

## Evaluation of *In Vitro* Antimicrobial and Anti-Inflammatory Potentials of Crude Methanolic Extracts of *Excoecaria agallocha* (Leaves)

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**Abstract.** *Excoecaria agallocha* plant (Leaves) was analyzed to explore in-vitro antimicrobial and membrane stabilizing activities as a crude methanolic extract. Antimicrobial activity was performed against a wide range of Gram positive (+Ve) and Gram negative (-Ve) bacteria by using disc diffusion method and various methanolic extracts of leaves of *E.agallocha* was tested for determining membrane stabilizing activity at hypotonic solution and heat induce condition and standard acetyl salicylic acid (0.10 mg/mL) was employed as standard. The zones of inhibition created was found to be 1.3 cm at a concentration of 100 µL/ disc in case of Gram negative (-Ve) bacteria *Salmonella typhi*. The results obtained were compared with that of a standard Ampicillin (10 µL) and imipenem (10µL), penicillin (10µl), cefoxitine (30µl). Crude methanolic extract of 10mg/ml concentration showed maximum value of 17.67±0.0102 and 18.92±0.086% respectively under hypotonic solution and heat induced condition. Thus the result suggest that, crude methanolic extracts of *E.agallocha* possessed slight to moderate antimicrobial and membrane stabilizing properties.

### Introduction

*Excoecaria agallocha* is a nice plant, belonging to the family “Euphorbiaceae”, locally known as *gewa* (Bangladesh), It is locally used to treat sore and stings from marine creatures, smoke from bark is used to treat leprosy, purgative and an emetic agent. The roots are used to treat toothache and swellings.

Microorganisms are responsible for several infectious diseases throughout the world, especially in developing countries and become the major causes of mortality and morbidity in immunocompromised patients [1]. Antimicrobial like antibiotic have used for treating these diseases. But due to the rapid emergences of bacterial resistance, the use of antibiotic has become a greater challenge to modern medical treatment [2]. Again most of our currently used antibiotic possess remarkable toxic effects. So it is badly need to analyze the natural products in order to explore new antibacterial agent to combat against these condition [3-4].

Inflammation is one of the major pathological disorders that cause inflammation of cells at various region of our body. Unlike normal body cell, inflammatory cell produces a wide range of cytokines as well as several physiologically active arachidonate metabolites [5-6]. It is usually characterized by heat, redness, pain, swelling and disturbed function. It includes wide array of extravasations of fluid, cell migration, tissue breakdown, and repair enzyme activation and mediator release [7]. Lysosomal enzymes produce during inflammation produce a variety of disorders including heart attacks, septic shocks and rheumatoid arthritis. Some clinical study suggested that, inflammation is also responsible for initiation and progression of atherosclerosis [8-10]. Non-steroidal anti-inflammatory drugs (NSAIDs) are mainly used for the treatment of pain, fever, inflammation and rheumatic disorders associated with inflammation. Which have several adverse

effects especially dyspeptic symptoms, gastrointestinal erosions and peptic ulcers to more serious complications, such as over bleeding or perforation and gastric irritation leading to formation of gastric ulcers [11-13]. So the development of new anti-inflammatory drugs is still necessary to overcome the adverse effect of NSAID and in such case natural product such as medicinal plants could be a good source of new anti-inflammatory drugs with less undesirable effects [14].

From the ancient civilization, human beings are largely depending upon nature and nature is always a good source of medicine. Most of our currently used medicine came from animal or plant i.e. nature. For the treatment of diseases and injuries, mankind started using medicinal plants throughout the world since the beginning of the civilization, because medicinal plants and its products have proved to be an important therapeutic aid for alleviating the ailments of human kind [15]. Moreover, medicinal plants also serve as a natural laboratory [16], wealth of a country and store house of drugs [17-18]. Natural products and its related drugs are used to treat 87% of all categorized human diseases (48/55), mainly as antibacterial, anticancer, anticoagulant, antiparasitic, and immunosuppressant agent [19].

