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Original Research Article

Preliminary phytochemical analysis, formulation and evaluation of *in vitro* anti-inflammatory potential of the formulated gel obtained from an Ethanolic leaves extract of *Coleus amboinicus* Lour

Praveen Sekar[1,*], **Vignesh Natarajan**[2], **Nivetha Shanmugam**[2], **Thilagasundari Kandasamy**[3]

[1]*Dept. of Pharmaceutical Chemistry, Swamy Vivekanandha College of Pharmacy, Tiruchengode, Tamilnadu, India*
[2]*Dept. of Pharmaceutics, Swamy Vivekanandha College of Pharmacy, Tiruchengode, Tamilnadu, India*
[3]*Dept. of Pharmacology, Swamy Vivekanandha College of Pharmacy, Tiruchengode, Tamilnadu, India*

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ABSTRACT

Infectious microbes like bacteria, viruses, or fungi commonly cause inflammation when they enter the body, live in certain tissues or circulate in the circulation. For long years ago, natural sources have been used for the treatment of various diseases. In this way, *Coleus amboinicus* belonging to the family Lamiaceae was found to be having various pharmacological activities like anticancer, antimicrobial, antioxidant, anti-inflammatory, anti-diabetic, larvicidal and cardiovascular activities. The objective of the study is to formulate and evaluate the *in vitro* anti-inflammatory potential of the topical gel using an Ethanolic leaves extract of *Coleus amboinicus.* The formulated gel F3 showed better spreadability with a net content of 99 % and a drug release of 94.84 %. The *in vitro* anti-inflammatory potential of the formulation F3 was evaluated using the inhibition of albumin denaturation assay and Human red blood cells (HRBC) membrane stabilization assay. The formulated gel and standard exhibited 45.86-82.32 % and 55.25-88.07 % of inhibition at the concentration of 50-250 μg/ml, respectively at albumin denaturation assay and 25.35-75.49 % and 30.70-87.18 % of inhibition at 50-250 μg/ml concentration, respectively. Thus, the formulated topical gel using an Ethanolic leaves extract of *C. amboinicus* was found to be possessing potent anti-inflammatory activity.

1. Introduction

The primary method in which the body reacts to infection, irritation, or other harm, with redness, warmth, swelling, and pain as prominent features. Inflammation is now understood to be a generic immunological response. "Inflammation is the fundamental process by which tissues of the body respond to injury." Inflammation has a crucial part in a variety of complications, including rheumatoid arthritis, atherosclerosis and asthma, all of which have a high global frequency. Pro-inflammatory cytokines similar to interleukin (IL)- 1, tumor necrosis factor (TNF), interferon (INF)- c, IL- 6, IL- 12, IL- 18 and the granulocyte-macrophage colony-stimulating factor are released during an inflammatory response, this response is inhibited by anti-inflammatory cytokines similar as IL- 4, IL- 10, IL- 13, IFN- a and the transubstantiating growth factor.[1] For many years, salicylate-containing plants were used therapeutically, leading to the development of Aspirin, a key anti-inflammatory medicine. Aspirin, an anti-inflammatory drug produced from natural sources, is widely utilized in modern therapeutic use. Many

* *Corresponding author*.
E-mail address: praveenrsp222@gmail.com (P. Sekar).

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additional aspirin-like medications, such as nonsteroidal anti-inflammatory drugs (NSAIDs), are now accessible. For thousands of years, people have researched inflammation in an attempt to find a cure. In 30 A.D., Celsius defined the four famous indications of inflammation (rumor, calor, dolor, and tumor or redness, heat, pain, and swelling) and utilized willow leaf extracts to alleviate them.[2] Nowadays people are seeking to combat the treatment of diseases with natural sources, in that way *Acanthus ilicifolius*,[3]*Allium sativum* ,[4] Curcumin[5] which is the most important ethnobotanical drug insulated from *Curcuma longa* and is reported to have a variety of medical applications including anti-inflammatory activity. Others like *Zingiber officinale,*[6]*Tinospora cordifolia,*[7]*Silybum marianum,*[8] *Pluchea lanceolata*[9] and *Garcinia mangostana*[10] are the few herbs for Inflammation.[11]

