Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2025; 14(1): 135-143
Received: 28-11-2024
Accepted: 30-12-2024

B Udayabhanu
Assistant Professor,
Pharmaceutical Chemistry,
Pullareddy Institute of
Pharmacy, Hyderabad,
Telangana, India

Kiran Kumar
Assistant Professor, Department
of Pharmaceutical Chemistry,
Pulla Reddy Institute of
Pharmacy, Hyderabad,
Telangana, India

Meraj
Assistant Professor, Department
of Pharmaceutical Chemistry,
Pulla Reddy Institute of
Pharmacy, Hyderabad,
Telangana, India

Thrilochan
Assistant Professor, Department
of Pharmaceutical Chemistry,
Pulla Reddy Institute of
Pharmacy, Hyderabad,
Telangana, India

Rahman
Assistant Professor, Department
of Pharmaceutical Chemistry,
Pulla Reddy Institute of
Pharmacy, Hyderabad,
Telangana, India

Corresponding Author:
B Udayabhanu
Assistant Professor, Department
of Pharmaceutical Chemistry,
Pulla Reddy Institute of
Pharmacy, Hyderabad,
Telangana, India

Evaluation of the synergistic effect of herbal drugs *Embelia ribes, Cinnamomum verum* and *Zingiber officinale* by *in vitro* anti-inflammatory studies

B Udayabhanu, Kiran Kumar, Meraj, Thrilochana and Rahman

DOI: https://doi.org/10.22271/phyto.2025.v14.i1b.15231

Abstract

Herbal drugs have a great significance in the field of therapeutics. In this work we have selected three herbal drugs i.e.; *Embelia ribes, Cinnamomum verum and Zingiber officinale* which have proven anti-inflammatory property individually, now our study focuses on the synergistic effect of the above three drugs by in-vitro Anti-inflammatory studies. Preliminary phytochemical screening has been performed primarily and followed by the evaluation for the proposed activity. The main action of anti-inflammatory agents is the inhibition of Cyclooxygenase enzymes which are responsible for the conversion of Arachidonic acid to prostaglandins. Since human red blood cell (HRBC) membranes are similar to these lysosomal membrane components, the prevention of hypotonicity induced HRBC membrane lysis was taken as a measure in estimating the anti-inflammatory property of extracts of *Embelia ribes, Cinnamomum verum and Zingiber officinale*. Thus, Human red blood cell membrane stabilization (HRBC method) has been used as a method in estimating the anti-inflammatory property. The present study aimed to authenticate that traditional information by *in vitro* anti-inflammatory screening.

Keywords: HRBC membrane, anti-inflammatory, synergistic effect

1. Introduction

Herbal drugs shall mean a dosage form consisting of one or more herbs or processed herbs in specified quantities to provide specific nutritional, cosmetics benefits, or other benefits meant for use to diagnose, treat, mitigate diseases of human beings or animals or to alter the structure or physiology of human beings or animals. *In vitro* studies use cells derived from animals or cell lines which have an infinite lifespan. These model systems are relatively cheap and simple to procure, enabling reliable and efficient drug discovery studies. Synergy is defined as the interaction of two or more agents to produce a combined effect greater than the sum of their individual effects. In the present study we are planning to evaluate Synergistic Effect of Herbal Drugs (*Embelia ribes, Cinnamomum verum* and *Zingiber officinale*) by *In vitro* Anti-Inflammatory Activity Studies."

Inflammation is a reaction of living tissues towards injury, and it comprises systemic and local responses. In spite of our dependence on modern medicine and the tremendous advances in synthetic drugs, a large number of the world populations (80% of people) cannot afford the products of the western Pharmaceutical industry and have to rely upon the use of traditional medicines, which are mainly derived from plant material. The fact is well recognized by the WHO which has recently compiled an inventory of medicinal plants listing over 20 000 species. The family Myrsinaceae, Zingiberaceae, Lauraceae consists of several important medicinal plants with wide range of pharmacological, biological activities and interesting Phyto chemical constituents. The main action of anti-inflammatory agents is the inhibition of Cyclooxygenase enzymes which are responsible for the conversion of Arachidonic acid to prostaglandins. Since human red blood cell (HRBC) membranes are similar to these lysosomal membrane components, the prevention of hypotonicity induced HRBC membrane lysis was taken as a measure in estimating the anti-inflammatory property of extracts of *Embelia ribes, Cinnamomum verum and Zingiber officinale*. Thus, Human red blood cell membrane stabilization (HRBC method) has been used as a method in estimating the anti-inflammatory property. The present study aimed to authenticate that traditional information by *in vitro* anti-inflammatory screening.