By considering this entire outcome from natural product our present study was undertaken to determine the antimicrobial and anti-inflammatory potentials of methanolic extract of *Excoecaria agallocha*.

## Materials and method

**Collection of plant specimen:** The whole plant of *Excoecaria agallocha* was collected from Sonadia Deep, a coastal region of Bangladesh on 06<sup>th</sup> April 2015 and it was identified by Bangladesh National Herbarium, Mirpur, Dhaka (Accession number- 38326).

**Extraction:** Plants are separated into leaves, fruits, stems and undergo sun-dry for ten days. Then the leaves of plants were powdered by means of a suitable grinder. About 400 gm of powered material was taken into a suitable clean, flat-bottomed glass container and moisten in 1600 ml of 80% Methanol. Then the container with its contents was sealed and kept at room temperature for a period of 14 days accompanying occasional shaking and stirring. It was then filtered through a piece of clean, white cotton material for coarse filtration and finally pass through Whatman filter paper. The filtrate (Methanol extract) obtained was evaporated at a suitable rotary evaporator and subjected to water-bath until dried. It rendered into a sticky concentrate of brownish black color. The sticky concentrate was designated as crude extract of Methanol.

## Antimicrobial activity:

**Test organism:** Single strains of Gram positive (*Staphylococcus aureus*) and three strains of Gram negative (*Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*) bacteria were used as a test organism for antimicrobial activity. These organisms were collected from the Department of Microbiology, Noakhali Science and Technology University, Sonapur-3814, Noakhali, Bangladesh and are sub-cultured in nutrient broth and nutrient agar culture media.

**Procedure:** The antimicrobial screening which is the first stage of antimicrobial drug research is performed by several ways, but we used Kirby-Bauer's Disc diffusion method which is widely accepted method for in-vitro antimicrobial screening [20]. Previously sterilized four petri dishes were flooded with Mueller-Hinton Agar media and after solidification of the media desired culture of micro-organism was inoculated on the plate through cotton burg stick which is already soaked into the desired microbial culture broth. Sample was prepared by dissolving 1 mg extract in 1 ml methanol (1mg/ml) and loaded into dried and sterilized filter paper discs with different volume (25  $\mu$ l/disc, 50  $\mu$ l/disc, 75  $\mu$ l/disc, and 100  $\mu$ l/disc respectively) using micropipette. These discs were transferred to each petri dish carefully using a sterile forceps and allowed to dry for 24 hours at room temperature. The dishes were then incubated at 37°C for 24 hours to allow optimal growth of microorganism. Then the dishes were observed for bacterial growth, which can be expressed in term of zone of inhibition (in millimeter) by using antibiotic zone scale. Different antibiotics (Ampicillin,

Imipenem) and sterile filter paper disc with respective solvent (methanol) of 25  $\mu$ l were used as positive and negative control respectively. If the test sample possesses any antimicrobial activity, it will reduce the growth of the microorganisms and a clear, distinct zone of inhibition will be appeared surrounding the medium. The antimicrobial activity of the test sample was calculated by measuring the diameter of zone of inhibition expressed in centimeter [21].

#### Assay of membrane stabilization:

**Preparation of test sample:** For this study we used methanolic extracts of *Excoecaria agallocha* with a various concentration of (2, 4, 6, 8, 10 mg/mL) as a representative sample to evaluate anti-inflammatory effect.

**Drug:** Aspirin or acetyl salicylic acid (ASA) was used as standard drug in comparison with different methanolic extracts of *Excocaria agallocha*.

**Collection of RBC:** Human RBCs were collected from the healthy volunteer and were kept in a test tube containing anticoagulant EDTA under standard conditions of temperature  $23\pm 2^\circ\text{C}$  and relative humidity  $55\pm 10\%$ .

**Preparation of phosphate buffer solution:** Phosphate buffer with a pH of 7.4; strength 10 mM was obtained by using 0.0352% monosodium phosphate dehydrate and 0.1099% disodium phosphate anhydrate. The buffer was prepared by adding 0.352 gm monosodium phosphate dehydrate and 1.099 gm disodium phosphate anhydrate in 1000 mL water.

