


Meriandra dianthera Aqueous Extract and Its Fraction Prevents Blood Coagulation by Specifically Inhibiting the Intrinsic Coagulation Pathway: An in vitro Study

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Background: Currently, cardiovascular disorders are the primary cause of mortality in the world and constitute a serious medical problem. Blood coagulation is an essential process to prevent excessive blood loss through injured blood vessels; however, abnormal blood clotting in the blood vessels can result in fatal cardiovascular disorders. This study investigated the in vitro anticoagulant activity of *Meriandra dianthera* crude extract and its fractions and their erythrocyte membrane stabilizing activity.

Methods: The plant leaves were extracted by a decoction method and were further fractionated by a liquid–liquid partition with a solvent of crescent polarity. The in vitro anticoagulant activity of the plant extract and its fractions was assessed by PT and APTT assays, while the membrane stabilizing activity was determined through hypotonic induced hemolysis.

Results: The crude aqueous leaf extract of *Meriandra dianthera* significantly ($P < 0.001$) prolonged the intrinsic clotting pathway measured by APTT by specifically acting on the intrinsic coagulation pathway. By using liquid–liquid fractionation, the residual aqueous fraction was identified as the fraction responsible for the anticoagulant activity of the crude extract as it significantly ($P < 0.001$) prolonged APTT while the other fractions failed. Both the crude extract and its aqueous residue fraction did not affect the extrinsic coagulation pathway measured by PT. In the membrane stabilizing assay, crude extract and aqueous residue fraction showed the highest membrane stabilizing activity.

Conclusion: The crude extract and its aqueous residue fraction showed a potent in vitro anticoagulant and membrane stabilizing activity, which shows the potential of the plant's leaves as a new source of bioactive molecules for coagulation-related disorders.

Keywords: blood coagulation, in vitro, anticoagulant, fractionation, medicinal plant, prothrombin time, APTT, *Meriandra dianthera*

Introduction

The critical mechanism to maintain the delicate equilibrium between hemorrhage and thrombosis through anticoagulants and pro-coagulants and thereby to retain blood in the blood vessels during injury is achieved through the process of hemostasis. Hemostasis is attained through primary and secondary mechanisms. Primary hemostasis is the formation of a platelet plug at the site of damage in response to vascular injury. The secondary hemostasis includes a proteolysis process in which precursors of the coagulation factors are activated to accelerate the formation of the subsequent clotting factor,¹ which is accomplished via the intrinsic and extrinsic pathways,² which converge in the common pathway to convert soluble fibrinogen into insoluble fibrin polymers. The polymers result in the formation of a clot by creating a meshwork that is reinforced by platelets and other blood components.³ This coagulation process is vital to avoid excessive blood loss through the injured blood vessels; however, any abnormal blood clotting in the blood vessels can result in fatal cardiovascular disorders.⁴ Currently, cardiovascular

disorders are the primary cause of mortality in the world and constitute a serious medical problem and it is predicted that these disorders will cause mortality of almost 23.6 million people by 2030.⁵

Anticoagulants are a class of drugs that inhibit the formation of a clot in the blood vessels by affecting either the availability or activation of blood coagulation factors and platelets. However, these treatments frequently result in life-threatening side effects like thrombocytopenia, bleeding events, and osteopenia.⁶ Therefore, the alarming limitations of the currently used anticoagulants have stimulated the search for alternative new agents from natural sources.⁷

Plants, due to their effect on the biological process, could be a new source for the development of novel anticoagulant agents. Medicinal plant-based drugs in addition to being simple and effective, they also show a broad spectrum of activity with special attention on preventive action. There is compelling scientific evidence that indicates the intake of dietary anticoagulants or phytochemicals with anticoagulant activity can eventually diminish or eliminate the risk of thromboembolic diseases.⁸ Herbs like garlic are commonly sold in the market as blood thinners and their anticoagulant activity has been shown by studies.⁸ However, despite the popular use of *Meriandra dianthera* in traditional medicine no study has been done, to the best of the author's knowledge, to scientifically determine its in vitro anticoagulant activity.

