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In vitro Anti-inflammatory Activities of Milk Weed (*Euphorbia heterophylla* Linn.)

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ABSTRACT

Leaves of Euphorbia heterophylla (Milk weed) is frequently used in herbal medicine for curing of several conditions in many parts of Nigeria. The study was aimed at elucidating the leaves' anti-inflammatory potentials and mechanisms of action. The leaves were assessed for their phytochemical contents. The in vitro anti-inflammatory activities of methanol extract of the leaves were evaluated for inhibition of albumin denaturation, proteinase inhibitory activity, membrane stabilization, and anti-lipoxygenase activity, which are associated with inflammatory processes. Aspirin, diclofenac sodium and indomethacin were used as standard drugs. Phytochemical analyses indicated the presence of high concentrations of alkaloids, saponin, and flavonoids; low concentrations of phenol and tannin, with a ferric reducing antioxidant power of 10.7 mg GAE/g. The methanol extract of the leaves significantly inhibited albumin denaturation and proteinase activity, stabilized membrane of red blood cells from hemolysis in heat and hypotonic conditions, and inhibited lipoxygenase activity. The overall results suggest that E. heterophylla is a prospective source of anti-inflammatory agents.

Keywords: *Euphorbia heterophylla*, EHME, Anti-inflammation

INTRODUCTION

The use of herbal medication for the treatment of illnesses and diseases started centuries ago. The World Health Organization supports the use of traditional medication provided they are confirmed to be effective and nontoxic [1]. In developing countries, a large number of inhabitants live in intense poverty and some are anguishing and dying for want of safe water and medication, they have no alternative for major health care [2]. Plants of *Euphorbia* are widespread in environments extending from herbs and shrubs to trees in tropical and temperate regions all over the world [3]. The family Euphorbiaceae encompasses 280 genera and 730 species with the largest genus *Euphorbia* having about 1600 species. Generally, they have typical milky latex, sticky sap; some are co-carcinogenic, cause severe skin irritation and are lethal to livestock and humans. *Euphorbia heterophylla* leaf is used in herbal medical practices as laxative, anti-gonorrheal, migraine and wart cures. The plant matrices have been used as fish poison, insecticide and ordeal poisons. In some parts of Kogi State, Nigeria, the leaves are used as anticonvulsant and cough remedy. The leaves *of E. heterophylla* have been reported to contain quercetin, a bioflavonoid complex with antioxidant and anti-inflammatory properties. Also diterpenoids, which are believed to have tumor inhibitor properties as well as a stimulating effect on the immune system, have been described in the root of *E. heterophylla* [3]. The principal objective of this study was to investigate the anti-inflammatory property of the leaves of *E. heterophylla.*

MATERIALS AND METHODS

Collection of plant samples

The leaves of *E. heterophylla* used for this study were purchased early hours of the day in October 2016 from the local markets of Ado-Ekiti, Ekiti State, Nigeria and transported to the Microbiology Laboratory of Afe Babalola University, Ado-Ekiti (ABUAD). Dr. O.T. Ogunmefun, a botanist in the Department of Biological Sciences, Afe Babalola University, identified the plants.

Preparation of plant extracts

The leaves of *E. heterophylla* were washed to remove sand. The Plant extract was prepared by cold percolation technique described by Akinpelu and Onakoya (2006) [4]. The test plant species was well dried under the shade and then crushed into fine powder using an electrical blender. A 200 g portion of the powdered plant sample was separately soaked in 500 ml of methanol in a glass container and covered with their lids. The mixture was then kept at room temperature for 2 days to allow complete extraction of the active ingredients or the chemical constituents. The fluids were then filtered using Whatman No 1 filter paper into beakers. The extract was obtained by concentrating in a rotary evaporator and the concentrate dried at 50°C on a water bath.

Phytochemical screening of plant extracts

A preliminary qualitative phytochemical screening of all extracts was carried out using standard phytochemical procedures to ascertain the presence of plant secondary metabolites including alkaloids, flavonoids, steroids, saponins, phenols, tannins, glycosides, cardiac glycosides and reducing sugar [5].

Quantitative phytochemical analysis

Quantitative analysis for the plant secondary metabolites such as alkaloids, flavonoids, steroids, saponins, phenols, tannins, glycosides, cardiac glycosides and reducing sugar were carried out on the pulverized samples following the methods of Harbone [6] and Mayuri [7].

Test for flavonoids

One gram of the sample was measured into 10 ml of 80% Methanol; left to stand for 2 h, filtered into a weighed petri dish and left to dry in the oven at 40°C until it attained a constant weight. The weight of the petri dish was recorded.