Inflammation

Definition: Inflammation is a local response (reaction) of living vascularized tissues to endogenous and exogenous stimuli. The term is derived from the Latin "in flam mare" meaning to burn. Inflammation is fundamentally destined to localize and eliminate the causative agent and to limit tissue injury. Thus, inflammation is a physiologic (protective) response to injury. Inflammation is itself not to be considered as a disease but as a salutary operation consequent either to some violence or to some diseases".

Causes: Causes of inflammation are apparently causes of diseases such as:

1. **Physical agents:** Mechanical injuries, alteration in temperatures and pressure, radiation injuries.
2. **Chemical agents:** Including the increasing lists of drugs and toxins.
3. **Biologic agents (Infectious):** Bacteria, viruses, fungi, parasites
4. **Immunologic disorders:** Hypersensitivity reactions, autoimmunity, immunodeficiency states etc
5. **Genetic/metabolic disorders:** Examples gout, diabetes mellitus etc.

Examples of diseases with specific inflammation

- Syphilis
- Tuberculosis
- Leprosy
- Scleroma

Nomenclature

The nomenclatures of inflammatory lesion are usually indicated by the suffix 'itis'. Thus, inflammation of the appendix is called appendicitis and of meninges as meningitis, etc. However, like any rule, it has its own exceptions examples pneumonia, typhoid fever.

Classification

Inflammation is classified crudely based on duration of the lesion and histologic appearances into acute and chronic inflammation.

According to the course

- Acute,
- Subacute,
- Chronic

According to the predominant phase

- Alterative,
- Exudative,
- Proliferative (Productive)

According to the causative factors

- Trivial,
- Specific

Acute inflammation

- Acute inflammation is an immediate and early response to an injurious agent and it is relatively of short duration, lasting for minutes, several hours or few days.
- It is characterized by exudation of fluids and plasma proteins and the emigration of predominantly neutrophilic leucocytes to the site of injury.

The five cardinal signs of acute inflammation are

1. Redness (rubor) which is due to dilation of small blood vessels within damaged tissue as it occurs in cellulitis.
2. Heat (calor) which results from increased blood flow (hyperemia) due to regional vascular dilation
3. Swelling (tumor) which is due to accumulation of fluid in the extravascular space which, in turn, is due to increased vascular permeability.
4. Pain (dolor), which partly results from the stretching & destruction of tissues due to inflammatory edema and in part from pus under pressure in, as abscess cavity. Some chemicals of acute inflammation, including bradykinins, prostaglandins and serotonin are also known to induce pain.
5. Loss of function: The inflamed area is inhibited by pain while severe swelling may also physically immobilize the tissue.

Events of acute inflammation

Acute inflammation is categorized into an early vascular and a late cellular response.

1. The Vascular response has the following steps

- Immediate (Momentary) vasoconstriction in seconds due to neurogenic or chemical stimuli.
- Vasodilatation of arterioles and venules resulting in increased blood flow.
- After the phase of increased blood flow there is a slowing of blood flow & stasis due to increased vascular permeability that is most remarkably seen in the post-capillary venules. The increased vascular permeability oozes protein-rich fluid into extravascular tissues. Due to this, the already dilated blood vessels are now packed with red blood cells resulting in stasis. The protein-rich fluid which is now found in the extravascular space is called exudate. The presence of the exudates clinically appears as swelling. Chemical mediators mediate the vascular events of acute inflammation.

2. Cellular response

The cellular response has the following stages:

- Migration, rolling and adhesion of leukocytes
- Transmigration of leukocytes
- Chemotaxis
- Phagocytosis

Normally blood cells particularly erythrocytes in venules are confined to the central (Axial) zone and plasma assumes the peripheral zone. As a result of increased
vascular permeability, more and more neutrophils accumulate along the endothelial surfaces (Peripheral zone).

- **Migration, rolling, pavementing, and adhesion of leukocytes**
- Margination is a peripheral positioning of white cells along the endothelial cells.
- Subsequently, rows of leukocytes tumble slowly along the endothelium in a process known as rolling.
- In time, the endothelium can be virtually lined by white cells.
- Thereafter, the binding of leukocytes with endothelial cells is facilitated by cell adhesion molecules such as selectins, immunoglobulins, integrins, etc which result in adhesion of leukocytes with the endothelium.

