Assessing The Anti-Inflammatory Potential Of *Vitex negundo*: An In-Vitro Experimental Study

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Abstract
Vitex negundo is an aromatic plant from the Verbenaceae family, native to India, which has been known for its therapeutic properties since ancient times. In the current study, we investigated the anti-inflammatory properties of *Vitex negundo* (VN) extract via in-vitro laboratory experiments such as HRBC (Human red blood cell) membrane stabilisation assay, albumin denaturation inhibition assay, and proteinase inhibition assay. The results exhibited that, in the HRBC membrane stabilisation test, the VN extract significantly reduced cell damage, exhibiting 79.95% protection at a concentration of 200 µg/ml against the control anti-inflammatory drug diclofenac sodium.
In the protein denaturation assay, VN effectively inhibited protein denaturation, with its highest inhibition rate of 72.29% at 200 µg/ml concentration. The proteinase inhibition assay also demonstrated satisfactory results, with maximum inhibition (58.24%) observed at 200µg/ml.
These results confirm the strong anti-inflammatory properties of VN, particularly at a concentration of 200 µg/ml, where it was most effective in mitigating inflammation-related mechanisms. Our results propose that VN has significant potential to be developed into a natural, plant-based anti-inflammatory therapeutic agent and is a promising alternative to synthetic drugs. The current study will be a foundation for future research efforts that aim to explore the therapeutic properties of VN, and future research endeavours should focus more on advanced phytochemical characterisation, drug formulations and human clinical trials to exploit the use of these properties.

INTRODUCTION

Inflammation is considered an integral component of the body's defence mechanism against pathogens, foreign bodies, and tissue injuries. It primarily functions to repair damaged tissues and restore them to a healthy state (Sivapalan et al., 2023). These processes are vital for maintaining homeostasis, but it can sometimes also have harmful effects, like tissue damage, angiogenesis, oxidative stress, and fibrosis in various tissues (Gusev et al., 2022). Under normal circumstances, these inflammatory responses resolve quickly after removing harmful agents and pathogens. However, dysregulation of the inflammatory process has been identified as a significant factor in the onset and progression of numerous chronic diseases, such as autoimmune diseases (Sivapalan et al., 2023; Gusev et al., 2022). Inflammatory diseases resulting from immune system dysregulation pose a challenge for treatment, as they can lead to tissue damage, progressive degeneration, and in some cases, systemic complications as well (Ciesielski et al., 2022).

To manage this illness, both chronic and acute, glucocorticoids are the most commonly used medications due to their immediate action (Reichardt et al., 2021).

A significant number of individuals with chronic inflammatory conditions, including autoimmune disorders, rely on glucocorticoid therapy. While these medications are powerful anti-inflammatory agents with a broad spectrum of activity, frequent and long-term usage leads to a range of adverse effects, such as osteoporosis, weight gain, hypertension, and an increased risk of infections, etc. These pose significant challenges for proper treatment and management (Hardy et al., 2020).

Throughout history, natural medicines derived from medicinal plants have been highly valued for their therapeutic potential. In India, plant-based remedies have been effectively used to treat various diseases. this knowledge about natural herbs and medicines also contributed to the very foundation of modern medicine. While synthetic drugs

are essential in certain cases, they often carry the risk of side effects, which are unavoidable. Therefore, exploring traditional medicinal alternatives to synthetic drugs could help reduce their adverse effects and the huge treatment costs involved (Noetzlin et al., 2022). The benefits of plant-based medicines and their properties are well known in India, but more advanced research is needed in this field to develop these herbal formulations into standardised and effective drugs, ensuring their efficacy and safety for widespread use (Cunh et al., 2024).

The aim of our study is to examine the in-vitro anti-inflammatory properties of Vitex negundo, which is an aromatic plant from the Verbenaceae family that is native to India and is generally distributed across the subcontinent (Singh et al., 2020). Vitex negundo, also known as "chaste tree", is a shrub that can grow up to 3 meters tall in height and can be easily recognised by its distinctive quadrangular branches. Every part of the plant is rich in beneficial secondary metabolites, making it an important ingredient of traditional and folk medicine in India (Nath et al., 2024).

