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Influence of different cultivars of *Phoenix dactylifera* L−date fruits on blood clotting and wound healing

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ABSTRACT

Objective: To investigate different types of dates and medical properties of influencing blood clotting and wound healing in an animal model. **Methods:** Three different cultivars of dates (Ajwa, Khalas, and Fardh) were examined *in−vivo*, for blood clotting and wound healing using CD1 mice of both sexes. Study of toxicity to animals was performed accordingly prior to further investigations. The ethanolic extracts were given orally to animals as a constituent in their daily water. Blood samples were obtained from the mice inferior vena cava to carry out the prothrombin time (PT) assay using the manual method and confirmed using a semi-automated machine. The bleeding time (BT) assay was performed using the cutting technique. In the wound healing analysis, a small cut (5-10 mm) in the skin overlying the thigh was conducted in all mice under anesthesia. The diameter of the cut and healing status were measured on a daily basis throughout the time of the experiment using a roller. **Results:** Ajwa was able to elevate both PT and BT (P<0.05), significantly in a time-dependent manner followed by Khalas date (P<0.05). The results of PT and BT of Fardh date were found to be very close to those of the control group (P<0.05). Despite its activity as an anticoagulant, Khalas date showed a potential property to enhance wound healing in contrast to other dates and the control groups in this study. **Conclusions:** Omani Khalas date fruit has both antithrombotic as well as wound healing properties. The results open a new gate with these fruits for exploring the potential component(s) that may play an important role in antithrombotic as well as wound healing process.

1. Introduction

Skin integrity can be disrupted leading to a wound formation that heals spontaneously. Wound healing involves four overlapping stages (hemostasis, inflammation, proliferation, and maturation). Right after an injury, a blood clot forms a scab in the injured area to stop bleeding. Then chemical mediators are released resulting in a neutrophils-mediated acute inflammatory response followed by macrophages and lymphocytes. Subsequently, a granulation tissue

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is formed by transforming the undifferentiated mesenchymal cells to fibroblasts which synthesize the components of extracellular matrix containing proteoglycans, fibronectin, and type Ⅰ and Ⅲ collagen fibers. This process is essential for the restoration of normal skin appearance[1].

The involvement of this restoration process is underlining the term hemostasis. Hemostasis is a physiological process that hinders any bleeding within the place of injury without disturbing the blood flow in other portions of the circulation which consists of primary and secondary hemostasis. Primary hemostasis involves platelet adhesion, platelet aggregation leading to a platelet plug formation. Secondary hemostasis simultaneously accrues with the primary hemostasis and is built on the creation of insoluble fibrin by the extrinsic and intrinsic proteolytic coagulation pathways. This fibrin can stabilize the blood clot. To inhibit excessive clot formation, the enzyme plasmin, which is produced by plasminogen, can dissolve fibrin. This is known as the fibrinolytic system, which must be kept in balance with the procoagulant system as any disruption, because one of these pathways will result in either bleeding or thrombosis[2]. Anticoagulant treatment is necessary for patients who have too much clotting (thrombosis) as an outcome of a disorder in the hemostasis pathways. Warfarin and heparin are the main anticoagulant drugs used nowadays. Unfortunately, both cannot be considered as ideal anticoagulant drugs because of their clinical drawbacks. One of their clinical side effects is causing bleeding in addition to their interaction with other drugs. Therefore, researchers and scientists started to explore alternative medicine with no or minimum drawbacks if any[3].

Alternative medicine refer to any practice having a healing impact initiated by people's belief. Dates (*Phoenix dactylifera* L.) have been used as a stable food as well as a traditional remedy for decades. Hence, approximately 60%-80% of the world's population are attracted to plant-derived drugs as crucial drugs resource, especially in developing countries, to combat serious diseases[4,5]. Unfortunately, alternative medicine is neither originated nor confirmed by scientific methods, *i.e.*, part of biomedicine[6].

Doubtlessly, the practice of using plants as a source of medicine has long history[7]. In the Middle East, for example, dates (*Phoenix dactylifera* L.) have been cited in the Holy Quran in Suraht Maryam "Mary" (PUH) telling the story of consuming dates as a nutrition source for Mary after the process of delivering the prophet "Jesus" (PUH) (Qur'an 19: 25-26). Furthermore, prophet Mohammed (PUH) mentioned that dates had the potential to treat several diseases and were recommended to Muslims[8]. In Judaism, they celebrate Palm Sunday and date is believed as one of the seven holy fruits[9].