Transmigration of leukocytes

- Leukocytes escape from venules and small veins but only occasionally from capillaries. The movement of leukocytes by extending pseudopodia through the vascular wall occurs by a process called diapedesis.
- The most important mechanism of leukocyte emigration is via widening of inter-endothelial junctions after endothelial cells contractions. The basement membrane is disrupted and resealed thereafter immediately.

- **Chemotaxis**
- Chemotaxis is a unidirectional attraction of leukocytes from vascular channels towards the site of inflammation within the tissue space guided by chemical gradients (including bacteria and cellular debris).
- The most important chemotactic factors for neutrophils are components of the complement system (C5a), bacterial and mitochondrial products of arachidonic acid metabolism such as leukotriene B4 and cytokines, Interleukin-L(IL-8). All granulocytes, monocytes and to lesser extent lymphocytes respond to chemotactic stimuli.

Phagocytosis

Phagocytosis is the process of engulfment and internalization by specialized cells of particulate material, which includes invading microorganisms, damaged cells, and tissue debris. These phagocytic cells include polymorphonuclear leukocytes (particularly neutrophiles), monocytes and tissue macrophages.

Phagocytosis involves three distinct steps.

1. **Recognition and attachment:** The particle to be ingested by the leukocytes: Phagocytosis is enhanced if the material to be phagocytosed is coated with certain plasma proteins called opsonins. These opsonins promote the adhesion between the particulate material and the phagocyte's cell membrane.
2. **Engulfment:** During engulfment, extension of the cytoplasm (pseudopods) flow around the object to be engulfed, eventually resulting in complete enclosure of the particle within the phagosome created by the cytoplasmic membrane of the phagocytic cell. As a result of fusion between the phagosome and lysosome, a phagolysosome is formed and the engulfed particle is exposed to the degradative lysosomal enzymes.
3. **Killing or degradation:** The ultimate step in phagocytosis of bacteria is killing and degradation. There are two forms of bacterial killing.

a) **Oxygen-independent mechanism:** This is mediate by some of the constituents of the primary and secondary granules of polymorphonuclear leukocytes. These include: Bactericidal permeability increasing protein (BPI), Lysozymes, Lactoferrin, and Defenses.
It is probable that bacterial killing by lysosomal enzymes is inefficient compared with the oxygen dependent mechanisms. The lysosomal enzymes are, however, essential for the degradation of dead organisms within phagosomes.

b) **Oxygen-dependent mechanism:** There are two types of oxygen- dependent killing mechanisms

i) **Non-myeloperoxidase dependent**
The oxygen - dependent killing of microorganisms is due to formation of reactive oxygen species such as hydrogen peroxide (H_2O_2), super oxide (O_2) and hydroxyl ion (HO-) and possibly single oxygen ($1O_2$). These species have single unpaired electrons in their outer orbits that react with molecules in cell membrane or nucleus to cause damages.

ii) **Myeloperoxidase-dependent**
The bactericidal activity of H_2O_2 involves the lysosomal enzyme myeloperoxidase, which in the presence of halide ions converts H_2O_2 to hypochlorous acid (HOCI). This H_2O_2 - halide - myeloperoxidase system is the most efficient bactericidal killing system in neutrophils. A similar mechanism is also effective against fungi, viruses, protozoa and helminths. Like the vascular events, the cellular events (i.e. the adhesion, the transmigration, the chemotaxis, & the phagocytosis) are initiated or activated by chemical mediators.

Chemical mediators of inflammation

Chemical mediators account for the events of inflammation. Inflammation has the following sequence:
Cell injury → Chemical mediators → Acute inflammation (i.e. the vascular & cellular events).

