

In-vitro Anticoagulant and Antiplatelet Activity of *Artemisia herba-alba* Assoextract

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Abstract: *Background:* Cardiovascular diseases involving deep vein thrombosis (DVT), strokes, and heart attacks are the main causes of morbidity and mortality in countries throughout the world. In recent years, many people have begun to use traditional (alternative) medicine in the treatment of the majority of diseases because it is natural, cheap, and safe with minimal or no side effects when compared to chemical substances. *Objective:* The present study aimed to determine the in vitro anticoagulant and antiplatelet activity of the *Artemisia herba-alba* Asso extract. *Method:* The blood samples used in the evaluation of the anticoagulant and antiplatelet activity of the plant extract in this study were taken from healthy volunteers. Prothrombin time (PT) and clot retraction (CR) tests were performed before the addition of *A. herba-alba* Asso extract (as controls) and after the addition of the extract at different concentrations (2%, 4%, and 8%) as the test group. The results were analyzed statistically using SPSS. *Results:* A significant increase in the means of the PT and CR tests was revealed upon incubation of the *A. herba-alba* Asso extract with normal blood samples ($p < 0.05$) for all concentrations in both the PT and clot retraction tests. *Conclusion:* This study has demonstrated that the *A. herba-alba* Asso extract has strong anticoagulant and antiplatelet activity, so it may be used for the management of thrombotic diseases.

Keywords: DVT: Deep Vein Thrombosis, PT: Prothrombin time, CR: Clot Retraction

1. Introduction

Worldwide human health is facing a number of challenges such as stroke, acute coronary syndrome and other ischemic events. Although industrialized countries are witnessing a decrease in cardiovascular disease incidences, there has been rapid increase in these cases in developing nations. The foundation of cardiovascular disorders is from acute atherothrombotic events. Arterial thrombosis is a process wherein overly active hemostatic system leads to excess production of platelets resulting in abatement of blood circulation in cerebral, peripheral or coronary arteries. This obstruction may sometimes lead to ischemic stroke, myocardial infarction or limb gangrene (1).

Platelets are an essential component of human body required for repair of endothelium and primary hemostasis. However, when in excess, platelets lead to formation of occlusive platelets thrombus (2,3). Even during vascular injuries, these platelets by stimulating the blood coagulation contribute to the repair of damaged blood vessels. However, the presence of coagulation cascade produces factor Xa which in turn catalyzes prothrombin into thrombin (4). This conversion in turn transforms fibrinogen into fibrin which promotes platelet aggregation and causes vascular atherothrombotic disorders. Coagulation and platelet activation inhibition is the strategy used for treating thrombotic disease via biologically or chemically tested drugs. The downside to this phenomenon is that these drugs lead to adverse effects such as neutropenia, thrombotic thrombocytopenia, or hemorrhaging. Thus, recent development has supported the usage of herbal medicines for treating cardiovascular diseases (5–7).

The World Health Organisation (WHO) in 2008 stated that about 80% of the African and Asian population uses traditional medicines for treatments of different disorders. There are various plants which are used ethno medicinally for blood related treatments in order to treat hemorrhoids,

control excessive bleeding, and have dressing of wound to staunch blood flow. The usage of garlic saponins increases membrane fluidity and taxanes for microtubule stabilization in maintenance of disaggregated platelets. Harmaline and Harmaline-induced reduction of tyrosine phosphorylation limits arachidonic acid liberation and calcium mobilization for decreasing platelets aggregation. Many photochemicals like sulfated polysaccharides, allicin, polyphenols, or lapachol can be used for coagulation cascade attenuation through decreased activity or inhibition of thrombin, plasminogen activator, thiol enzymes, tissue factor or other clotting factors. These sources help in developing cheap, toxic, and new therapeutic approaches and controlling the adverse effects of chemical and biological drugs (8,9).

Among the various herbal medicines used for dealing with platelets activation and coagulation, *Artemisia Herba-alba* is one of them. Belonging to the *Asteraceae* family, it is a perennial dwarf shrub that grows in semi-arid and arid climates i.e. deserts of Middle East, Spain, North Africa, and even the north western Himalayas (10). The *Asteraceae* family consists of many medicinal and aromatic plants such as *Santolina*, *Artemisia*, *Chrysanthemum*, or *Centaurea*. These plants belong to *Anthemideae* tribe which is largest *asteraceae* family with around 300 herbs and shrubs (11,12). *A. herba-alba* being a native plant of Jordan (known as Shih), it is locally used for folk medicine due to its richness in essential oils, lactones, sesquiterpene, and other chemical compounds (10). Named as “*chih*” or “*desert wormwood*” in North Africa and other areas, this plant is a popular traditional medicine herb used to cure diabetes, calm abdominal pain, abscess, bronchitis, diarrhea, and even work as antispasmodic, analgesic, or diuretic agent (13).

