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Pedalium murex Linn (Pedaliaceae) fruits: a comparative antioxidant activity of its different fractions

DK Patel, R Kumar, SK Prasad, S Hemalatha*

Pharmacognosy Research Laboratory, Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Varanasi-221005, Canada

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ABSTRACT

Objective: To examine the antioxidant activity and total phenolic content of different solvent fractions of *Pedalium murex* (*P. murex*) Linn fruits (Family: Pedaliaceae) as well as the correlation between the total antioxidant capacity and total phenolic content. **Methods:** In the present study, the antioxidant activities of *P. murex* were evaluated using six *in-vitro* assays, namely total antioxidant assay, DPPH assay, reducing power, nitric oxide scavenging, hydrogen peroxide scavenging and deoxyribose scavenging assays, and total phenol contents were also investigated. **Results:** The ethyl acetate (EA) fraction was found to have high levels of phenolic content (298.72 ±2.09 mg GAE/g). The EA fraction exhibit higher total antioxidant capacity, higher percentage of DPPH radical scavenging activity (135.11±2.95 μg/mL), nitric oxide (200.57±4.51 μg/mL), hydrogen peroxide (217.91±6.12 μg/mL), deoxyribose (250.01±4.68 μg/mL) and higher reducing power. Correlation coefficient (r^2=0.914) was found to be significant between total phenolic content and total antioxidant activity. **Conclusions:** In general, the results indicate that the EA fractions are rich in phenolic antioxidants with potent free radical scavenging activity implying their importance to human health.

1. Introduction

An antioxidant is a compound that inhibits or delays the oxidation of substrates even if the compound is present in a significantly lower concentration than the oxidized substrate. Natural antioxidants, which are ubiquitous in fruits, teas, vegetables, cereals, and medicinal plants, are employed not only for the prevention and treatment of various diseases caused by oxidative damage, but also for improving the shelf life of food products, and they have received great attention since they are effective free radical scavengers, by donating hydrogen to highly reactive radicals, thereby preventing further radical formation[1-4].

Human bodies possess enzymatic and non-enzymatic antioxidative mechanisms and minimize the generation of reactive oxygen species. When the generation of the active oxygen-free radical is overgrown many degenerative diseases, such as brain dysfunction, cancer, heart diseases, age-related degenerative conditions, declination of the immune system, cancer, coronary arteriosclerosis, ageing processes, carcinogenesis, gastric ulcer and DNA damage arise[5-9]. Thus, it is essential to develop and utilize effective natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases. Epidemiological studies suggest that increased consumption of fruits are important dietary sources of antioxidant polyphenols to humans associated with a lower risk of degenerative diseases, therefore they play an important role in health care[10]. Phenolic substances have been the object of special interest because they are widely distributed in the plant kingdom and highly present in the human diet. They have the structure required to act as free radical scavengers and have potential as food antioxidants[11]. Many herbal infusions, exhibit antioxidative and some other biological activities related to the presence of phenolic compounds, like flavonoids. Crude extracts of fruits, herbs, vegetables, cereals and other plant materials rich in phenolics are increasingly of interest in the food industry, because they retard oxidative degradation of lipids and thereby improve the quality and nutritive value of food[12]. In the food industry, the attention

*Corresponding author: S Hemalatha, Pharmacognosy Research Laboratory, Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Varanasi-221005, Canada.
Tel: +91 9415256481
E-mail: shemalatha.phe@itbhu.ac.in
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of manufacturers has also been shifted from synthetic to natural antioxidants[10].

Pedalium murex (*P. murex*) commonly known to the world as "Large Caltrops" and in Hindi as "Gokhru" or "Bara Gokhru" is a member of sesame family Pedaliaceae. The fruits are rich in polyphenolics (flavonoids and phenolics), glycosides like sapogenin (diosgenin-0.06%) and soluble proteins (20.14 mg/g)[13,14]. Due to high polyphenolics present in this plant it is duly necessary to investigate its antioxidative activity by comparing with the standard compound. The aim of this paper was the evaluation of a potential new source of antioxidant from *P. murex* fruits. For that purpose, various processes of extraction and fractionation were performed in aqueous or organic solvents. Then, the total polyphenolic contents of different fractions were determined and their antioxidant activities were also evaluated through *in vitro* assays.

