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In Vitro Evaluation of Anti-Inflammatory Activity of *Vitex negundo*

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ABSTRACT

Inflammation is a complex biological response associated with tissue injury and several chronic diseases, and prolonged use of conventional anti-inflammatory drugs is often limited by adverse effects. Medicinal plants offer a promising alternative due to their safety and therapeutic potential. Vitex negundo Linn. (Lamiaceae), commonly known as Nochi, is widely used in traditional medicine for the treatment of pain, inflammation, and related disorders. The present study aims to evaluate the in vitro anti-inflammatory activity of Vitex negundo leaf and flower extracts. Dried leaves and flowers of Vitex negundo were subjected to cold maceration using isopropyl alcohol and hydroalcoholic solvents. The obtained extracts were evaluated for anti-inflammatory activity by the Human Uman Red Blood Cell (HRBC) membrane stabilization method and protein denaturation inhibition assay, using diclofenac sodium as the standard drug. Both assays were performed at various concentrations (10–50 µg/mL), and the percentage inhibition was calculated spectrophotometrically. The results demonstrated a concentration-dependent anti-inflammatory activity for both extracts. The isopropyl alcohol extract showed higher membrane stabilization and protein denaturation inhibition compared to the hydro-alcoholic extract. At 50 µg/mL, the isopropyl alcohol extract exhibited maximum inhibition, indicating significant protection against inflammatory processes. The observed activity may be attributed to the presence of flavonoids, terpenoids, and other bioactive phytoconstituents known for their anti-inflammatory properties.

Keywords: Vitex negundo, anti-inflammatory activity, HRBC membrane stabilization, protein denaturation, medicinal plants.

INTRODUCTION

Inflammation is a complex process, which is frequently associated with pain and involves occurrences such as the increase of vascular permeability, increase of protein denaturation, and membrane alteration. When cells in the body are damaged by microbes, physical agents, or chemical agents, the injury is in the form of stress. Inflammation of tissue is due to a response to stress. It is a defensive response that is characterized by redness, pain, heat, swelling, and loss of function in the injured area. Loss of function occurs depending on the site and extent of injury. Since inflammation is one of the body's nonspecific internal systems of defense, the response of a tissue to an accidental cut is similar to the response that results from other types of tissue damage caused by burns due to heat, radiation, or bacterial or viral invasion.

When tissue cells become injured, they release kinins, prostaglandins, and histamine. These work collectively to cause increased vasodilation (widening of blood capillaries) and permeability of the capillaries. This leads to increased blood flow to the messengers that attract some of the body's natural defense cells, a mechanism known as chemotaxis. Inflammation is a complex pathophysiological process mediated by a variety of signalling molecules produced by leukocytes, macrophages, and mast cells undergoing various cellular responses such a phagocyte uptake and the production of inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2),, and tumour necrosis factor (TNF) that bring about the edema formation as a result of extravasation of fluid and proteins and the accumulation of leukocytes at the inflammatory site. In addition, it is broadly accepted that cytokines, produced by either immune or central nervous system cells, might directly sensitize the peripheral nociceptors.

Inflammation is an important cellular response triggered by various mechanical, chemical, or immunological stress factors, and it is regulated by a delicate balance between local factors that finally determine the outcome of the disease process: progression or resolution. The inflammatory response is a complex and highly regulated sequence of event that starts an initial production of pro-inflammatory mediators that recruit professional inflammatory cells to the site of injury to clear the offending trigger. This is followed by an anti-inflammatory phase, in which resident tissue cells may acquire the potential for protecting themselves from further activation and injury. More recently inflammation was described as "the succession of changes that occurs in a living tissue

when it is injured, provided that the injury is not of such a degree as to at once destroy its structure and vitality" or "the reaction to injury of the living microcirculation and related tissues." Under inflammatory conditions, immune cells are also stimulated by adhesion molecule activation signals in order to enhance the migration capacity to inflamed tissue and finally to form heterotypic cell clustering between the immune cells, endothelial cells, and inflamed cells.

