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Phytochemical Investigation and Assessment of In-Vitro Anti-Inflammatory Activity of Hydroethanolic Extracts of *Combretum Indicum* and *Euphorbia Milii*: A Comparative Study

Prerna[1], Preeti Chaudhary[2]*, Nasiruddin Ahmad Farooqui[3], Shamim Ahmad[4]

[1,3,4]Department of Pharmacology, Translam Institute of Pharmaceutical Education and Research, Meerut, Uttar Pradesh

[2]Department of Pharmacy, NCRD's Sterling Institute of Pharmacy, Nerul, Navi Mumbai, Maharashtra, India

*** Corresponding Author:** Dr. Preeti Chaudhary, Associate Professor, NCRD'S Sterling Institute of Pharmacy, Nerul, Navi Mumbai, Maharashtra, India,

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KEYWORDS

Combretum indicum, *Euphorbia milii*, Diclofenac sodium, Egg albumin, Haemolysis, Anti-inflammatory activity

ABSTRACT:

In the present research work, phytochemical investigation and *in-vitro* anti-inflammatory activity of *Combretum indicum* and *Euphorbia milii* was evaluated. In this study, we compared the effects of hydroethanolic extracts of *Combretum indicum* and *Euphorbia milii* with the standard Diclofenac sodium at equivalent concentration (100-500 µg/ml). Phytochemical screening of each plant extracts was performed. The presence of alkaloids, flavonoids, phenolic compounds, tannins and terpenoids was confirmed by preliminary phytochemical screening in both plants. In-vitro anti-inflammatory activity was evaluated using two different methods: Inhibition of egg albumin denaturation and human red blood cell membrane stabilization assay. In case of inhibition of egg albumin denaturation assay, hydroethanolic extract of *Combretum indicum* and *Euphorbia milii leaves* showed an inhibition of 85.22 and 73.04%, respectively at 500 µg/ml concentration. While standard diclofenac sodium exhibited 83.77% inhibition at 500 µg/ml concentration. Investigation showed that with the increase in concentration, both plants extract has the ability to inhibit protein denaturation significantly and is comparable with the standard diclofenac sodium. In the case of human red blood cell membrane stabilization assay, the maximum percentage of protection of haemolysis of *Combretum indicum*, *Euphorbia milii* hydroethanolic extracts and standard diclofenac sodium was found to be 82.78, 68.97 and 78.31% respectively at 500 µg/ml of concentration. There was a statistical difference in the mean of % protection of haemolysis of both plants and standard diclofenac sodium.

1. Introduction:

Inflammation is a complex process which is associated with pain and involves occurrences such as increased vascular permeability, enhanced protein denaturation, and rearrangement of the membrane. When cells in the body are damaged by microbes, physical agents, or chemical agents, the injury is in the form of stress. [1] Inflammation of tissue is due to response to stress. It is a defensive response that is characterized by redness, pain, heat, and swelling and loss of function in the injured area. Loss of function depends on the area and severity of the condition occurring. A drug or substance that reduces inflammation (redness, swelling, and pain) in the body is called an anti-inflammatory agent. They block the biosynthesis pathway of prostaglandins and other substances in the body that cause inflammation by inhibiting the cyclooxygenase (COX) enzymes, producing anti-inflammatory, analgesic, and antipyretic effects. [2] Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely used to cure and control inflammation, fever, and pain. [3] However, their use has not been therapeutically efficacious in all types of inflammations. Furthermore, the chronic use of NSAIDs can cause severe adverse effects leading to hemorrhage and ulcers. [4] There is a massive need to explore new anti-inflammatory agents with selective action and lesser

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toxicity. Plants and isolated phytoconstituents are promising and interesting sources of new anti-inflammatory agents. So, an attempt has been made to search for such plants and evaluate the anti-inflammatory potential of these plants. *Combretum indicum* and *Euphorbia milii* were selected for our research work. Both plants were selected on the basis of their ethnomedicinal uses and the chemical constituents.

Combretum indicum, a member of the combretaceae family, is commonly known as Rangoon creeper or Madhu Malti. According to the several studies, *Combretum indicum* contains a variety of secondary metabolites as quisqualic acid, myristic acid, arachidonic acid, flavonoids glycoside, alkaloids, rutin, tannins, palmitic acid, kaempferol and two forms of the cysteine synthase, isoenzyme A and isoenzyme B with diverse pharmacological activities. [5] Different sections of the *Combretum indicum* have been utilized as traditional treatments for anti-inflammatory, antioxidant benefits, digestive aids, fever-reducing, and skin-beneficial properties. Additionally, it is used to support respiratory health, manage diabetes, and as an adaptogen to help the body adapt to stress. [6] *Euphorbia milii* is an ornamental and medicinal plant. This plant is native to the Euphorbiaceae family. It is widely cultivated in tropical and warm areas like China, Nepal, Myanmar, Philippines, India, Brazil, Thailand, Nigeria, and Pakistan. [7] The plants are characterized by the presence of milky latex which is more or less toxic. E. milli is used for ornamental purpose and have not been reported in folk therapy in Pakistan, however in Nepal the latex is used to treat sprains; while in China it is used to cure hepatitis and abdominal edema. In Brazil E. milii is commonly known as “Crown of Thorns”. Naturally, E. milli crude latex showed to be a potent plant molluscicide due to its toxic effect to mammals. Phytochemical studies of E. milli revealed the presence of β-sitosterol, cycloartenol, β-amyrin acetate, lupeol, euphol, triterpenes and flavonoids. [8]

2. Objectives:

The main objective of present study was to assess the in-vitro anti-inflammatory activity of hydroethanolic leaves extract of *Combretum indicum* and *Euphorbia milii* and to evaluate the major bioactive constituents present in the extracts of above-mentioned plants.

