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Phytochemical Profile of Phoenix rupicola T. Anderson Seeds and In Vitro Evaluation of their Estrogenic Activity using MCF-7 Cell Lines

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Abstract

The current study aimed to investigate the phytochemical profile and the in vitro estrogenic activity of *Phoenix rupicola* T. Anderson seeds' extracts. Gas chromatography/ mass spectrometry (GC/MS) of the petroleum ether (seed oil) and chloroform extracts revealed the characterization of 93 compounds. γ-Sitosterol (8.54%), n-dodecane (6.00%), n-undecane (5.87%) and (2E)-2-decenal (3.48%) represented the major compounds in the petroleum ether extract, whereas lauric acid (21.73%), γ-sitosterol (21.21%), palmitic acid (9.19%) and myristic acid (6.37%) were the main components in the chloroform extract. Moreover, investigation of the 70% aqueous methanol extract by high-performance liquid chromatography coupled with diode-array detector (HPLC-DAD) revealed the identification of 13 phenolic compounds, of which catechin (105.75 mg%), vanillic acid (58.26 mg%), *p*-hydroxybenzoic acid (32.84 mg%) and cinnamic acid (27.62 mg%) dominated The estrogenic activity of the extracts was tested in vitro via sulforhodamine B (SRB) assay on MCF-7 cells, where petroleum ether extract at a dose of 10 µg ug/mL exhibited the highest activity ($p < 0.05$) indicated by 1.17 % increase in cells' proliferation compared to 17β-estradiol. Consequently, *P. rupicola* seeds provide a promising source of phytoconstituents with estrogenic activity worthy for further detailed studies.

Keywords: Phoenix rupicola T. Anderson; GCMS; HPLC-DAD; *in vitro* estrogenic assay; MCF-7 cell lines.

1. Introduction

Genus *Phoenix* is one of the most common genera in the palm family (Arecaceae) with 17 species distributed around the world. *Phoenix* spp. have been used for food, fermented beverages, clothing, construction, fiber, and ornamental purposes. Their fruits, seeds and pollen grains have been used traditionally to treat various ailments viz. fever, inflammation, paralysis, nervous disorders, liver, and intestinal problems, in addition to their positive effects on both female and male fertility [1–6].

Phoenix rupicola T. Anderson (Cliff date palm) is a wild palm which grows in the hill slopes of Eastern Himalayas. Currently, *P. rupicola* has been deposited under near threatened category [NT] (IUCN 2006) because of its habitat destruction due to anthropogenic pressure [7]. The cliff date palm, unlike other date palms, does not retain the old leaf bases, thus possess a slender smooth trunk about 6.1 m tall. The leaves are long (3.1 m) and bright green. The fruits are purplish red dates composed of a pericarp, mesocarp, endocarp and one seed.

Estrogens belong to a family of steroidal molecules which regulate cellular processes that trigger the development and maintenance of female characteristics and sexual reproduction, such as regulating menses, bone metabolism and lipid levels. Menopause is the halting of menses and marks the end of women reproductive cycle. It is associated with undesirable symptoms like hot flashes, mood swings, sleep disturbances and weight gain, in addition to osteoporosis and cardiovascular diseases [8]. Thus, many women use traditional medicine to alleviate these symptoms, instead of using conventional hormonal replacement therapy (HRT) since they are associated with increased risk of blood clotting, breast, ovarian and endometrial cancer [9,10].

MCF-7 is a human breast cancer line, which has been used by scientists worldwide in anticancer drug

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research, as well as to screen the estrogenic and anti-estrogenic activity indirectly through measurement of the cells' proliferation. MCF-7 possesses estrogen, progesterone and glucocorticoid receptors and when grown in vitro, the cells are capable of forming domes and the epithelial-like cells which grow in monolayers. Thus, it is the best-characterized estrogen receptor (ER)-positive cell line in terms of known genes regulated by estrogens that promote proliferation. And therefore, it is the most widely used cell line for proliferation assays [11,12].

Recent studies proved that genus *Phoenix* exhibited estrogenic activity where n-butanol and petroleum ether fractions of *P. dactylifera* pollen showed a significant increase in FSH and estradiol levels [13]. Moreover, our previous study revealed that *P. rupicola* pulp had a potent estrogenic activity in ovariectomized rats compared to reference standard (17β-estradiol) [14]. Taking this into account, in addition to very few studies were found reporting the lipid profile of *P. rupicola* seeds. Daulatabad et al. [15], studied the fatty acid component of *P. rupicola* seed oil and reported that the oil contained 51.8% unsaturated fatty acids mainly oleic and linoleic, as well as 48.2% saturated fatty acids namely lauric, myristic, palmitic, stearic, arachidic and behenic acids. Consequently, the current study was designed to characterize the phytoconstituents of the of *P. rupicola* seeds, as well as, to study the in vitro estrogenic activity on MCF-7 cell lines.

2. Experimental

2.1. Plant material

P. rupicola dates were obtained from Cairo-Alexandria Desert Road in August 2018. Plant taxonomist Mrs. Therese Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and El-Orman Botanical Garden, Giza, Egypt, authenticated the obtained plant material. Voucher specimen (PHG-P-PR-264) was deposited in the herbarium of the Faculty of Pharmacy, Ain Shams University. The dates were cleaned; the pulps and seeds were separated manually, cut into small pieces, dried at 40 ºC, then ground into fine powder and kept in closed containers in the refrigerator at 4 ºC.

