Anticoagulant and Thrombolytic Activities of Leaf Extract of *Mangifera indica* in Smokers

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Abstract

In this study anticoagulant and thrombolytic activities of *Mangifera indica* have been investigated which were not present in literature because ethnobotanical evidences have not been done before. The thrombolytic and anticoagulant (*in vitro* and *in vivo*) activities also phytochemical screening and high performance liquid chromatography of aqueous-methanolic (70:30) leaves' extract of *M. indica* have been investigated. For *in vitro* experiment *M. indica* displayed a noteworthy ($p<0.05$) increment in prothrombin time, clotting time and activated partial thromboplastin time while *in vivo* experiment noteworthy ($p<0.05$) increase in clotting time, bleeding time, activated partial thromboplastin time and prothrombin time in a dose-dependent manner in rabbits after one week of treatment while heparin being taken as a positive control. For *in vitro* experiment, aqueous-methanolic extract in a dose-dependent manner displayed noteworthy ($p<0.05$) clot lysis while streptokinase being taken as a positive control. Moreover, HPLC showed the presence of mangiferin, quercetin and isoquercetin which down regulate the activity of factor Xa as well as act on antithrombin and plasminogen respectively, in coagulation cascade.

Keywords: *Mangifera indica*, anticoagulant, thrombolytic, prothrombin time, activated partial thromboplastin time.

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Introduction

A few cardiovascular maladies such as coronary artery diseases, DVT, pulmonary embolism, heart attacks and strokes are caused by coagulation and thrombus formation are the fundamental causes of death in developed countries (Dickneite *et al.*, 1995). Thromboembolic disorders may be caused by arteriosclerosis, infection or traumatic injury as well as slow blood flow that may lead to clot formation. The use of a tissue plasminogen activator has been shown much effective to dissolve the clot (Hall, 2016). In the process of coagulation, many different coagulation factors and enzymes are required in sequential cascade reactions. Activated Stuart-Prower factor by intrinsic and extrinsic pathways causes the production of prothrombin activator which catalyzes prothrombin to its activated form thrombin which catalyzes fibrinogen into fibrin (Dickneite *et al.*, 1995; Tortora & Grabowski, 1996). Thrombin also plays an essential role in the activation of the fibrin stabilizing factor and increases the production of the labile factor. As a result, thrombin is increased and platelets are activated which leads to the aggregation of platelets and clotting is accelerated (Grabowski & Tortora, 2000). Medicinal plants are also being used in the treatment and lowering the risks of CVDs, besides the use of pharmacological drugs. Some plant materials may also show anti-coagulant and thrombolytic activities but mostly the focus is on antioxidant properties of such plants (Gilani *et al.*, 2010).

Mangifera indica, commonly known as mango (Chaunsa), belongs to the family *"Anacardiaceae"*, of the plant kingdom native to the Indian subcontinent. Hundreds of developed assortments have been presented to other warm districts of the world (*Tropicos | Name - Mangifera Indica L.*, n.d.). Many countries have been used mango extract from leaves, fruit pulp, roots, bark, stem and seed kernel for curative purposes (Núñez-Sellés, 2005). The different chemical constituents are present in the leaf of *M. indica* such as flavonoids, alkaloids, phenols, saponins, minerals, vitamin C, B (Okwu & Ezenagu, 2008). The leaf extract is being used for different biological activities such as anti-diabetic (Aderibigbe *et al.*, 2001), anti-microbial (Akinpelu & Onakoya, 2006), immunomodulator (Shah *et al.*, 2010), anti-allergic (Rivera *et al.*, 2006), hepatoprotective (Das *et al.*, 2012), cardioprotective (Bhatt & Joshi, 2017), anti-inflammatory and analgesic (Ojewole, 2005).

The novel approach is to study the role of aqueous-methanolic leaves' extract of *M. indica* to treat cardiovascular diseases caused by coagulation and thrombus formation leading to its related risk factors.