Besides, it is believed that the consumption of Ajwa and Khalas dates can raise the sexual strength (libido). It is reported that Fardh date have a role in making the joints stronger particularly the knees and they are also recommended by traditional healers to people who suffer gastrointestinal problems, especially constipation. Moreover, these dates are claimed to have the potential to enhance the functions of the intestines, colons, and kidneys.

In the literature, dates have been illustrated in many studies to have a potential to prevent some diseases. Previous studies reveal that date has antioxidant, antitumor and anti-inflammatory activities. It is shown that dates have the highest amount of polyphenols, which is an antioxidant[10]. Respectively, flavonoid and phenol, which are other components of date fruits, can control diabetes on rats and have antitumor activity[11,12]. Furthermore, dates also have antibacterial activity against, Fusarium species and Alternaria species[13]. In addition, it has been demonstrated by a study that extracts from Ajwa which is originated from Saudi Arabia have an anti-inflammatory activity by inhibiting both lipid peroxidation cyclooxygenase enzyme1 (COX1) and COX2[14].

In this present study we have investigated the activities of three different cultivars dates based on their anticoagulant and wound healing capacities.

To the best of our knowledge, this is the first report that studied the combination efficacy of anticoagulant, bleeding and wound healing activity of different cultivars of dates. Hence, this study was to search for an agent that has a combination efficacy of anticoagulant in parallel of enhancing wound healing acceleration at the same time. Discovering the right candidate may contribute clinically by holding a future promise for those undergoing bypass (open-heart surgery) operations or hemophilia disorders.

2. Materials and methods

2.1. Collection and preparation of plant materials

Three different cultivars of dates have been selected and used in this study, Ajwa from Al-Madinah, Kingdom of Saudi Arabia, Khalas, and Fardh, from the Sultanate of Oman. These various types were bought from traditional markets. Authentication and the taxonomic identification of plant materials were confirmed by college of Agriculture, Sultan Qaboos University. Voucher specimens were deposited at the College of Agriculture, Sultan Qaboos University. Two hundred grams of each type of dates were used in this study. The dates were harvested at the same year. Each date was extracted using 96% ethanol. The dates were immersed in ethanol and homogenized in an electric blender for about 5 min then incubated at room temperature for approximately 3 d with continuous shaking. The prepared mixture was then filtered two times and ethanol was allowed to evaporate after each time. After that, the mixture was lyophilized, weighed and then kept at 4 ℃ till used.

2.2. Ethics

Due to the nature of using the animal model in this study, an ethical approval was acquired from the Ethics and Research Committee of Sultan Qaboos University (SQU) (SQU/AEC/2016-17-1). All experiments involving animals were performed according to the standards of ethical procedures.

2.3. Animal

Mice were obtained from the animal house, College of Medicine and Health Sciences, Sultan Qaboos University (SQU). Sixty-two CD1 mice (25-35 g) of both sexes were used in the study,

of which 10 mice were used for toxicity study, 8 mice were used for the wound healing experiments, 8 for the bleeding time (BT) experiments and 36 for the anticoagulation experiments. Approximately, 0.16 mg ethanolic date extract [equivalent to one raw date (0.56 mg)] was given to each mouse per day throughout the experiments (4 weeks). The extract was given as a suspension in the animal's drinking water. Mice were exposed to heat light (according to the standard procedures followed in the Animal House, SQU) to stimulate sweating and hence drive the mice to drink water.

2.4. Oral route acute toxicity evaluations of dates extracts

In order to evaluate the toxicity and the cumulative effect "safety protection", potentialities of the dates extracts composition; *in-vivo* assay using "oral" routes was performed. Ten CD1 mice (25-35 g) were used in this assay. The mice were given various amounts (*i.e.*, 0.5 mg to 100 mg) of the extracts to investigate the toxic and lethal doses. The ethanolic extract aqueous solution (dissolved in water) was given orally by intubation at variable dosages stated above to reach the dose 100 mg. The mice were observed continuously for any behavioral alteration for two weeks after administration. The observation was done hourly at day 1, and later on at 4–6 times per day. Consequently, mice were sacrificed and dissected at the end of the observation period. Their livers, spleens, eyes, and lungs were examined to look for any histopathological changes.