**Preparation of isotonic solution:** A solution that has same osmotic pressure relative to that of human cell, is known as isotonic solution. Either 0.16M sodium chloride (NaCl) solution (approximately 0.95% salt in water) or 0.3M nonelectrolyte solution is approximately isotonic with human red blood cells. For the preparation of isotonic solution of 154 mM strength, 4.5045 gm NaCl was added with 500 mL phosphate buffer solution and mixed gently.

**Preparation of hypotonic solution:** A solution that has an osmotic pressure lower than that of a reference solution or of an isotonic solution is called hypotonic solution. For the preparation of hypotonic solution of 50 mM strength, 1.4625 gm NaCl was added with remaining 500 mL phosphate buffer solution and mixed properly.

**Preparation of erythrocyte suspension:** 5 mL blood was collected from healthy volunteer under standard condition and taken in a test tube containing EDTA (2.2 mg/mL of blood) as anti-coagulant. Then it was centrifuged for 5 min at 3000 rpm to remove supernatant plasma layer and the blood cells were washed three times with isotonic solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifuge action for 10 min at 3000 rpm using the same volume as supernatant. Finally, it was re-suspended in the same volume of this isotonic buffer solution.

#### Hypotonic Solution- Induced Hemolysis:

The membrane stabilizing activity of the extracts was evaluated by using hypotonic solution induced human erythrocyte hemolysis, designed by Sikder et al. [22] with minor modification. Here 0.5 ml of this erythrocyte suspension was mixed with 5 ml hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffer saline (pH 7.4) containing either the different concentration of crude methanolic extracts (2, 4, 6, 8 and 10 mg/ml respectively) or Acetyl Salicylic Acid (0.10 mg/ml). The Acetyl Salicylic Acid was used as a reference standard. Again 0.5 ml of RBCs was mixed with 5 mL hypotonic buffered saline solution alone as a control sample. The mixture was incubated for 10 minute at room temperature, then centrifuged for 10 min at 3000 rpm and finally the optical density of supernatant was measured at 540 nm. The percentage inhibition of hemolysis was calculated by the following equation:

$$\% \text{ inhibition of hemolysis} = \left\{ \frac{(\text{OD}_{\text{control}} - \text{OD}_{\text{test sample}})}{\text{OD}_{\text{control}}} \right\} \times 100 .$$

### Heat induced hemolysis:

Aliquots (5 ml) of the isotonic buffer, containing different concentration of the crude methanolic extracts of *Excocaria agallocha* were put into two duplicate sets of centrifuge tubes [16]. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30  $\mu$ L) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54 °C for 20 min in a water bath. The other pair was maintained at 0-5 °C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm using UV spectrometer. The percentage inhibition or acceleration of hemolysis in tests and was calculated using the following equation:

$$\% \text{ inhibition of hemolysis} = 100 \times \{(\text{OD}_2 - \text{OD}_1) / (\text{OD}_3 - \text{OD}_1)\}$$

where, OD<sub>1</sub> = test sample unheated, OD<sub>2</sub> = test sample heated and, OD<sub>3</sub> = control sample heated.

### Statistical analysis:

All the above assays were conducted in triplicate and repeated threes for consistency of results and statistical purpose. The data were expressed as Mean  $\pm$  SD and analyzed by one-way analysis of variance (ANOVA) followed by Dunnett't' test using SPSS software of 10 versions. P<0.05 was considered statistically significant.

