

QUANTITATIVE AND QUALITATIVE ANALYSIS OF VARIOUS PHYTO-CONSTITUENTS IN *PHOENIX PUSILLA* UNRIPE FRUIT ETHANOLIC EXTRACT

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ABSTRACT

Objective: The aim of the present study was to perform preliminary phytochemical screening and in vitro antioxidant activity of *Phoenix pusilla* by using ethanol as solvent.

Methods: The ethanol extract was screened for its potential antioxidant activity by 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and lipid peroxidation (LPO) inhibitory activity, and compounds such as phenols, tannins, flavonoids, vitamin C, vitamin E, protein, carbohydrates and lipids were quantified using in vitro techniques.

Results: The total phenolic content of the ethanol unripe fruit extract was 4.22 ± 0.18 mg gallic acid equivalent (GAE)/g of extract. The total flavonoid and tannin content of the plant were 2.39 ± 0.22 mg quercetin equivalent/g of extract and 1.62 ± 0.02 mg GAE/g of the extract respectively. DPPH radical scavenging effect of the extract was determined spectrophotometrically. *P. pusilla* extract showed a good antioxidant potential with IC_{50} of $37.64 \mu\text{g/ml}$ and $8.88 \mu\text{g/ml}$ for LPO and DPPH assay respectively.

Conclusion: The greater amount of phenolic compounds leads to more potent radical scavenging effect as shown by *P. pusilla* unripe fruit. In conclusion, the obtained results of the tests demonstrated that this plant might be used in the prevention and in the treatment of different diseases related to oxidative stress.

Keywords: Oxidative stress, Medicinal plants, Date palm, 1-diphenyl-2-picrylhydrazyl, Lipid peroxidation, Phenols.

INTRODUCTION

Medicinal plants are the richest bio-resource of drugs for traditional systems of medicine, modern medicine, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Several studies have demonstrated pharmacological properties of medicinal plants and their isolated constituents to possess antioxidant, antidiabetic, antibacterial, antiviral, and antiulcer activity [1,2].

Living cells may generate free radicals and other reactive oxygen species by-products as a results of physiological and biochemical processes. Free radicals can cause oxidative damage to lipids, proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases in humans [3]. The ingestion of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing [4] and in recent years, there has been a worldwide trend toward the use of natural phytochemicals present in berry crops, teas, herbs, oil-seeds, beans, fruits and vegetables [5].

An antioxidant has been defined as any substance that when present in low concentrations compared with that of an anti-oxidisable substrate, significantly delays or inhibits the oxidation of that substrate [6]. Recently, the demand for fruit and fruit products has increased considerably, as fruits are well-known storehouse of polyphenols and phytonutrients which possess antioxidant activities [7]. Phytochemical screening of various plants has been reported by many workers. These studies have revealed the presence of numerous chemicals, including alkaloids, flavonoids, steroids, phenols, glycosides, and saponins. The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites. A number of studies have focused on the biological activities of phenolic compounds, which are antioxidants and free radical scavengers. The crude extracts of herbs, spices and other plant materials rich in phenolics and flavonoids are of increasing interest in the food

industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food [8]. *Phoenix pusilla* Gaertn., (Family: Arecaceae) a multipurpose palm species closely related to the date palm, is commonly known as the small date palm in India, as it only grows to 100 cm tall [9]. It is a beautiful shrubby suckering palm with a very short stem enveloped in persistent leaf sheaths. A crown of about 15-17 leaves is produced every year. Just like the true date palm, *Phoenix dactylifera* it is dioecious, producing male and female flowers on separate trees. It grows wild in dry areas in India at low elevations. Its flowering season starts in November and runs through January. Clusters of edible orange-red fruits turn into black drupes in the months of July and August [10]. The pulp of the fruit is fleshy, sweet and mealy. The tender part of the palm is often eaten by the poorer people as a meal called kanji. The leaflets are woven into mats and the split petioles into baskets. Brooms were also made out of the leaves of this palm. Its fruit is used in herbal medicines, as it is sweet, sour, cooling and laxative, cardiogenic, aphrodisiac, carminative and roborant. The fruit is also used for hyperdipsia, burning sensation, fevers, consumption, cardiac debility, seminal weakness, gasteropathy and general debility [11].

