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EVALUATION OF *INVITRO* ANTIOXIDANT ACTIVITY OF *Pedalium murex* L.

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ABSTRACT

Pedalium murex belonging to family Pedaliaceae was distributed throughout India. In the present study, to assess the in vitro anti-oxidant activity of the ethanolic extract of whole plant *Pedalium murex*. The antioxidant activities of the ethanol extracts from the *Pedalium murex* whole plant were assessed in efforts to validate the herb. The anti-oxidant activity of the plant has been studied using its ability to scavenger DPPH, total antioxidant capacity, superoxide radicals, hydrogen peroxide, hydroxyl radical and nitric oxide radical. Analysis of plant extracts revealed a high scavenging activity suggesting a possible role of these phyto constituents in the antioxidant property. The values are found to comparable to those of ascorbic acid as the standard. Moreover, the results were observed in a concentration and dose dependent manner. Studies clearly indicate that the *Pedalium murex* has significant anti-oxidant activity and also used in folkloric medicines.

Keywords: *Pedalium murex,* physicochemical characters, phytochemical screening, Antioxidant activity.

INTRODUCTION

Plants have been used as medicines for thousands of years. People depend on plants for several purposes like for wood, timber, non-timber forest products, food, medicine etc. (Jain et al., 2005).

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They have always been used as a rich source of biologically active drugs and have numerous traditional uses to serve mankind for many thousand years (Kirtikar *et al*., 1999). Now a day, they are used widely because of growing awareness of people toward sun wanted side effects and high cost of the allopathic medicines which makes them beyond the reach of common people.

In the present era of drug development and discovery of newer drug molecules many plant products are evaluated on the basis of their traditional uses. One of the many plants which are being evaluated for their therapeutic efficacies is *Pedalium murex* which is commonly known as Latjeera (Hindi) & Rough Chaff tree (English).

Concept of free radicals

Free radicals are an atom or molecule that bears an unpaired electron and is extremely reactive in its outermost atomic or molecular orbital, capable of engaging in rapid change reaction that destabilize other molecules and generate many more free radicals. Free radicals and oxidants play a dual role as both toxic and beneficial compounds, since they can be either harmful or helpful to the body. They are produced either from normal cell metabolisms in situ or from external sources. The sources of free radicals can be endogenous and exogenous in nature. Endogenous sources of free radicals are intracellularly generated from auto-oxidation or inactivation of small molecules. Exogenous sources of free radicals are tobacco smoke, certain pollutants, organic solvents, anesthetics and pesticides. Free radical reactions are initiated continuously in cells and tissues in the body from both enzymatic and non-enzymatic reactions.

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Enzymatic reactions serving as sources of free radical reactions include those involved in phagocytosis, prostaglandin biosynthesis and in the Cytochrome P450 system. Free radicals also arise in the non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionising radiation (Diplock *et al*, 1998). The sites of free radical generation encompass all cellular constituents including mitochondria, lysosomes, peroxisomes, endoplasmic reticulum, plasma membrane and sites within the cytosol. Free oxygen radicals play a cardinal role in the etiology of several diseases like arthritis, cancer, atherosclerosis etc. The oxidative damage to DNA may play a vital role in aging. Free radicals are generated in the human body when oxidation occurs during aerobic respiration. The destructive free radical nitrogen dioxide is the result of a reaction between nitric oxide (NO') and oxygen (O_2), is formed in cigarette smoke and vehicle exhaust and has been implicated in respiratory illnesses and irreversible lung damage (Kirtikar *et al*., 1999).

Reactive oxygen species

Reactive oxygen species (ROS) are the oxygen-centered free radicals. It refers to an array of metabolites derived from molecular oxygen (O2). A number of ROS are responsible for oxidative damage in the human body .Reactive nitrogen species (RNS, e.g. Nitric oxide, NO•) play a vital role in the generation of free radicals. NO• is an abundant reactive radical which has a role in diverse physiological processes like neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation. Overproduction of reactive nitrogen species is called Nitrosative stress (Valko *et al*., 2007).

Natural sources of antioxidants

Medicinal plants are an important source of antioxidants (Rice-Evans, 2004). Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of certain diseases such as cancer, heart diseases and stroke (Prior and Cao, 2000). The secondary metabolites like phenolics and flavonoids from plants have been reported to be potent free radical scavengers. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark (Mathew and Abraham, 2006).