One such recognized plant is *Coleus amboinicus*, which belongs to the Lamiaceae family, which comprises roughly 200 genera and 3200 species with a history of disease treatment.[12] Numerous studies have found antibacterial,[13] anti-inflammatory and analgesic,[14] anticancer,[15] wound healing,[16] anticonvulsant,[17] larvicidal[18] and antioxidant[19] effects. It has also been shown to be useful in the treatment of respiratory, cardiovascular, dental, skin, digestive, and urinary illnesses. *C. amboinicus* has a bright future in the pharmaceutical and nutraceutical sectors, where there is a high need for natural, cost-effective and safer bioactive compounds.[19]

One of the human body's easiest organs to administer topical medications to is the skin, which also serves as the primary conduit for their distribution. Transparent gels are among the topical formulations with a wide range of options for the therapy, from solid dosage to semisolid dosage forms and to liquid dosage formulation.[20] Gels offer more promise as a vehicle based on a phytopharmaceutical approach. Topically administering drugs as opposed to ointments since they are non-sticky and need less energy during formulation. For a long time, drug administration through the skin has been a promising notion since the skin is easy to approach and has a huge surface area. The topical gel provides a potent effect through extensive contact with the circulatory and lymphatic systems and the path is non-intrusive. The existence of a network produced by the interlocking of gelling agent particles causes a gel to be stiff. The structure of the network and the properties of the gel are determined by the nature of the particles and the kind of form that is responsible for the links.[21] Thus, this study focuses on the anti-inflammatory potential of the formulated topical gel using an Ethanolic extract of *C. amboinicus*.

2. Materials and Methods

All the chemicals used in this study are of analytical grade, which is bought from Sigma Chemical Co. (St. Louis, Missouri, United States of America (USA)). Double distilled water is used throughout the experimentation and Whatman No. 1 filter papers are used for filtration purposes.

2.1. Plant collection and authentication

The leaves of the *C. amboinicus* were collected from Nainamalai, Namakkal, Tamilnadu during the month of January. The collected leaves were authenticated by the Botanical Survey of India, Tamilnadu Agricultural University, Coimbatore, Tamilnadu, India (BSI/SRC/5/23/2020/Tech/562). The leaves were then washed with running water, dried under shade conditions and dried leaves are used for further processing.

2.2. Preparation of extract

The extraction was carried out in accordance with Oladeji et al., 2019, with minor adjustments. 50 g of powdered leaf sample was precisely weighed and defatted with petroleum ether using a maceration procedure. The defatted material was then macerated in ethanol for 48 hours at room temperature to extract the phytochemicals. The extract was refined using a sterile muslin cloth, concentrated, and utilized to make the gel.[22]

2.3. Phytochemical screening[22,23]

According to Oladeji et al., 2019 and Yadav et al., 2014, the presence of several phytochemicals in the produced extract was identified. The presence of phytochemical elements such as alkaloids, tannins, terpenoids, saponins, steroids, flavonoids, phlobatannins, anthraquinones, coumarins, and others was tested using the protocols outlined below.

2.3.1. Alkaloids

In a test tube, the extract was mixed with 2 ml of chloroform. Wagner's reagent was added to the admixture in the quantity of 3 - 4 drops, and the presence of alkaloids was indicated by a sanguine-brown coloration.

2.3.2. Tannins

A test tube containing 10 ml of distilled water and 5 g of extract was boiled and filtered. The filtered samples were treated with iron (III) chloride (0.1) to produce a blue-green precipitate. This indicates the presence of tannins.

2.3.3. Terpenoids

5 ml of the extract, 3 ml of concentrated sulphuric acid and 2 ml of chloroform were added in drops. The appearance of a sanguine-brown layer indicates the presence of terpenoids.

2.3.4. Saponins

A test tube containing 10 ml of distilled water and 5 g of extract was warmed. Upon the production of froth, the presence of saponins is confirmed.

2.3.5. Steroids

5 g of extract was added to 2 ml of chloroform and upon the addition of sulphuric acid, the blowfish green interface will be formed, which indicates the presence of steroids.

