

## Antioxidant and enzyme inhibitory activities of *Cissampelos pareira* L. leaf extracts

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### Abstract

Plants have remained as a valuable source of traditional medicine since ages to treat various ailments. The phytochemical screening, antioxidant properties and  $\alpha$ -glucosidase inhibitory activity of fresh leaves of *Cissampelos pareira* L., used in the management and treatment of different diseases, was evaluated by several *in vitro* systems. The capability of crude polar and non-polar extracts to scavenge free radicals was studied using different free radical generating systems to assess the peroxidative damage in biomembranes. The  $IC_{50}$  values were calculated to evaluate the antioxidant efficiency of each plant extract. Among the four different extracts, alcohol (CPA) and ethyl acetate (CPE) extracts of *C. pareira* exhibited significant antioxidant properties by presenting much lower  $IC_{50}$  values in comparison to other two extracts. Furthermore, *in vitro* assay of the  $\alpha$ -glucosidase property of CPA showed an  $IC_{50}$  of  $98.23 \pm 0.47$   $\mu$ g/ml. Results obtained indicated that *C. pareira* could be employed as a natural antioxidant agent due to its ability to quench the over production of free radicals in various diseases. Therefore, the results obtained in our study may account for a scientific evidence of antioxidant and antidiabetic properties of this plant for which this plant is known in the traditional and folk systems.

**Keywords:** *Cissampelos pareira* L., medicinal plants, oxidative stress, antioxidant, antidiabetic, traditional medicine

### 1. Introduction

The phenomenon of oxidative stress resulting from the imbalance of a critical balance of pro-oxidants and antioxidants in the organism has scientifically been established as a vital player in the pathology of chronic ailments. The unabated generation of free radicals, resulting in oxidative damage to biological molecules, finally manifested in the form of various pathological or degenerative conditions like ageing, diabetes mellitus, atherosclerosis, Alzheimer's diseases, cancer (Ali *et al.*, 2008). Therapeutic strategies involving the use of antioxidant phytochemicals to halt the proliferation of free radical chain reactions and shield the human body from illnesses have gained prominence in the recent times (Gul *et al.*, 2011). The identification and isolation of antioxidant principles from natural sources has gained fresh impetus recently, owing to their beneficial properties. Many of the modern drugs which are from natural sources, mainly of plant origin have been identified from nature for thousands of years (Cowan, 1999). Since the ancient times, a large number of plants have been used as traditional medicines against various bacterial and/or viral infections and ailments, caused by oxidative stress all over the world. In last 20-25 years, there is a

significant increase in the use of plants as complementary and alternative medicine (Rios and Recio, 2005). Plants and plant products have been mainly used as medical supplements all over the world, due to their potent biological properties with less or no toxicity and cost effectiveness (Agarwal and Prabakaran, 2005; Borris, 1996). Antioxidant, free radical scavenging, antidiabetic, anti-inflammatory and antitumour abilities from the plants have been mainly attributed to polyphenol compounds such as flavonoids and phenolic groups (Cerutti, 1985; Moskovitz *et al.*, 2002). There is a significant interest towards the discovery of natural antioxidants of plant origin which can protect the human body from free radical-mediated oxidative damage. Medicinal plants have been extensively studied by different research groups during the recent past for their antioxidant potential which were attributed due to the presence of novel antioxidants. Keeping this in view, we have made an endeavour to calculate the phytoconstituents and antioxidant capacities of *C. pareira*, which is a potent medicinal herb with varied uses in the traditional system of medicine, to find incipient potential sources of antioxidants. The results from this study will help better understand the antioxidant capacity profile and antidiabetic properties of this medicinal plant. *C. pareira*, belonging to the family Menispermaceae, is a perennial creeping herb, found commonly in semi-dry areas of tropics (Saldanha, 1984). It has been used in the traditional system of medicine in the management of various diseases like dysentery, diarrhea and skin disorder. Antiseptic, insecticidal and parasiticidal properties have also been attributed to the leaves from this plant, and it is used to check hemorrhage from cuts, burns and wounds (Singh *et al.*, 2010). Given

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its usage in traditional medicine, the current investigation was aimed to assess the antioxidant and  $\alpha$ -glucosidase ability of *C. pareira* including the composition of their antioxidant components like total phenolics and total flavonoids.

## 2. Materials and Methods

### 2.1 Plant materials

The healthy leaves of *C. pareira* were collected from the University of Hyderabad Campus, Hyderabad, India during its flowering period, in the month of July, 2011. The plant was authenticated and a voucher specimen of the plant (UoH/VS/CP 03) has been preserved for reference.

### 2.2 Chemicals and instrument

The analytical grade chemicals were purchased from Hi-Media and Merck, India. Standard chemicals were procured from Sigma-Aldrich Chemicals Co. (Germany). A Shimadzu Multispect- 1501 spectrophotometer (Kyoto, Japan) was used for absorbance measurements.

### 2.3 Preparation of extracts

The air dried leaves were made into coarse powder, using a mechanical grinder, which was then subjected to successive extraction in a Soxhlet apparatus, using hexane, ethyl acetate, ethanol and water. The plant residue was dried in an oven at 40°C to remove the previous solvent completely before extracting with the next solvent. Extracts were then filtered through a Whatman No.1 paper filter and concentrated to dry mass with the aid of rotary evaporator. The extracts were stored in a dark container after weighing for further analysis. The different extracts obtained were designated as CPH (for hexane extract), CPE (for ethyl acetate extract), CPA (for ethanol extract) and CPW (for water extract). Dried extracts of 20 mg/ml stock solution were prepared and different concentrations were used in various experiments.