Macrophages in the inflammatory reaction initially require an interaction between surface receptors such as Toll-like receptors (TR) and stimuli and subsequent up regulation of intracellular signalling events mediated by enzymes such as phosphoinositide 3-kinases (P(PI3K) and mitogen-a-activated protein kinases (MAPKs) as well as transcription factors (e.g., nuclear factor [N[NF]-κ and activator protein [AP]-1). Overall, these events lead macrophages to express pro-inflammatory genes such as inducible NO synthase (iNOS) and cyclooxygenase (COX)-, cause large amounts of macrophage-derived inflammatory mediators can cause collateral or severe damage such as septic shock, rheumatoid arthrthritis, and arteriosclerosis, the effective blockade of these inflammatory responses is an important therapeutic target. Inflammatory diseases are a major cause of morbidity of the workforce throughout the world.

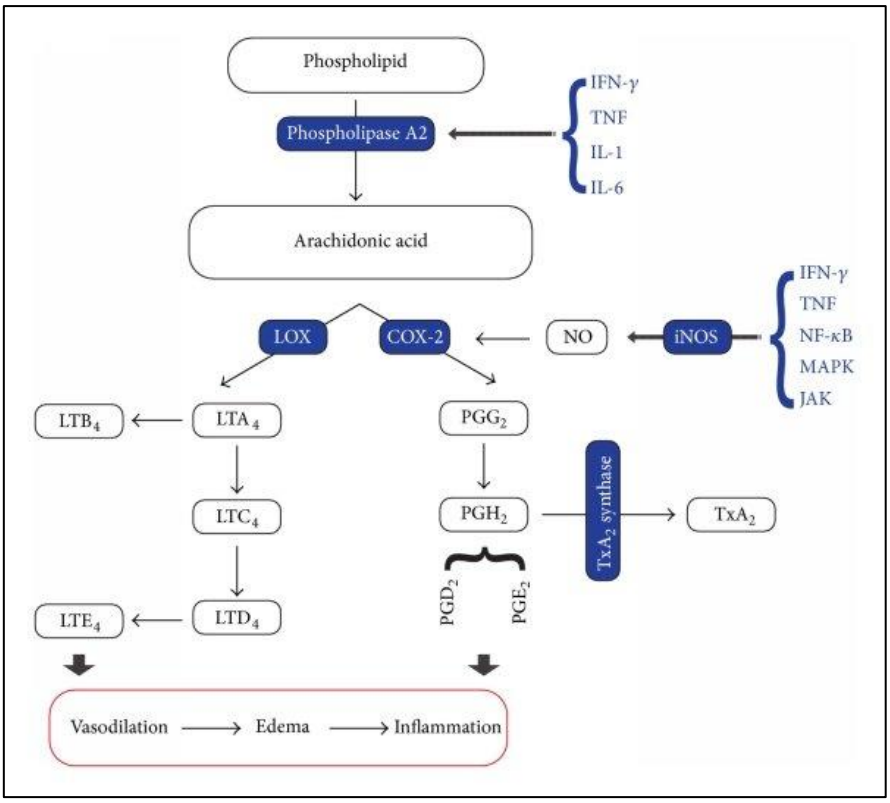

Figure 01: Inflammation pathway

TYPES OF INFLAMMATION

Inflammation can be classified into two types. They are,

- Acute inflammation
- Chronic inflammation

ACUTE INFLAMMATION:

Acute inflammation is rapid in onset (seconds or minutes), of relatively short duration (minutes, hours, or at most a few days), characterized by the exudation of fluid and plasma proteins & the emigration of leukocytes, predominantly neutrophils.

CHRONIC INFLAMMATION:

Chronic inflammation in contradistinction, is of insidious onset, of longer duration, and is associated histologically with the presence of lymphocytes, macrophages, plasma cells, Proliferation of blood vessels and fibroblasts.

SIGNS OF INFLAMMATION

The five classic signs of acute inflammation are redness, heat, swelling, pain, and loss of function. These symptoms result from increased blood flow, vascular permeability, and chemical mediator release in response to injury or infection.

❖ **Redness (Rubor):**

Increased blood supply to the area.

❖ **Heat (Calor):**

Increased blood flow making the skin warm to the touch.

❖ **Swelling (Tumor):**

Fluid accumulation and, in some cases, edema.

❖ **Pain (Dolor):**

Stimulation of nerve endings by inflammatory mediators.

❖ **Loss of function (Functio laesa):**

Reduced mobility or inability to use the area.

Chronic inflammation may present more subtly with fatigue, fever, or skin rashes.