3. Materials and Methods:

3.1 Chemicals Required:

Standard Drug: Diclofenac sodium was chosen as the standard drug in both models as given in previous research papers and it was purchased from the local medical store (Som Medical Store, Modipuram, Meerut, Uttar Pradesh).

Other Solvents: The solvents and other chemicals used were analytical grade and were obtained from local dealers.

3.2 Collection, Authentication and Drying of Plant Leaves:

The mature green leaves of *Combretum indicum* were collected from the garden of Somnath Enclave, Kanker Khera Road, Meerut Cantt and the leaves of *Euphorbia milii* were collected from Vrindaven Garden, Roorkee Road Meerut in the month of December. The sample of both plants was verified by Prof. Vijai Malik, Head, Department of Botany, Chaudhary Charan Singh University, Meerut (250004). He provided specimen no. Bot/PB/550 for reference. After authentication, plant material was rinsed with tap water to remove dust particles and soil. After washing material was dried firstly in air and at room temperature for three to four days.

3.3 Preparation of Hydroethanolic Leaves Extract:

About 100 gm of dried coarsely powdered leaves of *Combretum indicum* and *Euphorbia milii* were packed in the thimble chamber of two distinct Soxhlet apparatus. Firstly, leaves of both plants were extracted using petroleum ether for about 24 hrs. After defatting, the marc was dried in hot air oven at 50ºC, packed again in Soxhlet apparatus, and further extracted by using 500 ml of 70% ethanol and 30% water (V/V) mixture for three days. The solvents were heated in the round bottom flask, vaporised into the sample thimble, condensed in the condenser, and then dripped back into the flask. The procedure was resumed once the liquid content reached the syphon arm and drained back into the bottom flask. After being concentrated by evaporation at 70°C for 8 hr, the extract was dried. At last, % yield of the extract was calculated and kept it under refrigeration at 4°C temperature prior to phytochemical screening. [9]

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3.4 Preliminary Phytochemical Screening:

Preliminary phytochemical examinations to check for the presence of different phytoconstituents such as alkaloids, tannins, phenolic compounds and flavonoids, glycosides, carbohydrates, fixed oil & resins compound present in the hydroethanolic extracts of *Combretum indicum* and *Euphorbia milii* were done by using various reagent and standard procedure given in the standards book and research papers. [10,11]

3.5 Qualitative Evaluation by Thin Layer Chromatography:

Hydroalcoholic extract of both plant sample was subjected to TLC studies. TLC plates were prepared by pouring silica gel G slurry. Slurry of silica gel G was prepared by dissolving the silica gel and water with ratio of 1:2 ratio. A 0.25 mm thick TLC plate was prepared and air-dried for 15-20 minutes. The activation of TLC plates was done by keeping the plates in hot air oven at 105ºC temperature for 30 minutes. Spotting of the sample was done with microcapillary. The solvent system Methanol: Chloroform: Hexane in the ratio of 7:2:1 (10 ml) was prepared according to hit and trial and by referring previous research papers. [12] For the detection of the spot all the plates were sprayed with iodine vapors. The colored spots developed on the stationary phase were marked and their distances were measured. The movement of the active compound was expressed by the retention factor (Rf).

Rf value was calculated by using the following formula:

$$Rf = \frac{Distance\ travelled\ by\ Solute}{Distance\ travelled\ by\ solvent}$$

3.6 Screening for In-vitro Anti-inflammatory Activity: To conduct in-vitro anti-inflammatory activity of hydroethanolic extracts of *Combretum indicum* and *Euphorbia milii*, two models were selected.

1. Egg Albumin Denaturation Assay
2. Human Red Blood Cell Membrane Stabilization Assay

3.6.1 Egg Albumin Denaturation Assay: The egg albumin denaturation assay measures a drug or compound's capacity to prevent or lessen egg albumin denaturation to evaluate its anti-inflammatory effects. [13,14]

Preparation of 1% of Egg Albumin Solution: Fresh hen's egg was used to make a 1% egg albumin solution and it was purchased from the local market. The inner white part of the egg was separated into a beaker without disturbing the yellow part. The collected whitish part of the inner egg is the egg albumin.

For the Preparation of Egg Albumin Solution, A Fresh Hen's Egg was Carefully Cracked.

⇩

Transferred 1 ml of the Translucent Portion of Egg (Egg albumin) to 100 ml of volumetric flask.

⇩

In the volumetric flask 100 ml of w/v cold water was added.

⇩

The solution was stirred thoroughly.

Procedure: The assay was conducted by following these steps:

1. 0.2 ml of 1% egg albumin solution, 2 ml of sample extracts (*Combretum indicum* and *Euphorbia milii*) or standard (Diclofenac sodium) at varying concentrations (100-500 µg/ml), and 2.8 ml of phosphate buffered saline (pH 7.4) were mixed to form a reaction mixture of a total volume of 5 ml.