In ancient Vedic traditions, Vitex negundo was revered for its therapeutic properties, such as its ability to reduce inflammation, alleviate pain, and promote wound healing. It has traditionally been used to treat various ailments, including pain, skin disorders, and other health conditions. The leaves, roots, and seeds of this plant contain potent phytochemicals and hence have been widely exploited in traditional healing practices. Even today, Vitex negundo continues to be a key element of Ayurveda and traditional medicine in India, where its uses and benefits are still being explored and appreciated (Sharma et al., 2023).

The current in-vitro study will investigate the anti-inflammatory properties of Vitex negundo, and the findings will provide valuable insights into its mechanisms of action, enhancing our understanding of its pharmacological potential. Moreover, the findings of this study will be a foundation for future research efforts to reveal the therapeutic properties of VN.

MATERIALS AND METHODS

Sample Collection

The *Vitex negundo* plant was collected from the Alappuzha district of Kerala, India, where this plant is traditionally used for many medicinal purposes and is readily available in all nearby areas. The leaves of Vitex negundo were thoroughly cleansed to remove any adhering dirt or matter and then properly washed with water, followed by air-drying only under the shade and making it powdered using a domestic grinder to a crude extract. The whole study was conducted in the Department of Biochemistry, St.Gregorios College of Health Science, Parumala, Kerala, India.

Reagents

The following chemicals and materials were used in the study: Methanol (99.9%, analytical grade, Merck, India), fresh whole human blood (collected under ethical approval), Alsever solution (Himedia, India), Tris-HCl buffer with the pH 7.4, (Sigma-Aldrich, USA), trypsin (analytical grade, SRL, India), casein (analytical grade, Sigma-Aldrich, USA), perchloric acid (analytical grade, Merck, India), fresh egg albumin, phosphate-buffered saline (PBS) with the pH of 7.4, (Himedia, India), and diclofenac sodium (analytical grade, Sigma-Aldrich, USA) at different concentrations as control. All chemicals were in analytical grade and procured from reliable suppliers, ensuring high purity for experimental accuracy.

Preparation of plant extract

The powdered leaves were extracted using a Soxhlet apparatus, following the procedure outlined by Dharmasiri et al. (2003) study. Ten grams of the powdered leaves were placed in an extraction thimble, and 50 ml of methanol (99.9%) was added to the solvent vessel. The extraction was carried out at 65°C, for the methanol to vaporise, condense, and extract the desired compounds, thereafter cooling, the extract was filtered using Whatman No. 1 filter paper and was stored in a sterile container at -20°C (Dharmasiri et al., 2003).

The HRBC (Human Red Blood Cell) Membrane Stabilization Assay

The HRBC assay is used to evaluate the ability of VN extracts to protect red blood cell membranes from damage caused by physical (heat) and chemical stress. Here, plant extracts are mixed with human red blood cells (HRBCs) and exposed to heat. The amount of hemoglobin released into the supernatant is then measured calorimetrically to

evaluate the extent of membrane damage. A lower concentration of haemoglobin in the supernatant indicates that the plant extract is effective in protecting the cell membrane in stress conditions.

Preparation of Erythrocyte Suspension:
The red blood cell suspension was prepared according to the method described by Gandhidasan et al. (1991), with a few modifications. Initially, 10 ml of whole blood was added to the heparinized centrifuge tube to prevent clotting and was then mixed with an equal amount of Alsever solution, these mixture was centrifuged with iso-saline, to separate the red blood cells.

Heat-Induced Hemolysis:
The test was conducted following the method of Rao et al., and Ajithkumar et al. (2020). Prepared RBC suspension was mixed with plant extract (both at four mL) at different concentrations such as 10, 20, 50, and 100 µg/mL. The mixtures were then incubated at 56°C for 30 minutes followed by centrifugation to separate the supernatant from the red blood cells settled. The absorbance of the supernatant was measured at 560 nm using a spectrophotometer to assess the amount of hemoglobin released. The percentage of hemolysis was calculated using a standard formula to determine the protective effect of the plant extracts on red blood cell membranes.

