

**ANTIOXIDANT AND ANTICANCER ACTIVITIES OF
FRUIT WATER EXTRACT Of *Hyphaene thebaica* L. (DOUM)**

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ABSTRACT

The antioxidant capacity and the total phenol content were analyzed in the fruit extract of *Hyphaene thebaica* L. The antioxidant capacity was estimated by DPPH and iron chelating assays. Quercetin, ascorbic, BHT and tannic were used as positive controls. Phenolic compounds of doum fruit extract were identified by GC-Mass. Also the effect of doum extract on the of acute myeloid leukemia cells (AML) was studied. The results showed that the total phenolic content was 0.7 μ g/ g dried doum fruit sample as quercetin and 0.6 μ g/ g as gallic acid. Nine major phenolic compounds were identified by GC-Mass as; Gallic acid, P-coumaric, Catechol, Apiginin, ferulic acid, carvacrol, resorcinol, pyrogallol and cinnamic acid. In iron chelating assay the results showed that 8 μ g/ml doum extract gave the best antioxidant activity (21% inhibition). In DPPH assay 10 μ g/ml extract exhibited 50% antioxidant activity (IC₅₀) but 15 μ g/ml extract exhibited 80 % antioxidant activity. In the viability test of AML cells, the results showed that the half maximal inhibitory concentration (IC₅₀) of doum extract was 3 μ g/ml. The results indicated that the water doum extract could be an important dietary source of phenolic compounds with high antioxidant and anticancer activities.

Key words: AML, antioxidant and anticancer activities, Hyphaene thebaica L., total phenols

Abbreviations: BHT : butylated hydroxytoluene , DPPH : 1,1-diphenyl-2-picrilhydrazyl , AML : acute myeloid leukemia, GC-MS : Gas chromatography –Mass spectroscopy

1. INTRODUCTION

The oxidative stress, defined as the imbalance between oxidants and antioxidants in favor of the oxidants potentially leading to damage has been suggested to be the cause of aging and various diseases in humans. In modern western medicine, the balance between antioxidation and oxidation is believed to be a critical concept in maintaining a healthy biological system (Dreosti 1991; Ahmad 1995; Davies 2000; Tiwari 2001 and Katalinic *et al.*, 2006). In traditional Chinese medicine for more than 2000 years, a general recommendation to the consumer is to increase the intake of foods rich in antioxidant compounds (*e.g* polyphenols, carotenoids) due to their well-known healthy effects. As a consequence, the evidence accelerated the search for antioxidant principles, which led to the identification of natural resources and isolation of active antioxidant molecules. Many plants have been identified as having potential antioxidant activities and their consumption is recommended (Kitts *et al.*, 2000 ;

Lee *et al.*, 2003 ; Kilani *et al.*,2008; Piao *et al.*, 2008 and Wang *et al.* , 2009).

Bioactive phenols are very interesting as antioxidants because of their ability to act as efficient free radical scavengers (Langley-Evans, 2000) In the last two decades the number of publications on the potential health benefits of polyphenols has increased enormously (Tiwari 2001; Lee *et al.*, 2003; Hinneburg *et al.*, 2006 and Katalinic *et al.*, 2006).

Doum is one of the commonly consumed beverages in traditional places in Egypt and is rich in polyphenolic compounds (Eldahshan *et al.* , 2008). The current focus is toward natural antioxidants, especially plant polyphenols (Katalinic *et al.*, 2006 and Eldahshan *et al.*, 2008, 2009).

Physiologically, antioxidants play a major role in preventing the formation of free radicals, which are responsible for many oxidative processes. Antioxidants may be synthetic, such as butylated hydroxyanisole (BHA), propyl gallate (PG) and

butylated hydroxytoluene (BHT) or of natural origin such as α -tocopherol ; phenolic compounds as well as polyphenolics (Abas *et al.*, 2006).

The aim of this study was to investigate the activities of the water doum fruit extract as an antioxidant and anticancer and to determine its total phenols content. In addition, the total phenols were identified by GC-MS.

