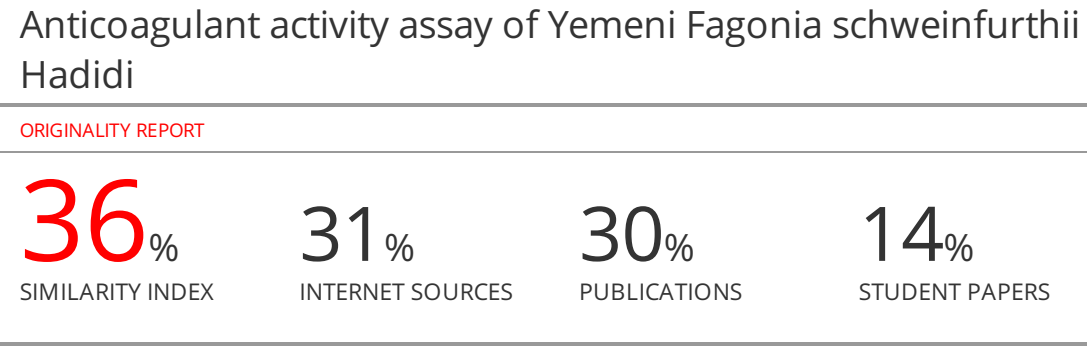
**Reviewer’s Comments**

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**Anticoagulant activity assay of Yemeni *Fagonia schweinfurthii* Hadidi**

**Abstract:**

Blood coagulation is a quick and effective process that results in the creation of clots, which demands to monitor.Many illness disorders include an abnormality in blood coagulation.This study examined the *in vitro* effects of methanol, ethyl acetate, and n-hexane extracts from aerial parts of *Fagonia schweinfurthii* Hadidi on healthy human volunteers' blood coagulation.

Moreover, the ~~Secondary~~ metabolites were extracted from dried and crushed *F. schweinfurthii* aerial parts using n-hexane, ethyl acetate, and methanol respectively.The extracts were examined *in vitro* on the blood coagulation profile, prothrombin time (PT), and activated partial thromboplastin time (aPTT) of apparently healthy human volunteers at various concentrations (10-100 µg/ml). of *F. schweinfurthii* aerial parts ethyl acetate and n-hexane extracts at 25, 50, 75, 100µg/ml showed a significant (P<0.05) prolongation of PTand aPTTin the blood obtained from the volunteers. On the other hand,a significant prolongation(P<0.05) of PT with n-hexane extract was observed at 25,50,75,100 µg/ml while PTT was significantly prolonged(P<0.05) with n-hexane extract at 50,75, 100 µg/ml. The highest prolongation effect was recorded with ethyl acetate(PT) and methanol extract(PTT).These results showed that *F. schweinfurthii* aerial parts possess bioactive components with anticoagulant properties, which may be exploited in the treatment of blood coagulation disorders.

**Keywords:**Blood coagulation,*F. schweinfurthii*,Partial thromboplastin time,Prothrombin time.

**Introduction:**

Herbal medicines have been utilized as a form of medical treatment for a wide variety of conditions since ancient times.Herbal remedies have been extremely important to the overall health of people all over the world1. Many secondary metabolites that plants produce have significant biological activities. Among these, the anticoagulant activity might be highlighted.

Anticoagulants are agents that interact with the body's natural blood-clotting system to treat and prevent blood clots.They are used in patients with Deep Vein Thrombosis ( DVT),Pulmonary Embolism (PE), blood clots in the arteries or veins, atrial fibrillation that causes strokes and mechanical heart valves2.

*Fagonia schweinfurthii*Hadidi belongs to the family Zygophyllaceae. In Ayurveda it is called Dhanvayaasa, Dhanvayavaasa, Dhanvayaasaka, Duraalabhaa, Samudraantaa, Gaandhaari, Kachhuraa, Anantaa,Duhsparshaa 3 .