### 2.4 Determination of phytoconstituents

#### 2.4.1 Determination of total phenols

The Folin-Ciocalteu reagent assay was used to determine the total phenolic content in different plant extracts (Yang *et al.*, 2007). About 100  $\mu$ l of extracts were mixed with 125  $\mu$ l of Folin-Ciocalteu reagent and 300  $\mu$ l of 20%  $\text{Na}_2\text{CO}_3$  were then added. The volume was made up to 1  $\mu$ l with double distilled water and incubated at room temperature for 2 h, the absorbance was measured at 760 nm. Total phenolics were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight.

#### 2.4.2 Determination of total flavonoids

Total flavonoid content was estimated by a colorimetric method (Barreira *et al.*, 2008) with slight modifications. Extract samples (10  $\mu$ l) were diluted with 500  $\mu$ l of distilled water and 30  $\mu$ l of 5%  $\text{NaNO}_2$  were added to the sample together with 60  $\mu$ l of 10% aluminum chloride. After mixing and incubation for 10 min, 350  $\mu$ l of 1 M NaOH were added to the reaction mixture and a total volume of 1 ml was made up with distilled water. Following vigorous mixing and incubation of 20 min at room temperature, the absorbance was read at 510 nm against blank. The results obtained were expressed as quercetin equivalents (mg QE)/g of dry weight (dw). A standard curve was prepared with known concentrations of quercetin.

### 2.5 Antioxidant ability assays

#### 2.5.1 Determination of total antioxidant capacity

Phosphomolybdenum assay described by Prieto *et al.* (1999) was employed to evaluate the ability of plant extracts to potentially reduce transition metal ions. The reagent solution contained 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate mixed with samples diluted in dimethyl sulfoxide (DMSO). The incubation was carried out at 90°C in a dry thermal block for 1 h. and 30 min, subsequently, allow to cool down to room temperature and finally the absorbance of the green phosphomolybdenum complex was measured at 695 nm. The reducing capacities of the analyzed extracts were expressed as mg of ascorbic acid equivalents/g dry weight (mg AAE/g) of dw.

#### 2.5.2 Determination of ferric reducing power (FRAP)

The reducing power of different extracts was determined as per the given method with minor changes (Oyaizu, 1986). Briefly, 10  $\mu$ l of each sample was mixed with phosphate buffer (250 ml, 200 mM, pH6.6) and potassium ferricyanide [ $\text{K}_3\text{Fe}(\text{CN})_6$ ] (2.5ml, 1% w/v). The mixture was incubated at 50°C for 20 min. A 250  $\mu$ l of trichloroacetic acid (10% w/v) was added to the reaction, which was then centrifuged for 10 min at 1000 rpm. The supernatant (500  $\mu$ l) was mixed with distilled water (250  $\mu$ l) and  $\text{FeCl}_3$  (100  $\mu$ l, 10% w/v). The absorbance was measured at 700 nm and reducing the power of extracts was expressed as mg ascorbic acid equivalents (AAE) per gram (g) of dry weight (dw).

#### 2.5.3 DPPH radical assay

The DPPH free radical scavenging activity of extracts was determined according to the method described by Braca *et al.* (2002). Briefly, 0.004% w/v solution of DPPH in 95% methanol was prepared. A 20  $\mu$ l aliquot of each plant extract (with appropriate dilutions) was added to 1  $\mu$ l of methanolic DPPH solution and thoroughly vortexed. The absorbance of the solution was measured at 517 nm after incubating at room temperature in dark conditions for 45 min. For the sake of blank and reference, methanol (95%) and L-ascorbic acid were used, respectively.

#### 2.5.4 Nitric oxide radical scavenging activity

This activity of the extracts was measured following the protocol of Sreejayan and Rao (1997) after minor changes. 40  $\mu$ l of sodium nitroprusside (10 mM) solution was added to 100  $\mu$ l of the extract with concentration ranging from 40-400  $\mu$ g/ml. The mixture was incubated for 15 min under light conditions, followed by dilution with 300  $\mu$ l of Griess reagent (1% sulfanilamide in 2%  $\text{H}_3\text{PO}_4$ ). An addition of 10  $\mu$ l of 0.1% naphthylethylene diamine dihydrochloride in 2.5%  $\text{H}_3\text{PO}_4$  was made to the mixture after incubation for 45 min under light conditions at 30°C. In all the samples, the final volume was adjusted to 1ml with double distilled water. Immediately after the colour development, the final volume was made to 1  $\mu$ l with double distilled water and the absorbance reading was taken at 546 nm and a comparison with standard ascorbic acid was made.

