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Original Research Article

Anticoagulant potential and total phenolic content of six species of the genus *Ficus* from Azad Kashmir, Pakistan

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Abstract

Purpose: *To investigate the total phenolic and flavonoid contents of Ficus benghalensis, Ficus elasticaa, Ficus palmata, Ficus religiosa, Ficus semicordata and Ficus auriculata, and to determine their anticoagulant potential.*
Methods: *Crude methanol extracts were prepared from the plant leaves, and fractionated using liquid-liquid partition with n-hexane, chloroform and ethyl acetate. The total flavonoid and total phenolic contents of the extracts and their fractions were determined. The anticoagulant potential of the six Ficus species were evaluated in healthy human plasma, using activated partial thromboplastin time (APTT) and prothrombin time (PT) methods.*
Results: *Phytochemical analysis showed the presence of considerable amounts of flavonoids ranging from 5.3 ± 0.7 to 11.8 ± 0.3 mg rutin equivalents (RE)/g, and phenolic compounds ranging from 8.0 ± 0.7 to 86.5 ± 1.5 mg gallic acid equivalents (GAE)/g in each fraction of the six species. Results from in vitro anticoagulant potential assays showed significant anticoagulant properties, with prothrombin time (PT) ranging from 17.7 ± 0.7 to 26.7 ± 2.2 s, and activated partial thromboplastin time (APTT) varying from 47.7 ± 3.3 to 72.3 ± 5.4 s.*
Conclusion: *The results indicate that F. semicordata and F. Religiosa have higher anticoagulant potential than the other Ficus species studied.*

Keywords: *Medicinal plants, Anticoagulant, Polyphenols, Flavonoids, Ficus*

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INTRODUCTION

Cardiovascular diseases (CVDs) are associated with poor health and high incidence of death worldwide [1]. The major cause of death in CVD patients is thrombus-induced myocardial infarction which also contributes to the pathogenesis of atherosclerotic CVDs [1]. Abnormal coagulation leads to the formation of thrombus in blood vessels resulting in ischemia [1]. In atherosclerotic arteries, platelet activation induces arterial thrombosis [2]. Therefore, it is important to regulate platelet function in order to prevent thrombotic events. However, the currently available antithrombotic and antiplatelet drugs produce unsatisfactory results, and often

lead to vascular relapses. Indeed, most thrombo-embolic processes require anticoagulant therapy. Therefore, there is need for development of novel, more effective and less toxic bioactive anticoagulant and antithrombotic drugs with different mechanisms of action. Anticoagulants arrest coagulation after the initial platelet aggregation with blood clotting factors [3]. Generally, anticoagulation therapy reduces the severity of CVDs, and prevents existing clots from growing larger [4]. Anticoagulants reduce the formation of blood clots in other vital organs such as lungs and brain [3]. Moreover, they diminish the risks of developing other diseases such as atrial fibrillation, deep vein thrombosis, myocardial infarction, stroke and pulmonary embolism [3,4]. Although anticoagulants such as heparin, aspirin and warfarin are often used in clinical practice, some patients may develop aspirin resistance. Therefore, natural sources of anticoagulants (plants/herbs) may provide alternative and safer therapy for thrombotic disorders [3]. Herbal drugs have shown great efficacy in the treatment of many diseases. According to the World Health Organization (WHO), about 25% of drugs used in the world are plant-based [5]. Indeed, in many parts of the world, there is a significant preference for herbs over orthodox drugs for primary health care. The high cost of synthetic medicines and their limited supply to rural areas in many developing countries have driven the continuous dependence on traditional herbal medicines [6]. Medicinal plant-based products are used as raw material in several multibillion-dollar industries all over the world [7].

The genus *Ficus* of the Moraceae family has about 800 species of woody trees, shrubs and vines [8]. These species are found mainly in tropical areas, and are used in medical practice worldwide [8,9]. The medicinal plants of genus *Ficus* contain triterpenes, polyphenols, flavonoids, sterols, alkaloids, coumarins and other secondary metabolites [10]. These phytochemicals are used as anti-rheumatic, antidiabetic, anti-helminthic, digestive, mild-laxative and anti-dysentery agents [11]. In the present study, six species of genus *Ficus,* namely, *Ficus benghalensis, Ficus elasticaa, Ficus palmata, Ficus religiosa, Ficus semicordata* and *Ficus auriculata* were screened for their anticoagulant potential.

