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Effects of antioxidant and antibacterial proferties of polyphenol extract of *Cissampelos pareira*

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Abstract
The present study assessed the antioxidant and antibacterial activities of polyphenol extract of *Cissampelos pareira* leaves. The antioxidant activity was determined by ABTS radical lipid peroxidation inhibition, superoxide scavenging and metal chelating assay and also antibacterial activity was evaluated by the disc diffusion method. The polyphenol extract of *C. pareira* showed high antioxidant activity ABTS (67.32%), lipid peroxidation inhibition (73.32%), superoxide scavenging (75.32%) and had antibacterial activity against both Gram positive and Gram negative bacteria. Higher inhibition zone was detected against *S. aureus* (17.3 mm). The extract of this plant showed high phenolic, flavonoids contents and it can be concluded that these compounds may be responsible for antioxidant and antibacterial activity. The results indicated that the polyphenol extract of *C. pareira* leaves possessed strong antibacterial and antioxidant properties and could be an important source of natural compounds for development of new drugs.

Keywords: antioxidant; antibacterial; polyphenol extract and *C. pareira*

Introduction

Plants have played a significant role in providing the human race with remedies. Plants play important roles in human life not only as suppliers of oxygen but also as a fundamental resource to sustain the human race on this earthly plane. Plants also play a major role in our nutrition by converting energy from the sun during photosynthesis. In addition, plants have been used extensively in traditional medicine since time immemorial. Medicinal properties of few such plants have been reported but a good number of plants still used by local folklore are yet to be explored. Siddha, Ayurveda and Unani systems of medicine provide good base for scientific exploration of medicinally important molecules from nature. Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to humans against infections and degenerative diseases; however, recent concern has been paramount regarding the potential detrimental side effects of synthetic additives in humans (Mercy *et al*., 2018) [9].

The potential of the antioxidant constituents of plant materials for the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists and food manufacturers as consumers move toward functional foods with specific health effects (Huang *et al*., 2011) [6]. The antioxidative effect is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes. Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury. Moreover, knowledge and application of such potential antioxidant activities in reducing oxidative stresses *in vivo* has prompted many investigators to search for potent and cost-effective antioxidants from various plant sources (Boulekbache *et al*., 2013) [2]. Polyphenols are naturally occurring compounds found largely in the fruits, vegetables, cereals and beverages. Fruits like grapes, apple, pear, cherries and berries contains up to 200–300 mg polyphenols per 100 grams fresh weight. The products manufactured from these fruits, also contain polyphenols in significant amounts. Typically a glass of red wine or a cup of tea or coffee contains about 100 mg polyphenols. Cereals, dry legumes and chocolate also contribute to the polyphenolic intake (Graf *et al*., 2005). Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens (Yolmeh and Sadeghi 2016) [18]. In food, polyphenols may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability. Towards the end of 20th century, epidemiological studies and associated meta-analyses strongly suggested that long term consumption of diets rich in plant polyphenols offered some protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Santharam *et al*., 2015) [15].

Cissampelos pareira, a well-known medicinal climber-plant of the Menispermaceae family, has been extensively used in the traditional medicinal system since the ancient time for the treatment of numerous diseases such as ulcer, wound, rheumatism, fever, asthma, cholera, diarrhoea, inflammation, snakebite, malaria, rabies, and also recommended for blood purification. *C. pareira* including mainly isoquinoline alkaloids along with few flavonoids, flavonoid glycosides, and fatty acids. The crude extracts of *C. pareira* have shown various pharmacological activities such as antipyretic, anti-inflammatory, antiarthritic, antiulcer, antidiabetic, anticancer, antifertility, anti-microbial. To our understanding, there are no efficient studies on the *in vitro* antioxidant activity of polyphenol extract of *C. pareira* so far, and in previous studies the antimicrobial properties of the leaf extract have been less widely considered. Therefore, the main objective of the present study was polyphenol extract of *C. pareira* evaluate the antibacterial and antioxidant activity.

