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Research Article

Antioxidant and Free Radical Scavenging Activities of Leaf Extract of *Cayratia pedata* (Lam.) Juss. Family: Vitaceae

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ABSTRACT

Many metabolic, detoxification and biotransformation reactions taking place within the body may lead to production of free radicals and reactive oxygen species (ROS). The anti oxidant system present in the body will neutralize these molecules and protect the cells from the hazardous effects of them. ROS is believed to be involved in many pathological conditions including cancer, diabetes and neurological diseases. Many natural products and dietary supplements possess antioxidant as well as free radical scavenging activities. In the present study we have evaluated the antioxidant and free radical scavenging activities of the ethanol extract of *Cayratia pedata* (Lam.) Juss.Family Vitaceae. The extract showed significant anti oxidant and reducing power activity as well as hydroxyl, super oxide and nitric oxide scavenging activities.

Keywords: *Cayratia pedata*, Free radical scavenging, anti-oxidant, Flavonoids, Total phenol content.

INTRODUCTION

The role of oxidative stress in the etiology of many diseases is well known today. Many researchers are now focusing on the deleterious effects of reactive oxygen species (ROS) and the beneficial effects of anti-oxidants against these effects. Literature[1,2] tells that oxidative stress contributes to cardiac disorders like myocardial infarction and cardiomyocyte apoptosis.

A free radical is a radical containing unpaired electron. Free radicals are highly reactive due to the presence of unpaired electrons. In normal individuals the antioxidant system within the body can scavenge the free radicals produced. However, in some conditions, large amounts of free radicals are generated in the body and the production exceeds removal[3]. The most important ROS includes super peroxide anion, hydroxyl radical and singlet oxygen[4].

The free radicals play an important role in the etiology of many diseases like cancer, alzheimer's disease, parkinsonism and cardiovascular disorders. The relation between inflammatory bowel diseases and the related intestinal damage is related to low concentration of antioxidant defense[5]. The antioxidant and free radical scavenging properties of plants may be attributed to the presence of multiple components acting differently but sometimes synergistically.

Cayratia pedata (Lam.) Juss. Family: Vitaceae is a weak woody climber naturalized in tropical evergreen and semi-evergreen forests of India, Andaman-Nicobar Islands, Sreelanka and Malaysia[6]. In folklore medicine, *C.pedata* is used for treating many conditions like scabies, wounds, boils, skin ailments, diarrhoea, and fever and in many inflammatory conditions. It is used also as an emetic, anthelmintic, astringent and rubifaceint[7]. Preliminary phytochemical analysis of the extract revealed the presence of carbohydrates, flavonoids, sterols, terpenes, tannins and phenolic compounds and alkaloids[8]. The anti-inflammatory activity of C.pedata in carrageenan induced rat hind paw edema has been reported[9]. Since free radicals are involved in many pathological processes an attempt has been made to study the free radical scavenging activity of the plant.

MATERIALS AND METHODS

Preparation of extract of leaf of Cayratia pedata

Leaves of *C.pedata* were collected from the forest areas of Palode village of Thiruvananthapuram district. The leaves were cleaned, dried at shade, powdered, pre-extracted with petroleum ether and further extracted with ethanol (95%) by Soxhlet extraction. The alcoholic extract was dried *in vaccuo* to get the final soft extract (CPEE).

Estimation of total phenolic content (Gallic Acid Equivalence method)[10]

The Folin-Ciocalteu reagent (a mixture of phosphomolybdate and phosphotungstate) was used for the colorimetric determination of phenolic and polyphenolic antioxidants. It works by measuring the amount of the substance being tested needed to inhibit the oxidation of the reagent. The reaction mixture was prepared by mixing 1 ml of each of gallic acid (standard) solutions (in concentrations of 10,20,40,80 and 100 µg/ml) with 0.25 ml of Folin-Ciocalteu reagent and 1.25 ml of 20% sodium carbonate solution. The mixture was then allowed to react for 40 min. at room temperature. After 40 minutes the contents were again mixed well and measured the optical density (blue colour) at 725 nm. The test solution consisted of 1mg /ml solution of CPEE in

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Table 1: Evaluation of hydroxyl radical scavenging actitity of *C.pedata* leaf (ethanol) extract.