2. MATERIALS AND METHODS

2.1. Plant material and chemicals

Dried doum fruit was obtained from a local supermarket in Giza, Egypt. Authenticated by Dr. Fathy M. Soliman, Professor of Phycology, Pharmacognosy Department, Faculty of Pharmacy, Cairo University and Prof. Dr. Mahmoud H. M. Abdel-Rahman, Botany Department, Faculty of Science, El-Fayoum University. Chemicals were purchased from Sigma Chemical Co. (USA). All chemicals and reagents were of analytical grade

2.2. Sample preparation and extraction

The eaten part of doum fruit was separated and then ground to powder. Fifty grams of ground dried doum fruit were suspended in 600 ml ultra pure water at 37°C overnight. The aqueous extract was then filtered, and the obtained filtrate was concentrated under vacuum using a rotary evaporator, The concentrated extract was kept in refrigerator to dryness yielding the crude extract (11.5 g), which was then suspended in ultra pure water to prepare the water extract of doum fruit, and stored at 4°C prior to use.

2.3. Total phenols

The total phenols of doum extract were estimated according to the Folin-Ciocalteu method (Singleton *et al.*,1999). To 50 μ l of doum extract, 250 μ l of undiluted Folin-Ciocalteu-reagent were added . After 1 min, 750 μ l of 20% (w/v) aqueous Na₂ CO₃ were added, and the volume was made up to 5.0 ml with H₂O. The control contained all reaction reagents except the extract. After 2h of incubation at 25°C, the absorbance was measured at 760nm using a spectrophotometer (Thermoscientific-UK) and compared to a quercetin calibration curve. Total phenols were determined as quercetin and gallic acid equivalents, and the values were presented as means of triplicate analyses

2.4. GC-Mass determination

Doum fruit extract was analyzed by gas chromatography coupled to a mass and FID detectors (Agilent GC System 6890 Series, Mass Selective Detector, Agilent 5973 Network, FID detector). Samples were injected with an

autosampler (Agilent 7683 Series). The inlet temperature was 180°C, HP-101 (25 m x 0.2mm x 0.2 mm) column, programmed temperature, 50°C held for 1 min, rising at 8°C/min. to 180°C 3 min, The helium flow rate was 34cm/sec. Individual compound identifications were made by matching spectra with those from mass spectral library (Wiley 275.L), and the identity of each compound was confirmed by its Kovats index (Jennings and Shibamoto,1980).

2.5. Iron chelation activity

The chelation of iron (II) ions by the doum fruit water extract was carried out as described by Carter (1971). Different concentrations of the extract were added to 100 μ l of 2.0 mM aqueous FeCl₂ and 900 μ l methanol. The control contained all the reaction reagents except the extract or positive control substances. After a 5min incubation, the reaction was initiated by 400 μ l of 5.0 % ferrozine (0.5 gm ferrozine/10ml methanol). After a 16 min equilibrium period, the absorbance at 562nm was recorded using a spectrophotometer (Thermoscientific-UK). The iron chelation activities were calculated from the absorbance of the control (A_c) and of the sample (A_s) using equation (1). The values are presented as the means of triplicate analysis.

$$\text{Inhibition (\%)} = (A_c) - (A_s) / (A_c) \times 100 \quad (1)$$

Where A_c (0) is the absorbance of the control at t = 0 min.

and (t) is the absorbance of the antioxidant at t =16 min

2.6. DPPH radical (1,1-diphenyl-2-picrilhydrazyl)

The ability of the extracts to scavenge DPPH free radicals was determined by the method of Gyamfi *et al.* (1999). The control contained all the reaction reagents except the extract or positive control substance. Different concentrations of the tested sample were placed in a cuvette, and 2 ml of methanol solution of DPPH radical were added. Absorbance measurements commenced immediately. The decrease in absorbance at 517 nm was determined after 16 min incubation in dark for all samples using a spectrophotometer (Thermoscientific-UK) . Special care was taken to minimize the loss of free radical activity of the DPPH radical stock solution. Methanolic solutions of pure compounds (vitamin C, tannic and quercetin) were tested too at different concentrations. All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated according to the equation (1)

2.7. Viability of acute myeloid leukemia (AML)

Acute myeloid leukemia cells (AML) were taken from patients in the National Cancer Institute (NCI) Cairo, Egypt after clinical diagnosis. The mononuclear cells were separated from the whole blood samples of AML patients according to Hofman *et al.* (1982).

2.7.1. Medium and reagents

The culture medium was prepared using RPMI 1640, 10% fetal bovine serum and 10 % L-glutamine. Trypan blue (0.4%) was prepared by dissolving of 0.4g of the dye in 100 ml distilled water then kept in brown closed glass bottles.