METHODS

Plant material and extraction

The fruits of *P. pusilla* were collected from Vellore District, Tamil Nadu. The plant was authenticated by Prof. P. Jayaraman, Plant Anatomy Research Center, Chennai and a voucher specimen was deposited in the herbarium (PARC/2014/2220). The plant material was dried at room temperature and coarsely powdered and subjected to soxhlet extraction with ethanol for 24 hrs. The extract was filtered, and solvent was completely removed by using a rotary evaporator. The residue *P. pusilla* unripe fruit ethanol extract (PPUF) was stored at 4°C until required for further use.

Preliminary phytochemical screening

Preliminary phytochemical screening of PPUF was carried out as per the standard textual procedure for the presence or absence of

chemical constituents such as phenolic compounds, reducing sugars, flavones, glycosides, saponins, alkaloids, anthroquinones, proteins and tannins [12].

Determination of primary metabolites

Total protein content

Pipette out 0.1 ml of the extract in test tubes. The volume of the test tubes was made to 1 ml with distilled water. A tube with 1 ml of water serves as a blank. 5 ml of reagent C was added to each tube including the blank. The contents were mixed well and allowed to stand for 10 minutes. 0.5 ml of reagent D was added, mixed well and incubated at room temperature in the dark for 30 minutes. Blue color was developed. The colour intensity was read at 660 nm. A standard graph of protein was plotted, from which the protein content of the extract was determined [13].

Total carbohydrate content

About 25 mg of extract was weighed and hydrolyzed by boiling with 2.5 N HCl for 2 hrs and then cooled to room temperature. This mixture was then neutralized using solid sodium carbonate until the effervescence ceases. The volume was made up to 100 ml with water and centrifuged at 3,500 rpm for 10 minutes. 0.5 ml of supernatant was pipetted out with duplicates in two other test tubes. The volume was made up to 1 ml with water in all test tubes. A tube with 1 ml of water served as a blank. 4 ml of anthrone reagent was added and heated for 8 minutes in the water bath and cooled. The green color developed was read at 630 nm. A standard graph of glucose was plotted, from which the carbohydrate content of the extract was determined [14].

Total lipid content

A volume of 0.1 ml of the supernatant was pipetted out with duplicates in two test tubes. The volume was made up to 1 ml with working FeCl₃ acetic acid reagent (0.05%). To this, 4 ml of FeCl₃ acetic acid reagent was added and kept at room temperature for 10 minutes. To this, 3 ml of conc. sulfuric acid was added. The tubes were kept in ice cold condition for 20 minutes. Pink colour was formed. The color intensity was read at 540 nm. A standard graph of cholesterol was plotted, from which the lipid content of the extract was determined [15].

Quantification of secondary metabolites

Determination of total phenolic compound

Total phenol was determined by Folin-Ciocalteu reagent. A dilute extract of PPUF or gallic acid was mixed with Folin-Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous 7.5% Na₂CO₃ (4 ml). The mixtures were allowed to stand for 15 minutes and the total phenols were determined at 765 nm. A standard curve was prepared using gallic acid. Total phenol values were expressed in terms of gallic acid used as a reference compound [16].

Determination of total flavonoid compound

Aluminum chloride colorimetric method was used for flavonoids determination. The extract was prepared in 70% ethanol and separately mixed with 4.5 ml of methanol, 0.1 ml of 10% aluminum chloride and 0.1 ml of 1 M sodium acetate. It remained in room temperature for 30 minutes; the absorbance of the reaction mixture was measured at 415 nm. A standard graph of quercetin was plotted, from which the flavonoid content of the extract was determined [17].