EXPERIMENTAL

Plant materials

Leaves of *F. benghalensis, F. elastica, F. palmata, F. religiosa, F. semicordata, and F. auriculata* were collected from Azad Kashmir, Pakistan at coordinates 34.22°N 73.28°E, in August 2016. The material was identified by Dr. Ajaib of the Department of Botany, Mirpur University of Science and Technology, Mirpur, Azad Jammu and Kashmir, Pakistan. The voucher specimens (no. MUST722 – MUST727) were deposited at the Herbarium of the Department of Biotechnology. The leaves were shade-dried at room temperature for 2 weeks, triturated with a blender and kept in sealed containers away from light and humidity until used.

Extract preparation

The dried leaf powder was soaked in methanol at a plant material: methanol ratio of 1:5 (w: v) for 6 days. The soaked samples were occasionally shaken to ensure proper mixing. The mixture was then filtered using a muslin cloth and Whatman filter paper. The filtrate was further concentrated using a rotary evaporator, to obtain a semi-solid material i.e. crude methanol extract (MCE). A portion of the crude extract was dissolved in phosphate buffered saline (PBS) and used for biological screening.

Fractionation of MCEs

The MCEs were subjected to liquid–liquid partitioning using solvents of increasing polarity to obtain n-hexane, chloroform and ethyl acetate fractions. The fractions were dried through solvent evaporation in a rotary evaporator under reduced pressure.

Phytochemical analysis

Determination of total phenolic content (TPC)

The TPCs of the plant extracts were determined using the Folin-Ciocalteau method as reported by Harborne [12]. The principle involved in this assay is that polyphenols are reduced by Folin-Ciocalteau reagent to produce a blue-coloured complex, the intensity of which is proportional to the amount of polyphenol present. The concentrations of polyphenols in the plant extracts were calculated from a gallic acid standard calibration curve. For the calibration curve, different concentrations of gallic acid (12.5, 25, 50, 100, 200, and 400 µg/mL) were prepared. Then, 0.5 mL of each solution was added to 2.5 mL of 10 % Folin-Ciocalteau reagent and 2.5 mL of 7.5 % sodium carbonate solution, followed by incubation at room temperature for 1 h. Thereafter, the absorbance of the solution was read at 765 nm in a UV-visible spectrophotometer. The readings were

taken in triplicate for each sample and the mean value was calculated. The same procedure was used for plant extracts. The TPC content was expressed as mg GAE/g of plant extract as shown in Equation 1:

$$TPC = \frac{(C \times V)}{m} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where *C* is the concentration of gallic acid obtained from the calibration curve in mg/ml; *V* is the volume of sample solution in ml, and *m* is the weight of plant extract.

Determination of total flavonoid content

The total flavonoid contents of the plant samples were measured using the method of Kumaran and Karunakaran [13]. In essence, 1 mL of extract solution was mixed with 1 mL of ethanolic solution of $AlCl_3$ and 1.5 mL of 5 % sodium acetate solution, followed by incubation at room temperature for 2.5 h. The absorbance of the solution was measured at 440 nm in a UV-visible spectrophotometer. Triplicate measurements were made for each sample, and the mean of three values was taken. The same procedure was repeated for standard solution of rutin at different concentrations i.e. 12.5, 25, 50, 100, 200, and 400 µg/mL to obtain a calibration curve. The total flavonoid content was expressed as mg rutin equivalent/g of sample, using the regression equation (Equation 2):

$$Y = 0.0067x - 0.1331, R^2 = 0.9781 \quad \ldots\ldots\ldots (2)$$

where *x* is the absorbance and *y* is the rutin equivalent.