2.5. Prothrombin time assay

Thirty-six CD1 mice were divided into four groups (both sexes, six mice per group): control group, Ajwa dates group, Fardh dates group and Khalas dates group. Before the extract was given, two mice from each group were sacrificed and their blood was collected for analysis. This process was repeated at the end of the 1st and 2nd week. To provide an adequate demonstration that changes in the prothrombin times were clearly influenced by the oral intake of the dates's extract we ceased the extract oral intake at the end of the 2nd week and replaced it completely with water only (this was also performed for other investigated parameters illustrated in the subsequent sections). The process of sacrificing and collecting the blood samples was repeated at the end of the 3rd week All mice were anesthetized by Ketamine (200 μL) and Xylazine (100 μL). The blood samples were obtained from the inferior vena cava, using 0.5 mL insulin syringes, and collected in sodium citrate anticoagulated (1 mL) tubes. Plasma was then separated by centrifugation for 5 min at 4 000 rpm. The PT test was performed to investigate the factors of the extrinsic pathway (mainly for factor Ⅶ) using the standard manual procedure[15]. Briefly, the PT reagent was pre-warmed at 37 ℃ for 5 min simultaneously. A volume of 100 μL of the PT reagent was pipetted into a test tube. A volume of 50 μL of the sera sample was added to the tubes prepared in the previous step and then mixed in a water bath at 37 ℃. Once the sample mixed with the prewarmed PT reagent, clotting time was recorded as soon as a clot was observed. Each test was performed in duplicate for each sample and then the results were confirmed using a semi-automated machine Diagnostica Stago ST4 Coagulation Analyzer.

2.6. In-vivo bleeding time assay

Animals were divided into four groups (both sexes, six mice per group): control group, Ajwa dates group, Fardh dates group and Khalas dates group. Before the extract was given, the BT test was performed. This test was repeated at the end of the 1st and 2nd week. Subsequently, after the 2nd week the extract was replaced completely by water throughout the 3rd week. At the end of the 3rd week the BT test was performed accordingly. Animals were placed in a restraint chamber that inhibits their movements while performing the BT test and then the tails were wiped with ethanol for sterilization purposes. The next step was cutting the tip of the tail of each mouse to cause bleeding. A stopwatch was started as soon as the mice began to bleed. A filter paper was used to wipe off blood every 30 s. As soon as the bleeding ceased the time was recorded.

2.7. Evaluation of wound healing efficacy

Eight mice were divided into 4 groups: control group, Ajwa dates group. Fardh dates group and Khalas dates group, with 2 mice in each group. At the end of the 1st week, a cut (5-10 mm) in the skin overlying the thigh was induced in all mice. The cut method described by Moreira *et al.*[16] was followed. At first, the mice were anesthetized using Ketamine (200 μL) and Xylazine (100 μL). Once the mouse is completely anesthetized, the animals were shaved at the site where the cut was performed. Next, the skin was raised to form a sandwiched skinfold and then the skin was cut by a sharp scissor in a form of a fine circle. The cut diameter (mm) was then measured on a daily basis throughout the 2nd week using a standard roller. The measurements method described by Moreira *et al.*[16] was followed and based on the formula:

$$(\text{Larger diameter}/2) \times (\text{minor diameter}/2) \times \pi$$

At last, the percentage of what is remaining of the wound was obtained using the formula:

$$(\text{Diameter of actual wound}/\text{diameter of an original wound}) \times 100$$

2.8. Data analysis

The data were analyzed using Excel software (Microsoft Corporation, USA, 2016 version). The average and standard deviation were used for the continuous variables. The correlations among the variables were assessed using two-tailed *t*-tests. All the results were considered significant when the probability (*P*-value) is less than 0.05.

3. Results

Dates were tested for their acute toxicity dose for oral administration by introducing an increasing dose of the ethanolic extract. All animals were alive after 2 weeks of giving the dates extract. No abnormal behaviour was observed. Inspection of the

eyes, livers, lungs, and spleens showed no extraordinary signs. Animals also showed normal body weight increase during the two weeks period.

