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Original Research Article

Effect of Extraction Method and Solvent Power on Polyphenol and Flavonoid Levels in *Hyphaene Thebaica* L Mart (*Arecaceae*) (Doum) Fruit, and its Antioxidant and Antibacterial Activities

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Abstract

Purpose: *To evaluate the influence of extraction method and solvent type on extractable polyphenols and flavonoids in Doum Hyphaene Thebaica L. Mart. (Arecaceae) fruit, as well as to examine the antioxidant and antibacterial activities of the fruit extracts.*
Methods: *The extraction procedures were performed separately in an ultrasonic bath or shaking water bath for 30 min (70 °C for ethanol and 60 °C for methanol) at agitation speed of 50 Hz and 70 rpm, respectively. The antioxidant potential of the extracts was investigated using β-carotene bleaching, 2,2-diphenylpicrylhydrazyl (DPPH) and reducing power ability assays. In vitro antibacterial activity of the extracts against Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, and Salmonella typhi was assessed using agar disc diffusion assay.*
Results: *Total polyphenol content (TPC) and total flavonoid content (TFC), as well as antioxidant capacity were maximized using methanol as the extraction solvent, particularly with the ultrasonic method. The half maximal inhibitory concentration (IC_{50}) values of the methanol/ultrasonic (MU), methanol/water bath (MW), ethanol/ultrasonic (EU), and ethanol/water bath (EW) extracts in the DPPH assay were 107.6 126.7, 172.7, and 196.3 µg/mL, respectively. The extracts showed strong antibacterial activity against Staphylococcus aureus and Salmonella typhi, while MU extract inhibited the growth of all pathogenic bacteria used in this study.*
Conclusion: *The antioxidant and antibacterial activities of Doum fruit extracts are significantly affected by the type of extracting solvent and equipment used. The findings further demonstrate that MU extract had stronger antioxidant and antibacterial activity than the other extracts.*

Keywords: *Doum fruit, Hyphaene thebaica, Ultrasonic extraction, Antioxidant, Phenolic, Flavonoid, Antibacterial*

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INTRODUCTION

It is becoming increasingly apparent that antioxidants are important in health and disease prevention. Therefore, the interest in food and food supplements as a source of antioxidants is growing worldwide [1]. Various factors, such as temperature, solvent extracting power, extraction time, and extraction method, significantly affect the composition of the extract [2]. The total

polyphenol level is significantly influenced by the nature of the extraction solvent, sonication extraction time, as well as the interaction between these factors. However, solvent extracting power is the most important factor affecting antioxidant capacity [3]. Antimicrobials of plant origin are effective treating infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials [4]. Antimicrobial activity may involve complex mechanisms, such as inhibition of cell wall, cell membrane, nucleic acid, and protein synthesis, as well as the inhibition of nucleic acid metabolism. The substances identified in the extracts may act separately or in concert to exert these activities [5].

Hyphaene thebaica (Doum fruit) is a desert palm native to Egypt, Sub-Saharan Africa, and Western India [1]. Doum fruit is a good source of essential minerals such as potassium, sodium, calcium, magnesium, and phosphorus [6]. Furthermore, Doum fruit contains B-complex vitamins, carbohydrates, and fiber, which are essential for good nutrition. The aqueous extract of Doum fruit shows antioxidant and anticancer activities. These activities are likely due to the substantial amount of water-soluble phenolic compounds, whereas the aqueous ethanol extract of Doum leaves can scavenge reactive oxygen species [1,7]. The objective of the present study was to optimize the extraction of polyphenols and flavonoids from Doum fruit using different extraction methods. Furthermore, we assessed the antioxidant and antibacterial activities of Doum fruit extracts.

EXPERIMENTAL

Sample preparation

Doum fruit was collected in March 2014 from a local market in Aljazeera Aba City, The Republic of Sudan. The plant was identified by Dr. Haidar Abd Algadir, a taxonomist at the herbarium of the Medicinal and Aromatic Plants Research Institute (MAPRI), National Center for Research, Khartoum, Sudan. The voucher herbarium number of the sample is HYth014 and is available for future reference at the National Center for Research. First, seeds were removed from the Doum fruit and it was sun dried for one week. Pre-dried Doum fruit was brought to the National Engineering Research Center for Functional Food, Jiangnan University, Wuxi, China. Samples were kept dry in desiccators at room temperature and milled into a fine powder using a laboratory scale hammer mill (Debarker Co Ltd, Beijing, China).

