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Phytochemical and Biological Studies of Date Palm Extracts *phoenix dactylifera* Siwi Variety

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THE investigation of four extracts of Siwi date fruit (70% methanol, ethyl acetate, *n*-butanol and chloroform) showed good antibacterial, antifungal activities. This study includes evaluation of the antioxidant, and antimicrobial activities with determination of MIC for the four prepared extracts. Moreover, their cytotoxicity was determined against HeLa and HEK293T cancer cell lines by MTT 3-(4 , 5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) assay. The antioxidant activity was determined by diphenyl-(2,4,6-trinitrophenyl) iminoaxonium (DPPH) method. New findings are the important elements as Ni and Si beside the essential valuable amino acid valine, and the pentoses xylose and arabinose all proved to exist in Siwi fruit never reported in any other Egyptian date varieties. Other important elements and secondary metabolites are seven unsaturated fatty acids, one phenolic acid, five flavonoids, sixteen amino acids, twenty monoterpenes and five sesquiterpenes beside an appreciable amount of dietary fibers, were also detected. No aflatoxins or pesticides were found in Siwi date fruit. Separation of all compounds was done through extensive chromatographic analysis on PC, CC and HPLC, Identification of all compounds was performed by UV, Co-PC with standards, amino acid analyzer, atomic absorption, GCMS and ESIMS. HPLC was used for the test of aflatoxins with aflatoxins standard, according to association of analytical communities AOAC. Furthermore, the test for pesticides was done using LC/MS/MS according to the European Standard method EN 15662: 2008.

Keywords: Siwi date, Phoenix dactylifera, Antibacterial, Antifungal, Antioxidant, Cytotoxicity, Hela, Hek293T.

Introduction

Phoenix dactylifera L. or date palm (family Arecaceae is one of the oldest known fruit crops. It has been cultivated in North Africa and the Middle East for more than 5000 years [1], it has nutritional and biological values [2,3]. It has been cultivated in North Africa and Middle East for more than 5000 BC. There are more than 600 variety of dates classified according to its shape and organoleptic properties of the fruit [4]. Except for its storage and areas of production, Siwi variety has not been studied before [5] so it initiates us to do more phytochemical and biological studies on semi dry Siwi variety of *Phoenix dactylifera*. Date palm. *P.dactylifera* spreads outside through arabian countries and middle east countries as Spain [6]. From the systematic point of view, *P.dactylifera* belongs to Angiospermsm, family Arecaceae, genus Phoenix, species dactylifera. Palm dates has a great economic importance as it is used as food, shelter, clothing, producing different products as oil, alcohol, sugar, organic acids, lipids and protein [7], Although date palm is primarily of nutritional value yet many varieties of *P.dactylifera* showed antimicrobial, antifungal and hemolytic activities [8,9] besides as an antioxidant [10], for improving male and female fertility [11] hepatoprotecive, nephroprotective [12] and for reduction of tumor growth [13-15]. It is our aim here to investigate the phytochemical

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constituents and to measure its possible biological activity.