Meriandra dianthera (or its synonym name *Meriandra bengalensis*) is a plant belonging to the Lamiaceae family, locally known as Nihba/Mezaguf. Traditionally, its leaves are reported to be used to treat hypertension, malaria, gastritis, bronchial asthma, and infectious hepatitis.⁹

Materials and Methods

Plant Collection and Authentication

Fresh leaves of *Meriandra dianthera* were collected from Adi-nfas, North of Asmara, Capital city of Eritrea. The botanical identification and authentication of the plant was done by a botanist from the Eritrean Institute of Technology (EIT). A voucher specimen (No MD-R-0012) was placed in the herbarium, Asmara College of Health Sciences, Department of Biochemistry.

Plant Preparation

The collected plant leaves were thoroughly washed with distilled water to remove dirt, mud, or filthy particles present on the surface and were shade dried at room temperature for 2 weeks. The shade dried plants were then powdered using mortar and pestle for further extraction purposes.

Hot Aqueous Extraction (Decoction)

The hot water extraction from the leaves of *Meriandra dianthera* was performed according to the method described by a previous study⁷ with some modifications. A total of 100 g of powdered *Meriandra dianthera* leaves were extracted for 5 h at 100°C with 1 L distilled water. Then, the hot extract was filtered using grade No. 1 filter paper. Rotary evaporator was used to concentrate the filtrate under reduced pressure. Finally, the semi-liquid product was further dried in a water bath at 60°C, and was stored at -20°C until further analysis.

Fractionation of *Meriandra dianthera* Hot Crude Extract

The leave crude extract was further fractionated by a liquid-liquid partition with increasing polarity of solvents (ie, chloroform, ethyl acetate, butanol) to separate the crude extract compounds according to their solubility in their respective solvents to obtain four different fractions of chloroform, ethyl acetate, butanol, and aqueous residue fractions.

Blood Sample Collection and Preparation of Pooled Plasma

Blood was obtained from 10 healthy individuals by using sterile syringes from the median cubital vein on their left arm. Volunteers who were suffering from any cardiovascular diseases, diabetes, obesity, dyslipidemic disorders, or smokers and those who recently used nonsteroidal anti-inflammatory drugs, oral contraceptives, or anticoagulant therapy were excluded from the blood sample collection. The collected blood sample was transferred into a vacutainer containing 3.2% trisodium citrate to prevent the clotting process, and centrifugation was done at a rate of 3000 rpm for 15min using the

HERMLE benchtop centrifuge to obtain platelet-poor plasma. The obtained plasma sample of each individual was pooled in a plain tube using a pipette and was analyzed immediately.

In vitro Blood Coagulation Study

In this study, the action of the plant extracts in the intrinsic and common blood coagulation pathways was evaluated by the Activated Partial Thromboplastin Time (APTT) assay and that of the extrinsic pathway was determined by the Prothrombin Time (PT) assay.

Activated Partial Thromboplastin Time (APTT) Assay

In vitro intrinsic anticoagulant pathway was measured by Activated Partial Thromboplastin Time (APTT). Human normal pooled plasma (200 μ L) was mixed with 100 μ L of different concentrations of *Meriandra dianthera* crude extract and fractions (500 μ g/mL, 1 mg/mL, 2 mg/mL, 5 mg/mL, and 10 mg/mL). Meanwhile, APTT automated reagent was reconstituted and subjected to APTT assay, together with 0.025 M calcium chloride. The clotting time was then recorded by an automated coagulometer (ACL elite coagulation analyzer, Instrumentation Laboratory Company). Plasma with vehicle (0.9% normal saline) was used as a negative control (absence of anticoagulant activity) and heparin (1 IU/mL) was used as a positive control. The tests were done in triplicate for each concentration.