Extraction process

To extract phenolic and flavonoid compounds, equal amounts of dried Doum fruit powder were separated into two groups, with two samples in each group. The first group of samples was extracted using ethanol (70 %, v/v) at 70 °C (sample-to-solvent ratio of 1:10) by putting one sample in a shaking water bath (HZ-8812 S-B Hulida Laboratory Co, Ltd) at 70 rpm for 30 min and the second sample in an ultrasonic cleaner bath (KQ-500DE 220V; 50 Hz Numerical Control Ultrasonic Cleaner, Ultrasonic Equipment Co, Ltd, Kun Shan, China) for 30 min. The second group of samples was extracted by the addition of methanol (80 %, v/v) at 60 °C (sample-to-solvent ratio of 1:10) using the same apparatuses. After 30 min, mixtures were filtered using cheese-cloth and sintered glass filters (16 and 10 μm pore size, respectively). Ethanol and methanol were removed from the extracts under reduced pressure at 40 °C using a rotary vacuum evaporator (High-speed centrifuge, Kai Te Experimental Equipment Co, Ltd, Shanghai, China). Each dried extract was dissolved in ethanol (25 mL) and stored at 4 °C until analysis.

Total phenolic content

The total polyphenol content (TPC) of Doum fruit sample extracts was estimated colorimetrically using Folin–Ciocalteu reagent [8]. The appropriately diluted extract (200 μL) was mixed with 15 mL distilled water in a 25 mL volumetric flask; subsequently, 1.25 mL undiluted Folin–Ciocalteu reagent was added. After 1 min, 3.75 mL 20 % sodium carbonate was added to the mixture. Distilled water was added to a final volume of 25 mL. After incubation for 30 min at room temperature, the absorbance was measured at 760 nm using an ultraviolet and visible spectrophotometer (Unico Euippment Co, Ltd, Shanghai, China). The phenolic content of the samples was calculated based on the standard curve for gallic acid. Results are expressed as Gallic Acid equivalents (GAE) in mg/g DW (dry weight of the sample).

Total flavonoid content

The total flavonoid content (TFC) of Doum fruit extracts was determined as described previously [9] with slight modifications. Doum fruit extracts (2.5 mL) were diluted with distilled water (10 mL), followed by the addition of 5 % $NaNO_2$ solution (0.75 mL). Thereafter, 10 % $AlCl_3$ solution (0.75 mL) was added, and the reaction mixture was incubated at room temperature for 5 min. After incubation, 5 mL 1 M NaOH was added, and

distilled water was added to a final volume of 25 mL. The mixture was shaken vigorously, and the absorbance was measured at 510 nm using a spectrophotometer. Results are expressed as milligrams (mg) rutin equivalents (RE)/grams (g) DW.

Determination of the reducing power

The reducing power of the extracts was measured according as described by Xiang and Ning [10]. Samples with different concentrations (0.025–0.5 mg/mL) were mixed with 2 mL phosphate buffer (0.2 M, pH 6.6) and 2 mL 1 % potassium ferricyanide. The mixture was incubated for 20 min at 50 °C. After incubation, 2 mL 10 % trichloroacetic acid was added to the mixture, followed by centrifugation at 3000 × g for 10 min at room temperature. Aliquots of the supernatant (2 mL) were mixed with 2 mL distilled water and 0.4 mL 0.1 % ferric chloride. The absorbance was measured at 700 nm against the blank that contained all reagents except sample extracts. BHT (0.025–0.5 mg/mL) was used as a positive control.