Percentage of hemolysis= $\frac{\text{OD of Control}-\text{OD of test}}{\text{OD of Control}} \times 100$

Protease inhibitory action
Method described by Bijina et al. (2011) was used to measure the proteinase inhibitory activity of Vitex negundo leaf extract. Reaction mixtures were prepared by combining 0.06 mg of trypsin and 1 mL of 25 mM Tris-HCl buffer (pH 7.4), and then adding 1 mL vitex negundo extract in different concentrations. These mixtures were incubated at 37°C for 5 minutes to activate the enzyme and after activation, 1.0 mL of 0.8% (w/v) casein was added to each reaction mixture. Thereafter the reaction mixture was incubated for an additional 20 minutes to enable trypsin to hydrolyse the casein. The enzymatic reaction was terminated by adding 2.0 mL of 70% (v/v) perchloric acid, which precipitated the proteins. The mixtures were then centrifuged to separate the cloudy suspension containing protein precipitates from the supernatant. The supernatant transferred to a new container, and its absorbance was measured at 280 nm using a spectrophotometer, with a buffer used as a control. The % inhibition was calculated using the standard formula given below.

Percentage of inhibition= $\frac{\text{OD of Control}-\text{OD of test}}{\text{OD of Control}} \times 100$

Albumin denaturation method
"The protein denaturation assay" was conducted following the procedure outlined by Gambhire et al., with certain modifications made as per the procedure described by Gunathilake et al. The reaction mixture (5 mL) of 0.2 mL of fresh egg albumin, 2.8 mL of 'phosphate-buffered saline' (PBS, pH 6.4), and 2.0 mL of concentrations of the test samples. The positive control was prepared by mixing 0.2 mL of fresh egg albumin and 2.8 mL of PBS (pH 6.4), and diclofenac sodium at different concentrations (10, 30, and 100 µg/mL). The negative control consisted of the same amount of albumin and PBS, then the distilled water replacing the test sample. The whole mixture was then incubated at 37°C for 15 minutes and heated at 70°C for 5 minutes to induce denaturation (Gambhire et al., 2009). Then, allow it to cool, the absorbance has been measured at 660 nm, with the blank. The percentage of inhibition in protein denaturation was calculated by using the formula:

Percentage of inhibition= $\frac{\text{OD of Control}-\text{OD of test}}{\text{OD of Control}} \times 100$

RESULTS AND DISCUSSION

HRBC (Human Red Blood Cell) Membrane Stabilization Assay
The "HRBC Membrane Stabilization Assay" was performed to evaluate the protective effect of Vitex negundo extract on the membrane of human red blood cell (HRBC) under heat-induced stress. The results indicated that Vitex negundo exhibited a concentration-dependent membrane stabilizing effect. At the lowest tested

concentration of 20 µg/ml, the extract provided 37.87% protection, with 62.13% hemolysis, indicating moderate membrane protection. As the concentration increased, the protective effect also improved, with 44.00% protection observed at 50 µg/ml and 47.01% protection at 100 µg/ml, corresponding to 56.00% and 52.99% hemolysis, respectively. At the highest concentration of 200 µg/ml, Vitex negundo exhibited the strongest protective effect, providing 64.15% protection and only 35.85% hemolysis, which indicates significant stabilization of the HRBC membrane. When compared with Diclofenac sodium, one of the standard drugs, which provided 79.95% protection at 200 µg/ml, Vitex negundo demonstrated comparable effects, especially at higher concentrations. These findings suggest that Vitex negundo extract effectively stabilizes HRBC membranes, with concentrations of 100 µg/ml and above showing promising membrane protective properties. The 200 µg/ml concentration displayed the most substantial protective effect, making Vitex negundo a potential candidate for formulation as an alternative therapeutic option to synthetic drugs (See Figure 1, Table 1& Graph 1).

The results indicate that the extract of Vitex negundo, shows a clear dose-dependent trend, with the 200 µg/mL concentration showing the highest membrane protection (64.15%) and the lowest hemolysis (35.85%). This suggests that Vitex negundo possesses significant membrane-stabilizing effects, which could indicate its anti-inflammatory and antioxidant properties (See Table 1 & Chart 1).