Materials and Methods

Plant materials

The leaves of *Cissampelos pareira* were collected from Government siddha medical college, herbal garden, Arumbakkam, Chennai, Tamilnadu, during January 2017 and it was taxonomically identified and authenticated as flower of *Cassia alata* by Dr. S. Sankaranarayanan, Head, Department of Medicinal Botany, Government Siddha Medical College, Arumbakkam, Chennai-600 106, Tamil nadu. A voucher specimen was deposited in the herbarium for future reference (Ref.No. MB/2017/Ceasal-24).

Preparation of Extracts

The leaves of *Cissampelos pareira* was thoroughly cleaned, dried under the shade and coarsely powdered. The polyphenol extract was prepared according to a previously reported method Kumarappan *et al*. (2012). The powdered plant material was mixed with 70% aqueous-methanol and stored at room temperature for 5 days. After 5 days, it was filtered and the solvent was evaporated. The residue was dis-solved in water, and the aqueous layer was washed with petroleum ether several times until a clear upper layer of petroleum ether was obtained. The lower layer was then treated with ethyl acetate containing glacial acetic acid (10 mL/L). Extraction of the polyphenols was performed for 36 h at room temperature, and the combined ethyl acetate layer was then concentrated. The residue was lyophilised. The extract obtained was dried and stored in an airtight container at 4◦C. The yield of the dry poly-phenolic extract was 30.5% (w/w). The dried extract was dissolved in Milli-Q water and used for further study.

Phytochemical Screening

The aqueous leaves extract of *C. pareira* were subjected to phytochemical screening to determine the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, glycosides, saponins and polyphenols using standard procedures (Harborne 1973).

Thin Layer Chromatography

Thin layer chromatography of polyphenol extract of *C. pareira* was performed using standard procedures (Harborne 1973). The polyphenol extract was placed carefully in precoated aluminum silica gel 60 F, Merck F 254 using a microcapillary tube. The spots were allowed to dry for few minutes and the TLC plate was placed in the solvent mixture, Toluene, acetone and Formic acid (6:6:1). After drying, the TLC plates were observed under UV at 240nm and 360 nm in UV TLC viewer. The Rf value of the spots was calculated by using the standard formula,

$$\text{Retention factor Rf} \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

ABTS *(*2, 2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) Radical Scavenging Assay

ABTS radical scavenging activity of polyphenol extract of *C. pareira* was followed by Re *et al*. (1999) [14]. ABTS radical was newly prepared by addition 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to produce an absorbance of 0.70 at 734 nm and the same was used for the antioxidant activity. The final solution of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Ascorbic acid. Correspondingly, in the experiment group, 1 ml reaction mixture encompassed 950 µl of ABTS solution and 50 µl of different concentration of each extracts. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734 nm against distilled water by using a Deep Vision (1371) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

ABTS Scavenging Effect (%) = $[(A_0 - A_1/A_0) \times 100]$ Where A_0 is the absorbance of the control reaction and A_1 is the absorbance of extract.

Inhibition of Lipid Peroxidation Activity

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al*. (1979). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of polyphenol extract of *C. pareira* in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard.

Inhibition of lipid peroxidation (%) by the each extracts was calculated according to 1-(E/C) × 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample.

Superoxide Radical Scavenging Assay

This assay was based on the capacity of the polyphenol extract of *C. pareira* to inhibit the photochemical reduction of Nitroblue tetrazolium (NBT) in the presence of the riboflavin-light-NBT system (Tripathi and Pandey Ekta, 1999; Tripathi and Sharma, 1999). Each 3 ml reaction solution contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM Ethylene diamine tetra acetic acid (EDTA), NBT (75 µM) and different concentration of extracts. It was kept visible in fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV–Vis Spectrophotometer. Identical tubes with reaction mixture were kept in the dark served as blanks. The percentage inhibition of superoxide radical activity was measured by comparing the absorbance of the control with test sample solution:

$$\% \text{ Super oxide radical scavenging capacity} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 was the absorbance of control and A_1 was the absorbance of both plant extracts fraction.