#### 2.5.5 Superoxide radicals scavenging activity

The scavenging activity of *C. pareira* leaf extracts towards superoxide anion radicals was measured by following the method of Liu *et al.* (1997). Phenazine methosulfate nicotinamide adenine dinucleotide (PMS-NADH) system generated non-enzymatic

superoxide anions through the reaction of PMS, NADH and oxygen. The activity was examined by the decrease of nitroblue tetrazolium (NBT) in the reaction samples. The superoxide anion generation was measured in 3 ml of Tris-HCl buffer (100 mM, pH 7.4) containing 750  $\mu$ l of NBT (300  $\mu$ M) solution, 750  $\mu$ l of NADH (936  $\mu$ M) solution and 300  $\mu$ l of different concentrations (40 - 400  $\mu$ g/ml) of extracts. L-ascorbic acid was used as a positive control. The start of the reaction was triggered by adding 750  $\mu$ l of PMS (120  $\mu$ M) to the mixture. After 5 min of incubation at room temperature, the absorbance was measured at 560 nm against blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

### 2.5.6 Hydroxyl radical scavenging activity assay

The scavenging activity of hydroxyl radicals was measured with Fenton reaction as per the protocol given by Hinneburg *et al.* (2006). Different concentrations of the extracts ranging from 40 - 400  $\mu$ g/ml and are action mixture containing FeCl<sub>3</sub> (10 mM), L-ascorbic acid (1 mM), H<sub>2</sub>O<sub>2</sub> (10 mM), deoxyribose (28 mM) were mixed in 500  $\mu$ l phosphate buffered saline (20 mM, pH 7.4). The mixture was incubated for 30 min at 37°C and subsequently boiled for 15 min after the addition of 1 ml of trichloro acetic acid (10%, w/v) and 1 ml thiobarbituric acid (2.8% w/v; in 25 mM NaOH). The extent of oxidation was estimated at 532 nm, and the percentage inhibition of the deoxyribose degradation to malonaldehyde represents the scavenging activity of the test sample. L-ascorbic acid was used as the positive control.

### 2.5.7 Hydrogen peroxide scavenging assay

The ability of the extracts to scavenge hydrogen peroxide was evaluated by the method of Long *et al.* (1999). A 40 mM of the H<sub>2</sub>O<sub>2</sub> solution was mixed with different concentrations of extracts ranging from 20 - 200  $\mu$ g/ml, followed by incubation at room temperature for 3.5 h. An aliquot of 90  $\mu$ l of the H<sub>2</sub>O<sub>2</sub> from sample solution was mixed with 10  $\mu$ l of HPLC-grade methanol and final volume was adjusted to 1ml by adding 0.9 ml of FOX reagent was added (9 ml of 4.4 mM butylated hydroxytoluene (BHT) in methanol and 1 ml of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H<sub>2</sub>SO<sub>4</sub>). The reaction mixture was subsequently vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 560 nm. L-ascorbic acid was used as the reference compound.

### 2.5.8 Lipid peroxidation

The extent of lipid peroxidation by modified thiobarbituric acid-reactive species (TBARS) assay was estimated by taking a rat liver homogenate as lipid rich media (Ohakawa *et al.*, 1979). Healthy albino rats of the Wistar strain (250 g) were sacrificed (procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were reviewed and approved by Institutional Animal Ethics Committee of University of Hyderabad vide approval No. (LS/IAEC/IAG/11/10; dated 15/4/2011). The liver was pulverized with 0.15 M KCl, homogenate obtained was centrifuged at 800 g for 15 min at 4°C and the supernatant was used for thiobarbituric acid assay. To the liver microsome preparations, the extracts were added at different concentrations (40 - 400  $\mu$ g/ml) and incubated at room temperature for 10 min. Then, 50  $\mu$ l Fenton's

reagent (10 mM FeCl<sub>3</sub>; 10  $\mu$ l of 2.5 mM H<sub>2</sub>O<sub>2</sub>; 0.1 M L-ascorbic acid) in phosphate buffer (0.2 M, pH 7.4) were added, and the final volume was made to 1 ml. For the induction of induction of lipid peroxidation process, the tubes were incubated for 30 min. at 37°C. After that, 2 ml of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid, 0.5% thiobarbituric acid were added to each sample, followed by heating at 70-90°C for 15 min. The mixture was allowed to cool down to room temperature, subsequently centrifuged at 1000 rpm for 10 min. The extent of lipid peroxidation in each mixture was monitored by the appearance of a pink coloured chromogen which represents thiobarbituric acid reactive substances (TBARS). The optical density reading of the supernatant taken at 532 nm and diminishing of the formation of pink chromogen in pretreated reaction mixtures was considered as the indication of lipid peroxidation inhibition.

### 2.6 Effects of extracts on $\alpha$ -glucosidase activity

The  $\alpha$ -glucosidase inhibitory activity of the extracts was determined by quantifying the release of 4-nitrophenol from 4-nitrophenyl  $\alpha$ -D-glucopyranoside (4-NPGP), according to Matsui *et al.* (1996) with slight modifications. Briefly, the reaction mixture was prepared by using p-nitrophenyl-  $\alpha$ -D-glucoside (PNP-glycoside) as a substrate in 0.1 M phosphate buffer (pH 6.8). The appropriate concentration of the sample solutions along with enzyme solution (0.01 units) were added to premixed solutions of PNP-glycoside (10 mM) and 10  $\mu$ l of GSH (3 mM) along with the suitable concentration of samples were added to and the final volume was adjusted to 1 ml. The progress of the reaction was ceased by adding 5  $\mu$ l of 100 mM sodium carbonate solution. The quantification of enzymatic activity was achieved by measuring the p-nitrophenol released from PNP-glycoside by taking the absorbance 400 nm spectrophotometrically. Metformin was used as a positive control.