2.2. Preparation of plant extracts

Powdered *P. rupicola* seeds (213.4 g) were, successively, exhaustively extracted in a Soxhlet apparatus using petroleum ether (60-80°C), chloroform and 70% aqueous methanol. The obtained extracts were then filtered, and the filtrate was concentrated under reduced pressure using rotatory evaporator (Heidolph, Germany) till dryness at 55°C to yield 4 g (1.87%) petroleum ether (seed oil), 3.2 g (1.5%) chloroform and 30 g (14.05%) 70% aqueous methanol extracts. The prepared extracts were used in the assessment of the biological activity. All solvents used were of the highest grade commercially available.

2.3. Gas chromatography–mass spectrometry (GC/MS) analysis of petroleum ether and chloroform extracts of P. rupicola seeds

GC/MS analysis of petroleum ether (seed oil) and chloroform extracts of *P. rupicola* seeds was carried out using a Shimadzu GCMS-QP2010 (Koyoto, Japan) equipped with Rtx-5MS fused bonded column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Restek, USA) equipped with a split–splitless injector. The initial column temperature was kept at 50 °C for 3 min (isothermal) and programmed to 300 °C at a rate of 5 °C/min and kept constant at 300 °C for 10 min (isothermal). Injector temperature was 280 °C. Helium carrier gas flow rate was 1.37 mL/min. All the mass spectra were recorded applying the following condition: (equipment current) filament emission current, 60 mA; ionization voltage, 70 eV; ion source, 220°C. Diluted samples (1% v/v) were injected with split mode (split ratio 1: 15). Compounds were identified based on the compounds' mass spectrometric data and retention indices in comparison with those of the National Institute of Standards and Technology (NIST) Mass Spectral Library (December 2011), the Wiley Registry of Mass Spectral Data,8th edition and literature.

2.4. Determination of phenolics and flavonoids in 70% aqueous methanol extract of P. rupicola seeds by high-performance liquid chromatography coupled with diode-array detector (HPLC-DAD)

HPLC analysis was carried out according to [16] with slight modifications. The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 mL/ min for a total run time of 70 min and the gradient program was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. There was 10 min of post-run for reconditioning. The injection volume was 10 µL and peaks were monitored simultaneously at 280, 320 and 360 nm for the benzoic acid, cinnamic acid derivatives and flavonoids, respectively. All samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectrum and compared with those of the available authentic standard compounds.

2.5. Cell culture and proliferation assay

Human breast adenocarcinoma cell line (MCF-7) was obtained from Nawah Scientific Inc., (Mokatam, Cairo, Egypt). Cells were maintained in DMEM media supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of

heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO_2 atmosphere at 37 °C. Cell viability was assessed by SRB assay. Aliquots of 100 µL cell suspension ($5x10^3$ cells) were incubated in 96-well plates in complete media for 24 h. Cells were treated with another aliquot of 100 µL media containing petroleum ether, chloroform and 70% aqueous methanol extracts of *P. rupicola* seeds at concentrations (0.001, 0.01, 0.1, 1, 10 µg/mL) for a five-dose assay using estradiol (0.001 µg/mL) as reference standard (positive control). After 72 h of drug exposure, cells were fixed by replacing media with 150 µL of 10% TCA and incubated at 4 °C for 1 h. The TCA solution was removed, and the cells were washed 5 times with distilled water. Aliquots of 70 µL SRB solution (0.4% w/v) were added and incubated in a dark place at room temperature for 10 min. Plates were washed three times with 1% acetic acid and allowed to air-dry overnight. Then, 150 µL of TRIS (10 mM) was added to dissolve protein-bound SRB stain; the absorbance was measured at 540 nm using a BMG LABTECH®- FLUO star Omega microplate reader [17, 18].

2..6. Statistical analysis

Each set of experiment was repeated at least three times. Values were expressed as mean ± standard deviation of the proliferative effect, which represented the maximum proliferation induced by the extracts. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test. P value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. GC/MS analysis

GC/MS analysis of the petroleum ether (seed oil) and chloroform extracts of *P. rupicola* seeds revealed the annotation of 87 and 16 compounds, respectively, as illustrated in Table 1. Hydrocarbons including saturated, unsaturated, cyclic and aromatic predominated in the petroleum ether extract, representing 62.53% of the extract, with n-dodecane (6.00%) being the major compound, followed by n-undecane (5.87%) and 3,7-dimethyldecane (2.07%). On the other hand, fatty acids and/or esters dominated in the chloroform extract accounting for 37.29% of the extract, with lauric (21.73%), palmitic (9.19%) and myristic acids (6.37%) representing the most abundant components. While fatty acids and/or esters in the petroleum ether extract accounted for 3.23%, in which lauric (1.59%) and palmitic (0.64%) acids, as well as ethyl oleate (0.52%) were the major components. These results were in accordance with Daulatabad et al. [15], who reported that *P. rupicola* seed oil contained oleic (41.2%), lauric (20.8%), myristic (12.4%), palmitic (10.9%) acids, however linoleic (10.6%), stearic (3.3%), arachidic (0.5%) and behenic (0.3%) acids could not be detected under the conditions used in our study. Additionally, phytosterols represented 10.77 and 24.73% of petroleum ether and chloroform extracts, respectively, where γ-sitosterol was the most major phytosterol in both extracts (8.54 and 21.21%, respectively).