Sources of mediators

The chemical mediators of inflammation can be derived from plasma or cells.

a) Plasma-derived mediators:

i) Complement activation

- Increases vascular permeability (C3a, C5a)
- Activates chemo-taxis (C5a)
- Opsoninization (C3b, C3bi)

ii) Factor XII (Hageman factor) activation

Its activation results in recruitment of four systems: the kinin, the clotting, the fibrinolysis and the compliment systems.

b) Cell-derived chemical mediators

Cell-derived chemical mediators include:

Cellular mediators	Cells of origin	Functions
Histamine	Mast cells, basophiles	Vascular leakage & platelets
Serotonin	Platelets	Vascular leakage
Lysosomal enzymes	Neutrophils	Bacterial & tissue destruction macrophages
Prostaglandins	All leukocytes	Vasodilatation, pain, fever
Leukotriene	All leukocytes	LB4
Chemo-attractant	LC4, LCD4, & LE4	Broncho and vasoconstriction
Platelet activating factor	All leukocytes	Bronchoconstriction and WBC priming
Activated oxygen species	All leukocytes	Endothelial and tissue damage
Nitric oxide	Macrophages	Leukocyte activation
Cytokines	Lymphocytes, macrophages	Leukocyte activation

Most mediators perform their biologic activities by initially binding to specific receptors on target cells. Once activated and released from the cells, most of these mediators are short lived. Most mediators have the potential to cause harmful effect

2. Plant Profile
2.1 Plant Profile

Botanical Name	*Embelia ribes*
Family	Myrsinaceae

- **Habits and Habitats**
  A large tree which is distributed throughout India from the Central Himalayas to peninsular India ascending to 1700m, also common in the lower hills. The fruits, seeds, leaves, root bark and roots of ribes are useful as medicine.
- **Plants description**
  It is a creeper which has a brittle stem. Leaves are ovate in shape having both the ends sharp. It is about 3-inch-long and 1 ½ inches broad, shiny and modulated. Flowers are white, small having petiole 3 to 4 inch in length. Fruit is small like that of a pepper. Its color varies from red to blackish. It is found in bunches. The outer covering of the fruit is fragile and inside the seed is spotted.
- **Parts Used:** Fruits and dried seeds

- **Medicinal properties and uses**
  Alternative, anti-diarrhoeal, antidysentric, anti hemorrhagic. The decoction of seeds is beneficial in fevers, skin diseases and chest complain. Young leaves in combination with ginger used as a gargle for aphthae and indolent ulcers in the mouth as well as sore throat. The paste of root bark is applied to chest in pneumonia.
- **Chemical Composition**
  Embelin is present in seeds. It consists of an alkaloid, christembine, homoembelin and homoraparone, vilangine and guercitol.

- **Production Technology**
  Cultivated in well-drained soil in sun with high humidity. Propagation by seeds sown when ripe. Whole plants are collected during summer and autumn and used fresh or dried in decoctions, or pounded with roots as a poultice. Fruits are collected when unripe or ripe for decoctions, powder, and confectionery. Roots and bark are collected as required for decoctions.

2.2 Plant Profile

Botanical Name: *Zingiber officinalis*
Family: Zingiberaceae

- **Habits and Habitats**
  Ginger is a large tuberous perennial plant which is cultivated extensively in almost all tropical and subtropical countries like India, China, Africa, and Australia. India and China are the world's leading producers of Ginger. Ginger has a tendency to grow horizontally and the soil can be hilled around the growing stems to force a more vertical growth habit. Native to humid, partly-shaded habitats in moist tropical and subtropical forests of Southeast Asia. Mostly grown in states of India like Madhya Pradesh, Karnataka.
- **Plant description**
  The ginger plant has a thick, branched rhizome (underground stem) with a brown outer layer and yellow centre that has a spicy, citrusy aroma**.** The stem sticks up about 12 inches above ground with long, narrow, ribbed, green leaves, and white or yellowish-green flowers.

- **Parts used:** Rhizome

- **Medicinal properties and uses**
  Ginger also contains antioxidants and anti-inflammatory properties. Ginger has been used for a wide array of unrelated ailments such as arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever, infectious diseases, and helminthiasis.
- **Chemical compositions:** The main chemical components identified included Zingiberene, (+)-β-Cedrene, Farnesene, α-Curcumene, β-Elemene and (-)-Borneol.

- **Production technology**
Ginger is propagated by portions of rhizomes known as seed rhizomes. Carefully preserved seed rhizomes are cut into small pieces of 2.5 - 5.0 cm length weighing 20 - 25 g each having one or two good buds.