The *A. herba-alba* is the greenish silver perennial herb, which grows to the height of 20-40 cm and is a chamaephyte i.e. its bud fosters new plant growth every year close to ground. The stem of this plant is erect and rigid and the leaves of flowering plants are small. Leaves of the plant are alternate, grey, ovate, 2-3 pinnatisect, sessile,

Volume 9 Issue 12, December 2020

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and covered with glandular hairs. Flowering head are oblong, tapering at base, and sessile. The plant flowers from September to December and is found in Iberian Peninsula and centre of Spain in abundance (14–16). It is considered that the plant has 8 flavonoid glycosides i.e. vicenin-2, isovitexin, schaftoside, 3-glucosides, isoschaftoside, and 3-rutinosides of patuletin and quercetin (17–20). As the plant extract has shown many biological activities like insecticidal, antibacterial, neurological, and antioxidant activities (21), this study aims at evaluating the *in vitro* anticoagulant and antiplatelet activity of *Artemisia Herba alba* extract.

2. Materials and methods

This experimental study was done at Najran area from January 2020 to October 2020. The study population were 50 blood samples collected from healthy individual from Najran University.

Inclusion criteria

Samples were collected from apparently healthy individuals included both sex with age between (18–30) were included. Smokers, history of bleeding or thrombosis and people under anticoagulant therapy were excluded.

Study design and plant materials

This experimental study was evaluated the anticoagulant and antiplatelets activity of *artemisiahherba alba asso* methanolic extract at a concentration (2%, 4%, 8%). The plant of *Artemisia herba alba asso* was collected from herbal market in Najran.

Preparation of the methanolic extract

200 g of the powder was soaked for 5–7 days with 2000 ml of 80% methanol at 25°C. After filtration, the filtrate was evaporated with a rotary evaporator to remove the methanol under reduced pressure at 50°C. The dry crude extract of the plant samples was stored in the refrigerator in a dark glass bottle until use.

Preparation of platelet poor plasma (PPP)

Whole blood was collected from healthy volunteers into 3.2% sodium citrate Vacutainer. Then the citrated whole blood was centrifuge for 15 min at 3500–4000r at room temperature and the supernatant was obtained as platelet-poor plasma (PPP). For accurate results, a proportion of blood and citrate in the test tube must be fixed (1:10), before being placed in a centrifuge. The sample was visually verified in order to determine if there were any clot formation within the tube, because in that case the test results would not be valid. All samples were stored at 25°C until the time of testing.

Preparation of different concentrations (2%, 4%, 8%)

Three diluted solutions were prepared from the stock solution of plant extract as follows: 8 gram of the stock was dissolved in 100ml of 80% methanol to make 8% concentration, then 50ml from first diluents (8%) was added to 50ml of methanol to prepare 4% concentrate. Finally 50ml of 4% concentration was added to 50ml methanol to achieve 2% concentration.

Coagulation assay

The human plasma coagulation assay were performed using the prothrombin time kits and equipment. The assay were performed according to the diagnostic kit's manufacturer's instructions from BLOOD COMPANY, coagulation assay were performed by using water path at Najran University.

Artemisia Herba Alba extract was added to the normal blood samples as solutions in different concentrations (2%, 4%, 8%) and coagulation test was done to assess *in vitro* anticoagulant effect of the extract.

All samples separated into four different groups, group one serve as a control in which the samples assayed into reagent protocol purely (100µl of sample +200 µl of reagent). Group Two, three and four in which samples were mixed with extract concentration (2%, 4%, 8%) respectively, then incubated in water path 3–5 minutes and then assayed according to reagent protocol (10µl extract concentration +90µl plasma+200µl reagent). The result obtained by observing formation of fibrin strand and the time was recorded. As quality control, samples were mixed with methanol and incubated 3–5 minutes, then assayed according to reagent protocol (10µl methanol+90µl plasma+200µl reagent) to exclude the effect of methanol.

