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Study on the Phytochemical Screening and Biological Activity of Pheonix Slyvestris Seeds

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Abstract: ***Seeds of plant extract Phoenix Slyvestris (commonly known as Indian date palm) are studied for its therapeutic properties and undergone phytochemical screening. The samples (Phoenix silvestris oil extract of seeds) were tested in duplicates for their MIC property against organisms Stephylococcus aureus and E. coli. Sample is undergone phytochemical screening to indentify secondary metabolites. The antioxidant property was conducted by invitro method of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was conducted on the crude leaves ethanolic extract and ethyl extract. The antioxidant activity of the samples was compared to the gallicacid as a standard. The sample is also undergone gas chromatography technique.***

Keywords: *Phoenix Slyvestris,* Phytochemical screening, Gallic acid, DPPH radical scavenging, MIC, Gas chromatography

1. Introduction

Phoenix sylvestris (*sylvestris*-Latin, of the forest) also known as silver date palm, Indian date, sugar date palm or wild date palm, is a species of flowering plant in the palm family native to southern Pakistan, most of India, Sri Lanka, Nepal, Bhutan, Myanmar and Bangladesh. Chemical composition analysis revealed a high carbohydrate (85.83%) content. Sap also contains appreciable amount of reducing sugar (3.95%), crude lipid (1.15%), crude protein (1.08%), crude fibre (0.18%) and ash (0.46%). In mineral composition potassium (80mg/100gm) was found to be maximum followed by sodium (18.23mg/100gm), calcium (4.76mg/100gm) and magnesium (2.23mg/100gm). The sap is rich in Vitamin B3 (12.3mg/100gm) and Vitamin C (12.75mg/100gm). The calorific value of sap is 358 kcal. The results revealed that sap is a good nutrient supplement and is opulent in carbohydrate, protein, potassium, sodium, vitamin B-complex and vitamin C. [10] [12]

2. Materials and methods

2.1. Sampling of Seeds

The fresh seeds of Phoenix sylvestris were collected from **Chikkanahalli village, Kolar district, Karnataka, India** during fruiting season i.e., in the month of April to August. Collected seed material was immediately sprayed with ethanol to cease the enzymatic degradation of secondary metabolites.

2.2. Grinding of seeds

The seeds were shade dried and chopped into small fragments using iron rod which is non rusty. Powdered in traditional grinding stone using glass rod itself in the village home within 10-15 days at room temperature (28-30˚C).

2.3. Soxhlet extraction

The seed material was subjected to Soxhlet extraction successively and separately from non-polar to polar solvents i.e., diethyl ether (b. p.34.6°C), and ethanol (95%) (b. p.78.37°C) in a Soxhlet extractor. The powered 80g is taken in a pouch that was made up of chromatographic thick sheet. Then it is inserted into a thimble of soxhlet apparatus of 500ml quantity. Total 550ml of Ethanol is taken for extraction. The Round Bottom flask contains 250ml of Ethanol solvent and the pouch is inserted into the thimble chamber, filled with 350ml of Ethanol solvent.

The Boiling point of Ethanol is 78° C. Therefore the mantel temperature should be thermostat at 60°C. By using the chill water circulator the temperature of water condenser was maintained at 5°C. Then the water circulation started from inlet to outlet. The solvent in RB flask heated to reflux. [2]

The same procedure is followed for Ether solvent (non polar). The total 600ml of diethyl ether (boiling point 34.6°C) is used and the temperature of thermostat should be 20°C. The solvent vapor travels up a distillation arm. The condenser ensures that any solvent vapor cools and drips back down into the thimble chamber. [3]

The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask (RB). This cycle may be allowed to repeat 4 times, over 4 hours or a day. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the RB flask after extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound the material in RB flask was taken then subjected to evaporation process to separate an Ethanol from the crude.