2.3.6. Flavonoids

A 5 g extract was precisely weighed, then it was mixed with a few drops of magnesium strips. When strong hydrochloric acid was added, a yellowish color was seen, confirming the presence of flavonoids.

2.3.7. Phlobatannins

The sample turned reddish brown when hydrochloric acid (1%) was added, indicating the presence of phlobatannins.

2.3.8. Anthraquinone

5 g of extract was precisely weighed and 10 ml of benzene was added, agitated and filtered. 5 ml of ammonia was introduced and the admixture was filtered after being supplemented with a grey of benzene. Again 5 ml of ammonia was introduced and the conformation of greenish-unheroic colour indicates the presence of anthraquinone.

2.3.9. Coumarins

To 2 ml of excerpt, 3 ml of 10 NaOH was added, the conformation of unheroic color indicates the presence of coumarins.

2.4. Preparation of Gel from an Extract

Weighed the needed quantity of Carbopol 943 and dispersed slowly in the 50 ml of distilled water in a 250 ml beaker. The admixture was stirred in the magnetic stirrer and warmed for 30 mins at 15 °C. The surfactant Tween 80 and the emulsifier HPMC was dissolved in the Glycerol and stirred well, also mix both fusions with continuous stirring. Further, the needed quantity of *C. amboinicus* extract was mixed into the below admixture and volume made up to 100 ml by adding remaining distilled water. Triethanolamine was added dropwise in the expression to maintain the pH of the skin and the gel was attained with needed thickness.[24]

2.5. Evaluation of gel[25]

2.5.1. Estimation of active constituents in gel formulation (Drug content)

Each formulation (1 g) was placed in a 50 ml volumetric beaker, filled to capacity with methanol, and thoroughly shaken to dissolve the active components in methanol. The filtrate was pipetted out and diluted to 10 ml with methanol after being filtered using Whatman sludge paper. The active component concentration was determined spectrophotometrically using a standard wind plotted at 254 nm.

2.5.2. Measurement of pH

A digital pH meter was used to determine the pH. 1g of gel was dissolved in 100 ml of distilled water and kept for 2 hours. pH was measured in triplet and the average values were calculated.

2.5.3. Homogeneity

After the prepared gels were set in the vessel, all generated gels were visually examined for uniformity. They were examined for the appearance and presence of aggregates.

2.5.4. Washability

The prepared formulation was applied to the skin, and the ease of washing with water was tested.

2.5.5. Viscosity

The density of gel was determined using a Brookfield viscometer (S- 62, model LVDV- E) at 25 °C with a spindle speed of the viscometer rotated at 12 rpm.

2.5.6. Spreadability

Two sets of normal confines glass slides were taken. One of the slides was covered with the herbal gel mixture. The other slide was put on top of the gel, and the gel was pressed between the two slides in a region submerged by 7.5 cm along the slides. A 100-gram weight of gel was put on the top slides, and the gel between the two slides was softly squeezed to produce a thin sub caste. The weight was removed, and the excess gel on the slides was scraped away. The two slides in place were connected to a stage with the least amount of disturbance and in such a way that only the higher slides fell off freely due to the force of weight tied on them. A 20 g weight was carefully connected to the top slide. The time required for the upper slide to travel 7.5 cm and separate from the lower slide under the influence of the weight was recorded. The experiment was performed three times, and the meantime was computed. The following formula was used to calculate spreadability.

$S = m \times l/t$

Where, S= spreadability, m-weight tied to upper slides (20 g), l- length of the glass slide (7.5 cm), t- time taken in sec

2.6. In vitro drug permeation study[26]

The Franz diffusion cell was used to conduct *in vitro* diffusion tests on all formulations. As receptor medium, phosphate buffer (pH 7.4) was employed. The medicine penetration was determined using a dialysis membrane. The diffusion membrane was connected to the diffusion cell (donor cell) so that the skin membrane was in direct contact with the formulation's release face in the donor cell. Before mounting the donor compartment on the diffusion cell, a 100- ml isotonic phosphate buffer result at pH 7.4 was added. A counted quantum of formulation, corresponding

to 1 g of gel, was taken and gently submerged in 100 ml of receptor medium while being constantly agitated. The entire system was kept at 37 °C. An aliquot of 5 ml was taken at defined time intervals of up to 5 hours and spectrophotometrically measured at 254 nm. The diffusion medium was replaced with an equal quantum of new diffusion medium after each withdrawal. For each time interval (in hours), the cumulative percent release was determined.