Concentration in µg/ml	Optical density at 532 nm			Percentage inhibition	
	Control (A)	Ascorbic acid (B)(Standard)	CPEE (C)	Ascorbic acid	CPEE
50	0.054±0.001	0.028 ±0.002	0.048±0.001	46.67*	11.11*
100		0.024±0.0001	0.035±0.0001	55.93*	17.04*
200		0.020±0.0002	0.040±0.0001	62.07*	26.29*

* Significant, p<0.01, N=3, ANOVA, against control

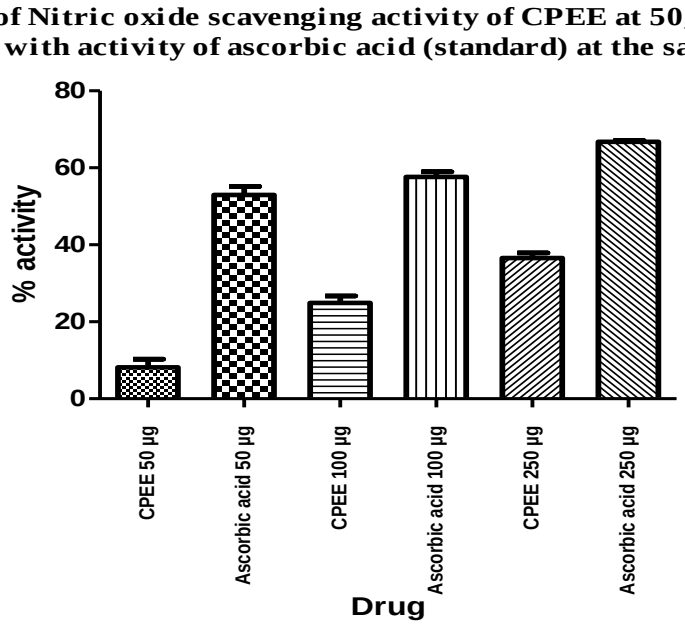

Figure 1: Evaluation of nitric oxide scavenging activity of *C.pedata* leaf extract.

methanol.

Determination of total flavonoids content[11]

The flavonoids content was determined by interpolation from the regression line of a standard graph obtained by plotting the absorbance of various concentrations of Quercetin (standard) against the corresponding concentrations. The reaction mixture consists of 1 ml of extract solution, 4 ml distilled water and 0.3ml of 5% sodium nitrite. After 6 minutes 0.3ml of 10 % aluminium chloride solution and 2ml of 1M sodium hydroxide were added. The total volume was made up to 10ml with distilled water. A blank was prepared without addition of aluminium chloride solution. The solutions were mixed well and the absorbance was measured against the blank at 510nm using UV-Visible spectrophotometer.

Hydroxyl radical scavenging activity

This assay was based on the measurement of competition between the extract and 2-deoxyribose for hydroxyl radical formed by the Fenton's reaction by the method originally described by Gulhan *et al*[12] and modified by Elizabeth and Rao 1990[13]. The reaction mixture contained 0.1ml of (3 mM) deoxyribose, 0.5 ml of (0.l mM) Ferric chloride solution, 0.5 ml of (1 mM) H_2O_2 and 0.8 ml phosphate buffer (20mM, pH 7.4). The reaction mixture (0.9ml) was mixed with 0.1 ml ascorbic acid at concentrations of 50µg, 100 µg and 200µg/ml (each in triplicate). CPEE solutions were also tested in same concentrations. Buffer (0.1 ml) was added to positive control. Contents of all the tubes were incubated for 1 hour at 37º C. After incubation, 800 µl each of that reaction mixture were transferred from all the tubes to another group of test tubes and added 200 µl of 8% SDS, 1 ml trichloro acetic acid (2.8%, pH 3.5) and 1 ml of 1% thiobarbituric acid to all the tubes. The tubes were kept in boiling water bath for 1 hour, cooled and added 1 ml distilled water and 5 ml pyridine-butanol mixture (1:15) each to all the tubes and mixed well and centrifuged. The optical density of the mixtures was measured at 532 nm. Percentage activity was calculated by the formula:

% Activity= $[(OD_c - OD_t / OD_c] \times 100$.

Where, OD_c and OD_t are the optical densities of the control and test respectively.