Prothrombin Time (PT) Assay

In vitro extrinsic anticoagulant pathway was measured by Prothrombin Time (PT) assay. Human normal pooled plasma (200 μ L) was mixed with 100 μ L of different concentrations of *Meriandra dianthera* crude extract and fractions (500 μ g/mL, 1 mg/mL, 2 mg/mL, 5 mg/mL, and 10 mg/mL). The PT automated reagent was reconstituted and subjected to PT assay, together with 0.025 M calcium chloride. The clotting time was then recorded by an automated coagulometer (ACL elite coagulation analyzer, Instrumentation Laboratory Company). Plasma with vehicle (0.9% normal saline) was used as a negative control and heparin (1 IU/mL) was used as a positive control. The tests were also done in triplicate for each concentration.

Membrane Stabilizing Assay

The membrane stabilizing activity of the extract and its fractions was evaluated by using hypotonic-solution induced human erythrocyte hemolysis, as previously done with slight modification.¹⁰ The assay mixture consisting of 0.5 mL of 2% (v/v) human erythrocyte suspension mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract at different concentrations or the reference drug, acetylsalicylic acid (0.1 mg/mL). The mixtures were incubated for 10 minutes at room temperature and centrifuged at 3000 rpm for 10 minutes. The absorbance (OD) of the supernatant was measured at 560 nm using a spectrophotometer. The percentage inhibition of hemolysis was calculated according to the following equation:

$$\% \text{ Inhibition of hemolysis} = 100 \times (\text{OD control} - \text{OD test sample} / \text{OD control})$$

Qualitative Phytochemical Analysis

The crude extract and all its fractions were subjected to preliminary phytochemical screening for the presence of alkaloids, flavonoids, anthraquinone, C-glycosides, O-glycosides, saponin, phenol, tannin, sterols, Terpenoids following the procedure.^{11,12}

Data Analysis

All experiments were performed in triplicate, and the results were expressed as mean \pm SEM. The data were analyzed by using one-way analysis of variance (ANOVA) with Tukey's HSD as the post hoc in SPSS software version 20. Significant differences for all data sets were measured and designated as * $P < 0.05$ and ** $P < 0.01$.

Ethical Consideration

This research received ethical clearance from the Asmara College of Health Science research ethical committee with Approval Number of ACHS/REC/2571. The volunteers that participated in the study were ahead informed regarding the full scope of the study and verbal consent followed by written informed consent was obtained.

Result and Discussion

The study was designed to investigate the in vitro anticoagulant activity of hot aqueous extract of *Meriandra dianthera* and its fractions. The percent extraction yield for crude extract and fractions of the plant is shown in Table 1. A total of 300 g leaf part of *Meriandra dianthera* was extracted by decoction method and gave a yield percent of 16.88% crude extract. Thirty grams of crude extract were then used for liquid–liquid partition to obtain a different percent of the four fractions.

The in vitro anticoagulant activity was measured by APTT and PT assays. APTT is able to measure the inhibition of intrinsic factors of the blood coagulation pathway such as factor XII, XI, IX, VIII, and V, while the PT assay gives a clear picture of the extrinsic pathway factors such as factors I, II, V, X and VII.¹³

The crude extract significantly prolonged the intrinsic clotting time to 52.63±0.26 s and 59.16±4.7 s at a concentration of 5 mg/mL and 10 mg/mL respectively compared with the control of 30.76±1.87 s (Figure 1). Aqueous residue fraction showed the most potent anticoagulant activity as it significantly prolonged ($P < 0.01$) the intrinsic clotting time even at the lowest concentrations of 500 µg/mL, 1mg/mL and 2 mg/mL into 42.8±1.77 s, 49.73±4.09 s, 52.8±7.31 s, respectively. At higher concentrations of 5 mg/mL and 10 mg/mL, the aqueous residue fraction also showed a highly significant increase ($P < 0.001$) in the intrinsic clotting time into 74.7±3.41 s and 125±13.98 s, respectively (Figure 2).

