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Research Article

A Preliminary Novel Findings on *Invitro* Anti-Coagulant Activity of *Acalypha Indica* Linn Leaf Extracts

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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, tannin, phenols, 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.

INTRODUCTION

Thrombotic disorders increases the risk of cardiovascular diseases and represents a major health problem in world wide. 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 disorder[1,2.] 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[3,4]. Warfarin, heparin, aspirin, vitamin K-antagonists and theirderivatives 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[5-9]. Among the anticoagulants, heparin plays a pivotal role in the treatment. The main complication related with heparin therapy is that it causes internal bleeding[10] and also some disadvantages like heparin – induced thrombocytopenia, poor bioavailability and contamination by animal –derived pathogens since its origin is from animal[11-13].Such that, there is still an urge for new anticoagulants that are effective and safe when used in cardiovascular diseases[14]. 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[15,16]. Since there is a compelling scientific evidences that consumption of dietary anticoagulants with anti-coagulant properties reduces the risks of thrombotic disorders[17,18]. Thus, in the current study, the anticoagulant activity of *Acalyphaindica* 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

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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 comfirmed 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 flavonoids

Alkaline Reagent Test

1ml of extract, few drops of sodium hydroxide solution wad 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 wad added to 1drop of blood and placed on a glass side 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 wad 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 H_2SO_4 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

Borntrager's test

2ml of extract, 3ml of chloroform was added, mixed and shaken well. Add 10% ammmonia solution and shake well. A rose pink to red colour change in ammonical 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 prothromin 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 [19,20]. 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-calcification

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 $CaCl_2$ were added to the above mixture. And all the test tubes were shaken and mixed well and incubated for 1minute 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 $CaCl_2$ 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 $CaCl_2$ 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

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

Extracts	Parts	Phytochemical test					
		Alkaloids	Flavanoids	Saponin	Tannin and phenolic compound	Steroids And terpenoids	Glycosides
Petroleum ether	Dry leaves	-	-	-	-	+	-
Chloroform	Dry leaves	-	-	-	-	+	-
Ethyl acetate	Dry leaves	-	-	-	+	-	-
n-butanol	Dry leaves	-	+	-	+	-	-
Ethanol	Dry leaves	+	+	-	-	+	-
Aqueous	Dry leaves	-	-	-	-	-	-

+ =Positive ,- =Negative

Chloroform crude extract showed the presence of steroids and terpenoids while alkaloid, Flavanoids, Saponins, Tannin, Phenolis compound and Glylosides are absent. Additionally, ethyl acetate revealed the presence of tannin and phenolic compounds. Alkaloids, Flavanoids, Saponins, Steroids, Terpenoids and Glycosides are absent in the crude extract.

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

Ethanol crude extract of *A.indica* leaves showed the positive result for alkaloids, Flavanoids, 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 acitivity 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 viz., 2min: 19sec, 4min: 3sec, 5min: 40sec and 11min: 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)[22] 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, alkaloids, glycosides and polyphenols in aqueous extract of *S.argel*. On the other hand *A.indica* aqueous extract showed the presence of alkaloids, flavanoids, 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 Fenugreek seeds (Trigonella foenumm-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 *Pentacslethra 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 *Bouldia Laevis* produced a most profound bleeding effect at a high dose of 100-200mg/kg [25]. Anticoagulant activity of aqueous, methanol, acetone

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

Experiment		Amount of plasma	Amount of plant extract	Amount of calcium	Time of coagulation
Positive control 0.05gm/ml		0.1ml	0.05ml of EDTA	0.15ml	-
Negative control		0.1ml	0.05ml of saline	0.15ml	1min:40sec
AIPE (gm/ml)	0.062	0.2ml	0.1ml	0.3ml	3min:2sec
	0.125	0.2ml	0.1ml	0.3ml	10min:3sec
	0.25	0.2ml	0.1ml	0.3ml	30min:15sec
	0.5	0.2ml	0.1ml	0.3ml	60min:5sec
AICF (gm/ml)	0.062	0.2ml	0.1ml	0.3ml	4min:33sec
	0.125	0.2ml	0.1ml	0.3ml	6min:23sec
	0.25	0.2ml	0.1ml	0.3ml	8min:17sec
	0.5	0.2ml	0.1ml	0.3ml	10min:5sec
AIEA (gm/ml)	0.062	0.2ml	0.1ml	0.3ml	3min:33sec
	0.125	0.2ml	0.1ml	0.3ml	5min:47sec
	0.5	0.2ml	0.1ml	0.3ml	7min:15sec
AInB (gm/ml)	0.062	0.2ml	0.1ml	0.3ml	2min:19sec
	0.125	0.2ml	0.1ml	0.3ml	4min:3sec
	0.25	0.2ml	0.1ml	0.3ml	5min:40sec
	0.5	0.2ml	0.1ml	0.3ml	11min:40sec
AIET (gm/ml)	0.062	0.2ml	0.1ml	0.3ml	1min:25sec
	0.125	0.2ml	0.1ml	0.3ml	3min:10sec
	0.25	0.2ml	0.1ml	0.3ml	5min:12sec
	0.5	0.2ml	0.1ml	0.3ml	7min:2sec
AIAQ (gm/ml)	0.062	0.2ml	0.1ml	0.3ml	5min:3sec
	0.125	0.2ml	0.1ml	0.3ml	9min:23sec
	0.25	0.2ml	0.1ml	0.3ml	15min:50sec
	0.5	0.2ml	0.1ml	0.3ml	23min:6sec

