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Abstract

The present research work was conducted to appraise the antithrombotic activity of *Mentha longifolia* using *in vitro* and *in vivo* experiments. Aqueous methanolic (70:30) extract produced significant ($p < 0.01-0.001$) and dose-dependent increase in *in vitro* blood clotting time, bleeding time, prothrombin time and activated partial thromboplastin level with maximum effect at highest concentration. While in *in vivo* experiment, aqueous methanol extract showed noteworthy ($p < 0.01-0.001$) prolongation in bleeding time and clotting time after 30, 60 and 90 min of administration except for 25 mg/kg at 30 min which is non-significant. Moreover, plant extract exhibited considerable increase ($p < 0.1-0.001$) in bleeding time, clotting time, prothrombin time as well as activated partial thromboplastin time in rabbits after seven days of treatment. Additionally, HPLC analysis of *M. longifolia* aqueous methanolic extract illustrated the presence of various valuable phytoconstituents. In a nutshell, *M. longifolia* possesses potential antithrombotic activity and hence systematically proved to be beneficial in patients with vascular diseases.

Introduction

Thrombosis is a multifaceted physiological process associated with pathogenesis of ischemic brain diseases and ischemic heart diseases (Rajput et al., 2012). In many developed countries, thromboembolic complications such as heart attack, deep vein thrombosis, pulmonary emboli, and strokes are the major causes of morbidity and mortality (Kumar et al., 2011). Recent antithrombotic agents consist of anticoagulants, thrombolytics and antiplatelets which reduce blood clotting by dissolving previously developed clot or preventing new clot formation. Nevertheless, these drugs are allied with severe adverse effects like bleeding, thrombocytopenia, drug-drug interactions, allergic reactions and cerebral hemorrhage. Reocclusion may occur in some cases and increase the mortality and morbidity associated with thrombotic complications (Albuquerque et al., 2004). Moreover, direct thrombin and factor Xa inhibitors are the new drugs as alternative to

vitamin K antagonists for stroke prevention in atrial fibrillation but associated with various clinical limitations such as unpredictable dose-response relationship and need of intense monitoring. Owing to above mentioned shortcomings, there is need to seek out newer drugs with safety, effectiveness and lack of severe toxicity (Moll et al., 2002).

Medicinal plants such as *Triclisia dictyophylla*, *Dictyota menstrualis*, *Berberis calliobotrys*, *Orbignya phalerata* have shown to have antithrombotic effects (Ajugwo and Ezimah, 2013; Albuquerque et al., 2004; Alamgeer et al., 2016; Azevedo et al., 2007).

Mentha longifolia L. belonging to Lamiaceae family is a perennial herb and locally known as Junglee Pudina in Pakistan (Shah et al., 2009; Bertoli et al., 2011). Traditionally it is also used to treat rheumatism, neuralgia, gall stone, cardiovascular problems, gastric disorders, bladder stone, jaundice, toothache, dyspnea, sedative, insect repellent and headache (Haq et al., 2011; Naghbi



et al., 2010). Hence, the current study was designed to corroborate the antithrombotic activity of *M. longifolia* to be used effectively in the cardiovascular diseases.

Materials and Methods

Experimental animals

Healthy rabbits of either sex weighing 1.0-1.5 kg were used in this study. Animals were fed with seasonal green leafy vegetables and Alfa Alfa leaves with tap water *ad libitum*. They were housed under standard laboratory conditions of $21 \pm 2^\circ\text{C}$, relative humidity 55% and 12 hours light/dark cycle. The *in vitro* tests were performed on blood taken from the rabbits.

Chemicals

Methanol, (Merck®, Germany), heparin (Leo Pharmaceuticals®, Denmark), prothrombin reagent, (Macc and Rains Pharmaceuticals (Pvt.) Ltd. Pakistan), activated partial thromboplastin reagent (Mac and Rains Pharmaceuticals (Pvt.) Ltd. Pakistan), trisodium citrate, (Sigma Chemicals, USA) and EDTA (Sigma Chemicals, USA) were purchased.

Plant collection and extraction

Aerial-parts of the *M. longifolia* were collected from a village of District Gilgit, Shikyote, from Gilgit-Baltistan, Pakistan and authenticated by Dr. Sheer Wali Khan, taxonomist, Department of Botany, Karakoram International University, Gilgit-Baltistan. The aqueous methanol extract was prepared by cold maceration technique by soaking finely powdered plant material in a mixture of aqueous-methanol (70:30) at room temperature for 3 days followed by filtration. Finally, filtrate was evaporated using rotary evaporator and then dried by lyophilizer (Gilani et al., 2005).