Platelet function assay

The human platelet function assay was performed using the simple clot retraction test and their equipment by using water path.

Artemisia herba alba asso extract was added to the normal whole blood sample by using different concentration (2%, 4%, 8%) from the stock, and the clot retraction test was done to assess the *in vitro* antiplatelet function of the extract.

All samples were separated into four different group, group one serve as a control in which there were no addition (1ml of blood incubated in water path at 37°C and read the time which the clot was take to retract). Group two, three and four which mixed samples with different concentration of extract (2%, 4%, 8%) respectively (100µl extract +1ml blood incubated in water path and read the time of clot retraction). As quality control samples were mixed with 80% methanol (100µl methanol+1ml blood incubated in water path at 37°C and the time of clot retraction was recorded .

Ethical considerations

The Ethics and Research Committee of Najran University approved the study protocol. The purpose of this study was clarified and discussed with the participants. Samples were taken with informed consent from donor.

Statistical analysis

Data were reported as mean ± SD, and a one-way ANOVA test was used to compare the mean values of the control and the different concentrations of extracts, as well as to calculate the p-values. Correlations were used to compare the different concentrations. A p-value of less than 0.05 was considered statistically significant.

3. Results

Impact of A. herba-alba on PT test

Multiple comparisons of mean values of PT when Incubated with A herba-alba methanolic extract in different concentrations (2%, 4%, 8%) were (19.30 ± 3.56, 22.30±4.11, 26.30 ± 5.27) respectively as compared with the mean value of controls (14.30 ± 1.94), the results were statistically significant with (P. value < 0.001) for 4% and 8% concentrations and (P. value = 0.034) for 2% concentration as shown in figure (1), table (1) and (2). There were no statistical significant differences between methanolic extract of different concentration (2% and 4%) and (4% and 8%) P. value were (0.331 and 0.120) respectively. Comparison between 2% and 8% concentration show statistical significance with (p. value = 0.002) table (3).

Table 1: The base line statistics of Prothrombin time (sec) for control and three different concentrations of the extract

	NO	Minimum	Maximum	Mean± S.D
Control	50	10.00	16.00	14.30±1.95
2%	50	14.00	27.00	19.30±3.56
4%	50	17.00	30.00	22.30±4.11
8%	50	22.00	36.00	26.30±5.27

Table 2: Multiple Comparisons between mean PT value (sec) of control and varied concentrations

I	II	Mean (I)	Mean (II)	P.value
PT (Sec) control	2%	14.30±1.95	19.30±3.57	0.034
	4%		22.30±4.11	< 0.001
	8%		26.30±5.27	< 0.001

One way anova test was used P-value ≤0.05: considered significant

Table 3: Multiple Comparisons of mean of PT (sec) in different concentrations of methanolic extraction

I	II	Mean I	Mean (II)	P. value
2%	4%	19.30±3.56	22.30±4.11	0.331
	8%		26.30±5.27	0.002
4%	8%	22.30±4.11	26.30±5.27	0.120