Min-minutes,sec-seconds

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 acitivity. 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), *Acherunthus 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

1. Leng GC, Lee AJ, Fowkes FG, Whiteman M, Dunbar J, Housley E., Ruckley CV. Incidence, natural history and cardiovascular events insymptomatic and asymptomatic peripheral arterial disease in the general population. International Journal of Epidemiology 1996; 25(6):1172-81.
2. Anand S, Shivashankar R, Ali MK, Kondal D, Binukumar B, Montez-Rath ME., *et al*. Prevalence of chronic kidney disease in two major Indian cities and projections for associated cardiovascular disease. Kidney International 2015; 88(1): 178-85.
3. Hirsh J, O'Donnell M, Weitz JI. New anticoagulants. Blood 2005;105(2): 453-63.
4. Hirsh J, O'Donnell M, Eikelboom JW. Beyond unfractionated heparin and warfarin: current and future advances. Circulation, 2007;116: 552-60.
5. Stone WM, Tonnessen BH. Money SR. The new anticoagulants. Perspectives in Vascular Surgery and Endovascular Therapy 2007;19: 332-335.
6. Bounameaux H. The novel anticoagulants: entering a new era. Swiss Medical Weekly 2009; 139(5-6): 60-64.
7. Rosendaal FR, Cannegieter SC, Van der Meer J, Briët E. A method to determine the optimal intensity of oralanticoagulant therapy. Thrombosis and Haemostasis 1993; 69:236-9.
8. Hurlen M, Abdelnoor M, Smith P, Erikssen J, Arnesen H.Warfarin, aspirin, or both after myocardial infarction. The New England Journal of Medicine 2002; 347:969-74.
9. Antman EM, Van de Werf F. Pharmacoinvasive therapy: thefuture of treatment for ST-elevation myocardial infarction.Circulation 2004; 109:2480-6.

10. Levine MN, Hirsh J, Kelton JG. Heparin-induced bleeding. In: Lane DA, Lindahl U,editors. Haemostasis chemical and biological properties, clinical applications. London: Edward Arnold, 1988; 455–73.
11. Hirsh J. Drug therapy: Heparin. The New England Journal of Medicine 1991; 324:1565–74.
12. Beijering RJ, Tencate H, Tencate J.Clinical applications of new antithromboticagents. Annals of Hematology 1996; 72:177–83.
13. Mourão PAS, Pereira MS. Searching for alternatives to heparin. Sulfated Fucans From Marine Invertebrates. TCM 1999; 9(8):225–32.
14. Hirsh J. New anticoagulants. American Heart Journal 2001; 142:S3–8.
15. Chua TK, Koh HL. Medicinal plants as potent sources of lead compounds with antiplatelet and anti-coagulant activities. Mini - Reviews in Medicinal Chemistry 6:611–24.
16. World Health Organization. WHO Fact sheet. http://www.who.int/mediacentre/factsheets/fs134/en 2003.
17. Matsubara K, Matsuura Y, Bacic A, Liao M, Hori K, Miyazawa K. Anticoagulant properties of a sulfatedgalactan preparation from a marine green alga, *Codiumcylindricum*. International Journal of Biological Macromolecules 2001; 28: 395-399.
18. Guglielmone HA, Agnese AM, Nunez Montoya SC, Cabrera JL. Anticoagulant effect and action mechanism of sulphated flavonoids from *Flaveriabidentis.* Thrombosis Research 2002; 105: 183-8.
19. Biggs R, McFarlane R. Human Blood Coagulation and theirdisorders, Blackwell Scientific Publications, Oxford, 1962; 430-436.
20. Hull R, Delmore T, Carter C, Hirsh J, Genton E, Gent M. Adjusted subcutaneous heparin versus warfarin sodium in the long-term treatment of venous thrombosis. The New England Journal of Medicine 1982; 306(4):189-94.
21. Schved JF, Biron-Andréani C. Hématologie:Exploration de l'hémostase. Montpellier 2005; . 19.
22. Harborne JB. Phytochemical methods, 2nd edition, Chapman and Hall publications, London, NewYork, 1984; 288.
23. Imaduddin M, TajEldin Majed M, Abdulmutalab Hyder E, Bikir. An in vitro anticoagulant effect of Fenugreek(Trigonella foenum – graecum) in blood samples of normal Sudanese individuals. Sudanese Journal of Paediatrics 2013;.13(2).
24. Anslem O, Ajugwo Teddy C, .Adias Felize NO, Suala, Kevin Aghatise, *et al*., Anosike: In vitro studies of anticoagulation activity of pentaclethro macrophylla. World Journal of nutrition and health 2013; 1(1):10-12.
25. Chinaka O, Nwaenujor, Rita I, Udegbunam, Julius O, Ode, Stella A. Madubuike anti thrombotic activities of New bouldia laevis(P.Beauv)seem ex bureau leaves. Journal of applied Pharmaceutical Science 2015; 5(05): 075-079.
26. Uma R, Srinivasan PT, Arivoli A, Vicky M. Evaluation of invitro anticoagulant activity of leaf extrats of *murraya koenigii* (LINN)and *Bavhinia tomentosa*. International Journal of Institutional pharmacy and life science 2014; 4(6): 121-126.
27. Dandjesso C, Klotoe JR, Dougnon TV, Segbo S, Ategbo SM, Gbaguidi F*, et al*., Phytochemistry and hemostatic properties of some medicinal plants sold as anti haemorrhagic in cotonou markets (Benin) 2012; 5(8): 3105-3109.