2.4. Rotary evaporator:

Turn the cooling water on through the condenser coils, a gentle flow is sufficient. The sample solution to be evaporated is placed in a Round Bottom flask (no more than half full). This Round Bottom flask is carefully attached to the ground glass joint and secured with a clip and placed in thermostatic evaporator bath and solvent collector is clipped

to the condenser to collect the solvent (Ethanol), set the temperature at 60°C. Now turn on the vaccum, place your finger over the vent hole and use it to control the vaccum. If the sample starts to boil release the pressure by removing your finger. Once the volume of Ethanol is minimized, repeat the process until it "Calms down". Until the Ethanol is evaporated and collected in collector flask, turn off the rotary evaporator, release the vaccum. Once the vaccum is released, carefully remove the round bottom flask which contains a sample.

The same procedure is used for the separation of Ether from Ether extract. The temperature should be maintained at 34.6°C.
The crude is used for phytochemical screening, Gas chormatographical analysis and biological activity. [4]

3. Phytochemical Screening

Phytochemical screening of plant extracts from different plant sample for secondary metabolites.

Secondary metabolites are organic compounds that are not directly in the normal growth, development (or) reproduction of an organism. Unlike primary metabolites, absence of secondary metabolites does not result in immediate death, but rather in long term impairment of the organisms survivability, fecundity (or) aesthetics or perhaps in no significant change at all secondary metabolites are often restricted to a narrow set of species with in a phytogenic group, secondary metabolites often play an important role in plant defense against herbivory and other inter species defenses. Humans use secondary metabolites as medicines, flavouringsand recreational drugs. Some of the secondary metabolites are Alkaloids, Flavonoids, Terpenoids, Saponins, Tannins, Glycosides, Lignin and other Phenolic compounds. [1]

Phytochemical Screening Tests:
Test for steroids: Acetic anhydride and H_2SO_4were added to each extract. The change of color from violet to blue or green showed the presence of steroids.

Test for terpenoids: The extract was mixed with $CHCl_3$ and carefully con. H_2SO_4 was added to form a layer. Reddish brown coloration at the interface was formed which indicate positive results for the presence of terpenoids.

Test for alkaloids: The fraction of extract added to Wagner's reagent the formation of brown precipitation showed the presence of alkaloids moiety.

Test for tannins: The extract in small quantity was mixed with water and heated on water bath and filtered. To the filtrate, few drops of $FeCl_3$, a dark green solution was obtained which indicates the presence of tannins.

Test for flavonoids: The extract was dissolved in dilute NaOH and few drops of HCl were added. A yellow solution turned into colorless which indicates the presence of flavonoids.

Test for glycosides: The extract was hydrolyzed with HCl and neutralized with NaOH solution and few drops of Fehling's solution A and B were added. Red precipitate was formed which indicate the presence of glycosides.

Test for proteins and amino acids: To the fraction of extracts, few drops of 0.2% Ninhydrin was added and heated for 5 minutes, formation of blue colour indicates the presence of proteins.

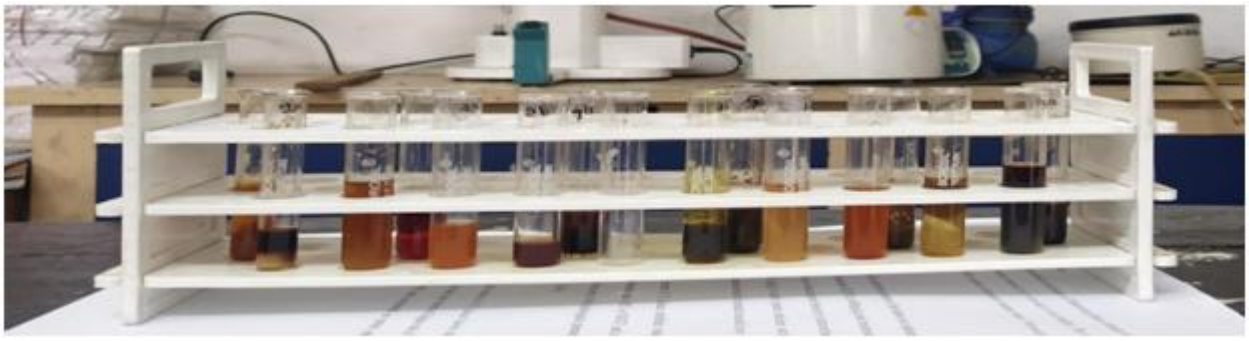

Figure 3.1: Tests for ether

Figure 3.2: Tests for ethanol

Phytochemical screening of Ether and Ethyl alcohol fractions of Pheonix sylvestris:

Natural Product	Ether Extract	Ethanol Extract
Steroids	+	+
Terpenoids	+	+
Alkaloids	-	+
Tannins	+	-
Flavonoids	+	+
Glycosides	+	+
Carbohydrates	+	+
Proteins	-	-
Amino acids	-	+

KEY: + = Present,-= Absent

Result

The preliminary phytochemical screening of Phoenix sylvestris Ether and Ethanoic fractions have discovered the presence of secondary metabolites of therapeutic importance. The major phytochemicals found were steroids/phytosterols and terpenoids, Alkaloids, Tannins, Flavonoids, Glycosides, Carbohydrates and Amino acids.

4. DPPH assay

The DPPH ((2, 2-diphenyl-1-picrylhydrazyl) assay is popular in natural product antioxidant studies. One of the reasons is that this method is simple and sensitive. This assay is based on the theory that a hydrogen donor is an antioxidant. It measures compounds that are radical scavengers. Figure 1 below, shows the mechanism by which DPPH$^{\bullet}$ accepts hydrogen from an antioxidant. DPPH$^{\bullet}$ is one of the few stable and commercially available organic nitrogen radicals. The antioxidant effect is proportional to the disappearance of DPPH in test samples.

Monitoring DPPH with a UV spectrometer has become the most commonly used method because of its simplicity and accuracy. DPPH shows a strong absorption maximum at 517 nm (purple). The colour turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm. Figure1. DPPH$^{\bullet}$ free radical conversion to DPPH by anti-oxidant compound.

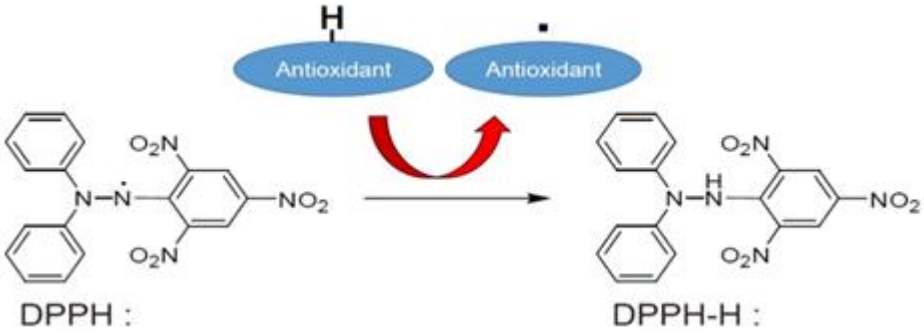

Figure 4.1: DPPH· free radical conversion to DPPH by anti-oxidant compound

Materials

1) DPPH-2, 2-Diphenyl-1-picrylhydrazyl (Cat No: D9132, Sigma)
2) Gallic Acid (Cat No: A5960-10mG, Sigma)
3) Adjustable multichannel pipettes and a pipettor (Benchtop, USA)
4) Methanol (Cat No: 34860-1L-R, Sigma)
5) DMSO (#PHR1309, Sigma)
6) 50 ml centrifuge tubes (# 546043 TORSON)
7) 10ml Borosil Glass tubes (TORSON)
8) 10 ml serological pipettes (TORSON)
9) 10 to 1000 ml tips (TORSON)

Equipments

1) Pipettes: 2-10µl, 10-100µl, and 100-1000µl.
2) UV-Vis Spectrophotometer (Systronics)

Assay Controls

1) Blank control (Only Methanol)
2) Negative control (Only DPPH)
3) Positive control (Gallic Acid)

DPPH assay:

Free radical scavenging capacity of the extracts from different samples was estimated using the stable DPPH radical. Different volumes (10-50 µL) of the samples were taken in the test tubes and the volume in each test tube was made up to 0.1mL with methanol. To all the tubes, 3mL of DPPH solution (whose absorbance was pre-set to 1) was added and incubated in dark condition for 15minutes. After incubation, the absorbance was read at 517nm spectrophotometrically with methanol as a blank. Percentage inhibition was calculated using the formula.