In vitro Methods

Various methods are used to investigate the antioxidant property of sample (diets, plants extracts, commercial antioxidant etc.). The objective of this review article is to accumulate all probable methods that are used to evaluate the antoxidant property of various samples. Two review articles have been published (Chanda and Dave, 2009) on in vitro evaluation of antioxidant activity. In this article, attempts have been taken to include in vivo too and to analyze the frequency of the use of different methods.

Pedalium murex is a member of the sesame family, Pedaliaceae. It is found in different parts of the world such as tropical Africa, Srilanka, India, Mexico and Pakistan. In India, it occurs mainly in the Western and Coromandel coasts as a weed of waste places and is generally called under the Hindi name "Gokhru or gokhar" and in Sanskrit as "gaja-daunstraka, gokshura or tittagokshura". Its names vary from one region to another ranging from North to South and from East to West part of the country. It is also called in Kannada (doddaneggilu), Malayalam (motha-malvi-gokharu), Tamil (Ananerinnil), Marathi (Gokhara), Gujarati (Gokhura), Oriya (Yanai nerunjil), Arabic (Khasake kabir), Burmese (Sulegi), Singapore (Ati neranchi), Persian (Khasake Kalan). The fruits of this plant are rich in flavonoids, sapogenin (diosgenin-0.06%) and soluble proteins (20.14 mg/g) (Badarinath *et al*., 2010).

The present study was to evaluate the antioxidant activity of ethanolic extract of *Pedalium murexin vitro* models.

MATERIALS AND METHODS

Collection, Identification and Authentication of plant materials

The plant species namely *Pedalium murex* plant was collected by in and around Koothanallur, Thiruvarur District, Tamil Nadu, India. The plant was identified with the help of the Flora of Presidency of Madras and authenticated by Dr. S. John Britto, RAPINAT Herbarium and Centre for

Molecular Systematics, St. Joseph's college, Tiruchirappalli (Voucher number of the specimen, AMTA 001) (Gamble, 1997). The plant was air dried under shade for 10-15 days. Then the dried material was grinded to fine powder using anelectric grinder and stored in air tight bottles. The powder matter was used for further analysis.

Preparation of the Ethanol extract

Ethanolic extracts was prepared according to the methodology of |Indian pharmacopoeia (Anonymous, 1996). The coarse powder material was subjected to Soxhlet extraction separately and successively with 210ml ethanol and 90ml distilled water. These extract were concentrated to dryness in flash evaporator under reduced pressure controlled at a temperature (400C – 500C). The paste form of the extracts was put in an air tight container stored in refrigerator.

In vitro antioxidant activity

Total antioxidant capacity

To determine the total antioxidant capacity was performed by the method of Sirwaikar Annie *et al*. (2004).

Reagents required

- 0.6 M sulphuric acid.
- 28 mM sodium phosphate.
- 4 mM ammonium molybdate.

Procedure

To 1 ml of extract of different concentration was treated with 1 ml of reagent solution (0.6mM sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) in eppendorf tube. Capped tubes were incubated in thermal block at 95^{o}C for 90min. after cooling to room temperature; the absorbance was measured at 695nm against blank. The activity was compared with ascorbic acid standard.

% of scavenging total antioxidant activity = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100

DPPH scavenging Activity

The DPPHScavenging Activity was determined by the method of Gulcin *et al*., (2004).

Reagents Required

- 1mM DPPH in 95% Ethanol

Principle

The DPPH is reacted with Methanol or Absolute Ethanol to yield purple colour DPPH radical. The presence of antioxidants which include polyphenolics and flavonoids in the sample will scavenge the formed DPPH radical and thereby a decreased color will be observed which is spectrophotometrically measured at 517 nm.

Procedure

The solution of DPPH was prepared by adding 4.3 mg of DPPH (1, 1-Diphenyl –2-picrylhydrazyl) was dissolved in 3.3 µl methanol; it was protected from light by covering the test tubes with aluminum foil. 150 µl DPPH solutions were added to 3ml methanol and absorbance was taken immediately at 517nm for control reading. Each of the samples of various concentrations was then further diluted with methanol up to 3µl and to each 150 µl DPPH was added. Absorbance was taken after 15 min. at 517nm using methanol as blank on UV-visible spectrometer. The IC50 values for each drug compounds as well as standard preparation were calculated. The DPPH free radical scavenging activity was calculated using the following formula:

% of DPPH scavenging activity = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100

Nitric Oxide scavenging activity

The Nitric OxideScavenging Activity was determined by the method of Kamble *et al*. (2011).

