ORIGINAL ARTICLE

In Vitro Assessment of Coagulation Activities in Human Plasma Treated With Ajwa Date (Phoenix dactylifera L.) Extracts

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ABSTRACT

Introduction: Arterial and venous thromboses contribute to significant morbidity and mortality rate, thus an anti-thrombotic agent is needed for prevention and treatment of thrombosis. Ajwa dates (Phoenix dactylifera L.) reportedly contain a high level of salicylic acid which is a compound responsible for anticoagulation via antagonism of vitamin K. The present study was designed to assess coagulation activities in human plasma treated with Ajwa date extracts in vitro. Methods: Platelet-poor plasma samples from 27 donors were treated with ethanol crude date extract (ET) or aqueous crude date extract (AQ) of Ajwa dates at different concentrations to generate the following seven test groups from each donor: control (normal saline), ET-I (0.1 g/mL), ET-II (0.5 g/mL), ET-III (1.0 g/mL), AQ-I (0.1 g/mL), AQ-II (0.5 g/mL) and AQ-III (1.0 g/mL). In vitro coagulation activities of Ajwa dates were assessed based on prothrombin time (PT, an assessment of extrinsic coagulation pathway), activated partial thromboplastin time (APTT, an assessment of the intrinsic coagulation pathway), and thrombin time (TT, an evaluation of level and function of fibrinogen). Results: A very significant prolongation of PT, APTT and TT were observed for the ET-II and ET-III groups and very significant prolongation of PT and TT was observed for the AQ-II and AQ-III groups. Significant prolongation of TT was observed in the AQ-I group. Conclusion: In conclusion, Ajwa date extracts had an anticoagulation effect on human plasma.

Keywords: Phoenix dactylifera L., Prothrombin time (PT), Activated partial thromboplastin time (APTT), Thrombin time (TT)

INTRODUCTION

Haemostasis is a dynamic, ordered and well-regulated body process of blood fluid maintenance, vascular damage repair and bleeding arrest (1). Haemostasis functions to prevent intravascular blood clot formation and to stop blood loss from blood vessels (2). Abnormality in normal haemostasis disrupts the balanced state, giving rise to two extreme and distinct conditions: obstructive intravascular clotting (thrombosis) or excessive bleeding (haemorrhage) (1)(3). Thrombosis formation in blood vessel is harmful when protective mechanism of coagulation inhibitor, blood flow and fibrinolysis become inoperative (4).

Arterial and venous thrombosis are responsible for significant morbidity and mortality (5). Arterial thrombosis commonly causes acute myocardial infarction, ischemic stroke and limb gangrene, which can progress to fatal pulmonary embolism and postphlebitic syndrome when deep-vein thrombosis occurs (5). Venous thrombosis is rare but occurs at sites of vascular disruption, post-surgical trauma, and indwelling venous catheters (5). Thrombosis can be prevented and treated using various types of antithrombotic agents, including antiplatelets, anticoagulants, and fibrinolytic drugs (5). Antithrombotic agents have been used in clinical settings for more than 50 years and are among the most frequently prescribed medications (6).

Data on adverse drugs events showed that warfarin (anticoagulant) and antiplatelet agents are among the main factors contributing to emergency hospitalization in older patients (7). Anticoagulant therapy requires meticulous and detailed monitoring to provide the best therapeutic benefits while also maintaining a safe balance between side effects and preventing thromboembolic events (6). Two serious issues associated with antithrombotic therapy are failure to prescribe anticoagulants when clinically indicated and insufficient monitoring once therapy has started (6). Natural products represent alternatives to pharmaceuticals used to treat coagulation disorders. They can be beneficial remedies because they are inexpensive and readily available (8). Salicylic acid is a compound that has the ability to block the action of vitamin K during activation of the coagulation pathway, thus functioning as a vitamin K antagonist (9).

Dates contain a high concentration of salicylic acid...
MATERIALS AND METHODS

Ajwa Date Collection
Ajwa dates were purchased from a local distribution centre. Dates weighed about 4–8 g per fruit, and for each extraction approximately 100 g (~16 dates) were used. Dates were selected based on size, colour, ripening stage, and quality. The edible part of dates (100 g) were pitted and oven-dried for 7 days.

