INTRODUCTION

Thrombotic disorders increases the risk of cardiovascular diseases and represents a major health problem in worldwide. Those anticoagulants which block the clotting factors or coagulation factors and in turn inhibit the clot formation are extensively used in the treatment of thrombotic disorders. The thrombotic disorders includes arterial thrombosis, atrial fibrillation, myocardial infarction, unstable angina, deep vein thrombosis, pulmonary embolism and cerebral stroke. Thus, anticoagulants play an effective role in the prevention and treatment of thrombotic disorders. Warfarin, heparin, aspirin, vitamin K-antagonists and their derivatives are some of the anticoagulants used. Unfortunately, these anticoagulants causes several deleterious life-threatening side effects like lack of reversibility, drug – drug interaction, internal bleeding, birth defects and miscarriage. Among the anticoagulants, heparin plays a pivotal role in the treatment. The main complication related with heparin therapy is that it causes internal bleeding and also some disadvantages like heparin – induced thrombocytopenia, poor bioavailability and contamination by animal –derived pathogens since its origin is from animal. Such that, there is still an urge for new anticoagulants that are effective and safe when used in cardiovascular diseases. Therefore, researchers are at the challenging task to identify a novel anticoagulant agents with more therapeutic effects and without side effects. Hence, at present, lavish focus is being carried out on plant material to acts as a potent source for this kind of research. Since there is a compelling scientific evidences that consumption of dietary anticoagulants with anti-coagulant properties reduces the risks of thrombotic disorders. Thus, in the current study, the anticoagulant activity of Acalypha indica leaf extracts were investigated by invitro method and further the results are furnished.

MATERIALS AND METHODS

Collection, preparation and extraction of plant materials

The leaf of Acalypha indica was collected, identified and a voucher specimen was deposited for further reference. The leaves were separated and washed with distilled water to remove dust particles. Then shade dried for 20 – 30 days at environmental temperature. The grounded fine powders were extracted with different solvents like petroleum ether, chloroform, ethyl acetate, n-butanol, ethanol and aqueous. The different crude extracts were stored at 4°C and used for further studies.

Phytochemical Analysis

Test for alkaloids

Mayer’s Test

ABSTRACT

The current study focuses on anticoagulant activity of leaf extract of Acalypha indica (A.indica) and to identify the active constituents present and responsible for the anti-coagulation activity. On sequential extraction of plant materials with petroleum ether, chloroform, ethyl acetate, n-butanol, ethanol and aqueous, crude extracts were obtained and screened for anti-coagulant activity. Anticoagulant activity of six different leaf extracts of A.indica was tested using prothrombin time (PT). In vitro anticoagulation assays were performed with different concentrations of the leaf extract on citrated plasma obtained from healthy volunteer donors. The different concentrations of crude extract tested in the present study were 0.062, 0.125, 0.25 and 0.5 gm/ml. The anti-coagulant activity of six extracts exhibited a concentration dependent activity. Among the six tested extracts, petroleum ether exhibited a highest activity by increased prothrombin time of 60min and 5 sec at 0.5gm/ml compared to positive and negative control. This is followed by aqueous, n-butanol, chloroform and ethyl acetate extract. It was also noted that ethanol extract showed no prolonged prothrombin time and it was within the normal level as compared to the control. Phytochemical screening of different extracts revealed the presence of steroids, terpenoids, flavonoids and alkaloids as secondary metabolites. From the results, for the first time it was highlighted that the A.indica leaf extracts affects the intrinsic pathway of coagulation cascade and thus prolongs the clotting time, hence this plant can be used in the management of blood clotting diseases.

Keywords: Thrombotic disorders, clotting factors, prothrombin time, anti – coagulant activity, phytochemicals.
1ml of extract, few drops of mayer’s reagent was added and precipitate was observed. A white creamy or yellow precipitate indicates the positive result to alkaloids.

Wagner’s Test
To 1ml of extract few drops of wagner’s reagent was added. Formation of reddish brown precipitate confirmed the test as positive.

Hager’s Test
To 1ml of extract 1ml of Hager’s reagent was added and observed. The positive report is indicated by a formation of prominent yellow precipitate.

Test for flavanoids
Shinoda test
1ml of extract was dissolved in 5ml of 95% alcohol and few fragments of mg turning and con Hcl (drop Wise) was added. Pink or crimson red colour was noted down to confirm the presence of flavanoids.