### Results and Discussion

**Antimicrobial Activity:** We have already mentioned that, the purpose of this study was to ascertain antimicrobial effect of crude methanolic extract of *Excoecaria agallocha*. The antimicrobial activity of the extracts of *Excocaria agallocha* were studied in different concentrations (25, 50, 75 and 100  $\mu$ g/ml) against four pathogenic bacterial strains, one Gram-positive (*Staphylococcus aureus*) and three Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*), the antibacterial activities of the extracts increased linearly with increase in concentration of extracts ( $\mu$ g/ml). After performing the experiment, we have seen that, methanolic extract of *Excoecaria agallocha* was resistant against gram positive (+Ve) bacteria, but show moderate activity against gram negative (-Ve) *Salmonella typhi* bacteria, with a zone of inhibition of 1.3 cm at a concentration of 100  $\mu$ L/disc, which is summarized in the following table-1. As compared with standard drugs penicillin, imipenem, cefixitine, ampicillin the results revealed that, *S. typhi* was more sensitive than the other.

**Table 1:** Antimicrobial effect of crude methanolic extract of *Excoecaria agallocha*.

Test Bacteria		Zones of inhibition (cm) of extracts (Mean $\pm$ SD)				Response of standard against microorganism
		25 $\mu$ l	50 $\mu$ l	75 $\mu$ l	100 $\mu$ l	
Gram Positive (+ve)	<i>Staphylococcus aureus</i>	-	-	-	-	Cefoxitine(30 $\mu$ l) (+++)
	<i>Salmonella Typhi</i>	-	-	-	1.3 $\pm$ 0.041 (+)	Penicillin(10 $\mu$ l) (+++)
Gram Negative (-ve)	<i>E. coli</i>	-	-	-	-	Ampicillin(10 $\mu$ l) (+++)
	<i>Pseudomonas Aeruginosa</i>	-	-	-	-	Imipenem(10 $\mu$ l) (+++)

(+++)= Highly active; (++) = moderately active; (+) = slightly active; (-) = Not active against microorganism.

Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. WHO reported that, almost 80% of world's population uses medicinal plant extracts or their constituents as folk medicine in traditional therapies [23]. In the present work, the extracts obtained from *Excocaria agallocha* show moderate activity against Gram negative bacterial

strains. The results were compared with standard antibiotic drugs penicillin, cefoxitine, imipenem, and ampicillin. In this screening work, extracts of *Excocaria agallocha* were found to be not inactive against Gram positive bacterial strains.

The above results show that the activity of methanolic extracts of *Excocaria agallocha* shows moderate antibacterial activities. This study also shows the presence of different phytochemicals with biological activity that can be of valuable therapeutic index. The result of phytochemicals of this plant was found to have alkaloids, glycosides, flavonoids, carbohydrates, anthraquinone, tannins, phenols, terpenoids, fixed oil and fats [24]. The previous results have been shown that plant rich in tannins, phenols compounds have been shown to possess antimicrobial activities against a number of microorganisms.

#### Anti-inflammatory effect:

The crude methanolic extract of leaves of *Excoecaria agallocha* were subjected to explore anti-inflammatory effect following standard procedure and the attained results were statistically represented in Tables 2-3 and Figures 1-2. Table 2 and Figure 1 revealed that plant methanolic extract showed % inhibition of hemolysis dose dependently. Almost similar result found when study carried out on heat induce condition (Table3 and figure 2). By considering the result, it is clear that the extracts were moderately potent on human erythrocyte, adequately protecting it against hypotonic solution and heat induced lyses, when compared with the standard acetyl salicylic acid (0.10 mg/ml). Where 10mg/ml of plant extract showed maximum value for % inhibition of hemolysis that was 17.67 and 18.62 for hypotonic solution and heat induce condition respectively (Figures 1-2). But, is found to be statistically significant ( $p < 0.05$ ) for only heat induce condition. However, 2mg/ml and 6mg/ml of extract showed statistically significant ( $p < 0.05$ ) value for both hypotonic solution and heat induce condition (Tables 2-3). When result found for both hypotonic solution and heat induce condition they sound to possess almost similar result shown at figure 3. It is now established truth that the vitality of cells depends on the integrity of their membranes [25]. When red blood cell is exposed to injurious substances such as hypotonic medium results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin [25, 26]. Excessive accumulation of fluid within the cell is related to the haemolytic effect of hypotonic solution thus resulting in the rupturing of its membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical -induced lipid peroxidation [25, 26]. This observation is also consistent with the breakdown of bio-membranes leads to the formation of free radicals which in turn enhance cellular damage [27, 28]. It is therefore expected that compounds with membrane-stabilizing properties, should offer significant protection of cell membrane against injurious substances [29, 30]. The results of the study showed that *Excoecaria agallocha* leaf extract possesses anti-inflammatory property, as it showed membrane stabilizing effect, as it offers significant protection of the erythrocyte against lysis induced by hypotonic solution and heat induce condition.