Determination of LD_{50} and rationalization of dose

To determine the effective therapeutic dose, acute toxicity test of the crude methanol extract (MCE) was carried out on New Zealand rabbits. The rabbits were divided into four groups of four animals each (n = 4), and MCE was administered orally at doses of 1.0, 2.0, 3.0 and 4.0 g/kg body weight to groups 1, 2, 3 and 4, respectively. The rabbits were kept under close observation for about 6 h after the MCE administration, and they remained under periodic observation for 5 days to monitor delayed toxicity, if any. The animals were monitored for changes in skin, fur, eyes, mucous membrane, CNS, respiration and circulation. Median lethal dose (LD_{50}) greater than 2.0 g/kg was considered safe, while one-tenth of the LD_{50} was taken as the effective therapeutic dose.

Evaluation of anticoagulant potential

The anticoagulant potential of each plant sample was tested on human blood sample. For this purpose, six healthy human donors were selected. Care was taken to ensure that the blood donors had not taken any drugs for at least seven days prior to sampling period. The blood samples were collected in Eppendorf tubes containing 400 µL of sodium citrate solution (3.2 %) in order to avoid blood clotting. Following centrifugation at 3000 rpm at 5 °C for 15 min, the resultant plasma samples were refrigerated at 4 °C prior to use.

Prothrombin time (PT) assay

The effect of plant extracts on the extrinsic pathway of coagulation was determined using the prothrombin time (PT) test as previously described by Felix-Silva *et al* [14], with slight modifications. The PT test was performed with commercially available PT reagent kit (Singapore Biosciences, Singapore). In essence, 90 µL of plasma was mixed with 10 µL of plant extract at concentration of 1 µg/µL, followed by incubation at 37°C for 5 min. Then, 100 µL of pre-warmed thromboplastin reagent (PT assay reagent) was added, and the clotting time was recorded with a digital timer. Plasma alone (with vehicle only) was used as normal control, while 0.5 U/mL of heparin was used as positive control.

Activated partial thromboplastin time (APTT) assay

The coagulation pathway is a cascade of events that lead to haemostasis. The intrinsic pathway consists of factors I, II, IX, X, XI, and XII otherwise known as fibrinogen, prothrombin, Christmas factor, Stuart-Prower factor, plasma thromboplastin, and Hageman factor, respectively. The common pathway consists of factors I, II, V, VIII, X which circulate in the bloodstream as zymogens and are activated by serine proteases, ultimately resulting in the activation of fibrinogen by conversion to fibrin. The effects of the plant extracts on the intrinsic pathway and common coagulation pathway were measured using the APTT assay according to a slight modification of the procedure outlined by Mao *et al* [15]. The test was similar to the PT test except that 100 µL APTT assay reagent (cephaloplastin) was used. The reaction mixture was incubated at 37 °C for 5 min, followed by addition of 100 µL of 25 mM

$CaCl_2$. Then, the coagulation time was recorded.

Statistical analysis

The data collected were transferred to Graphpad prism 7 and plotted. Values are expressed as mean ± standard error of the mean (SEM). Unpaired *t*-test was used for statistical analysis. Statistical significance was fixed at $p \leq 0.05$.

RESULTS

Phytochemical profile

Total phenolic contents

In this study, the six species of *Ficus* investigated were analysed for polyphenol contents using the Folin-Ciocalteu method. Although the FC method alone is not enough to determine the total phenolic content due to the diverse nature of polyphenols, it can give a considerably good estimation of the levels of these compounds. The results in Figure 1 show that the TPC content of the crude methanol extract of *F. auriculata* was 83.5±0.9 mg GAE/g. This was followed, in decreasing order of TPC content, by *F. palmata* (49.5±0.3), and then *F. religiosa* (47.0±0.4) mgGAE/g. Thus, total phenolic content was significantly higher *($p < 0.05$)* in *F. auriculata* than in any other tested *Ficus* species.