Ethanol Extraction
Pitted dates (100 g) were dry-blended with an analytical mill (IKA, A11 Basic, Janke und Kunkel-StràBe, Staufen, Germany). Ground dates were extracted two times with 200 mL of 99.7% ethanol (QreC) at room temperature (20 °C, 1 h) using an ultrasonic cleaner (WiseClean, WUC-A10H, Rikonerstrasse, Grafstal, Switzerland). The extract was centrifuged (6000 rpm, 10 min) and filtered. The supernatant was kept at 4 °C until used for analysis.

Aqueous Extraction
The aqueous extraction method was adapted from Vayalil et al., (2002) (17) with few modifications. Ground dates were extracted two times with 200 mL of distilled water at room temperature (20 °C, 1 h) using ultrasonication (WiseClean, WUC-A10H, Rikonerstrasse, Grafstal, Switzerland). The extract was centrifuged (6000 rpm, 10 min) and filtered. The supernatant was collected and lyophilized using a freeze dryer (Christ, Alpha 1-4 LSC, An der Unteren Suse, Osterode, Germany) to obtain the aqueous crude extract (AQ). The crude extract was kept at 4 °C until used for analysis.

Yield Percentage (%) Calculation
The percentage of extraction yield was calculated using the following formula (18).

\[
\text{Extraction yield } (\%) = \frac{\text{Weight of dried extract} \times 100}{\text{Weight of original sample}}
\]

Derivatisation Crude Extracts
The chemical derivatisation method was adapted from Scotter et al., (2007) (19) with few modifications. A 1 mL aliquot of crude extract solution (1 mg/mL) was transferred to a 2 mL glass vial, and solvent was removed under a stream of N2 on an evaporator system (Glas-Col, Terre Haute, Indiana, USA) at 35 °C. The vial was removed as soon as the residue solvent dried. A 200 µL aliquot of BSTFA/TMCS derivatising agent (Supelco, Bellefonte, Pennsylvania, United States) was added to the vial. The vial was capped immediately and heated at 60 °C for 1 h with occasional swirling. The solution was cooled to room temperature prior to analysis by gas chromatography-mass spectrometry (GC-MS, Agilent, 7890A, Santa Clara, California, United States).

Analysis of Compounds
The GC-MS analysis method was adapted from Deng et al., (2003) (20). Analysis was performed using a HP-5 ms capillary column (30 m, 0.25 mm, 0.25 µm) equipped with a MSD 5975C detector and spitless injection system. Helium gas (99.999%) was used as the carrier gas at a flow rate of 1 mL/min. The oven temperature was set at 100 °C for 2 min, then programmed to increase at 15 °C/min to 300 °C, which was maintained for 10 min. The results were analysed qualitatively in full-scan acquisition mode with a mass range of 45–500 amu. The compounds were identified by comparing mass spectra of each compound with the compound in the National Institute of Standards and Technology (NIST) database.

Sample Size Calculation
Power and Sample software (21) was used to determine the subject sample size needed for this study. The analysis was based on the paired t-test formula, and results were as follows: \( \alpha = 0.05 \), \( \delta = 0.345 \) (detectable difference at 60%), \( \sigma = 0.575 \) (based on coagulation activity results (22), power = 0.8 (80%), n = 27 (plus 10% drop out rate) for the subject group.

Donor Recruitment
Twenty-seven donors were recruited at the Clinical Trial Complex, Advanced Medical and Dental Institute, Universiti Sains Malaysia (USM). All donors were informed about the objective of this study and signed an informed consent form prior to blood collection. The protocol was authorized by the Human Research Ethics Committee at USM (Protocol Code : USM/
JEPeM/14120489). Inclusion criteria included all the criteria for blood donation as stated by the National Blood Centre (PDN). Inclusion criteria were as follows: male or female; age between 18 and 60 years old; healthy and free from clinical disorders; body weight > 45 kg; had eaten food before donating blood; not pregnant, not breast-feeding and not menstruating; and not involved in any risky activities (homosexual relationship, bisexual relationship, commercial sex relationship, multiple sexual partners, intravenous drug user, and sexual partner to the aforementioned risky groups). Exclusion criteria included having taken antiplatelet/anticoagulant/fibrinolytic drugs, vitamin/mineral supplements or medication for at least 7 days before blood donation.