Percentage inhibition = [Abs of control - Abs of sample/ abs of control] * 100

Conc of sample in µg	Gallic Acid	
	Absorbance at 517 nm	Percentage Inhibition
100	0.874	12.6
200	0.778	22.2
300	0.695	30.5
400	0.637	36.3
500	0.555	44.5

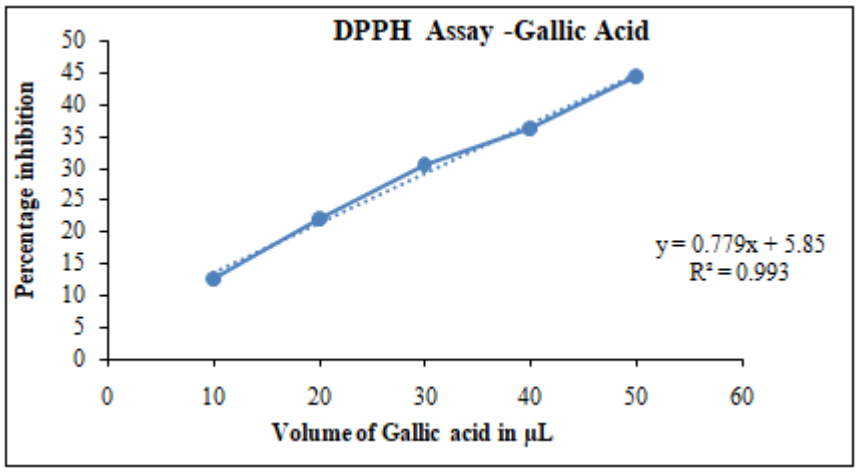

Figure 4.2: Graph of Volume of Gallic acid v/s Percentage inhibition

Dilutions: Ethanol sample 10 µL was diluted to 1mL with methanol.
Ether sample 100 µL was diluted to 1mL with methanol.

Conc of sample in µg	Absorbance at 517nm		Percentage Inhibition	
	Ethanol	Ether	Ethanol	Ether
100	0.529	0.972	47.1	2.8
200	0.454	0.937	54.6	6.3
300	0.333	0.897	66.7	10.3
400	0.198	0.850	80.2	15.0
500	0.107	0.809	89.3	19.0

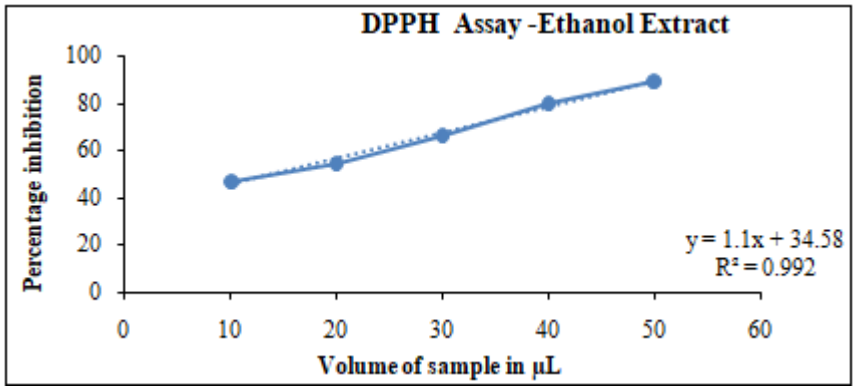

Figure 4.3: Graph of Volume of sample v/s Ethanol extract

Result:

Sample	IC50 value (µL)
Gallic acid	56.68
Ethanol Extract	14.02
Ether Extract	125.16

5. Biological activity of seeds extract of Phoenix sylvestris:

Antimicrobial activity:
Antimicrobial activity can be defined as a collective term for all active principles (agents) that inhibit the growth of bacteria, prevent the formation of microbial colonies, and may destroy microorganisms. An antimicrobial is a substance that either kills or inhibits the growth of microorganisms such as bacteria, fungi or protozoan's.