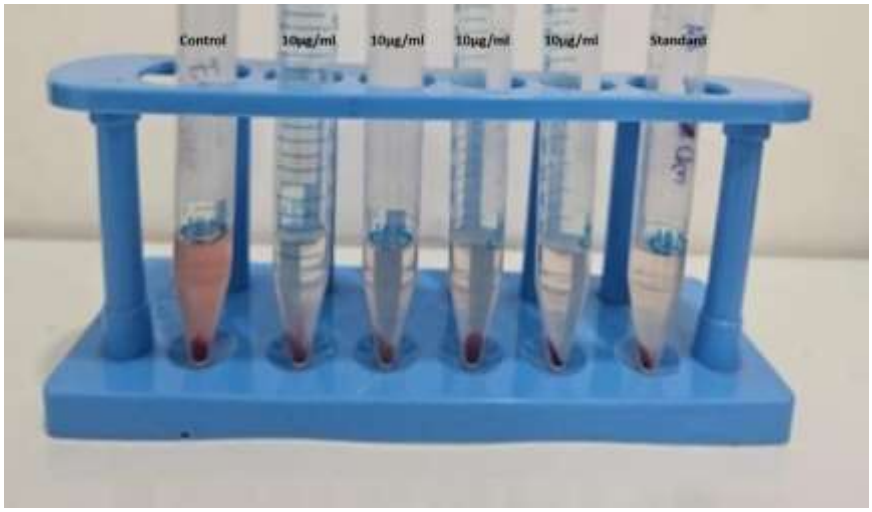

Figure 1: This figure shows the results of the HRBC membrane stabilisation assay using *Vitex negundo* extract at various concentrations, with hemolysis control and a standard drug (diclofenac sodium) for comparison. In this assay, HRBC suspension was added to all tubes at the concentration of 10 µg/mL. The *Vitex negundo* extract was tested at different concentrations, and the membrane stabilization effect was observed by measuring the percentage of hemolysis. The hemolysis control (distilled water) exhibited high hemolysis, while diclofenac sodium, the standard drug, showed significant protection. The figure shows the varying levels of red blood cell integrity under the influence of *Vitex negundo* and its ability to prevent hemolysis, indicating its potential membrane-stabilizing properties.

Table 1. Percentage hemolysis in different concentrations of *Vitex negundo*.

Sample	Concentration (µg/ml)	% Hemolysis	% Protection
Vitex Negundo	20	62.13	37.87
	50	56.0	44.0
	100	52.99	47.01
	200	35.85	64.15
Diclofenac	200	20.05	79.95

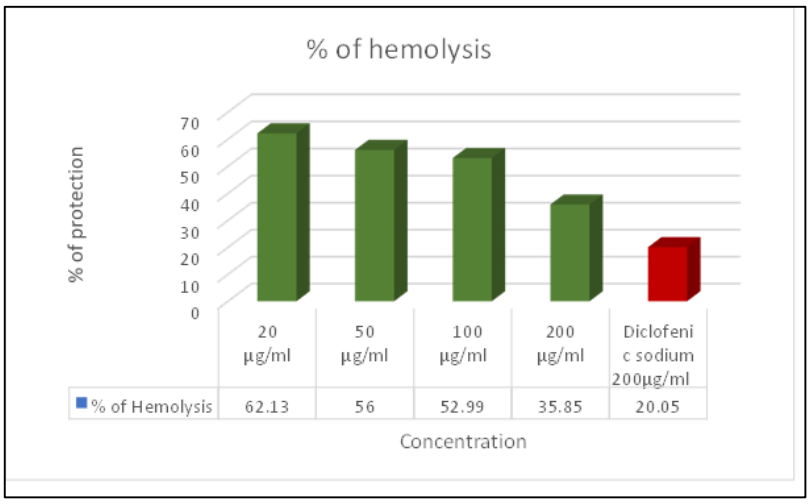

Chart 1. Graphical representation of % hemolysis obtained by HRBC Membrane Stabilization Assay