In vitro experiments on rabbit blood

Clotting time

In vitro anticoagulant activity of *M. longifolia* on blood clotting was determined by following the method as described by Nwaehujor et al. (2013). In brief, 1.0 mL of blood was drawn from the marginal vein of rabbit's ear and then added in 0.2 mL of 2.5, 5 and 10% w/v solutions of *M. longifolia* extract in different test tubes respectively after incubation at 37°C . Clotting time of the blood was recorded. Distilled water and blood were used as control for baseline clotting time.

Thrombolytic activity

The extract solution of varying concentrations 10, 20, 40 and 80 mg/mL were prepared. Then 0.5 mL of blood from the jugular vein of the rabbit was transferred in the previously weighed and sterilized eppendorff tubes after that incubated at 37°C for the period of 45 min to

clot. Afterwards the serum was completely removed from the tube and weighed the tube again. 100 μL of test solution was added to each Eppendorff tube containing pre-weighed blood clot. In negative control tube, distilled water was added instead of the extract solution. All the tubes were again incubated for 90 min at 37°C and monitored for clot lysis (Alamgeer et al., 2016). Subsequent to incubation, the released fluid was removed completely and the tube was weighed over again. The percentage of clot lysis was expressed as the difference in weight taken before and after clot lysis and calculated by the following formula:

$$\% \text{lysis} = \frac{\text{Weight of released clot}}{\text{Weight of clot}} \times 100$$

Where, weight of released clot = weight of clot before lysis - weight of clot after lysis

Determination of in vitro prothrombin time and activated partial thromboplastin time

Trisodium citrate was added in all the centrifuge tubes followed by the addition of 3 mL of blood sample from the rabbit ($n = 5$) and subjected to centrifugation at 3,000 rpm for 5 min. The plasma was separated using micropipette into the eppendorff tubes. 250 μL of the *M. longifolia* extract solution of different concentrations 2.5, 5 and 10% was added into the eppendorff tubes separately. To determine prothrombin time, samples were incubated at 37°C for 5 min afterward 200 μL of prothrombin time reagent was added to 100 μL of test plasma and clotting time was measured as prothrombin time. Moreover, for appraisal of activated partial thromboplastin time, 100 μL of activated partial thromboplastin reagent was added to 100 μL of test plasma (platelet poor plasma + plant extract of various concentration) and the mixture was incubated for 1 min after which 100 μL calcium chloride (25 mmol) was added and again incubated for 15 sec. Then clotting time was recorded as activated partial thromboplastin time (Alamgeer et al., 2016; Jagtap et al., 2012).

Determination of in vivo bleeding time and clotting time

In vivo bleeding time was appraised by following the procedure of Nwaehujor et al. (2013) with minor modification. Rabbits were segregated into four groups of five rabbits in each group. Aqueous methanol extract was given at 25, 50, and 100 mg/kg dose levels to Group I, II and III respectively. Group IV was taken as control group. Then marginal ear vein was pricked after intervals of 0, 30, 60, and 90 min and after every five seconds filter paper was used to check bleeding on scarred ear vein. Moreover, capillary tube method was used to determine the effect of *M. longifolia* aqueous methanolic extract on clotting time. Extract was administered to four groups of rabbits of five rabbits in each group. Group I was treated with 25 mg/kg, Group II was treated with 50 mg/kg whereas 100 mg/kg was

administered to Group III. One group was served as control. After piercing marginal ear vein, clotting time was noted after 0, 30, 60 and 90 min of dose administration. Blood was collected from pierced vein with the help of capillary tube by horizontally placing it. After every 30 sec, capillary tube was broken till the coagulated blood was shown in the form of thread.

Assessment of coagulation parameters after seven days of in vivo treatment

In determination of anticoagulant activity, seven day treatment was done with aqueous methanolic extract of *M. longifolia* by following the method of Alamgeer et al. (2016) with some modifications. Rabbits were divided into four groups of five rabbits in each group. First group was taken as control group whereas remaining groups were given different doses (25, 50 and 100 mg/kg) of aqueous methanolic extract of *M. longifolia* for a week. After seven days of treatment effect on clotting time was determined by capillary tube method and bleeding time was determined by filter paper method as described by Elg et al. (2001). Moreover, at day 7 blood sample was collected from each rabbit of each group and PT and APTT tests were performed (Alamgeer et al., 2016).