2. Materials and methods

2.1. Chemicals and instruments

DPPH free radicals were procured from Sigma Aldrich. Sodium carbonate, sodium phosphate, potassium ferricyanide, ammonium molybdate, standard rutin, ascorbic acid and gallic acid, 2-thiobarbituric acid (TBA), butylated hydroxy anisole (BHA), Follin-Ciocalteu, 2- deoxyribose and H_2O_2 (30%, v/v) were purchased from Merck India Ltd or Qualigens Fine Chemical Co. (India). All other chemicals and solvents were of analytical grade. The absorbance measurements were recorded using the ultraviolet-visible spectrophotometer of Shimadzu, Pharmaspec-1700.

2.2. Preparation of plant extract and its sub fractions

The plant material (fruit) was procured from market (herbal vendors) of Varanasi, and identified by Professor SD Dubey, Department of Dravyaguna, Institute of Medical Science, Banaras Hindu University, Varanasi, India and the voucher specimen (COG/PM/01/08) was kept for further reference at our Laboratory Herbarium, Department of Pharmaceutics, IT-BHU, Varanasi. The powdered material (1 kg) of air-dried fruit was extracted using 95% ethanol in a Soxhlet apparatus for 24 h (8 h per day for 3 days). After filtration of the solvent, the filtrate was concentrated to dryness under a vacuum evaporator. The ethanol extracts thus obtained were finally fractionated with the use of solvent in increasing polarity which was shown in the flowchart (Figure 1).

2.3. Antioxidant assays

2.3.1. Determination of total antioxidant activity

Total antioxidant activities of fractions were determined according to the method of Prieto *et al*[8]. Briefly, 0.3 mL of sample was mixed with 3.0 mL reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95 ℃ for 90 min under water bath. Absorbance of all the sample mixtures was measured at 695 nm. Total antioxidant activity was expressed as the number of equivalents of ascorbic acid in milligram per gram of extract.

2.3.2. Determination of reducing power

The reducing power of methanolic extracts was determined according to the method of Yildirim *et al*[15]. Different amounts of fraction (50-250 mg) in 1mL of methanol were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6), 2.5 mL of potassium ferricyanide (10 g/L) and 2.5 mL of trichloroacetic acid (100 g/L). The mixture was incubated at 50 ℃ for 20 min after centrifugation for 10 min at 3 000 rpm, Further 2.5 mL of the supernatant solution, 2.5 mL of distilled water and 0.5 mL $Fecl_3$ (1 g/L) was mixed properly and absorbance was measured at 700 nm, higher absorbance indicating stronger reducing power.

2.3.3. Determination of DPPH radical scavenging activity

1, 1-diphenyl-2-picryl-hydrazil (DPPH) method was used to determine the free radical scavenging activity of *P. murex* fractions, where 5 mL of DPPH (100 μM/mL) solution was added to 1mL of different fractions as well as standard (25-200 μg/mL)[7]. The absorbance was then measured 30 min later at 517 nm and the free radical scavenging activity was calculated in the form of IC_{50} value according to the standard equation using ascorbic acid as control which was mentioned here. The IC_{50} value is the concentration where 50% inhibition occurs.

$$\text{Percentage inhibition} = [(1-A1/A0)] \times 100 \quad \text{(i)}$$

Where, A0: Absorbance of the blank, A1: Absorbance test sample.