In the APTT assay, it is noteworthy to highlight that the aqueous residue fraction was more active compared to the crude extract. This indicates that the active anticoagulant constituents were concentrated in the aqueous residue fraction after the crude extract was purified by a liquid–liquid partition. The above explanation was substantiated by the fact that the remaining fractions, ie, the chloroform, ethyl acetate, and n-butanol fractions failed to increase the clotting time significantly even at higher concentrations (Figures 3–5). A similar result was obtained from a previous study conducted on aqueous leaf extract of *Jatropha gossypifolia* which also found the residual aqueous fraction to be the fraction responsible for the anticoagulant activity in the crude extract of the plant.¹⁴ Considering the fact that prothrombin time was not affected by the crude extract and its fractions rule out the effect of the plant's active constituents on the extrinsic and common pathway, clotting factors and also calcium chelating activity. Heparin, the standard drug used in this study, in the existence of antithrombin prolongs the APTT assay by inactivating factor XII, XI and IX.² Although further study is required to identify the exact mechanism of action of the plant and its effect on individual coagulation factors, this study shows that the plant *Meriandra dianthera* exerts its potent anticoagulant activity by specifically acting/inhibiting on any of the factors involved in the intrinsic pathway.

In addition to their anticoagulant activity, the crude extract and its fractions were also evaluated for their red blood cell membrane stabilizing activity. The anti-inflammatory activity of the *Meriandra dianthera* extract and its fractions

Table 1 Percent Yield of Extraction and Fractions of *Meriandra dianthera* Leaves

Extract Type	Weight of Dried Powdered Leaves/ Crude Extract	% Yield
Crude extract	300 g*	16.68
Chloroform fraction	30 g**	4
Ethyl acetate fraction	30 g**	11.6
Butanol fraction	30 g**	20
Aqueous residue fraction	30 g**	64.4

Notes: *Indicates yield is calculated after extraction from the initial dried powdered plant leaves while. **Indicates yield is calculated after fractionation from the initial crude extract obtained from the plant leaves.

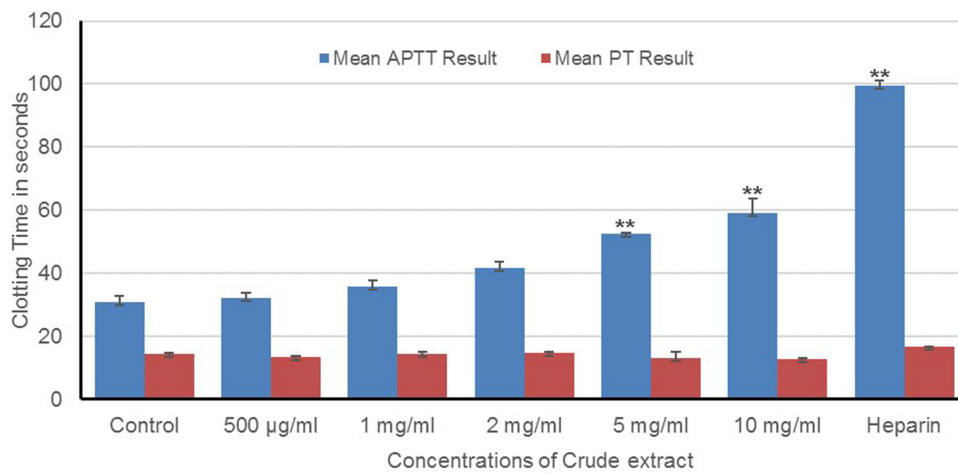


Figure 1 Prothrombin time (PT) and activated partial prothrombin time (APTT) of normal human plasma treated with different concentrations of crude extract. Results are expressed as Mean ± SEM, **P<0.01.