Alkaline Reagent Test
1ml of extract, few drops of sodium hydroxide solution was added and an intense yellow colour was formed which turned to colour test or decolorization on addition of few drops of dilute acid. This colour change indicates the presences of flavanoids.

Test for saponin
Foam of froth test
1ml of extract was diluted with 20ml of distilled water and the suspension was shaken in a graduated cylinder for 15minutes. A 1cm height foam or froth stable for 10min indicates the presence of saponins.

Haemolytic test
1ml of extract was added to 1drop of blood and placed on a glass slide and observed for haemolytic zone formation.

Test for tannins and phenolic compounds
Ferric chloride test
1ml of extract was dissolved in 5ml of distilled water. To this few drops of neutral 5% ferric chloride solution was added, heated. A change in colour from blue, green, violet colour signified the phenolic compound presence.

Lead Acetate Test
1ml of extract and 3ml of 10% lead acetate solution was added and noted. Bulky white or yellow colour precipitate confirmed the presence of both phenolic and tannin compounds.

Test for steroids and terpenoids
Salkowski reaction
1ml of extract, 2ml of chloroform and 2ml of concentrated sulphuric acid was added and shaken well. Then allow the tubes of stand for 5min. A red colouration in the lower layer indicate the presence of steroids and yellow colour or golden yellow in the upper layer confirmed the presence of triterpenoids.

Libermann-Burchard’s Test
2ml of extract, few ml of chlorofom and 1-2ml of acetic anhydride was added boiled and cooled. This is followed the addition of con H₂SO₄ along the sides of the test tube. A brown ring formed at the junction of two layers with a green colour in the upper layer showed the presence of steroids and deep red colour in lower layer indicated the triterpenoids presence.

Test for glycosides

Borntreger’s test
2ml of extract, 3ml of chloroform was added, mixed and shaken well. Add 10% ammonia solution and shake well. A rose pink to red colour change in ammonial layer indicated the presence of anthraquinone glycosides.

Legals test
2ml of the extracts were treated with sodium nitroprusside in pyridines and sodium hydroxide solution. A change in colour from pink to blood red indicates the presence of cardiac glycosides.

In vitro anticoagulant activity
Determination of prothrombin time
Collection of blood sample
The Sample of blood are obtained from the healthy volunteers using disposable polypropylene syringe withdrawn from superior cubital vein of right arm of each person and dispensed into the polypropylene container containing 3.8% tri sodium citrate to prevent the process of clotting (9 parts of blood to 1 parts of tri sodium citrate). Now the blood sample was immediately centrifuged at 3000rpm for 15 minutes to separate blood cell and plasma. The plasma was separated and used for determination of Prothrombin time test. The freshly prepared plasma was stored at 4°C until its use.

Stock solution
The crude extracts (Petroleum ether, Chloroform, n-Butanol, Ethyl acetate, Ethanol and Aqueous) were dissolved in DMSO solution to obtain the final concentration of 0.5gm / ml, 0.25gm / ml, 0.125 gm / ml and 0.062 gm / ml.

Collection of blood and plasma re-calification
To a clean test tube 0.2ml of plasma, 0.1ml of petroleum ether extracts, chloroform, extracts, n-Butanol extracts, ethyl acetate extracts, ethanol extracts and aqueous extracts of A.indica leaves extract at different concentration was added. 0.3ml of 25mm CaCl₂ were added to the above mixture. And all the test tubes were shaken and mixed well and incubated for 1 minute at 37°C in a water bath.

For Negative Control tube, 0.2ml of plasma, 0.1ml of 0.9% Saline water and 0.3ml of 25mm CaCl₂ were taken and incubated at 37°C in a water bath. The plant extract was replaced by saline. 0.01ml of 50mg/ml of EDTA and 0.2ml of plasma was used as positive control tube. 0.3 of CaCl₂ was also put into the test tube and placed immediately in the water bath pre warmed at 37°C. The clotting time was measured by tilting the tubes at an angle of 45°C every 5second to recognize the presence or absence of coagulum 21 until a clot was formed. A stopwatch was started to record the coagulation time in separate concentration of the extracts, control in seconds and stopwatch was stopped as soon as the clot formation began. The activity is expressed in term of clotting time in seconds. Each test was performed three times to obtain three determinants in coagulation time exerted with different concentration of extracts.