### 2.7 Statistical analysis

The percentage inhibitions of radicals, lipid peroxidation and enzymatic activities of the extracts were calculated using the formula:

$$\text{Percentage inhibition} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

All samples were analyzed in triplicate, and experimental results were expressed as Mean  $\pm$  standard deviation of mean (SD) of three replicates. IC<sub>50</sub> value (the concentration of the extracts required to scavenge 50% of radicals) was calculated for different extracts of *C. pareira*. The concentration-response curves were obtained by plotting the percentage inhibition versus concentration. Where appropriate, the data were tested by one-way ANOVA, using Graph Pad Prism 6v software. p-values were used to show correlations and their significance differences of p<0.05 were considered significant.

## 3. Results and Discussion

### 3.1 Determination of phytoconstituents

The quantitative estimation of *C. pareira* showed that the medicinal herb is rich in total phenolics and total flavonoid content according to the data shown in Table 1. The content of total phenolic of different extracts was significant and varying between 2.44 $\pm$ 0.05 to 10.14 $\pm$ 0.14 mg GAE/g dw. Among the tested extracts, the highest

**Table 1:** Quantitative estimation of phytochemicals and antioxidant activities of different extracts of *C.pareira*

Type of extract	Yield (%)	Phenolic content (mg GAE/mgdw)	Total flavonoid (mg QE/mgdw)	Total antioxidant capacity (mg AE/mgdw)	Ferric reducing antioxidant power (mg AAE/mgdw)
CPH	4.14	02.44 ± 0.05	6.92 ± 0.19	8.72 ± 0.13	3.40 ± 0.12
CPE	13.85	10.14 ± 0.14**	28.49 ± 0.75**	30.52 ± 1.07**	14.65 ± 0.32**
CPA	17.58	06.09 ± 0.15*	20.24 ± 0.69**	27.43 ± 0.61**	16.81 ± 0.55**
CPW	2.71	7.00 ± 0.18*	10.62 ± 0.32*	18.06 ± 0.14*	11.41 ± 0.40**

a: Gallic acid; b: Quercetin; c: Ascorbic acid equivalents mg/g dw plant material, respectively. Each value is expressed as a Mean ± standard deviation (n = 3). Averages followed by asterisk in each column differ by Tukey test at p<0.05.

amount of total phenolics was observed in CPE (10.14±0.14 mg GAE/g dw) whereas CPH showed least (2.44±0.05 mg GAE/g dw) content of phenolics. The flavonoid contents of the extracts in terms of standard quercetin equivalent (QE) were between 6.92±0.19 and 28.49±0.75 mg QE/g dw, highest being in CPA (28.49±0.075 mg QE/g dw) and lowest in CPH (6.92±0.19 mg QE/g dw).

There has been cumulative attention in the research on polyphenolic and flavonoid compounds, present in plant parts due to their health aspects and are widely used in combating oxidative stress related diseases (Chen *et al.*, 2002; Djeridane *et al.*, 2006). This property is thought to arise from their protective abilities by counter acting ROS, which are believed to play an important role in etiology and pathogenesis of diseases (Yingming *et al.*, 2004). The presence of these classes of phytochemicals as important bioactive agents in *C. pareira* leaves and these could be accountable for the witnessed antioxidant activity in particular or the therapeutic property of this plant as a whole.

### 3.2 Antioxidant ability assays

#### 3.2.1 Determination of total antioxidant ability

The total antioxidant ability of plant extracts was assessed using the most extensively used phosphomolybdate method (Gul *et al.*, 2011; Prasad *et al.*, 2009). The green phosphomolybdenum V complex formed due to reduction of Mo (VI) is to Mo (V) in the presence of extracts is measured at 695 nm. Total antioxidant ability of *C. pareira* leaf extracts expressed as equivalents of ascorbic acid (µg/ml of extract) is presented in Table 1. CPE has higher antioxidant capacity (30.52±1.07 mg ascorbic acid equivalent/g dw) than other three extracts which presented antioxidant ability in the order: CPA (27.43±0.61 mg AAE/g dw) > CPW (18.06±0.14 mg AAE/g dw) > CPH (8.72± 0.13 mg AAE/g dw). The antioxidant capacity of the extracts as shown by phosphomolybdenum method complements the results obtained by the DPPH radical scavenging assay where ethyl acetate extract leads in terms of activity as compared to other tested extracts. This difference in the degree of molybdenum reduction may be due to variation in the content of phytoconstituents. It is reported that phosphomolybdate scavenging activity is due to the contribution of many flavonoids and related polyphenols of medicinal plants (Sharififar *et al.*, 2009). Therefore, the results obtained imply that the CPE and CPA extracts have distinguished antioxidant potential as compared to standard compound ascorbic acid, seems to open an avenue for exploitation of cost effective natural antioxidants.

#### 3.3 Determination of ferric reducing power (FRAP)

The antioxidant potential of plant extracts is principally influenced by the composition of the extracts as well as the experimental conditions or methods and, hence, cannot be completely assessed by one single method. Therefore, to measure the various mechanisms of antioxidant action more than one type of antioxidant capacity test has to be performed (Frankel and Meyer, 2000). Accordingly, antioxidant capacities of *C. pareira* extracts were assessed by ferric reducing antioxidant power (FRAP) assay. Fe (III) reduction is often employed as an indicator of electron-donating ability, which is an essential mechanism of phenolic antioxidant reaction (Hinneburg *et al.*, 2006). Phenolics have hydroxyl and carboxyl groups which are able to bind particularly iron and copper (Gordon, 1990) and by this means, effect the reduction of the Fe<sup>3+</sup>/ferricyanide complex to the ferrous form (Oyaizu, 1986). Thus, the concentration of Fe<sup>2+</sup> was observed by measuring the formation of Perl's Prussian blue at 700 nm (Amarowicz *et al.*, 2004). The reducing power of *C. pareira* leaves ranged from 3.40±0.12 to 16.81± 0.55 mg AAE/g dw (Table 1). The CPA extract (16.81±0.55 mg AAE/g dw) exhibited advanced reducing power compared to the other extracts.