2. The pH of the reaction mixture was adjusted to 7.4 by adding a small amount of 1 N HCl.

3. A total volume of 5 ml of the control was formed by combining 2 ml of triple-distilled water, 0.2 ml of 1% egg albumin solution, and 2.8 ml of phosphate-buffered saline.

4. The reaction mixtures were then incubated at 37±2°C for 30 min and was heated in a water bath at 70±2°C for 15 min.

5. After cooling, the absorbance was measured at 660 nm by a suitable UV/Vis spectrophotometer using triple distilled water as the blank. [15]

6. The following formula was used to calculate the % inhibition of protein denaturation.

$$\%\ Inhibition = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}}\ X\ 100$$

3.6.2 Human Red Blood Cell Membrane Stabilization Assay: Inflammation can cause lysis of the lysosomal membrane, releasing enzymatic components. Leakage of

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lysosomal components from cell leads to further inflammation and tissue damage. Red blood cell membranes share similarities with lysosomal membranes, which are involved in inflammation. Mechanism of action of non-steroidal anti-inflammatory drug is to inhibit lysosomal enzymes or to stabilize lysosomal membrane. [16,17,18]

Preparation of Human Red Blood cells (HRBCs) Suspension: The human red blood cells required for the experiment was collected from a healthy human volunteer who had not consumed any anti-inflammatory drugs (NSAID's) for 2 weeks prior to the experiment. The fresh whole human blood (10 ml) collected was transferred to the heparinized centrifuged tubes with equal volume of Alsever's solution. The tubes were centrifuged at 3000 rpm for 10 min and the packed cells were washed thrice with equal volume of normal saline solution till the supernatant was clear and colorless. The volume of the cells was measured and a 10% v/v suspension was reconstituted with normal saline. [19, 20]

Procedure: The assay was conducted by following these steps:

1. 1 ml of the test extracts each of concentration ranging from 100-500 µg/ml was taken in a separate test tube.

2. To this 1 ml of freshly prepared phosphate buffer was added along with 2 ml of saline solution.

3. Finally, 0.5 ml of freshly prepared HRBC suspension was added.

4. Diclofenac sodium was used as a standard drug of varied concentration (100-500µg/ml) to determine the absorbance.

5. In the control tube normal saline was taken to produce 100% hemolysis.

6. All the tubes containing the reaction mixture were incubated at 37°C for 30 minutes and cooled under running tap water.

7. After incubation, the contents were then centrifuged at 3000 rpm for 20 min.

8. The hemoglobin content in the supernatant solution was collected and the absorbance of the supernatant solution was estimated spectrophotometrically at 560 nm.

9. The experiments were conducted in triplicates. [21]

10. The percentage of hemolysis was estimated by considering the percentage of hemolysis of control as 100%. The percentage of protection/percentage inhibition of hemolysis were evaluated using the formula

$$\% \text{ of Protection } = 100 - \frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} X\ 100$$

3.7 Statistical Analysis:

All Statistical calculations were performed using Microsoft excel. The data are expressed as Mean ± SEM. Statistical significance of the differences between the groups was analyzed using paired t-test. P values were calculated in each case and accordingly interpretation was carried out. A value of $p<0.05$ was considered as significant.

4. Results

4.1 % Yield of Hydroethanolic Extracts: The % yield of hydroethanolic leaves extract of *Combretum indicum* and *Euphorbia milii* was calculated by using following formula:

$$\% \text{ yield} = \frac{\text{Weight of dry extract}}{\text{Weight of dry plant biomass}} \text{X } 100$$

The % Yield of *Combretum indicum* and *Euphorbia milii* was found to be 27.78 and 33.78 % respectively.

Table 1: % Yield of Hydroethanolic Leaves Extracts of *Combretum indicum* and *Euphorbia milii*

S. No.	Plant	Weight of Dry Plant Taken (gm)	Weight of Dry Extract Obtained (gm)	Yield (%)
1	*Combretum indicum*	100	27.78	27.78
2	*Euphorbia milii*	100	33.78	33.78

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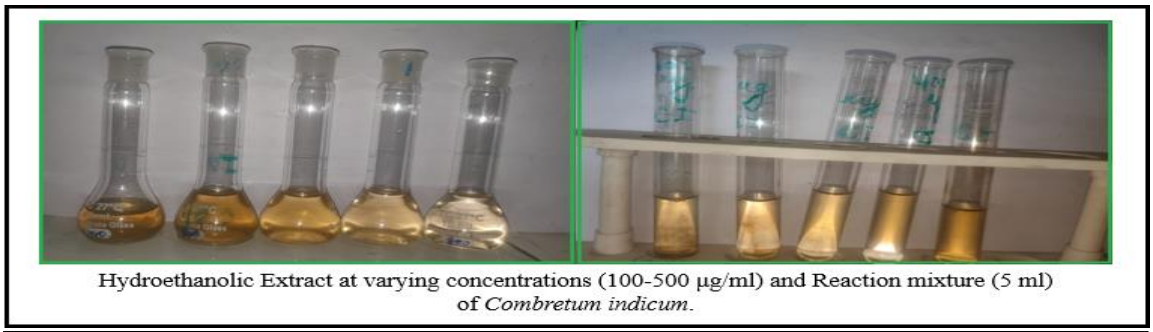
Figure 1: Hydroalcoholic Leaves Extract of *Combretum indicum* and *Euphorbia milii*

4.2 Preliminary Phytochemical Screening of Hydroethanolic Extracts: The preliminary phytochemical screening revealed that the hydroethanolic leaves extract of *Combretum indicum* contains alkaloids, flavonoids, tannins, phenolic compounds, phytosterols, glycosides, resins and terpenoids. The Phytochemical screening of hydroethanolic leaves extract of *Euphorbia milii* demonstrated the presence of alkaloids, glycosides, flavonoids, phenols (tannins), terpenoids and phytosterols. Interestingly, saponins, carbohydrates and fixed oils were absent in both plants.