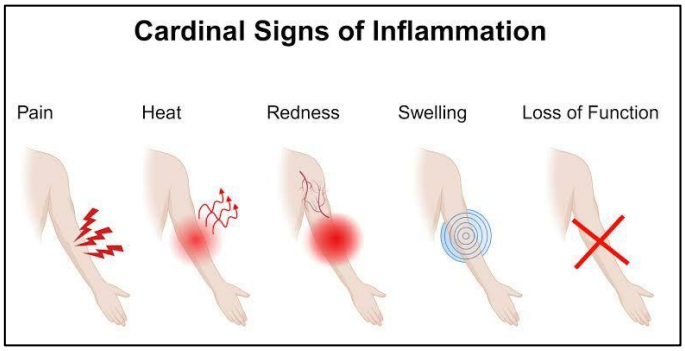

Figure 02: Signs of Inflammation

STANDARD DRUGS FOR INFLAMMATION

Standard drugs for inflammation include Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) such as ibuprofen, naproxen, and aspirin for mild-to-moderate pain and swelling, along with prescription options like celecoxib or diclofenac. Corticosteroids like prednisone are used for severe, chronic inflammation, while topical creams (e.g., diclofenac) treat localized soft-tissue injuries.

- **Aspirin:**

Aspirin is the most widely used drugs in the world today, because of its ability to act as anti-inflammatory medicine. However, patients with a history of peptic ulcer or other gastrointestinal disorders, are prone to gastroduodenal lesions on prolonged use of aspirin. The toxicity of aspirin is both dose- and disease-dependent.

- **Ibuprofen:**

Ibuprofen is also a commonly and successful used NSAIDs. However, long term use of ibuprofen, sulindac, phenylbutazone, and piroxicam has been associated with hepatotoxicity. The coxibs like rofecoxib, lumiracoxib, celecoxib and etoricoxib were reported to be associated with reduced gastrointestinal toxicity from the upper gastrointestinal tract when compared to non-selective NSAIDs; however, there are also reports that coxibs are associated with serious cardiovascular events and hepatotoxicity. Based on all these findings, the US Food and Drug Administration (FDA) in 2005 mandated that all NSAIDs should include a warning to highlight the potential increase in the risk of serious cardiovascular events, along with the warning about potential severe life-threatening gastrointestinal events. The same has been delivered by the European Medicines Agency (EMEA) as well as by a large number of national drug agencies all over the world. Thus, a careful evaluation of the risk profiles for adverse events before prescribing non-selective NSAIDs and coxibs is strongly recommended.

- **Diclofenac:**

Diclofenac reduces inflammation, swelling and arthritic pain by inhibiting prostaglandin synthesis and/or production. The drug also affects polymorphonuclear leukocyte function in vitro, thereby reducing chemotaxis, superoxide toxic radical formation, oxygen-derived free radical generation, and neutral protease production. Diclofenac has also been reported to suppress inflammation induced by various phlogistic agents in experimental animal models. However, it may cause side effects, including gastrointestinal disorders when administered by the oral route and cutaneous lesions by intramuscular injection. There are several published reports of cases of diclofenac-associated hepatotoxicity.

PLANT PROFILE

Vitex negundo

Figure 03: Leaves

Figure 04: Flower

BOTANICAL INFORMATION:

Botanical Name: *Vitex negundo L*

Family: Lamiaceae

VERNACULAR NAMES:

English: Chaste tree, Five leaved chaste

Tamil: Nochi

Hindi: Sambhala

Malayalam: Karinochi, vennochi

Nepali: Simali

Manipuri: Urik shibi

Kannada: Lakkigida, karilakki

TAXONOMIC CLASSIFICATION:

Kingdom: Plante

Subkingdom: Tracheobionta

Super division: Spermatophyta

Division: Magnoliophyta

Class: Dicotyledons

Superorder: Lamianae (Lamiids)

Order: Lamiales

Family: Lamiaceae

Genus: Vitex

Species: Vitex negundo

VARIOUS SPECIES OF CHASTE TREE:

Vitex trifolia

Vitex lucens

Vitex agnus – castus

DISTRIBUTION:

Throughout the India, Pakistan, Srilanka, China, Japan, Africa, Asia, Europe, Philippines.