**Table 2:** Effect of various concentrated extract of leaves of *Excoecaria agallocha* on hypotonic solution-induced haemolysis of erythrocyte membrane

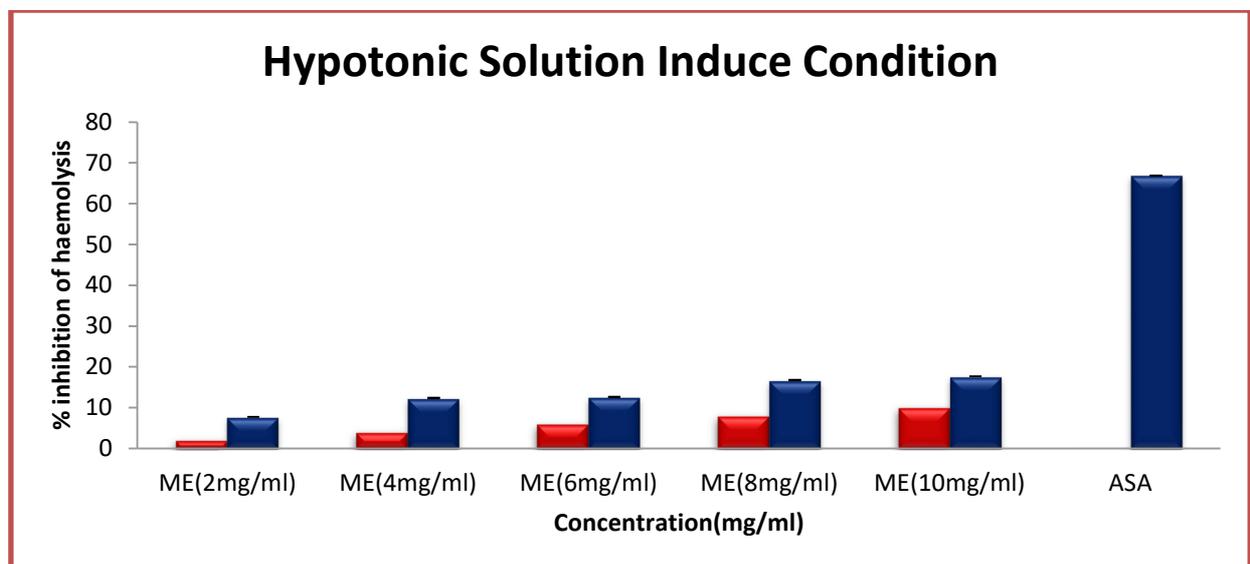
Treatment	Concentration	Optical density of samples in hypotonic solution (Mean $\pm$ SD)	% inhibition of haemolysis (Mean $\pm$ SD)
Control	---	3.768 $\pm$ 0.0514	—
ME	2 mg/ml	3.476 $\pm$ 0.0524	7.74 $\pm$ 0.0137**
ME	4 mg/ml	3.302 $\pm$ 0.0313	12.36 $\pm$ 0.0481
ME	6 mg/ml	3.291 $\pm$ 0.0402	12.65 $\pm$ 0.0289*
ME	8 mg/ml	3.137 $\pm$ 0.0317*	16.74 $\pm$ 0.0793
ME	10 mg/ml	3.102 $\pm$ 0.0433	17.67 $\pm$ 0.0102**
Acetyl salicylic acid	0.10 mg/ml	1.249 $\pm$ 0.0094***	66.85 $\pm$ 0.0265

Level of Significance \*\*\* =  $P < 0.001$ ; \*\* =  $P < 0.01$ ; \* =  $P < 0.05$