The PT results are illustrated in Figure 1. The highest PT [(23.30±0.76) s] was found at the end of the 1st week in group of Ajwa dates (P<0.05), followed by Khalas date [(13.70±0.50) s, P=0.04] in contrast to result of that of the Fardh date group where the results was found to be very close to the control group [(10.30±0.57) s]. At the end of the 2nd week, results of the PT had increased slightly. However, the results were not significant in all group. Interestingly, a significant decline was observed at the end of the 3rd week where Ajwa showed (15.30±0.93) s, Khalas (10.20±0.76) s, Fardh (9.00±0.50) s and the control (10.50±1.20) s. This decline may explain the role of the extract as in the 3rd week the extracts being completely replaced by water.

Further investigations of the three dates were performed based on the BT analysis as shown in Figure 2. The Ajwa dates were found to cause a significant prolongation of the mice BT [(15.16±1.00) min, P<0.05)] at the end of the 1st-week followed by Khalas date group [(8.33±0.76) min, P<0.05] in contrast to that obtained by the Fardh date [(6.50±0.50) min] or that of the control groups. Moreover, although the prolongation of the BT was increased in the second week, it was not significant in all groups. A subsequent potential decrease was observed at the end of the 3rd week. This may be related to the extract which has been completely replaced by water [Ajwa (9.50±1.00) min, Khalas (6.75±0.35) min, Fardh (6.25±1.10) min, control (4.75±0.36) min].

Figure 3 demonstrated the efficacy trend of wound healing acceleration properties of the three dates used within six consecutive days compared to the control group. The results showed that Khalas date could enhance the healing of wounds faster than the other groups. As a matter of fact, mice treated with Fardh dates enhance wounds by 50% in the second day of treatment and on the 6th day, only 4% of the wound remained (P<0.05) when compared to the other dates and the control.

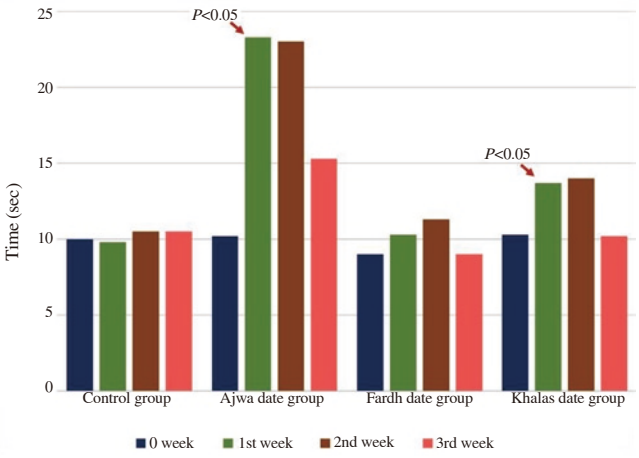

Figure 1. Influence of different dates on prothrombin time (PT) using CD1 mice.

Prothrombin time-international normalized ratio values in mice after 0, 1st, 2nd and the 3rd weeks where date fruits administration in drinking water. Data are expressed as mean±SD. P<0.05 when compared with 0 week and control group.

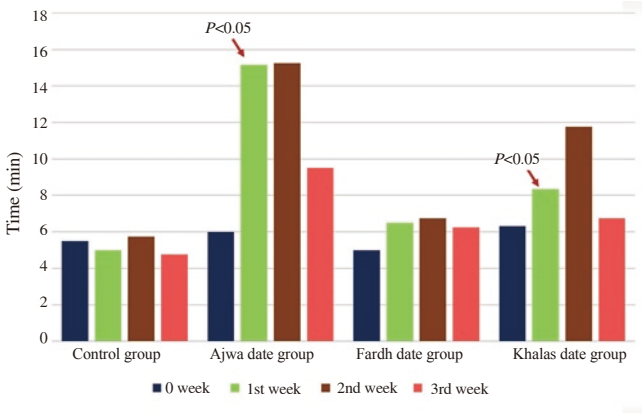

Figure 2. Tail bleeding time in dates treated mice.

The bleeding time was measured after amputation of a 3-mm portion of the tail. Data were compared after after 0, 1st, 2nd and the 3rd weeks where date fruits administration in drinking water. Data are expressed as mean±SD. P<0.05 when compared with 0 week and control group. Mice treated with the Ajwa and Khalas date fruits exhibited a fourfold prolonged bleeding time in week one and two compared with control mice (P<0.05). A decline was observed when dates being ceased.