Metal Chelating Activity

Metal chelating capacity of polyphenol extract of *C. pareira* was measured according to Iihami *et al*., (2003) [7]. 1 ml of different concentrations of flavonoid rich fraction was added to 0.05 ml of 2 mM ferric chloride solution. The reaction was initiated by the addition of 0.2 ml of 5 mM Ferrozine and the mixture was shaken vigorously. After 10 min, the absorbance was measured at 562 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The % inhibition of ferrozoine-Fe2+complex was calculated by following equation.

$$\% \text{ Inhibition of ferrozoine-Fe2+complex} = [(A_0-A_1)/A_0] \times 100$$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Culture Collection and Maintenance

The bacterial strains of *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa.* These standard strains were obtained from Microbial Type Culture Collection and gene bank (MTCC); Institute of Microbial Technology, Chandigarh, India. The stock culture was maintained on Mueller Hinton agar medium at 4 ◦C.

Antibacterial Activity

The antibacterial activities of the crude alkaloid extracts were assayed using the disc diffusion method (Bajalan *et al*., 2017) [1]. Bacteria were grown overnight on Mueller Hinton agar plates, five colonies were suspended in 5 ml of sterile saline (0.9%) and the bacterial population in the suspension was adjusted to ~$3x10^8$ CFU/ml. A sterile cotton swab was dipped into the suspension and the swab rotated several times with firm pressure on the inside wall of the tube to remove the excess fluid. The swab was used to inoculate the dried surface of MH agar plate by streaking four times over the surface of the agar, rotating the plate approximately by 90º to ensure an even distribution of the inoculums. The medium was allowed to dry for about 3 min before adding a sterile disc of 9 mm diameter. Each disc was placed firmly on to the agar to provide uniform contact with the bacteria. The different concentration of polyphenol extract of *C. pareira* was introduced on to each disc and the control disc received only 7% ethanol. The plates were incubated at 37°C for 24 h and the inhibition zone was measured and calculated. The experiments were carried out in duplicate three times. The results (mean value, *n*=3) were recorded by measuring the zones of growth inhibition surrounding the discs.

Statistical Analysis

All the experiments were done in triplicate. The SPSS software version 20 was used for data analysis. The results are expressed as mean values with standard deviation (±SD) from three experiments. The experimental data obtained were analyzed for multiple comparisons using one-way analysis of variance (ANOVA) and when the results were significant, Duncan's test was also used.

Result and Discussion

Phytochemical analysis

The phytochemical screening of aqueous leaves extract *C. pareira* studied presently showed the presence of alkaloids, flavonoids, polyphenol, terpenoids, and absence of glycosides and tannin (Table -1).

Table 1: Phytochemical screening of aqueous leaves extract *C. pareira*

Sl. No.	Phytochemical Constituents	Observation	Aqueous leaves extract C. pareira
1	Alkaloids -Dragendorff's Test-Mayers test	Orange /red precipitate Yellow or white precipitate	+ +
2.	Flavonoids-Alkalai Reagent-Lead acetate test	Intense yellow colour Precipitate formed	+ +
3.	Glycosides Keller-Killiani test	Reddish brown colour ring formed	-
4.	Tannin-$FeCl_3$ test	Blue black coloration	-
5.	Saponins-Frothing test	Foam	+
6.	Terpenoids-Salkowski test	Dark reddish brown color in interface	-
7.	Polyphenols-Ferrozine test	Raddish blue	+
8.	Anthocyanin test Ammonia	Ammonia layer yellow in color	+