**Blood Collection**

For the coagulation studies, 6 mL of blood were collected from each donor and placed in a trisodium citrate tube (3.2%) in a 9:1 ratio. Blood was centrifuged (6000 rpm, 10 min) to obtain platelet-poor plasma (PPP) and analysed immediately.

**Measurement of coagulation factors**

The method used to measure anticoagulant activity was adapted from Karim *et al.*, (2013) (23). Prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT) tests were conducted using an automated coagulation analyser (Diagnostica Stago, STA Compact, Parsippany, New Jersey, United States). PPP samples from the 27 donors were pre-incubated (37 °C, 7 min) with crude ET or AQ Ajwa date extracts at 1:1 (v/v) at different concentrations to generate the following seven test groups from each donor: control (normal saline), ETI (0.1 g/mL), ETII (0.5 g/mL), ETIII (1.0 g/mL), AQI (0.1 g/mL), AQII (0.5 g/mL), and AQIII (1.0 g/mL). The tests were run in duplicate from each donor. The results of coagulation tests were expressed in seconds (s).

**Statistical Analysis**

Statistical analysis was performed using SPSS Version 22.0 (IBM Corp, North Castle, New York, United States). Data were expressed as median with minimum and maximum range of duplicate determination. Kruskal-Wallis with Bonferroni correction was used to compare data between control and each treatment group. Values were considered to be significantly different at p < 0.05 and very significantly different at p < 0.001.

**RESULTS**

**Extract Yield**

Percentage of w/w extraction yield for Ajwa dates by ET and AQ extracts were 22% and 38%, respectively.

**Prothrombin Time**

**Ethanol Extract**

Table I and Figure 1 show that PT clotting time differed very significantly (p < 0.001) between the control and treatment groups ETII and ETIII. Compared to the control, PT in groups ETII and ETIII was prolonged by 6.1 s and 23.1 s, respectively (Table 1). However, no significant difference (p > 0.05) in PT was detected between the control and treatment group ETI. PT for ETI was only 0.2 s longer than that of the control group.

**Aqueous Extract**

Table 1 and Figure 1 also show very significant differences (p < 0.001) in PT between the control and treatment groups AQII and AQIII. Clotting time was prolonged by 7.0 s and 26.0 s in the AQII and AQIII groups, respectively, compared to the control (Table 1). No significant difference (p > 0.05) in PT was detected between the control and the AQI group, as both values were 18.8 s.

**Activated Partial Thromboplastin Time**

**Ethanol Extract**

Table 1 and Figure 2 illustrate that APTT was very significantly different (p < 0.001) between the control and treatment groups ETII and ETIII, with APTT prolonged

**Table 1.** Clotting time for coagulation parameters of PPP treated with Ajwa date ethanol (ET) and aqueous (AQ) extracts at concentrations of 0.1 g/ml (I), 0.5 g/ml (II), and 1.0 g/ml (III).