RESULTS
Phytochemical
Chloroform crude extract showed the presence of steroids and terpenoids while alkalioid, Flavonoids, Saponins, Tannin, Phenolins compound and Glycosides are absent. Additionally, ethyl acetate revealed the presence of tannin and phenolic compounds. Alkaloids, Flavonoids, Saponins, Steroids, Terpenoids and Glycosides are absent in the crude extract.

Phytochemical screening of n-Butanol crude extract revealed the presence of Flavonoids, Tannin and Phenolic compound except steroids, terpenoids, glycosides, Alkaloids and saponins.

Ethanol crude extract of *A.indica* leaves showed the positive result for alkalioids, Flavonoids, Steroids and Terpenoids where as Saponin, Tannin, Phenolic compounds and glycosides showed negative result.

To the best of our knowledge there are no phytochemical constituents present in aqueous as solvent for the extraction of *A.indica*. Steroids and terpenoids were the major phytochemical compound detected in petroleum ether extract of *A. indica* (Table 1).

**Anticoagulant**

Petroleum ether, chloroform, ethyl acetate, n-Butanol, ethanol, aqueous extracts of leaves of *A.indica* were tested for blood coagulation effects. Petroleum ether extract of *A.indica* exhibited greater prolonged clotting time 60min : 5sec, 30min : 15sec, 10min : 3sec, 3min : 2sec, at 0.5gm, 0.25gm, 0.125gm and 0.062gm/ml concentration respectively. As compared to four concentration 0.062gm exhibited lower activity whereas 0.125gm, 0.25gm and 0.5gm/ml showed greater promising anticoagulant activity than the EDTA.

Aqueous extract extended the clotting time 23min : 6sec, 15min : 50sec, 9 min : 23sec and 5min : 3sec at 0.5gm, 0.25gm, 0.125gm and 0.062gm/ml concentration respectively. More over concentration 0.062gm/ml demonstrated lower activity than other higher concentration tested viz., 0.125gm, 0.25gm and 0.5gm/ml. The result showed that n-Butanol extract exhibited better activity by extending the clotting time 2min : 19sec, 4min: 3sec, 5min: 40sec and 11 min: 40sec at 0.062gm, 0.125gm, 0.25gm and 0.5gm/ml respectively. 0.5gm/ml exhibited greater potency with prolonged clotting time of 11min : 40sec while 0.25gm, 0.125gm, and 0.062gm/ml showed poor activity.

Anticoagulant activity of chloroform extract of *A.indica* was carried out by invitro method. From the result 0.5gm/ml and 0.05gm/ml concentration delayed the coagulation time by 10min : 5sec, and 8min : 17sec, respectively whereas 0.125gm/ml and 0.062gm/ml showed moderate activity of 6min : 23sec and 4min : 33sec respectively. From the presence study chloroform extract have remarkable anti-coagulant activity than the control. The ethyl acetate extract of *A.indica* leaves slightly lengthened prothrombin time by 8min : 30sec at 0.5gm/ml concentration. However 0.25, 0.125g and 0.062gm/ml concentration presented a weaker anticoagulant activity of 7min : 15sec, 5min : 47sec and 3min : 33sec.

The ethanolic extract did not show any prolongation of prothrombin time and it is identical to that of the control with a prothrombin time of 1min : 25sec (0.062gm/ml), 3min : 10sec (0.25gm /ml), 7min : 2sec (0.5gm/ml).

**DISCUSSION**

Harbone (1984) proved the presence of terpenoid and steroids in petroleum ether extracts as similar to our result. Some other investigations also showed the presence of saponin, alkalioid, glycosides and polyphenols in aqueous extract of *S.argel*. On the other hand *A.indica* aqueous extract showed the presence of alkalioids, flavonoids, steroids and terpenoids as the major components.