Protease inhibitory action

The in-vitro proteinase inhibition assay was conducted to analyse the potential of Vitex negundo to inhibit proteinase activity and to compare its efficacy with a formulated drug. The assay was performed at varying concentrations of Vitex negundo extract such as "10, 20, 50, 100, and 200 µg/ml" and the inhibition percentage was then calculated. It is observed that, there is a concentration-dependent increase in proteinase inhibition, at 10 µg/ml, Vitex negundo exhibited 29.01% inhibition, and increased progressively with higher concentrations, reaching 58.24% inhibition at 200 µg/ml. The control drug exhibited a proteinase inhibition of 64.82% and the inhibition percentage of Vitex negundo at its highest tested concentration (200 µg/ml) is also close to that of the control drug. The similarity in the inhibitory activity of Vitex negundo and the control drug suggests that Vitex negundo has significant bioactive properties that make it capable of inhibiting proteinase activity effectively.

Chart 2 & Table 2 represent the in vitro proteinase inhibition assay compared to the efficacy with a formulated drug. The assay was performed at varying concentrations of Vitex negundo extract and the inhibition percentage was calculated. The results demonstrated an increase in proteinase inhibition based on the concentration. At 10 µg/ml, Vitex negundo showed 29.01% inhibition, and this increased progressively with higher concentrations, reaching 58.24% inhibition at 200 µg/ml.

The control drug exhibited a proteinase inhibition of 64.82%, the inhibition percentage of Vitex negundo at its highest tested concentration (200 µg/ml) is quite close to the control drug's effect. The similarity in the inhibitory activity of Vitex negundo and the formulated drug suggests that Vitex negundo possesses significant bioactive properties, capable of inhibiting proteinase activity effectively.

Table 2. Percentage of Inhibition of proteinase inhibition assay

Sample	Concentration (µg/ml)	% Inhibition
1	10	29.01
2	20	35.99
3	50	47.9
4	100	53.99
5	200	58.24
	Control	64.82

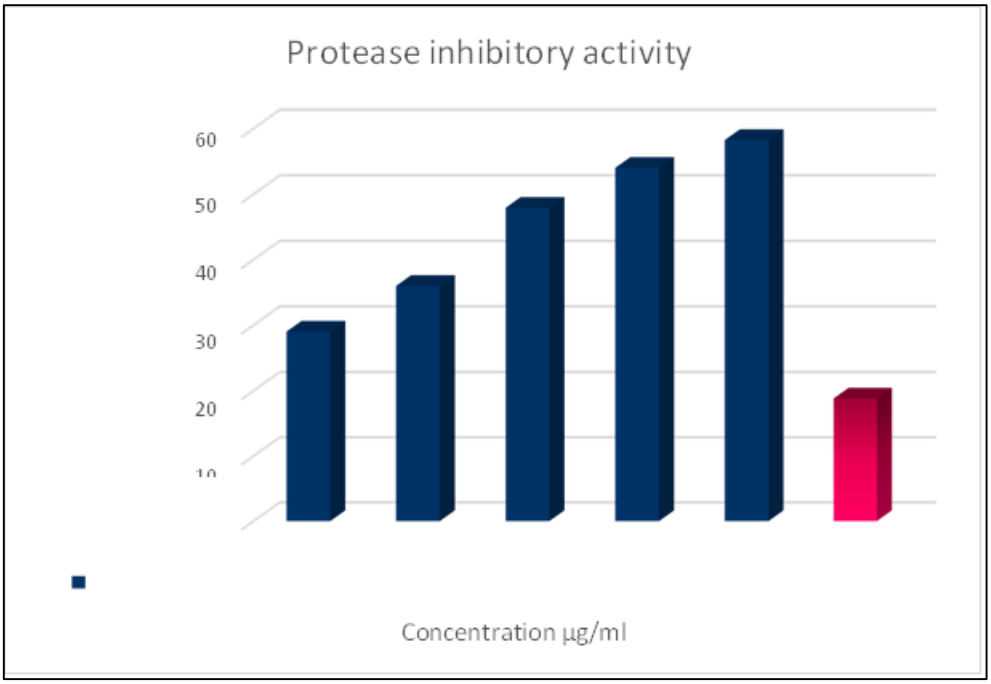

Chart 2. A graphical representation of % inhibition of proteinase inhibition assay