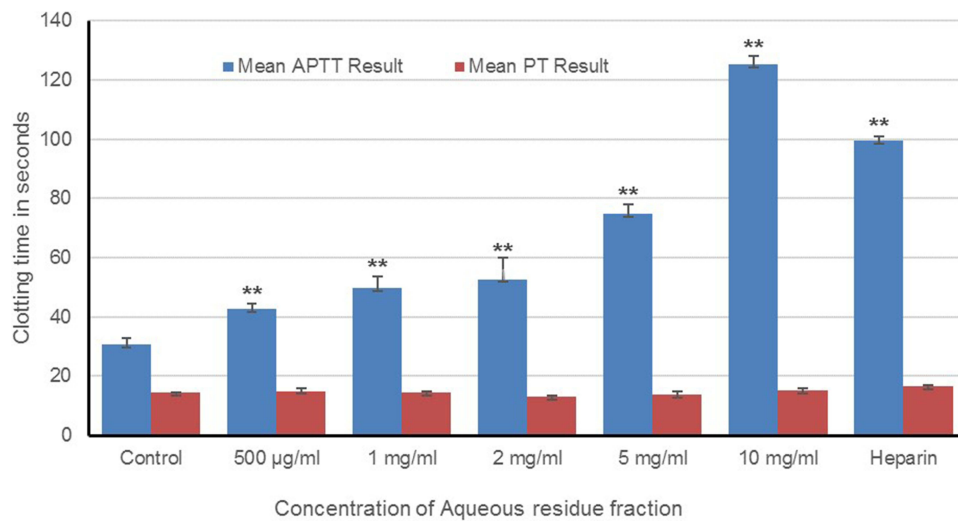


Figure 2 Prothrombin time (PT) and activated partial prothrombin time (APTT) of normal human plasma treated with different concentrations of aqueous residue fraction. Results are expressed as Mean ± SEM, **P<0.01.

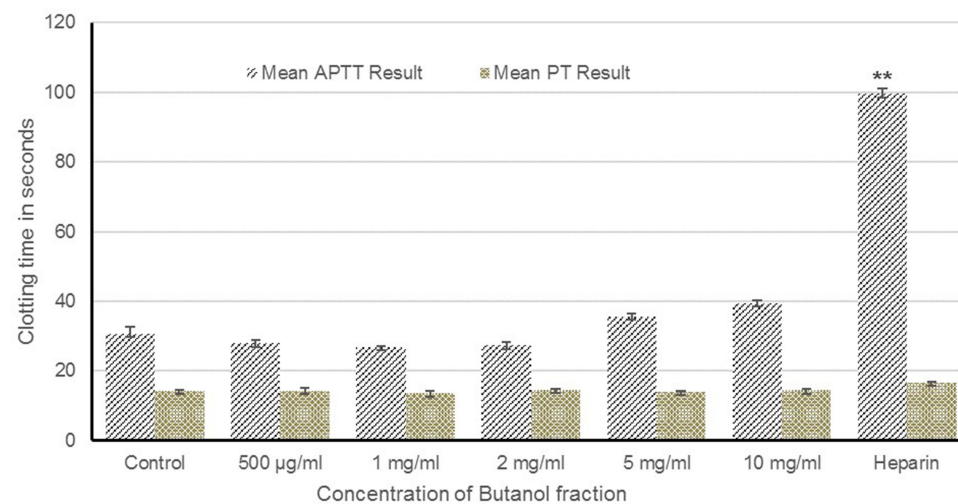


Figure 3 Prothrombin time (PT) and activated partial prothrombin time (APTT) of normal human plasma treated with different concentrations of butanol fraction. Results are expressed as Mean ± SEM, **P<0.01.

