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# Evaluation of *Ex-vivo* Cardioprotective and Anti-inflammatory Investigation of Bangladeshi Plants Extract

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author MKM designed the current project, performed the experiments; author Anaytulla carried out the experimental process and participated in data collection and in the sequence alignment; author PA participated in experimental process, wrote the manuscript, responsible for data interpretation and statistical analysis. Authors MMR, TKM, MMH and AKA conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final version of the manuscript.*

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## ABSTRACT

**Background:** The phytochemical screening of methanolic extract of *Dalbergia stipulacea* and *Hymendictyon excelsum* has indicated the presence of steroid, flavonoid and glycoside like anti-oxidative and cardioprotective compounds.
**Methods:** Since these compounds are of pharmacological interest, we got curious to check *D. stipulacea* and *H. excelsum* for their possible anti-inflammatory activity by HRBC (Human Red Blood Cell) membrane stabilization method, anti-arthritic activity by the inhibition of protein

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denaturation method and anti-thrombotic activity.
**Results:** The methanolic extracts of these plants exhibited notable anti-inflammatory activity and remarkable anti-arthritic, anti-thrombotic action. The maximum membrane stabilization of *D. stipulacea and H. excelsum* was found to be 72.33±2.52% and 69.33±2.52% at a dose of 1000 µg and that of protein denaturation was found to be 73.50±1.32% and 70.17±3.01% at the same dose, correspondingly. On the other hand when compared to Streptokinase for their anti-thrombotic activity, it was found that they were close to standard with p<0.0001 (from paired t-test), *D. stipulacea* and *H. excelsum* results 46.79±2.43% and 39.01±2.24%, respectively.
**Conclusion:** Therefore, our studies support the isolation and the use of active constituents from *D. stipulacea and H. excelsum* in treating inflammations and rheumatism as well as we should check for reason of its thrombolytic activity.

*Keywords: Anti-inflammatory; anti-thrombotic; Dalbergia stipulacea; Hymendictyon excelsum; protein denaturation.*

## 1. INTRODUCTION

There is a large source of anti-oxidants present in nature, use of them in traditional medicine is wide spread and serving from many diseases like inflammatory, digestive, psychotic, neuro-degenaration, jaundice etc. Drugs that are using in traditional medicine have no known mechanism of action and also crude, but if they are researched with recent method, we may got leads of novel drugs with good scavenging power. To know the mechanism of inflammation we need to know its mediator. The mechanism of inflammation is related to release of Reactive Oxygen species from activated neutrophil and macrophages. This over production leads to tissue injury by damaging the macromolecule and lipid peroxidation of membranes. In addition, ROS proliferate inflammation by stimulating the release of the cytokines such as interleukine- I, tumor necrosis factor-α (TNF-α), and interferon-γ, which stimulate recruitment of additional neutrophil and macrophages. Thus free radicals are important mediators that initiate or protract inflammatory processes, their neutralization by antioxidants and radical scavengers can ease inflammation. Flavonoids are one of most common anti-oxidative molecule. The beneficial effects of this molecule include anti-inflammatory, anti-thrombic, anti-vasodilator, anti-oxidant and anti-tumor property [1-4]. Most clinically important medicine belongs to steroidal or non-steroidal anti-inflammatory chemical therapeutics for treatment of inflammation related diseases. We have a bunch of steroidal and non-steroidal agents that are serving well, but these drugs have various and severe adverse effects, that's why we are looking for potent drugs from natural source that will replace present synthetic molecule and ensure patient safety profile.

Thrombus formation in circulatory system is due to failure of natural anticoagulant (protein C or Protein S) deficiency. This incident causes the vascular blockage, in a consequence can cause myocardial or cerebral infarction, leads to death [5]. Some agents include plasminogen activator (t-PA) that can be used to treat this kind of disease, in South Asian region we prefer Streptokinase (SK) as anti-thrombi agent, due to its affordability than other thrombolytic drugs [6,7]. But it has high risk of hemorrhage [8], as well as severe anaphylaxis and low specificity also reported. There is also restriction in treatment with SK in case of some given patient [9]. Although these drugs have some critical lacking, yet we have no other choice, but investigation is on-going to develop recombinant variants of these agents [10-14]. Human being from the very beginning trust herbal source for the treatment of different diseases, because they are harmonic to our body [15]. For this reason considerable efforts have been given by scientists to discover and develop new leads from plant and animal sources, special concern given towards anti-platelet [16,17], anti-coagulant [18,19], anti-thrombotic [20] etc.

The aim of our present study is to screen out phytochemical identity and to find out the anti-thrombotic and anti-inflammatory effect of *D. stipulacea* and *H. excelsum.*

## 2. METHODS AND MATERIALS

### 2.1 Plant Preparation

Those studied plant was collected from Chittagong Hill Tracts (Bandarban) region of Bangladesh and was identified by expert taxonomist Dr. Shkeikh Bakhtiar Uddin, Department of Botany, University of Chittagong.