2.7.2. Viability of tumor cells

The viability percentages of tumor cells were measured by the modified cytotoxic trypan blue-exclusion technique of Bennett *et al.* (1976). The viability percentages of tumor cells were measured after incubation with doum extract as

3. RESULTS AND DISCUSSION

There are many different antioxidant components in plants, and it is relatively difficult to measure each antioxidant component separately. Therefore, several different methods have been developed to evaluate the antioxidant activity of biological samples (Lopez, *et al.*, 2003). The total phenol content, the antioxidant capacity and anticancer activity of doum fruit water extract were determined in this study.

3.1. Extract yields

The amount of materials that can be extracted from a plant depends on the extract procedure and the possibility exists of sample-to- sample variation in the extracted materials. A double extraction of doum fruit was employed for this study. The extract yield was 230 mg extract/g dried plant material (Table1).

Table (1): Total phenols and raw materials of doum fruit extract.

Raw materials (mg extract/g plant material)	Phenols content (µg/ g dried plant material)	
	As gallic	As quercetin
230 ± 5	0.6 ± 0.02	0.7 ± 0.05

well as saline as the control. Two ml of media containing AML (2 x10⁴ cells) were transferred into a set of tubes each, then different concentrations (0.0, 1, 2, 3, 4, 8, 10 µ g / ml) of doum fruit extract were added into the appropriate tube as well as saline. The tubes were incubated at 37°C for 2 h then centrifuged at 1000 rpm for 5 min and the separated cells were suspended in 2 ml saline. For each examined material (and control), a new clean, dry small test tube was used and 10 µl of cell suspension, 80 µl saline and 10 µl trypan blue (0.4 %) were added and mixed, then the number of living cells (non stained) was calculated using a homocytometer slide by microscope (Nikon, TMS).

2.8. Statistical analysis

The direction and magnitude of correlation between variables were done using analysis of variance (ANOVA) and quantified by the correlation. The P-values less than 0.05 were considered statistically significant. Data are presented as mean values from three independent experiments made in triplicate. Different letters indicated significant differences at P≤ 0.05 level among treatments according to Duncan’s multiple range test.

3.2. Total phenolic content

Phenolic substances have been shown to be responsible for the antioxidant activity of plant materials (Rice-Evans *et al.*, 1996). Therefore, the amount of total phenols in the extract was investigated by Folin-CioCalter method. The total phenols content is expressed as quercetin and gallic. The results showed that the total phenolic content in the fruit extract consisted of 0.7 µg / g dried plant as quercetin and 0.6 µg / g as gallic acid (Table 1). Many herbs and spices are an excellent source of phenolic compounds which have been reported to show good antioxidant activity (Rice-Evans *et al.*, 1996 and Zhang and Wang, 2001). Doum fruits showed an antioxidant activity due to the substantial amount of their water-soluble phenolic contents.

3.3. Profile of the phenolic compounds

GC-Mass identification as described in Table (2) of the aqueous extract of doum fruit, revealed the presence of nine major phenolic components, viz.: gallic acid, P-coumaric, catechol, apiginin, ferulic acid, carvacrol, resorcinol, pyrogallol and cinnamic acid. These results are similar to those of Eldahshan *et al.* (2008) who identified fourteen phenolic compounds in the extract of doum leaves

Table (2): Phenol contents (mg/kg) of water doum fruit extract identified by GC-Mass.

Compounds	Control (mg/kg dried plant materials)
Gallic acid	39.66d
P-Coumaric acid	15.04f
Catechol	7.42g
Apiginin	133.8b
Carvacrol	42.2d
Resorcinol	70.7c
Pyrogallol	27.6 e
Cinnamic acid	68.84c
Ferulic acid	207.2a
Total phenols	572.8
Phenol compounds isolated using GC-MS . LSD = 10	

such as quercetin, gallic, apiginin, luteolin and kaempferol.

3.4. Iron II chelating activity

In the iron chelation assay, the general ability of the extract to donate electrons is tested, whereas in the DPPH assay, hydrogen atoms are involved. Therefore, the ability of the extract to chelate iron (II) ions was evaluated. The results in Table (3)

between the concentration of the extract and iron chelating ability of the extract. Standard antioxidant compounds were used to evaluate the iron chelating activity (ascorbic acid, tannic acid and quercetin). The results showed no significant correlation between the concentration of standard materials and iron chelating activity.

3.5. DPPH radical (1,1-diphenyl-2-picrylhydrazyl)

Table (3): The effect of doum fruit extract on iron chelating activity.