One way anova test was used P-value ≤0.05: considered significant

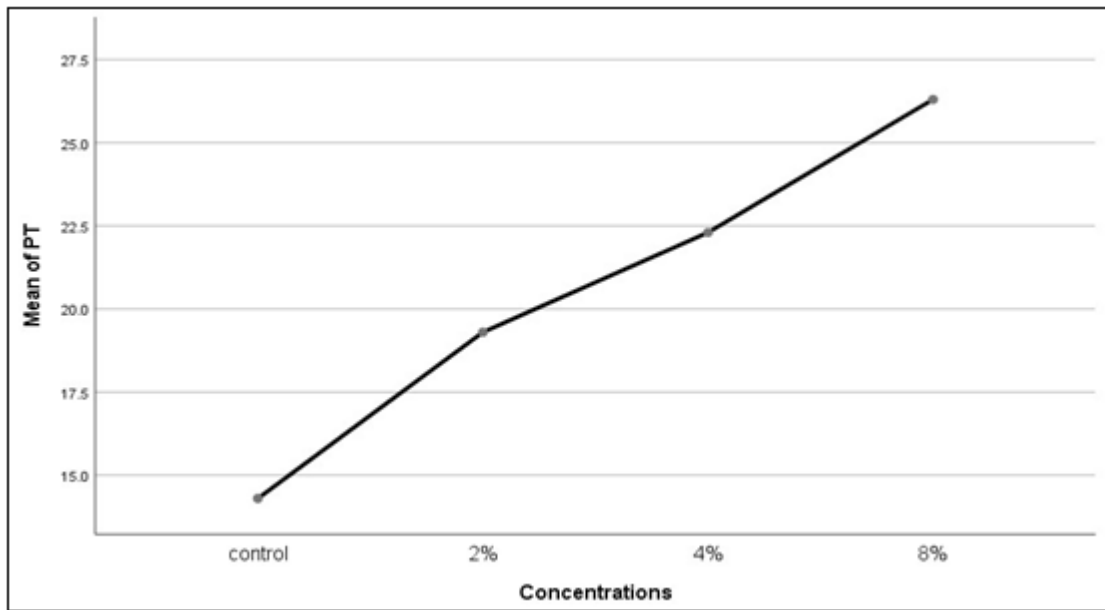


Figure 1: Show the mean of PT (second) at control and different concentrations of the extract (n=50)

Impact of A. herba-alba on Clot Retraction test:

The highest mean of clot retraction time were found when plasma incubated with A herba-alba extraction in 8% concentration (23:31± 0:58) hours as explained in table (4) & figure (2). Multiple Comparisons of mean of clot retraction when Incubated with A herba-alba methanolic extract in different concentrations (2%, 4%, 8%) the mean were (15:19±6:08, 17:20±6:04, 23:31±0:58) respectively as compared with mean control (3:05±0:36), the results were statistically highly significant with (P. value < 0.001) for all concentrations as shown in table (5). As shown in table (6) there were statistical significant difference between methanolic extract of different concentration (2% and 8%) and (4% and 8%) P. value were (< 0.001 and 0.016)

respectively. Comparison between 2% and 4% concentration show insignificant (P. value = 0.730).

Table 4: The mean (SD) of Clot retraction (CR) time (hours) for control and three different concentrations of the extract

Control (hrs)	No	Minimum	Maximum	Mean± S.D
	50	2:10	4:00	3:05±0:36
2%	50	7:40	24:00	15:19±6:08
4%	50	9:10	24:00	17:20±6:04
8%	50	21:00	24:00	23:31±0:58

Table 5: Multiple Comparisons of mean of CR (hrs) in varied concentrations and control

I	II	Mean (I)	Mean (II)	P. value
Control	2%	3:05±0:36	15:19±6:08	< 0.001
	4%		17:20±6:04	< 0.001
	8%		23:31±0:58	< 0.001

One way anova test was used, p-value ≤0.05: considered significant

Table 6: Multiple Comparisons of mean of CR(hrs) in different concentrations of methanolic extraction

I	II	Mean I	Mean (II)	P. value
2%	4%	15:19±6:08	17:20±6:04	0.730
	8%		23:31±0:58	0.001
4%	8%	17:20±6:04	23:31±0:58	0.016

