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PHYTOCHEMICAL AND ANTIOXIDANT PROPERTIES OF DATE PALM POLLEN (*PHOENIX DACTYLIFERA* L.).

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ABSTRACT

Phytochemical screening procedures along with structural analysis of many plant materials provided the basis for their therapeutic potencies and increased the relevance of many plants in pharmacological studies and drug developments. The present study was conducted to investigate some phytochemical and antioxidant properties of date palm pollen (DPP). The experiments were done using the conventional methods for extraction of plant materials (by methanolic maceration), preliminary phytochemical screening, Gas chromatography - mass spectrometry (GC-MS) analysis, and antioxidant 1,1-Diphenynl-2-Picrylhydrazyl (DPPH) assay. The preliminary phytochemical screening of the plant extract revealed the presence of tannins, flavonoids, alkaloids, steroids, terpenes, cardiac glycosides and carbohydrates, while further analysis by GC-MS analysis confirmed the presence of 29 compounds covering a wide range of fatty acid, phenols, triterpenoid and steroidal compounds including estrone, β-amyrin and 3-ketofucosterol. Moreover, the findings of the antioxidant DPPH assay indicated that, the plant extract produced a concentration dependent antioxidant activity. It can be concluded that, the presence of some phytochemical compounds in the plant material may justify its pharmacological properties and may provide the rationale for its traditional and ethnomedicinal uses which can be further evaluated by well-designed controlled studies.

INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is dioeciously, medium-sized tree occasionally found cultivated or self-grown [1]. The plant belongs to the Aracaceae family and is indigenous to the Arabian Peninsula, the Mediterranean, North Africa countries, parts of India and hotter parts of the USA [2]. In conversational languages dates are known as Sugar Palm (English), Nakhal (Arabic), Khajur (Hindi and Urdu), Karchuram (Tamil, Malayalam) and Karjura in Kannada language [3]. The fruits which are the most commonly used part are an important source of nutrition, especially in the arid regions with extreme conditions where very few plants can grow [4]. In traditional medicine, suspension of date palm pollen (DPP) is used to restore sexual potency and to cure male infertility [5]. Chemical analysis of pollen grains revealed the presence of a wide range of biochemically and nutritionally important minerals, trace elements, wide range of carbohydrates, organic acids, lipids, sterols, nucleic acids, proteins, free amino acids, water and lipid soluble vitamins, as well as cover different kinds of enzymes and cofactors [6]. As reported by Zaki *et al*. [7], the pollen grains of palm trees were used in Egyptian folk medicine to treat male and female infertility. Amer and Saharan [8] mentioned that, pollen grains contained estrogen, quercetin, β- amyrin, β- sitosterol, steroid, cholesterol, and estrone.
This study was aimed to determine the phytochemical content along with the antioxidant capacity of DPP extract which may aid further elucidation of the therapeutic potential of this plant product.

Materials and methods

Plant material:
Mature flower cluster of the male plant of date palm containing pollen were collected from Karema City, El-Shamalia State, Sudan.

The plant material was identified at the Herbarium of Medicinal and Aromatic Plant and Traditional Medicine Research Institute, National Center for Research, Khartoum, Sudan.

Chemicals and reagents:
All chemicals and reagents used in the study were of analytical grade and were purchased from reliable firms and institutes.

Extraction of plant material:
Pollen (white colour dust) was obtained by shaking the split spathe that contains pollen sacs. The powdered plant material was extracted by maceration using methanol (70 %) as solvent system in conical flasks (1.5 Litters) for 72 hours, with intermittent shaking, and then filtered under vacuum using Buchner funnel. The filtrate is then allowed to evaporate at room temperature and the extract was collected, freeze dried, and stored in an amber glass container (in refrigerator) until use.

Preliminary phytochemical screening:
Five grams of the plant extract was macerated in 50 ml of ethanol 70 % for 24 hours in conical flask at room temperature with intermittent shaking, then filtered and used for the following preliminary phytochemical screening tests as described by Nagy [9], and Sasidharan *et al*. [10].

1. Test for tannins
Two ml of 10 % ferric chloride solution was added to 2 ml of ethanolic extract of DDP that contained in test tube. Blue-black precipitate indicates the presence of tannins.

2. Test for alkaloids
One ml of Hcl (1 %) was added to 2ml ethanolic extract of DPP in test tube and then heated in a water bath for 10 minutes. 1 ml from the solution was taken and 6 drops of Dragendorff's, Wagner's and Mayer's reagents were separately added. Orange precipitate, brownish-red precipitate and creamish precipitate respectively, indicates the presence of alkaloids.