Determination of total tannins compound

A volume of 1 ml with duplicates of sample extract of concentrations (500 µg/ml) was pipetted out in test tubes. The volume was made up to 1 ml with distilled water, and 1 ml of water served as a blank. To this, 0.5 ml Folin-phenol reagent (1:2), followed by 5 ml of 35% sodium carbonate was added and kept at room temperature for 5 minutes. Blue color was formed. The color intensity was read at 640 nm. A standard graph of gallic acid was plotted, from which the tannin content of the extract was determined [18].

Dietary antioxidants

Determination of reducing power (vitamin C)

Reducing capacity of the extract was evaluated. To the extract, a drop of thiourea (10%) and 0.25 ml of 2% dinitro phenyl hydrazine (in 9N H₂SO₄) were added and incubated at 37°C for 3 hrs. After incubation, 1.25 ml of 85% H₂SO₄ was added under ice-cold condition and kept at room temperature for 30 minutes. The absorbance was measured at 540 nm against a blank. The experiments were repeated in triplicates [19].

Determination of total antioxidant content (vitamin E)

The total antioxidant activity was evaluated. An aliquot of the extract/vitamin E (equivalent to 500 mg) was combined with a reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In the case of blank, ethanol was used in place of extract. The tubes were capped and incubated in boiling water bath at 95°C for 60-90 minutes. Samples were cooled to room temperature, and the absorbance of the aqueous solution of each was measured at 695 nm against the blank [20].

Antioxidant analysis

1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

About 10 µl of each concentration (1.9-1000 µg/ml) of the extract was added to 190 µl DPPH (150 µM) in ethanol solution. After vortexing, the mixture was incubated for 20 minutes at 37°C. The control blank contains solvent without extract. The decrease in absorbance of test mixture due to quenching of DPPH free radicals was measured at 517 nm and the percentage inhibition was calculated. The inhibition concentration 50 (IC₅₀) value was determined as the concentration of the test mixture that gave 50% reduction in the absorbance from a control blank [21].

Lipid peroxidation (LPO) inhibition assay

Liver homogenate was prepared from commercially available chicken liver. The liver was quickly excised after decapitation and washed several times with ice-cold saline solution. A 10% liver homogenate was prepared using ice-cold KCl (0.15 M) in a Teflon tissue homogenizer and the test system containing homogenate with protein content was adjusted to 500 mg/ml. In the control system, to 1 ml of tissue homogenate, the LPO was initiated by the addition of 0.1 ml of FeSO₄ (25 mM), 0.1 ml of ascorbate (100 mM) and 0.1 ml of KH₂PO₄ (10 mM) and the volume was made up to 3 ml with distilled water and incubated at 37°C for 1 hr. Then, 1 ml of 5% 2,4,6-trichloroanisole and 1 ml of thiobarbituric acid (TBA) was added to this reaction mixture, and the tubes were boiled for 30 minutes in a boiling water bath. This was centrifuged at 3500 rpm for 10 minutes. In the test system, homogenate was incubated with various concentrations of extracts (1.9-1000 µg/ml). The extent of inhibition of LPO was evaluated by the estimation of TBA reactive substances (TBARS) level by measuring the absorbance at 532 nm [22]. The percentage inhibition of LPO was calculated by the formula:

$$(\%) \text{ Inhibition} = \left(\frac{[\text{control-test}]}{\text{control}} \right) \times 100.$$

Statistical analysis

Data were expressed as Mean±Standard Error Mean (SEM) of the mean of triplicates. IC₅₀ value was calculated using Graph Pad Prism 5.0.3 (Graph Pad Software, San Diego, CA, USA). Absorbance was read using ultraviolet/visible spectrophotometer (Perkin-Elmer; Lambda 25, USA).

RESULTS

Quantification of phytochemical constituents

The results obtained from the primary phytochemical screening showed that PPUF is rich in phenols, flavonoids, proteins, saponins, steroids and tannins (Table 1).

Quantified primary and secondary metabolites

Among the primary metabolites, protein content was the highest 40.46±3.53%, followed by lipids: 12.35±2.34% and carbohydrate 9.38±0.69% (Table 2). Vitamin C and E content of PPUF were

5.94±0.11 mg/g and 1.64±0.01 mg/g, respectively. The secondary metabolites were quantified, and the total phenol was 4.22±0.18 GE/g; total tannin was 1.62±0.02 GE/g; total flavonoid was 2.39±0.18 QE/g (Table 3).