4. Discussion

Plant-derived drugs remain an important resource, especially in developing countries, where 60%-80% of the world's population are still relying on traditional medicines to combat serious diseases[4,17]. About 60%-90% of patients with 'arthritis' have used complementary and alternative medicine; most of which used traditional Chinese medicine. Therefore, the use of medicinal plants as a source for relief from illness can be traced back to five millennia ago, hence doubtless an art as old as human civilization. It is well known that the *Phoenix dactylifera* L. fruit has a considerable amount of these phytochemical compounds. The use of these natural remedies to treat human diseases is due to that many of the current drugs used in modern medicine unfortunately have potential drawbacks.

Although atherosclerotic diseases like, cerebral infarction (stroke) and myocardial infarction can be prevented by both anticoagulants such as heparin and warfarin to suppress the coagulation system along with t antiplatelet drugs such as aspirin to inhibit primary hemostasis. These drugs, especially the heparin and warfarin, causes several clinical complications[18]. Hence, looking for an alternative agent becomes crucial for many types of research nowadays. Therefore, this study was performed to examine the anti-thrombotic and wound-healing activities of different date fruits from different *Phoenix dactylifera* L. plant species.

In this study, we have evaluated the efficacy of different *Phoenix dactylifera* L. date cultivars on the PT and BT activities. For the PT activity, investigation was based on the coagulation factors of the extrinsic pathway of the coagulation cascade factors Ⅴ, Ⅶ, and Ⅹ. Thus, a prolongation of PT would be an indication of the influence of the date fruits in either the extrinsic pathway, common pathway or the pathway involved in the formation of these factors[19].

After the first week of a consecutive administrating the dates' extracts, significant increase in PT was observed in a time-dependent

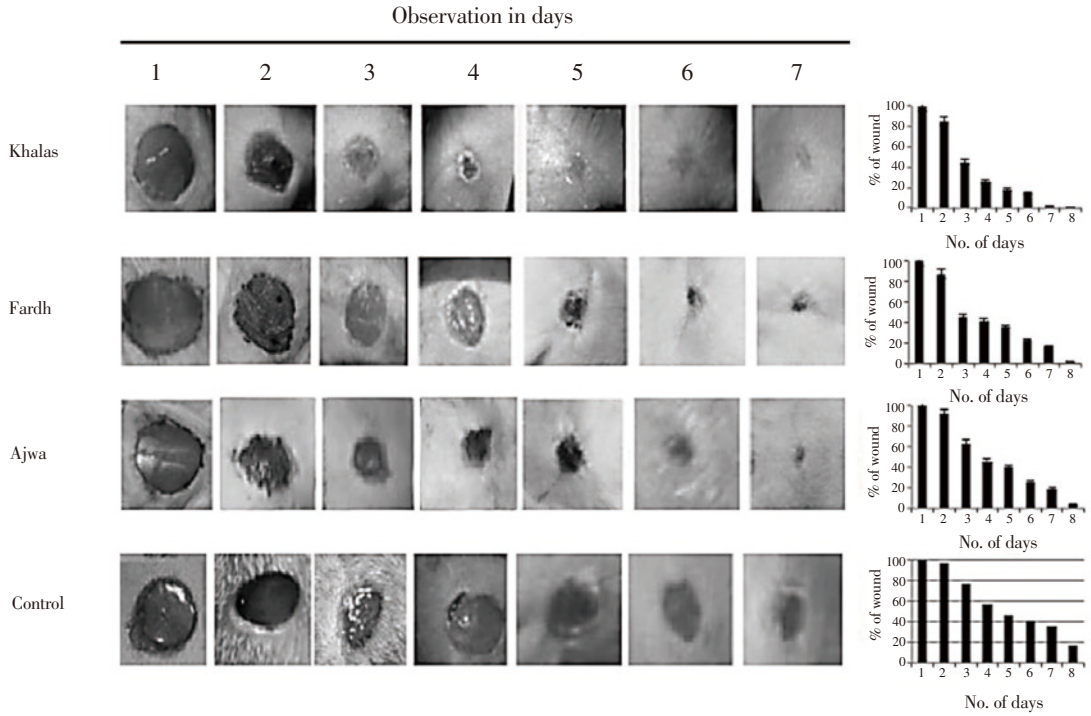

Figure 3. Comparison trend of healing rates of cutaneous full thickness wounds and photographical representation of wound healing trend in CD1 mice subjected to skin excision wounds on different days.