2.7. In vitro Anti-inflammatory activity

2.7.1. Protein denaturation assay

The *in vitro* anti-inflammatory activity of the formulated gel was studied using the bovine serum albumin denaturation system by using the reported procedure with minor variations.[27,28] In brief, the samples of colourful attention (50, 100, 150, 200 and 250 μg/ ml) were made and incubated with 0.5 w/ v of bovine serum albumin at 37 °C for 20 min. Also, the temperature was increased to 57 °C and heated for 30 min. After cooling to room temperature,2.5 ml of phosphate-softened saline was added and the turbidity was measured at 660 nm using a UV-Visible spectrophotometer. Aspirin was used as the reference standard. The chance inhibition of protein denaturation was calculated by using the following formula.

$$\% \text{ Inhibition} = 1 - At/Ac \times 100$$

2.7.2. Human red blood cell (HRBC) membrane stabilization assay

The established protocol with minimal modifications was utilized to examine the *in vitro* anti-inflammatory activity of formulated gel utilizing the human red blood cell (HRBC) membrane stabilization method.[29,30]

2.7.3. Preparation of blood samples for membrane 247 stabilization assays

The blood was drawn from the blood bank of Vivekanandha Medical Care Hospital and combined with an equivalent volume of Alsever solution (2g dextrose, 0.8g sodium citrate, 0.5g citric acid, and 0.42g NaCl). Before usage, the blood samples were kept at 4 °C for 24 hours. The supernatant was removed by centrifugation at 2500 rpm for 5 minutes. The cell suspension was rinsed with sterile saline solution (0.9 w/v NaCl) and centrifuged for 5 minutes at 2500 rpm. This was done three times until the supernatant was clear and colorless, and the packed cell volume was calculated. The cellular element was reconstituted in isosaline to a 10 suspension (v/ v) and employed in the test.

2.7.4. Hypotonicity-induced hemolysis

1 ml of each of the formulated compounds, reference (50, 100, 150, 200, and 250 μg/ml), and control were mixed individually with 1 ml phosphate buffer, 2 ml hyposaline, and 0.5 ml HRBC suspension. After 30 minutes at 37°C, the reaction mixture was centrifuged at 3000 rpm for 10 minutes. At 560 nm, the absorbance of the supernatant was measured spectrophotometrically. Aspirin was employed as a reference standard. The proportion of hemolysis was calculated by taking the control's percentage of hemolysis as 100%. The formula was used to calculate the proportion of protection vs the percentage of inhibition of hemolysis.

$$\% \text{ Inhibition} = 1 - At/Ac \times 100$$

3. Results and Discussion

3.1. Phytochemical analysis

Traditional medicines almost always contain raw plant extracts with a variety of chemical constituents. For a more in-depth examination of the plant material, the identification of these chemical components is required. Therefore, a preliminary phytochemical examination of fresh *C. amboinicus* leaves and an Ethanolic extract was carried out and compared. [32] Secondary metabolites have an important role in the biological conditioning of medicinal plants, such as hypoglycemic, anti-diabetic, antioxidant, antimicrobial, anti-inflammatory, anticarcinogenic, antimalarial, anticholinergic, antileprosy conditioning, and so on.[23] The existence of alkaloids, terpenoids, tannins, steroids, saponins, and phenolic composites was validated using qualitative phytochemical screening, and the findings are shown in table 1.