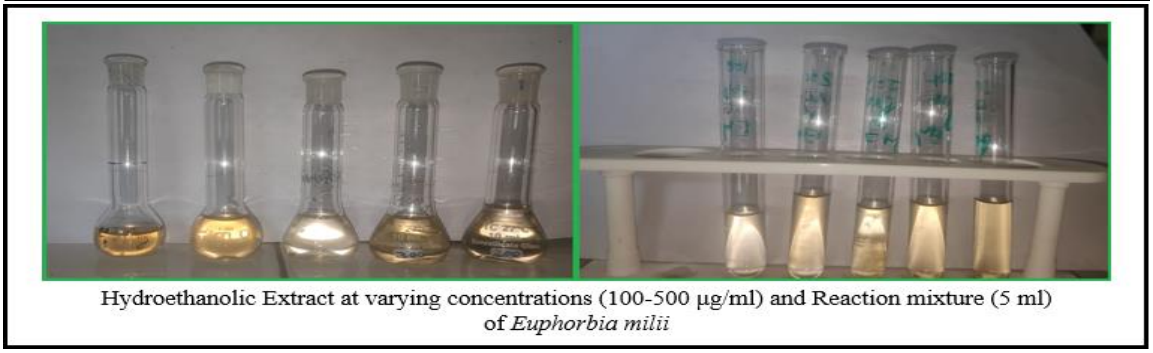
Qualitative Investigation by TLC: TLC analysis was done using the selected solvent of Methanol: Chloroform: Hexane in the ratio of 7:2:1 by the help of standard book & hit and trial and the research paper in which solvent system for phytoconstituents of anti-inflammatory activity are already mentioned. Rf value of hydroethanolic extracts of *Combretum indicum* and *Euphorbia milii* was found to be 0.86 and 0.90 respectively.

4.3 Results of In-vitro Anti-inflammatory Activity:

4.3.1 Egg Albumin Denaturation Assay: The percentage inhibition of egg albumin denaturation of hydroethanolic extract of *Combretum indicum* and *Euphorbia milii* was compared with the standard diclofenac sodium. The study was performed in triplicate. The percentage inhibition of protein denaturation was given in Table 2.



Hydroethanolic Extract at varying concentrations (100-500 µg/ml) and Reaction mixture (5 ml) of *Combretum indicum*.



Hydroethanolic Extract at varying concentrations (100-500 µg/ml) and Reaction mixture (5 ml) of *Euphorbia milii*

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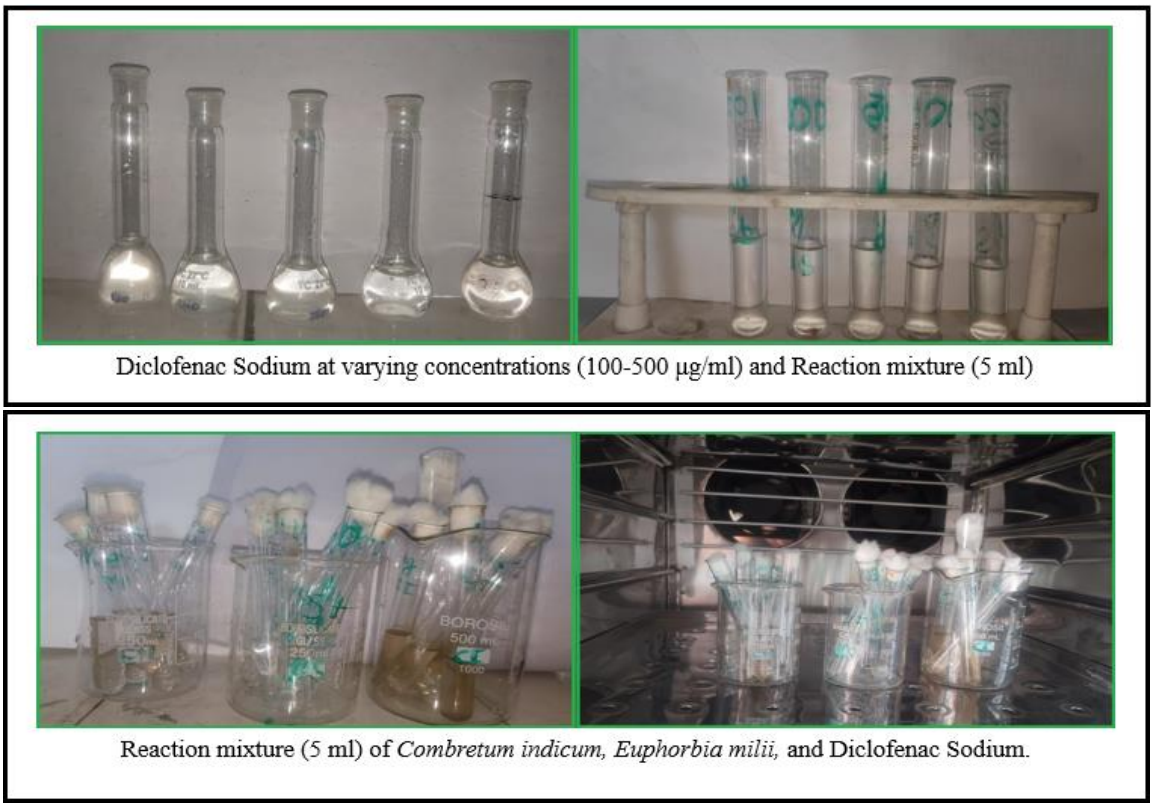