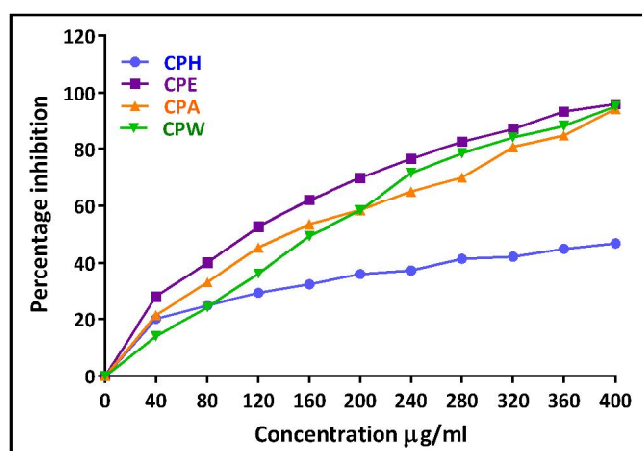
#### 3.4 DPPH radical scavenging activity

The stable, 1-diphenyl 1-2-picryl-hydrazyl (DPPH) radical is extensively used, fairly rapid and accurate technique for the assessment of free radical quenching. DPPH radical quenching ability by antioxidants is primarily because of their hydrogen-donating ability. DPPH is a free radical with nitrogen in the centre and possessing an odd, unpaired electron which upon acceptance of hydrogen or electron converts to a stable diamagnetic molecule entity (Soares *et al.*, 1997). According to the results, established in this investigation, it is found that all of the test plant extracts showed notable scavenging activities against DPPH model in a concentration-dependent manner, that is, the higher the concentration, the highest scavenging potential (Figure 1, Table 2). CPE extract with an IC<sub>50</sub> value of 113.65±0.52 µg/ml showed particularly high free-radical scavenging activity. CPH showed the lowest activity among the extracts, as the IC<sub>50</sub> value was found to be 425.53±0.33. The differences in scavenging activities of the extracts against DPPH might be explained by the variability in the mechanisms of the radical antioxidant reaction.

**Table 2:** IC<sub>50</sub> values obtained in the antioxidant/enzymatic activity assays

Sample	IC <sub>50</sub> µg/ml						
	DPPH	·OH	H <sub>2</sub> O <sub>2</sub>	NO·	O <sub>2</sub> <sup>·-</sup>	Lipid peroxidation	α-glucosidase
CPH	425.53±0.33	374.11±0.94	100.18±1.32	175.25±0.95	580.88±0.35	178.77±0.28**	—
CPE	113.65±0.52**	241.50±2.65	75.79±1.63**	142.27±1.23	150.85±0.52	209.20±0.81	—
CPA	149.03±0.44	255.05±1.08	102.82±1.65	85.98±0.56**	142.92±0.38**	230.55±0.76	98.23±0.47**
CPW	161.32±0.48	182.36±1.13**	314.07±0.77	284.40±1.10	444.84±0.46	288.36±3.23	—
Ascorbic acid	6.86±0.92	62.40±3.72	—	19.90±2.30	32.86±3.78	48.72±3.20	—
Quercetin	14.34±1.64	—	245.30±4.60	21.09±2.16	—	—	—
Metformin	—	—	—	—	—	—	696.04±7.54

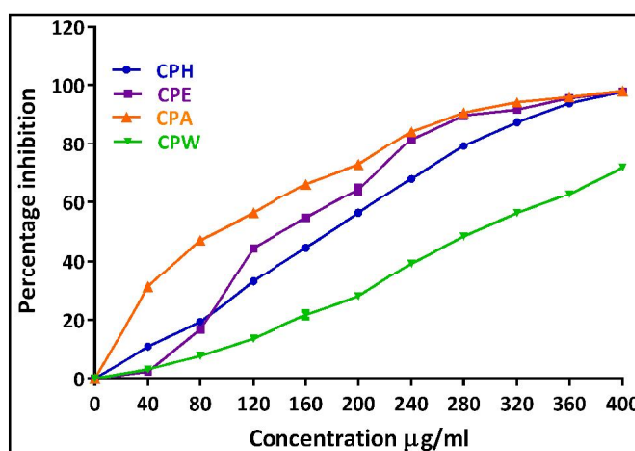
Values of IC<sub>50</sub> are expressed as mean of triplicate determinations ±standard deviation. Each value is expressed as a Mean ± standard deviation (n = 3). Averages followed by asterisk in each column differ by Tukey test at p<0.05.