Experimental

Date fruits were collected from the new valley governorate 400 Km. south west part of Egypt. Fruits (500 g) were cutted to small pieces then separately extracted with 70% methanol, ethyl acetate, *n*-butanol and chloroform. Amino acids were determined by Kieldhl analysis Gerhard Vadodest 20 S LC 300 amino acid analyzer EpendorfBiotronik, Germany. UV Shimadzu spectrophotometer Tokyo-Japan 1.00 cm^3 quartz cells was used for UV measuring purposes. All elements were determined through Atomic absorption spectrometer Buck model 210 VGP with detector model 190-930 nm, absorption emission 0.082 to 3.20. High performance liquid chromatography HPLC with Diode array detector DAD Agilent technologies USA system was used for separation and identification of flavonoids, sugars, vitamins and other phenolics. HPLC column of the type Zorbax-C18 (150g x 4.5 mm 1.D x 5 µm particle size) was used for the separation of flavonoids. Volatile oils were extracted through steam distillation and solvent-solvent extraction with n-hexane and chloroform, then identified by GC/MS Agilent 6800 gas chromatography with an Agilent mass spectrometric detector ionization energy 70 EV for identification of unsaturated fatty acids. LC/MS/MS of the type Triple stageQ system was used for identification of volatile oil mono-, sesqui- and triterpenes. The used fungal and bacterial species were previously isolated from cases of human dermatophytosis [16]. The fungi were grown in sterilized 9 Cm. petri dishes containing Sabouraud's Dextrose Agar (SDA) supplemented with 0.05% chlortimazoleas to suppress bacterial contamination [17]. From these cultures, agar disc (10 mm diam.) containing spores and hyphase were transferred specially to screw trapped vials containing 20 ml. sterile distilled water. After thorough shaking 1 ml samples of spore suspension were pipetted into sterile petri dishes, followed by addition of 15 ml. liquefied SDA, which was then left to solidify. The tested extract mixtures in dimethyl sulphoxide (DMSO) to give 2.0% concentration. Antifungal and antibacterial activities were determined according to the method reported in the literature [16] using 3mm diameter filter paper discs (Whattman NO.3) located with 10 ml. of the solution under investigation (200 ml. disc, 2.0%). The discs were placed on the surface of the fungal cultures, which were incubated at 30℃. The diameter of the inhibition zone around each disc was measured. The previous method was used for determining antibacterial activity using chloramphenicol antibacterial standard [18].

Cytotoxicity

Four concentrations of chloroform, ethyl acetate, *n*-butanol and 70% methanol extracts were used for measuring cytotoxicity on two cancer cell lines (Hela and HEK293T cells) after 24 hrs., (MTT), was mixed with the treated cells to give blue precipitate, which was dissolved in DMSO, then the absorbance is measured using UV-spectrophotometer for determination of the lowest effective concentrations that can reduce the cancer cell. The cell viability was calculated by measuring the absorbance of each concentration. Dimethyl sulphoxide was used as control [4, 13-15, 19].

Antioxidant activity

The antioxidant activity was measured using the DPPH method [19] thus different dilutions of phenolic extract were prepared for each variety. An aliquot of 25 µl. of diluted sample was added to 975µl. Of DPPH solution ($6x10^{-5}$M). The decrease in the absorbance was determined at 520 nm.

Test for Phenolics

Phenolics were determined by Folin-Ciocalteu solution using gallic acid as standard. Total carbohydrates were determined by anthrone test method using UV-Visible spectroscopic technique (61g/100g date fruit), while HPLC was used for its qualitative and quantitative determination.

Test for pesticide residues

Analysis of pesticides residues were done at central laboratory of pesticides and heavy metals food residual analysis using LC/MS/MS according to QuEChERs method (Quick, Easy, Cheap, Effective, Rugged and Safe) [3].

European method EN 15662: 2008.

Test for aflatoxins B1, B2, G1 and G2

Analysis of aflatoxins was done using HPLC with aflatoxins standards according to association of analytical communication (AOAAC) official method (19th Edition, 2012), the test was done at

TABLE1. Separated elements from Siwi Date

Element	mg/100g.
Mg	64.42
Si	7.56
P	21.17
S	18.25
K	507.95
Na	14.38
Mn	0.52
Co	0/09
Ni	1.71
Cu	0.42
Zn	0.57
Pb	0.02
Cd	0.23
Fe	1.79