Tannins

One gram of the sample was extracted with 25 ml of the solvent mixture of 80:20 Acetone: 10% glacial acetic acid for 5 h. The supernatant was purified and the Absorbance of the filtrate as well as the reagent blank measured at 500 nm Absorbance. A standard graph was produced with 10, 20, 30, 40, 50 mg/100 g of tannic acid. The concentration of tannin was read off taking into consideration dilution factors.

Alkaloids

One gram of the sample (W) was added to 20 ml of 10% acetic acid in ethanol, shaken, allowed to stand for 4 h and filtered. The filtrate was allowed to vaporize to about a quarter of its original volume and one drop of concentration ammonia added. The precipitate formed was filtered through a weighed (W1) filter paper. The filter paper was then dried in the oven at 60°C, and weighed when it had attained a constant weight (W2).

$$\%\text{Alkaloid} = \frac{W2-W1}{W} \times 10$$

Phenols/Phenolics/Phenolic Acids

A 2 ml of methanol extract is mixed with 0.5 ml of Folin-Ciocalteau reagent and 1.5 ml sodium carbonate (20%). Mix for 15 sec and allowed to stand at 40°C for 30 min to develop color. Measure $A_{765.}$ Expressed as GAE/g (Gallic Acid Equivalent).

Saponins

One gram of sample was added to 5 ml of 20% ethanol in a conical flask and placed in a water bath at 55°C for 4 h. Filtering and washing the residue with 20% ethanol twice and reducing the extract to about 5 ml in the oven followed this. The extract was further treated successively petroleum ether, butanol and 5% sodium chloride.

Steroids

Five gram of the sample was added to 100 ml of water and drops of 0.1 M ammonium hydroxide was added to take the pH to 9.1, then 2 ml pet ether, followed by addition of 3 ml acetic anhydride and concentrated $H_2SO_{4,}$ and the absorbance measured at 420 nm.

Cardiac glycosides/Cardenolides

One gram of the sample was extracted with 40 ml water and place in the oven 100°C for 15 min, added to this was 1 ml extract with 5 ml water to 2 ml glacial acetic acid plus one drop of $FeCl_{3.}$ This was followed with 1 ml concentrated H_2SO_4, the absorbance of the resulting solution was measured at 410 nm.

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay uses antioxidants as reductant in a redox-linked colorimetric method employing an easily reduced oxidant, Fe(III). Reduction of a ferric tripyridyltriazine complex to ferrous (2,4,6-tripyridyl-s-triazine) $_2$ i.e., Ferric(III) (colorless) to Ferrous II (blue) can be monitored by measuring absorbance at 593 nm. The absorption readings are related to the reducing power of the electron-donating antioxidants present in the test compound. Hence the FRAP assay can rank the reducing power and the antioxidant potential of the compound. The FRAP equation is:

$$\text{FRAP value of sample } (\mu\text{M}) = \frac{Abs\ (Sample) \times FRAP\ value\ of\ Std\ (\mu\text{M})}{Abs\ (Std)}$$

Assessment of *in vitro* anti-inflammatory activity of *E. heterophylla*

The *in vitro* anti-inflammatory activity of *E. heterophylla* methanol extract (EHME) was evaluated using albumin denaturation, proteinase inhibitory activity, membrane stabilization, and antilipoxygenase activity.

Inhibition of albumin denaturation

The anti-inflammatory activity of the plant extract by using inhibition of albumin denaturation mechanism was carried out according to methods described by Mizushima et al., [8] and Sakat et al., [9]. The reaction mixture consisted of 100 µl (100-500 µg/ml) test extract and 500 µl of 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was regulated to 6.3 using small amount of 1 N HCl. The sample extracts were then incubated at 37°C for 20 min and then heated to 51°C for 20 min. After cooling the samples the turbidity was measured at 660 nm (Spectrophotometer Model Spectronic 20). The tests were done in triplicate. The Percentage inhibitions of protein denaturation were then calculated as follows:

$$Percentage\ Inhibition = \frac{(Absorbance\ of\ Control - Absorbance\ of\ Sample)}{Absorbance\ of\ Control} \times\ 100$$

Proteinase inhibitory action

The test was completed according to the approach of Oyedepo et al. [10] and Sakat et al., [9]. The reaction mixture (2 ml) comprised 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of altered concentrations (100-500 µg/ml). The mixture was then incubated at 37°C for 5 min which was followed by the addition of 1 ml of 0.8% (w/v) casein. The mixture was then incubated for an additional 20 min. A 2 ml volume of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiments were performed in triplicate. The percentage inhibition of proteinase inhibitory activity was then calculated as:

$$Percentage\ Inhibition = \frac{(Absorbance\ of\ Control - Absorbance\ of\ Sample)}{Absorbance\ of\ Control} \times 100$$

Membrane stabilization

The blood was obtained from a healthy human volunteer who had not taken any Non-steroidal Anti-inflammatory Drugs (NSAIDs) for 2 weeks prior to the experiment and transferred to centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and washed three times with equal volume of normal saline. The volume of blood was then measured and reconstituted as 10% v/v suspension with normal saline.