Materials and Methods

Experimental animal: Rabbits (*Oryctolagus cuniculus*) of either sex with an average weight of 1.5kg were acquired from pet Market Hussain Agahi, Multan, Pakistan. Rabbits were kept in the animal house of stainless steel cages under standard laboratory conditions of 25°C and 12 hours light and dark cycle and entertained with ad libitum in Muhammad Institute of Medical and Allied Sciences, Multan. The experiments have been done as recommended by the National Institute of Health Guide for Care and Use of Laboratory Animal (*National Institute of Health. Guide for Care and Use of Laboratory Animal - Google Search*, n.d.) and approved by the Animal Ethical Committee of Muhammad Institute of Medical and Allied Sciences, Multan, Pakistan (10/DPT/MIMAS/Oct/20).

Drugs and chemicals: Streptokinase was purchased from Highnoon Laboratories Ltd. Pakistan. Heparin vial was purchased from Mehran Traders Ltd. Pakistan. Meyer's reagent, Folin-Ciocalteu reagent, prothrombin time and activated partial thromboplastin time reagents were purchased from Javaid Pharmaceuticals (Pvt.) Ltd. Pakistan. Methanol, HCl, H_2SO_4 and NaOH were purchased from Merck, Germany. Ferric chloride was purchased from BDH Laboratory, England.

Plant collection and extract preparation: *M. indica* was collected from the garden located in Muhammad Institute of Medical and Allied Sciences, Multan, South Punjab. The plant was identified with the assistance of an expert taxonomist (R.R.Steward, F.W. Pak. 625-3). The fresh leaves of the plant were left for shade drying. Dirt and debris were cleared before grinding of dried leaves by the special herb grinder to the coarsely powdered form. The airtight jar was used for the preservation of powdered plant. For extract preparation from powdered material was done by a standard reported method including maceration procedure in aqueous-methanolic (70:30) mixture. The evaporation of crude extract pool to a thick paste as stock solutions was done on a rotatory evaporator at 37℃ under low pressure (Alamgeer *et al.*, 2018). The estimated 10% yield of extract was taken using the formula.

% yield = (weight after evaporation × 100) / dry weight of leaves

Its 20%, 10% and 5% dilutions were stored in airtight jars in a lab refrigerator at -2°C.

In vitro experiments on human blood

Anti-coagulant activity: Blood samples (2mL) received from healthy human volunteers (n=7), taking no contraceptives and analgesics, were transferred in 4 separate test tubes. 0.2 ml of 20%, 10% and 5% dilutions of aqueous-methanolic extract and heparin (250 IU/mg) as a positive control was mixed in these 4 test tubes then subjected to incubation at 37℃. Then clotting time (CT) was measured with help of a stopwatch (Alamgeer *et al.*, 2018).

Determination of activated partial thromboplastin time and prothrombin time: Blood samples (3mL) received from healthy human volunteers (n=7), taking no contraceptives and analgesics, were transferred to sodium citrate containing tubes and centrifugation done for 5min at 3000rpm. Plasma was transferred to 8 distinctive eppendorf tubes of every member of the group by micropipettes. 20%, 10% and 5% dilutions of aqueous-methanolic extract (100uL) were mixed with the same amount of plasma in distinctive eppendorf tubes of each member of the group. 100uL heparin (250IU/mg) was mixed as a positive control. To determine the prothrombin time (PT) sample was subjected to incubation for 5 minutes at 37℃. Then prothrombin time reagent (200uL) was mixed in each eppendorf tubes being tested and time measured as PT with help of a stopwatch. To determine activated partial thromboplastin time (APTT), activated partial thromboplastin time reagent (100uL) mixed to plasma being tested. The mixture was incubated for 1 minute, then calcium chloride solution (100uL) was mixed and hatched for 15 seconds and clotting time was measured as APTT with help stopwatch (Alamgeer *et al.*, 2016).