3.2. Determination of phenolic acids and flavonoids by HPLC-DAD

The relative concentration of the detected phenolic acids and flavonoids were determined from the peak areas and compared to the available authentic standards, including gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, gentisic acid, catechin, chlorogenic acid, caffeic acid, syringic acid, vanillic acid, ferulic acid, sinapic acid, *p*-coumaric acid, rutin, rosmarinic acid, apigenin-7-*O*-glucoside, cinnamic acid, quercetin, apigenin, kaempferol and chrysin. The compounds were identified by matching the retention times and UV spectra against those of standards. Results in mg% (mg/ 100 g sample) were recorded in Table 2. Structures of the main phytoconstituents in *P. rupicola* seeds' extracts were expressed in Figure 1.

Ten phenolic acids have been identified in the 70% aqueous methanol extract of *P. rupicola* seeds, with vanillic acid being the major one (58.26 mg%), followed by *p*-hydroxybenzoic (32.84 mg%) and cinnamic (27.62 mg%) acids. Three flavonoids were also identified in the extract namely catechin (105.72 mg%), rutin (19.30 mg%) and quercetin (1.77 mg%). Gentisic, rosmarinic and sinapic acids, as well as, apigenin-7-O-glucoside, apigenin, kaempferol and chrysin were totally absent in the extract. These results were parallel to previous studies performed on the most common member of the genus *Phoenix; P. dactylifera.* Eid et al. identified and quantified via HPLC the hydroxybenzoic acids: gallic acid, protocatechuic, hydroxybenzoic, vanillic, isovanillic, and syringic acids, as well as the hydroxycinnamic acids: chlorogenic, caffeic, *p*-coumaric, ferulic, sinapic, and isoferulic acids, in addition to, the flavanol catechin in *P. dactylifera* dates' cultivars (Ajwa, Barni, and Khalas) at the main ripening stages (kimri, khalal, rutab, and tamr) [19]. The flavonoid aglycones quercetin, naringenin, myricetin, apigenin, kaempferol, and luteolin were also identified in the same study after acid hydrolysis. It is worth mentioning, that phenolic compounds have a potential to scavenge free radicals, thus, increasing antimutagenic effects and stimulating the immune system which in turn may contribute to different pharmacological effects such as anti-inflammatory, anticancer, antimicrobial and antiviral effects. Further research should be carried out to provide detailed information about the various pharmacological effects attributed to *P. rupicola* extracts.

Table 1:GC/MS analysis of petroleum ether and chloroform extracts of *P. rupicola* seeds