Heat induced hemolysis

A reaction mixture (2 ml) consisting of 1 ml EHME at varying concentrations (100-500 µg/ml) and 1 ml of 10% Red Blood Cells (RBCs) suspension. Instead of test sample, only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes holding reaction mixture were incubated in water bath at 56°C for 30 min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants were taken at 560 nm. The experiments were performed in triplicates for all the test samples [9]. The Percentage inhibition of Hemolysis was calculated as follows:

$$Percentage\ Inhibition = \frac{(Absorbance\ of\ Control - Absorbance\ of\ Sample)}{Absorbance\ of\ Control} \times\ 100$$

Hypotonicity-induced hemolysis

A volume of 0.5 ml of different concentration of plant extract (100-500 µg/ml), standard drug (100 µg/ml), and control (distilled water instead of hyposaline to produce 100% hemolysis) were separately mixed with 1 ml of phosphate buffer (pH 7.4, 0.15 M), 2 ml of hyposaline (0.36%) and 0.5 ml of Human Red Blood Cells (HRBC) suspension. Diclofenac sodium (100 µg/ml) was used as a standard drug. All the assay mixtures were incubated at 37°C for 30 min and centrifuged at 3000 rpm. The supernatant liquid was decanted and the hemoglobin content estimated by a spectrophotometer at 560 nm. The percentage hemolysis was assessed by assuming the hemolysis produced in the control as 100% [9].

$$Percentage\ protection = \frac{(Absorbance\ of\ Control - Absorbance\ of\ Sample)}{Absorbance\ of\ Control} \times\ 100$$

Antilipoxygenase activity

Antilipoxygenase activity was studied using linoleic acid as substrate and lipoxidase as enzyme. Test samples were suspended in 0.25 ml of 2M-borate buffer (pH 9.0) and added 0.25 ml of lipoxidase enzyme solution (20,000 U/ml) and incubated for 5 min at 25°C. Afterwards, 1.0 ml of linoleic acid solution (0.6 mM) was added, mixed well and absorbance measured at 234 nm. Indomethacin will be used as reference standard [9]. The percent inhibition will then be calculated from the following Equation:

$$Percentage\ Inhibition = \frac{(Absorbance\ of\ Control - Absorbance\ of\ Sample)}{Absorbance\ of\ Control} \times\ 100$$

Statistical analysis

Results were expressed as Mean ± SD. The difference between experimental groups were compared by One-Way Analysis of Variance (ANOVA) followed by Dunnet Multiple comparison test (control *vs.* test) using the software Graph Pad Instat

RESULTS

Phytochemical analysis of *Curcuma longa* and *E. heterophylla*

The extractive yield of 250 g of dried pulverized plant material of *E. heterophylla* was 2.77%. Quantitative phytochemical analyses of the dried leaves of *E. heterophylla* revealed 0.219 mg/g concentration of phenol, 0.143 mg/g of tannin, 2.285 mg/g of flavonoid, 12.000 mg/g of saponin and 19.700 mg/g of alkaloid (Table 1). The FRAP of *E. heterophylla* was found to be 10.712 mg GAE/g of plant sample (Table 1).

Table 1: Phytochemical content of leaves of *Euphorbia heterophylla*

Parameter (mg/100 g)	*Euphorbia heterophylla*
Phenol	21.9
Tannin	14.3
Flavonoid	228.5
Saponin	1200.0
Alkaloid	1970.0
FRAP (mg GAE/g of sample)	10.712

***In vitro* anti-inflammatory activity of *E. heterophylla* Methanol Extract (EHME)**

As part of the examination on the method of anti-inflammatory activity, capability of extracts to inhibit protein denaturation was considered. The extract was effective in inhibiting heat induced albumin denaturation at various concentrations as shown in Figure 1. The EHME showed maximum inhibition with 81.7% at 500 µg/ml. Aspirin, a standard anti-inflammatory drug showed the maximum inhibition with 80.7% at the concentration of 100 µg/ml.