3. Test for saponins
From the prepared plant extract, 0.5 ml was taken in a test tube, and then 5ml of distilled water was added to the tube. The mixture was shacked vigorously. Persistent froth volume produced, check each 10 minutes for 30 minutes, indicates the presence of saponins.

4. Test for cardiac glycosides (Keller-Kilani test)
Glacial acetic acid (1 ml), ferric chloride 10 % solution (6 drops) and concentrated sulphuric acid (6 drops) were added to test tube (contains 2 ml taken of the plant extract). Green-blue color indicates the presence of cardiac glycosides.

5. Test for steroides and triterpenes

- Lieberman-Burchard reaction
Acetic anhydride (2 ml) and few drops of concentrated sulphuric acid were added to test tube containing 2 ml of the plant extract. Blue-green ring between layers indicates the presence of steroids and pink-purple ring indicates the presence of triterpenes.

- Salkowski test
Few drops of concentrated sulphuric acid were added to test tube containing 2 ml of DDP extract and shaked. Yield of red colour indicates the presence of sterols, while turns lower layer to yellow indicates the presence of triterpenes.

6. Test for flavonoids
In test tube containing 0.5 ml of the plant extract, 5 - 10 drops of diluted Hcl and few pellets of magnesium turning were added, and the solutions was boiled for a few minutes. Reddish-pink or dirty brown colour indicates the presence of flavonoids

7. Test for carbohydrates
To 2 ml of the plant sample that placed in test tube 2 drops of Molisch reagent were added. The solution was then poured slowly into tube containing 2 ml of concentrated sulphuric acid and two layers were formed. Formation of a purple product at the interface of the two layers indicate the presence of carbohydrates.

Gas chromatography - mass spectrometry (GC-MS) analysis:
Based on the methods described by Al-Anber [11], Abirami and Rajendran [12] and Anees *et al*. [13], 2 g of the plant extract was mixed thoroughly with 7 ml of alcoholic sodium hydroxide (prepared by dissolving 2 g in 100 ml methanol). 7 ml from alcoholic sulphuric acid (1 ml H_2SO_4 to 100 ml methanol) was then added. The mixture was then shaked for 5 minutes. The content of the test tube was left to stand overnight. 1 ml of super saturated sodium chloride was then added and the content being shaken. 2 ml of n-hexane was added and the contents were shaked thoroughly for three minutes. Then the n-hexane layer was taken using disposable syringe. 5 µl from the n-hexane extract was diluted with 5 ml of diethyl ether. Then the mixture was filtered using 0.45 µm syringe filter and dried with 1 g of anhydrous sodium sulphate and 1 µl of the diluted sample was injected in GC-MS instrument.
Chromatographic separation was carried out with gas chromatograph coupled with mass detector spectrometer. Sample was injected spilt mode. Helium was used as a carrier gas with a flow rate of 1.61 mL/min. The GC-MS was programmed as follows:

- The instrument was set to an initial temperature of 60 C^o with rate 10 C^o/minute to 300 C^o as final temperature degree.

- The injection port temperature was 300 C°, and the ion source temperature was 200 C° and interface temperature was 250 C°.
- The sample was analyzed using scan mode in the range of m/z 40-500 charges to ratio and the total run time was 20 minutes.

The chemical constituents were identified by comparing their retention index and mass fragmentation patent with those available in the library of the National Institute of Standards and Technology (NIST).

***In vitro* DPPH (1,1-Diphenynl-2-Picrylhydrazyl) antioxidant assay:**

The antioxidant activity of DPP was estimated according to the procedure that described by Bahman *et al*. [14]. One ml of DPPH solution (0.3 mM) in ethanol 90 % was mixed with 2.5 ml taken from different concentrations (250, 125, 50, 25, and 10 µg/ml) of the plant extract. After 30 minutes incubation in dark at room temperature, absorbance was measured in a spectrophotometer at 518 nm. The concentrations were prepared in triplicates and the percentage of the radical scavenging activity (RSA) was calculated by the following equation:

RSA % = [Control – (Sample – Blank)] / Control × 100

Each 2.5 ml taken from the different concentrations of the plant extract plus 1 ml of 0.3 mM DPPH solution considered as sample, and 1 ml of ethanol plus 2.5 ml of the plant extract was used as blank, while 1 ml of 0.3 mM DPPH solution plus 2.5 ml of ethanol was used as control. Quercetin was diluted to final concentrations of 250, 125, 50, 25, and 10 µg/ml in ethanol and used as reference standard.