The n-hexane fractions of *F. benghalensis, F. elastica, F. palmata, F. semicordata, F. semicordata, and F. auriculata* had TPC contents of 86 ± 1.6, 64 ± 2.7, 71 ± 1.4, 57.5 ± 2.8, 58.5 ± 5 and 32.1 ± 1 mg GAE/g, respectively; while the ethyl acetate fractions contained TPC levels of 86.5 ± 1.5, 41 ± 5, 26 ± 0.05, 31 ± 3, 85.5 ± 1 and 77.5 ± 1.6 mg GAE/g, respectively. The TPC contents of the chloroform fractions of *F. semicordata* (78.5 ± 2.5 mg GAE/g) and *F. religiosa* (51 ± 1 mg GAE/g) were significantly higher than the corresponding level in the chloroform fraction of *F. benghalensis* (32.5 ± 0.6 mgGAE/g). Therefore, the results of the present study revealed that in the six *Ficus* species investigated, the amounts of TPC were higher in the n-hexane fractions than in the ethyl acetate and chloroform fractions. The chloroform fractions contained the least amounts of TPC.

Total flavonoid contents

The results of total flavonoid contents are shown in Figure 2. All the six species of *Ficus* investigated contained appreciable amounts of flavonoids ranging from 5.3 ± 0.7 to 11.8 ± 0.3 mg RE/g. The flavonoid contents of the crude methanol extract of *F. elastica* (10 ± 0.48 mgRE/g) and *F. auriculata* (7.35 ± 0.44 mgRE/g) were significantly higher than the corresponding values in the other species used in this study. The n-hexane fractions of *F. benghalensis, F. elastica, F. palmata, F. semicordata, F. semicordata, and F. auriculata* had flavonoid contents of 7.13 ± 0.1, 10.93 ± 0.58, 8.37 ± 0.33, 6.9 ± 0.2, 6.26 ± 0.16 and 7.05 ± 0.32 mg RE/g, respectively.

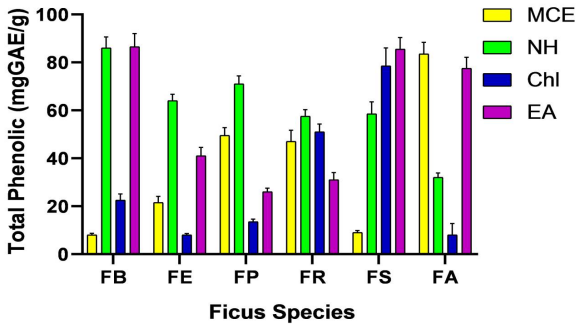

Figure 1: Total phenolic contents of the crude methanol extracts and other fractions of *Ficus* species expressed as mgGAE/g. A relatively high value of TPC was seen in each plant extract. All experiments were run in triplicate, and values are expressed as mean ± SEM. FB: *Ficus benghalensis*; FE: *Ficus elastica*; FP: *Ficus palmata*; FR: *Ficus religiosa*; FS: *Ficus semicordata*; FA: *Ficus auriculata*; MCE: methanolic crude extract; NH: n-hexane; Chl: chloroform; EA: ethyl acetate

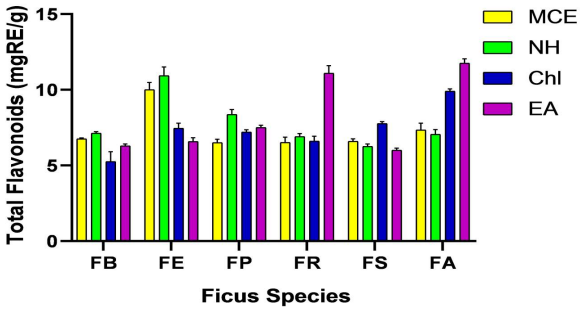

Figure 2: Total flavonoid contents of the crude methanol extracts and other fractions of six *Ficus species* expressed as mg RE/g. Fairly good amounts of total flavonoids were obtained. The assays were run in triplicate, and values are expressed as mean ± SEM. FB: *Ficus benghalensis*; FE: *Ficus elastica*; FP: *Ficus palmata*; FR: *Ficus religiosa*; FS: *Ficus semicordata*; FA: *Ficus auriculata*; MCE: crude methanol extract; NH: n-hexane; Chl: chloroform; EA: ethyl acetate