Figure 2: Egg Albumin Denaturation Assay

Table 2: Effect of Hydroethanolic Extracts of *Combretum indicum* and *Euphorbia milii* on Egg Albumin Denaturation

S. No.	Treatment	Concentration (µg/ml)	Absorbance Recorded at 660 nm	% Inhibition on Egg Albumin Denaturation ± SEM
1	Diclofenac Sodium	Control	0.690	---
		100	0.275	60.14 ± 0.583
		200	0.232	66.38 ± 0.514
		300	0.187	72.90 ± 0.542
		400	0.153	77.83 ± 0.952
		500	0.112	83.77 ± 0.610
2	Hydroethanolic Extracts of *Combretum indicum*	Control	0.690	---
		100	0.260	62.32 ± 1.117
		200	0.215	68.84 ± 1.158
		300	0.172	75.07 ± 1.128
		400	0.140	79.71 ± 0.473
		500	0.102	85.22 ± 0.525
3		Control	0.690	---

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	Hydroethanolic Extracts of *Euphorbia milii*	**100**	0.327	52.61 ± 0.626
		200	0.301	56.38 ± 0.462
		300	0.253	63.33 ± 0.545
		400	0.211	69.42 ± 0.798
		500	0.186	73.04 ± 0.757

All values are expressed in terms of Mean of % Inhibition ± SEM and are found to be significant when compared to control $p<0.05$

The percentage inhibition of hydroethanolic leaves extracts of *Combretum indicum* and *Euphorbia milii* increased with increasing concentration like standard diclofenac sodium. Hydroethanolic extract of *Combretum indicum* had percentage inhibition of protein denaturation of 62.32, 68.84, 75.07, 79.71% at concentration of 100, 200, 300 and 400 µg/ml respectively. At concentrations of 100, 200, 300, and 400 µg/ml, the hydroethanolic extract of *Euphorbia milii* showed a percentage inhibition of protein denaturation of 52.61, 56.38, 63.33 and 69.42%, respectively. For the standard drug (Diclofenac sodium) it was found to be 60.14, 66.38, 72.90 and 77.83% for same doses. The maximum inhibition of protein denaturation of *Combretum indicum*, *Euphorbia milii* hydroethanolic extracts and standard diclofenac sodium was found to be 85.22, 73.04 and 83.77% respectively at 500 µg/ml of concentration. Investigation showed that with the increase in concentration, both plants extract has the ability to inhibit protein denaturation significantly and is comparable with the standard diclofenac sodium. The decrease in the absorbance value with the increasing concentration taken at 660 nm shows significant anti-inflammatory activity.

A comparison of the effects of the hydroethanolic extracts of *Combretum indicum*, *Euphorbia milii* and standard diclofenac sodium on the inhibition of egg albumin denaturation is shown in Figure 3.

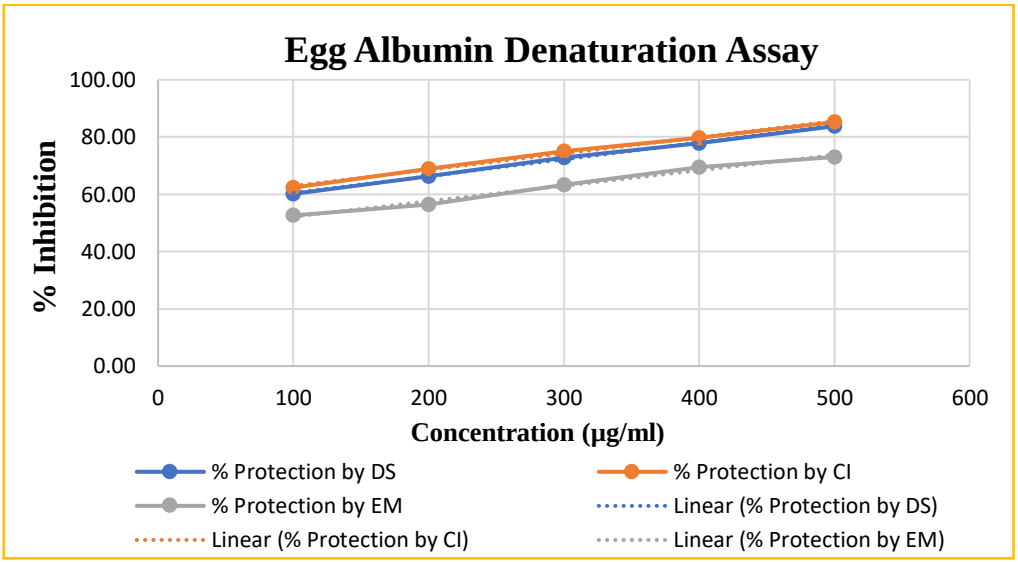

Figure 3: Inhibition of Egg Albumin Denaturation by Hydroethanolic Extracts of *Combretum indicum* and *Euphorbia milii*