2.3 Plant Profile
Botanical Name: *Cinnamomum verum*
Family: Lauraceae

- **Habits and habitats**
Cinnamomum verum is an evergreen tree that reaches a height of 8-17 m in the wild. In an unharvested state, the trunk is stout, 30-60 cm in diameter. Leaves stiff, extipulate somewhat variable in form and size. Cinnamon is native to Sri Lanka (formerly Ceylon) and is also cultivated in South America and the West Indies**.**

- **Plant description**
Cinnamon (*Cinnamomum verum*) also called Ceylon cinnamon, is a bushy evergreen tree of the laurel family (Lauraceae) and the spice derived from its bark. The spice consists of the dried inner bark and has a delicately fragrant aroma and a warm sweet flavor.

- **Parts used:** bark

- **Medicinal properties and uses**
Cinnamomum verum has been reported to have anti-diabetic, antibacterial, antioxidant, anti-inflammatory, and anticancer effects. cinnamon has also been reported to have activities against neurological disorders, such as Parkinson's and Alzheimer's diseases.
- **Chemical compositions**
Cinnamon consists of a variety of resinous compounds, including cinnamaldehyde, cinnamate, cinnamic acid, and numerous essential oils. Reported that the spicy taste and fragrance are due to the presence of cinnamaldehyde and occur due to the absorption of oxygen.
- **Production technology**
Production of cinnamon bark entails cutting the stems down low after an initial establishment period and harvesting the bushy regrowth stems at regular intervals thereafter. Stems are cut during the rainy season to facilitate peeling of the bark in 2 longitudinal strips.

3. Review of literature
- **Ginger:** Ginger (*Zingiber officinale* Rosc.), a member of the Zingiberaceae family, is a medicinal herb utilized for its anti-inflammatory and antioxidant qualities. Ginger's influence on health was discovered due to its high phytochemical content, which includes compounds that eliminate free radicals created by biological systems. Gingerol, shogaol, and other related ginger chemicals limit the body's production of prostaglandins and leukotrienes. They can also suppress the production of pro-inflammatory cytokines, such as IL-1, TNF-, NF-B, and IL-8. According to our knowledge, NF-B activation is associated with a number of inflammatory disorders, including cancer, kidney injury, and Alzheimer's disease.
- **Embelia:** Dried fruits of *Embelia ribes (E. ribes*) belong to family Myrsinaceae is one of the most significant plants used from the prehistoric time in the form of the drug Baibidanga or Vidanga. It has been used as an ingredient in most of the Ayurvedic formulation for the treatment of various ailments. Various formulation of *Embelia ribes* are used in ayurvedic system of medicine like asava, aristha, lauha and taila. Commonly it is known as false black pepper. It is listed in red book as threatened species. In various literatures, it is found that the fruits of that plant used as an anthelmintic, diuretic, carminative, contraceptive, anti-bacterial, anti-inflammatory astringent, antioxidant, anticancer agents and seed possessed antibiotic and antitubercular properties.
- **Cinnamon:** Cinnamon is a spice obtained from the inner bark of several tree species from the genus Cinnamomum. Cinnamon is used mainly as an aromatic condiment and flavouring additive in a wide variety of cuisines, sweet and savoury dishes, breakfast cereals, snack foods, tea and traditional foods. There are four major types of cinnamon. Darker-colored cassia cinnamon is the one most commonly sold in the United States. It's grown in southeastern Asia. Ceylon cinnamon, also known as true cinnamon, is frequently used in other countries. Cinnamon usually causes no side effects. But heavy use could irritate your mouth and lips, causing sores. Some people are allergic to it. It might cause redness and irritation if you put it on your skin.
- Literature review shows that already proven anti-inflammatory activity individually, Now the present study focuses on the synergistic effect of the extracts i.e; *Embelia ribes*, *Cinnamomum verum* and *Zingiber officinale*.

4. Materials and Methods
- **Preparation of Extracts**
- **Preliminary phytochemical studies**
- **Chemicals and instruments**
- ***In vitro* anti-inflammatory activity**
- **Preparation of extracts**

The selected plants materials were separately extracted successively with selected solvent with increase order of polarity using suitable extraction process and preliminary

phytochemical study was performed on various liquid extracts.

The fresh plant materials of *Embelia ribes* (seeds), *Cinnamomum verum* (bark) and *Zingiber officinale (rhizome)* were collected from young matured plants and authenticated. After authentication, the plant materials were collected in bulk, washed under running tap water to remove adhering dirt followed by rinsing with distilled water. The plant materials were then shade dried and separately pulverized in a mechanical grinder to obtain coarse powder.

