Exploring The Therapeutic Potential: In-Vitro Assessment of Antioxidant and Anti-Inflammatory Activity Of methanolic extract from *Tabernaemontana divaricata* Leaves

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Abstract

Introduction: Medicinal plants have various applications in the field of medical sciences. Plants are wide source of numerous phyto-constituents which have been proved to have multiple health benefits. Literature says that leaves of *Tabernaemontana divaricata* are loaded with such phyto-chemicals which can be a target in case of treatment of various infectious diseases. In Ayurvedic Medicine, the juice form of flower buds is mixed with oil and applied to the skin to treat inflammation. is also used in dental care, for scabies, as cough medicine and for eye ailments

Aim: To evaluate in-vitro antioxidant and anti-inflammatory activity of the methanolic extract of the leaves of *Tabernaemontana divaricate.*

Method: Methanolic extraction of the leaves of *Tabernaemontana divaricata* was made by using maceration method for 5 days and solvent was evaporated using rotary evaporator. To evaluate the anti-oxidant property, total phenolic content and total flavonoid content was measured against gallic acid and quercetin as reference. The in-vitro anti-inflammatory study was performed using techniques like heat induced hemolysis, hypotonicity induced hemolysis, protein denaturation study and protease inhibitory activity using goat blood cell.

Result: The total flavonoids content and total phenolic content were 38.57µg/ml and 45.48µg/ml respectively. The IC_{50} (Inhibitory concentration 50%) value was measured for each anti-inflammatory study. The IC_{50} value were **416 µg/ml, 166.66 µg/ml, 188 µg/ml and 280 µg/ml for the methods** heat induced hemolysis, hypotonicity induced hemolysis, protein denaturation study and protease inhibitory activity respectively

Conclusion: In conclusion, the methanolic extract of *Tabernaemontana divaricata* leaves exhibited significant antioxidant and anti-inflammatory properties. The high content of flavonoids and phenolic compounds underscores its potential therapeutic value. The extract demonstrated promising inhibitory effects against various inflammation-inducing mechanisms, as evidenced by the low IC50 values in the in-vitro anti-inflammatory assays. These findings support the traditional uses of *Tabernaemontana divaricata* in treating inflammation-related conditions. Further research exploring its mechanisms of action and potential clinical applications is warranted

Keywords: *Tabernaemontana divaricate*, tagar, anti-inflammatory, inflammation, anti-oxidant, radicle scavenging.

Introduction

Tabernaemontana divaricata, belongs to the family Apocynaceae popularly known as crepe Jasmine, is an evergreen flowering shrub and a popular house plant. The plant is found in India's upper Gangetic plain, Garhwal, East Bengal, Kasia Hills Assam, Burma, and Vishakhapatnam hills China, Thailand Japan and Myanmar. *Tabernaemontana divaricata* is an ornamental and useful medicinal plant. This, found mostly in South Asian region. Average height of the plant is around 4 to 6 feet. The leaves are large glossy and dark green [1,2]. *Tabernaemontana divaricata* plant also have anti-inflammatory, anti-asthmatic activity, antitussive activity and anti-cancer activity. *Tabernaemontana divaricata* has several uses in medicine. In Ayurvedic Medicine, the juice form of flower buds is mixed with oil and applied to the skin to treat inflammation. is also used in dental care, for scabies, as cough medicine and for eye ailments [3]. The constituent of *Tabernaemonta divaricata* was shown to be a potent inhibitor of ras functions and, very recently, it has also been found to induce morphological change as well as insulin production in pancreatic acinar carcinoma AR42J cells [4]. Flowers of plant contain α- Amyin acetate, β-amyrin acetate, lupeol, β–sitosterol, stigmasterol, & several other alkaloids like, dregamine, 20-epiervatamine, tabernamontanine, vobasine, voacangine, voacamine, flavonoid,

aglycocones, and flavanol glycosides like isovoacristine, voaphyllinehydroxyindolenine, janetine (tetrahydrolivadine), N-methylvoaphylline (hecubine), kaempferol [5]. It has been reported that alcoholic extract *Tabernaemontana divaricata* root and stem have a very high inhibitory activity against AChE, implying that it may be practitioners in the field for many neurodegenerative disorders, particularly myasthenia gravis and Alzheimer's disease. [6]

Fig 1: *Tabernaemontana divaricata* plant

Aim & Objectives

Background Concept of Work

Consumption of medicinal herbs is tremendously increasing over a past decade as an alternative approach to improve the quality of life and maintain a good health. The acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led researchers to investigate the antimicrobial activity of the herbal extracts. Likewise, treatment with herbal extracts can give long term benefit in case of inflammations and as Natural antioxidant supplements. From the botanical point of view, it is known that *Tabernaemontana divaricata* has been used in the treatment of the disease caused by, Inflammatory diseases. *Tabernaemontana divaricata* plant are also easily available in the nature. That is why this plant is chosen for this research work.