DESCRIPTION:

Vitex negundo (commonly known as the five-leaved chaste tree or Nochi) is an aromatic shrub or small tree belonging to the family Lamiaceae. It usually grows up to 2–5 meters in height and has quadrangular stems with a grayish bark. The leaves are palmately compound, generally with five lanceolate leaflets, arranged oppositely on the stem. Each leaflet is lanceolate to ovate-lanceolate with serrated margins, an acute apex, and a cuneate base, covered with fine hairs that give a grayish-green appearance.

CHEMICAL CONSTITUENTS:

The principal constituents of the leaf juice are casticin, isoorientin, chrysophenol D, luteolin, p–hydroxybenzoic acid and D-fructose.

The main constituents of the oil are sabinene, linalool, terpinen-4-ol, β-caryophyllene, α-guaiene and globulol constituting 61.8% of the oil.

Vitex negundo contains various chemical constituents, including flavonoids, terpenoids (like viridiflorol, caryophyllene, and sabinene), iridoid glycosides (such as negundoside and nishindaside), and lignans. Other compounds found in the plant include triterpenes, diterpenes, and various acids and alcohols. The specific compounds can vary depending on the plant part, geographical location, and season.

MATERIALS AND METHODS

COLLECTION AND AUTHENTICATION OF PLANT MATERIAL:

Dried flower and leaves of the Vitex negundo was collected from the field of JAMBAI near Erode and authenticated by Dr. P. Radha, Research officer (Botanist), Sci-II &I/C with product code V041225192N Siddha Medical Plants Garden, Cauvery Nagar, Mettur dam, Salem District, Tamil Nadu. Voucher specimen (No: SSMCOP/1896/2025-2026) has been deposited in the Department of Chemistry, SSM College of Pharmacy, Jambai, Tamil Nadu, India.

PREPARATION OF PLANT MATERIAL:

The collected leaves and flowers were washed thoroughly with running tap water followed by distilled water to remove extraneous matter. The leaves and flowers were shade-dried at room temperature for 7–10 days. The dried leaves and flowers were pulverized using a mechanical grinder to obtain coarse powder and stored in airtight containers until extraction.

EXTRACTION:

Dried flower and leaves of the Vitex negundo were dried under the shade and made it in to a fine power and passed it in to the Sieve No 10 and collected and loaded into a round bottom flask. Required chemicals are added and the extraction process is done.

COLD EXTRACTION (MACERATION) PROCESS:

- **Extraction Using Isopropyl Alcohol**

About 50 gm of powdered Vitex negundo leaves was transferred into a clean, dry conical flask. To this, 500 mL of isopropyl alcohol was added in a ratio of 1:10 (w/v). The mixture was sealed and kept for maceration at room temperature for 72 hours with intermittent shaking at regular intervals to ensure proper extraction.

After completion of the maceration period, the extract was filtered using Whatman No.1 filter paper. The filtrate was concentrated under reduced pressure using a rotary evaporator. The dried extract was weighed, labelled, and stored at 4 °C for further studies.

- **Extraction Using Hydro-Alcoholic Solvent**

A separate portion of 50 gm of powdered Vitex negundo leaves was macerated with hydro-alcoholic solvent (ethanol: water, 50:50 v/v) in a ratio of 1:10 (w/v). The mixture was kept at room temperature for 72 hours with occasional shaking.

The extract was filtered using Whatman No.1 filter paper. The filtrate was concentrated using a rotary vacuum evaporator and dried to obtain the hydro-alcoholic extract. The extract was weighed, labelled, and stored in airtight containers at 4 °C until further use.

Figure 05: Extraction process

ANTI INFLAMMATORY ACTIVITY

Determination of in vitro anti-inflammatory activity

HRBC Membrane Stabilization Method

Procedure:

The anti-inflammatory activity of various extracts of leaves and flower of Vitex negundo was assessed by in vitro HRBC membrane stabilization method. Blood was collected from healthy volunteers. The collected blood was mixed with equal volume of Alsever solution (dextrose 2%, sodium citrate 0:8% citric acid 0.05%, sodium chloride 0.42%, and distilled water 100ml) and centrifuged with iso saline solution. To 3ml of HRBC suspension, equal volume of extracts in different concentrations 10, 20, 30, 40 and 50µg/ml and standard was added. All the assay mixtures were incubated at 37°C for 30 minutes and centrifuged. The haemoglobin content in the supernatant solution was estimated by using a spectrophotometer at 560nm. The percentage of protection can be hence calculated from the equation is given below.