Reagents Required

- Sodium nitroprusside
- Phosphate-buffer
- Greiss Reagent
- Methanol

Principle

In this spectrophotometric method the absorbance of chromophore formed during the diazotization of the nitride with sulphanilamide and the subsequent coupling with naphthy ethylenediamine dihydrochloride was measured.

Procedure

Sodium nitroprusside (SNP-5mM) in phosphate-buffer saline was mixed with an equivalent amount of methanol to get the control. Methanol served as blank. Methanol was added to test solutions at different concentrations to make up a volume of 3ml and incubated at room temperature (27^0C) for 90 minutes. This incubated solution (1.5 ml) was added to 1.5 ml of Greiss Reagent. Absorbance at 546 nm was noted using UV–VIS spectrophotometer.

% of Nitric oxide Scavenging activity = {(Control Abs.—Sample Abs.)/Control Abs} × 100

Superoxide radical scavenging activity

The Superoxide radical Scavenging Activity was determined by the method of Nishimiki *et al.* (2004).

Reagents Required

- Nitroblue tetrazolium
- Phosphate buffer
- NADH

Principle

Phenazine methosulfate - nicotinamide adenine dinucleotide (PMS-NADH) system was used for the generation of superoxide anion. It was assayed by the reduction of nitroblue tetrazolium (NBT).

Procedure

About 1 ml of nitro blue tetrazolium, 1ml NADH in 100mM phosphate buffer at pH 7.8 and 0.1 ml of sample solutions of different concentrations were mixed. The reaction was started by adding 100µl PMS and the reaction mixture was incubated at 25°C for 5 minutes. The absorbance of the mixture was measured at 560nm.

% of Superoxide radical scavenging activity = {(Control Abs. - Sample Abs.)/Control Abs} × 100

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity of the extracts was assessed using spectrophotometric deoxyribose method (Kamble *et al.*, 2011) with modifications.

Reagents Required

- Potassium dihydrogen phosphate
- Potassium hydroxide
- Ethylene diamine tetra-acetic acid
- Thiobarbituric acid
- Trichloroacetic acid
- .Hydrogen peroxide

Procedure

100µl of extract (100 µg/ml) was added to the reaction mixture consisting of 200 µl of 100mM potassium dihydrogen phosphate potassium hydroxide, 200 µl of 15 mM 2-deoxyribose, 200 µl of 500 mM ferric chloride (FeCl3), 100 µl of 1 mM ethylene diamine tetra-acetic acid (EDTA), 100 µl of 1 mM L-ascorbic acid, 100 µl of 10 mM hydrogen peroxide (H2O2). The whole reaction mixture was incubated at 37±1°C for 1h. After incubation, 1 ml of each 1% (w/v) thiobarbituric acid (TBA) and 2.8% (w/v) trichloro acetic acid (TCA) were added into the reaction mixture. The reaction solutions were heated on a water bath at 80±1°C for 20 min and ascorbic acid was used as the standard hydroxyl scavenger. The absorbance of the solution was measured at 532 nm using UV-visible spectrophotometer and the scavenging activity (%I) was calculated.

% of Hydroxyl Radical Scavenging Activity= {(Control Abs.—Sample Abs.)/Control Abs} × 100

Hydrogen Peroxide Radical Scavenging Activity

Scavenging of hydrogen peroxide (H_2O_2) by the extracts was estimated as per theprotocol described by Gulcin *et al*., (2004).

Reagents Required

- Hydrogen peroxide
- Phosphate buffer

Procedure

0.6 ml of 40 mM H_2O_2 in phosphate buffer (pH 7.4) solution was added to the test tubes containing 3.4 ml of extract (500 µg/ml)in phosphate buffer (pH 7.4). After thoroughly mixing the reaction mixture, absorbance was noted on spectrophotometer at 230 nm. Same protocol was followed for standard antioxidant ascorbic acid and deionized water was used as blank. The percentage of H_2O_2 scavenging of the extracts was calculated.