<table>
<thead>
<tr>
<th>Coagulation Parameters</th>
<th>Control (0.1 g/ml)</th>
<th>Ethanol (0.1 g/ml)</th>
<th>Ethanol (0.5 g/ml)</th>
<th>Ethanol (1.0 g/ml)</th>
<th>Aqueous (0.1 g/ml)</th>
<th>Aqueous (0.5 g/ml)</th>
<th>Aqueous (1.0 g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT</td>
<td>18.8</td>
<td>19.0</td>
<td>24.9**</td>
<td>41.9**</td>
<td>18.8</td>
<td>25.8**</td>
<td>44.8**</td>
</tr>
<tr>
<td></td>
<td>(16.4–23.2)</td>
<td>(17.6–25.8)</td>
<td>(22.4–28.6)</td>
<td>(22.4–53.5)</td>
<td>(17.2–24.4)</td>
<td>(18.1–32.0)</td>
<td>(17.6–69.3)</td>
</tr>
<tr>
<td>APTT</td>
<td>50.8</td>
<td>52.2</td>
<td>64.4**</td>
<td>91.3**</td>
<td>55.7</td>
<td>54.2</td>
<td>57.5</td>
</tr>
<tr>
<td></td>
<td>(38.60–64.40)</td>
<td>(44.00–67.60)</td>
<td>(55.60–83.20)</td>
<td>(74.20–152.30)</td>
<td>(42.70–68.20)</td>
<td>(36.80–79.00)</td>
<td>(22.30–105.20)</td>
</tr>
<tr>
<td>TT</td>
<td>16.2</td>
<td>18.7</td>
<td>37.0**</td>
<td>64.5**</td>
<td>19.9</td>
<td>51.1**</td>
<td>91.2**</td>
</tr>
</tbody>
</table>

Results are expressed as median time in seconds (s) with maximum and minimum range (n = 27). Values were considered to be significantly different at p < 0.05 vs. control (*) and very significantly different at p < 0.001 vs. control (**).
Aqueous Extract

Table I and Figure 2 show that no significant differences (p > 0.05) in APTT clotting time were found between the control and all three AQ treatment groups. Although not significant, groups AQI, AQ II, and AQ III showed prolonged APTT compared to that of the control group.

DISCUSSION

Arterial and venous thromboses are responsible for significant morbidity and mortality (5)(24), especially in the case of venous thromboembolism and arterial thromboembolic events (25). Thrombosis is also a major problem in cancer patients, as 50% of solid...
Table II: Compounds in the Ajwa date ethanol extract as determined by GC-MS at 1 mg/mL.

<table>
<thead>
<tr>
<th>Component</th>
<th>t (min)</th>
<th>Percentage area (%)</th>
<th>Compounds identified</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.346</td>
<td>4.37</td>
<td>Sinensetin tricarballyl phosphate</td>
<td>C21H11O9P</td>
</tr>
<tr>
<td>2</td>
<td>8.789</td>
<td>2.83</td>
<td>Benzene, 1,1- (1,3-propanediyl) bis</td>
<td>C16H16</td>
</tr>
<tr>
<td>3</td>
<td>9.379</td>
<td>0.64</td>
<td>1,2-Diphenylcyclopropane</td>
<td>C16H16</td>
</tr>
<tr>
<td>4</td>
<td>9.987</td>
<td>0.59</td>
<td>1,3-Benzodioxane, 1,4-diphenyl - (E,E) -</td>
<td>C16H16</td>
</tr>
<tr>
<td>5</td>
<td>13.867</td>
<td>100.00</td>
<td>Benzoquinone, m-phenyl -</td>
<td>C20H16N</td>
</tr>
</tbody>
</table>

Table III: Compounds in the Ajwa date aqueous extract as determined by GC-MS at 1 mg/mL.

<table>
<thead>
<tr>
<th>Component</th>
<th>t (min)</th>
<th>Percentage area (%)</th>
<th>Compounds identified</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.228</td>
<td>2.95</td>
<td>Trichloroacetic acid, tridecyl ester</td>
<td>C13H11Cl3</td>
</tr>
<tr>
<td>2</td>
<td>8.789</td>
<td>3.94</td>
<td>Benzene, 1,1- (1,3-propanediyl) bis</td>
<td>C16H16</td>
</tr>
<tr>
<td>3</td>
<td>9.379</td>
<td>0.94</td>
<td>1,2-Diphenylcyclopropane</td>
<td>C16H16</td>
</tr>
<tr>
<td>4</td>
<td>9.987</td>
<td>0.77</td>
<td>1,3-Benzodioxane, 1,4-diphenyl - (E,E) -</td>
<td>C16H16</td>
</tr>
<tr>
<td>5</td>
<td>13.867</td>
<td>100.00</td>
<td>Benzoquinone, m-phenyl -</td>
<td>C20H16N</td>
</tr>
</tbody>
</table>

Therapeutic benefits of dates documented in the literature include potent antioxidant, antitumour, and anti-inflammatory activities (8). The current study also showed that both ethanol and aqueous extracts of Ajwa dates have significant anticoagulation effects on PPP, as PT, APTT, and TT were prolonged in a concentration-dependent manner. PT measures factors involved in common and extrinsic pathways, APTT measures factors involved in common and intrinsic pathways, and TT is an evaluation of level and function of fibrinogen (4)(22) (30).