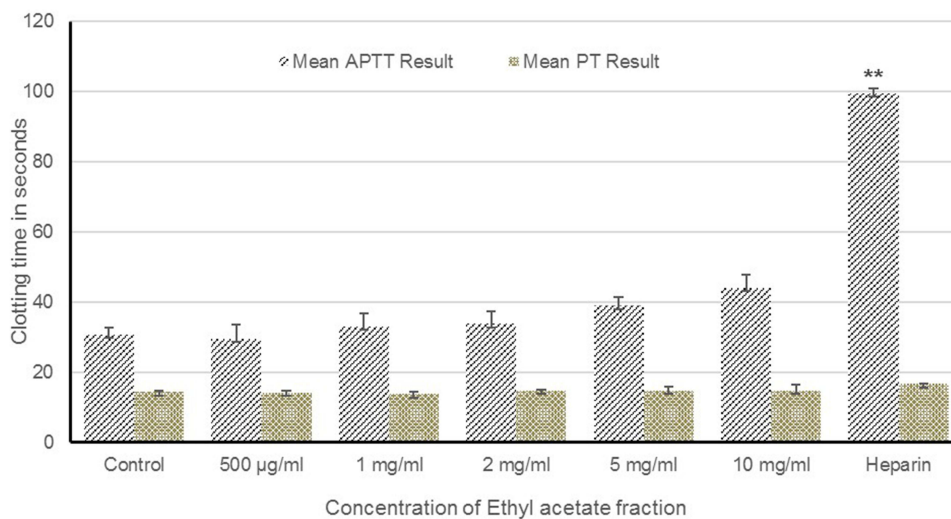


Figure 4 Prothrombin time (PT) and activated partial prothrombin time (APTT) of normal human plasma treated with different concentrations of ethyl acetate fraction. Results are expressed as Mean \pm SEM, ** $P < 0.01$.

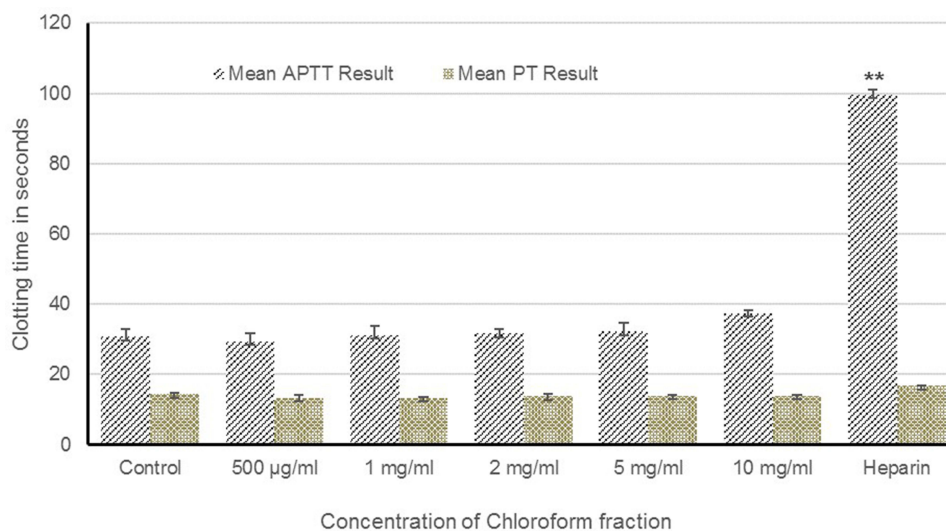


Figure 5 Prothrombin time (PT) and activated partial prothrombin time (APTT) of normal human plasma treated with different concentrations of chloroform fraction. Results are expressed as Mean \pm SEM, ** $P < 0.01$.

were assessed by the hypotonic solution-induced RBC membrane lysis inhibition assay. The osmotic fragility of the red blood cell is usually used to assess the effects of chemicals on the cell membrane as an *in vitro* assay.¹⁵ The erythrocyte membrane also functions as a model for lysosomal membrane and the RBC membrane stabilizing assay serves as a technique for the rapid screening of potential anti-inflammatory compounds. This is due to the observation that numerous agents capable of damaging the erythrocytes also result in releasing hydrolytic enzymes from lysosomes.¹⁶ In this study, as can be seen in Figure 6, the aqueous residue fraction followed by crude extract showed the highest membrane stabilizing activity of 76% and 73% respectively at a concentration of 3 mg/mL indicating their potency in preventing erythrocyte and lysosomal membrane lysis and also showing that the plant leaves also possess anti-inflammatory activity. The other fraction showed different levels of protection.