Objectives

The Objective of the present study is to select the suitable solvent and suitable method for extraction of the powdered leaves of *Tabernaemontana divaricata* to get high effectiveness for its anti-inflammatory and phytochemical screening. This project also aims to find and identify the Active phytochemical constituents found in *Tabernaemontana divaricate (Pinwheel plant)* leaves.

Materials & Methods

Collection and Authentication of Plant

The leaves of *Tabernaemontana divaricata* (about 3kg) were collected from local area plant during the month of September 2022 and the plant was identified by the help of regional nursery and finally authenticated by Botanical Survey of India, Howrah, West Bengal. The plant is identified with specimen no **JISU/PHARM.TECH/SSAHA-002**. Also identified that *Tabernaemontana divaricata* plant belong to family **Apocynaceae**.

Fig 2: Herbarium Sheet of *Tabernaemontana divaricata*

Extraction:

Fresh *Tabernaemontana divaricata* leaves, after collection was sun dried for 3 days. Plant material was then grinded in a mixer and grinder. Then they were milled to a coarse powder transferred through sieve and stored in an air tight container.

About 150g of Coarse powder was taken into a conical flask and 1000ml of Methanol(solvent) was added to the conical flask. Then it was wrapped in aluminum Foil and allowed to macerate for about 6 days with periodical shakings. After cold maceration, the extract was filtered and the excess solvent was reduced using a Soxhlet apparatus. The semisolid extract was refrigerated at 4°C for further analysis. [7]

Fig 3: Extraction procedure

Antioxidant Study:

Total phenolic content:

The total phenol content was determined with the Folin Ciocalteu's assay using gallic acid as standard. In the procedure, 0.5 ml of plant extracts at concentration 1 mg/ml was mixed with 1.5 ml Folin- Ciocalteu's reagent (FCR) diluted 1:10 v/v than after 5 minutes 1.5 ml of 7% sodium carbonate solution was added. The final volume of the tubes was made upto 10 ml with distilled water and allowed to stand for 90 minutes at room temperature. Absorbance of the sample was measured against the blank at 750 nm using a spectrophotometer and compared with the standard curve. [8]

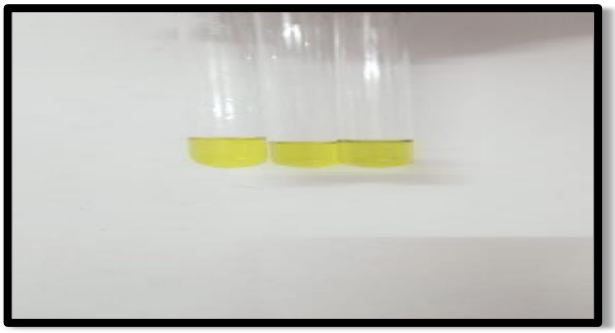

Fig 4: Study of Total Phenolic Content

Total Flavonoid content:

Total flavonoid content was determined by Aluminum chloride method using quercetin as a standard. 1ml of plant extract at concentration 1 mg/ml and 4 ml of water was added to a volumetric flask (10 ml volume). Add 0.3 ml of 5 % Sodium nitrite, 0.3 ml of 10% Aluminum chloride was added after 5 minutes. After 6 minutes of incubation at room temperature, 1ml of 1 M Sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to 10 ml with distilled water. Absorbance of the sample was measured against the blank at 510 nm using a spectrophotometer. [9]

Fig 5: Study of Total Flavonoid ContentAnti-Inflammatory Activity:

Preparation of red blood cell suspension:
The goat red blood cell membrane stabilization method has been used as a method to study the in vitro anti-inflammatory activity. The blood was collected and mixed with equal volume of Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.5 % citric acid and 0.42 % NaCl). All the blood samples were stored at 4 °C for 24 h before use. It was centrifuged at 2500 rpm for 5 min and the supernatant was removed. The cell suspension was washed with sterile saline solution (0.9 % w/v NaCl) and centrifuged at 2500 rpm for 5 min. This was repeated three times till the supernatant was clear and colorless and the packed cell volume was measured. The cellular component was reconstituted to a 40 % suspension (v/v) with phosphate buffered saline (10 mM, pH 7.4) and was used in the assays. [10]