%Inhibition of haemolysis 100 x (A1-A2/A1)

Where,

Al= Absorption of Control

A2 =Absorption of test sample

Here, the positive control was Diclofenac sodium. The negative control used was the Alsever's solution with blood in it and it contained no Diclofenac sodium or extracts of the plant material in it.

Effect on protein denaturation

Procedure:

Test solution (1ml) containing different concentrations of herbal extracts (10-50µg) or diclofenac sodium (100µg/ml) was mixed with 1ml of bovine albumin solution (1mM) and incubated at 27 ± 1° C for 15 min. Denaturation was induced by keeping the reaction mixture at 70° C in a water bath for 10 min. After cooling, the turbidity was measured spectrophotometrically at 660 nm. Percentage inhibition of denaturation was calculated from the control where no drug was added.

RESULTS AND DISCUSSION:

Anti-inflammatory Activity:

Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation of the mechanism of the anti-inflammation activity, the ability of different solvent plant extract protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation.

Table No. 01: HRBC membrane stabilizing activity

Effect of herbal extracts in erythrocyte haemolysis concentration		Absorbance at 560nm Average ± SEM	%Inhibition of haemolysis
Control		0.124±0.0012	----------
Diclofenac sodium(100µg|ml)		0.007±0.001	94.35
Isopropyl alcohol	10µg|ml	0.112±0.002	9.67
	20µg|ml	0.106±0.002	14.51
	30µg|ml	0.094±0.001	24.19
	40µg|ml	0.087±0.001	29.83
	50µg|ml	0.078±0.002	37.09
Control		0.120±0.004	----------
Diclofenac sodium(100µg|ml)		0.007±0.001	94.16
Hydro alcohol	10µg|ml	0.118±0.002	1.66
	20µg|ml	0.109±0.002	9.11
	30µg|ml	0.099±0.001	17.5
	40µg|ml	0.086±0.001	28.33
	50µg|ml	0.080±0.002	33.33

Effect on protein denaturation:

Table No. 02: Anti denaturation of protein in presence of Vitex negundo extracts

Effect of herbal extracts in erythrocyte haemolysis concentration		Absorbance at 660nm Average ± SEM	%Inhibition of haemolysis
Control		0.120	----------
Diclofenac sodium(100µg|ml)		0.009 ±0.0012	92.5
Isopropyl alcohol	10 µg |ml	0.110	8.33
	20 µg |ml	0.104	13.3
	30 µg |ml	0.099	17.5
	40 µg |ml	0.082	31.6
	50 µg |ml	0.069	42.5
Control		0.118	----------
Diclofenac sodium(100µg|ml)		0.009 ±0.0012	92.37
Hydro alcohol	10 µg |ml	0.116 ±0.002	1.69
	20 µg |ml	0.105 ±0.001	11.01
	30 µg |ml	0.096 ±0.002	18.64
	40 µg |ml	0.079 ±0.001	33.05
	50µg|ml	0.070 ±0.002	40.67

GRAPHICAL REPRESENTATION:

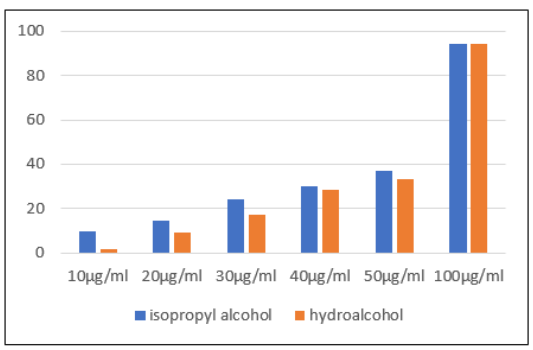

Figure 06: Effect of different extract on HRBC

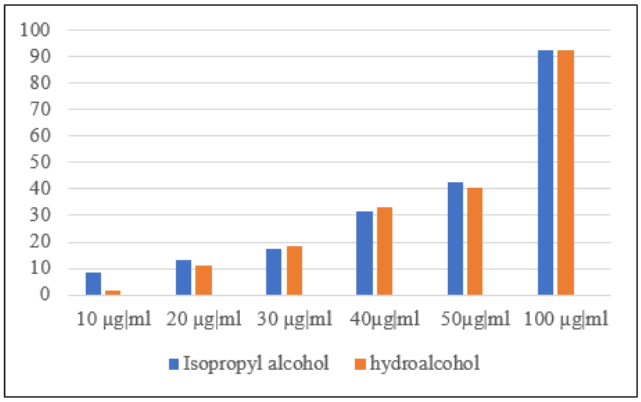

Figure 07: Denaturation of bovine protein by different extract

Figure 08: ISOPROPYL CONCENTRATION

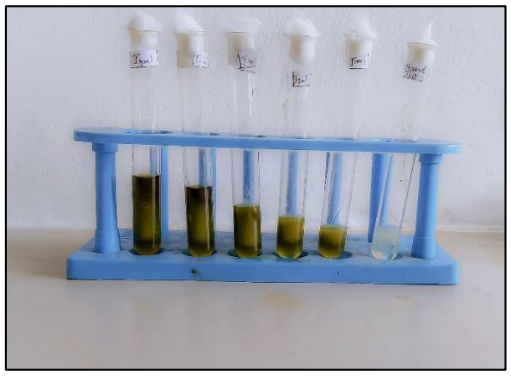

Figure 09: HYDROALCOHOL CONCENTRATION

CONCLUSION:

The attention of pharmacologists throughout the world has been focused on finding out safer and potent anti-inflammatory drug. This is not surprising since inflammatory disorders like rheumatoid arthritis have worldwide prevalence, occur in all races and ethnic groups and have onset early adulthood, sometimes crippling the afflicted person to render him economically nonproductive.

Anti-inflammatory drugs offer symptomatic relief in the inflammatory diseases when the underlying cause of inflammation is unidentified. Anti-inflammatory drugs, presently available for the treatment of joint inflammation of various kinds, have undesirable side effects such as causing peptic ulcers, GI complications, including bleeding and perforation due to inhibition of prostaglandin synthesis.

Therefore, the search for safe and effective drug is up surged. Therefore, plant remedies have become increasingly popular and are often preferred to synthetically derive pharmaceuticals. The natural products today symbolize safety in contrast to the synthetic drugs that are regarded as unsafe to humans and the environment. So, people are returning to the natural products with the hope of safety and security. Numerous evidences have shown that increased consumption of fruit and vegetables reduces the risk of various pathological events such as cancer, cardiovascular, cerebrovascular.

It is believed that current anti-inflammatory drugs such as opioids and non-steroidal anti-inflammatory drugs are not useful in all cases because of their side effects and low potency. As a result, search for other alternatives became necessary and imperative. Novel anti-inflammatory agents could be discovered from medicinal plants containing a wide variety of phytoconstituents. Traditional medicine for the treatment of various diseases is becoming more popular. Many medicinal plants provide relief of symptoms compared to that of conventional medicinal agent. Therefore, the present study was aimed at evaluating the scientific basis for the traditional use of Vitex negundo using in vitro anti- inflammatory models.

Vitex negundo is a plant that belongs to the family *Lamiaceae*. The constituents of the leaf juice are casticin, isoorientin, chrysophenol D, luteolin, p–hydroxybenzoic acid and D-fructose. The main constituents of the oil are sabinene, linalool, terpinen-4-ol, β-caryophyllene, α-guaiene and globulol constituting 61.8% of the oil. Vitex negundo contains various chemical constituents, including flavonoids, terpenoids (like viridiflorol, caryophyllene, and sabinene), iridoid glycosides (such as negundoside and nishindaside), and lignans. Other compounds found in the plant include triterpenes, diterpenes, and various acids and alcohols. The specific compounds can vary depending on the plant part, geographical location, and season.

In view of this, the present study was aimed to evaluate the Anti-inflammatory activity of isopropyl alcohol and hydro alcohol fractions of Vitex negundo leaves and flower by two methods like Protein denature method and human blood cell membrane stabilizing method. In anti-inflammatory activity both proteins denature method and in membrane stabilizing method isopropyl alcohol shows more activity than Hydro alcohol.

Hence it was concluded that isopropyl alcohol extract of Vitex negundo exhibits promising in vitro anti-inflammatory activity.

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