No.	Compound	Rt (min.)	Molecular Formula	%RA [a]		RI exp. [b]	RI rep. [c]	Base peak (m/z)	Class	Identification [d]
				Petroleum ether	Chloroform					
1.	1,1,2,3-Tetramethylcyclohexane	7.59	$C_{10}H_{20}$	0.26	-	952	957	69.10	Monoterpenoid	KI, MS
2.	1-Heptanol	7.75	$C_7H_{16}O$	0.48	-	957	957	57.10	Fatty alcohol	KI, MS
3.	2-Methylnonane	7.90	$C_{10}H_{22}$	0.89	-	962	962	43.05	Saturated HC	KI, MS
4.	3-Methylnonane	8.11	$C_{10}H_{22}$	1.14	-	969	968	57.05	Saturated HC	KI, MS
5.	β-Pinene	8.16	$C_{10}H_{16}$	-	3.09	971	971	43.00	Monoterpenoid	KI, MS
6.	trans-p-Menthane	8.45	$C_{10}H_{22}$	0.44	-	981	978	97.10	Monoterpenoid	KI, MS
7.	cis-p-Menthane	8.54	$C_{10}H_{22}$	0.30	-	984	982	55.05	Monoterpenoid	KI, MS
8.	2-Octanone	8.59	$C_8H_{16}O$	-	0.81	986	990	43.00	Ketone	KI, MS
9.	4-Ethyl-2,3-dimethyl-2-hexene	8.61	$C_{10}H_{20}$	0.14	-	986	913	69.05	Unsaturated HC	MS
10.	α-Phellandrene	8.73	$C_{10}H_{16}$	-	0.96	990	996	43.00	Monoterpene	KI, MS
11.	Mesitylene	8.83	$C_{10}H_{12}$	0.66	-	994	997	105.10	Aromatic HC	KI, MS
12.	n-Decane	9.01	$C_{10}H_{22}$	1.31	-	1000	1000	43.05	Saturated HC	KI, MS
13.	Ethyl 2-methyl-2-pentenoate	9.64	$C_8H_{14}O_2$	0.23	-	1020	1020	57.10	Fatty ester	KI, MS
14.	1,2,4-Trimethylbenzene	9.73	C_9H_{12}	1.41	-	1023	1025	43.05	Aromatic HC	KI, MS
15.	n-Butylcyclohexane	9.99	$C_{10}H_{20}$	0.79	-	1031	1032	83.10	Cyclic HC	KI, MS
16.	n-Pentylcyclo-pentane	10.06	$C_{10}H_{20}$	0.56	-	1033	1033	69.10	Cyclic HC	KI, MS
17.	3,7-Dimethylnonane	10.24	$C_{11}H_{24}$	0.89	-	1039	1042	57.05	Saturated HC	KI, MS
18.	1,1-Dimethyl-2-propylcyclohexane	10.33	$C_{11}H_{20}$	0.24	-	1042	1114	69.05	Cyclic HC	MS
19.	1-Ethyl-2-propylcyclohexane	10.43	$C_{11}H_{20}$	0.32	-	1045	1140	69.05	Cyclic HC	MS
20.	trans-Decahydronaphthalene	10.70	$C_{10}H_{18}$	1.99	-	1053	1053	67.05	Cyclic HC	KI, MS
21.	5-Methyldecane	10.84	$C_{11}H_{24}$	0.49	-	1058	1058	57.05	Saturated HC	KI, MS
22.	4-Methyldecane	10.94	$C_{11}H_{24}$	0.68	-	1061	1061	43.05	Saturated HC	KI, MS
23.	2-Methyldecane	11.04	$C_{11}H_{24}$	1.45	-	1064	1064	43.05	Saturated HC	KI, MS
24.	3-Methyldecane	11.25	$C_{11}H_{24}$	1.37	-	1071	1071	57.05	Saturated HC	KI, MS
25.	5-Undecene	11.39	$C_{11}H_{22}$	1.00	-	1075	1078	55.05	Unsaturated HC	KI, MS
26.	4-Undecene	11.52	$C_{11}H_{22}$	0.40	-	1079	1080	55.05	Unsaturated HC	KI, MS
27.	1-Butyl-2-Ethylcyclopentane	11.65	$C_{11}H_{22}$	0.97	-	1083	1083	97.15	Cyclic HC	KI, MS
28.	1-Ethyl-2-propylcyclohexane	11.84	$C_{11}H_{22}$	1.01	-	1089	1140	69.05	Cyclic HC	MS
29.	1-Methyl-3-pentyl cyclohexane	12.02	$C_{11}H_{22}$	0.93	-	1095	1094	97.10	Cyclic HC	KI, MS
30.	n-Undecane	12.18	$C_{11}H_{24}$	5.87	-	1100	1100	57.05	Saturated HC	KI, MS
31.	Nonanal	12.35	$C_9H_{18}O$	0.84	-	1105	1105	57.05	Aldehyde	KI, MS
32.	trans-Decalin, 2a-methyl	12.47	$C_{11}H_{20}$	1.12	-	1109	1109	81.10	Saturated HC	KI, MS
33.	2,6-Dimethyldecane	12.73	$C_{12}H_{26}$	1.82	-	1118	1119	57.05	Saturated HC	KI, MS
34.	3,7-Dimethyldecane	13.03	$C_{12}H_{26}$	2.07	-	1127	1127	43.05	Saturated HC	KI, MS
35.	1,1,2,3,3-Pentachloropropane	13.14	$C_9H_{12}O$	-	3.17	1131	1151	96.00	Miscellaneous	MS
36.	Pentylcyclohexane	13.24	$C_{11}H_{22}$	1.07	-	1134	1034	83.05	Cyclic HC	KI, MS
37.	Hexyl-cyclopentane	13.39	$C_{11}H_{22}$	0.81	-	1139	1136	69.05	Cyclic HC	KI, MS
38.	cis-Decalin	13.68	$C_{11}H_{20}$	0.62	-	1148	1146	55.00	Cyclic HC	KI, MS
39.	5-Methyl undecane	13.92	$C_{12}H_{26}$	1.45	-	1156	1156	57.10	Saturated HC	KI, MS
40.	4-Methyl undecane	14.05	$C_{12}H_{26}$	0.88	-	1160	1160	43.05	Saturated HC	KI, MS
41.	2-Methyl undecane	14.18	$C_{12}H_{26}$	1.85	-	1164	1164	43.05	Saturated HC	KI, MS
42.	3-Methyl undecane	14.38	$C_{12}H_{26}$	1.08	-	1171	1171	57.05	Saturated HC	KI, MS
43.	4-Dodecene	14.98	$C_{12}H_{24}$	0.35	-	1190	1190	55.05	Unsaturated HC	KI, MS
44.	n-Dodecane	15.29	$C_{12}H_{26}$	6.00	-	1200	1200	57.10	Saturated HC	KI, MS
45.	2,6-Dimethyl undecane	15.70	$C_{13}H_{28}$	1.42	-	1214	1214	57.05	Saturated HC	KI, MS
46.	3,7- Dimethyl undecane	15.94	$C_{13}H_{28}$	0.29	-	1222	1222	43.05	Saturated HC	KI, MS
47.	1-Hexylcyclohexane	16.43	$C_{12}H_{24}$	0.28	-	1239	1239	83.10	Cyclic HC	KI, MS
48.	(2E)-2-Decenal	17.17	$C_{10}H_{18}O$	3.48	2.06	1264	1264	43.05	Aldehyde	KI, MS
49.	3-Methyl dodecane	17.44	$C_{13}H_{28}$	0.41	-	1273	1273	57.05	Saturated HC	KI, MS
50.	Carvacrol	18.14	$C_{10}H_{14}O$	1.51	-	1297	1298	135.15	Monoterpene	KI, MS
51.	n-Tridecane	18.23	$C_{13}H_{28}$	0.86	-	1300	1300	57.10	Saturated HC	KI, MS
52.	Deca-2,4-dienal	18.79	$C_{10}H_{16}O$	0.95	-	1320	1320	81.05	Unsaturated HC	KI, MS
53.	2-Undecen-1-al	20.09	$C_{11}H_{20}O$	0.93	-	1367	1369	41.00	Saturated HC	KI, MS
54.	1-Tetradecene	20.80	$C_{14}H_{28}$	0.28	-	1393	1389	41.00	Saturated HC	KI, MS