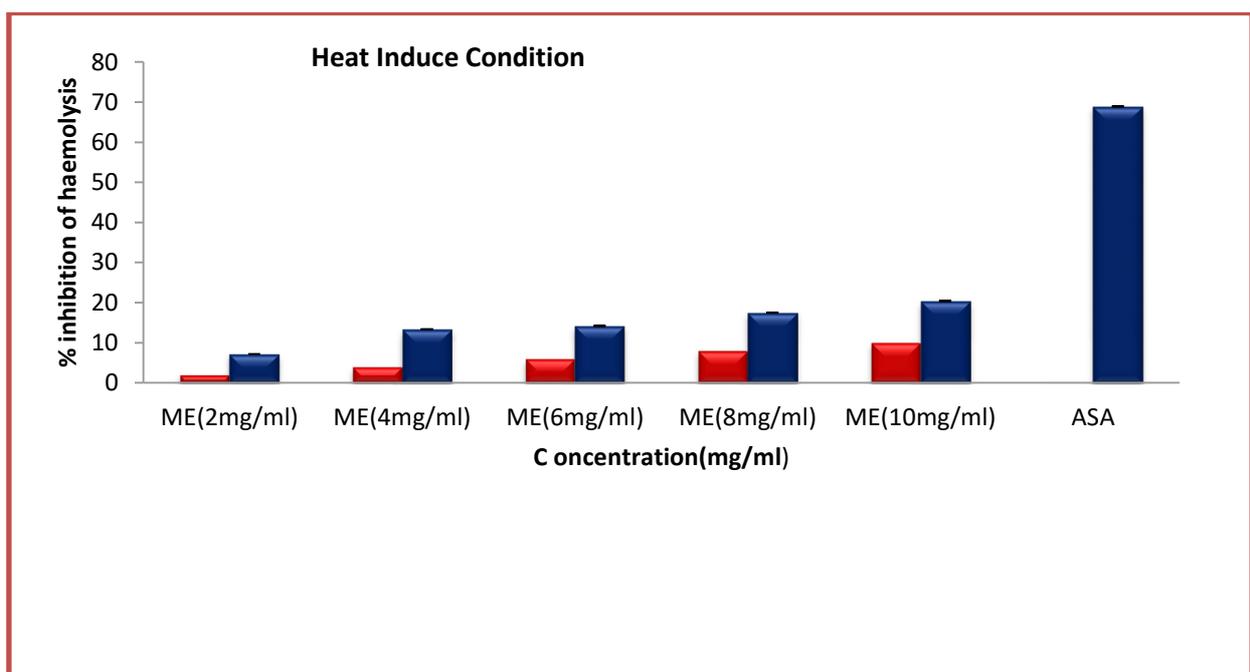
**Table 3:** Effect of various concentrated extract of leaves of *Excoecaria agallocha* on heat induced hemolysis of erythrocyte membrane

Treatment	Concentration	OD of sample $\pm$ SD		% inhibition of Haemolysis
		Heated Solution	Unheated Solution	
Control	---	1.0528 $\pm$ 0.063*		
ME	2 mg/ml	0.873 $\pm$ 0.179	0.863 $\pm$ 0.047	5.26 $\pm$ .037*
ME	4 mg/ml	0.819 $\pm$ 0.059	0.788 $\pm$ 0.216**	11.71 $\pm$ .018
ME	6 mg/ml	0.791 $\pm$ 0.131**	0.745 $\pm$ 0.062	14.94 $\pm$ .053**
ME	8 mg/ml	0.659 $\pm$ 0.085	0.585 $\pm$ 0.164*	15.81 $\pm$ .080
ME	10 mg/ml	0.513 $\pm$ 0.072	0.387 $\pm$ 0.075**	18.92 $\pm$ .086
Acetyl Salicylic Acid	0.10mg/ml	0.797 $\pm$ 0.026	0.316 $\pm$ 0.0014**	65.28 $\pm$ 1.63*