As we studied with their leaves, so after collection leaves were dried at room temperature to free from moisture. After that they were mashed and dissolved in sufficient amount of methanol (95%). The extract was obtained following cold extraction procedure.

## 2.2 Phytochemical Evaluation

Methanolic extract of *D. stipulacea* & *H. excrlsum* was studied for their phytochemical compounds such as alkaloid, steroid, tannin, glycoside and flavonoid (Table 2) [21].

## 2.3 Blood Sampling

4 ml blood sample was collected from median cubital vein of volunteers (n = 30). Volunteers, non-smoker, no history of anti-coagulant, anti-inflammatory or any other medication, was selected for our project and ethical approval for sampling was given by IIUC ethical committee. 500 µl of blood sample was drawn to each previously weighted micro tube for each separate sample.

### 2.3.1 Extract preparation for thrombolytic study

10 mg of MEDS and MEHE was dissolved in 10 ml distilled water at each separate test tube and shaken robustly for proper mixing. The mixture was kept overnight at room temperature for complete mixing and insoluble and sedimented part of solution was removed by filtration.

### 2.3.2 Thrombolysis

Experiments for clot lysis were carried as reported earlier [22]. In brief, 4 ml previously drawn venous blood from healthy volunteers was distributed in eight different pre weighed sterile micro centrifuge tube (0.5 ml/tube) and incubated at 37°C for 45 minutes. As clot formed, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To each tube containing pre-weighed clot, 100 µl of aqueous extract of herbs (*D. stipulacea* and *H. excelsum*) was added separately. As a positive control, 100 µl of SK and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After this time, fluid released was removed and tubes were again weighed to observe the difference in weight to measure clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The experiment was repeated 3 times with the blood samples of 30 volunteers.

### 2.3.3 Anti-inflammatory activity by Protein (BSA) denaturation

The mixture was consisting of test extract at different concentration and 1% aqueous solution of bovine albumin fraction. Tiny amount 1N HCl was added to the mixture to adjust the pH of the reaction. The samples were incubated at 37ºC for 20 min, in addition 20 min more heated at 57ºC. The solution was cooled and turbidity was measured spectrophotometrically at 660 nm for evaluation of percent of inhibition. The calculation was done by following equation,

$$\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$ [23]

### 2.3.4 Anti-inflammatory activity by membrane stability method

The lysosomal enzyme released during inflammation produces a variety of disorders. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane. Since HRBC (Human Red Blood Cell) membrane is similar to lysosomal membrane, the study was undertaken to check the stability of HRBC membrane by the extracts to predict the anti-inflammatory activity In-vitro. The various extracts at the concentration of 125, 250, 500 and 1000 µg/ml were incubated separately with HRBC solution [24].

Blood was collected from healthy volunteers. The collected blood was mixed with equal volume of Alsever solution (Dextrose 2%, Sodium citrate 0.8%, Citric acid 0.05%, Sodium chloride 0.42% and Distilled water 100 ml) and centrifuged with is osaline. To 1 ml of HRBC suspension equal volume of test drug in three different concentrations was added. All the assay mixtures were incubated at 37ºC for 30 minutes and centrifuged. The haemoglobin content in the supernatant solution was estimated by using spectrophotometer at 560 nm [25]. The percentage of haemolysis was calculated then, by the formula as given below:-

$$\text{Percent of hemolysis} = \frac{\text{OD of test}}{\text{OD of control}} \text{ X } 100$$

The percentage of protection can be hence calculated from the equation as given below,

$$\text{Percentage of protection} = 100 - \frac{\text{OD of test}}{\text{OD of control}} \text{ X } 100$$

Here 'OD of test'= optical density or the test sample's absorbance and 'OD of control'= optical density or absorbance of the negative control.

Here, the negative control used was Alsever's solution with blood in it and it contained no Aspirin or methanolic extract of the plant material in it.

### 2.4 Statistical Analysis

The significance limit between two extracts and standards were estimated with the help of paired t-test using SPSS-17 software. Results are expressed as mean±SD.

## 3. RESULTS AND DISCUSSION

### 3.1 Thrombolytic Test

SK (100 µl, 30,000 IU) as positive control showed 67.65±2.21% of clot lysis. Water, as negative control, showed only 9.72±1.06% of clot lysis. The extracts, *D. stipulacea* and *H. exelsum* (46.79±2.43% & 39.01±2.24%, respectively) were quite good enough when compared with both positive and negative control, Fig. 1 & Table 1.