β-Carotene bleaching assays

The reaction mixture containing 1 mL of 0.2 mg/mL β-carotene (dissolved in chloroform), 20 µL linoleic acid, and 200 µL Tween 20 was transferred to a round-bottomed flask. Each mixture was dosed with 0.2 mL of the corresponding doum fruit extracts (200 µg/mL) or positive control (BHT). Chloroform was removed under vacuum using rotary evaporator at room temperature. Thereafter, oxygenated distilled water (25 mL) was added to the mixture and shaken vigorously to form an emulsion. Aliquots (2 mL) of the emulsion were pipetted into test tubes and immediately placed in a 50 °C water bath for 1 h. Absorbance was measured at 470 nm [11]. The antioxidant activity (AA %) was calculated using Eq 1.

$$AA\ \% = \{1-[(A_0-A_t)/(A_{00}-A_{0t})]\} \times 100 \quad \ldots\ldots\ldots(1)$$

where A_0 and A_{00} are the absorbance values at time zero of the incubation for the test sample and control, respectively. A_t and A_{0t} are the absorbance values measured for the test sample and control, respectively, after 30, 60, and 90 min incubation.

DPPH free radical scavenging assay

The antioxidant activity of synthesized compounds, or the capacity to scavenge the "stable" free radical 2,2-diphenylpicrylhydrazyl (DPPH), was determined using DPPH free-radical scavenging assays [12]. Briefly, 1.5 mL DPPH solution (0.004 % in methanol) was incubated with 1.5 mL of the extracts at various concentrations (16-250 µg/mL). The reaction mixture was shaken and incubated for 30 min in the dark at room temperature. The control was prepared as above without the addition of extract. The absorbance of the solution was measured at 517 nm against a blank. DPPH radical scavenging activity was calculated using Eq 2.

$$\%\ inhibition = [(A_c-A_s)/A_c] \times 100 \quad \ldots\ldots\ldots\ldots(2)$$

where A_s is the absorbance of the sample extract and A_c is the absorbance of the control.

Assessment of antibacterial activity

The following bacteria strains were used: *Staphylococcus aureus* DQ269498.1, *Listeria monocytogenes* GE305625.1, *Escherichia coli* CP009273.1, and *Salmonella typhi* GU390666. All strains were purchased from the China General Microbiological Culture Collection Center (Beijing, China). The antibacterial activity of Doum fruit extracts was measured using disk diffusion assays as described previously [13] with some modifications. The test bacterial suspension (100 µL; approximately 1.5 × 10^8 CFU/mL bacteria) was aseptically spread on nutrient agar media using a sterile spreader. A 20 µL sample containing 40 µg/mL of each extract was impregnated into 6-mm sterilized paper discs (Whatman no. 1). Treatments included ampicillin (10 µg) and gentamicin (10 µg) as standard reference antibiotics. Inoculated plates were incubated for 24 h at 37 °C. The zone of inhibition was measured in millimeters (mm) and recorded as the mean ± standard deviation (SD) of triplicate experiments.

Statistical analysis

All experiments were performed in triplicate. One-way analyses of variance (ANOVA) were performed, and significant differences in mean values were evaluated using Duncan's tests ($p < 0.05$). All analyses were conducted using SPSS version 16.0 (SPSS, Chicago, Illinois, USA).

RESULTS

Table 1 shows that the TPC and TFC of Doum fruit extracts varied considerably. The TPC of pitted fruit extracts varied from 116.26 to 139.48 mg GAE/g DW. The TFC in different extracts varied widely, ranging from 24.04 to 47.17 mg RE/g DW (Table 1). These results revealed higher flavonoid levels were observed in MU extracts, followed by MW, EU, and EW extracts.

Table 1: Total phenolic and flavonoid content of Doum (*Hyphaene thebaica*) fruit

Solvent	Extraction method	TPC (mg/g DW)	TFC (mg/g DW)
Methanol 80%	Ultrasonic cleaner bath	139.48±1.18[a]	47.17±0.17[a]
	Shaking water bath	132.51±0.51[b]	41.55±0.17[b]
Ethanol 70%	Ultrasonic cleaner bath	123.36±1.48[c]	28.62±0.12[c]
	Shaking water bath	116.26±0.43[d]	24.04±0.17[d]

Results represent the mean ± standard deviation (SD, n = 3); values with different superscript letters in the same column are significantly different (p ≤ 0.05)

Reducing power

The reducing potential of the tested extracts was recorded over a concentration range of 0.025 to 0.5 mg/mL (Figure 1). The results showed that there were significant differences ($p < 0.05$) in some reducing power of doum fruit extracts. The reducing power of the samples varied from 0.064 to 2.14.