Thrombolytic activity: Blood samples (2.5mL) collected from healthy human volunteers (n=7), taking no contraceptives and analgesics, transferred in 4 different already weight eppendorf tubes of each member. Time was allowed for thrombus formation, after 45 minutes serum was removed from eppendorf tubes. Clots in eppendorf tubes were weighed. 20%, 10% and 5% dilutions of aqueous-methanolic extract (100uL) were applied in 3 distinctive eppendorf tubes of each member. 100uL streptokinase (30000IU) was mixed in the 4th eppendorf tubes as positive. The time allowed for thrombolytic activity. After 90 minutes liquid was removed and the in tubes remaining clots were weighed over again. The alteration between before and after clot lysis was taken as % clot lysis (Alamgeer *et al.*, 2018)

$$\% \text{ clot lysis} = \frac{\text{weight of clot before lysis - weight of clot after lysis}}{\text{Weight of clot before lysis}} \times 100$$

***In vivo* experiment on rabbit**

Determination of coagulation parameter: Rabbits were isolated into 4 groups (n=5) and dosing 20%, 10% and 5% dilutions of the aqueous-methanolic extract (100mg) were given to rabbits of the 1st, 2nd and 3rd group and heparin (50units/mg) was injected intravenously to rabbits of the 4th group for 7 days as a positive control. On the 7th day blood sample was received from the external jugular vein of rabbits of each group (Santos *et al.*, 2019) and PT and APTT tests have been performed (Alamgeer *et al.*, 2016). After seven days of treatment to decide the impact, the bleeding time (BT) was measured by prickling the marginal ear vein of each rabbit after an interval of 0, 30, 60 and 90 minutes and after each 5 sec filter paper was utilized (Nwaehujor *et al.*, 2013). And clotting time (CT) was measured by piercing the marginal ear vein of each rabbit of each group with the assistance of capillary tubes by putting it horizontally. The capillary tubes were broken after every 30 seconds until the thread was shown as coagulated blood (Alamgeer *et al.*, 2018).

Phytochemical screening: Flavonoids distinguished through Alkaline reagent test in which few drops of NaOH solution 2ml in aqueous-methanolic extract added, the yellow color disappeared when concentrated HCl added to it. Saponins distinguished through Foam test in which 2ml of distilled water added in test tube to 0.2g aqueous-methanolic extract after 15 minutes vigorous shaking 1cm foam formed. Alkaloid distinguished when 0.2g aqueous-methanolic extract dissolved in dilute H_2SO_4 and filtered thoroughly, 1ml filtrate treated with few drops of Meyer's reagent then white or creamy precipitates formed. Tannins distinguished through Ferric Chloride test in which a few drops of 5% ferric chloride solution added to 2ml aqueous-methanolic extract then bluish-black color appeared (Osibemhe and Onoagbe, 2015). Phenolics distinguished through Liquid-liquid extraction method in which Folin-Ciocalteu reagent added in aqueous-methanolic extract then it produced blue color (Jahromi, 2019). Ascorbic acid was distinguished through a method in which few drops of aqueous-methanolic extract were used on indophenol which produced discoloration of indophenol (Jahromi, 2017).

High performance liquid chromatography analysis of flavonoids: HPLC was used to estimate the flavonoids in aqueous-methanolic leaves' extract of *M. indica*. A binary gradient solvent system was used in HPLC, paired with a C-18 column with dimensions (250 4.6 mm), capable of separating 2 flavonoids in 36 minutes at a flow rate speed of 0.0008 µL/min and a film thickness of 5 µm, with an oven set at 30°C. The replicability for separation of components was good with (run-to-run), mangiferin, quercetin and isoquercetin were prepared as reference (purity > 99 percent), obtained from Aldrich (St. Louis, USA), and the dilutions were prepared with methanol to achieve 50 ug/mL. Samples were distinguished by comparing the sample retention times to standards. The separation factor and resolution were used to evaluate the efficiency of separated components using HPLC as shown in Figure-I.

Statistical analysis: The results were communicated as mean $\pm$ SEM and investigated by using one-way analysis variance (ANOVA) followed by Dunnett's t-test, 95% confidence interval $p<0.05$ considered as significant.

Results

Effect on *in vitro* activities

Thrombolytic activity: The 20% and 10% dilution of aqueous-methanolic leaves' extract of *M. indica* have shown noteworthy clot lysis ($p<0.01$-0.001) but 5% aqueous-methanolic extract has

displaced less clot lysis as displayed in Table-1.