Preparation of Extracts

The dried powdered plants materials (500 g each) were separately defatted with petroleum ether and successively extracted with methanol and Hydro alcoholic mixture (1:10 ratio) using a Soxhlet extractor. The period of extraction was fixed at 5 h for every solvent at every stage of the extraction process. The solvents were purified by distillation prior to extraction. After completion of extraction, the extractive value was determined with respect to the dried plant material. After the filtrate has obtained, it was then transferred into a weighed Petry plates. The obtained extracts were concentrated to dryness by keeping filtrate for complete evaporation of solvent. The extractive value in percentage was calculated by using following formula and recorded.

Extractive value (%) = Weight of dried extract /Weight of plant material X 100

Aqueous, methanolic, extracts of *Embelia ribes, Cinnamomum verum* and *Zingiber officinale*. The above extracts were studied for their colour, consistency and extractive values and reported in

Table 1: Percentage extractive value (%w/w)

Solvent Extract	Colour	Consistency	%w/w Extractive Value
Methanol (*Embelia ribes*)	Dark brown	Sticky with light brown stain	2.0
Aqueous (*Cinnamomum verum*)	Brownish red	Waxy residue	1.4
Methanol (*Zingiber officinale)*	Brick red	Amber coloured waxy residue	3.8

Preliminary phytochemical studies

A plant may be considered as a biosynthetic laboratory, not only for the chemical compounds such as carbohydrates, proteins and lipids that are utilized as food by man, but also for a multitude of a compounds like glycosides, alkaloids, volatile oils, saponins, etc., that exert a physiological effect. The compounds that are responsible for therapeutic effects are usually the secondary metabolites. A systematic study of a crude drug embraces through consideration of both primary and secondary metabolites derived as a result of plant metabolism. The plant material may be subjected to preliminary phytochemical screening for the detection of various plant constituents. (Harborne J.B., 1984 and Wagner H., Bladt S., 1996) [9].

Tests for alkaloids

a) **Mayer's reagent:** Dissolve 1.36 g of mercuric chloride in 60 ml. Distilled water (a) Dissolve 5 g potassium iodide in 60 ml. distilled water (b) Mix (a) & (b) and adjust the volume to 100 ml with distilled water. With alkaloids, it produces white to buff coloured precipitate.
b) **Wagner's reagent:** Dissolve 1.27 g of iodine and 2 g of potassium iodide in 5 ml of water and make up the volume 200 ml with distilled water. With alkaloids, it produces reddish brown precipitate.
c) **Dragendroff's reagent:** Boil 14 g of sodium iodide with 5.2 gm of bismuth carbonate in 50 ml glacial acetic acid for a few minutes. Allow it to stand overnight and filter of the precipitate of sodium acetate crystals. Preserve the stock solution in amber coloured bottle. When needed, add 20 ml of acetic acid to 10 ml of this stock solution and make up to 100 ml with water. With alkaloids, it produces orange brown precipitate.
d) **Hager's reagent:** A saturated aqueous solution of picric acid used for detection of alkaloids. It gives characteristics crystalline precipitate with many alkaloids.

Test for glycosides

Test for cardiac glycosides

a) **Keller-Kiliani test:** To an extract of the drug in glacial acetic acid, few drops of ferric chloride and conc. Sulphuric acid are added. A reddish-brown colour is formed at the junction of two layers and the upper layer turns bluish green. The test confirms presence of cardiac glycosides with presence of digitoxose as the glycone moiety.

Test for anthraquinone glycosides

a) **Borntrager's test:** Boil 0.1 g of the powdered drug with 5 ml. 10% sulphuric acid for 2 min. Filter while hot, cool the filtrate and shake gently with equal volume of benzene. Allow the benzene layer to separate completely from the lower layer. Pipette out and transfer the benzene layer to a clean test tube. Add about half its volume of aqueous solution of ammonia (10%). Shake gently and allow the layer to separate. The lower ammoniacal layer will acquire pink to red colour due to the presence of free anthraquinones.
b) **Modified Borntrager's test:** The C-glycosides of anthraquinones requires more drastic conditions for hydrolysis and thus a modification of the above test is used. Ferric chloride and hydrochloric acid are used to effect oxidative hydrolysis. 0.1 gm of the drug is boiled with 5ml of dil. HCl and 5 ml of 5% solution of ferric chloride for five minutes cool the solution and filter. This filtrate is shaken with benzene. Separate the benzene layer and add an equal volume of dilute solution of ammonia. This ammonical layer shows pink to red colour.