β-Carotene bleaching

In the β-carotene bleaching assays, all Doum fruit sample extracts had lower antioxidant activities than BHT. The antioxidant activities of extracts were: MU > MW > EU > EW (Figure 2). There were significant differences ($p < 0.05$) among the antioxidant activities of all extracts.

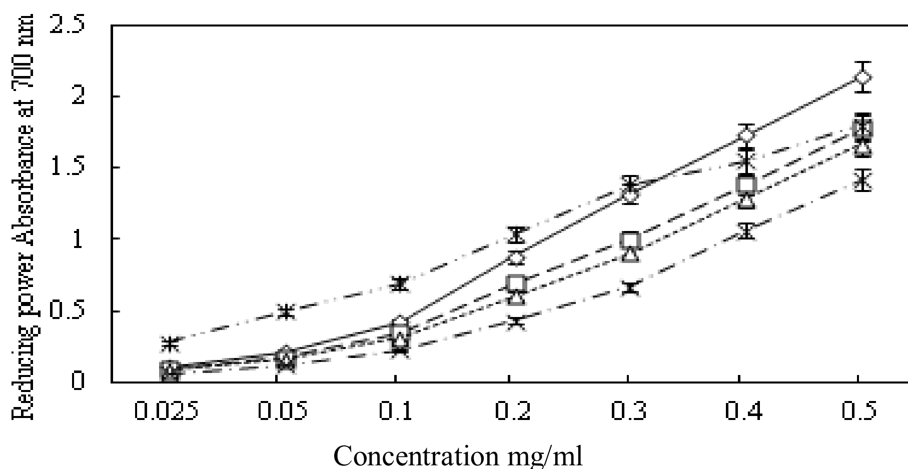

Figure 1: Reducing power of different extracts from the Doum fruit at different concentrations. MU: methanol/ultrasonic, MW: Methanol/water bath, EU: ethanol/ultrasonic, EW: ethanol/water bath, BHT: positive standard BHT. Data represent the mean ± SD (n = 3)

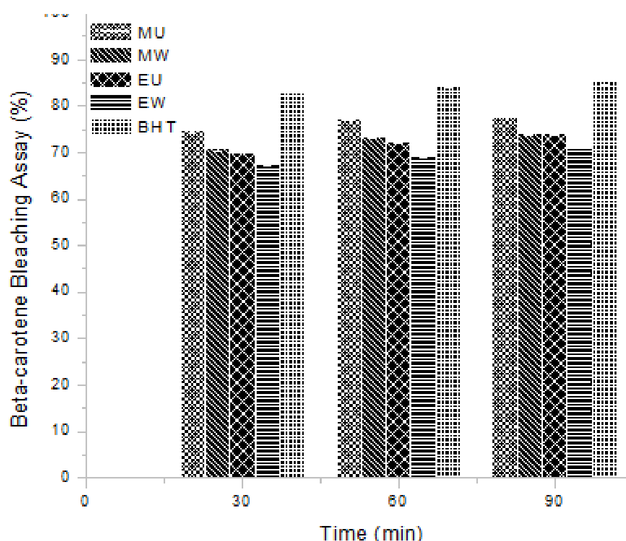

Figure 2: β-carotene bleaching results. MU: methanol/ultrasonic, MW: methanol/water bath, EU: ethanol/ultrasonic, EW: ethanol/water bath, BHT: positive standard BHT. Values are the mean ± SD (n = 3)