**Figure 1:** DPPH scavenging activity of different extracts from *C. pareira* by different solvents at different concentrations. Each value represents a Mean ± SD (n = 3)

### 3.5 Nitric oxide radical scavenging activity

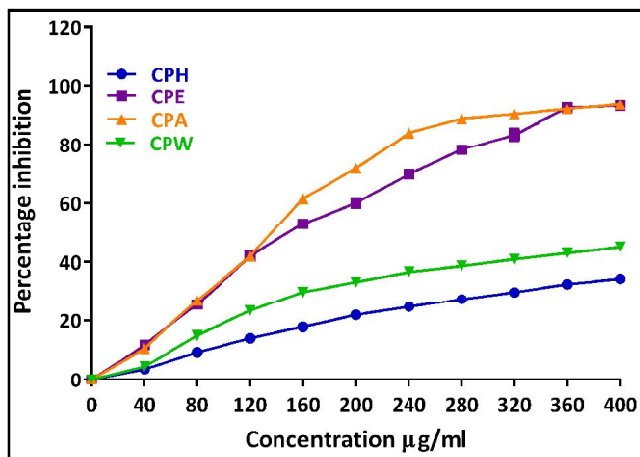
The generation of nitrite ions by the interaction of oxygen and NO produced due to sodium nitroprusside in an aqueous solution at physiological pH is the basic principle of nitric oxide radical scavenging activity (Sreejayan and Rao, 1997). Reduced production of nitric oxide from scavenging of nitric oxide which acts against oxygen can be monitored at 546 nm. As shown in Figure 2, the four extracts under study reduced the absorbance, and the concentration of extracts was directly proportional to the reduction. The results of this study clearly identified CPA (IC<sub>50</sub> = 85.98±0.56 µg/ml) as better NO scavengers as it had the highest efficiency to quench the NO radical. CPE and CPH had almost similar scavenging activities and possessed moderate effectiveness to scavenge the NO radical with an IC<sub>50</sub> value of 142.27±1.23 µg/ml and 175.25±0.95 µg/ml, respectively, while CPW (IC<sub>50</sub>=284.40±1.10 µg/ml) showed the lowest activity among the extracts. Direct NO scavenging activity of these extracts can be partially attributed to suppression of released NO as these extracts decreased the amount of nitrite generated from the decomposition of SNP *in vitro*. Hence, it appears that of *C. pareira* extracts contains active compounds which react with nitric oxide radical and are soluble in both solvents of extraction.



**Figure 2:** Nitric oxide scavenging activities of different extracts from *C. pareira* by different solvents at different concentrations. Each value represents a Mean ± SD (n = 3).

### 3.6 Superoxide scavenging activity

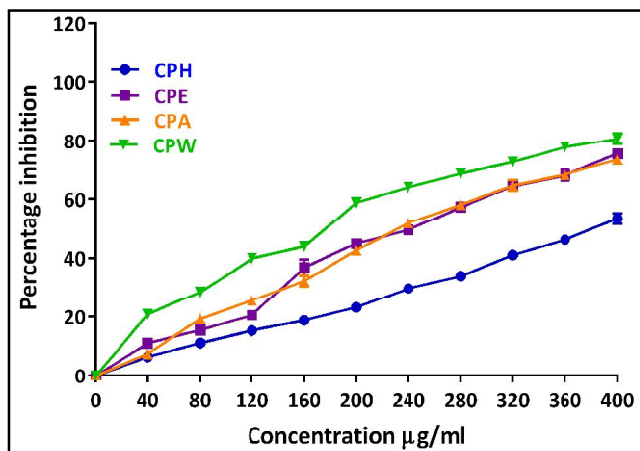
Superoxide (O<sub>2</sub><sup>·-</sup>) radical is recognized as far more destructive to cellular components as it triggers the chain of reactive oxygen species, thus, significantly contribute to the tissue damage and other ailments. This radical has a significant role to play in lipid peroxidation (Dahl and Richardson, 1978). Under the conditions of oxidative stress, not only a dramatic increase in the concentration of this species takes place but also its transformation into other reactive oxygen species creates a severe pathophysiological damage to the tissue (Gülçin *et al.*, 2007; Lee *et al.*, 2008). The superoxide radicals can be estimated by their capability to decrease NBT which are produced as a result of dissolved oxygen by PMS-NADH coupling. Figure 3 shows that *C. pareira* leaf extracts (CPE and CPA) effectively inhibited O<sub>2</sub><sup>·-</sup> dependent NBT reduction in this experiment in a concentration-dependent manner than other two extracts (CPH and CPW), thus, indicating their capacities to reduce superoxide radicals in the reaction. The values of superoxide radical scavenging abilities of CPH, CPE, CPA and CPW were 34.43±0.35, 93.27±0.55, 93.64±0.27 and 44.96±0.46%, respectively at the highest concentration of 400 µg/ml. IC<sub>50</sub> values of ascorbic acid, CPH, CPE, CPA and CPW are presented in Table 2. Such significant antioxidant activity may be due to the presence of natural antioxidants such as polyphenols in *C. pareira* leaves (Castro and Freeman, 2001).



**Figure 3:** Superoxide scavenging activity activities of different extracts from *C. pareira* by different solvents at different concentrations. Each value represents a Mean  $\pm$  SD (n = 3).