Anticoagulant potential

The effects of the crude methanol extracts of the six *Ficus* species and their fractions on haemostasis were evaluated using their anticoagulant potential through prothrombin time

(PT) and the activated partial thromboplastin time (APTT) measurements. The PTs of crude methanol extracts of *F. benghalensis, F. elastica, F. palmata, F. religiosa, F. semicordata, and F. auriculata* were 21.7 ± 1.2, 18.3 ± 0.9, 17.3 ± 1.1, 20.3 ± 1.4, 22.7 ± 1.7 and 26.7 ± 2.2 s, respectively. The PT values of n-hexane ranged from 17.3 ± 0.9 to 21.0 ± 1.0 s. The corresponding ranges for chloroform and ethyl acetate were 17.7 ± 0.7 to 22.0 ± 1.1 and 17.8 ± 0.9 to 24.0 ± 1.5 s, respectively. Therefore, the results showed that all fractions of the six *Ficus* species delayed prothrombin times, when compared to the negative control or normal control (13.3 ± 0.6 s), as shown in Figure 3. The n-hexane fraction of *F. palmata* had the lowest PT of 17.3 ± 0.9 s, while the ethyl acetate fraction of *F. auriculata* had the highest PT of 24.0 ± 1.5 s.

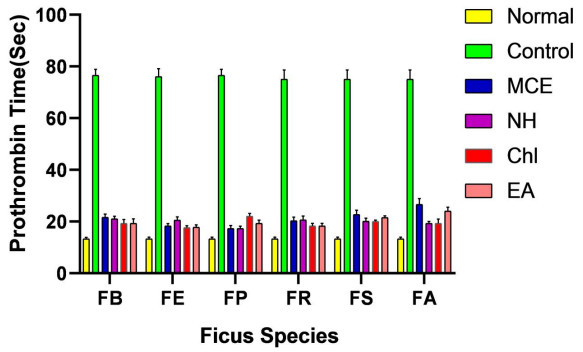

Figure 3: Prothrombin times of the crude methanol extracts and fractions of the six *Ficus* species. A relatively delayed PT was observed with each plant sample, relative to the normal group. The experiments were repeated three times, and values are expressed as mean ± SEM. FB: *Ficus benghalensis*; FE: *Ficus elastica*; FP: *Ficus palmata*; FR: *Ficus religiosa*; FS: *Ficus semicordata*; FA: *Ficus auriculata*; MCE: methanolic crude extract; NH: n-hexane; Chl: chloroform; EA: ethyl acetate

Activated partial thromboplastin time

The activated partial thromboplastin times of the crude methanol extracts of the six *Ficus* species and their n-hexane, chloroform and ethyl acetate fractions were determined using APTT assay reagent. The results (Figure 4) showed delayed APTT, when compared to normal. The activated partial thromboplastin time of crude methanol extracts of *F. benghalensis, F. elastica, F. palmata,, F. religiosa, F. semicordata, and F. auriculata* were 67.3 ± 3.2, 58.7 ± 4.5, 65.7 ± 5.0, 71.0 ± 3.3, 64.3 ± 4.7 and 70.3 ± 5.5sec, respectively (Figure 4). The APTT values for n-hexane, chloroform and ethyl acetate fractions ranged from 51.7 ± 2.4 to 72.3 ± 5.4, 49.7 ± 6.1 to 71.7 ± 5.5, and 47.7 ± 3.3 to 69.7 ± 2.9 s, respectively. Thus, the fractions of all six *Ficus* species showed significantly higher APTT than normal (43.3 ± 3.1 s). The ethyl acetate fraction of *F. religiosa* had the lowest APTT of 47.7 ± 3.3 s, while n-hexane fraction of *F. religiosa* produced the highest APTT of 72.3 ± 5.4 s.