Table-1: Thrombolytic activity

Drugs/crude extracts	Clot lysis (%)
Streptokinase	78.71±1.48
20% aqueous-methanolic extract	65.81±1.37
10% aqueous-methanolic extract	41.52±1.42
5% aqueous-methanolic extract	18.62±1.31

Anti-coagulant activity: Aqueous-methanolic leaves' extract of *M. indica* has shown noteworthy ($p<0.01$-0.001) increment at 20%, 10% and 5% dilutions in CT as displayed in Table-2.

Prothrombin time and activated partial thromboplastin time: Aqueous-methanolic leaves' extract of *M. indica* has shown a noteworthy ($p<0.01$-0.001) increment in at 20%, 10% dilutions but 5% dilution aqueous-methanolic extract has shown insignificant ($p<0.1$) increment in PT and APTT as displayed in Table-2

Table-2: Anti-coagulant activity, prothrombin time and activated partial thromboplastin time

Blood parameters	Heparin	Aqueous-methanolic extract 20%	Aqueous-methanolic extract 10%	Aqueous-methanolic extract 5%
CT (min)	25.7±3.4	$22.8±3.2^{l}$	$17.5±2.9^{l}$	$13.6±3.1^{m}$
PT (sec)	157.6±0.5	$103.7±0.8^{l}$	$84.8±0.6^{l}$	$43.5±0.4^{n}$
APTT (sec)	398.7±5.0	$245.6±4.8^{l}$	$203±4.5^{l}$	$97±4.6^{n}$

*Notation ^{l} $p<0.001$, ^{m} $p<0.01$ and ^{n} $p<0.1$.

Impact on different coagulation parameters after one week dosing in rabbits: Aqueous-methanolic leaves' extract of *M. indica* has shown noteworthy ($p<0.01$-0.001) increment in BT, CT, APTT and PT at 100mg/kg and 50mg/kg concentration but insignificant at 25mg/kg concentration as displayed in Table-3.

Table-3: Impact on different coagulation parameters after one week dosing in rabbits

Blood parameters	Heparin 50 units/kg	Aqueous-methanolic extract 100mg/kg	Aqueous-methanolic extract 50mg/kg	Aqueous-methanolic extract 25mg/kg
BT (min)	13.6±4.3	$11.5±3.8^{l}$	$7.2±4.2^{l}$	$3.6±4.0^{m}$
CT (min)	7.8±1.6	$5.6±1.9^{l}$	$4.4±1.2^{l}$	$3.4±0.9^{n}$
PT (sec)	35.6±4.3	$32.3±4.7^{l}$	$29.8±4.2^{l}$	$14.2±3.9^{n}$
APTT (sec)	73.7±5.4	$67.8±4.9^{l}$	$53.4±4.5^{l}$	$42.5±4.5^{m}$

*Notation ^{l} $p<0.001$, ^{m} $p<0.01$ and ^{n} $p<0.1$.

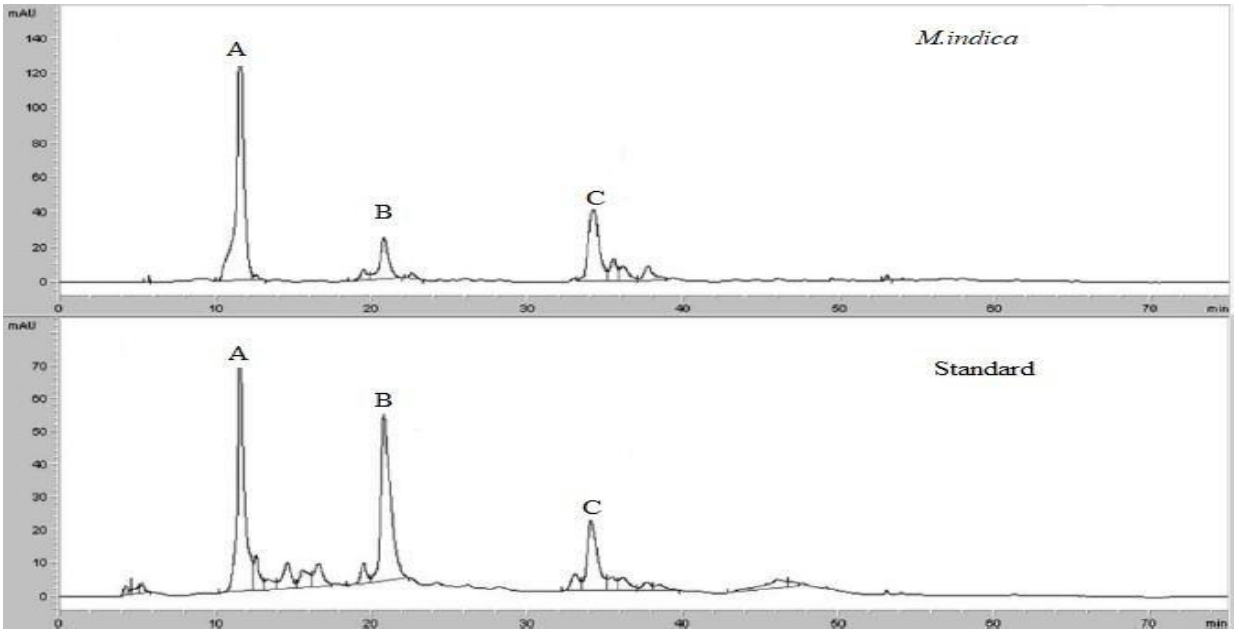
Phytochemical screening: Phytochemical screening of aqueous-methanolic leaves' extract of *M. indica* was displayed in Table-4.