Nitric oxide scavenging activity[14,15]

In this estimation, the ability of the extract to remove the nitric oxide generated at physiological pH from an aqueous solution of sodium nitroprusside by Griess reaction is measured. This estimation is based on the reaction between nitrate, sulphanilamide and naphtylethylenediamine in acidic medium to form an azo dye. (Griess Illosvory reaction). Nitric oxide scavenging activity was measured spectrophotometrically.

The extract and standard (ascorbic acid) was tested in concentrations of 50, 100 and 200 µg/ml. The reaction mixture containing 0.1 ml solution of the test substance in

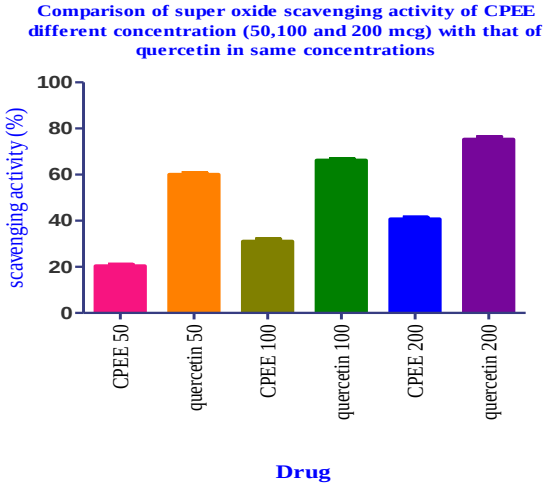

Figure 2: Estimation of superoxide scavenging activity of C.pedata leaf extract (ethanolic). Standard used was quercetin.

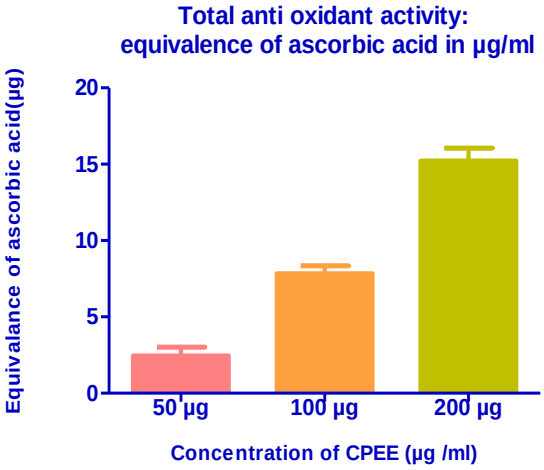

Figure 3: Estimation of total anti-oxidant activity of CPEE

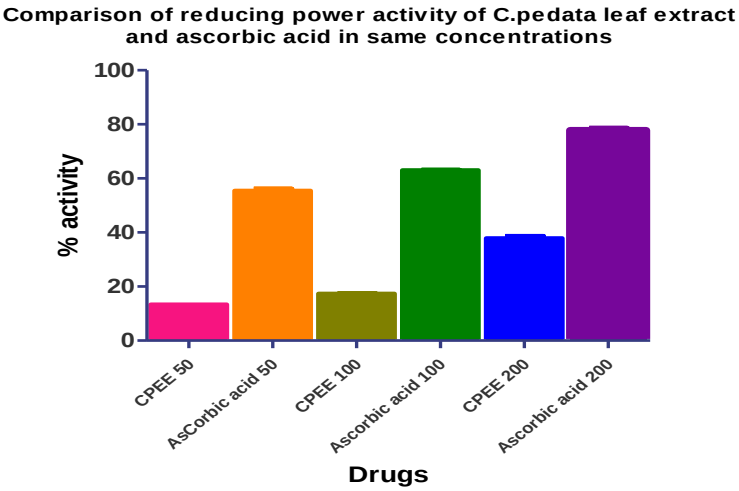

Figure 4: Evaluation of reducing power activity of *C.pedata* leaf extract in ethanol.

methanol-water mixture and 2.9 ml sodium nitroprusside solution (5mM) in PBS were incubated at 25 °C for 150 minutes. After incubation, 1.5 ml of solution was removed from each tube to another set of tubes and then added 1.5 ml of Griess reagent to all. The absorbance of the chromophore formed during the diazotization of sulphanilamide with nitrate and subsequent coupling with naphthyl ethylene diamine dihydrochloride was read immediately at 546nm.