One way anova test was used, p-value ≤0.05: considered significant

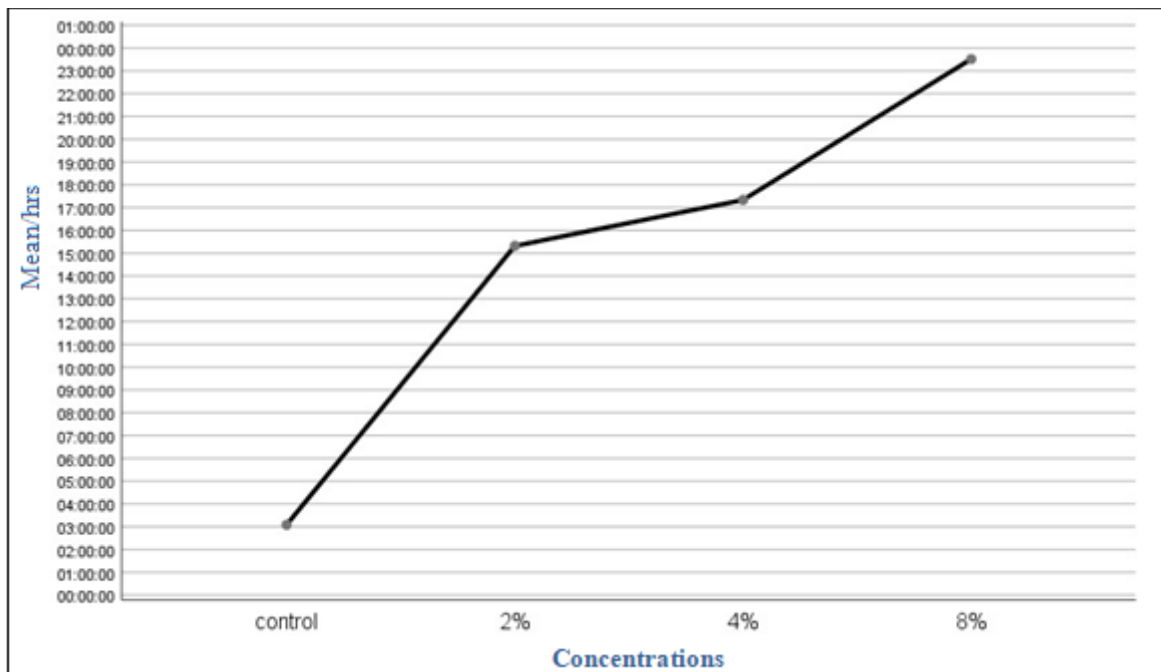


Figure 2: Show the mean of clot retraction (hrs) at different concentrations of the extract (n=50)

4. Discussion

Thrombotic drugs are widely used to manage these diseases, but they are expensive and have many side effects. Therefore, we need a cheap, safe, and natural treatment. Previous studies have indicated that some medicinal plants have shown anticoagulant activity, and they may provide a new therapeutic way to treat a range of people who are affected by these diseases. All around the world, research was being conducted on phytochemicals with anticoagulant properties. These are considered to be therapeutically better, have more effective anticoagulant or antiplatelet agents, and handle multiple targets without many side effects (22)

Artemisia has been reported to contain a rich store of compounds like phenols, antioxidant nutrients, and flavonoids, which are thought to be responsible for its observed antiplatelet and anticoagulant action(7). This study aimed to determine an in vitro anticoagulant and antiplatelet effect of *A. herba-alba*Asso extracts on PT and CR tests. The results showed significant mean differences in PT and CR at different concentrations (2%, 4%, and 8%) of *A. herba-alba* extract (p= 0.05).There was an increase in PT and CR as the concentration increased, which indicates there was an effect of the *Artemisia* extract on these tests.

Several previous studies have assessed the effect of different types of flavonoids on coagulation and platelet aggregation; one demonstrated that jaceosidin and eupatilin are associated with a significant prolongation in PT(p<0.001) and also

inhibit platelet aggregation(7). Another study done on hispidulin found that it inhibits human platelet aggregation by increasing cAMP levels (23). This previous study suggests that the prolongation of PT and CR in this study may be due to the effect of either or both hispidulin and cirsilineol in one or more of the coagulation factors that affect PT.

The result showing a significant increase in PT(p <0.05) was in agreement with previous studies done on other types of *Artemisia*; one of these studies was conducted by Kemal *et al.* in the Department of Pharmacognosy, and it aimed to assess the invitro anticoagulant activity of *Artemisia dracuncululus* leaf extracts, with the results showing a significant increase in the PT (p <0.05) (24). The results presented here were also consistent with those reported in another study done by Jie-LiLv *et al.* in 2014, which demonstrated the anticoagulant activity of *Artimisiaargyi*, with the result showing that there was a significant increase in PT (p <0.05)(25). Another study conducted by Ri Ryu *et al.* in 2013, which aimed at assessing the anticoagulant effect of *Artemisia princeps pampanini*, also showed a significant increase in PT (p <0.05) (7).

The present study revealed a significant increase in CR time (p <0.05), and this result is in accordance with the previous study by Ri Ryu *etal* mentioned above, the results of which showed significant inhibition of platelet aggregation (p <0.05)(7). The findings presented here are also in agreement with those of the study by Brigitte *et al.* on hispidulin, a

natural flavone, with the results showing inhibition of human platelet aggregation with increased cAMP levels(23).

5. Conclusion

A. *herba-alba* Asso extract had significant invitro anticoagulant and antiplatelet activity, and these findings may justify the use of this plant in the management of CVD. It could be a cost-effective way to combat disease complications. Further in vivo and invitro studies with large sample sizes are needed to confirm our results. Also, sophisticated techniques for determining platelet function must be applied in further studies.

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