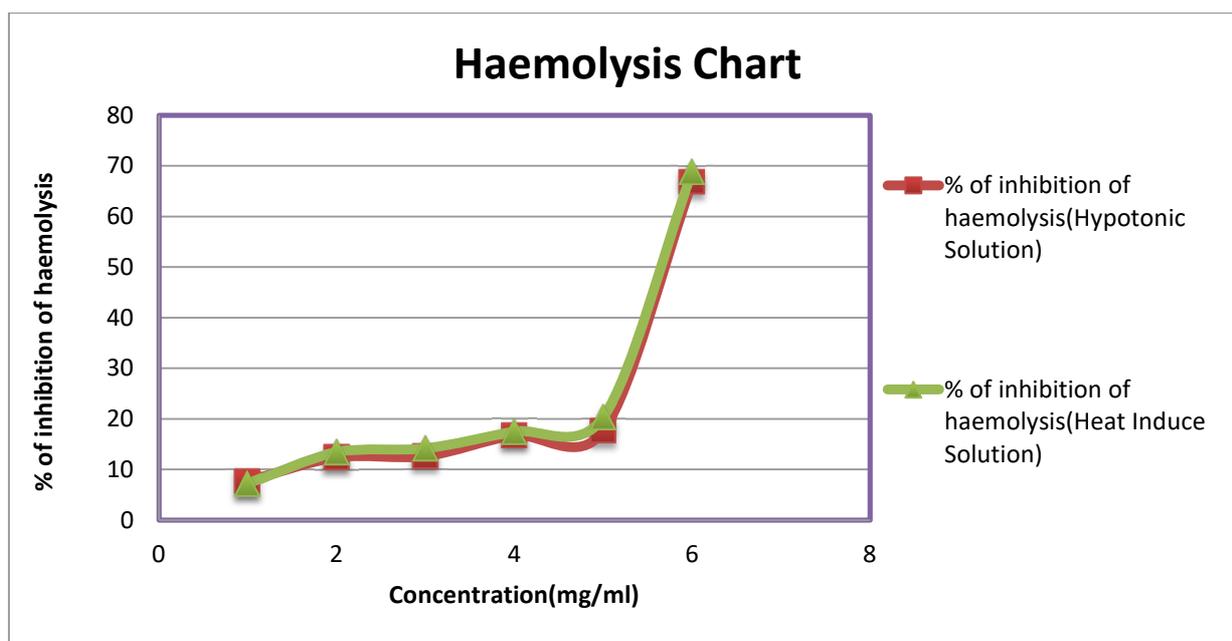
Level of Significance \*\*\* = P < 0.001; \*\* = P < 0.01; \* = P < 0.05



**Figure 1:** % of inhibition of haemolysis by crude methanolic extract on hypotonic solution-induced condition. Here, ME= Methanolic extract of *E.agallocha*, ASA= Acetyl Salicylic Acid



**Figure 2:** % of inhibition of hemolysis by crude methanolic extract on heat induce solution condition.



**Figure 3:** Graphical comparison of two results indicating % of inhibition of hemolysis by methanolic extract of *E. agallocha* at two different condition (hypotonic solution and heat induce solution)

## Conclusion

From the above experiments we could terminated that the crude methanolic extracts of *E. agallocha* (leaves) showed moderate in-vitro anti-inflammatory activities at higher concentration. We also confreres that, it also revealed moderate antimicrobial activities against gram negative (-ve) bacteria but were resistant against gram positive (+ve) bacteria.

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**Conflict of interest:** None declared.

## List of the Abbreviation's:

RBC=Red Blood Cell  
NaCl= Sodium Chloride  
EDTA= Ethylene Diamine Tetra Acetic acid  
UV= Ultra Violet  
OD= Optical Density  
ANOVA= Analysis of Variance  
SD= Standard Deviation  
WHO= World Health Organization  
ME= Methanolic Extract  
ASA= Acetyl Salicylic Acid  
NSAID= Nonsteroidal Anti-inflammatory Drug

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