A hypertensivebial activity against microorganism (Stephylococcus aureus, and Escherichia Coli. The Phoenix Sylvestris seeds oil was extracted by using Ether and Ethanol as solvent. The antimicrobial activity was estimated by using the disc diffusion method. Finally, the extract revealed that, they have antibacterial activity against tested microorganism and zone of inhibition is observed. The in-vitro antibacterial activity against few gram-positive bacteria and gram-negative bacteria was examined. The extraction is done by using Ether and Ethanol by Soxhlet extraction method. The antibacterial activity was investigated by using well diffusion method. (8)

Well diffusion method to check the Minimum Inhibition Concentration (MIC)
The sample (Phoenix silvestris oil extract of seeds) were tested in duplicates for their MIC property against organisms (Stephylococcus aureus and E. coli)

Culture Media Preparation for Bacteria
Luria Bertani (LB) broth (Tryptone 10g, Sodium chloride 10g, Yeast extract 6g, Distilled water 1000mL) 30mL was prepared in 4 Erlenmeyer flasks by adding Tryptone 0.3g, Sodium chloride 0.3g, Yeast extract 0.18g, Distilled water

30mL and autoclaved at 121°C for 15 minutes. Later, Staphylococcus aureus strain and E. coli strain was inoculated respectively in 30mL of sterilized LB broth and incubated at 37˚ C for 24h.

Bacterial Culture preparation

Cultured organism (S. aureus and E. colj) were centrifuged at 6000rpm for 10 minutes respectively, supernatant was discarded and the pellets were dissolved in 1% (w/v) Sodium chloride and adjusted to absorbance 1.000 at 600nm under UV spectrophotometer (Genesys 10S UV-VIS Spectrophotometer)

Sample preparation

The sample 10ml (Phoenix silvestris oil extract of seeds) and Control 10mg (Tetracycline) was dissolved in 1mL Dimethyl sulfoxide (DMSO) respectively. Different aliquots of the sample and control was prepared by pipetting 10µL (100µg), 20µL (200µg), 30µL (300µg) and 40µL (400µg) and the final volume was made upto 50µL by adding DMSO.

Media preparation for MIC

Luria Bertani (LB) agar media (Tryptone 10g, Sodium chloride 10g, Yeast extract 6g, Agar 20g, Distilled water 1000mL) 500mL was prepared by adding Tryptone 5g, Sodium chloride 5g, Yeast extract 3g, Agar 10g, Distilled water 500mL in Erlenmeyer flask and autoclaved at 121°C for 15 mins.

Platting for MIC against organisms

Approximately 25mL of LB agar was poured into the sterilized petriplates and allowed it to solidify. 200µL prepared inoculum (S. aureus and E. coli) was poured into the agar plates respectively and spread thoroughly using a plate spreader. Five wells measuring 0.6 cm was made in each plates using the borer and 50µL of prepared sample and control (Tetracycline containing 100µg, 200µg, 300µg, 400µg were loaded into the respective wells and 50µL of DMSO was loaded in the middle well as control blank.

Figure 5.1: Test samples

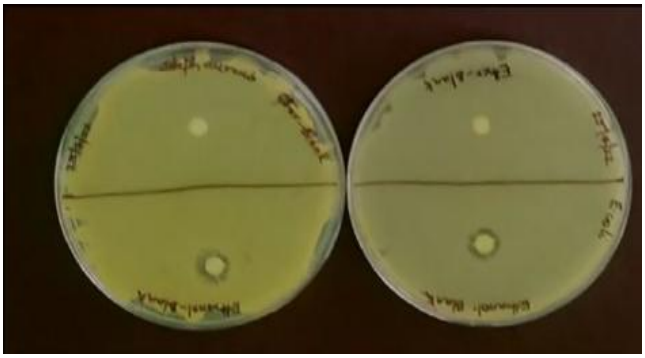

Figure 5.2: Ether and Ethanol samples

Sample	Test strain	Zone of Inhibition
Labelled Ether	Stephylococcus aureus	Observed
	Escherichia coli	Observed
Labelled Ethanol	Stephylococcus aureus	Observed
	Escherichia coli	Observed

Result: The antimicrobial activity is checked for Phoenix sylvestris using two bacterias namely stephylococcus aureus and Escherichia coli. From the below data, I can conclude that there is an Antimicrobial activity for seeds of Phoenix sylvestris.