### 3.7 Hydroxyl radical scavenging activity assay

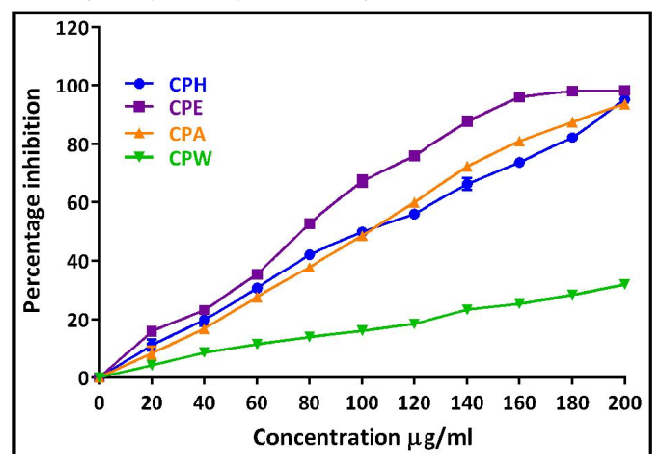
The hydroxyl radical is the extremely mercurial and has been implicated as highly damaging to adjacent biological molecules of the living cells (Frankel, 1999). Carcinogenesis, mutagenesis and cytotoxicity are caused by these radicals due to their direct effect on the DNA. Antioxidant capacity of an extract is directly relative to hydroxyl radical ( $\cdot$ OH) scavenging capacity. Thus, the hydroxyl radical-scavenging activity of crude extracts was evaluated by the 2-deoxyribose method. The *C. pareira* extracts revealed considerable quenching properties against  $\cdot$ OH, and the percentage inhibition was relative to the concentration of each extract (Figure 4, Table 2). The  $IC_{50}$  values were found in the range from  $182.36 \pm 1.13$  to  $374.11 \pm 0.94$   $\mu$ g/ml. CPW showed the highest activity against hydroxyl radicals, whose concentration of  $182.36 \pm 1.13$   $\mu$ g/ml were sufficient to inhibit 50% of hydroxyl radical-mediated deoxyribose degradation. Slightly less activity was shown by CPE and CPA ( $IC_{50} = 241.50 \pm 2.65$  and  $255.05 \pm 1.08$   $\mu$ g/ml, respectively). This activity might be attributed to the transition metal mainly iron chelating capacity of the phenolics and flavonoids present in these extracts as this could lead to obstruction for the generation of free radicals *via* Fenton reaction (Heim *et al.*, 2002; Arulmozhi *et al.*, 2010).



**Figure 4:** Hydroxyl radical scavenging activities of different extracts from *C. pareira* by different solvents at different concentrations. Each value represents a Mean  $\pm$  SD (n = 3).

### 3.8 Hydrogen peroxide scavenging assay

Hydrogen peroxide itself is not very reactive but it can diffuse through cell membrane directly and reacts with  $Fe^{2+}$  and  $Cu^{2+}$  ions to form  $\cdot$ OH radicals which initiated toxic effects (Aruoma, 1999). High concentration of hydrogen peroxide is lethal to the cells, and its build-up causes oxidation of cellular targets like DNA, lipids, proteins, *etc.*, eventually leading to cell death (Yen and Duh, 1994). Moreover,  $\cdot$ OH radicals perform the peroxidic reaction of lipids by abstracting hydrogen atoms from membrane lipids (Van Wijk *et al.*, 2008). Thus, elimination  $H_2O_2$  is prime criteria for the defence of living systems (Sinclair *et al.*, 1990). Figure 5 explains a substantial reduction in the concentration of  $H_2O_2$  due to the  $\cdot$ OH scavenging ability of the extracts. The extracts exhibited concentration-dependent hydrogen peroxide scavenging activities. The scavenging efficiency increased as follows:  $CPW < CPA < CPH < CPE$ . CPE was most efficient, with the lowest  $IC_{50}$  value,  $75.79 \pm 1.63$   $\mu$ g/ml (Table 2). Therefore, *C. pareira* extracts quench hydrogen peroxide which can be due to the existence of phenolic groups that exhibit proton donating ability, thereby, neutralizing it into water.

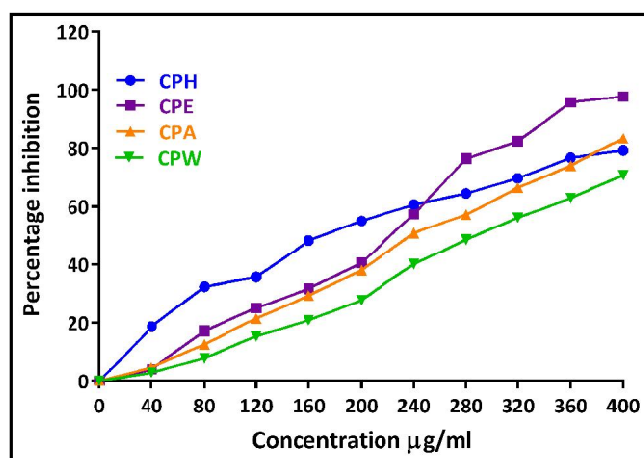


**Figure 5:** Hydrogen peroxide scavenging activities of different extracts from *C. pareira* by different solvents at different concentrations. Each value represents a Mean  $\pm$  SD (n = 3).