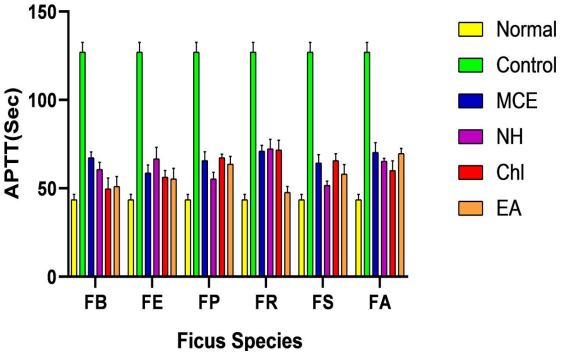

Figure 4: Activated partial thromboplastin times of the crude methanol extracts of the six *Ficus* species and their solvent fractions. A relatively delayed APPT was observed in each test sample, when compared to the normal group. The experiments were carried out in triplicate, and values are expressed as mean ± SEM. FB: *Ficus benghalensis*; FE: *Ficus elastica*; FP: *Ficus palmata*; FR: *Ficus religiosa*; FS: *Ficus semicordata*; FA: *Ficus auriculata*; MCE: methanolic crude extract; NH: n-hexane; Chl: chloroform; EA: ethyl acetate

DISCUSSION

The results of the current study showed that all the six *Ficus* species investigated had anticoagulant potential. Moreover, the fractions obtained from the leaf extracts of these *Ficus* species contained variable concentrations of phenolic and flavonoid compounds. Polyphenols constitute the largest group of phytochemicals, with more than 8000 different phenolic compounds of which more than 4000 are flavonoids [16]. Due to their diverse chemical nature, polyphenols exhibit antimicrobial, antioxidant, anti-allergic, anti-inflammatory, antiaging, and anticancer properties [17]. Thus, they are gaining popularity in the pharmaceutical, food and cosmetic industries [18]. The results of the present study revealed that the *Ficus* species investigated contained significant amounts of phenolic compounds. The crude methanol extract of *F. auriculata* had the highest total phenolic content amongst the six *Ficus* species.

Flavonoids have great potential in medical care due to their broad-ranging anti-inflammatory, anticancer, antibacterial and antioxidant activities. Moreover, flavonoid-rich foods are extensively employed in traditional medicines for treating different diseases [18]. The results obtained in this study showed that the six species of *Ficus* investigated contained appreciable amounts of flavonoids ranging from

5.25 ± 0.65 to 11.8 ± 0.29 mg RE/g. The flavonoids were significantly higher in the crude methanol extracts of *F. elastica* and *F. auriculata* than in any of the other *Ficus* species studied.

The crude methanol extracts and their n-hexane, chloroform and ethyl acetate fractions showed anticoagulant properties, as seen in delayed prothrombin times ranging from 17.3 ± 0.9 to 26.7 ± 2.2 sec. Similarly, the crude extracts and their n-hexane, chloroform and ethyl acetate fractions produced delayed APTT, when compared to control. This is the first study to report the anticoagulant potential of six species of genus *Ficus.* These results are strongly supported by the presence of significant amounts of phenolic and flavonoid compounds. Moreover, previous studies have reported strong antioxidant potential of other species of the genus *Ficus* [19-21].

CONCLUSION

The therapeutic potentials of medicinal plants have made alternative medicine research one of the most promising fields of biomedical research. The findings of the current study indicate the good anticoagulant potentials of crude methanol extracts of leaves of six *Ficus* species and their fractions, with *F. semicordata* and *F. religiosa* demonstrating higher anticoagulant activity than the other *Ficus* species.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this manuscript, and that all liabilities pertaining to claims relating to the content of this manuscript will be borne by the authors. Sumaira Ambreen contributed to the design of the study and participated in carrying out the experiments. Muhammad Tariq conceived the idea, participated in the design of the study, analysed the data, and prepared the manuscript. Muhammad S, Masoud took part in designing of the study and drafting of the final manuscript. Imran Ali contributed to data analysis and helped in drafting the manuscript. Muhammad Qasim participated in the analysis of data and drafting of the manuscript. Aamar Mushtaq conducted the sampling and the extraction of plant material and helped in in vitro studies as well. Maqsood Ahmed helped in preparing the manuscript. Rehana Asghar provided the facility and proofread the manuscript. All authors read and approved the final manuscript for publication.

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