4.3.2 Human Red Blood Cell Membrane Stabilization Assay: To perform human red blood cell membrane stabilization assay, fresh human blood (10 ml) was collected from a healthy human volunteer and it was transferred to the heparinized centrifuged tubes with equal volume of Alsever's solution. The tubes were centrifuged at 3000 rpm for 10 minutes.

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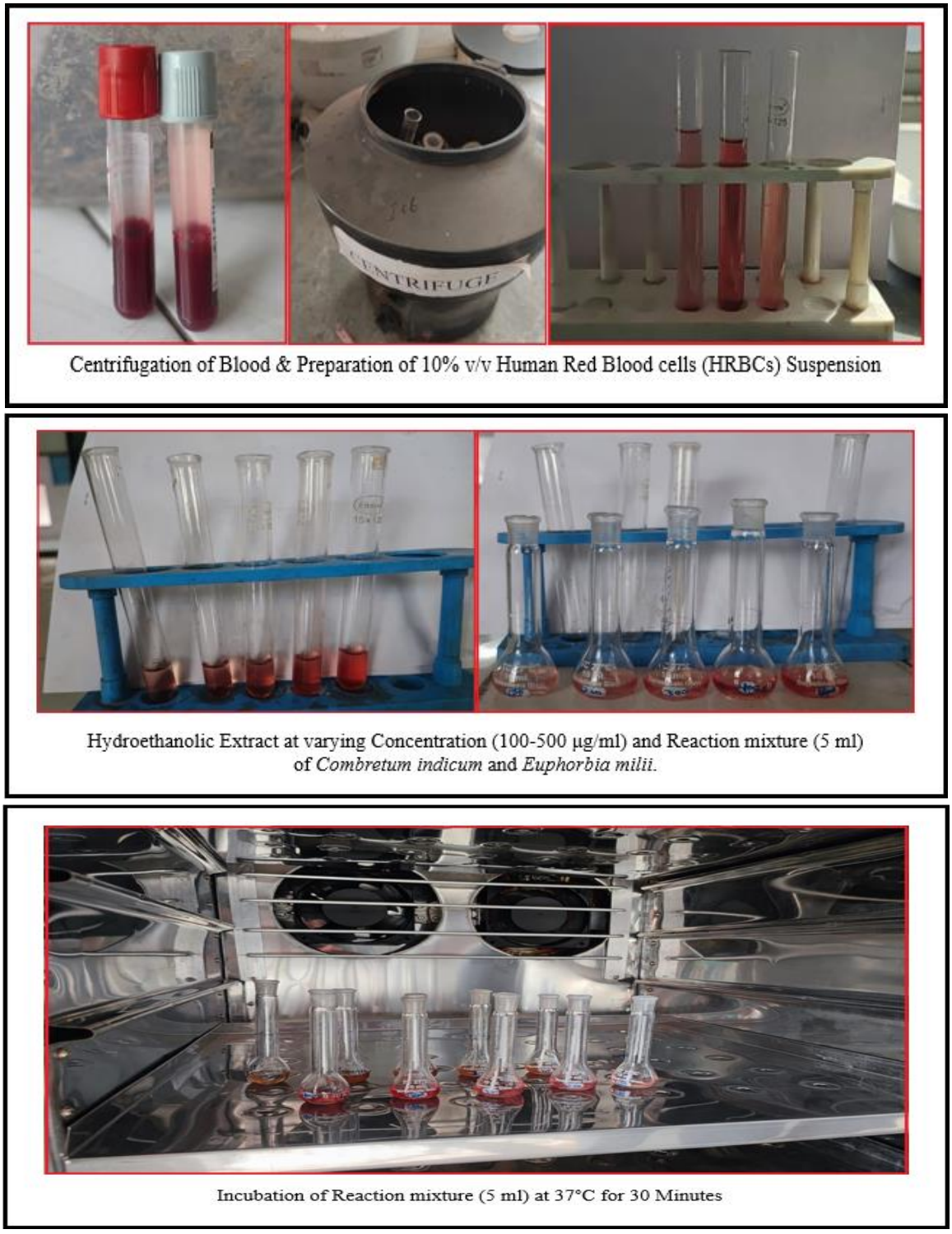

Figure 4: Human Red Blood Cell Membrane Stabilization Assay

The percentage of protection of hemolysis by hydroethanolic extract of *Combretum indicum* and *Euphorbia milii* was compared with the standard diclofenac sodium. The study was performed in triplicate. The results of percentage of protection of hemolysis were shown in Table 3.