Albumin denaturation method

The results from the protein albumin (EA) denaturation inhibition assay, as shown in Table 3, and Graph 3, demonstrate the potential of Vitex negundo extract on inhibition of protein denaturation, a key mechanism in inflammation. At 10 µg/ml, the extract exhibited 29.89% inhibition, which increased progressively with higher concentrations. At 20 µg/ml, the inhibition rose to 34.78%, and at 50 µg/ml, it reached 52.92%, showing a moderate anti-inflammatory effect. Further, the concentrations of 100 µg/ml and 200 µg/ml provided even stronger inhibition, with 63.01% and 72.29% inhibition, respectively. This indicates a concentration-dependent increase in the extract's ability to prevent protein denaturation. In comparison, the control showed only 12.53% inhibition, while Diclofenac sodium, the standard anti-inflammatory drug, exhibited a high inhibition of 89.95%, highlighting its potent effect as it is a formulated drug composition. Overall, the data suggest that Vitex negundo extract effectively inhibits protein denaturation, with its activity comparable to Diclofenac sodium.

Based on the results of all the performed assays, the effective concentration of Vitex negundo demonstrating anti-inflammatory activity appears to be in the range of 100 to 200 µg/ml. At these concentrations, Vitex negundo shows substantial anti-inflammatory effects by stabilizing cell membranes, inhibiting proteinase activity, and preventing protein denaturation. The concentration of 200 µg/ml is the critical concentration of Vitex negundo where it showed the most robust effects across the assays, suggesting that it could be an optimal dose for therapeutic use in reducing inflammation while still maintaining an acceptable level of efficacy.

The VN extract exhibited a concentration-dependent response across the assays, in the HRBC Membrane Stabilization Assay, Vitex negundo exhibited 47.01% membrane stabilization at 100µg/ml and 64.15% at 200µg/ml. This result suggests that VN is effective in protecting cell membranes from damage caused by heat-induced stress, proving its potential as a reliable membrane-stabilizing agent. These results are similar to the findings of Sharma et al. (2023), where in their study investigators emphasized the traditional use of Vitex negundo and its potential as a therapeutic option for treating conditions related to membrane damage (Sharma et al., 2023).

In the Proteinase Inhibition Assay, Vitex negundo showed notable activity, with 53.99% inhibition at 100 µg/mL and 58.24% inhibition at 200 µg/mL, indicating its potential to reduce proteinase-induced tissue damage. Similarly, in the Albumin Denaturation Assay also, the VN extract demonstrated 63.01% inhibition at 100 µg/mL and 72.29% inhibition at 200 µg/mL, This shows its effectiveness in preventing protein denaturation, which is a key contributor to inflammation.

According to the opinion of Gill et al. (2018), bioactive substances in VN have the ability to alter or inhibit several signalling pathways, such as COX-1, TNF-α, these pathways have a significant role in inflammation related to various diseases (Gill et al., 2018).

Several studies have explored the anti-inflammatory, analgesic, antioxidant, and anti-androgenic activity of VN seed extracts, therefore it is notable that this plant species has a variety of medicinal properties in every part of it, and it needs to be explored to a larger extent (Sharma et al., 2023).

The results of our study provide strong evidence supporting the role of VN as a natural anti-inflammatory agent and these results will be a valuable addition to future research efforts to develop safe natural therapeutics. In addition, future research efforts should focus on exploring the detailed phytochemical analysis and biochemical pathway inhibition mechanisms involved. Addressing this limitation through further research will help us to exploit the holistic properties of VN for therapeutic use.