Free radical scavenging activity

Figure 3 shows the scavenging effects of methanol extracts (MU, MW) and ethanol extracts (EU, EW) of Doum fruit on DPPH. The scavenging ability of the Doum fruit extracts on DPPH increased with increasing concentrations compared with results obtained using tertiary butylhydroquinone (TBHQ) and ascorbic acid standards. The IC_{50} of the MU extract was 107.6 ± 0.15 µg/mL, followed by MW with an IC_{50} of 126.7 ± 0.23 µg/mL, EU with an IC_{50} of 172.7 ± 0.51 µg/mL, and EW with an IC_{50} of 196.3±0.47 µg/mL. The positive controls, TBHQ and ascorbic acid, showed high scavenging activity with IC_{50} values of 8.9 ± 0.02 µg/mL and 49.7 ± 0.07 µg/mL, respectively.

Antibacterial activity

Table 2 shows the antibacterial activity of the methanol and ethanol extracts of Doum fruit. Based on these data, it is evident that the fruit extracts have variable inhibition effects against pathogenic bacteria.

DISCUSSION

Natural antioxidants for medical and food applications are obtained through extraction procedures to provide maximum yield while maintaining high quality extracts. Extraction procedures are optimized according to the vigor of the extraction procedure, and there is the possibility of sample-to-sample variation in the extracted material, sample/solvent ratio, type of solvent, and time and temperature of extraction [14]. In this study, we employed double extractions on Doum fruit samples to systematically compare the extracts using two extracting solvents and two types of apparatuses for the extraction. There was a significant difference in TPC and TFC between the two extracting solvents (ethanol and methanol), and to a lesser extent, between the two apparatuses (ultrasonic cleaner bath and shaking water bath). The polyphenol levels obtained using ethanol varied from 123.36 to116.26 GAE/g DW. Using methanol, the polyphenol levels in extracts varied from 139.48 to 132.51 mg GAE/g DW.

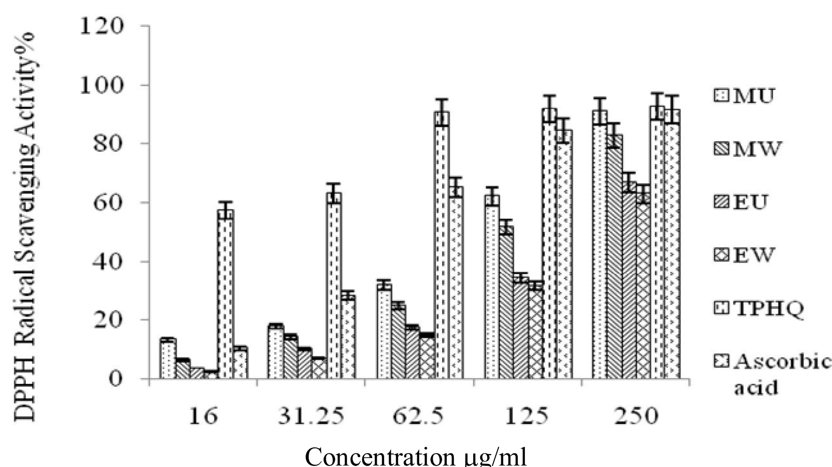

Figure 3: Inhibition of DPPH radical by MU, MW, EU, EW, tertiary butylhydroquinone (TBHQ) and ascorbic acid. Data represent the mean ± SD (n = 3)

Table 2: Antibacterial activity of Doum fruit extracts

Test bacteria	Doum fruit extracts (zone of inhibition, mm)				Standard antibiotic (zone of inhibition, mm)
	MU	*MW*	*EU*	*EW*	
Gram positive bacteria					Ampicillin
S. aureus	$14.2\pm 0.72_A^c$	$11.4\pm 0.84_A^d$	$16\pm 0.75_A^b$	$14.7\pm 0.70_A^c$	24.9 ± 0.81^a
L. monocytogenes	$8.2 \pm 0.56_B^b$	$5.3\pm 0.64_B^c$	$5.5\pm 0.65_C^c$	ND	20.3 ± 0.75^a
Gram negative bacteria					Gentamicin
E. coli	$4.6 \pm 0.53_C^b$	ND	ND	ND	25.3 ± 1.06^a
S. typhi	$15.5\pm 0.81_A^b$	$11.5\pm 0.70_A^{cd}$	$12.5\pm 0.93_B^c$	$10.6\pm0.60_B^d$	26.2 ± 0.86^a