55.	n-Tetradecane	21.01	$C_{14}H_{30}$	0.26	-	1400	1400	57.10	Saturated HC	KI, MS
56.	n-Pentadecane	23.63	$C_{15}H_{32}$	0.29	-	1500	1500	57.05	Saturated HC	KI, MS
57.	n-Dodecanoic acid (Lauric acid)	25.41	$C_{12}H_{24}O_2$	1.59	21.73	1572	1570	73.05	Fatty acid	KI, MS
58.	1-Hexadecene	25.93	$C_{16}H_{32}$	0.77	-	1593	1589	43.05	Unsaturated HC	KI, MS
59.	Ethyl dodecanoate	26.03	$C_{14}H_{28}O_2$	0.19	-	1597	1597	88.10	Fatty ester	KI, MS
60.	n-Tetradecanoic acid (Myrsitic acid)	29.95	$C_{14}H_{28}O_2$	0.35	6.37	1761	1761	73.05	Fatty acid	KI, MS
61.	1-Octadecene	30.54	$C_{18}H_{36}$	1.27	-	1786	1786	43.05	Unsaturated HC	KI, MS
62.	Hexahydro-farnesyl acetone	31.70	$C_{18}H_{36}O$	0.20	-	1842	1845	43.05	Sesquiterpene ketone	KI, MS
63.	Methyl hexadecanoate	33.39	$C_{17}H_{34}O_2$	0.23	-	1929	1929	74.05	Fatty ester	KI, MS
64.	n-Hexadecanoic acid (Palmitic acid)	34.19	$C_{16}H_{32}O_2$	0.64	9.19	1968	1968	43.05	Fatty acid	KI, MS
65.	1-Eicosene	37.72	$C_{20}H_{40}$	1.46	-	1994	1995	43.05	Unsaturated HC	KI, MS
66.	Ethyl oleate	38.10	$C_{20}H_{38}O_2$	0.52	-	2171	2171	55.05	Fatty ester	KI, MS
67.	1-Docosene	38.52	$C_{22}H_{44}$	1.05	-	2195	2195	57.10	Unsaturated HC	KI, MS
68.	4,8,12,16-Tetramethylheptadecan-4-olide	41.44	$C_{21}H_{40}O_2$	0.59	-	2361	2364	99.05	Diterpene lactone	KI, MS
69.	1-Tricosene	42.02	$C_{23}H_{46}$	0.66	-	2395	2293	57.10	Unsaturated HC	KI, MS
70.	n-Pentacosane	43.74	$C_{25}H_{52}$	0.29	-	2500	2500	57.10	Saturated HC	KI, MS
71.	2-Monopalmitin	43.99	$C_{19}H_{38}O_4$	-	1.23	2516	2519	43.05	Monoacylglycerol	KI, MS
72.	Bis(2-ethylhexyl) phthalate	44.64	$C_{24}H_{38}O_4$	1.44	-	2557	2550	149.10	Miscellaneous	KI, MS
73.	1-Hexacosene	45.25	$C_{26}H_{52}$	0.57	-	2596	2596	57.05	Unsaturated HC	KI, MS
74.	1-Heptacosene	46.49	$C_{27}H_{54}$	0.54	-	2678	2684	57.05	Unsaturated HC	KI, MS
75.	n-Heptacosane	46.83	$C_{27}H_{56}$	0.91	3.70	2701	2700	57.05	Saturated HC	KI, MS
76.	1-Octacosene	48.24	$C_{28}H_{56}$	0.47	-	2797	2797	57.05	Unsaturated HC	KI, MS
77.	n-Nonacosane	49.71	$C_{29}H_{60}$	0.88	-	2901	2900	57.10	Saturated HC	KI, MS
78.	Chondrillasterol acetate	50.70	$C_{31}H_{50}O_2$	0.35	-	2974	-	95.10	Sterol	MS
79.	n-Untriacontane	52.40	$C_{31}H_{46}$	1.16	-	3101	3100	57.10	Saturated HC	KI, MS
80.	24-Norursa-3,12-diene	52.45	$C_{29}H_{46}$	-	1.56	3105	3105	135.20	Triterpene	KI, MS
81.	Dodecanoic acid, 2-hydroxy-1,3-propanediyl ester (1,3-Dilaurin)	53.39	$C_{27}H_{52}O_5$	0.93	4.96	3177	3202	183.25	Diacylglycerol	MS
82.	n-Dotriacontane	53.70	$C_{32}H_{66}$	0.26	-	3200	3200	57.10	Saturated HC	KI, MS
83.	Triacontanal	54.41	$C_{30}H_{60}O$	0.36	-	3250	3251	57.10	Aldehyde	KI, MS
84.	Campesterol	54.60	$C_{28}H_{48}O$	0.74	-	3263	3131	43.05	Sterol	MS
85.	n-Tritriacontane	55.15	$C_{33}H_{68}$	1.44	-	3301		57.10	Saturated HC	KI, MS
86.	γ-Sitosterol	56.05	$C_{29}H_{50}O$	8.54	21.21	3355	3351	43.05	Sterol	KI, MS
87.	1-Dodecanoyl-3-myristoylglycerol	56.41	$C_{29}H_{56}O_5$	2.07	4.32	3377	3401	57.10	Triacylglycerol	KI, MS
88.	Lup-20(29)-en-3-one	57.21	$C_{30}H_{48}O$	1.66	-	3484	3384	95.10	Triterpenoid	MS
89.	9,19-Cyclolanost-24-en-3-ol, (3β)	57.42	$C_{30}H_{50}O$	0.48	3.52	3509	3466	69.10	Sterol	KI, MS
90.	Lupeol	57.65	$C_{30}H_{50}O$	0.63	-	3525	2484	207.05	Triterpenoid	MS
91.	Dotriacontanal	57.83	$C_{32}H_{64}O$	0.74	-	3538	3390	57.10	Aldehyde	MS
92.	Sitostenone	58.62	$C_{29}H_{48}O$	0.66	-	3592	3458	124.10	Sterol	MS
93.	1-Heptatriacontanol	60.29	$C_{37}H_{76}O$	2.97	2.03	3707	3942	55.05	Fatty alcohol	MS
Hydrocarbons				62.53%	3.70%					
Fatty acids/ esters				3.23%	37.29%					
Sterols				10.77%	24.73%					
Alcohols/ aldehydes/ ketones				8.87%	4.90%					
Terpenes/ terpenoids				5.59%	5.61%					
Acylglycerols				3.00%	10.51%					
Miscellaneous				1.44%	3.17%					
Total identified compounds				95.43%	89.91%					