Test for carbohydrates

a) **Molisch's test:** To aqueous or alcoholic solution of the substance in a test tube add 10% alcoholic solution of α-napthol. Shake well and add a few drops of Conc. Sulphuric acid along the side of the test tube. A violet ring at the junction of two liquids confirms the presence of carbohydrates.
b) **Fehling's test:** Add 2 ml of Fehling's solution A and 2 ml of Fehling's solution B to 2 ml of liquid extract in a test tube and boil. If yellow or bricked precipitate appears, then reducing sugars are present.
c) **Benedicts test:** Add 5 ml of Benedict's reagent to 3 ml of test solution in a test tube and boil on a water bath.

Appearance of brick red precipitate at the bottom of the test tube shows presence of monosaccharides.

Test for gums and mucilages

- **Precipitation with 95% alcohol:** Gums and mucilages precipitate with addition of 95% alcohol, being insoluble in alcohol.
- **Molisch's test:** To aqueous or alcoholic solution of the substance in a test tube add 10% alcoholic solution of α-napthol. Shake well and add a few drops of Conc. Sulphuric acid along the side of the test tube. A violet ring at the junction of two liquids confirms the presence of carbohydrates, gums and mucilages.
- **Ruthenium red test:** Dissolve 0.8gm of ruthenium red in 10 ml of 10% solution of lead acetate. It stains mucilage to red colour.

Test for proteins and Amino acids

a) **Biuret test:** To 2 ml of extract, 2 ml of 10% NaOH solution and 2 to 3 drops of 1% CuSO4 solution is added and mixed. Appearance of violet or purple colour confirms presence of proteins.
b) **Ninhydrin test:** To 2 ml. of extract add 0.5 ml of ninhydrin solution. Boil for 2 minutes and cool. If blue colour appears then proteins are present.
c) **Xanthoproteic test:** To 2 ml of extract add 1 ml of Conc. HNO_3, boil, cool and add 40% NaOH drop by drop. Appearance of coloured solution indicates presence of proteins.
d) **Millon's test:** To 2 ml of extract add 2 ml of Millon's reagent, boil, cool and add few drops of $NaNO_2$ solution. Appearance of red precipitate or colouration indicates presence of proteins.

Test for tannins and phenolic compounds

a) **Ferric chloride test: -** A 5% W/V solution of ferric chloride in 90% alcohol are used for detection of phenols.
b) **Lead acetate test: -** Tannins are precipitated with lead acetate.
c) **Gelatin solution test:** To a solution of tannins (0.5-1%) aqueous solution of gelatins (1%) and sodium chloride (10%) are added. A white to buff coloured precipitate is formed.

Test for steroids and sterols:

a) **Lieberman Burchard reagent:** To about 2 ml of a solution extract in chloroform in a dry test tube, add 2 ml of acetic anhydride and 2-3 drops of Conc. Sulphuric acid Mix and stand for a few minutes. An emerald green colour develops if steroids or sterols are present.
b) **Salkowski's test:** To 5 ml of a solution of extract in chloroform in a dry test tube add gently along the sides, on equal volume of conc. Sulphuric acid. Observe the upper chloroform layer and the lower acid layer. The acid layer develops a yellow colour with a green fluorescence. The chloroform layer will give a play of colours first from bluish red to gradually violet red.

Test for saponins

- **Foam test (1ml of extract + 9 ml of water):** About 1 ml of alcoholic or aqueous extract is diluted separately with distilled water to 10 ml and shaken in a graduated cylinder for 15 minutes and kept aside. About one cm layer of foam after standing for 30 minutes indicates the presence of saponins.
- **Haemolysis Test (3 drops of blood + 1 drop of extract): -** Haemolysis occurs if saponins are presents.

Test for flavonoids:

a) **Sodium hydroxide test: -** The extract dissolved in water, filtrate treated with sodium hydroxide a yellow colour is observed if flavonoids are present.
b) **Sulphuric acid test: -** A drop of conc. Sulphuric acid when added to the above, the yellow colour disappears.
c) **Shinoda's test: -** To the aqueous or alcoholic solution of the extract, add a piece of magnesium ribbon and few drops of Conc. HCl. A pink colour develops which indicates presence of flavonoids.