Results and discussion

Plant extraction:

Methanolic extraction of DPP powder yielded a brownish gummy texture semisolid extract weighing 56.1 g (18.7 % from the total 300 g).

Extraction is an important step because it is necessary to obtain the desired chemical components from the plant materials for further investigations, and proper actions must be taken to assure that these potential constituents are not lost, distorted or destroyed during the preparation of samples [15]. The basic operation steps may include pre-washing, drying, grinding and/or freeze drying of plant materials to obtain a homogenous sample, and as the target compounds may be non-polar to polar and/or thermally labile, the suitability of the method and selection of solvents for extraction must be considered [10].

From the previous studies, different solvents and extractive procedures were applied. However, no one has compared the qualitative or quantitative yields of these methods which may necessitate a conduction of further researches to optimize the extraction method for this plant material. Therefore, the plant material was extracted by maceration using methanol as solvent system to ensure extraction of both polar and non-polar compounds, and to guarantee that thermolabile constituents are not affected.

Phytochemical tests:

Preliminary phytochemical screening:

The qualitative preliminary phytochemical screening of DPP extract revealed the presence of tannins, flavonoids, alkaloids, steroids, terpenes, cardiac glycosides and carbohydrates, while saponins were not found. Results of the preliminary phytochemical screening were shown in Table 1.

Table 1: Preliminary phytochemical screening of DPP methanolic extracts.

Phytochemical Test	Result
(1) Tannins (ferric chloride test)	**+**
(2) Alkaloids	
Dragendorff's reagent	**+**
Wagner's reagent	**+**
Mayer's reagent	**+**
(3) Saponins (froth test)	**-**
(4) Cardiac glycosides (Killer-Kilani test)	**+**
(5) Steroides	
Salkowski test	**±**
Liberman-Burchard test	**+**
(6) Terpenes	
Salkowski test	**±**

Liberman-Burchard test	±
(7) Flavonoids	+
(8) Carbohydrates (Molisch test)	+

Key: (+) present, (±) slight, (-) absent.

The qualitative analysis for preliminary phytochemical constituents of DPP extract were in accordance with those reported by Tauqeer *et al*. [16] and Bosila *et al*. [17]. The presence of flavonoids and steroids were in close agreement with other findings [1, 4]. However, some differences in the estimation of some constituents (high or small values) such as alkaloids, steroids and cardiac glycosides in the obtained results compared to the previously reported data may be due to the differences in the methods that used for extraction or may be due to climatic and environmental factors. Generally, the presence of some phytochemical compounds in the plant material may justify its pharmacological properties and may provide the rationale for its traditional and ethnomedicinal uses.

Gas chromatography - mass spectrometry analysis:

In the present study, GC-MS analysis of DPP methanolic extract revealed the presence of 29 compounds, from them 3 fatty acid compounds were in abundance and share about 87 % from the total content (about 35 % palmitic acid esters, about 31 % linoleic acid esters and about 21 %, linolenic acid esters), while the other compounds were in trace amount. The detected compounds covered a wide range of fatty acids, phenols, and terpenoid compounds. Several steroids, including estrone, β-amyrin and 3-ketofucosterol, were detected in the plant sample as shown in Table 2.

Table 2: Compounds detected in GC–MS analysis of DPP methanolic extract.

Peak	Retention Time	Percentage	Name
1	9.580	0.11	Caryophyllene
2	13.049	0.20	Methyl tetradecanoate
3	13.811	0.05	Methyl isomyristate
4	14.117	0.07	Pentadecanoic acid, methyl ester
5	14.944	7.52	Methyl palmitoleate
6	14.983	0.75	1,5-Cyclooctadiene, 1-ethyl-
7	15.147	18.11	Palmitic acid, methyl ester
8	15.607	1.53	Ethyl 9-hexadecenoate
9	15.798	8.98	Palmitic acid, ethyl ester
10	16.110	0.22	Heptadecanoic acid, methyl ester
11	16.800	25.11	Linoleic acid, methyl ester
12	16.870	20.57	Linolenic acid, methyl ester
13	17.042	2.01	Methyl stearate
14	17.383	6.29	Linoleic acid, ethyl ester
15	17.457	4.67	Ethyl 9,12,15-octadecatrienoate
16	17.636	0.74	Stearic acid, ethyl ester
17	18.590	0.17	cis-13-Ecosenoic acid, methyl ester
18	18.793	0.36	Eicosanoic acid, methyl ester
19	20.409	0.55	Docosanoic acid, methyl ester
20	20.904	0.23	Docosanoic acid, ethyl ester
21	21.172	0.06	Tricosanoic acid, methyl ester