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Table 3: In-vitro Anti-inflammatory Activity of Hydroethanolic Extracts of *Combretum indicum* and *Euphorbia milii* by HRBC Membrane Stabilization Assay

S. No.	Treatment	Concentration (µg/ml)	Absorbance Recorded at 560 nm	% Protection
1	Diclofenac Sodium	Control	1.231	---
		100	0.520	57.76 ± 0.577
		200	0.421	65.80 ± 0.545
		300	0.402	67.34 ± 0.526
		400	0.354	71.24 ± 0.588
		500	0.267	78.31 ± 0.408
2	Hydroethanolic Extracts of *Combretum indicum*	Control	1.231	---
		100	0.512	58.41 ± 0.516
		200	0.411	66.61 ± 0.815
		300	0.363	70.51 ± 0.611
		400	0.261	78.80 ± 0.553
		500	0.212	82.78 ± 0.553
3	Hydroethanolic Extracts of *Euphorbia milii*	Control	1.231	---
		100	0.640	48.01 ± 0.540
		200	0.523	57.51 ± 0.469
		300	0.509	58.65 ± 0.646
		400	0.451	63.36 ± 0.616
		500	0.382	68.97 ± 0.509

All values are expressed in terms of Mean of % Protection ± SEM and are found to be significant when compared to control $p<0.05$

From the results, it was observed that the standard drug diclofenac sodium has a percentage protection of 57.76, 65.80, 67.34, 71.24, and 78.31%. The hydroethanolic extract of *Combretum indicum* has 58.41, 66.61, 70.51, 78.80, 82.78%, and the hydroethanolic extract of *Euphorbia milii* has 48.01, 57.51, 58.65, 63.36, and 68.97% at concentrations of 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, and 500 µg/ml, respectively. It indicates that hydroethanolic extract of *Combretum indicum* at concentration of 500 µg/ml shows maximum anti-inflammatory activity (% protection) as compared to standard drug Diclofenac sodium and hydroethanolic extract of *Euphorbia milii.*

A comparison of % protection of hemolysis by hydroethanolic extracts of *Combretum indicum*, *Euphorbia milii* and standard diclofenac sodium is shown in Figure 5.

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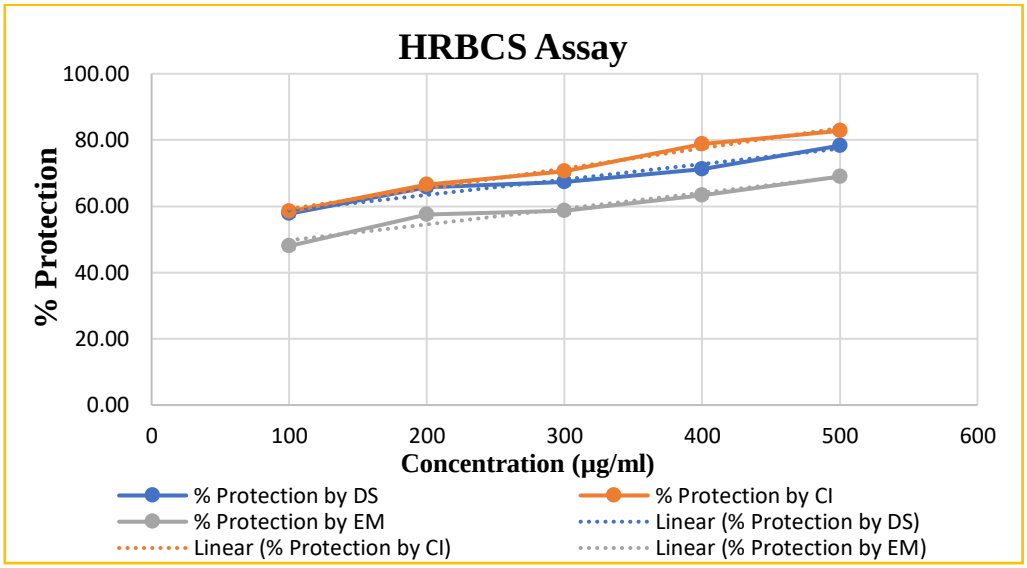

Figure 5: Effect of Hydroethanolic Extracts of *Combretum indicum* and *Euphorbia milii* on HRBC Membrane Stabilization

5. Discussion

Protein denaturation is a process by which protein loses its secondary and tertiary structure and its biological function may also be lost. Denaturation of protein is acclaimed as a marker for inflammation. Denaturation of proteins causes the production of autoantigens which ultimately leads to the inflammation and inflammatory diseases like rheumatic arthritis, cancer, diabetes etc. Hence, by inhibiting the protein denaturation, inflammatory activity can be inhibited. [22] Egg albumin protein denaturation assay and human red blood cell membrane stabilization assay are the widely accepted models used to evaluate the anti-inflammatory property of the products. Egg albumin method provides a cheap alternative method of testing the anti-inflammatory activity of herbal medicine using denaturation technique. [23] In this assay, denatured proteins are generated by subjecting it to extremes of heat, pH, or other denaturing agents. Anti-inflammatory property of the substance can be identified by adding the substance to the inflammation (through the generation of denatured proteins). Protein denaturation inhibition by substances indicate anti-inflammatory property. Higher the degree of its inhibition, greater would be the anti-inflammatory potential. Some literature reported that the plants extracts may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophils lysosomal constituents include bactericidal enzymes and proteinases, which upon extracellular release cause further tissue inflammation and damage. The precise mechanism of this membrane stabilization is yet to be elucidated. Now-a-days NSAIDs are generally used as reference drugs. NSAIDs prevent inflammation by blocking the cyclooxygenase enzyme activity. NSAIDs also has a property of preventing protein denaturation and membrane stabilization. Even though synthetic chemical drugs like diclofenac sodium, indomethacin have potent anti-inflammatory property, on prolonged use they can cause serious side effects like ulceration, hemorrhage, perforation and obstruction. Because of these complications induced by prolonged conventional synthetic drug treatment, the search towards natural agents which has anti-inflammatory properties are increasing nowadays. Several plant extracts were previously tested for anti-inflammatory efficacy utilizing an egg albumin denaturation assay and human red blood cell membrane stabilization assay with diclofenac sodium as the standard drug. Studies have shown that substances derived from plants are found to be effective, safe and alternative as an anti-inflammatory agent. The present study involves phytochemical investigation and evaluation of in-vitro anti-inflammatory activity of hydroethanolic extracts of *Combretum indicum* and *Euphorbia milii* against egg albumin denaturation assay and human red blood cell membrane stabilization assay. In this study, inhibition of protein denaturation and