+ indicate positive result; -- Indicate negative result

Free Radical-Scavenging Ability

The antiradical activity of polyphenol extract of *C. pareira* leaves, as the representative of dietary food source, were assessed *in vitro* by ABTS assay, as well as by evaluation of potential to decoloration of ABTS. In table-2 the results of antioxidant activity obtained for tested samples, as well as Vitamin-C used as standard are shown. It can be clearly seen that polyphenol extract of *C. pareira* displayed notable antioxidant activity, significantly higher than Vitamin-C. However, in present study showed that these activities were mainly due to presence of polyphenol compounds. Even though the ABTS radical has slight significance to biological systems and living organisms, this technique is extensively observed as revealing of the ability of plant extracts to scavenge free radicals, and will mention to hydrogen atom or electron donation ability, autonomously of any enzymatic activity (Mileva *et al*., 2014) [10].

Table 2: Free radical-scavenging ability by polyphenol extract of *C. pareira*

Different concentration of extract	Percentage of ABTS radical activity	
	Polyphenol extract of *C. pareira*	Standard Vitamin-C
25 µl/ml	16.34±0.23	14.63±0.78
50 µl/ml	31.23±2.78	26.31±2.39

75 µl/ml	46.32±0.89	41.32±1.89
100 µl/ml	67.32±2.45	62.34±2.47
EC50 value	58.31	64.32

[a] Results are expressed as percentage inhibit of ABTS ability with respect to control. Each value represents the mean+SD of three experiments

Inhibition of Lipid Peroxidation Activity

In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Polyphenol extract of *C. pareira* leaves also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in polyphenol extract of *C. pareira* 73.32% with EC_{50} value 53.21 µl/ml and lowest inhibition percentage ascorbic acid 70.32% with EC_{50} 56.34 (Table-3). As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). Normally, the mechanism of flavonoid compounds for neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals (Parejo *et al*., 2002) [13].

Table 3: Inhibition of lipid peroxidation activity of polyphenol extract of *C. pareira*

Different concentration of extract	Inhibition percentage of Lipid peroxidation	
	Polyphenol extract of *C. pareira*	Standard Vitamin-C
25 µl/ml	20.31±2.36	17.32±0.78
50 µl/ml	42.36±0.48	40.32±2.34
75 µl/ml	56.31±2.78	54.31±1.89
100 µl/ml	73.32±2.45	70.32±2.45
EC_{50} value	53.21	56.34

[a] Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

Superoxide Scavenging Activity

Polyphenol extract of *C. pareira* leaves exhibited powerful scavenging activity for superoxide radicals in a concentration dependent process than positive control. Polyphenol extract of *C. pareira* showed highest radical activity in the percentage of 75.32% with EC_{50} value 49.32 µl/ml when compared to positive control 69.32% with EC_{50} Value 58.31 µl/ml (Table-4). One of the standard method to produce Superoxide radicals is through photochemical reduction of nitro blue tetrazolium (NBT) in the presence of a riboflavin-light-NBT system. These superoxide radicals are extremely toxic and may be produced either through xanthine activity or through mitochondrial reaction. Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive specie. The superoxide radical is known to be produced *in vivo* and can result in the formation of hydrogen peroxide *via* dismutation reaction (Liyana *et al*., 2006) [8]. The result clearly indicates that the plant extract has a noticeable effect as scavenging superoxide radical.

Table 4: Superoxide scavenging activity of polyphenol extract of *C. pareira*

Different concentration of extract	Percentage of Superoxide scavenging activity	
	Polyphenol extract of *C. pareira*	Standard Vitamin-C
25 µl/ml	17.32±2.34	15.34±1.78
50 µl/ml	31.26±0.78	28.64±2.48
75 µl/ml	52.34±1.56	48.34±1.39
100 µl/ml	75.32±2.45	69.32±2.89
EC50 value	49.32	58.31

[a] Results are expressed as percentage of Superoxide scavenging activity with respect to control. Each value represents the mean+SD of three