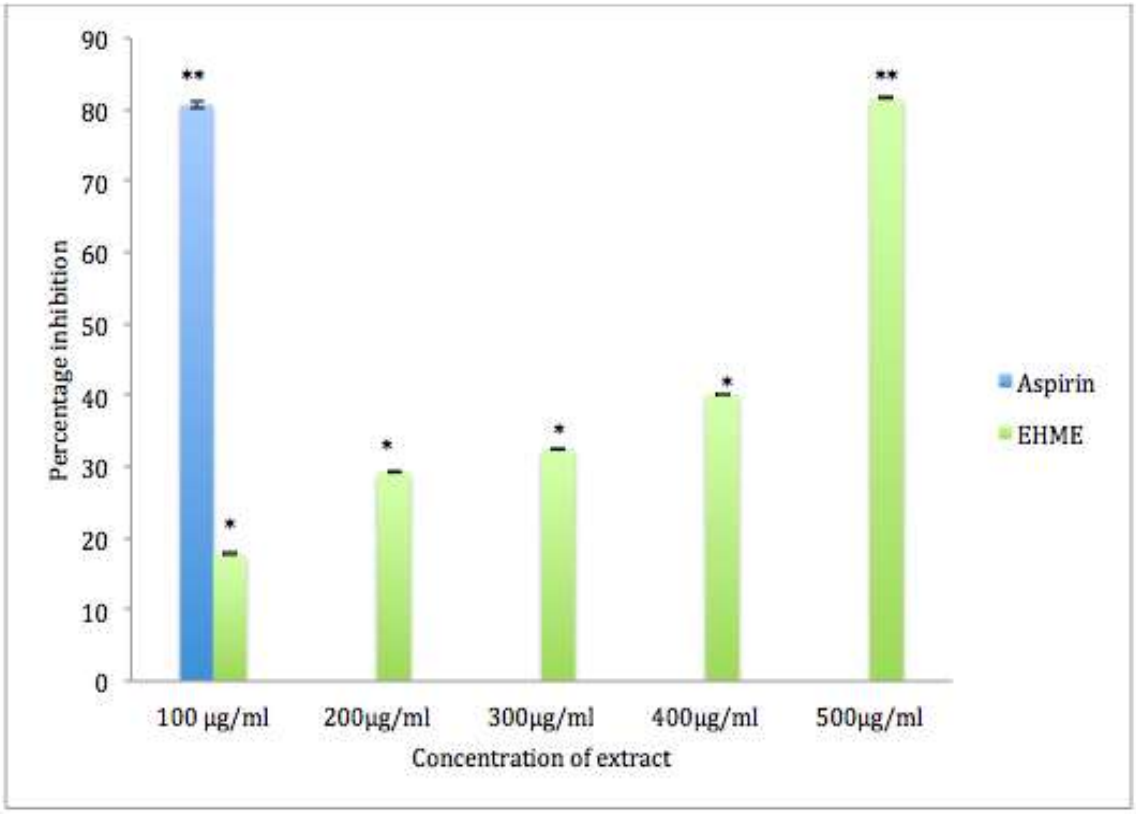

Figure 1: Percentage Effect of *Euphorbia heterophylla* on heat induced protein denaturation

Extracts compared with control, $^{**}p \leq 0.01$ was considered as extremely significant; $^{*}p \leq 0.05$ considered significant; and $^{ns}p > 0.05$ considered not significan

The methanol extract of *E. heterophylla* exhibited significant anti-proteinase activity at different concentrations as shown in Figure 2. The methanol extracts of *E. heterophylla* showed maximum inhibition with 56% at 500 µg/ml. Aspirin showed the greatest inhibition with 62.2% at 100 µg/ml.