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protection of hemolysis by hydroethanolic extracts of both plants were evaluated and the results of both methods were compared with the standard Diclofenac sodium. It was followed up with the identification of the plant's bioactive constituents using phytochemical analysis tests. According to previous studies, plants have secondary metabolites as major constituents through secondary metabolism. These secondary metabolites have various medicinal properties. Secondary metabolites like phenolic compounds such as flavonoids, tannins, and alkaloids, saponins, terpenoids can be used as anti-inflammatory agents. Phytoconstituents of *Combretum indicum* and *Euphorbia milii* were studied and found that they have more than 20 phytoconstituents including phenolic compounds, flavonoids, tannins, terpenes, alkaloids, which were known to possess anti-inflammatory property. Results of egg albumin denaturation assay revealed that standard drug Diclofenac sodium showed 60.14, 66.38, 72.90, 77.83, and 83.77% inhibition of protein denaturation at 100, 200, 300, 400 and 500 µg/ml of sample concentration whereas hydroethanolic leaves extracts of *Combretum indicum* showed 62.32, 68.84, 75.07, 79.71 and 85.22% at 100, 200, 300, 400 and 500 µg/ml of sample concentration in the concentration dependent manner. Hydroethanolic extract of *Euphorbia milii* leaves showed a percentage inhibition of protein denaturation of 52.61, 56.38, 63.33, 69.42, and 73.04 % at concentrations of 100, 200, 300, 400 and 500 µg/ml respectively. This study proves that the % inhibition of protein denaturation were significantly increased with hydroethanolic leaves extract of *Combretum indicum* and *Euphorbia milii* when compared with standard drug diclofenac sodium. The percentage inhibition of protein denaturation was increased with increasing concentration like standard diclofenac sodium. Maximum inhibition of protein denaturation was observed at 500 µg/ml of sample concentration. As per the results of egg albumin denaturation assay, *Combretum indicum* was found to be more effective to cure inflammation as it exhibits highest % inhibition of protein denaturation when compared to that of conventional standard drug diclofenac and hydroethanolic extract of *Euphorbia milii*.

Human red blood cell membranes are similar to lysosomal membrane components, the prevention of HRBC membrane lysis was taken as a measure of anti-inflammatory activity of drugs. The results obtained demonstrated that hydroethanolic extract of leaves of *Combretum indicum* can statistically significant ($p<0.05$) inhibit HRBC haemolysis. Results of human red blood cell membrane stabilization assay revealed that the hydroethanolic leaves extract of *Combretum indicum* at concentration range from 100 μg/ml to 500 μg/ml protects the human erythrocyte membranes against lysis. The hydroethanolic leaves extract of *Combretum indicum* exhibited the percentage of protection 58.41, 66.61, 70.51, 78.80, 82.78% of RBC haemolysis as compared with 57.76, 65.80, 67.34, 71.24, and 78.31% produced by Diclofenac sodium at concentration of 100, 200, 300, 400 and 500 μg/ml respectively. The hydroethanolic extract of *Euphorbia milii* showed the percentage of protection of hemolysis of 48.01, 57.51, 58.65, 63.36, and 68.97% at concentrations of 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, and 500 µg/ml, respectively. The investigation suggested that the hydroethanolic leaves extract of *Combretum indicum* showed little higher anti-inflammatory activity when compared with standard diclofenac sodium and hydroethanolic extract of *Euphorbia milii*.

6. Conclusion

In-vitro anti-inflammatory activity of *Combretum indicum* and *Euphorbia milii* have not been assessed till date. The hydroethanolic leaves extracts of both plants has considerably high phenolic and flavonoid contest. The therapeutic applications of flavonoids on inflammation and as anti-oxidant have previously been reported. The anti-inflammatory property is highly related to the antioxidant capacity. The results concluded that hydroethanolic leaves extracts of *Combretum indicum* have the highest ability to control the denaturation of protein related generation of auto-antigen, which relates to the inhibition of the denaturation of proteins. In both in-vitro anti-inflammatory models, hydroethanolic leaves extract of *Combretum indicum* was found to be more effective than standard diclofenac sodium and hydroethanolic leaves extract of *Euphorbia milii*.

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