Doum fruit (dried) and positive control concentration $\mu\text{g/ml}$	(% Inhibition)			
	Doum fruit	Quercetin	Ascorbic acid	Tannic acid
2	$10^b \pm 0.67$	$26^a \pm 1.76$	$22^a \pm 1.76$	$17^b \pm 1.48$
3	$13^b \pm 0.58$	$19^b \pm 0.67$	$14^b \pm 2.6$	$21^a \pm 2.06$
4	$18^a \pm 1.17$	ND	ND	ND
5	$19^a \pm 1.61$	ND	ND	ND
8	$21^a \pm 1.79$	$19^b \pm 0.58$	$9^c \pm 0.57$	$19^a \pm 2.50$

Iron Chelating activities were calculated as % inhibition values for Doum fruit. The values for the pure compounds (quercetin, ascorbic acid, and tannic) were calculated from data obtained from similar experiments .
LSD = 3.5, ND: not determined

show that there is a low correlation between the total phenols in the examined extract and iron chelation activity. These results are in agreement with Halliwell (1997). In complex systems, such as food and food preparations, various different mechanisms may contribute to oxidative processes, such as in Fenton reactions, where transition metal ions play a vital role as catalysts of oxidative processes. Different reactive oxygen species might be generated and various target structures such as lipids, proteins and carbohydrates, can be affected (Halliwell, 1997). These processes can be delayed by iron chelating and deactivation. Therefore, the ability of the extract to chelate iron (II) ions was evaluated. The results in Table (3). show that 8 $\mu\text{g/ml}$ doum extract gave the best iron chelating (21% inhibition). No significant correlation was found

The good correlation between the results from the total phenolics analysis and the antioxidative assays have been previously reported (Zhang and Wang, 2001). The role of an antioxidant is to remove free radicals. One important mechanism through which this is achieved is by donating hydrogen to a free radical causing its reduction to an unreactive species. Addition of hydrogen would remove the odd electron feature which is responsible for radical reactivity. The hydrogen-donating activity, measured using DPPH radicals as hydrogen acceptors, showed that there was a significant association between the concentration of the extract and the percentage of inhibition (Table 4). It was observed that at the concentration of 10 $\mu\text{g/ml}$, the extract exhibited 50% antioxidant activity (IC_{50}) while the 15 $\mu\text{g/ml}$ extract exhibited 80% antioxidant activity. Also there was a

significant association between the concentration of quercetin, but there was a non significant association between the concentration of ascorbic, and BHT as shown in (Table 4). These results are

when compared to the control (2 % death). Also doum extract reduced the viability from 98 to 40% (60% death) at 4 µg/ml and the dead cells reached 92% by 8 µg/ml. From these results it was

Table (4): The effect of doum extract on DPPH free radical – scavenging.

Doum fruit (dried) and positive control concentration µg/ml	(% Inhibition)			
	Doum extract	Quercetin	Ascorbic acid	BHT
2	10 ^c ± 0.67	11 ^c ± 0.10	13 ^{bc} ± 1.01	9 ^c ± 0.89
3	24 ^c ± 0.58	38 ^b ± 1.17	15 ^{bc} ± 0.89	14 ^b ± 0.89
4	25 ^{bc} ± 2.06	ND	ND	ND
5	30 ^{bc} ± 5.13	ND	ND	ND
6	39 ^b ± 2.06	ND	ND	ND
10	50 ^b ± 10.54	69 ^a ± 3.41	18 ^a ± 0.89	42 ^a ± 2.01
15	80 ^a ± 2.44	ND	ND	ND

DPPH free radical - scavenging was calculated as % inhibition values for doum fruit. The values for the pure compounds (quercetin, ascorbic acid, and BHT) were calculated from data obtained from similar experiments. LSD = 11, ND: not determined.

in agreement with the findings of Hsu *et al.* (2006), Lantto *et al.* (2009) and Wang *et al.* (2009). Also Eldahshan *et al.* (2008 and 2009) showed that the aqueous ethanolic extract of doum leaves appeared to be a potent scavenger of reactive oxygen species.

3.6. The effect of doum extract on the viability of AML (*in vitro* study)

The effect of doum extract on acute myeloid leukemia cells is recorded in (Table 5). It can be

clear that the doum extract has an inhibitory effect on AML cells. There was a significant association between the concentration of doum extract and the inhibitory effect as shown in Table (5). The inhibitory effect of doum extract could be due to the presence of phenolic compounds in the extract (Fabiani *et al.*, 2006 and Feng *et al.*, 2007) who found that phenolic compounds can induce apoptosis in HL-60 leukemia cell line.