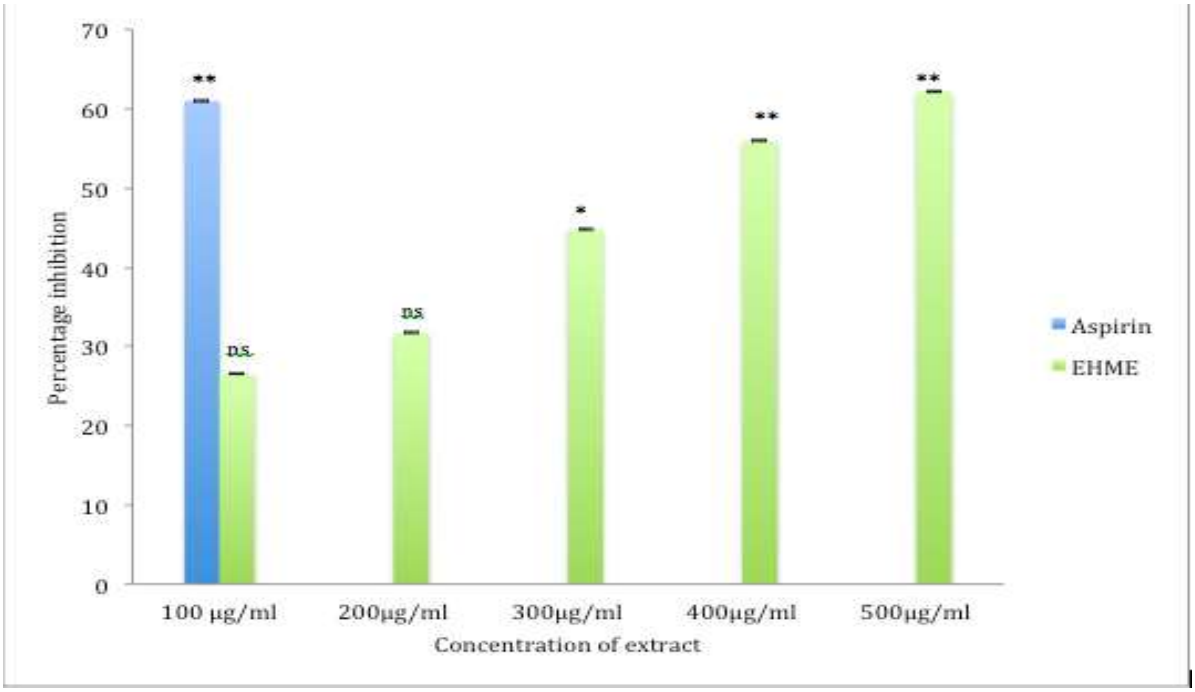

Figure 2: Percentage effect of *Euphorbia heterophylla* methanol extract on proteinase inhibitory action
Extracts compared with control, $^{**}p \leq 0.01$ was considered as extremely significant; $^{*}p \leq 0.05$ considered significant; and $^{ns}p > 0.05$ considered not significant

The methanol extract of *E. heterophylla* was also effective in inhibiting the heat-induced hemolysis of RBC to varying degrees as shown in Figure 3. The results revealed that at concentration range of 300-500 µg/ml, methanol extract of *E. heterophylla* protect significantly ($p < 0.05$) the erythrocyte membrane against lysis induced by heat. Aspirin, at 100 µg/ml, offered a significant ($p<0.01$) protection against damaging effect of heat solution.

EHME at concentration range of 200-500 µg/ml was also found to protect the erythrocyte membrane against lysis induced by hypotonic solution significantly ($p<0.01$) (Figure 4). Diclofenac sodium (100 µg/ml) offered significant ($p<0.01$) protection against the detrimental effect of hypotonicity solution. At the concentration of 500 µg/ml, *E. heterophylla* showed maximum protection with 77%, whereas, Diclofenac sodium (100 µg/ml) showed 57.3% inhibition of RBC hemolysis when compared with control.

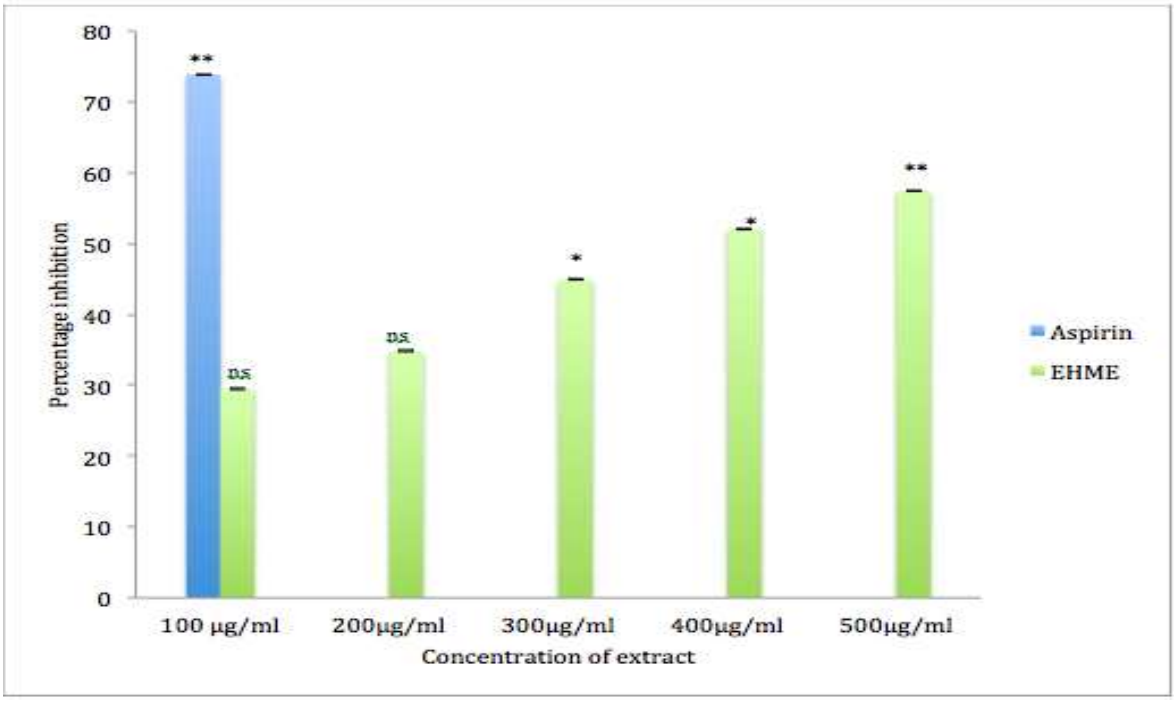

Figure 3: Effect of *Euphorbia heterophylla* methanol extract on heat induced haemolysis of erythrocyte
Extracts compared with control, $^{**}p \leq 0.01$ was considered as extremely significant; $^{*}p \leq 0.05$ considered significant; and $^{ns}p > 0.05$ considered not significant