It's obvious since ancient era that man trusts in herbal preparations for cure by any means, with some modifications, yet now we depend on this section for different critical treatment. About 30% of pharmaceuticals are prepared from plant source all over the world [26]. A significant number of researches have been conducted on anti-thrombotic effect and also evidence of foods that can prevent coronary diseases and stroke [27-30].

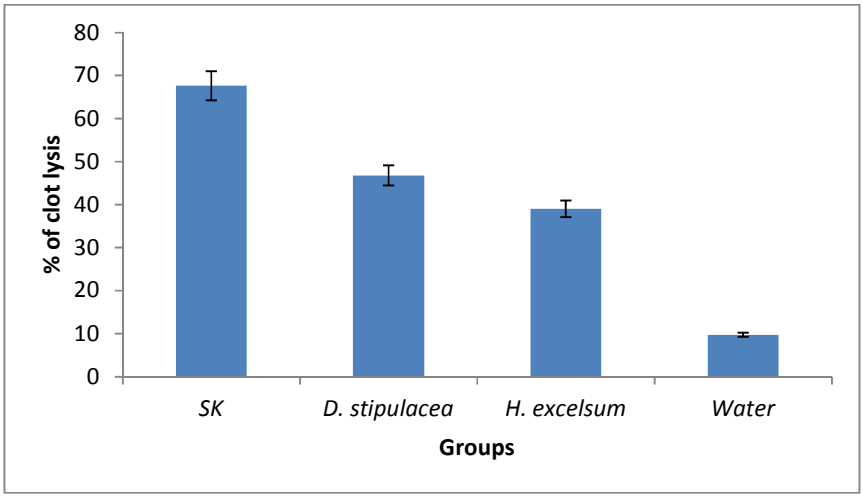


**Fig. 1. Clot lysis by SK, water and two different herb extracts, water has negligible effect in comparison with other preparations**

**Table 1. Effect of two studied plant extracts at 1mg/ml dose**

| Drugs/extracts | % of clot lysis (mean±SD) | *p*-value from paired t-test (compared with water) |
|---|---|---|
| SK | 67.65±2.21 | <0.0001* |
| *D. stipulacea* | 46.79±2.43 | <0.0001** |
| *H. excelsum* | 39.01±2.24 | <0.0001*** |
| Water | 9.72±1.06 | |

*p-value less than 0.0001 are statistically considered as significant*

Major problem with anti-thrombotic drugs are its side-effects, most common is bleeding and embolism, sometime patient dies due to this complication [31-35]. In context with the above study and result it would be interesting to investigate for responsible chemicals and their mechanism for clot lysis. Our studied extracts must go through its toxicity test first and both of these plants have something interesting that can be used as future lead for thrombolytic agent.

**Table 2. Phytochemical analysis of methanolic extract of DS and HE**

| Compounds | *D. stipulacea* | *H. excelsum* |
|---|---|---|
| Steroid | ++ | + |
| Phenolic content | ++ | ++ |
| Tannin | + | + |
| Glycoside | ++ | ++ |
| Carbohydrate | ++ | ++ |
| Alkaloid | + | ++ |
| Flavonoid | ++ | + |

### 3.2 Anti-inflammatory Activity by Protein (Albumin) Denaturation

Protein denaturation is a well known and established cause of inflammation. Drugs like salicylic acid, phenylbutazone (anti-inflammatory drugs) have shown dose dependent response to thermally induced protein denaturation. As a part of our investigation in a search of anti-inflammatory activity, potency of MEDS & MEHE in inhibition of protein denaturation, it was found effective at different concentration (Fig. 2). They results 73.50±1.32% and 70.17±3.01% of inhibition at maximum concentration, correspondingly. Aspirin, an established anti-inflammatory drug showed 84.77±1.42% of inhibition, it was close to our studied plant part.

### 3.3 Anti-inflammatory Activity by Membrane Stabilizing

Membrane stabilizing activity was studied to further ensure the anti-inflammatory property of MEDS & MEHE. The extracts are very much potent in inhibiting heat induced hemolysis. The actual mechanism of this extract may be due to inhibit the release of lysosomal content of neutrophils at the site of inflammation. These lysosomal constituents are involved in bactericidal action by containing bactericidal enzyme and protease, these when release extracellularly cause damage to tissue and inflammation [36]. Test extracts inhibited hemolysis at different degree according to the concentration (Table 3 & Fig. 3). The percent of inhibition found was 72.33±2.52% and 69.33±2.52%, in that order, at 1000 µg concentration where as the compared standard (Aspirin) results 88.33±1.53%.