Also İşgör *et al.* (2008) studied the effects of

Table (5): Effect of doum extract on the viability of AML.

Treatment	Doum extract Concentration (µg/ml)	% Dead cells
Tumor + saline (control)	0.0	2 ^f ± 0.58
Tumor + doum extract	1	7 ^f ± 1.17
Tumor + doum extract	2	17 ^e ± 2.06
Tumor + doum extract	3	50 ^d ± 5.88
Tumor + doum extract	4	60 ^c ± 5.88
Tumor + doum extract	8	82 ^b ± 2.44
Tumor + doum extract	10	92 ^a ± 2.35

Data are presented as mean values from three independent experiments made in triplicate. Different letters indicated significant differences at P ≤ 0.05 level among treatments according to Duncan's multiple range test. LSD = 7.5.

found that the incubation of tumor cells with doum extract significantly reduced the viability of these cells and the dead cells were significantly increased with high extract concentration. At concentration of 2 µg/ml the extract reduced the viability from 98 to 83% (17% death). The dead cells produced by extract reached 50 % by 3 µg/ml

garlic extract on human leukemia HL60 cell lines. They found that the growth inhibition exerted by extracts was in a dose dependent manner. The aqueous doum extract has cytotoxic effect according to the guidelines from the American National Cancer Institute, which considered that IC50 for any potential plant should be < 30 µg/ml

(Alenka *et al.*, 2000). From the results obtained, it was observed that doum extract has shown antitumor effects towards leukemia cancer cells, which could be determined at 3µg/ml (Table 5) .

Finally, the results from the antioxidant assays showed that the examined extract could act as a radical scavenger . One important mechanism of antioxidant action may be DPPH scavengers. In DPPH assays Doum extract showed a higher antioxidant activity than BHT and ascorbic, but a lower antioxidant activity than quercetin, whereas in iron chelating assay the extract showed a low antioxidant activity than all positive control antioxidants, (quercetin, ascorbic and tannic). The results suggest that phenolic compounds might be contributors to the antioxidant activity of doum fruit extract. The results obtained from viability assay revealed that doum extract has a significant anticancer activity against acute myeloid leukemia cells. This anticancer activity could be due to the antioxidant activity of doum extract and presence of phenolic compounds. In future experiments it would be interesting to investigate other effects of doum extract in different food systems. Also many studies must be done to understand the mode of action of doum extract as an anticancer agent.

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تأثير مستخلص ثمار الدوم كمضاد للاكسدة وللسرطان

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ملخص

تم قياس قدرة مستخلص ثمار الدوم كمضاد للاكسدة، كذلك تم تقدير محتوي و قدرة الفينولات الكلية كمضاد للاكسدة. أجري ذلك بطريقتين هما طريقة DPPH وطريقة الحديد المخيلية. استخدم الكورستين والاسكوربيك وبيوتيلاند هيدروكسي تولوين كمعاملة كنترول موجبة. وتم التعرف علي المركبات الفينولية بجهاز GC-Mass. تم أيضا دراسة تأثير مستخلص الدوم على مدى حيوية اللوكيميا. أوضحت النتائج أن الفينولات الكلية كانت 0.7 ميكروجرام/ جرام مادة جافة من ثمار الدوم ككورستين و 0.6 ميكروجرام كحامض الجاليك. تم التعرف علي تسعة مركبات فينولية باستخدام جهاز GC-Mass وهي حامض الجاليك، وكوماريك، وكاتيكول، وابعجينين، حامض الفيروليك، وكارفاكول، وريزورسينول، وبيروجالول وحامض السيناميك. أوضحت النتائج في طريقة الحديد المخيلية أن 8 ميكروجرام /مل اعطت افضل نشاط كمضاد للاكسدة (21% تثبيط) وفي تجربة ال DPPH سبب 1 مجم مستخلص الدوم 50% كمضاد للاكسدة وتركيز 10 ميكروجرام/مل سبب 80% كمضاد للاكسدة. في تجربة الحيوية اوضحت النتائج ان التركيز المسبب لموت نصف الخلايا السرطانية كان 3ميكروجرام / مل. دلت النتائج على ان مستخلص ثمار الدوم مصدر غذائي جيد للمركبات الفينولية وله نشاط كمضاد للاكسدة و للسرطان.