2.3.4. Determination of nitric oxide scavenging activity

The procedure was based on the method, where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide. Nitric oxide interacts with oxygen to produce nitrite ions that can be estimated by Greiss reagent[16]. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. The reaction mixture consisted of 2 mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) with 0.5 mL of fraction at different concentrations and then incubated at 250 ℃ for 150 min. Further, 0.5 mL of this solution was added into 1.0 mL sulfanilic acid reagent which was prepared by mixing 33% H_2SO_4 in 20% glacial acetic acid. Later on it was incubated at room temperature for 5 min and to this 1.0 mL naphthylethylenediamine dihydrochloride (0.1% w/v) was added which was further incubated at room temperature for 30 min. Finally absorbance was measured

at 546 nm and nitric oxide radical scavenging activity was calculated using this equation (i) followed by calculating the IC_{50} values.

2.3.5. Determination of Hydrogen peroxide scavenging activity

The scavenging activity was evaluated following the proposed method described by Jayaprakasha *et al*[17]. 1mL of the methanolic solution of different fraction as well as standards with 2 mL (20 mM) of hydrogen peroxide prepared in phosphate buffered saline (PBS, pH 7.4). Absorbance was measured at 230 nm after 10 min. According to the equation (i) percentage inhibition was calculated in terms of IC_{50}.

2.3.6. Determination of Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of different fractions of the *P. murex* was evaluated by the deoxyribose method described by Halliwell *et al*[18]. A mixture of 500 μL of different fraction, 1 mM $FeCl_3$, 1 mM EDTA, 20 mM H_2O_2, 1 mM L-ascorbic acid, and 30 mM deoxyribose in potassium phosphate buffer (pH 7.4) were incubated for 1 h at 37 ℃, after addition of 1 mL of 2.8% (w/v) trichloroacetic acid and 1 mL of 1% (w/w) 2-thiobarbituric acid. Again it was heated in a boiling water-bath for 15 min and finally absorbance was measured at 532 nm against a blank.

2.4. Determinations of total phenolics contents

The total phenolic estimation was done as per the Folin-Ciocalteu (FC) method[11]. 1 mL of the various fraction was taken into 10 mL volumetric flask to which 8 mL of distil water, 0.5 mL of FC reagent was added and mixed for 15 min. After that 1.5 mL of 20% sodium carbonate solution was added to this solution, kept for 2 h at ambient temperature. Finally the absorbance of the colored reaction product was measured at 765 nm, standard gallic acid solutions were used for calibration curve and results were expressed as mg of gallic acid equivalent per gram (mg GAE/g) of dried extract.

2.5. Statistical analysis

Results were expressed as mean value ± standard error mean (SEM) of three independent determinations. Linear regression analysis was performed, quoting the correlation coefficient r^2.

3. Results

3.1. Yield of fractions

Yields of different fractions of *P. murex* plant extract were given in Table 1. Among the all fraction petroleum ether fraction was found to be maximal followed by ethyl acetate.

3.2. Total phenolic content

On the basis of the result the ethyl acetate fraction was observed to possess the highest amount of phenolics while petroleum ether fraction was shown to be the least. The content of the total phenolics in fractions was determined using the linear regression equation of the calibration curve (y=0.008 × + 0.092, r^2= 0.995) and was expressed as Gallic Acid Equivalent (Table 1).

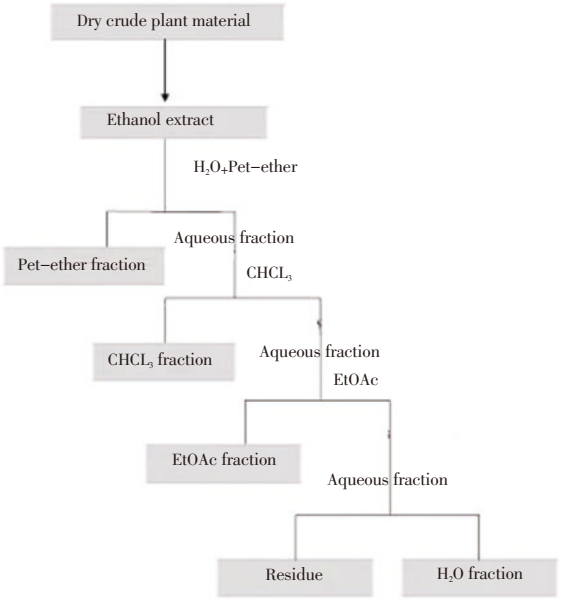

Figure 1. Schematic diagram of fractionation of *P. murex* extract.