6. Gas Chromatography

Gas chromatography is the process of separating compounds in a mixture by injecting a gaseous or liquid sample into a mobile phase, typically called the carrier gas, and passing the gas through a stationary phase. The mobile phase is usually an inert gas or an unreactive gas such as helium, argon, nitrogen or hydrogen. The stationary phase is a microscopic layer of viscous liquid on a surface of solid particles on an inert solid support inside a piece of glass or metal tubing called a column. The surface of the solid particles may also act as the stationary phase in some columns. The glass or metal column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled and the eluent coming off the column is monitored by a computerized detector. (15)

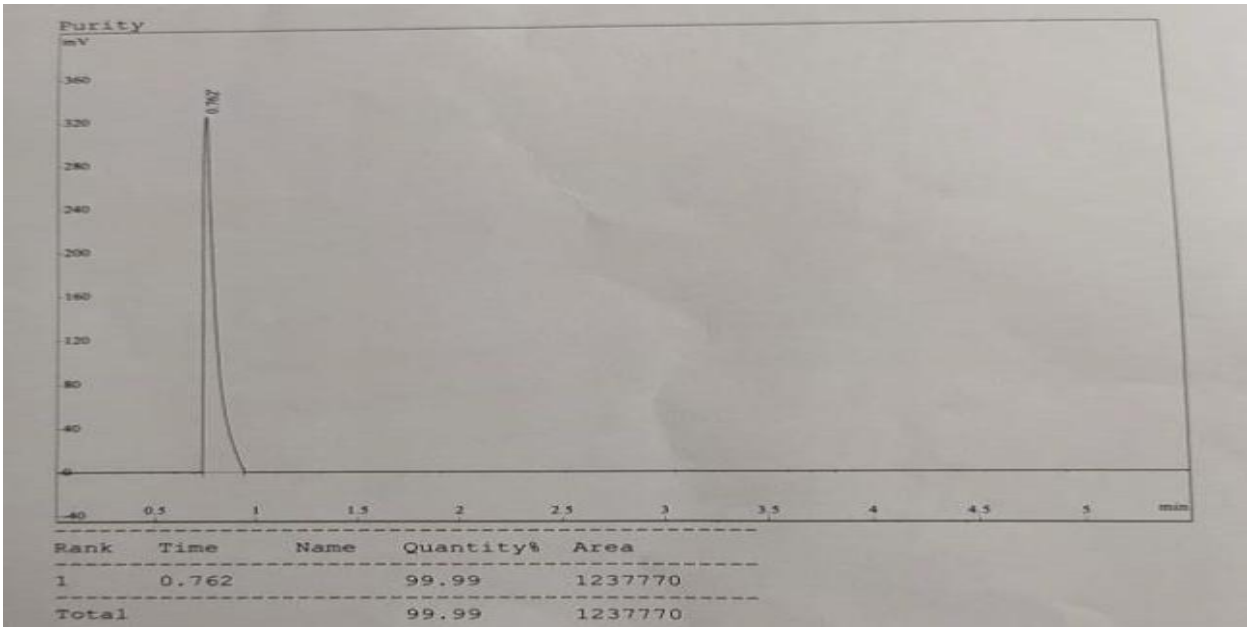

Figure 6.1: Gas chromatography of the sample

7. Conclusion

All stages of extraction from the pre-extraction are equally important in the study of medicinal plants. The sample preparation such as grinding and drying affected the efficiency and phytochemical constituents of the final extractions that eventually have an effect on final extracts. The data obtained from this study implies that the Ethanoic and Ether extract of Phoenix sylvestris seed is rich in alkaloids, steroids, terpenoids, glycosides, carbohydrates etc.

In addition, this plant requires extensive phytochemical and pharmacological studies. The use of these plants in folk medicines suggests that they represent an economic and safe alternative to treat infectious diseases. The investigation of chemical compounds from natural products is fundamentally important for the development of new drugs. However, the mechanism of action of these compounds should be elucidated. Phoenix sylvestris is thought to have therapeutic characteristics and has been used to treat abdominal pain, fever, loss of consciousness, constipation, heart problems, toothaches, nervous disability and helminthiasis. The phytochemical, pharmacological and traditional uses of the wild date palm were the subject of this review (Phoenix sylvestris).

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