Table 1: Qualitative phytochemical analysis of an Ethanolic extract of *C. amboinicus* leaves (+ indicates the presence and – indicates the absence)

Phytochemicals	Presence/absence
Alkaloids	+
Tannins	+
Terpenoids	+
Flavonoids	-
Steroids	+
Saponins	+
Phlobatannin	-
Phenolics	+
Anthraquinone	-
Coumarin	-

3.2. Evaluation of formulated gel

The developed gel formulations were tested for a variety of pharmaceutical parameters, and the results are shown in the table. The net content, pH, washability, spreadability, and unity of all manufactured gels were estimated. Visual evaluation for their appearance and *in vitro* drug releases. The *C. amboinicus* gel was green in color and transparent. The unity was considered to be acceptable. The gel formulation is dependent on gelatinizing agent attention

increases. The gelatinizing agent improves density and diminishes spreadability. F1- F4 batches are distinct. After evaluation, the F4 batch outperforms the other batches. These optimized formulation drug-excipient studies are carried out to ensure that no drug-excipient commerce occurs in the formulation.

Table 2: Formulation of herbal gel containing *C. amboinicus* extract

Ingredients	F1	F2	F3	F4
Carbopol 940	0.1g	0.1g	0.2g	0.2g
HPMC	0.2g	0.4g	0.2g	0.4g
Glycerol	5ml	5ml	5ml	5ml
Tween 80	0.1ml	0.1ml	0.1ml	0.1ml
Triethanolamine	2ml	2ml	2ml	2ml
water	70ml	70ml	70ml	70ml

3.3. *In vitro drug permeation study*

It shows the *in vitro* diffusion characteristics of F1 through F4 formulation in figure 1. Because the pH of the membrane utilized ranged from 5 to 7.8, phosphate buffer saline pH7.4 was used for the gel formulations' *in vitro* release tests. The *in vitro* release patterns of all four Carbopol 940 formulations generated approximately 100 % releases from the formulation within 6 h. The *in vitro* release properties of the developed topical herbal gel formulations were encouraging and consistent with retailed diclofenac gel. Among the four formulations, F3 had stronger release qualities (94.84) than F1, F2 and F4.

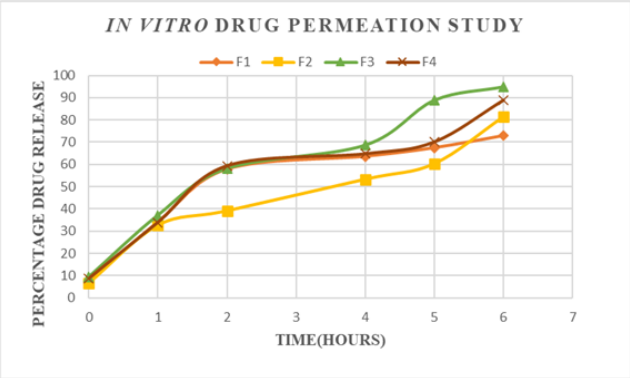

Fig. 1: *In vitro* drug permeation study of the formulated gel using an Ethanolic leaves extract of *C. amboinicus*

From the above results, it has been demonstrated that *C. amboinicus* extract can be prepared and developed as a topical gel using polymers of Carbopol 940 and HPMC. The gel was formulated in the varying ratios of Carbopol 940: HPMC as F1 containing 1:1, F2-1:2, F3-2:1 and F4-2:2. The above results showed that increase in Carbopol 934(polymer) ratio results in decreased drug release and less spreadability. The mixture of these two gelling agents in the ratio of 2:1 i.e., F3 showed better physiochemical and drug release characteristics and it was subjected to the evaluation of *in vitro* anti-inflammatory activity.