[a] %RA: Relative area (peak area relative to the total peak area).
[b] $RI_{exp.}$: Retention index determined experimentally on an RTX-5MS bonded column.
[c] $RI_{rep.}$: Published retention indices
[d] Compounds were identified based on the compounds' mass spectrometric data and retention indices in comparison with those of the National Institute of Standards and Technology (NIST) Mass Spectral Library (December 2011), the Wiley Registry of Mass Spectral Data,8th edition and literature.

Table 2 :Phenolic and flavonoid compounds identified in the 70% aqueous methanol extracts of *P. rupicola* seeds and their concentrations as detected by HPLC-DAD against standards

No.	Compound	Rt (min.)	Concentration (mg% sample)
1.	Gallic acid	3.8	14.64
2.	Protocatechuic acid	6.7	6.55
3.	Gentisic acid	9.6	ND
4.	*p*-hydroxybenzoic acid	9.7	32.84
5.	Catechin	11.5	105.75
6.	Chlorogenic acid	12.4	20.81
7.	Caffeic acid	13.1	0.63
8.	Syringic acid	13.9	4.63
9.	Vanillic acid	15.7	58.26
10.	Ferulic acid	20.1	3.69
11.	Sinapic acid	20.9	ND
12.	Rutin	22.9	19.30
13.	*p*-coumaric acid	25.8	12.01
14.	Rosmarinic acid	27.1	ND
15.	Apigenin-7-O-glucoside	28.9	ND
16.	Cinnamic acid	34.6	27.62
17.	Quercetin	36.0	1.77
18.	Apigenin	39.5	ND
19.	Kaempferol	39.9	ND
20.	Chrysin	52.4	ND

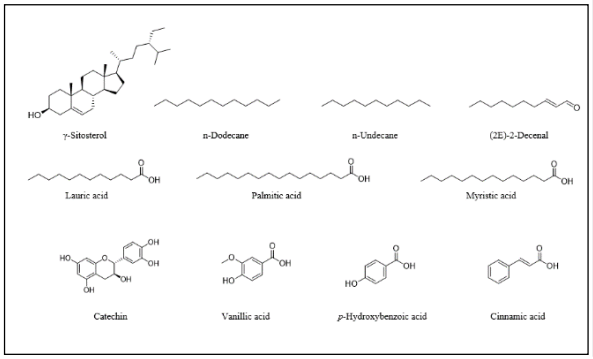

Fig. 1. Structures of the main phytoconstituents in *P. rupicola* seeds' extracts

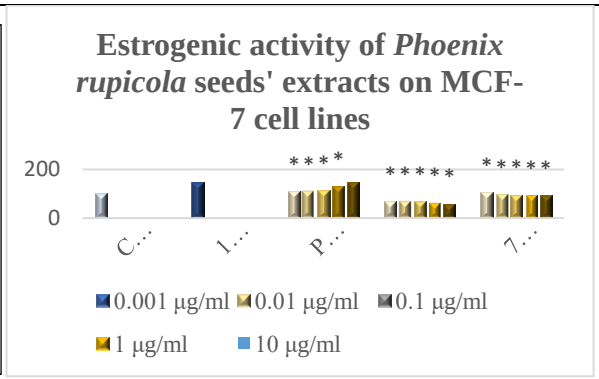

Fig. 2. Estrogenic activity of *P. rupicola* seeds' extracts on the proliferation of MCF-7 cell lines. Each value represented as the mean ± S.D of three independent experiments (n = 3). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test. P value < 0.05 was considered statistically significant. ∗Significantly different from 17β-estradiol.