*F. schweinfurthii* is annual to biennial, up to 25 cm tall, spiny, erect, undershrub, with more and less granular leaves; branches are thin, terete, triage, glabrous, and the leaves are opposite and 1-3 foliate. Petioles vary in length from 3 to 30 mm, are deeply striated, and are very slender; stipules have two pairs of sharp, very short petiolules. Geographically it distributed in India, Pakistan, Iran, Eritrea, Ethiopia, Sudan, Somalia, and Kenya4,5. In Yemen, *F. schweinfurthii* is distributed in Tihama, Shara’b, Muthaikhira, Aden, Lahj, Abyan, Qatabah, Demt, alHus’ein, Ashu’ib, Juban, Amran, Raydah, Huth, Haddah, E of Rada, Hadramout, Socotra6,7.

Despite claims of *F. schweinfurthii* use in several regions of the world for the treatment of fever, wounds, and skin diseases, there is little to no scientific research on the plant's effects on blood coagulation.Therefore, this work aims to investigate the assumption and the potential of *F. schweinfurthii*as a medicinal plant for the treatment of blood coagulation disorders is important.

**Materials and methods**

**Drugs and chemicals**

The reagents used were all of the analytical quality.

Prothrombin Time (PT) and activated partial thromboplastin time (PTT) reagent kits were obtained from SEIMENS.

**Plant Material:**

*F. schweinfurthii* aerial parts were collected in September 2019 from local areas in Sana'a, Yemen. The plant was identified by Dr. Hassan Ibrahim, the Biology Department, Sciences College, Sana'a University, and a voucher specimen was given to the pharmacognosy department.

**Preparation of plant extract:**

The fresh aerial parts were properly rinsed with tap water, dried at room temperature, and then using an electric blender, reduce to a fine powder. The powder was stored at 40C and protected from light prior to future uses.

In 3.76 L conical flasks, powdered aerial parts weighing 752g were sequentially soaked in hexane, ethyl acetate, and methanol. The samples were immersed at room temperature for three to seven days with continuous agitation. These procedures were repeated three times to obtain higher extraction yields. The extracts were then filtered and concentrated in a rotary evaporator set to 40◦C to 60°C. The crude extracts were collected and driedin oven at 37 °C. The dried extracts were weighed and stored at 4°C for later investigation8.

***In vitro* anticoagulant activity assay:**

The anticoagulant activity of *F. schweinfurthii aerial* parts extracts was investigated by the classical coagulant assays PT and PTT. Nine parts of human healthy blood were drawn into one-part sample which would be measured.

Different concentrations(100,75,50,25,10µg/ml) of methanol, ethyl acetate and n-hexane of *F. schweinfurthii aerial* parts extracts and Heparin were prepared, All the samples were dissolved in a 0.9% (w/w) NaCl aqueous solution. Heparin was used as positive control and Plasma alone was be used as negative control.

For PT assay, citrated normal plasma was mixed with a sample solution and incubated for 3 min at 37 ◦C. Then PT assay reagent 0.20 ml, pre-incubated for 3 min at 37 ◦C, was added to the mixture and clotting time was recorded. For PTT assay, citrated normal human plasma was mixed with a sample solution and incubated for 3 min at 37 ◦C. Then, PTT assay reagent 0.10 ml, pre-incubated for 3 min at 37 ◦C, was added to the mixture and incubated for 5 min at 37 ◦C. After that, 0.10 ml CaCl2 (0.025 mol/L) pre-incubated for 3 min at 37 ◦C, was added and clotting time was recorded9. The experiment was carried out in triplicate.

**Statistical analysis:**

SPSS version 26 was used to analyze the data. All results were done in triplicate and were expressed as the mean ± SD from three different experiments. One way ANOVA followed by Dunnett’s multiple comparison test was used to was used to measure statistical differences between the mean in all experiments. The statistical difference was indicated with value p <0.05, p <0.01, and p <0.001.