PT clotting time of more than 2 s is considered to be abnormal (3), and this test is regarded as the best way to rule out trauma, disseminated intravascular coagulation, and necessity for surgery when bleeding problems occur (24(31). In the current study, ethanol extracts of Ajwa dates very significantly prolonged PT when extract concentration was increased from ET I to ET II and ET III and from AQ I to AQ II and AQ III. These findings support the premise that these extracts affect the common and extrinsic pathways at concentrations of 0.5 mg/mL and above. However, ET I and AQ I (0.1 g/mL) did not result in significant prolongation of PT. Torres-Urrutia et al., (2011) (22) did not study anticoagulant activity of dates, but they measured PT in human plasma treated with 36 aqueous and methanolic extracts of fruits and vegetables at 0.001 g/mL. They found that only extracts of grapes caused small but insignificant PT prolongation (22).

APTT is a measure of the time required for plasma to clot in the presence of kaolin or other surface active agents, platelet substitute and calcium (3). In clinical and diagnostic settings, APTT is regarded as a very sensitive test whereby normal APTT result will rule out any clinically significant APTT factor deficiency (3); it is mostly used to diagnose sensitivity to lupus anticoagulants. Lupus anticoagulants are phospholipid-dependent coagulation inhibitor whose detection is affected by coagulation factor deficiencies (32).

In the current study, ethanol extracts of Ajwa dates very significantly prolonged APTT when extract concentration was increased from ET I to ET II and ET III, but none of the aqueous extracts significantly prolonged APTT. Thus, only ethanol extracts at 0.5 g/mL and higher impacted the common and intrinsic pathway. Torres-Urrutia et al., (2011) (17) reported that methanol and aqueous extracts of cancer patients experience thrombosis and 95% of cancer patients exhibit clotting activation (26). In fact, thrombosis is the second leading cause of mortality in cancer patients following cancer itself (25). Thus, correction of thrombotic-haemostatic imbalance by antithrombotic therapy is crucial in clinical practice, and many antithrombotic agents specific for thrombosis have been developed.

An ideal anticoagulant would be able to prevent pathological thrombosis and restrict reperfusion injury while allowing the normal haemostasis response to vascular injury and bleeding (1). To date, such an ideal drug does not exist, and increased bleeding risk remains the major challenge to developing effective anticoagulants and fibrinolytic drugs (1). Use of synthetic drugs is expensive, alters genetic and metabolic pathways, and can have adverse side effects (8). Thus, researchers seek complementary and alternative medicines for medical therapies and production of health care products such as dietary supplements. Dietary supplements contain one or more of the following: vitamins, minerals, herbs or botanicals, amino acids, and other dietary substances (27).

The most common dietary supplements related to haemostasis includes Echinacea, ginseng, gingko, garlic, glucosamine, St. John’s wort, peppermint, fish oil/omega-3 fatty acids, ginger, and soy (27). Antithrombotic effect of some medicinal plants has been documented using animal models (28)(29). Cercis chinensis leaves that contained an active ingredient, myricitrin, have demonstrated prolongation of APTT, PT and TT (28). Malus halliana Koehne flowers, a Chinese traditional medicine has also shown similar effects on APTT, PT and TT with four compounds; baohuoside II, kaempferol-3-O-a-L-furan arabinoside, phloretin-4’-O-glycosidase and afzeloside exhibited anticoagulant activity while afzeloside alone have inhibitory effect on thrombus formation (29).
of raspberries did not significantly prolong APTT at 0.001 g/mL. In clinical setting, most cases of abnormal APTT level occurs when single intrinsic or common factors in coagulation pathways fall below 30-40% of normal range (31).