TABLE 2. GC/MS monoterpenes and sesquiterpenes identified from Siwi date fruits

No.	Compound name	Method of separation of monoterpenes and sesquiterpenes (peak area) Steam distillation RT.in Mins.	(Peak area)	Percentage%	n-hexane R.T.	Peak area	%	$CHCL_3$ R.T.	Peak area	%
1	β-myrecene	6.55	(0.3)	1.6 %						
2	α-terpinolene	8.44	(0.33)	1.66 %						
3	Linalool	8.62	(0.64)	3.42 %				8.61	(3.82)	9.48%
4	Neoalloocimene	9.15	(0.64)	3.42 %						
5	Camphor	9.52	(0.9)	4.81 %						
6	Isomentole	9.87	(2.23)	11.93 %						
7	Menthol	10.02	(2.38)	12.73%						
8	α-terpineol	10.36	(029)	1.55%				10.35	(0.5)	1.24%
9	Dihydrocarvone				10.47	(1.61)	2.3%			
10	Isohydrocarveol				10.78	(1.59)	2,27%			
11	β-citronellol	10.98	(0.23)	1.23%						
12	Camphene				11.02	(2.33)	3.33%			
13	L-Carvone	11.31	(1.89)	10.11%	11.31	(60.5)	86.4%	11.31	(1.35)	3.35%
14	E-Citral							11.81	(24.7)	61.3%
15	Limonene	11.93	(0.01)	0.05%						
16	Isolimonene	12.82	(1.75)	5.35%						
17	Eugenol	13.29	(0.24)	1.28%				13.29	(4.02)	16.2%
18	Nopyl acetate	14.34	(0.52)	2.78%				14.34	(3.45)	8.56%
19	Trans-cinnamyl acetate							14.68	(1.45)	3.6%
20	O-Cymene	7.16	(2.27)							
1	β-elemene				13.84	(1.59)	2.27%			
2	α-humulene							14.89	(1.13)	2.8$%
3	Y-cadinene							15.28	(0.89)	2.21%
4	α-selinene				15.54	(1.84)	2.63%			
5	Calamenene				15.94	(0.56)	0.8%			
		One triterpene			Squalene RT. 31.35					

central laboratory of pesticides and heavy metals for food residual analysis. The test (according to association of analytical communities AOAC) proves the absence of any aflatoxins in Siwi date variety. The test for pesticides was done using LC/MS/MS according to the European Standard method EN 15662: 2008.

Statistical Analysis

All the experiments were repeated at least three times in three different days, and each experiment was conducted in triplicate and error bars represent standard deviation (SD). All the values were represented as mean± SD.

Results and Discussions

We here report for the first time the phytochemical profile and biological activity of four different extracts namely, 70%methanol, ethyl acetate, *n*-butanol and chloroform of date palm (*P.dactylifera*) Siwi variety. Surveying the quantities of elements in Siwi date variety (Table 1) proved it has the highest safely daily intake amounts of nutritional elements. Besides, Si and Ni are firstly reported in Siwi date palm fruits and not reported in any other date variety. Si prevents bones deformities: it assists calcium for the growth and maintenance of joints and bones, prevents alopecia and maintains skin appearance [20-22]. In addition to the fore mentioned elements, other nutritional elements as Mg, P, K, Na, Mn, Cu, Zn, Co, Fe, S and Cl were proved to present in sufficient amounts, however traces of Cd and Pb have been found, probably from water ground.

A total of twenty monoterpenes and five sesquiterpenes have been identified from n-hexane and chloroform extracts using steam distillation and their percentages recorded in Table 2..Moreover, seven fatty acids have been separated by GC (Table 3, Fig 1) and identified in the form of their esters by MS, namely the ethyl ester of dodecanoate, tetradecanoate, pentadecanoate, hexadecanoate and heptadecanoate besides ethyl oleate and 67% of the total fatty acids as the ethyl ester of the polyunsaturated fatty acid, i.e. linolenolate. Five different vitamins have been reported from the investigated Siwi date, namely, A, B1, B2, E and B3. The later one has the highest abundance, of four times more than the highest reported of all date fruit varieties ever known (Table4), however, vitamin E was never reported from any date variety. Flavonoid detection proved the presence of five compounds namely: the 3, 5, 6 and 7-hydroxyflavones beside to quercetin and one phenolic acid namely coumaric acid. The later acid is known for increasing the expression of antioxidant enzyme genes in rat cardiac tissue [23]. These findings explain the positive antioxidant activity of Siwi date fruit.

Siwi date has sixteen amino acids (Table 5, fig 2), among them valine and cysteine as the first detection, not reported at any maturation stage of any other non-Egyptian date variety [5]. Valine affects muscle growth and recovering tissues damaged during physical activity [24,25].

The tests proved the presence of higher amounts of monosaccharides, ever reported from other date fruit varieties namely: glucose, fructose, galactose, arabinose and xylose, the latter two pentoses have not been detected from any other date variety.