Phytochemical investigation by using HPLC technique

The sample was prepared by the method of Tokusoglu (2003) with minor modification. The sample was prepared by mixing 16 mL of distilled water with 24 mL methanol and 50 mg plant extract. Then 10 mL of 6M HCl was mixed. The mixture was kept in an oven at 95°C for 2 hours to isolate aglycones of flavonol (glyco-

sides) and then it was filtered through microfilter before injecting into the HPLC. An HPLC analysis was performed using HPLC model LC-10A fitted with SCL-10A system control unit, rheodyne injector, two LC-10 AT pumps, CTO-10A column oven, SPD-10A UV-Visible detector and data acquisition class LC-10 software. Then filtered sample (20 µL) was injected into an analytical Shim-Pack CLC-ODS reverse phase (C-18) column (25 cm × 4.6 mm; 5 µm particle size). Solvent A (H₂O: acetic acid- 94:6, pH=2.27) and solvent B (acetonitrile 100%) were used. Graded elution of mobile phase was used to perform chromatographic separation (mixture of solvent A and solvent B (50:50 v/v), which was filtered using 0.45 µm membrane under vacuum before use) at a flow rate of 1 mL/min at 30°C. Detection was performed at a wavelength of 280 nm (Sultana, 2008).

Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis was performed by One-way analysis of variance (ANOVA) with post test Dunnet's. $p < 0.05$ was considered as statistically significant.

Results

Effect on in vitro blood clotting time

The aqueous methanol extract produced significant ($p < 0.001$) and dose-dependent (2.5, 5 and 10%) increase in blood clotting time with maximum effect at 10% extract solution (Figure 1A).

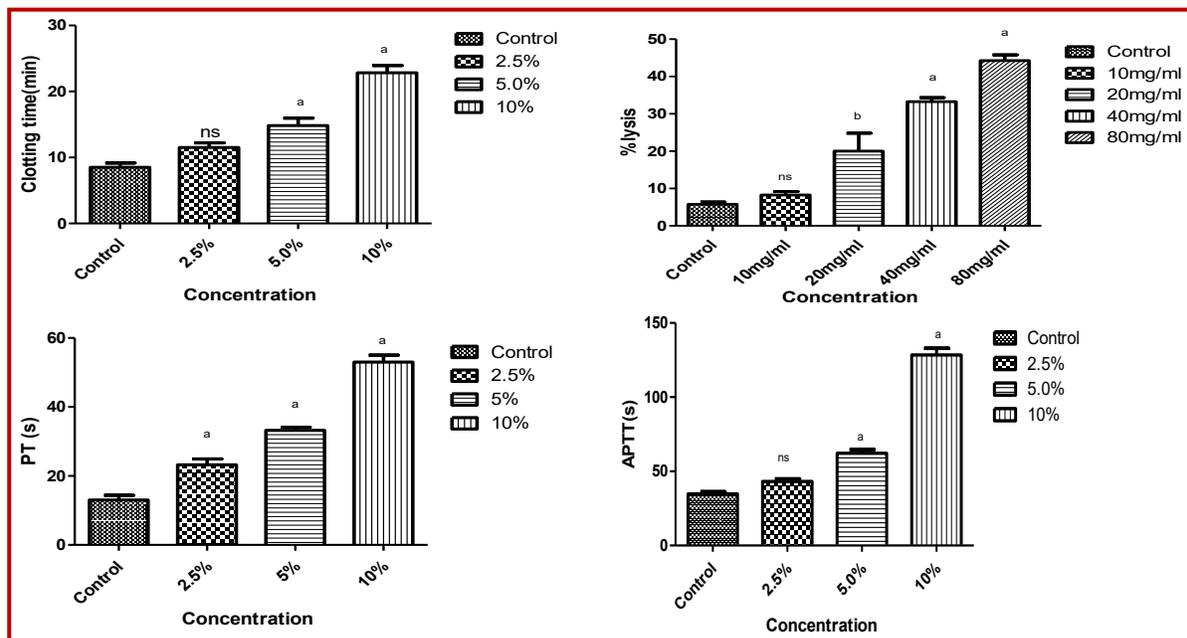


Figure 1: Effect of aqueous methanolic extract of *M. longifolia* on clotting time (A), clot lysis (B), prothrombin time (C), and activated partial thromboplastin time (D) of rabbit blood; ns = non significant and $p < 0.001$, $b p < 0.01$

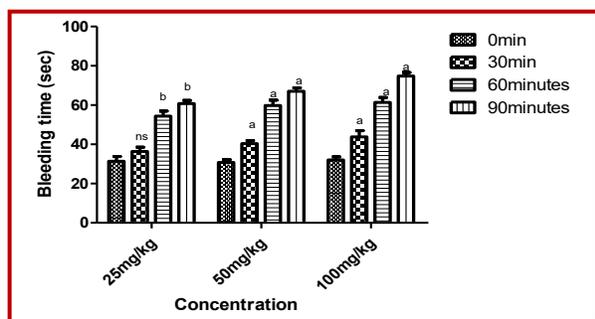


Figure 2: Effects of aqueous methanolic extract of *M. longifolia* on bleeding time of rabbit. Where, ^{ns}non significant, ^b $p < 0.01$ and ^a $p < 0.001$