TT measures the time required for plasma to clot in the presence of thrombin, and it represents the final coagulation stage. TT of more than 18 s signifies an abnormal result (3). In the clinical setting, prolonged TT may result from hypofibrinogenemia, antibody inhibitors of thrombin, and fibrin breakdown products (3). TT was very significantly prolonged when ethanol extract concentration increased from ET I to ET II and ET III. In addition, all three aqueous extracts significantly prolonged PT (AQ I, p < 0.05, AQ II and AQ III, p < 0.001). These findings suggest that both ethanol and aqueous extracts of Ajwa dates impact the final coagulation system at concentrations of 0.5 g/mL and above and 0.1 g/mL and above, respectively. None of the 36 aqueous and methanolic extracts of fruits and vegetables tested by Torres-Urrutia et al., (2011) (22) prolonged TT.

Different effects of fruit and vegetable extracts on coagulation activities may be due to the variety of fruit or vegetable, cultivation conditions, type of study (in vivo or in vitro), and concentration of extracts used in assays (22). This may explain the difference between the results of the current study (i.e., date extracts as anticoagulants) and those of Onuh et al., (2012) (33). They detected significant increases in platelet count in rats treated with both ethanolic and aqueous crude date extracts for 112 days. Because platelets are principle factors in haemostasis (2,4), the increased platelet count suggests that the extracts had a pro-coagulant effect.

In the current study, qualitative GC-MS screening and analysis identified five compounds from the ethanol and aqueous extracts of Ajwa date, but salicylic acid or acetylsalicylic acid were not detected. Ethanol (semi-polar) and water (polar) were chosen as the solvents to extract chemical compounds from dates because of their polarity indexes. Following the principle of “like dissolves like,” the extraction solvent can be chosen based on the polarity of the substances of interest (34), such as salicylic acid, which is a polar compound. In contrast to the present findings, Swain et al., (1985) (11) reported that both fresh dates and dried dates contain salicylic acid. Venema et al., (1996) (35) detected low salicylic acid and no acetylsalicylic acid in 30 foods thought to have high salicylate levels based on the published literature. Clearly, data on salicylate content in foods are contradictory (19,35,36). Janssen et al., (1997) (37) suggested that variation in salicylic acid content in foods reported in different studies may be due to dissimilar origin, storage, processing, analytical methods, and extraction methods.

None of the compounds identified by GC-MS in the current study are known to have anticoagulant activities relative to studies by He et al., (2019) (28) and Cui et al., (2018) (29). Therefore, the significant anticoagulant activity induced by both extracts may be due to other compounds as yet unidentified. Four of the compounds identified in the ethanol extract were matched in the NIST database with matching quality values ranging from 91% to 96%. However, the fifth compound, Benzonitrile, m - phenethyl -, had low matching quality but the highest abundance among the peaks detected. Similarly, four of the compounds identified in the aqueous extract had matching quality to the NIST database ranging from 89% to 97%. The fifth peak, Benzonitrile, m - phenethyl -, had low matching quality but the highest abundance among the peaks detected. The low matching quality may be due to the lack of established data on this compound in the database.

This study had several limitations. First, antiplatelet and fibrinolytic studies could not be conducted due to lack of necessary equipment (i.e., platelet aggregometer) and supplies (i.e., streptokinase, which is categorized as a poison in Malaysia). Second, time limitation affected the coagulation studies. PT, APTT, and TT must be tested within 4 h post-collection of plasma for accurate results. Even when plasma is stored frozen at –20 °C, the quality of coagulation factors can be maintained for only 2 weeks. Third, the duration for this study was only 6 months. A longer duration would have allowed recruitment of a larger sample size.

CONCLUSION

The purpose of this study was to assess in vitro coagulation activities in human plasma treated with Ajwa dates extracts. Both ethanol and aqueous extracts of Ajwa dates exhibited concentration-dependent anticoagulation activities. Very significant prolongation of PT, APTT, and TT was observed for ET I and ET II (> 0.5 g/mL). Very significant prolongation of PT and TT was observed for AQI and AQ III (> 0.5 g/mL), and significant prolongation of TT was observed for AQ III (0.1 g/mL).

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