Table 1
Antioxidant activities and total phenolic content of the different fraction of *P. murex* (Mean ± SEM) (μg/mL).

No	Extract/fraction	% yield	IC_{50} values*				Total phenol content
			DPPH	Nitric oxide	H_2O_2	Deoxyribose	
1	Petroleum ether	25.8	367.80±4.81	434.75±18.26	416.94± 8.95	446.68±14.03	67.83±0.74
2	Chloroform	11.2	325.59± 6.23	377.82±6.41	389.06±10.01	392.15±4.13	96.89±1.34
3	Ethyl acetate	18.4	135.11±2.95	200.57±4.51	217.91±6.12	250.01±4.68	298.72±2.09
4	Aqueous	15.6	210.8±4.16	300.23±6.63	262.11±6.78	330.88±6.99	183.07±2.55
5	Rutin	NA	NA	NA	87.07±0.51	NA	NA
6	BHA	NA	NA	NA	NA	123.55±1.61	NA
7	Ascorbic acid	NA	77.91±1.93	88.17±3.04	NA	NA	NA

NA: Not analyzed; *: The results were the average of three determination ± SEM.

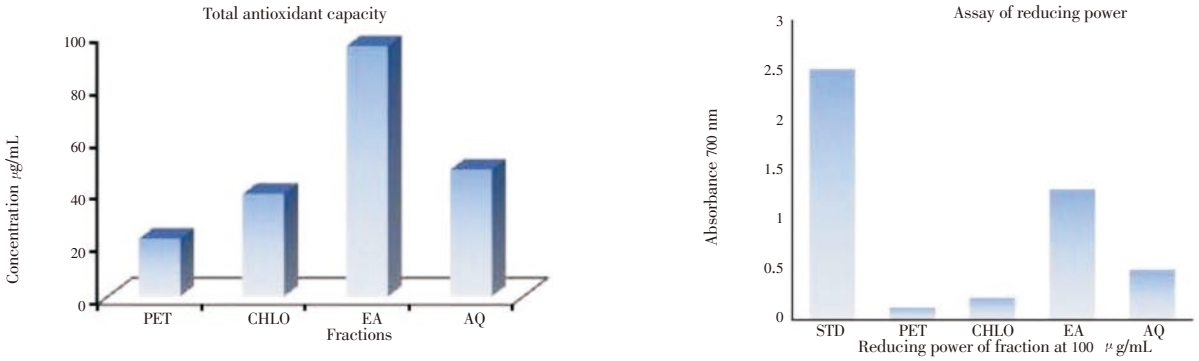

Figure 2. Total antioxidant capacity and assay of reducing power of different fraction of *P. murex*.
STD: Rutin; PET: petroleum ether; CHLO: chloroform; EA: ethyl acetate; AQ: aqueous.

3.3. Total antioxidant capacity

The linear regression equation of the calibration curve (y=0.005 × − 0.042, r^2=0.996) was incorporated to determine the total antioxidant capacity of the various fraction of *P. murex* (Figure 2) and was expressed as the number of equivalent of ascorbic acid (μg/mL plant extract and their fractions). The results in the Figure 2 showed that the highest capacity of antioxidant nature was found to be with that of ethyl acetate fraction (95.70±3.74 μg/mL Ascorbic Acid Equivalent) comparing with other fraction.