Dietary fibers (3.81 g/100g date fruit) were detected in Siwi date fruit, which promote physiological effects including laxation and/ or blood cholesterol and glucose attenuation (American association of Cereal Chemists, 2001). Neither aflatoxins nor pesticide residues were observed. Identification was done through PC with standards, UV, amino acid analyzer, GC/MS, atomic absorption spectrophotometer and HPLC/MS.

Chloroform and ethyl acetate extracts exhibited medium antibacterial activities against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Serratiam arcescens* using chloramphenicol as standard (Table 6) while methanol and n-butanol showed no activity. Ethyl acetate was the only extract that showed medium antifungal activity against *Fusarium oxysporum* and *Scopulariopsis brevicaulis* using clotrimazol as standard due to the presence of flavonoids, while other extracts did not exhibit any antifungal activity.

The four extracts showed different cytotoxic activities against HeLa and HEK293T cancer cell lines being higher for ethyl acetate and 70%methanol extracts and slightly less for both *n*-butanol and chloroform extracts (Tables 7-8) *n*-Butanol, ethyl acetate and chloroform extracts exhibited good antioxidant activity comparable to

TABLE 3. GC data of Fatty acid esters identified from Siwi date fruits

No.	Retention time R.T.(mins.)	Name	Yield (%)
1	15.78	Ethyl dodecanoate (M.wt.=228)	0.87
2	18.63	Ethylteradecanoate (M.Wt.=256)	2.57
3	20.9	Ethylpentadecanoate (M.Wt.=270)	1.2
4	21.32	Ethylhexaadecanoate (M.Wt.=284)	40.4
5	22.46	Ethylheptadecanoate (M.Wt.=298)	2.5
6	23.43	Ethyloleate (M.Wt.=310)	52.2
7	26.36	Ethyl linolinoloate (M.Wt.= 308)	0.29

TABLE 4. Vitamines found in Siwi variety date fruit and the highest reported in other varieties

Vitamin	Siwi fruit (µg/100g)	Highest reported (µg/100g)
Vitamin A	**3.64**	**44.70**
Vitamin B1	**307.1**	**120.00**
Vitamin B2	**607.00**	**160.00**
Vitamin B3	**5731.00**	**1610.0**

TABLE 5. Amino acids isolated from Siwi date fruit

No.	Amino acid	Retention Time In mins.	Conc. (**µg/ml.**)	Wt. (g/100g)	Amino acid %
1	Aspartic acid	11.53	42.29	0.19	5.99
2	Threonine	14.93	2.63	0.01	0.37
3	Serine	16.27	8.16	0.04	1.16
4	Glutamic acid	18.07	138.56	0.61	19,64
5	Glycine	25.7	27.43	0.12	3.89
6	Alanine	26.7	83.36	0.37	11.82
7	Cystine	29.35	17.62	0.08	2.5
8	Valine	32.08	31.86	0.14	4.52
9	Methionine	35.92	5.29	0.02	0.75
10	Isoleucine	36.97	10.06	0.04	1.43
11	Leucine	38.12	39.86	0.18	5.65
12	Tyrosine	41.37	20.88	0.09	2/96
13	Phenylalanine	42.85	39.53	0.17.	5.6
14	Histidine	52.87	7.15	0.03	1.01
15	Lysine	54. 27	17.73	0.08	2.51
16	(NH_4)+	58.62	228.42	1	32.38
17	Argenine	64.37	16.56	0.07	2.35
	Total		705.5	31	100

TABLE 6. Antibacterial activity and (MIC) of *phoenix dactylifera* Siwi variety extracts.

Bacterial strain	$CHCl_3$ MIC(mg/ml)	EtOAc MIC(mg/ml)	Chloramphenicol
Staphylococcus aureus +ve	8 (34.4)	8 (17.2)	10 (0.15)
Bacillus cereus +ve	10 (136)	8 (17)	16 (0.15)
Escherichia coli +ve	8 (17)	11 (68.8)	12 (0.3)
Pseudomonas aeruginosa +ve	10 (34)	10 (68.8)	10 (0.15)
Serratia marcescens –ve	10 (272)	8 (137.5)	20 (0.3)

TABLE 7. Cytotoxicity of Siwi extracts on Hek293T

Conc. Ext. Abs.	0.00125	0.0025	0.00375	0.005	0.0075	0.01
Control DMS	0.564	1.041	1.339	1.905		
$CHCl_3$	0.402	0.379	0.315	0.688		
EtOAc.	-------	0.835	--------	0.731	0.612	0.369
BuOH				0.313	0.289	0.826
MeOH				0.65	0.662	1.077

TABLE 8. Cytotoxicity of Siwi extracts on Hela cells.