Metal Chelating Activity

The metal chelating property of polyphenol extract of *C. pareira* leaves was displayed as per Table-6. Polyphenol extract of *C. pareira* were evaluated for their ability to compete with ferrozine for ferrous iron in the solution. In this evaluation, the polyphenol extract of *C. pareira* hindered the formation of ferrous and ferrozine complex, signifying that they have chelating activity and are capable of capturing ferrous iron before ferrozine. The polyphenol extract of *C. pareira* reduced the greenish blue color complex immediately and showed the highest chelating activity 78.34% With EC_{50} Value 46.34 µl/ml than positive control Vitamin-C 72.34% with EC_{50} value 54.23 µl/ml. In the presence of chelating agents the complex formation is dis-rupted, resulting in a decrease in the red colour of the complex. Measurement of colour reduction, therefore, allows estimating the metal chelating activity of the coexisting chelator (Elmastas*et al*., 2006).

Table 5: Metal chelating activity of polyphenol extract of *C. pareira*

Different concentration of extract	Percentage of Metal chelating activity	
	Polyphenol extract of *C. pareira*	Standard Vitamin-C
25 µl/ml	22.31±1.78	19.34±1.87
50 µl/ml	36.34±2.78	38.67±2.34

75 µl/ml	44.37±1.36	46.34±0.78
100 µl/ml	78.34±2.45	72.34±2.45
EC_{50} value	46.34	54.23

[a] Results are expressed as percentage of Metal chelating activity with respect to control. Each value represents the mean+SD of three experiments.

Effect of Polyphenol Extract of *C. Pareira* Leaves on the Growth of Pathogenic Bacteria by DISC Diffusion Method

Antibacterial activity of polyphenol extract of *C. pareira* leaves tested against *Klebsiella pneumoniae, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa* were screened and assayed as inhibition zones in the agar plates (Table-6).

Upon testing all the bacteria tested were found to be sensitive to the polyphenol extract of *C. pareira*. Furthermore, the zone of inhibition study revealed that the polyphenol extract possessed antibacterial activity in proportion to concentration gradient ranges 25-100 µl/ml against the tested microbes. Among the bacteria studied, *Staphylococcus aureus* (Gram positive) and *Escherichia coli* was diagnosed to be highly susceptible followed by *Pseudomonas aeruginosa* and *Enterococcus faecalis*. This may confirm the antibacterial property of polyphenol extract of *C. pareira*. Cowan (1999) [3] reported that phenolic compounds and especially flavonoids can act as antimicrobial agents via several different mechanisms, including inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function, inhibition of energy metabolism, inhibition of the attachment and biofilm formation, inhibition of the porin on the cell membrane, alteration of the membrane permeability, which can result in cell destruction, and attenuation of pathogenicity.

Table 6: The antibacterial activity of the polyphenol extract of *C. pareira* by disc diffusion method

Pathogenic organism	Different concentrations Crude extract (µl/ml)			
	25 µl/ml	50 µl/ml	75 µl/ml	100 µl/ ml
Pseudomonas aeruginosa	7.2±1.3	8.9±0.6	10.2±1.6	12.6±1.4
Staphylococcus aureus	9.5±0.4	13.1±0.9	14.8±1.1	17.3±0.5
Escherichia coli	8.1±1.3	11.1±0.3	13.7±0.6	15.8±0.7
Enterococcus faecalis	6.8±0.5	9.5±0.6	12.1±0.7	14.3±0.8

*The inhibitory Zone size measured included the 6.0 mm size of the well by means of caliper. All the assays were duplicated, and the mean values were recorded.

Conclusions

The present study confirmed that the polyphenol extract of *C. pareira* leaves possessed strong antioxidant and antibacterial possessions. This extract showed high phenolic, content.

It could be take a chance that phenolic compounds may be responsible for its antioxidant and antibacterial activity. With respect to the results, the polyphenol extract of *C. pareira* could be an important source of natural compounds with antioxidant capacity and antibacterial properties for development of new drugs.

Further studies are needed to isolate the bioactive compounds from the extract and to elucidate the exact mechanism of action of the free radical scavenging effect and the antibacterial activity.

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