In the preliminary phytochemical screening, the crude extract and aqueous residue fraction which showed the highest membrane stabilizing activity were found to contain flavonoids, tannin, and saponins which is in line with previous studies that showed the role of these phytochemicals in stabilizing the erythrocyte and lysosomal membrane.^{17,18} The

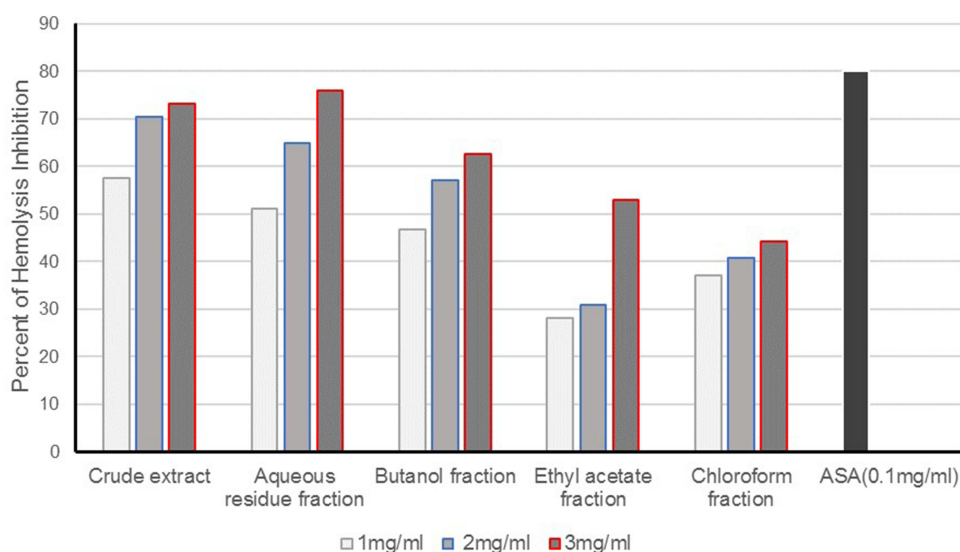


Figure 6 Membrane stabilizing activity of *Meriandra dianthera* crude extract and its fractions on hypotonic solution-induced hemolysis of erythrocyte membrane. ASA, acetylsalicylic acid. Values are expressed as mean \pm SEM.

preliminary phytochemical test results of the crude and its fractions indicate the varying presence of flavonoids, phenols, saponins, sterols, terpenoids, tannins, anthraquinone, c-glycosides, and o-glycosides (Table 2). The presence of this wide range of phytochemical constituents indicates that the *Meriandra dianthera* leaf possesses numerous biologically active compounds, which could serve as a potential source of drugs in herbal medicine.

Conclusion

This study has clearly shown that the hot aqueous leaf extract of *Meriandra dianthera* has a potent in vitro anticoagulant activity. Further purification of the crude extract showed that the residual aqueous fraction was the fraction with the highest anticoagulant activity by specifically acting on the intrinsic coagulation pathway. This shows the plants promising potential as a new source of bioactive molecules for therapeutic purposes. In addition to the anticoagulant activity, the crude extract and residual aqueous fraction also showed the highest activity in the inhibition of hemolysis in red blood cells. We, therefore, recommend further

Table 2 Result of the Preliminary Phytochemical Analysis on *Meriandra dianthera* Crude Extract and Its Fractions

Tests	Crude Extract	Aqueous Residue Fraction	Butanol Fraction	Ethyl Acetate Fraction	Chloroform Fraction
Alkaloids	+	+	+	+	+
Flavonoids	+	+	+	+	+
Anthraquinone	-	-	-	-	-
C-glycosides	+	+	+	+	+
O-glycosides	+	+	+	+	+
Saponins	+	+	-	-	-
Phenol	+	+	+	+	+
Tannins	+	+	+	+	-
Sterols	-	-	-	-	-
Terpenoids	+	+	+	+	+

Notes: (+) sign denotes the presence of corresponding phytoconstituents and (-) sign denotes the absence of corresponding phytoconstituents.

identification and biological characterization of the anticoagulant potent molecules from this plant and to identify the specific clotting factors in the coagulation cascade that are most sensitive to the action of this plant.

Abbreviations

APTT, activated partial thromboplastin time; PT, prothrombin time; OD, optical density; ASA, acetylsalicylic acid; mg, milligram; mL, milliliter.

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Disclosure

The authors report no conflict of interest in this work.

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