Effect on *in vitro* thrombolytic activity

The methanolic (70%) extract of *M. longifolia* revealed noteworthy increase ($p < 0.01-0.001$) in clot lysis at 10, 20, 40 and 80 mg/mL with maximum thrombolytic activity at 80 mg/mL (Figure 1B).

Effect on *in vitro* prothrombin time and activated partial thromboplastin time

Different concentrations of plant extract (2.5, 5 and 10%) exhibited considerable ($p < 0.001$) increase in prothrombin time and activated partial thromboplastin time on concentration reliant manner (Figure 1C, D).

Effect of plant extract on *in vivo* bleeding time and clotting time

Plant extract produced substantial ($p < 0.01-0.001$) and dose-dependent (25, 50 and 100 mg/kg) increase in bleeding and clotting time after 30, 60 and 90 min of administration except for 25 mg/kg at 30 min which was non-significant (Figure 2).

Effect on various coagulation parameters after seven days treatment of *M. longifolia* extract in rabbit

The aqueous methanolic extract of *M. longifolia* significantly ($p < 0.1-0.001$) and dose dependently (25, 50 and 100 mg/kg) increased the bleeding time, clotting time, prothrombin time and activated partial thromboplastin time in rabbits (Table I).

HPLC analysis of *M. longifolia* aqueous methanolic extract

The HPLC analysis of the *M. longifolia* aqueous methanolic extract showed the presence of quercetin, gallic acid, caffeic acid, p-coumaric acid, m-coumaric acid, ferulic acid and trans-4-hydroxy-3-methoxy cinnamic acid (Figure 3).

Discussion

From current findings, it has been avowed that the aqueous methanolic extract significantly increased the bleeding time, clotting time, prothrombin time and activated partial thromboplastin time. Although, exact mechanism of anticoagulant activity is not known but heparin, chelating agents and vitamin K antagonists exert their effects by acting at various steps in blood coagulation pathways. Moreover, increase in bleeding time might be considered owing to low platelet count or antilipid peroxidation effect as lipid peroxides cause platelet aggregation (Gadi et al., 2009). In effect inhibition of platelet aggregation increase the bleeding time in animals (De Caterina et al., 1994) as declared from the findings of current analysis.

Clotting time is another major determinant of intrinsic pathway (Dapper et al., 2007). It involves various intrinsic clotting factors e.g.; I, II, V, VIII, IX, X, XI and XII, and any defect in any of these factors may increase or decrease the blood clotting time (Weremfo et al., 2011). These clotting factors are proteinic in nature and are present in resting state in blood pool and when the vessels become damaged they come into action and so play their part in coagulation cascade (Alesci et al., 2009). Aqueous methanolic extract delayed blood clotting as corroborated by results of *in vitro* and *in vivo* studies. As in coagulopathy the most reliable test is prothrombin time test (Hinchcliff et al., 2013; Furlanello et al., 2006). Prothrombin time and activated partial thromboplastin time are the tests that differentiate between the changes in extrinsic pathway or intrinsic pathway of blood clotting. Intrinsic clotting factors are assessed by activated partial thromboplastin time and

Table I

Effect of aqueous methanolic extract on different blood coagulation parameters after seven days of treatment

Blood parameters	Dose			
	Control	25 mg/kg	50 mg/kg	100 mg/kg
Bleeding time (sec)	31.4 ± 2.4	70.6 ± 3.5 ^a	87.8 ± 1.9 ^a	95.4 ± 1.9 ^a
Clotting time (sec)	186.0 ± 3.6	201.6 ± 8.1 ^{ns}	261.2 ± 3.0 ^a	311.4 ± 3.5 ^a
Prothrombin time (sec)	11.2 ± 1.1	19.4 ± 1.9 ^c	27.0 ± 2.6 ^a	33.0 ± 1.6 ^a
Activated partial thromboplastin time (sec)	25.6 ± 1.8	42.4 ± 4.4 ^b	54.8 ± 1.5 ^a	65.8 ± 2.1 ^a

Results are expressed as mean ± SEM; One-way ANOVA followed by Dunnet's t-test; Where, ^{ns}non-significant, ^c $p < 0.1$, ^b $p < 0.01$ and ^a $p < 0.001$ as compared to control