3.4. Reducing power activity

The results in the figure 2 revealed that the highly polar fractions such as ethyl acetate and aqueous part were found to exhibit a significant reducing power as compared with that of the non polar fraction such as petroleum ether and chloroform. They can be arranged in the order as: ascorbic acid > ethyl acetate fraction > aqueous fraction > chloroform fraction > petroleum ether fraction.

3.5. DPPH radical scavenging activity

DPPH scavenging assay is one of the most preferred antioxidant method for determination of the radical scavenging activity of plant material. When DPPH converts to 2, 2-diphenyl-1-picryl hydrazine its purple color fades due to contact with a hydrogen donor. Different fraction exhibited considerable free radical scavenging activity as indicated by their IC_{50} values which was shown in Table 1. In comparison with ascorbic acid (IC_{50} value 77.91±1.93 μg/mL), ethyl acetate fraction was shown to have a reliable IC_{50} value (135.11±2.95 μg/mL.

3.6. Nitric oxide scavenging activity

Nitric oxide or reactive nitrogen species are highly reactive species which occur generally when their reaction occurs with oxygen or super oxides. Table 1 showed that ethyl acetate fraction had good nitric oxide scavenging activity (IC_{50} value 200.57±4.51 μg/mL) compare with petroleum ether fraction (IC_{50} value 434.75±18.26 μg/mL).

3.7. Hydrogen peroxide scavenging activity

Scavenging ability of various fractions was presented in Table 1 using rutin as control. The ethyl acetate fraction showed a good activity in depleting H_2O_2, with an IC_{50} value of 217.91±6.12 μg/mL as compared with the standard (87.07±0.51 μg/mL).

3.8. Deoxyribose scavenging activity

The scavenging capacity of hydroxyl radical by ethyl acetate fraction was found to have maximum (IC_{50} value 250.01±4.68) in comparison with other fractions (Table 1). This revealed that ethyl acetate had more power (less than the standard) to eliminate the hydroxyl radical as compared with the other tested fractions.

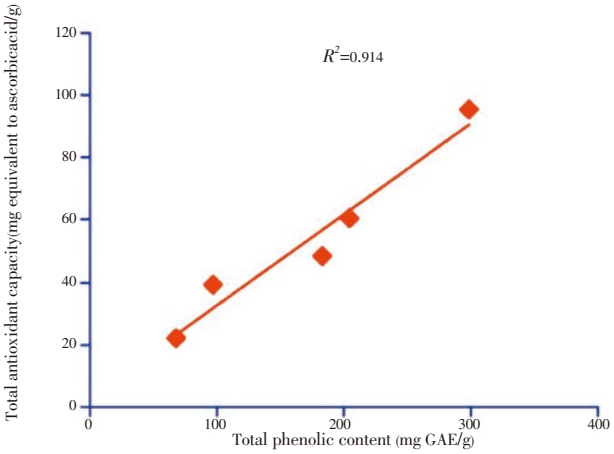

Figure 3. Correlations between the total antioxidant capacity and total phenolic content of different fraction of *P. murex*.

3.9. Co-relationship between the total antioxidant capacity and the total phenolic content

The best criteria to prove the antioxidant activity of a

plant is to correlate the total poplyphenolics with that of the antioxidant activity data. This can be estimated by comparing the r^2 value obtained by plotting a graph between the total antioxidant activities of various fractions versus its total phenolic content with that of the total antioxidant capacity (Figure 3). Our investigation showed that the r^2 obtained between the total antioxidant capacity and the total phenolic contents of all the fractions was found to have a linear property with a value of 0.914 (r^2) when compared with the total antioxidant capacity r^2=0.996.

4. Discussion

Natural substances exhibiting antioxidant properties can be supplied as food additives, or as specific preventive pharmaceuticals. Many antioxidant compounds, naturally occurring from plant sources, have been identified as free radical or active oxygen scavengers. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants[6]. It has been demonstrated that plants, containing high amounts of secondary metabolites like phenolic have been identified as a free radical or active oxygen scavengers and can prevent damages caused by oxidative stress[19-21]. Increasing experimental evidence has suggested that these compounds can affect a wide range of cell biological functions by virtue of their radical scavenging properties[6]. The intake of antioxidants such as polyphenols has been effective in the prevention of many diseases[6].