Conc. Ext. Abs.	0.0025	0.0050	0.00375	0.0075	0.0100	0.00125
Control Clotramizol	1.14	1.55	1.339	1.92	2.34	---------
$CHCl_3$	0.85	-----	0.79	-------	-------	1.02
EtOAc	0.59	0.48	---------	0.59	0.48	
BuOH	0.97	1.04	--------	0.88	0.82	----------
MeOH	0.40	0.52	----------	0.69	1.80	----------

TABLE 9. Antioxidant activities of Siwi date extract

Sample	Inhibition %
n-Hexane extract	4.04
$CHCl_3$	48.74
EtOAc.	15.86
n-Butanol	50.85
Rutin	84.87
Vitamin C	75.62

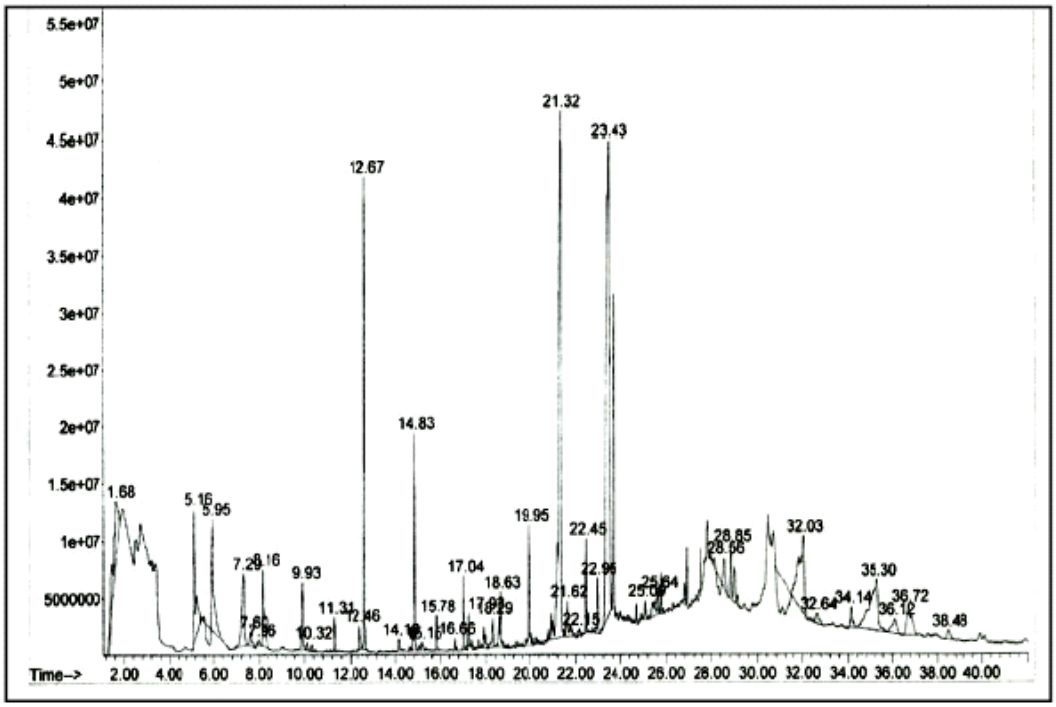

Fig 1. Gas chromatogram of fatty acid esters separated from Siwi date fruit

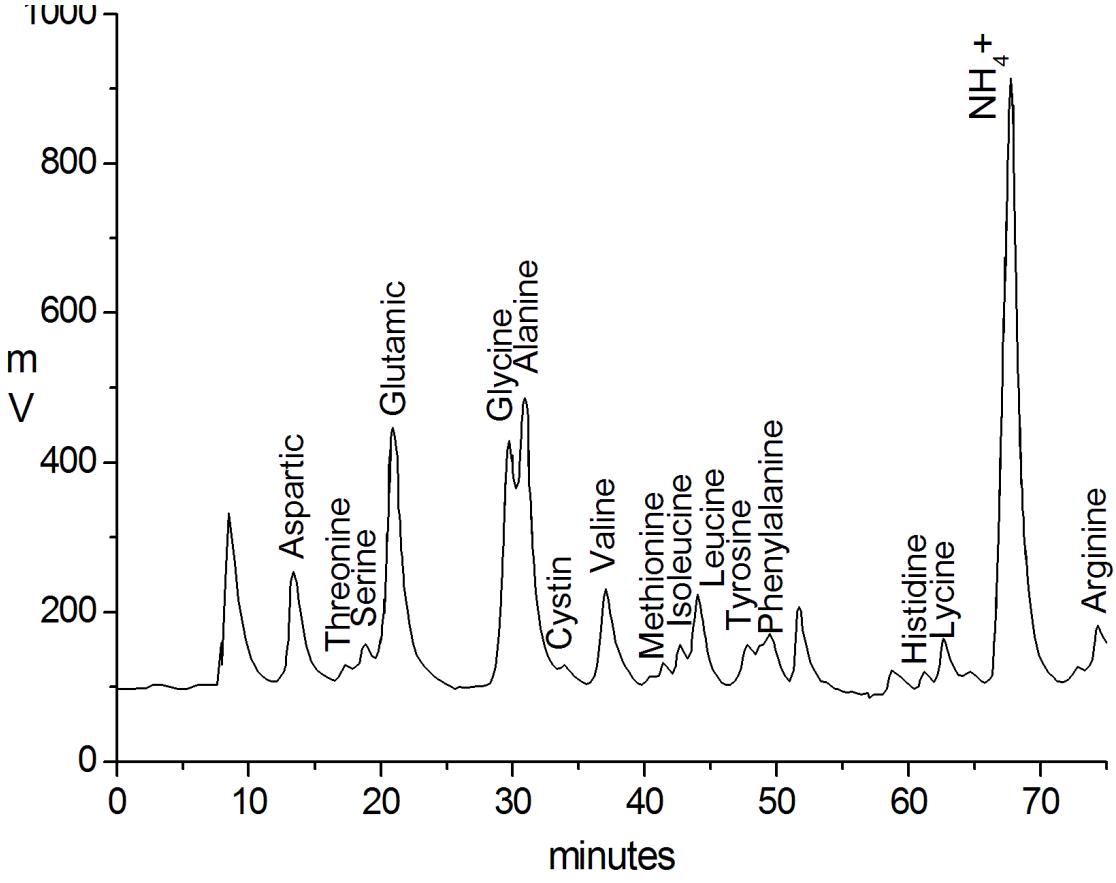

Fig. 2 LC3000 Chromatogram of separated amino acids from digested Siwi semi dry date fruit (El-Siwi)

rutin and ascorbic acid as positive controls, while *n*-hexane showed weak antioxidant activity (Table 9). In all cases MIC values were determined.

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دراسه فيتوكيميائيه وبيولوجيه لمستخلصات ثمار نبات البلح من النوع (سيوي)

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تم في هذا البحث دراسه المكونات الكيميائيه لاربعه مستخلصات عضويه (الكلوروفورم وخلات الايثيل والميثانول والبيوتانول) لثمار البلح من النوع سيوي. تبين وجود العديد من الاحماض الامينيه (سبعه عشر حمض اميني) والسكريات (خمسه انواع) والمركبات المتطايره (عشرون من المواد التربينيه وخمسه من السيسكويتربينيه)والمعادن (عشره عناصر)والفيتامينات (أ-ب1-ب2-ب3)والاحماض الفينوليه والفلافونيدات (سبعه مركبات)

تم دراسه مضادات الميكروبات ودرجه السميه للخلايا ومضادات الاكسده.

تم تعيين متبقيات المبيدات والافلاتوكسين . تم قياس درجه سميه المستخلصات علي خلايا سرطان الرحم والكلي .