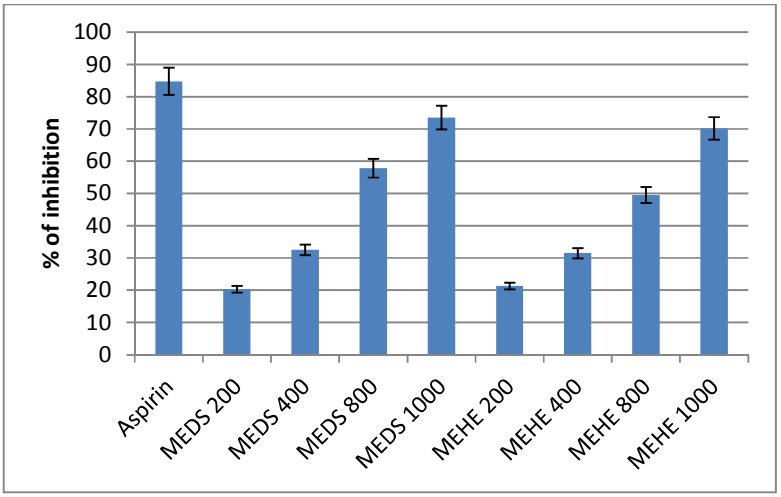


**Fig. 2. Protein denaturation inhibited by aspirin and two studied plant extract at different doses**

**Table 3. Effect of MEDS and MEHE in inhibition of inflammation**

| Group | % of inhibition (protein denaturation) | | | | % of inhibition (membrane stability) | | | |
|---|---|---|---|---|---|---|---|---|
| | 200 µg | 400 µg | 800 µg | 1000 µg | 200 µg | 400 µg | 800 µg | 1000 µg |
| Aspirin | 84.77±1.42 | …. | …. | …. | 88.33±1.53 | …. | ….. | ….. |
| MEDS | 20.33±2.08 | 32.50±2.29 | 57.83±2.02 | 73.50±1.32 | 26.12±1.70 | 33.65±2.17 | 56.67±1.53 | 73.33±2.52 |
| MEHE | 21.33±2.08 | 31.50±1.50 | 49.53±2.50 | 70.17±3.01 | 22.41±2.73 | 30.00±2.32 | 50.73±2.56 | 69.33±2.52 |

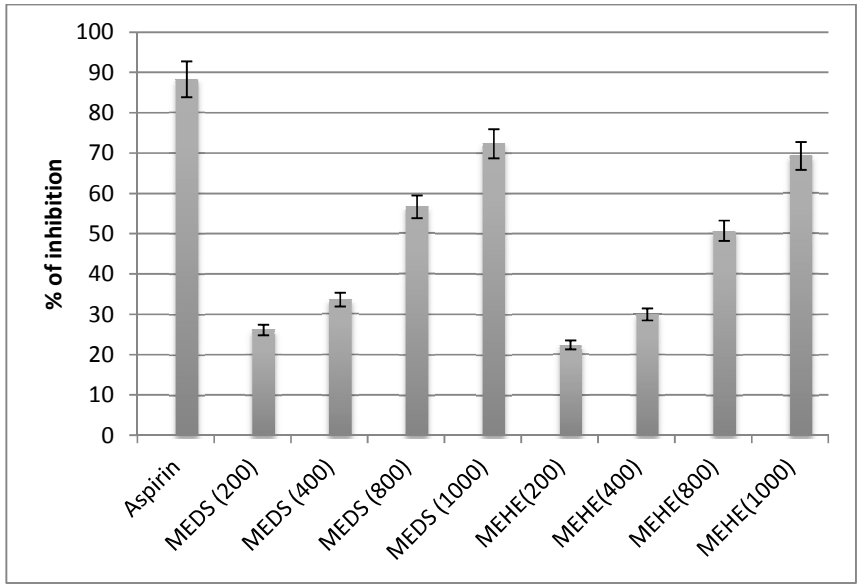


**Fig. 3. Percent of membrane stability given by aspirin and two studied plant at different dose in the anti-inflammatory study by HRBC membrane stabilizing**

## 4. CONCLUSION

This study revealed that MEDS and MEHE have effective anti-inflammatory potency as well as effective in clot lysis and they also contain some useful anti-oxidant compound that's important for human health. The actual mechanism of action for their anti-inflammatory and antithrombotic action is yet not confirmed but it is possible that the extracts produced anti-inflammatory effect due to surface area/volume ration of cells, which could be brought about by an expansion of membrane or the shrinkage of the cells and an interaction with membrane proteins [37]. Further investigation is required on these extract to confirm the mechanism of action and there is possibility to find something new that could be a lead compound for future cardioprotective as well as anti-inflammatory drug.

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## ETHICAL CONSENT

As we have used human blood sample, we needed to be approved by authorized person, we took our approval from our institutional ethical authority to collect blood and use in our designed protocol. All authors read this article and approved this article and they also declared nothing done in this research that violates any ethical statement.

## COMPETING INTEREST

The authors declare that they have no conflict interest.

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