Table-4: Phytochemical screening of aqueous-methanolic leaves' extract of *M. indica*

Phytochemicals	Results
Flavonoids	++
Saponins	+
Alkaloids	+
Tannins	+
Phenols	+
Ascorbic acid	++

HPLC analysis: HPLC of aqueous-methanolic leaves' extract of *M. indica* was displayed in Figure-1

Figure-1: HPLC of aqueous-methanolic leaf extract of *M. indica* indicating the presence of A, mangiferin: B, quercetin: C, isoquercetin regards to retention time.



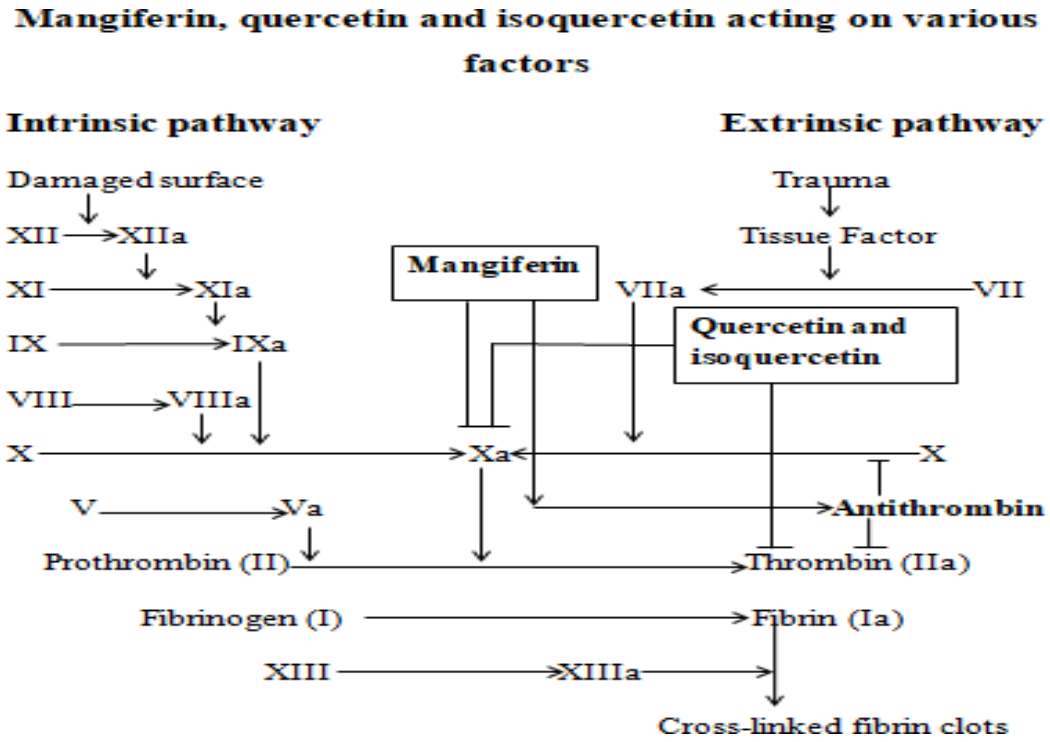
Discussion

Inhibition of platelet accumulation increment BT in animals (De Caterina *et al.*, 1994) as pronounced from finding of this study. Another major assessment of the intrinsic pathway is CT (Dapper *et al.*, 2007). PT test is the most trustworthy test in coagulopathy (Hinchcliff *et al.*, 2013; Furlanello *et al.*, 2006). PT and APTT are tests that differentiate alteration in intrinsic and extrinsic pathways of coagulation. Various clotting factors involve in the intrinsic pathway and the type of imperfection in these factors cause increment or diminish in CT (Weremfo *et al.*, 2011). These clotting variables are naturally proteinic in the resting state but when blood vessels harmed these factors activated and act in the coagulation cascade (Alesci *et al.*, 2009). By APTT intrinsic clotting factors are assessed and increment in APTT reflects the deformity in clotting components XII, XI, IX, VIII and V as well as Willebrand's figure and diminishes in PT shows deformity in clotting components VII, X and V (Alamgeer *et al.*, 2018). By inhibitors of coagulation components at the side restraint of phospholipid and Ca^{++} activity, these two variables are too affected (De Caterina *et al.*, 1994).

This study showed that aqueous-methanolic leaves' extract of *M. indica* has a significant increment in PT and APTT. Flavonoids, mangiferin, quercetin and isoquercetin have the ability against platelet aggregation (Okwu & Ezenagu, 2008). As mangiferin heptasulfate has been reported as it inhibit