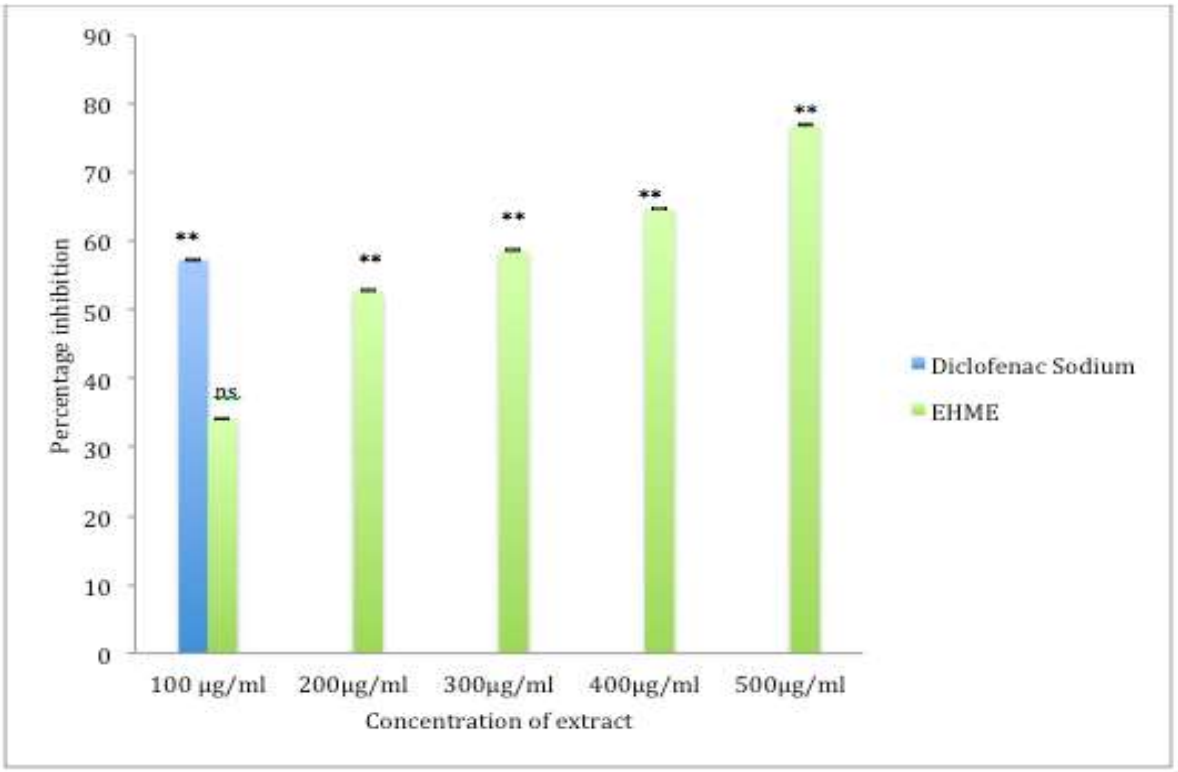

Figure 4: Effect of *Euphorbia heterophylla* methanol extract on hypotonicity induced haemolysis of erythrocyte
Extracts compared with control, $^{**}p \leq 0.01$ was considered as extremely significant; $^{*}p \leq 0.05$ considered significant; and $^{ns}p > 0.05$ considered not significant

EHME tested at 100, 200, 300, 400 and 500 µg/ml for lipoxygenase inhibitory action, produced 8.2, 16.6, 24.1, 46.5, and 59% inhibition of lipoxygenase, respectively. The standard indomethacin, showed 84.8% inhibition at a concentration of 100 µg/ml. At the concentration of 100 and 200 µg/ml, EHME did not show significant ($p>0.05$) inhibition when compared with control (Figure 5).