Natural anticoagulant agents that inhibit coagulation process are of greater potential interest for the prevention of atherosclerosis and coronary artery disease. This study demonstrated that *A.indica* extracts in different concentration 0.5gm, 0.25gm, 0.125gm and 0.062gm inhibits clot and there by increases prothrombin time. The result also shows that as concentration of *A.indica* extract increases it strongly inhibits the coagulation process and increases prothrombin time. This may be attributed to the presence of phyto compound that have been noted in the leaves extracts. These results confirm the observations that have previously noticed in Fensugreek seeds (Trigonons foenum-graecum) 23. *Pentaclethra macrophylla* seed extract exhibited anticoagulant activity at concentration of 0.2g/2ml (25min : 20sec) and 0.5g/2ml (105sec) while coagulation was not achieved in 0.1g/2ml. This indicates that *Pentaclethra macrophylla* extract at higher concentration exhibited a good anticoagulation activity as similar to our results obtained 24. Similar to our results leaves extract of New *Boudia Lavis* produced a most profound bleeding effect at a high dose of 100-200mg/kg 25. Anticoagulant activity of aqueous, methanol, acetone

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**Table 1: Phytochemical analysis of crude extracts of *A.indica* dry leaves.**

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Parts</th>
<th>Alkaloids</th>
<th>Flavanoids</th>
<th>Saponin</th>
<th>Tannin and phenolic compound</th>
<th>Steroids and terpenoids</th>
<th>Glycosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>Dry leaves</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Dry leaves</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>Dry leaves</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>n-butanol</td>
<td>Dry leaves</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Dry leaves</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Aqueous</td>
<td>Dry leaves</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = Positive, - = Negative
and ethyl acetate leaves extracts of *Murraya Koenigii* and *Bauhinia Tomentosa* were tested in normal human plasma. From the result it was showed that among the concentration tested 0.2mg/ml, 0.4mg/ml, 1mg/ml, 2mg/ml, 3mg/ml and 4mg/ml, the higher concentration 4mg/ml produced a potent anticoagulant activity. The present investigation agrees with the above reported finding 26.

In the presence study the aqueous leaves extracts of *A.indica* exerted greater anticoagulant activity as similar to the result reported by aqueous extract of the leaves of *Enicostemma Littorale* (whole plant), *Acheranthus aspera* (leaves), *Abbution indicum* (leaves) and *Tridax procumbens* (whole plants) . Hydro alcoholic extract of four Medicinal plants *Annona Senegalensis* (leaves) *New bouldia Laevis* (leaves), *Cassytha filiformis* (aerial part), *Cissampelos mucronata* (aerial part) revealed the presence of coagulant properties 27.

**CONCLUSION**

In conclusion, this study for the first time attempted to see the anti-coagulant properties of different extracts of leaves of *A.indica*. From the findings it was found that this plant may act as an promising alternative in the treatment of thrombotic disorders.

**REFERENCES**


### Table 2: Cloting time for different concentration of the different extract of *A.indica*.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Amount of plasma</th>
<th>Amount of EDTA</th>
<th>Amount of saline</th>
<th>Time of coagulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>0.1ml</td>
<td>0.05ml</td>
<td>0.15ml</td>
<td>-</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.062</td>
<td>0.2ml</td>
<td>0.1ml</td>
<td>1min:40sec</td>
</tr>
<tr>
<td>AIPE (gm/ml)</td>
<td>0.125</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>3min:25sec</td>
</tr>
<tr>
<td>AICF (gm/ml)</td>
<td>0.25</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>10min:3sec</td>
</tr>
<tr>
<td>AIEA (gm/ml)</td>
<td>0.062</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>6min:23sec</td>
</tr>
<tr>
<td>AInB (gm/ml)</td>
<td>0.25</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>8min:17sec</td>
</tr>
<tr>
<td>AIET (gm/ml)</td>
<td>0.062</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>10min:5sec</td>
</tr>
<tr>
<td>AIAQ (gm/ml)</td>
<td>0.125</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>3min:33sec</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>5min:47sec</td>
</tr>
<tr>
<td></td>
<td>0.062</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>2min:19sec</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>4min:3sec</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>5min:40sec</td>
</tr>
<tr>
<td></td>
<td>0.062</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>11min:40sec</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>3min:10sec</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>5min:12sec</td>
</tr>
<tr>
<td></td>
<td>0.062</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>7min:2sec</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>5min:3sec</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>9min:23sec</td>
</tr>
<tr>
<td></td>
<td>0.062</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>15min:50sec</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td>23min:6sec</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.2ml</td>
<td>0.3ml</td>
<td></td>
</tr>
</tbody>
</table>

Min-minutes, sec-seconds