Looking back to our results it was observed that ethyl acetate fraction was regarded as the most effective solvent for polypheloics like phenolic acids and flavonoids resides. The total phenolic contents of the ethyl acetate fraction were higher than those of any other fraction. Therefore, the high content of total phenols in ethyl acetate fraction of *P. murex* indicated the strong antioxidant properties of this part. The results confirmed the possibility of recovering high amounts of phenolics with antioxidant properties from fruit of *P. murex* which could be explained by the possible formation of complexes by a part of the phenolic compounds with other components[22].

The positive correlation between the total polyphenolic content and antioxidant potential of various plant extracts has been well demonstrated in prior reports and it is interestingly seen that our results correlate perfectly with the previous studies. High phenolic compounds from plant source lead to a positive activity as antioxidant. But this can be governed not only through the correlation coefficient but also through the scavenging activity in various antioxidant assays[20]. The antioxidant activity of the phenolic compounds was attributed to its redox properties, which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers, and metal chelating property[23]. In general, in accordance to the DPPH assay results, among the various fraction of *P. murex* ethyl acetate portion was shown to be a good scavenger of the free radical, indicating the DPPH radical scavenging activity of *P. murex* which may be mostly related to their phenolic hydroxyl group. Nitric oxide or reactive nitrogen species generally formed when their reaction occurs with oxygen or super oxides such as NO_2, N_2O_4, N_3O_4, NO_3^- and NO_2^- which are highly reactive in nature. Excessive production of NO leads to the ill effects and can cause detrimental to human body so that it can lead to disorders like inflammation, cancer and other pathological complications[6]. Our data shows that the polar fractions of *P. murex* have a good nitric oxide scavenging activity which is comparable with the standard rutin. This reveals that the polar fraction has better ability to scavenge the highly reactive nitric oxides as compared with the non polar ones. The same results occur with the potency to scavenge the hydrogen peroxide as well as the hydroxyl radical. Hydrogen peroxide are of less reactive in nature as compared with that of hydroxyl radical which are extremely reactive and capable of damaging almost every molecule found in living cells[24-27]. Hence it is necessary to prevent the imbalance of these radicals in the body. In addition to these radical scavenging assays the correlations fit well with both of the reducing power and the total antioxidant activities[11]. Our data on the reducing power of the tested fractions suggest that it is likely to contribute significantly to the observed antioxidant effect. However, the antioxidant activity of antioxidants has been attributed by various mechanisms, among which some are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging[28]. Reducing power is one mechanism for action of antioxidants and may serve as a significant indicator of potential antioxidant activity for antioxidants[18]. Therefore, the reducing capacity may be used as an indicator of the potential antioxidant activity that can terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. Hence, from our investigation it can be seen that polyphenols which belong to a heterogeneous class of compounds possess a variety of effects towards antioxidant behavior. In search of plant source of natural antioxidants, some medicinal plants and fruits have been extensively studied for their antioxidant activity and radical scavenging in the last few decades[29]. Moreover, the fruit of *P. murex* has shown to have extensive property to act as antioxidant.

It can be concluded that *P. murex* can be utilized as a source of natural antioxidant as their fractions exhibit antioxidant activity. The results indicate that ethyl acetate solvent fractions exhibit higher antioxidant activities as compared with the other fraction. The findings of this work are useful for further research to identify, isolate and characterize the specific compound which is responsible for higher antioxidant activity. Bioactive compounds found

in *P. murex* depict a major breakthrough for a variety of food/medical applications as they are mainly responsible for natural antioxidants in different food or pharmaceutical products.

Conflict of interest statement

We declare that we have no conflict of interest.

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