in fibroblast and low mortality in brine shrimp assays. Based on the findings, this formulation holds a promise for future development in cosmetic products, mosquito repellent gel, inhaler and herbal mouthwashes for oral healthcare applications.

Acknowledgements

We would like to thank Saveetha Institute of Medical and Technical Sciences for support.

Authors' contributions

Study design and conception was created by RS. Data Collection was carried out by SG. Analysis and interpretation of results was performed by RS, SG and KS. Draft manuscript preparation was executed by RS and SG. All authors reviewed the results and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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