Antioxidant potential

DPPH is one of the stable free radicals generally used for testing preliminary radical scavenging activity of a compound or a plant extract. The ethanol extract was found to exhibit better inhibition of DPPH radical with an IC_{50} value of 8.88 µg/ml than LPO. In the present study, the ethanolic extract of *P. pusilla* showed a good antiradical activity by scavenging DPPH radical. LPO is known to be very harmful to cellular components as a precursor of more reactive oxygen species. The peroxidation inhibitory activity of *P. pusilla* were studied and showed potent scavenging activity, as indicated by their IC_{50} value 37.64 µg/ml (Fig. 1).

DISCUSSION

To study the phytochemical composition of the extracts prepared from *P. pusilla*, a phytochemical screening was performed allowing to consider the possible medical uses that may have this plant as several studies have demonstrated the positive correlation between the phytochemical composition of plants and their medicinal uses [23].

The results obtained from the phytochemical screening show that PPUF is rich in various secondary metabolites at different concentrations. Indeed, we note the presence of proteins, saponins, flavonoids, phenolic compounds, reducing sugars, carbohydrates, amino acids, glycosides, steroids and tannins in the ethanolic extract. Consequently, *P. pusilla* by its richness in different secondary metabolites may have several medical importances such as anti-tumor especially the ethanol extract due to the presence of flavonoids [24] and antioxidant due to its richness in phenolic compounds [25].

Many reports support the use of antioxidant supplements in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases [26]. The process of LPO has been suggested to proceed via free radical chain reaction [27], which has been associated with cell damage in bio membranes [28]. The damage has been shown to precipitate different diseases like cancer, cardiovascular diseases and diabetes. The ability of the ethanolic extract of *P. pusilla* to inhibit the process of LPO was tested. The ethanol extract fraction showed that the highest inhibition of LPO in liver homogenate was with 87.59%. The relatively high inhibition of

LPO at 1000 µg/ml observed in liver homogenate could be attributed to the presence of glutathione and other antioxidants in hepatic cells. High LPO inhibitions showed by ethanol extracts could be related to the presence of phenolic compounds, which can be correlated to the antioxidant activity of natural plant product [29].

DPPH is a free radical stable at room temperature, and produce a purple color solution in ethanol. It is reduced in the presence of antioxidant

Table 1: Preliminary phytochemical analysis of PPUF

S. No.	Phytochemical	Present/absent
1	Phenolic compounds	++
2	Reducing sugars	++
3	Carbohydrates	++
4	Flavonoids	++
5	Glycosides	+
6	Saponins	++
7	Steroids	+
8	Alkaloids	-
9	Anthroquinones	-
10	Quinones	-
11	Proteins	+++
12	Amino acids	++
13	Tannins	+

+: Mild, ++: Moderate, +++: Significant. PPUF: Phoenix pusilla unripe fruit

Table 2: Quantitative analysis of primary metabolites

Assay	% w/w
Protein	40.46±3.53
Lipid	12.35±2.34
Carbohydrate	9.38±0.69

Values are expressed as mean±SEM (n=3). SEM: Standard error of the mean

Table 3: Quantitative analysis of secondary metabolites

Assay	% w/w
Phenol	4.22±0.18
Tannin	1.62±0.02
Flavonoid	2.39±0.22
Vitamin E	1.64±0.01
Vitamin C	5.94±0.11