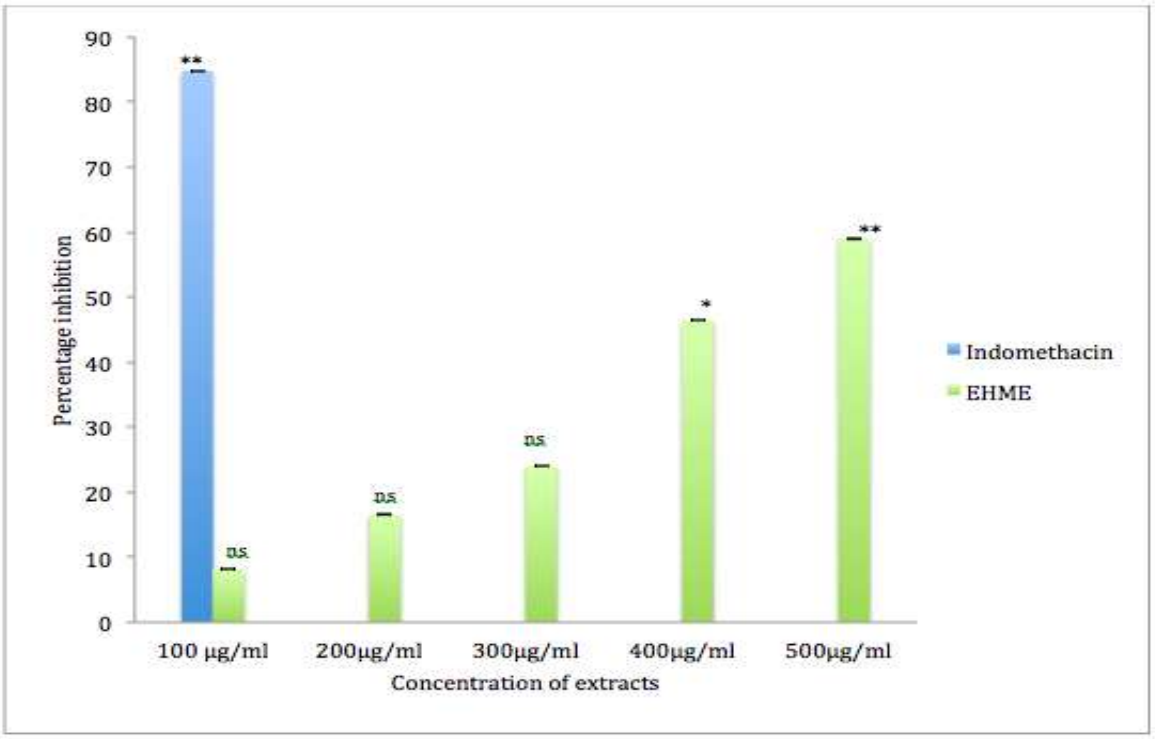

Figure 5: Effect of *Curcuma longa* methanol extract and *Euphorbia heterophylla* methanol extract on lipoxygenase inhibitory action
Extracts compared with control, $^{**}p \leq 0.01$ was considered as highly significant; $^{*}p \leq 0.05$ considered significant and $^{ns}p > 0.05$ considered not significant

DISCUSSION

Medicinal plants are important source of exploring novel pharmacological agents and can be natural composite sources of new anti-infectious agents [11]. *Euphorbia heterophylla* has been found to possess high antimicrobial along with phytochemical properties [11]. In this study the medicinal potentials of *E. heterophylla* were assessed through the phytochemical analyses, antimicrobial, and anti-inflammatory activities of the plants. The quantities of alkaloids, tannins, flavonoids, phenols, and saponins, detected in the phytochemical analysis (Table 1) justified their uses as medicinal plants in ethnobotany and can be useful in detecting lead compounds for the manufacture of both anti-inflammatory and antimicrobial drugs of plant origin. The cardioprotective, anticancer, anti-inflammatory, antimicrobial, antidiabetic, antiaging, neuroprotective and antioxidant properties of these plants could be attributed to these phytochemicals present [12]. Qualitative phytochemical analysis of the extract from *E. heterophylla* indicated that their methanol extracts contained alkaloids, tannins, flavonoids, saponins and phenols. The FRAP of *E. heterophylla* also revealed its richness in antioxidant properties (Table 1).

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structures by application of an outward pressure or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological functions when denatured [8]. Denaturation of proteins is a well-documented cause of inflammation. Phenylbutazone, salicylic acid, flufenamic acid (anti-inflammatory drugs) etc., have shown dose dependent ability to thermally induce protein denaturation [8]. In this study methanol extract of the plant was efficient in inhibiting heat induced albumin denaturation at different concentrations as shown in Figure 1. The EHME showed maximum inhibition, with 81.7% at 500 µg/ml and compared favorably with aspirin, a standard anti-inflammatory drug showed the maximum inhibition with 80.7% at the concentration of 100 µg/ml. Neutrophils are recognized as rich sources of serine proteinase and are concentrated at lysosomes. It was earlier narrated that leukocytes proteinase plays a vital role in the development of tissue damages during inflammatory reactions and significant level of protection was provided by proteinase inhibitors [13]. The EHME showed maximum inhibition, with 56% at 500 µg/ml. Aspirin showed the maximum inhibition with 62.2% at 100 µg/ml.