% inhibition =100 x [1-T/C]

Where, T and C are the absorbance of test and control solutions respectively.

Super oxide free radical scavenging activity[16,17]

Super oxide anions are generated in PMS-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan product. The reaction mixture contained EDTA (6 µM), NaCN (3µg), riboflavin (2µM) and NBT (50µM) in phosphate buffer. 2.9 ml reaction mixture was mixed with 0.1ml solution of test substance in various concentrations ((50, 100 and 200 µg/ml). The standard used was quercetin in similar concentrations. All of the tubes were kept under an incandescent lamp (so that they get uniform illumination) for 15 minutes. The absorbance of solution was measured at 560nm against control (2.9 ml reaction mixture+ 0.1ml distilled water)

% inhibition =100 x [1-T/C]

Where T and C are the absorbance of test and control solutions respectively

Total antioxidant activity[18]

The reaction mixture contained 0.6ml H_2SO_4, 28mM sodium phosphate and 4mM ammonium molybdate and 0.3 ml solution of the extract. The solutions were incubated at 95ºC for 90 minutes and the absorbance of the solution was measured at 695nm against blank (Methanol) after cooling to room temperature. The antioxidant activity is expressed as number of µg equivalent of ascorbic acid, the values of which are obtained from a standard graph plotted with OD of standard against the corresponding concentrations of ascorbic acid, treated in a similar manner.

Reducing power activity

Table 2: Comparative evaluation of reducing power activity of CPEE with ascorbic acid

Concentration µg /ml)	Optical density at 700 nm (Mean ± SEM)			Value of t and R^2, for N=3,4df, at 95%CL.
	OD of Control (A)	CPEE (B)	Ascorbic acid (C)	
50 (1)	0.049 ±0.001	0.056±0.0003	0.077± 0.0003	t=57.71,R^2=0.997(B1 & C1)*
100 (2)		0.0581±0.000	0.0807± 0.000	t=239.7,R^2=0.99(B2 & C2)*
200 (3)		0.07 ± 0.001	0.0.879 ±0.0004	t=34.18, R^2=0.99(B3 & C3)*

*significant, ,p<0.05

The reducing power of extract was determined by the method of Yen and Duh[19]. Different concentrations (50,100,200µg/ml) of extract were mixed with 2.5ml of phosphate buffer (pH 6.6) and 2.5ml of 1% potassium ferricyanide and boiled it for 20 minutes at 50°C. To that solution, added 2.5 ml of TCA and centrifuged for 10 minutes at 2000 rpm. To the supernatant 1ml of distilled water and 250µl of 0.1% ferric chloride solution were added and the absorbance was read at 700nm. The increase in optical density indicates reducing power activity.

RESULTS

Estimation of total phenolic content

Many plants are rich in phenolic contents. Many reports suggest that plants containing phenolic compounds and tannins possess anti-oxidant, cyto-protective, free radical scavenging anti-proliferative and anti-inflammatory activities[20,21]. The antioxidant and free radical scavenging properties of plants may be attributed to the presence of multiple components acting differently but sometimes synergistically. The total phenol content of a definite amount of CPEE was expressed as Gallic acid equivalents in µg/ml. The calculated value of total phenol content in 1 mg CPEE is equivalent to 86.78µg of Gallic acid

Determination of total flavonoids content

Flavonoids are a group of plant metabolites (plant pigments) sharing similar chemical structure. Flavonoids are reported to possess many biological and pharmacological activities such as anti-inflammatory, anti cancer[22,23], antibacterial and antidiabetic activities. They modify cell signaling and production of many enzymes like cycloxygenase, iNOS, LOX, etc. They are powerful antioxidants and free radical scavengers[24]. Many C-glycoflavonoids have already been synthesized or isolated from natural sources and their SAR have been studied[25].

The total flavonoids content in CPEE was determined from a standard calibration curve plotted by the observed absorbance versus the concentration of standard substance (Quercetin). The equivalence of quercetin was calculated from the equation of slope for the standard curve plotted with absorption against concentration. 100 µg of the extract was found to be equivalent to 34.43 µg of quercetin. (R^2=0.997)

Hydroxyl radical scavenging activity

Hydroxyl radicals play a potential role in degenerative diseases such as atherosclerosis, cancer, diabetes and in neurological diseases[26]. Hydroxyl radicals can cause damage to DNA structure and impairment of mitochondrial antioxidant enzymes[27,28].