Day 1 represents the excision day followed by observations on a daily basis.

manner. Mice treated with Ajwa and Khalas showed a prolongation of 23 s and 14 s, respectively, in contrast to that of the Fardh and the control groups.

This was further confirmed by BT assay. The results were interesting and showed a considerable prolongation in the BT of animal groups treated with both Ajwa and Khalas dates, which reached 14 and 8 min respectively, after the first week of administration of the extract. This finding suggests that potential influence effects of Ajwa and Khalas date fruits on the inhibition of the primary hemostasis involving platelet adhesion, platelet aggregation, and / or platelet mass formation. Owing to the very high PT of *Phoenix dactylifera* L. fruit, such dates *i.e.*, Ajwa and Khalas may be ideal and applicable agents as anti-snake venom against specific toxins that trigger very rapid clotting by activating the clotting factor cascade (specifically prothrombin) to form multiple blood clots in blood. Another potential beneficial use is that they can be used as an alternative source for the development of new anticoagulant agents. These findings were in agreement to finding of Satish *et al*.[20], which showed that the methanolic extract of Careya arborea, which is traditionally used as antitumor and anti-venom, could prolong PT, activated partial thromboplastin time (APTT) and thrombin time (TT)[20].

The efficacy of Ajwa and Khalas dates in relation to PT and BT may be due to their polyphenolic composition. A similar finding was demonstrated by Kumar *et al*.[21], which showed that owing to polyphenolic composition, both Porana volubilis and Erigeron canadensis can prolong TT by inhibiting thrombin-mediated activity via heparin cofactor Ⅱ. Due to its polysaccharide content, the methanol extract of the leaf of Eichhornia crassipes acts on the intrinsic pathway resulting in the prolongation of APTT alone. Another plant that prolongs all the clotting times is Viola yedoensis, which is traditionally used to reduce swelling and to treat carbuncle and boil[22]. In parallel to the PT and BT investigation we have assessed the wound-healing activity of these date fruits. The processes of wound healing consist of four overlapping stages, which are coagulation, epithelization, granulation, collegenation and tissue remodeling. Many growth factors including macrophage-derived growth factor, platelet-derived growth factor and monocyte-derived growth factor are obligatory for the initiation and advancement of wound healing[23]. It is reported that several substances like tissue extracts, vitamins, minerals and a number of plant products such as tannins, carbohydrate, saponins, diterpenes, flavonoids and polyphenolic compounds exhibit pro-healing effects[24]. Interestingly, our study showed that the Khalas date could accelerate wound healing despite its antithrombotic activity in contrast to the control as well as other date cultivars.

Furthermore, there are several mechanisms by which wound healing can be enhanced and accelerated. These include the formation of granulation tissue, decreasing period of epithelialization, increase in the rate of wound contraction and enhanced synthesis of collagen[1]. Despite its anticoagulant activity demonstrated above, the significant potential of Khalas date to initiate and accelerate wound-healing can be possibly explained by that the impairment of clotting in coagulation phase have lesser impact on wound healing process than the enhancement effect of this date fruit in other phases *i.e.*, epithelization, granulation, collagenation and tissue remodeling, resulting in the acceleration of wound healing. This is supported by the fact that wounds of patients who had bypass (open heart surgery) operations heal completely even though they take warfarin in their treatment regimen as an anticoagulant agent.

This finding is in agreement with several studies which show that there are few agents with the combination efficacy of antithrombotic

and enhancing wound healing acceleration activities. The natural honey, for example, was proved to have this combination of activities in two separate studies. Ahmed *et al*.[24] illustrated that the natural honey significantly prolonged PT, APTT, and TT in vivo. Another study performed by Sazegar *et al*.[25] described that honey and zinc supplement accelerate wound-healing *in-vivo* by increasing the tensile strength, epithelialization, collagen fibers, and revascularization. These findings are similar to what we have demonstrated in this study. However, the question to be addressed is whether the combination of both the Ajwa and Khalas may have a synergistic potential or not, which is yet to be investigated.

In conclusion, our results show that selected date fruits of the *Phoenix dactylifera* L. have antithrombotic as well as enhancing wound healing acceleration activities. These findings reflect that the selected dates can contribute as combination agents of anticoagulant and wound healing enhancer that can add a future promise for those undergoing bypass (open heart surgery) operations.

Conflict of interest statement

We declare that we have no conflict of interest.

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