**Results:**

The anticoagulant activity of methanol, n-hexane, and ethyl acetate extracts of *F. schweinfurthii* aerial parts was determined *in vitro* using the traditional coagulation assays prothrombin time (PT) and activated partial thromboplastin time (a PTT) assays, using normal citrated human plasma as a negative control and heparin as a reference. Many different concentrations (10, 25, 50, 75, and 100 µg/ml) of *F. schweinfurthii* aerial parts methanol, ethyl acetate, and n-hexane extract were prepared, and the results are given in the table (1 and 2). The normal value of PT and PTT from a healthy human was 11.8 and 30.20 seconds, respectively. Heparin was utilized as a positive control and showed considerable anticoagulant activity (PT > 60 s, PTT > 170 s).

Methanol, ethyl acetate, and n-hexane extracts of *F. schweinfurthii* aerial parts were assessed by PT at 10, 25, 50, 75, and 100µ g/ml. The results showed significant prolongation (p˂0.01, p˂0.0001) of PT by the methanol, ethyl acetate, and n-hexane extract at (25,50,75,100µg/ml) concentrations. In contrast, at a concentration of 10 µg/ml, methanol, ethyl acetate, and n-hexane extract did not significantly(p˃0.05) prolong PT compared with the plasma.

In the PTT assay, there was an insignificant prolongation(p˃0.05) of PTT at a concentration of 10µg/ml of methanol and ethyl acetate extracts compared with the plasma. However, n-hexane extract didn’t show a significant prolongation of PTT at 10 µg/ml,25µg/ml concentrations (p˃0.05). On the other hand, methanol and ethyl acetate extracts produced significant prolongation (p˂0.01, p˂0.0001) of PTT at 25,50,75,100µg/ml concentrations compared to the plasma. While, n-hexane extract showed a significant prolongation (p ˂0.01, p˂0.0001) of PTT at 50,75,100µg/ml compared to the plasma.

The highest PT and PTT prolongation times were recorded at 100 µg/mL compared with other tested concentrations. The methanol extract exhibited the highest PTT Prolongation activity compared with other tested extracts.

In contrast, ethyl acetate recorded the maximum PT prolongation activity compared with the other tested extracts.

**Table 4:** *Invitro* anticoagulant activity assay (PT) in a sec of *F. schweinfurthii* aerial parts extracts.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sample concentration (Mg/ml) | Methanol extract | Ethyl acetate  extract | Hexane  extract | Heparine |
| ~~Mean±SD~~ | ~~Mean±SD~~ | ~~Mean±SD~~ | ~~Mean±SD~~ |
| 10 | 12.91±0.22 c | 13.11±0.28 b | 12.74±0.74 | 63.32±1.70 a |
| 25 | 14.11±0.48 a | 16.07±0.17 a | 15.22±0.92 b | 93.52±0.60 a |
| 50 | 19.21±0.51 a | 21.88±0.23 a | 21.03±0.75 a | 115.02±1.14 a |
| 75 | 23.79±0.33 a | 25.69±0.46 a | 24.93±1.01 a | 139.73±2.87 a |
| 100 | 26.59±0.57 a | 29.44±0.77 a | 28.08±1.85 a | 157.06±1.98 a |
| Control | 11.80±0.07 | 11.80±0.07 | 11.80±0.07 | 11.80±0.07 |