3.3. In vitro estrogenic assay

SRB assay was used to study the proliferation of MCF-7 cells when treated with the successive extracts of *P. rupicola* seeds at conc. (0.001, 0.01, 0.1, 1, 10 µg/mL). Figure 2 illustrated the estrogenic activity of the studied extracts in MCF-7 cell lines. Petroleum ether extract showed significant (p < 0.05) dose-dependent increase in cells' proliferation with increase in extract concentration, where the dose of 10 µg/mL exhibited the most potent estrogenic activity of all the studied extracts, as indicated by the increase in cell proliferation (1.17%), compared to 17β-estradiol. On the other hand, chloroform and 70% aqueous methanol extracts unexpectedly showed significant dose-dependent decrease in cells' proliferation with increase in extract concentration.

The estrogenic activity of petroleum ether extract could be attributed to the abundance of secondary metabolites including saturated and unsaturated fatty acids which were previously reported to possess estrogenic activity through modulation of estrogen and/or estrogen receptors by altering estradiol binding to receptors [20]. On the other hand, the decrease in activity of the chloroform extract could be explained by the high concentration of γ-sitosterol which was reported to inhibit the proliferation of MCF-7 cells in a dose reported manner [21]. Similarly, flavonoids such as quercetin, catechin and rutin were also reported to possess

antiproliferative activities against MCF-7 cell lines [22], thus could explain the dose-dependent decrease in cells' proliferation with the increase in extract's concentration.

4. Conclusions

The current study investigated the phytochemical profile and the *in vitro* estrogenic activity of the successive extracts of *P. rupicola* seeds. GC/MS analysis of PE and chloroform DE extracts revealed the abundance of lipophilic constituents including saturated, unsaturated, and oxygenated hydrocarbons, as well as sterols, terpenes, fatty acids and acylglyecrols. Moreover, HPLC-DAD analysis showed the richness of in the 70% aqueous methanol extract with phenolic acids and flavonoids. *In vitro* bioactivity study of the seeds' successive extracts on MCF-7 cell lines demonstrated that the petroleum ether extract exhibited strong estrogenic activity. According to the above results, it is concluded that *P. rupicola* seeds could be considered a promising natural source of phytoestrogens. Further detailed studies should be conducted to isolate the bioactive constituents of the plant, as well as, to identify their underlying mechanisms of action. Additionally, the metabolites described in this study could be used as markers for future standardization of *P. rupicola* seeds via GC/MS and HPLC.

5. Conflicts of interest

The authors declare no conflict of interest.

6. Formatting of funding sources

National Research Centre, Grant/ Award Number: Thesis no. 2/5/3.

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8. References

[1] Elgindi M. R., Singab A. N., El-Taher E. M. M. and Kassem M. E. S. A., Comprehensive Review of *Phoenix* (Arecaceae). *Res. J. Pharm. Biol. Chem. Sci*., **6** (3), 966–974 (2015).

[2] Mehraban F., Jafari M., Toori M. A., Sadeghi H., Joodi B., Mostafazade M. and Sadeghi H., Effects of Date Palm Pollen (*Phoenix Dactylifera* L.) and Astragalus Ovinus on Sperm Parameters and Sex Hormones in Adult Male Rats. *Iran. J. Reprod. Med.*, **12** (10), 705–712 (2014).

[3] Moshfegh F., Baharara J., Namvar F., Zafar-Balanezhad S., Amini E.and Jafarzadeh L., Effects of Date Palm Pollen on Fertility and Development of Reproductive System in Female Balb/C Mice. *J. HerbMed Pharmacol*., **5** (1), 23–28 (2016).

[4] Tahvilzadeh M., Hajimahmoodi M. and Rahimi R. The Role of Date Palm (*Phoenix Dactylifera* L) Pollen in Fertility: A Comprehensive Review of Current Evidence. *J. Evidence-Based Complement. Altern. Med*., **21**(4), 320–324 (2016). https://doi.org/10.1177/2156587215609851.

[5] Ammar N.M., Al-Okbi S.Y., Mohamed D.A. and El-Kassem L.T., Antioxidant and Estrogen Like Activity of The Seed of *Phoenix dactylifera* L. Growing in Egyptian Oases. *Report and Opinion*, **1**(3), 1-8 (2009).

[6] Ammar N. M., El-Kassem L. T., El-Sayed N. H., Calabria L. M. and Mabry T. J., Flavonoid Constituents and Antimicrobial Activity of Date (*Phoenix Dactylifera* L .) Seeds Growing in Egypt. *Med. Aromat. Plant Sci. Biotechnol*.,**3**, 1–5 (2009).