Table 3. Percentage inhibition of Protein egg-albumin (EA) denaturation inhibition assay

Concentration (µg/ml)	% Inhibition
10	29.89
20	34.78
50	52.92
100	63.01
200	72.29
Control	12.53
Diclofenac sodium	89.95

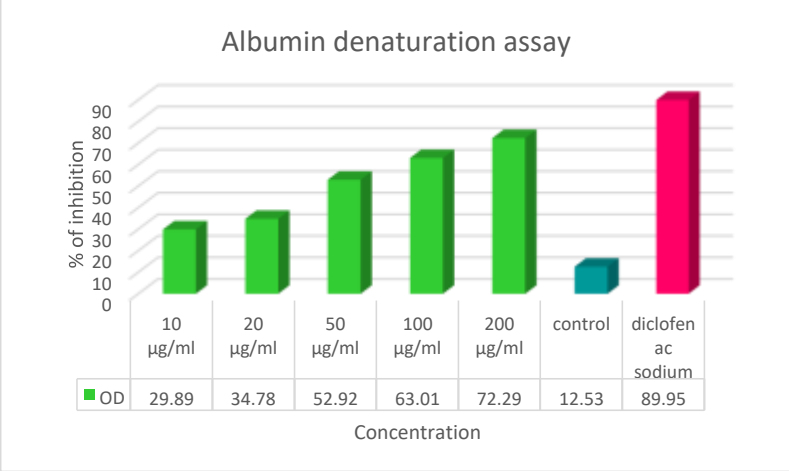

Chart 3. Represents the results of Albumin denaturation assay

Figure 2: Protein precipitation in proteinase inhibition assay, showing the appearance of the reaction mixture before centrifugation.

Figure 3: Protein precipitation in proteinase inhibition assay, showing the appearance of the reaction mixture after centrifugation

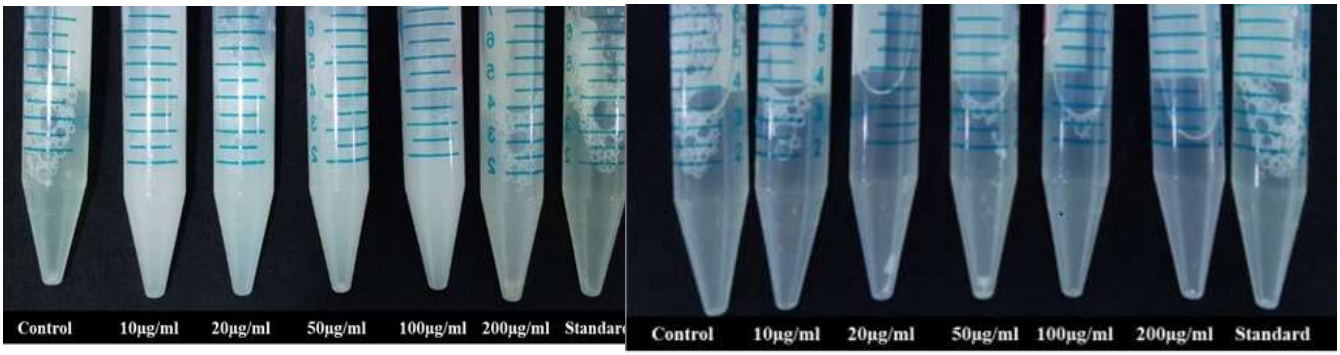

Figure 4: Reaction tubes before heating: In the initial stage, the reaction mixtures consist of egg albumin (EA) at varying concentrations of the Vitex negundo extract. The mixture is clear, and no denaturation has occurred, because the solution is at room temperature. At this stage, the egg albumin remains in its native, non-denatured form, and no protein aggregation is visible.

Figure 5: Reaction tubes after heating: Upon heating, the reaction mixture at 70°C for 5 minutes, the protein undergoes denaturation, resulting in the unfolding and aggregation of egg albumin molecules. The denatured proteins precipitate out of the solution, causing the mixture to become cloudy or turbid. The degree of turbidity directly correlates with the extent of protein denaturation. In the presence of the Vitex negundo extract, particularly at higher concentrations, the turbidity is reduced compared to the control (distilled water), indicating that the extract has inhibited protein denaturation to some extent and this indicates the Vitex negundo demonstrating its potential anti-inflammatory properties by preventing protein denaturation.

Conclusion

Vitex negundo has significant anti-inflammatory effects, at concentrations of 100 and 200 µg/ml. These results suggest that Vitex negundo could be developed into a naturally formulated anti-inflammatory drug that can be used as a potential alternative against synthetic drugs. This makes Vitex negundo a promising and safer therapeutic option for managing inflammation-related conditions.

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Conflict of interests

The authors declare that there is no competing interest.

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