% of Hydrogen Peroxide Radical Scavenging activity = {(Control Abs.—Sample Abs.)/Control Abs} × 100

RESULTS AND DISCUSSION

In vitro antioxidant activity

Radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems. Antioxidants orchestrate many biologic responses to inflammation and immunity, they function as signalling mechanisms for redox regulation, even minimal levels of oxidative stress is highly sensed and the protective antioxidant mechanism is set into action which is essential for the maintenance of the structural integrity of proteins. In recent years, attention has been focused on the anti-oxidant properties of plant- derived dietary constituents of food (Gulcin, 2004)

Antioxidants are believed to intercept the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate or propagate further oxidation of lipid. Ascorbic acid and oxalic acid are natural antioxidants present in *P.murex* extracts.

The antioxidant activity of ethanolic extract of *P.murex* was evaluated by 6 different in vitro models. Ascorbic acid was used as the standard antioxidant. All the assays were done in triplicate and expressed as mean ± SD value. It was observed that the extracts scavenged the free radicals in a dose dependent manner.

Total antioxidant capacity of *P.murex* extracts is expressed in terms of ascorbic acid equivalents. For the determination of total Antioxidant capacity, ascorbic acid is used as a reference standard from which plant extracts with potential antioxidant activity are evaluated Table 3. The extracts showed different range of total antioxidant capacity and this may be associated with the high amounts of polyphenol compounds extracted in the corresponding solvent in plant extracts. The ethanolic extract of *P.murex* plant is highest total antioxidant capacity .Thus this is an indication of different antioxidant capacity that may be due to different concentration of secondary metabolites in the extracts (Table 1).

DPPH• is one of the free radicals widely used for testing preliminary radical scavenging activity of the plant extract (Hageman, 1998). Scavenging of DPPH• radical is related to the inhibition of lipid peroxidation (Akter, 2011). DPPH decoloration assay is a simple and reliable antioxidant assay. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable phenoxy radical (Cotelle *et al*., 1996), with an absorption maximum band around 515- 528 nm and thus it is a useful reagent

for evaluation of antioxidant activity of compounds. (Sanchez., 2002). The assay is based on the conversion of purple colored DPPH free radical to yellow colored stable diamagnetic molecule by reaction with the hydrogen donating scavenger. In this model, the ethanolic extract 250mg was found to scavenge the DPPH radical to a greater extent. The maximum percentage of inhibition of alcoholic extract was 50.2 at 250mg where as the standard ascorbic acid showed 40.2 % inhibition at 250 mg. The alcoholic extract showed better DPPH scavenging potential than the standard ascorbic acid. (Table 2).

Total Antioxidant capacity (TAC) is the measure of the ability of substances extracted from food (plants or animals) to slow down oxidative stress. The antioxidant activity for the different extracts of *P.murex* leaf was evaluated by using phosphomolybdate method. It determines the total antioxidant capacity. The method is usually used to determine the total antioxidant capacity of plant extracts (Halliwell, 1994) the basic principle of the assay is based on the reduction of Mo(VI) to Mo(V) in presence antioxidant components from the substance being studied (Table 3)

Nitric Oxide (NO), produced endogenously from LArginine by nitric oxide synthetase, plays an important role in many physiological processes including vascular regulation, immune responses, and neural communication (Rackova*et al* .,2007) NO is extremely unstable and can interact with molecular oxygen to form nitrite (NO_2-) and nitrate (NO_3-), which acts as free radical. This model is based on the inhibition of nitrite free radical generated from sodium nitroprusside in buffer solution. Nitric oxide generated in physiological systems can be determined using Griess Reagent which is a mixture of N-(1-naphthyl) ethylenediamine and sulfanilic acid. Sulfanilic acid is quantitatively converted to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to N-(1-naphthyl) ethylenediamine dichloride to form a purple colored azo dye, which is detected by measuring the absorbance at 546nm.In nitric acid assay, the highest activity was observed with alcoholic extract with a high percentage inhibition. The extract was found to have better scavenging effect of NO than the standard ascorbic acid (Table 4).

Superoxide anion assay was carried out by alkaline DMSO assay. Superoxide is generated by the addition of NaOH to air saturated dimethyl sulfoxide (DMSO).The generated superoxide remains stable in solution, which reduces Nitroblue tetrazoliumin to formazan dye at room temperature. .Superoxide anion plays an important role in plant tissues and is involved in the formation of other cell-damaging free radicals. (Duan et al., 2007)All extract exhibited excellent superoxide anion scavenging activity, which can be correlated with high content of flavonoids like hibiscetin, cyanidine, cyclopropenoides in extracts (Table 5). Study suggested that the flavonoids may be involved in the dismutation of superoxide anion radical (Arora*et al*., 2011).