[7] Tag H., Kalita P. and Nimasow G., Status Report on *Phoenix rupicola* T . Anderson (Arecaceae) – an Endemic and Status Report on *Phoenix rupicola* T . Anderson (Arecaceae) – an En-Demic and Threatened Wild Palm in Pakhui Wildlife Sanctuary , East Kameng (Eastern Himalayas), India. 2010, No. April 2014.

[8] Peirotén Á., Bravo D. and Landete J. M., Bacterial Metabolism as Responsible of Beneficial Effects of Phytoestrogens on Human Health. *Crit. Rev. Food Sci. Nutr*., **60** (11), 1922–1937 (2020). https://doi.org/10.1080/10408398.2019.1622505.

[9] El-Halawany A., Salah El Dine R., Chung M. H., Nishihara T. and Hattori M., Screening for Estrogenic and Antiestrogenic Activities of Plants Growing in Egypt and Thailand. *Pharmacognosy Res*., **3** (2), 107–113 (2011). https://doi.org/10.4103/0974-8490.81958.

[10] Magne Nde C. B., Njamen D., Tanee Fomum S., Wandji J., Simpson E., Clyne C. and Vollmer G., In Vitro Estrogenic Activity of Two Major Compounds from the Stem Bark of *Erythrina lysistemon* (Fabaceae*). Eur. J. Pharmacol*., **674** (2–3), 87–94 (2012). https://doi.org/10.1016/j.ejphar.2011.10.031.

[11] Resende F. A., de Oliveira A. P. S., de Camargo M. S., Vilegas W. and Varanda E., A., Evaluation of Estrogenic Potential of Flavonoids Using a Recombinant Yeast Strain and MCF7/BUS Cell Proliferation Assay. *PLoS One*,

8 (10), 1–7 (2013). https://doi.org/10.1371/journal.pone.0074881.

[12] Comșa Ș., Cîmpean A. M. and Raica M., The Story of MCF-7 Breast Cancer Cell Line: 40 Years of Experience in Research. *Anticancer Res.*, **35** (6), 3147–3154 (2015).

[13] Otify A.M., Hammam A.M.M. and Farag M. A., Phoenix dactylifera L. date tree pollen fertility effects on female rats in relation to its UPLC-MS profile via a biochemometric approach. *Steroids*, **173,** 108888 (2021).

[14] Abouzeid E.M., Afifi A.H., Salama A., Hussein R.A., Youssef F.S., El-Ahmady S.H. and Ammar N.M., Comprehensive metabolite profiling of Phoenix rupicola pulp and seeds using UPLC-ESI-MS / MS and evaluation of their estrogenic activity in ovariectomized rat model. *Food Research International*, **157**, 111308 (2022). doi:10.1016/j.foodres.2022.111308

[15] Daulatabad C.D. and Ankalgi R.F., Component Acids of Palmae Seed Oils. *Fette, Seifen, Anstrichmittel*., **87**(3), 126-128 (1985). doi:10.1002/lipi.19850870310

[16] Kim K.H., Tsao R., Yang R. and Cui S.W., Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. *Food Chemistry*, **95**(3), 466-473 (2006). doi:10.1016/j.foodchem.2005.01.032

[17] Skehan P., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D., Warren J.T., Bokesch H., Kenney S. and Boyd M.R., New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst*., **82**(13), 1107-1112 (1990). doi:10.1093/jnci/82.13.1107

[18] Abdel -Naim A. B., Alghamdi A. A., Algandaby M. M., Al -Abbasi F. A., Al -Abd A. M., Eid B. G., Abdallah H. M. and El -Halawany A. M., Rutin Isolated from Chrozophora tinctoria Enhances Bone Cell Proliferation and Ossification Markers. *Oxid. Med. Cell. Longev*., **2018** (2018). https://doi.org/10.1155/2018/5106469.

[19] Eid N. M. S., Al -Awadi B., Vauzour D., Oruna -Concha M. J. and Spencer J. P. E., Effect of Cultivar Type and Ripening on the Polyphenol Content of Date Palm Fruit. *J. Agric. Food Chem*., **61** (10), 2453 –2460 (2013).https://doi.org/10.1021/jf303951e.

[20] Suzuki K. M., Isohama Y., Maruyama H., Yamada Y., Narita Y., Ohta S., Araki Y., Miyata T. and Mishima S., Estrogenic Activities of Fatty Acids and a Sterol Isolated from Royal Jelly. *Evidence -based Complement. Altern. Med*., **5** (3), 295 –302 (2008). https://doi.org/10.1093/ecam/nem036.

[21] Saeidnia S. The Story of Beta-sitosterol- A Review., *European Journal of Medicinal Plants*, **4**(5), 590-609 (2014). doi:10.9734/ejmp/2014/7764

[22] Delgado L., Fernandes I., González-Manzano S., De Freitas V., Mateus N. and Santos-Buelga C., Anti-proliferative effects of quercetin and catechin metabolites. *Food and Function*. **5**(4), 797-803 (2014). doi:10.1039/c3fo60441a