Values are expressed as mean±SEM (n=3). SEM: Standard error of the mean

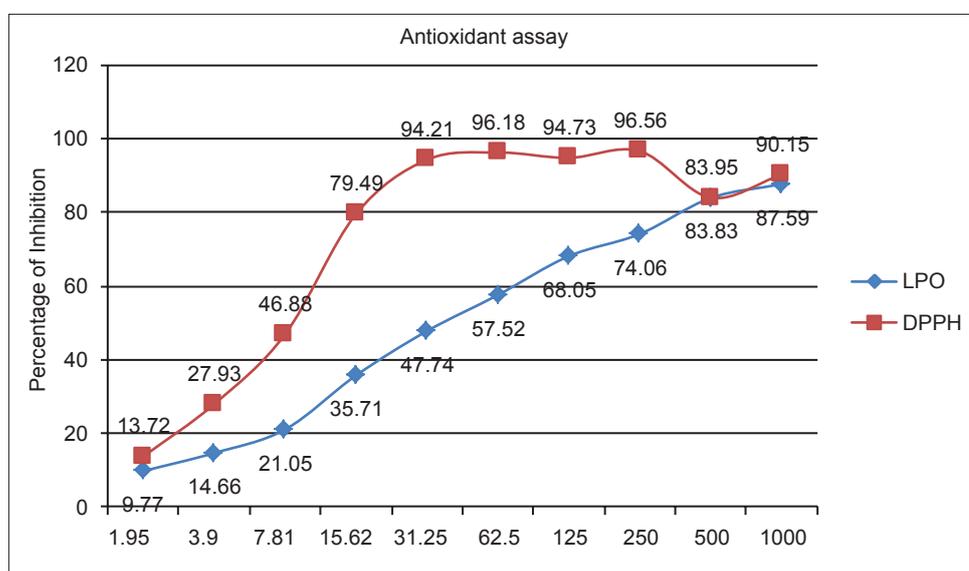


Fig. 1: Lipid peroxidation and 1-diphenyl-2-picrylhydrazil scavenging activity of *Phoenix pusilla* extract

molecule, giving rise to a yellowish ethanol solution. One of the mechanisms involved in antioxidant activity assay is the ability of a molecule to donate a hydrogen atom to a radical, and the propensity of the hydrogen donation is the critical factor involved in free radical scavenging [30]. Phenolic compounds may contribute directly to antioxidative action [29]. They are regarded to be the most important antioxidative components of plants; hence correlations between the concentration of plant phenolic and the total antioxidant capabilities have been reported [31]. The result in Table 3 shows the phenolic content of *P. pusilla* expressed as mg/g galic acid equivalent (GAE). The extract had the highest total phenolic content (4.22 mg/g GAE). Phenolic content of ethanol fraction corresponds to its LPO inhibition, and DPPH radical scavenging activities. This supports earlier reports, correlating the presence of phenolic compounds to antioxidative actions [29,31]. Variation observed in antioxidant activities solely depends on varieties, location and growth conditions [32].

Vitamin C and vitamin E are among the widely studied dietary antioxidants. Vitamin C (ascorbic acid) is considered the most important water soluble antioxidant in extracellular fluids. It is capable of neutralizing reactive oxygen species in the aqueous phase before LPO is initiated. Vitamin E, a major lipid-soluble antioxidant, is most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from LPO. Vitamin C has been cited as being capable of regenerating vitamin E [33].

Based on the results obtained in the present study, it is concluded that 70% ethanolic extract of PPUF, which contains large amounts of flavonoids and phenolic compounds, exhibits high antioxidant and free radical scavenging activities. Both these classes of compounds have good antioxidant potential and their effects on human nutrition and health are considerable. The mechanism of action of flavonoids is through scavenging or chelation. Phenolic compounds are also very important plant constituents because their hydroxyl groups confer scavenging ability.

CONCLUSION

The present study reveals that PPUF is a rich source of natural antioxidants which could be extracted efficiently with ethanol. The PPUF extracts showed a higher potency in scavenging of DPPH free radical. This may be related to the high amount of phenolic compounds and flavonoids in this plant extract. The data clearly indicated that the extracts ethanol (70%) of PPUF showed good antioxidant activity. The results of our work demonstrated the importance of this plant *P. pusilla* Gaertn., thus highlighting the possibility of its medical use, particularly its antioxidant activity due its richness in different secondary metabolites, especially the phenolic compounds and therefore can be used in the prevention of several diseases associated with oxidative stress.

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