Chemicals and instruments

Chemicals used:

i) Normal saline.
ii) Phosphate buffer P^H 7.4
iii) Hyposaline solution
iv) Alsever solution
v) Diclofenac sodium (200 µg/ml)

Instruments used

i) Ultra-centrifuge
ii) UV-Visible Spectrophotometer

***In vitro* anti-inflammatory activity**

***In vitro* anti- inflammatory activity by HRBC membrane stabilization method**

The dried powdered material was macerated in methanol and water (Aqueous extracts) receptively. The extract was concentrated to a small residue (5 gm) and the phytoconstituents in the synergistic mixture extract were identified to be Alkaloids, Flavonoids and saponins respectively. The synergistic mixture is taken in different ratios for the study of in-vitro anti-inflammatory activity.

This method followed as per *Sangeetha et al*., 2011. The blood was collected from healthy human volunteer who had not taken any NSAIDS for 2 weeks prior to the experiment and mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline and a 10% suspension was made. Concentrations of different extracts were prepared 100 µg/ml using distilled water and to each extract 1 ml of phosphate buffer, 2 ml hyposaline and 0.5 ml of HRBC suspension were added. It was incubated at 37^0C for 30 min and centrifuged at 3,000 rpm for 20 min. and the haemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. Diclofenac (200 µg/ml) was used as reference standard and a control was prepared by omitting the extracts.

Evaluation of in-vitro anti-inflammatory activity of various extracts in the present study.

The percentage hemolysis was estimated by assuming the hemolysis produced in the control as 100%.

The Percentage protection is calculated by the formula as follows and the results were tabulated in the Table 5.

The Percentage protection = 100- (OD sample/ OD control) × 100

5. Results and Discussion

The results of the Preliminary Phytochemical studies are tabulated.

Table 2: Phytochemicals of *Synergestic mixture*

Name of the chemical test	Methanolic extract
Alkaloids (Wagner's test)	+
Alkaloids (Hager's test)	+
Glycosides	-
Tannins	+
Flavonoids	+
Phenols	-
Carbohydrates	-
Steroids	-
Saponins	-
Terpenoids	-

Table 3: The percentage protection is calculated by the formula:

SAMPLE	Optical density	Control Optical density
Methanol *(Embelia ribes)*-100µg/ml	0.15	0.3
Aqueous (*Cinnamomum verum*)-100 µg/ml	0.20	0.3
Methanol (*Zingiber officinale)*-100µg/ml	0.10	0.3
Methanol (Synergistic mixture) -100µg/ml	0.09	0.3
Diclofenac sodium (200 µg/ml)	0.08	0.3

The Percentage protection = 100- (OD sample/ OD control) × 100

Table 3: *In-vitro* anti-inflammatory activity of various extracts in the present study.

S. No	Extract type (µg/ml)	Percentage protection
1.	Methanol *(Embelia ribes)*-100µg/ml	50%
2.	Aqueous (*Cinnamomum verum*)-100 µg/ml	33.3%
3.	Methanol (*Zingiber officinale)*-100µg/ml	66.6%
4.	Methanol (Synergistic mixture) -100µg/ml	70%
5.	Diclofenac sodium (200 µg/ml)	74%

6. Conclusion

Inflammation is a common phenomenon and it is a reaction of living tissues towards injury. Steroidal anti-inflammatory agents will lyse and possibly induce the redistribution of lymphocytes, which cause rapid and transient decrease in peripheral blood lymphocyte counts to affect longer term response.

HRBC method was selected for the *in vitro* evaluation of anti-inflammatory property because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release.

Preliminary phytochemical studies of the synergistic mixture reveal the presence of Alkaloids, Flavonoids, Saponins, and Carbohydrates by using various laboratory reagents which were freshly prepared for the present study.

In vitro anti-inflammatory activity by HRBC membrane stabilization method, in the present study the synergistic extract of *Embelia ribes, Cinnamomum verum, Zingiber officinale* showed significant anti-inflammatory activity in a concentration dependent manner. Methanol extract (Synergistic mixture) at a concentration of 100 µg/ml showed 70% protection of HRBC in hypotonic solution. All the results were compared with standard Diclofenac which showed 74% protection. The results indicated that the synergistic mixture has shown promising anti-inflammatory property. Further study can be done by in-vivo studies for the synergistic mixture.

7. References

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