The results of evaluation of hydroxyl radical scavenging activity is summarized in table 1. The *C.pedata* leaf extract shows significant (p<0.01) activity in scavenging hydroxyl radicals generated in the reaction system in vitro. The IC_{50} of ascorbic acid (standard) and CPEE determined from the graph plotted with the percentage activity against concentrations, were found to be 54.23µg/ml and 435.18 µg/ml respectively.

Nitric oxide scavenging activity

Endogenous nitric oxide acts as a vasodilator and signaling molecule. Nitric oxide reacts with superoxide anion to form peroxynitrite (-$ONOO^{-)}$ which is a highly reactive compound. Peroxynitrite can react with other endogenous molecules like amino acids, lipids, thiol group, DNA bases and proteins[29].

It was found that *C. pedata* leaf extract exhibited significant nitric oxide scavenging activity. (p<0.05). The percentage activity of the extract in concentrations of 50,100 and 250 µg/ml were found to be 8.32, 24.76 and 36.72 while that of ascorbic acid in similar concentrations were 53.16, 57.64 and 66.7. The calculated value of IC_{50} for nitric oxide scavenging activity of *C. pedata* leaf extract is 342.79µg/final volume. A comparison of the activities of ascorbic acid and *C. pedata* leaf extract is shown in fig 1.

Super oxide free radical scavenging activity.

Superoxide or hyper oxide anions is a dioxide formed by one electron reduction of molecular oxygen. Superoxides are immunologically important because one of its action is killing the invading microorganisms. Super oxides are involved in pathogenesis of many diseases and aging[30].

A comparative evaluation of superoxide free radical scavenging activity of *C.pedata* leaf extract and quercetin (standard) is shown in the fig 2. It was observed that the percentage activity of C.pedata extract in concentrations of 50, 100 and 200 µg / ml was 20.4±0.66, 31.1±1 and 41± 0.79 respectively. The percentage activity of quercetin standard at the same concentrations was 60 ± 82, 66.2± 0.71 and 75.11 µg/ml respectively. The calculated values of IC_{50} of *C.pedata* extract and quercetin were 260.64 and 55.8 µg/ml respectively.

Antioxidant Activity

The total anti-oxidant activity of CPEE in various concentrations was expressed as µg of ascorbic acid. The results are shown in fig. 3. It was observed that the antioxidant activity of CPEE in concentrations of 50, 100

and 200 µg /ml can be equated to the antioxidant activity of 2.45 ±0.441, 7.85± 0.604 and 15.22 ±1.03 µg /ml of ascorbic acid respectively.

Reducing power activity

The results of estimation of reducing power activity of *C.pedata* leaf extract is summarized in table 2. The increase in optical density shows increased activity with respect to the control. Fig.4 shows a comparison of the reducing power activity of *C.pedata* extract in various concentrations with ascorbic acid, which is used as the standard in same concentrations. The EC_{50} value of *C.pedata* extract calculated from the percentage activity was 276.82 µg/ml.

DISCUSSION

Reactive oxygen species and free radicals are produced as a result of many biochemical and biotransformation reactions undergoing in the body. The ill effects of ROS are nullified by the constitutional or endogenous anti-oxidant system within the body. However, excessive production of the free radicals and ROS lead to oxidative stress, which plays a role in the pathogenesis of several diseases. The role of ROS in cancer is extensively studied[31] and most currently available synthetic antioxidants produce side effects when they are consumed for this activity[32]. The anti-oxidant properties as well as the abilities of many herbs and plant based products to scavenge free radicals have already been studied.

In the present study, the antioxidant and free radical scavenging activities of ethanol extract of *Cayratia pedata* leaves were evaluated using various in vitro methods. The results of the study points that the ethanol extract of leaves of *Cayratia pedata* contains phytochemical agents with antioxidant and free radical scavenging activities. The results of estimation of total phenolic contents and total flavonoids content points to the fact that the antioxidant and scavenging activities may be attributed to the presence of these secondary metabolites. In order to use the leaves of *C.pedata* for its potential anti-oxidant activity, further studies like isolation, identification, chemical characterization and in vivo studies of these compounds are required.

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