### 3.9 Determination of inhibition of lipid peroxidation

Lipid peroxidation, which chiefly involves the oxidation of polyunsaturated fatty acids, the principle component of biomembranes result in the generation of certain degradation products. Among these products, MDA, which is one of the TBARS is commonly used as an index for the study of lipid peroxidation and subsequently for the oxidative stress (Yagi, 1991; Janero, 1990; Wu and Ng, 2007). *C. pareira* extracts were observed to be less effective in comparison to standard ascorbic acid. However, significant differences were noted between the ability of different extracts to inhibit lipid peroxidation in liver tissues. The scavenging of lipid peroxides by the extracts is presented in Figure 6. CPH had the maximum inhibitory activity against lipid peroxidation with an  $IC_{50}$  value of  $178.77 \pm 0.28$   $\mu$ g/ml, while CPE, CPA and CPW were also found to be active with  $IC_{50}$  values of  $209.20 \pm 0.81$ ,  $230.55 \pm 0.76$ ,  $288.36 \pm 3.23$   $\mu$ g/ml, respectively (Table 2). The uniqueness of *C. pareira* extracts in inhibiting lipid peroxidation in liver tissues may be ascribed to a particular flavonoid class that may be dominant in these extracts as flavonoids are known to exist indifferent categories (Pietta, 2000). Since the flavonoids exhibit

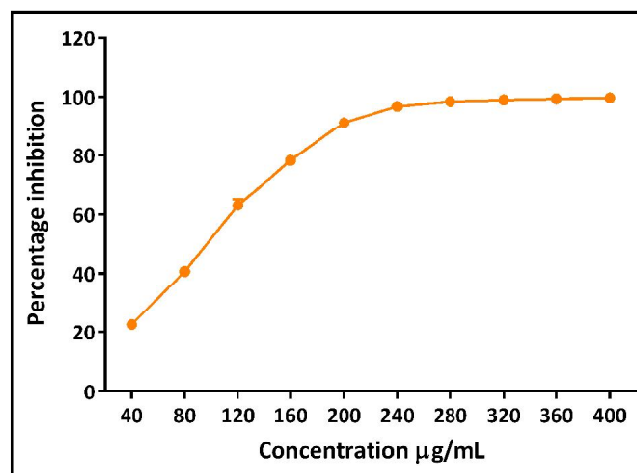
the metal chelating and free radical scavenging properties (Doss *et al.*, 2010), these plant extracts may, therefore, prove valuable in maintaining the membrane integrity of liver organ.



**Figure 6:** Lipid peroxidation activities of different extracts from *C. pareira* by different solvents at different concentrations. Each value represents a Mean  $\pm$  SD (n = 3).

### 3.10 Effects of extracts on $\alpha$ -glucosidase activity *in vitro*

The definitive role played by free radicals and the antioxidant defence system of the body in the pathology of diabetes has been extensively studied off late. The tissue damage resulting from the onslaught of oxidative stress plays a very critical role in the initiation as well as the progression of diabetes (Baynes, 1991). Drugs like a carbose that inhibit  $\alpha$ -glucosidase and amylase in the epithelium of the small intestine have been demonstrated to decrease postprandial hyperglycemia (Sima and Chakrabarti, 2004) and recover compromised glucose metabolism without stimulating insulin secretion in non-insulin-dependent diabetes mellitus (NIDMM) patients (Carrascosa *et al.*, 2001). These medications have proved as a bone which is ailing from type II diabetes. Thus, the hindrance offered to the carbohydrate absorption with a plant based  $\alpha$ -glucosidase inhibitors opens new avenues and mechanistic opportunities for the treatment of type II diabetes mellitus. Natural products are still one of the best and readily available sources of molecules which inhibit  $\alpha$ -glucosidase enzyme. In recent studies on medicinal plants, polymeric polyphenols were found to contribute to high  $\alpha$ -glucosidase inhibition (Subramanian *et al.*, 2008; Ma Cue *et al.*, 2004; Onal *et al.*, 2005). Therefore, we investigated the inhibition activity in different extracts of *C. pareira*. Our findings revealed that CPA extract of the herb efficiently inhibits a  $\alpha$ -glucosidase enzyme *in vitro*. It was observed that CPA achieved the  $\alpha$ -glucosidase inhibition, followed a concentration-dependent pattern. At a concentration of 200  $\mu$ g/ml, the percentage inhibition reached upto 91.35% and the  $IC_{50}$  value was found to be  $98.23 \pm 0.47$  (Figure 7). Although the activity was lesser in comparison to the standard drug, Metformin (100% at 10 $\mu$ g/ml), which may be due to the crude nature of the extract and this effect can be further enhanced by the purification. It is worth to mention that the other extracts of *C. pareira* did not show  $\alpha$ -glucosidase enzyme inhibitory activity in this study. The results of this study indicated the administration of CPA can probably manage the postprandial blood glucose levels and, thereby, confirmed the usage of this herb of its antidiabetic potential.



**Figure 7:**  $\alpha$ -glucosidase inhibitory activity of alcoholic extract of *C. pareira*. Each value represents a Mean  $\pm$  SD (n = 3).

## 4. Conclusion

The phytochemical study performed in our studies, revealed that *C. pareira* is a rich source of medicinally important phytochemical classes like phenolics and flavonoids. The pharmacological properties associated with *C. pareira* as well as the antioxidant activities demonstrated in the study may be attributed to these phytochemicals. The variation in free radical scavenging ability of the extracts with respect to the type of radicals can be explained by the difference in the mechanism of free radical quenching by different molecular entities. In addition to this, the alcoholic extract of *C. pareira* (CPA) was found to be very effective for inhibition of  $\alpha$ -glucosidase enzyme which is a prominent and key target for controlling type 2 diabetes. This study has provided an impetus for use of this herb as a nutritional supplement as well as a source of valuable phytochemicals in pharmaceutical and cosmetic industries. Further, direction of the study will include the validation of these biological activities *in vivo* as well as isolation and characterisation of bioactive compounds from this potent medicinal plant.

### Conflict of interest

We declare that we have no conflict of interest.

### Acknowledgment

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