Hypotonicity-induced hemolysis Study:
Sample extracts were prepared (100, 200, and 500 µg/ml), respectively using distilled water and to each concentration 1 ml of phosphate buffer, 2 ml hyposaline and 0.5 ml of goat blood suspension were added. It was incubated at 37 °C for 30 min and centrifuged at 3000 rpm for 20 min. The hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. A control was prepared by omitting the extracts. The percentage inhibition of hemolysis or membrane stabilization was calculated. [10] **% Inhibition of Hemolysis = [(A1 – A2)/ A1] x 100** where A1 = absorption of the control, and A2 = absorption of test sample mixture

Heat-Induced Hemolysis Study
0.05 mL of goat blood cell suspension and 0.05 mL of hydromethanolic extracts of leaves were mixed with 2.95 mL phosphate buffer (pH 7.4). The mixture was incubated at 54 °C for 20 min in a shaking water bath. After the incubation, the mixture was centrifuged (2500 rpm for 3 min), and the absorbance of the supernatant was measured at 540 nm using a UV/VIS spectrometer (Optima, SP-3000, Tokyo, Japan). Phosphate buffer solution was used as a control for the experiment. [11] **% Inhibition of hemolysis = [(A1 – A2)/ A1] x 100** where A1 = absorption of the control, and A2 = absorption of test sample mixture

Effect on Protein Denaturation
The reaction mixture (5 mL) consisted of 0.2 mL of 1% bovine albumin, 4.78 mL of phosphate buffered saline (PBS, pH 6.4), and 0.02 mL of extract, and the mixture was mixed, and was incubated in a water bath (37 °C) for 15 min, and then the reaction mixture was heated at 70 °C for 5 min. After cooling, the turbidity was measured at 660 nm using a UV/VIS spectrometer (Optima, SP-3000, Tokyo, Japan). Phosphate buffer solution was used as the control. [12,13] **% Inhibition of hemolysis = [(A1 – A2)/ A1] x 100** where A1 = absorption of the control sample, and A2 = absorption of the test sample.

Protease Inhibitory Activity
The reaction solution (2 mL) consisted of 0.06 mg trypsin, 1 mL of 20 mM Tris-HCl buffer (pH 7.4), and 1 mL test sample (0.02 mL extract 0.980 mL methanol). The solution was incubated (37 °C for 5 min), and then 1 mL of 0.8% (w/v) casein was added, and the mixture was further incubated for an additional 20 min. At the end of incubation, 2 mL of 70% perchloric acid was added to terminate the reaction. The mixture was centrifuged, and the absorbance of the supernatant was measured at 210 nm against buffer as the blank. Phosphate buffer solution was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula: [11,12] **% Inhibition of hemolysis = [(A1 – A2)/ A1] x 100** where A1 = absorption of the control sample, and A2 = absorption of the test sample.

Result and Observation:
Antioxidant Study:
Total Phenolic Content:
Total Phenolic Content (µg/ml) concentration was calculated in terms of Gallic acid equivalent using standard curve equation **(y = 0.0069x+ 0.0673)** of Gallic acid.
The leaves extract was taken 1mg/ml and absorbance was measured at 750nm in UV spectrophotometer and absorbance of the sample was 0.224. The concentration is calculated from the straight-line equation and value of X was 22.71014.
So, the total phenolic content concentration in the *Tabernaemontana divaricata* leaves was **22.71µg/ml**

Total Flavonoid Content:
Total Flavonoid Content (µg/ml) concentration was calculated in terms of Quercetin equivalent using standard curve equation (**y = 0.0031x+0.0159**) of Quercetin.
The leaves extract was taken 1mg/ml and absorbance was measured at 510 nm in UV spectrophotometer and absorbance of the sample was 0.122. The concentration is calculated from the straight-line equation and value of X was 34.22581.
So, the total flavonoid content concentration in the *Tabernaemontana divaricata* leaves was **34.225µg/ml.**

Anti-inflammatory study:
Hypotonicity Induced Hemolysis:

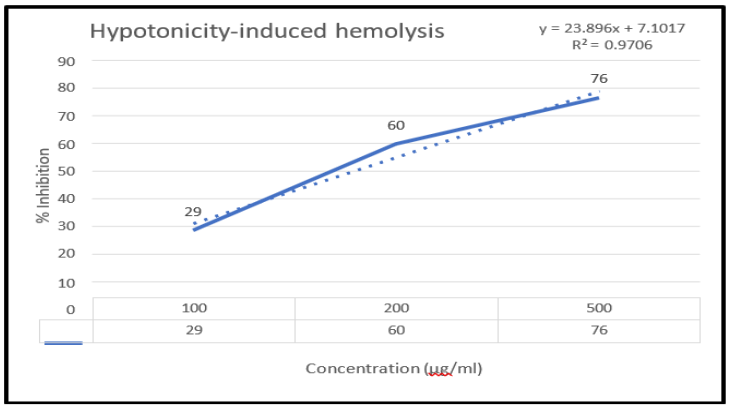

Fig 6: In-Vitro Anti-Inflammatory Study by Hypotonicity-induced hemolysis

From the UV visible spectroscopy statistical analysis through regression equation showed that good correlation exists between the concentration of extract and % Inhibition, with R2= 0.9706 for *Tabernaemontana divaricata* extract and **IC_{50} (Inhibitory concentration) value is 166.66 µg/ml.**

Heat Induced Hemolysis:

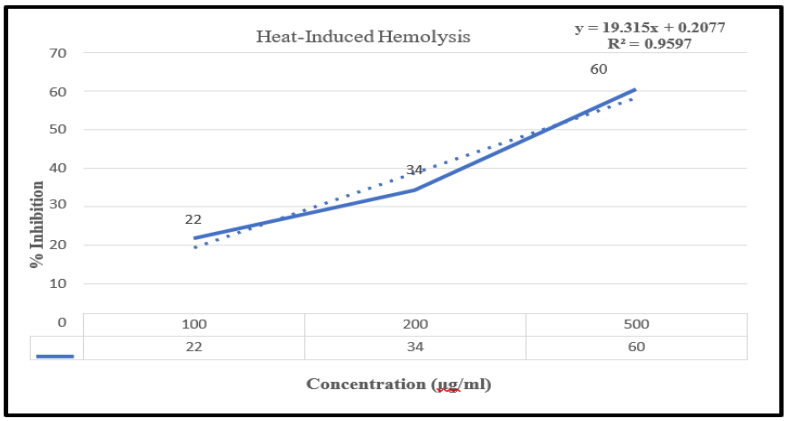

Fig 7: In-Vitro Anti-Inflammatory Study by Heat-Induced Hemolysis

From the UV visible spectroscopy statistical analysis through regression equation showed that good correlation exists between the concentration of extract and % Inhibition, with R2= 0.9597 for *Tabernaemontana divaricata* extract and **IC_{50} (Inhibitory concentration) value is 416 µg/mlEffect on Protein Denaturation:**

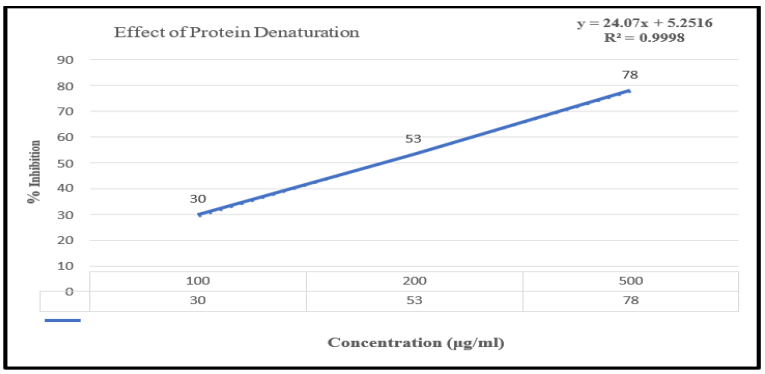

Fig 8: In-Vitro Anti-Inflammatory Study effect by Protein Denaturation

From the UV visible spectroscopy statistical analysis through regression equation showed that good correlation exists between the concentration of extract and % Inhibition, with R2= 0.998 for *Tabernaemontana divaricata* extract and **IC_{50} (Inhibitory concentration) value is 188 µg/ml.**

Protease Inhibitory Activity:

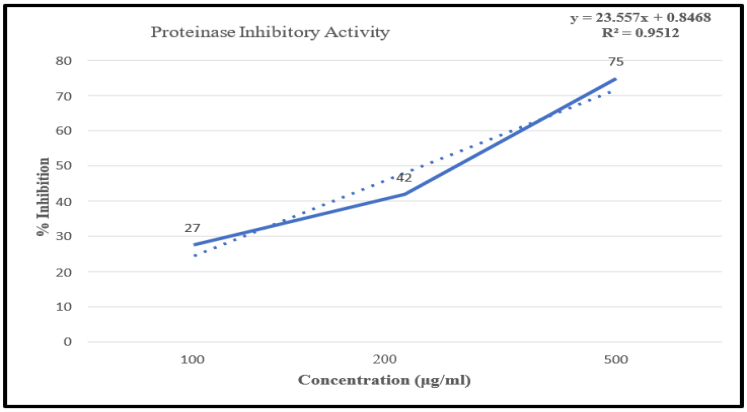

Fig 9: In-Vitro Anti-Inflammatory Study by Protease Inhibitory Activity

From the UV visible spectroscopy statistical analysis through regression equation showed that good correlation exists between the concentration of extract and % Inhibition, with R2= 0.9512 for *Tabernaemontana divaricata* extract and **IC_{50} (Inhibitory concentration) value is 280 µg/ml.**

Discussion and Conclusion:

The investigation into the phytochemical composition and anti-inflammatory properties of *Tabernaemontana divaricata* leaves provides valuable insights into the potential therapeutic benefits of this plant. The measured concentrations of total phenolic content (22.71 **µg/ml**) and total flavonoid content (34.225 **µg/ml**) underscore the rich bioactive profile of the plant extract. Phenolic compounds and flavonoids, known for their antioxidant and anti-inflammatory properties, are increasingly recognized for their potential role in the prevention and treatment of various disorders. The data obtained from in vitro anti-inflammatory studies further solidify the importance of *Tabernaemontana divaricata* in addressing inflammatory conditions. The hypotonicity-induced haemolysis assay revealed a notable anti-inflammatory effect with an IC50 value of 166.66 µg/ml. This suggests that the *Tabernaemontana divaricata* leaf extract possesses the ability to mitigate inflammation induced by hypotonic conditions, showcasing its potential in conditions related to cellular swelling and inflammation. The heat-induced haemolysis method displayed a moderate anti-inflammatory effect, as indicated by an IC50 value of 416 µg/ml. While this value is higher than that observed in the hypotonicity-induced haemolysis assay, it still signifies a significant inhibitory concentration, demonstrating the plant's potential in managing heat-induced inflammatory processes. The protein denaturation method, yielding the lowest IC50 value of 188 µg/ml, highlights the robust anti-inflammatory activity of *Tabernaemontana divaricata* against protein denaturation-induced inflammation. This result suggests that the plant extract may be particularly effective in preventing and alleviating inflammatory conditions associated with protein misfolding or aggregation. The protease inhibitory activity method demonstrated an IC50 value of 280 µg/ml, indicating the plant's ability to inhibit protease activity and potentially modulate inflammatory responses mediated by protease cascades.

In conclusion, the cumulative data strongly advocate for the therapeutic potential of *Tabernaemontana divaricata* in addressing inflammatory disorders. The plant's rich phenolic and flavonoid content, coupled with its effective inhibition of various inflammatory pathways, positions it as a promising candidate for the development of anti-inflammatory agents. Considering the prevalence of inflammatory disorders in modern society, the exploration of natural remedies such as *Tabernaemontana divaricata* becomes increasingly significant. Future studies should delve into the isolation and identification of specific bioactive compounds responsible for these effects, paving the way for targeted therapeutic interventions derived from this medicinal plant. *Tabernaemontana divaricata* emerges as a valuable natural resource with the potential to contribute to the development of novel anti-inflammatory strategies in contemporary healthcare.

Future Perspectives:

The future research on *Tabernaemontana divaricata* should aim to identify the specific beneficial compounds responsible for its antioxidant and anti-inflammatory properties. Advanced analytical techniques like chromatography and spectroscopy can help pinpoint and measure these individual compounds. Additionally, investigating the underlying mechanisms of its pharmacological effects would provide valuable insights into its therapeutic potential. Conducting in vivo studies in animal models of inflammation could validate the efficacy and safety of the methanolic extract, paving the way for clinical trials. Exploring new formulations and delivery methods to enhance the bioavailability and effectiveness of *Tabernaemontana divaricata* extracts would also be beneficial. Integrating traditional knowledge with modern scientific approaches will facilitate the development of evidence-based therapies utilizing this plant.

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