3.4. *In vitro anti-inflammatory activity*

3.4.1. *Inhibition of albumin denaturation assay*

The body tackles hazardous substances including infections, injuries, and toxins in an effort to heal itself through inflammation. When your cells get damaged, your body releases chemicals that activate your immune system. The inflammatory substance acts on the cell membranes, causing phospholipase A2 to become activated and release arachidonic acid and other metabolites. Leukocytes that are migrating can interact with any inflamed tissue site because of the increased vascular permeability caused by inflammatory mediators such as cytokines, histamine, serotonin, leukotrienes, and prostaglandins. The mediators' liberation is reduced when this chain of events is disturbed, causing the microcirculation to revert to a normal hemodynamic state.[31] One of the key factors contributing to inflammatory disorders is the denaturation of tissue proteins. In several inflammatory illnesses, protein denaturation may be the cause of the generation of auto-antigens. The generation of chemical compounds with the ability to inhibit protein denaturation would be beneficial for the production of anti-inflammatory medications.[32] Therefore, the anti-inflammatory potential of the formulated gel using an Ethanolic extract of *C. amboinicus* was determined by using the protein denaturation assay. The samples of various concentrations ranging from 50 μg/ml to 250 μg/ml were tested to determine the anti-inflammatory potential using the inhibition of protein denaturation assay and the inhibitory potential of the formulated gel and standard drug is given in table 4 and figure 2. Aspirin was used as the standard drug. Anti-inflammatory activities were performed in triplicate and the mean was taken as the final reading. The formulated gel exhibited the inhibition of protein denaturation within the range of 45.86 to 82.32 % at the concentration of 50-250 μg/ml, respectively. The standard drug also tested as like that of test samples and exhibited 55.25-88.07 % of inhibition at 50 to 250 μg.ml concentration. The inhibition rate of albumin denaturation was increased by increasing the concentration of the samples i.e., it exhibited significant activity in a dose-dependent manner. From the obtained results, it revealed that the formulated gel using an Ethanolic leaves extract of C. amboinicus exhibited potent anti-inflammatory action as such as the standard.

3.4.2. *Human red blood cell (HRBC membrane stabilization assay)*

The membrane of an erythrocyte resembles that of a lysosome. By oxidizing cell membrane lipids during inflammatory processes, lysosomes may play a significant

Table 3: Evaluation of herbal gel containing *C. amboinicus* extract

Formulation code	Appearance	pH	Homogeneity	Spreadability gm.cm/sec	Viscosity Cps	Net content (%)
F1	Light green& transparent	6.2	Homogenous	40.83	60100	88
F2	green & transparent	6.5	Homogenous	45.13	64300	94
F3	green & transparent	6.7	Homogenous	58.19	73900	99
F4	Light green & transparent	6.9	Homogenous	33.16	78500	97

Table 4: Percentage inhibition of the formulated gel and standard using inhibition of albumin denaturation assay

Concentration (μg/ml)	Absorbance at 660 nm		Percentage Inhibition (%)	
	Test	Standard	Test	Standard
50	0.386	0.319	45.86	55.25
100	0.295	0.283	58.62	60.30
150	0.211	0.214	70.40	69.98
200	0.162	0.139	77.27	80.50
250	0.126	0.085	82.32	88.07
Control	0.713		0.00	

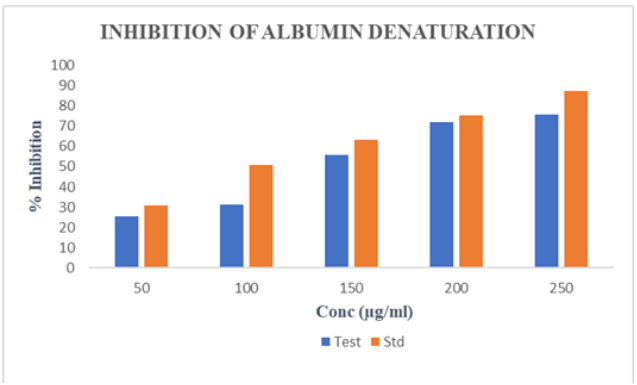

Fig. 2: Anti-inflammatory potential of gel and standard (Aspirin) using inhibition of albumin denaturation assay