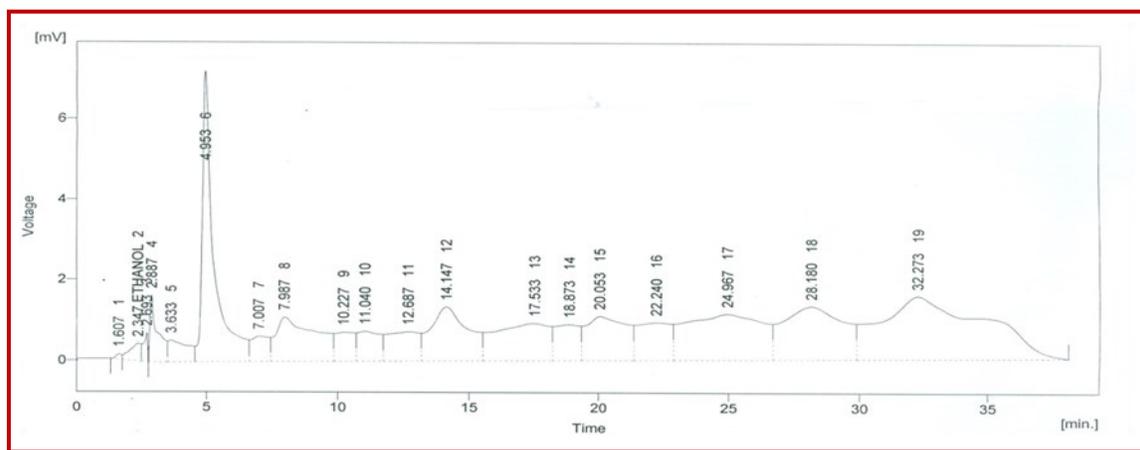


Figure 3: HPLC analysis of aqueous methanolic extract of *Mentha longifolia*

any defect in clotting factors (VIII, IX, XI, XII, and V) and Willebrand's factor is reflected by increase in APTT and decrease in PT reflects abnormalities in coagulation factors V, VII and factor X (Vogel, 2006). Though these two factors are also affected by inhibitors of coagulation factors along with inhibition of phospholipid and Ca^{++} activity (Azevedo et al., 2007). In view of the fact that plant extract delayed both *in vitro* and *in vivo* prothrombin time and activated partial thromboplastin time as declared in results of present study.

It is previously investigated that coumarins are involved to decrease the coagulation extent (Pochet et al., 2004). Likewise *M. longifolia* also contains coumarin as important part of its active constituents (Shah et al., 2010) as reported in HPLC results. Coumarin is the major organic compound in phytochemicals that is said to have particular anticoagulant activity (Morton, 1992) and its derivatives are reported to increase bleeding time in animals. In liver, synthesis of some clotting factors II, VII, IX and X depends on the presence of vitamin K where inactivated groups and coumarins present in anticoagulant drugs antagonize the synthesis of coagulant proteins and hence interferes with the formation of blood clot (Dejana et al., 1979). Furthermore these constituents interfere the metabolism of vitamin K and so stop the synthesis of vitamin K dependent coagulation factors (Meyer et al., 1992; Radositis et al., 1997). Some researchers have reported that thrombolytic action of plants may be attributable to presence of tannins, saponins and alkaloids. Seeing that *C. xanthocarpa* has shown inhibition of platelet aggregation on account of terpenes, saponins, tannins and flavonoids (Klafke et al., 2010) and flavonoids and coumarins are responsible for antithrombotic activity of *Peucedanum grande* (Nijveldt et al., 2001; Heim et al., 2002). In addition, numerous earlier studies advocate the presence of flavonoids (quercetin, quercitrin, myricetin and rutin), tannins and phenolic compounds in plants having antithrombotic activity (Schmeda-

Hirschmann, 1995; Markman et al., 2004; Hsu et al., 2008). It is hypothesized that plant exert thrombolytic activity which might be due to the presence of some flavonoids that inhibit platelet aggregation by binding to thromboxane A_2 receptors (Guerrero et al., 2005; Han et al., 2012).

Conclusion

Flavonoids, coumarins and other phytoconstituents may participate in the thrombolytic action of *M. longifolia* as stated in HPLC analysis.

Financial Support

University of Sargodha

Ethical Issue

The study protocol was approved by the Institutional Animal Ethics Committee, Faculty of Pharmacy, University of Sargodha. All experiments are designed in accordance with the standard procedures of National Research Council (NRC, 1996).

Conflict of Interest

Authors declare no conflict of interest

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