Hydroxyl radicals are important active oxygen species causing lipid peroxidation and enormous biological damage (Table 6). These radicals are formed in free solution by their ability to degrade deoxyribose into fragments (Babu *et al*., 2012) Ethanol extract of *P.murex* inhibited about 44.63% of the radicals at a concentration of in comparison to the known scavenger, ascorbic acid, which scavenged only about 40.2% hydroxyl radical at the same concentration.

Hydrogen peroxide though a weak oxidizing agent is important because of its ability to penetrate biological membranes, once inside the cell it can probably react with Fe^{2+} and Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects. Like superoxide anion, all extract showed excellent H_2O_2 scavenging activity. Leaves and stems contain β-sitsterol, stigmasterol, compounds and their derivatives responsible for antioxidant activity.

Table 1: Total Antioxidant scavenging activity of *Pedalium murex*

S.No	Concentration (µg/ml)	Ethanolic extract	
		% of Inhibition	IC_{50} Value(µg/ml)
1	50	21.9±1.6	18.69
2	100	25.8±0.4	
3	150	32.06±1.1	
4	200	39.2±0.9	
5	250	45.7±0.4	
6	Ascorbic acid (standard)	42.5±2.5	28.52

Table 2: DPPH scavenging activity of *Pedalium murex*

S.No	Concentration (µg/ml)	Ethanolic extract	
		% of Inhibition	IC_{50} Value(µg/ml)
1	50	22.4±1.4	17.281
2	100	32.5±2.6	
3	150	40.7±4.1	
4	200	44.8±2.4	
5	250	50.2±1.9	
6	Ascorbic acid (standard)	48.3 ± 1.2	32.56

Table 3: Nitric oxide scavenging activity of *Pedalium murex*

S.No	Concentration (µg/ml)	Ethanolic extract	
		% of Inhibition	IC_{50} Value(µg/ml)
1	50	30.5 ± 2.6	33.3
2	100	34.4 ± 1.5	
3	150	39.4 ± 0.6	
4	200	44.8 ± 0.3	
5	250	49 ± 0.8	
6	Ascorbic acid (standard)	54.5±2.5	62.04

Table 4: Superoxide dismutase scavenging activity of *Pedalium murex*

S.No	Concentration (µg/ml)	Ethanolic extract	
		% of Inhibition	IC_{50} Value(µg/ml)
1	50	12.8±2.01	1.98
2	100	18.1±1.05	
3	150	23.3±0.9	
4	200	26.1±1.8	
5	250	33.5±1.4	
6	Ascorbic acid (standard)	38.45.5±2.5	18.67

Table 5: Hydroxy Radical Scavenging Activity of *Pedalium murex*

S.No	Concentration (µg/ml)	Ethanolic extract	
		% of Inhibition	IC_{50} Value(µg/ml)
1	50	22.2±1.1	3.4
2	100	25.5±0.7	
3	150	32.4±0.7	
4	200	38.9±1.1	
5	250	44.63±0.4	
6	Ascorbic acid (standard)	54.5±2.5	28.8

Table 6: Hydrogen Peroxide Scavenging Activity of *Pedalium murex*

S.No	Concentration (µg/ml)	Ethanolic extract	
		% of Inhibition	IC_{50} Value(µg/ml)
1	50	19.9±1.9	8.36
2	100	22.5±2.2	
3	150	30.06±1.9	
4	200	35.5±0.4	
5	250	41.4±1.3	
6	Ascorbic acid (standard)	42.5±2.5	28.86

CONCLUSION

The outcome of the study, thus, provides strong evidence in establishing the antioxidant properties of *P.murex* , which, are proved by DPPH, total antioxidant capacity, superoxide radicals, hydrogen peroxide , hydroxyl radical and nitric oxide radical. The result of the present study clearly indicates that the ethanolic extract of *P.murex* whole plant possesses antioxidant activity, which claims its traditional use as a substitute for *P.murex* Further in depth studies are to be carried out to understand the molecular mechanisms of antioxidant action of the ethanolic extract of *P.murex* coupled with animal studies and clinical trials would result in the arrival of cost effective, safe, efficacious anticancer drug which is a boon for ailing human society.

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