22	21.907	0.40	Tetracosanoic acid, methyl ester
23	22.363	0.15	Ethyl tetracosanoate
24	22.629	0.04	Squalene
25	23.304	0.03	Hexacosanoic acid, methyl ester
26	26.898	0.11	β-amyrin
27	27.405	0.42	Lupeol
28	28.259	0.30	Estrone
29	28.471	0.25	Stigmasta-4,24(28)-dien-3-one, (24E)-

Attention was directed toward extracts and biologically active compounds isolated from popular plant species are due to many advantages and potentials gained from ingredients of these plants as nutritional values and in controlling of some diseases.
Various works had reported the presence of several constituents from DPP chemical analysis which is quite similar to our findings [11, 18]. Another data reported the presence of many chemical compounds in the plant material including essential oils, steroids, alkaloids, flavonoids, triterpenoids, and phenolic compounds with some differences in the quantitative estimation and/or the presence of some constituents compared to the obtained results [1, 4, 17, 19]. These variations in some chemical composition could be due to the differences in environmental conditions, age of the plant, extraction of plant materials, and methods used for chemical analysis.

***In vitro* DPPH antioxidant assay:**
Results of the *in vitro* antioxidant assay using DPPH for DPP extract using different concentrations (10, 25, 50, 125 and 250 µg/ml) showed an acceptable level of anti-oxidation compared to the standard. The plant extract exhibited concentration dependent radical scavenging activity (Table 3), and the highest scavenging activity was produced at the concentration of 250 µg/ml which scavenged 69.3 %, while the least scavenging activity was produced at the concentration of 10 µg/ml (7.6 %).

Table 3: DPPH radical scavenging activities of DPP methanolic extracts and Quercetin.

Concentration (µg/ml)	DPPH Radical Scavenging Activity %	
	DPP	Quercetin
250	69.3	89.8
125	62	85.3
50	36.1	62
25	34.2	42.2
10	7.6	37

Antioxidant tests could be based on the evaluation of lipid peroxidation or on the measurement of free radical scavenging potency. The radical scavengers donate hydrogen to free radicals, leading to non-toxic species and therefore to inhibition of the propagation phase of lipid oxidation. The use of DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidants and radical scavenging activities [14].
Medicinal plants and their constituents have been reported to exhibit antioxidant activity due to the presence of free radical scavengers like polyphenols, flavonoids, coumarin, and phenolic compounds [20].
The antioxidant activities of DPP extract may be attributed to the presence of high proportion of conjugated unsaturated fatty acids, flavonoid and tannins (phenolic compounds) that have been revealed by previous phytochemical investigation. These findings were in accordance with that reported by Fawkeya and Abdel-Monem [19], and Amany *et al*. [21]. Flavonoids are reported to exhibit antioxidant activities and are effective scavengers of superoxide anions, peroxynitrite, peroxyl, and hydroxyl radicals [22]. In addition, it has been reported that DPP is a rich plant in phenolic compounds such as cinnamic acids, flavonoid glycosides, flavanols, free phenolic acids and bound phenolic acids [1]. Also some phytochemical studies had confirmed that the plant contain vitamin C, vitamin B1, vitamin B2, nicotinic acid and vitamin A [23], and the antioxidant capacity could be attributed to the presence of all or some of these constituents.

Conclusion

As experimentally evident, it could be concluded that, the chemical analysis of the plant extract revealed the presence of tannins, flavonoids, alkaloids, steroids, terpenes and cardiac glycosides in the preliminary phytochemical screening, and a number of 29 compounds covering a wide range of fatty acids, phenols, triterpenoid and steroidal compounds detected by GC-MS analysis. The *in vitro* DPPH antioxidant assay revealed that the plant material possesses a concentration dependent antioxidant capacity.

In general, isolation and characterization of the active compounds of the traditionally used plants should be encouraged as it may impart a value addition to the traditional knowledge and use, enhance the pharmaceutical value, and evaluate the clinical importance of these natural products.

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