directly factor Xa, even though persulfated 3, 6-(O-glucopyranosyl) xanthone has been reported as a dual inhibitor, directly and by activation of antithrombin III (Da Silva *et al.*, 2011). Also quercetin and isoquercetin have been reported to inhibit the enzymatic activity of thrombin and factor Xa and defeat fibrin clot formation and blood clotting (Choi, 2016). In this study, HPLC has shown that mangiferin, quercetin and isoquercetin are also present in aqueous-methanolic leaves' extract of *M. indica*. So it is considered that extract exerts anticoagulant activity may be due to the presence of these flavonoids by acting on the mechanisms as shown below.

Figure-II: Mangiferin, quercetin and isoquercetin acting on various factors



Various earlier studies have appeared that the nearness of flavonoids (mangiferin, quercetin and isoquercetin), tannins and phenolic components in plants having thrombolytic activities (Markman *et al.*, 2004; Hsu & Yen, 2008). Leaves of *M. indica* have been reported as having a rich amount of flavonoids, tannins, saponins, alkaloids and phenolic components (Okwu & Ezenagu, 2008). Also in this study phytochemical screening and HPLC have shown that flavonoids, tannins, saponins, alkaloids and phenolic components are present in aqueous-methanolic leaves' extract of *M. indica*. So it is considered that extract exerts thrombolytic action may be due to nearness of a few flavonoids, hinder platelet aggregation by inhibition of Thromboxane A2 receptors (Guerrero *et al.*, 2005).

We use distilled water and methanol as solvents so that the maximum required ingredients dissolve in high quantity for maximum effects as some ingredients are highly dissolved in aqueous solution but not dissolved in alcohol and some ingredients are highly dissolved in alcohol but not dissolved in aqueous. It is easy to prepare that extract because solvents are easily available.

Conclusion

Results of this study declared that the phytochemical constituents in leaves of *Mangifera indica* may participate in anticoagulant and thrombolytic activities and its aqueous-methanolic extract have anti-coagulant and thrombolytic activity.

Ethical issues

The study was approved by Institutional Animal Ethical Committee, Ali-Ul-Murtaza, Department of Rehabilitation Sciences, Muhammad Institute of Medical and Allied Sciences, Multan (10/DPT/MIMAS/Oct/20).

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C**onflict of interest**

No conflict of interest among authors.

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