The results represent the mean ± SD (n = 3); values in the same row that do not share the same small letters are significantly different ($p < 0.05$); different capital letters in a column indicate significant difference $p < 0.05$); standard antibiotics were used as positive control; ND = not detectable

Importantly, these levels are almost two times higher than those previously reported for methanol extracts [15]. In contrast, the flavonoid quantity obtained using methanol varied from 41.55 to 47.17 mg RE/g DW. These results are in agreement with the findings of Mohamed *et al* [15].

The antioxidant capacities of Doum fruit extracts were evaluated using reducing power assays, β-carotene bleaching assays, and DPPH radical assays. Assessing the reducing power of a compound may be a good indicator of its potential antioxidant activity. As shown in Figure 1, compared to the MU, MW, and EU extracts, the EW extract exhibited the lowest antioxidant capacity in reducing power assays. Measurement of reducing power is primarily linked with the occurrence of reductants such as phenolics, which exert antioxidant action by donating their hydrogen atom or breaking free radical chains. Reducing power is the measurement of the reductive ability of an antioxidant, and is evaluated by the transformation of Fe^{3+} to Fe^{2+} in the presence of extracts [16].

β-carotene has biological activity and is an important physiological compound. The presence of different antioxidants can hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical formed during the system [17]. In β-carotene bleaching assays, the linoleic acid free radical formed after abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules. As shown in Figure 2, the MU extract showed the highest antioxidant activity in the β-carotene bleaching assays. However, the difference in the values for the MW and EU extracts was not significant.

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule [18]. The reduction capability of DPPH was determined by examining the decrease in its absorbance at 517 nm, which was induced by antioxidants. The scavenging ability of the Doum fruit extracts on DPPH was likely due to the hydrogen donating ability of the polyphenolic compounds in the extracts. In the present studies, the IC_{50} values of the Doum extracts varied from 107.6 to 172.7 µg/mL. These results are lower than what has previously been reported [7]. These differences referred to many factors such as type of solvent, composition of solvent and apparatuses that were used in the extraction. Overall, the results indicate that the solvent extracting power exerted the most pronounced effect on extract quality, followed by the apparatuses that were used in the extraction.

Antibacterial activity

The antibacterial activities against gram positive and gram negative bacteria suggest there is a spectra of antibiotic compounds or metabolic toxins in plant extracts [15]. Our results showed that ethanol extracts were most effective against *Staphylococcus aureus*, whereas methanol extracts showed stronger inhibition effects against *Salmonella typhi* (Table 2). We also found that lower antibacterial activities of all Doum fruit extracts against *L. monocytogenes* compared to *S. aureus*. Our data show that the MU extract inhibited the growth of all pathogenic bacteria in this study. Similarly, other authors have reported that methanol and aqueous extracts of Doum fruit showed higher antibacterial activity against Gram-positive bacteria [15]. However, all Doum fruit extracts demonstrated no antibacterial activity against *E. coli* colonies except the MU extract, which had slight activity. The mechanism of polyphenol toxicity against microbes may be related to inhibition of hydrolytic enzymes (proteases) or other interactions that inactivate microbial adhesins, cell envelope transport proteins, and non-specific interactions with carbohydrates [19]. Based on the properties of the organic solvent used for extraction, the extract appears to contain diverse substances, ranging from non-polar to polar compounds.

CONCLUSION

The findings of this study have shown that the antioxidant activity of Doum fruit is significantly affected by the quality of the extracting solvent and the apparatus used. The results also reveal that Doum fruit extracts contain high levels of phenols and flavonoids, and possess significant antioxidant and antibacterial activities. It is evident from these findings that Doum fruit can serve as a potential source of natural antioxidants and antibacterial agents, which can help prevent diseases related to oxidative stress and pathogenic bacteria. Future studies should identity the antioxidant and antibacterial compounds of Doum fruit and further determine their spectrum of activity.

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