Note: a p value <0.001, b p value <0.01, c p value <0.05 compared to control



**Figure1:** Prothrombin time of normal human plasma treated with*F. schweinfurthii*extracts:

a p value <0.001, b p value <0.01, c p value <0.05 compared to contr

**Table 2:** Invitro anticoagulant activity assay (PTT) in a sec

of *F. schweinfurthii*extracts

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sample concentration (Mg/ml) | Methanol extract | Ethyl acetate  Extract | Hexane  extract | Heparine |
| ~~Mean±SD~~ | ~~Mean±SD~~ | ~~Mean±SD~~ | ~~Mean±SD~~ |
| 10 | 31.29±0.44 | 31.08±0.67 | 30.33±0.84 | 115.66±0.72 a |
| 25 | 34.40±0.40 a | 33.06±0.30 b | 32.16±0.41 c | 132.33±1.71 a |
| 50 | 37.94±0.95 a | 36.26±0.41a | 33.33±0.19 a | 147.28±0.80 a |
| 75 | 46.23±0.88 a | 45.00±0.23 a | 39.59±1.10 a | 162.65±1.19 a |
| 100 | 55.60±0.84 a | 48.61±1.28 a | 43.17±0.68 a | 178.56±1.49 a |
| control | 30.20±0.44 | 30.20±0.44 | 30.20±0.44 | 30.20±0.44 |

Note: a p value <0.001, b p value <0.01, c p value <0.05 compared to control



**Figure2:**Partial thromboplastin time of normal human plasma treated *F. schweinfurthii*extracts : a p value <0.001, b p value <0.01, c p value <0.05 compared to control

**Discussion:**

Anticoagulant and procoagulant medications are commonly used in healthy and pathological states, including cardiovascular disease, diabetes mellitus, and bleeding disorders.Although many of these drugs have been developed over the years, most are usually accompanied by undesirable side effects. As a result, new anticoagulants and procoagulants are still required with fewer side effects. The present study investigated the *in vitro* anticoagulant effects of *F. schweinfurthii* aerial parts methanol, n-hexane, and ethyl acetate extract using classical P.T. and PTT assays.

The results showed significant prolongation of the PT and PTT by n-hexane , ethyl acetate and methanol extract in a concentration-dependentmanner with an optimum prolongation of PT and PTT at 100µg/mL and the minimal prolongation time was obtained at 10µg/m. These results agreed with the report by Ismail *et al*., 201710and Chourasia *et al*., 2011 11that Fagonia cretica leaves extract and aqueous extract of Fagonia arabica and its fractions exhibited a concentration dependent anticoagulant activity.A comparison of the effect of the three extracts on the P.T. and PTT showed that the ethyl acetate and methanol extract exhibited the highest anticoagulant activity. The highest anticoagulant activity was observed with the ethyl acetate (medium-polar) and methanol(polar) extracts compared with n-hexane(non-polar) extract, which suggests that the major anticoagulant components of *F. schweinfurthii aerial* parts are polar. These results agreed with Duric *et al*12 that methanol extract showed the highest anticoagulant activity. Anticoagulant activity *F. schweinfurthii* aerial parts extracts may be attributed to saponin, flavonoids, tannin, triterpenoids 11,13, and coumarins14,15.

P.Tand PTT are coagulation parameters used to determine the clotting mechanism. Prothrombin time (P.T.) is an effective assay for evaluating the activity of the factors of the extrinsic coagulation pathway. Simultaneously, the PTT is utilized to assess the activity of components involved in intrinsic and common pathways. Prolonged PTT and P.T. values in clinical evaluation suggest an abnormality in the activity of specific clotting factors; for example, an unusually long PTT but normal P.T. value indicates the need to assay contact pathway factors VIII, IX, and XI. If the P.T. and PTT are affected, this points to factors V, X, and prothrombin (factor II) of the commonpathway16.Thus, the prolonged P.T. and PTT by *F. schweinfurthii* treatment suggest inhibition of factors V, X, and prothrombin of the common coagulation pathwayactivity.

**LIMITATIONS OF THE STUDY**

**Conclusion:**

According to the study, *F. schweinfurthii* has anticoagulant effects that can be used to treat blood coagulation disorders.To explore the mechanism by which the anticoagulant components of the plant impact their efficacy, additional *in vivo* research is necessary.

**Author’s Contribution**

**Acknowledgements**

**CONFLICT OF INTEREST**

"No conflict of interest associated with this work”.

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