part in tissue destruction. Lysosomes can hinder the receptor Hsp 90 (also known as the asteroid anti-inflammatory receptor) from binding steroidal anti-inflammatory medicines by shrinking it, which heightens the inflammatory state. Therefore, inhibiting the release of proteases and minimizing inflammatory responses may both benefit from the stabilization of the lysosome membrane. The ability of the extracts to maintain the integrity of the erythrocyte membrane suggests that they can also maintain the integrity of the lysosome membrane. Therefore, the ability of plant extracts to inhibit hemolysis can be considered proof of their capacity to reduce inflammation.[33] Therefore, the anti-inflammatory potential of the formulated gel using an Ethanolic leaves extract of *C. amboinicus* was evaluated using a heat-induced hemolysis assay. The samples of varying concentrations ranging from 50-250 μg/ml were tested and the percentage inhibition of hemolysis is given in table 5 and figure 3. Aspirin was used as the standard. The experiments were performed in triplicate and the average was considered as the final reading. The formulated gel exhibited inhibition within the range of 25.35-75.49 % at the concentration of 50-250 μg/ml, respectively. The formulated gel exhibited maximum protection and minimum hemolysis of HRBC (75.49 % and 24.51 %, respectively) at a concentration of 250 μg/ml in a hypotonic solution. The standard drug also exhibited maximum protection of 87.78 % and minimum protection of 30.70 % at a concentration of 250 and 50 μg/ml, respectively. From the obtained results, it reveals that the percentage inhibition of the gel was comparable with that of the standard.

Table 5: Percentage inhibition of the formulated gel and standard HRBC membrane stabilization assay

Concentration (μg/ml)	Absorbance at 660 nm		Percentage Inhibition (%)	
	Test	Standard	Test	Standard
50	0.530	0.492	25.35	30.70
100	0.489	0.340	31.12	50.98
150	0.315	0.261	55.63	63.23
200	0.199	0.176	71.97	75.21
250	0.174	0.091	75.49	87.18
Control	0.710		0.00	

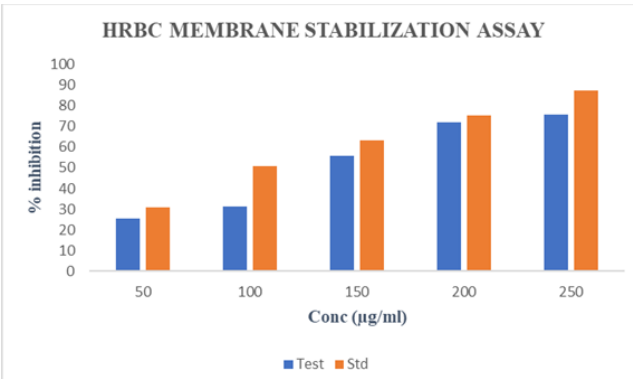

Fig. 3: Anti-inflammatory potential of gel and standard (Aspirin) using HRBC membrane stabilization assay

The samples demonstrated membrane stabilization by preventing erythrocyte membrane lyses caused by hypotonicity, and their stabilization indicated that the formulated gel may stabilize lysosomal membranes. Liposomal membrane stabilization plays a critical role in regulating the inflammatory response by preventing the release of lysosomal components from activated neutrophils, which contribute to systemic inflammation and damage via an extracellular release.[34] The prepared gel containing an Ethanolic extract of *C. amboinicus* may aid in these conducts, encouraging or lubricating the outflow of certain intracellular substances.

4. Conclusion

The current study shows that the gel was effectively formulated in an eco-friendly way utilizing an ethanolic leaves extract of *C. amboinicus.* The presence of anti-inflammatory phytochemicals such as alkaloids, terpenoids, tannins, and steroids was evaluated using qualitative phytochemical screening. Formulation F3 exhibited higher homogeneity and spreadability with a net content of 99% and better release of the four distinct formulations. The anti-inflammatory capacity of the formed gel was tested *in vitro*, and the formulation F3 with Carbopol 940 and HPMC in a 2:1 ratio was shown to be as effective as the reference medicine, aspirin. As a result, a topical gel containing an Ethanolic extract of *C. amboinicus* was shown to have strong *in vitro* anti-inflammatory action. Furthermore, preclinical, clinical and long-term stability studies are required.

5. Authors Contribution

All the authors have contributed equally.

6. Source of Funding

None.

7. Disclosure of Conflict of Interest

The authors hereby disclose no conflicts of interest regarding the publication of this paper.

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Author biography

Praveen Sekar, Assistant Professor https://orcid.org/0000-0002-3509-5981

Vignesh Natarajan, PG Scholar https://orcid.org/0000-0003-1414-6636

Nivetha Shanmugam, PG Scholar https://orcid.org/0000-0001-5030-0085

Thilagasundari Kandasamy, PG Scholar https://orcid.org/0009-0007-5335-4995

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