The HRBC membrane stabilization has been used as an approach to analyse the *in vitro* anti-inflammatory activity because the erythrocyte membrane is comparable to the lysosomal membrane [14] and its stabilization suggests that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal membrane is vital to regulating the inflammatory response by averting the discharge of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes additional tissue inflammation and damage upon extra cellular release. The lysosomal enzymes released during inflammation create various conditions. The extra cellular activities of these enzymes are said to be related to acute or chronic inflammation. Non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane [14].

EHME effectively inhibited the heat-induced hemolysis at different concentrations, providing confirmation for membrane stabilization as an extra mechanism of the plant anti-inflammatory potentials. The extract may conceivably inhibit the release of lysosomal content of neutrophils at the place of inflammation. These neutrophil lysosomal constituents include bactericidal enzymes and protease, which upon extracellular release

cause further tissue inflammation and damage [15]. The plant extract (300-500 µg/ml) inhibited the heat-induced hemolysis of RBCs to varying degree as shown in Figure 2. EHME showed maximum inhibition with 57.5% at 500 µg/ml, while aspirin showed the maximum inhibition with 73.9% at 100 µg/l. EHME (200-500 µg/ml) also showed inhibition of hypotonicity-induced haemolysis of RBCs (Figure 3) with maximum inhibition of 77% at 500 µg/ml. Though the exact mechanism of this membrane stabilization is yet to be clarified, it is likely that the extract produced this effect on surface area/volume ratio of the cells, which could be as a result of the expansion of membrane or the shrinkage of the cells and an interaction with membrane proteins [16]. The significant membrane stabilizing activity of the methanol extracts may be due to the presence of polyphenolic contents. Several reports have shown that herbal drugs are capable of facilitating the stabilization of red blood cell membrane and possess anti-inflammatory activity [17].

The plant Lipoxygenase (LOX) pathway is in many respects comparable to the 'arachidonic acid cascades' in animals [18]. For this reason, the *in vitro* inhibition of lipoxygenase constitutes a good model for the assessment of plants with anti-inflammatory potential [19]. LOXs are sensitive to antioxidants and most of their action may consist in inhibition of lipid hydroperoxide creation due to sifting of lipidoxy or lipid peroxy- radical created in course of enzyme peroxidation. This can check the accessibility of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX [19]. EHME showed significant inhibition of lipoxygenase activity at 300-500 µg/ml (Figure 5). Lipoxgenases are lipid-peroxidizing enzymes involved in the biosynthesis of leukotriene from arachidonic acid, mediators of inflammatory and allergic reactions. These enzymes catalyze the addition of molecular oxygen to unsaturated fatty acids such as linoleic and arachidonic acids [20]. There are four main isoenzymes already described, namely, 5-LOX, 8-LOX, 12-LOX and 15-LOX, depending on the site of oxidation in the unsaturated fatty acids [20]. The common substrates for LOX are linoleic and arachidonic acids.

During inflammation, arachidonic acid is metabolized via the COX pathway to produce prostaglandins and thromboxane A2, or via the LOX pathway to produce hydroperoxy-eicosatetraenoic acids and leukotrienes [18]. The LOX pathway is effective in leucocytes and many immune-competent cells including mast cells, neutrophils, eosinophils, monocytes and basophils. Upon cell activation, arachidonic acid is cleaved from cell membrane phospholipids by phospholipase A2 and provided by LOX activating protein to LOX, which then metabolises arachidonic acids in a series of reactions to leukotrienes, a group of inflammatory mediators [20]. Leukotrienes act as phagocyte chemo-attractant, recruiting cells of the innate immune system to sites of inflammation. For instance in an asthmatic attack, it is the production of leukotrienes by LOX that causes the constriction of bronchioles leading to bronchospasm [21].

Therefore, the selective inhibition of LOX is a vital remedial approach for asthma [20]. Inhibitors of the actions of LOX could offer potential therapies to manage many inflammatory and allergic responses. Medicinal plants may therefore be prospective sources of inhibitors of COX-2/LOX that may have fewer side effects than NSAIDs.

CONCLUSION

This study indicated that the biomolecules of *E. heterophylla* can be used for designing potent anti-inflammatory and that can be used for the treatment of various diseases such as bacterial infections and inflammations. The extract significantly inhibited albumin denaturation and proteinase activity, stabilized membrane of red blood cell from hemolysis in heat and hypotonic conditions, and inhibited lipoxygenase activity, all of which are associated with inflammatory processes. This study provided added evidence that the plant, *E. heterophylla* possesses anti-inflammatory potentials.

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