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In Vitro Antioxidant activity of leaf extracts of *Cissampelos pareira* L var. hirsuta (Buch.-Ham.ex DC.) Forman.

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ABSTRACT

The present investigation was undertaken to appraise the antioxidant properties and the total phenolic and flavonoid content of hexane, ethyl acetate and ethanol leaf extracts of *Cissampelos pareira* L var. hirsuta (Family: Menispermaceae). The total phenolic content calculated as gallic acid equivalent (GAE) was found to be high in ethyl acetate extract (62.3mg GAE equivalent/g of extract) and total flavonoid content was found to be high in ethyl acetate extract (26.80mg QE equivalent/g of extract). Antioxidant potential was evaluated by determining the activity of DPPH assay and H_2O_2 radical scavenging activity. In DPPH scavenging assay, the IC_{50} value of ethyl acetate extract was found to be (65.81 µg/ml) which was comparable to the standard ascorbic acid (51.06 µg/ml). In H_2O_2 scavenging assay, IC_{50} value of ethyl acetate extract was found to be (46.19 µg/ml), which was comparable to the standard ascorbic acid (34.67 µg/ml). The significant linear correlation was confirmed between the values for the total phenolic and flavonoid content and antioxidant activity of plant extracts. The high contents of phenolic compounds indicated that these compounds contribute to the antioxidant activity.

Keywords: *Cissampelos pareira,* antioxidant activity, total phenol, total flavonoid

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INTRODUCTION

Plant derived substances have recently become great interest owing to their versatile application[1]. Natural products present in the medicinal plants have been used as a source of drugs in the traditional medicine and some of them have been scientifically explored [2]. Medicinal plants contain antioxidant compounds which protects the cell against the damaging effect of reactive oxygen species. Reactive Oxygen Species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide play a crucial role in the development of various ailments such as arthritis, asthma, dementia, mongolism, carcinoma and parkinson's disease. The free radicals in the human body are generated through aerobic respiration or from exogenous sources[3]. Many plants often contain substantial amounts of antioxidants including vitamin C and E, carotenoids, flavonoids and tannis etc., and thus can be utilized to scavenge the excess free radicals from human body. Phenolic compounds from medicinal plants possess strong antioxidant activity and may help to protect the cells against the oxidative damage caused by free-radicals[4]. They are the class of antioxidant agents, which can act as free radical terminators[5] and possess scavenging ability due to their hydroxyl group[6]. They are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers[7]. The interest on the role of natural antioxidants due to the toxicity of synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) at fairly high doses which limits their therapeutic usage[8,9].

Cissampelos pareira L var. hirsuta. is a significant flowering plant of family Menispermaceae. This species is known as abuta or laghupatha in ayurvedic medicine. . It is found in subtropical parts of India, Asia, East Africa and America**.** It is perennial climbing herb/shrubs, 2-5m high with a thickened root. Leaves have an orbicular shape 7-14cm in diameter. They are membranous or leathery, veined, glabrous, to densely pilose. The stem is woody, flexible, and slender up to 1cm and twines for support. The root system consists of flexible, light brown, lateral roots with sinkers and moderately abundant fine roots. Flowers are green, male ones in short umbels, 10-12cm long, females in pendulous spikes, 7-10cm long with a little round leaflet at the base of very flower Fruits are short hairy orange to red drupe, one seeded. Seeds are horseshoe-shaped; embryo elongate, narrow, embedded in endosperm, cotyledons flattened[10]**.** It possesses antibacterial, anti-inflammatory, antihistamine, antioxidant, antispasmodic, diuretic, hypotensive, muscle relaxant, uterine relaxant, antiseptic,aphrodisiac, analgesic, ant hemorrhagic, cardio tonic, diaphoretic, expectorant[11].

Hence, the present study was aimed at measuring the relative content of phenol and flavonoid and antioxidant potential of leaf extracts of *Cissampelos pareira* var.hirsuta.

MATERIALS AND METHODS

Collection of Plant material

Cissampelos pareira L var.hirsuta fresh plant parts were collected from Velimalai hills, Kanyakumari district, Tamil Nadu, South India, in the month of April-July2015. The plant was identified and authenticated by Dr. A.G.Pandurangan, Botanist and Director, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI) Palode, Thiruvananthapuram, where voucher specimen has been deposited for future reference bearing the collection number is 76876.

Preparation of plant extract (Hot percolation process):

1kg air dried leaf powder of *Cissampelos pareira* L was subjected to successive solvent extraction carried out in several batches using different solvents in increasing polarity index in a soxhlet apparatus by hot percolation technique. The solvents used were hexane, ethyl acetate and ethanol. The powered materials were evenly packed in a soxhlet extractor for about 36 hours with different solvents. The temperature was maintained (25°c - 100°c) on an electric heating mantle with thermostat control. The extract was collected and evaporated to dryness by using vacuum distillation unit. The final extract thus obtained was used for determining total phenol and flavonoid content and also in *vitro* antioxidant assay.

Determination of total phenol content:

Total phenolic content was estimated by Folin Ciocalteu's method. 1 ml eac of aliquots and standard gallic acid (10, 20, 40, 60, 80, 100 µg/ml) was taken in the test tubes and 5 ml of distilled water and 0.5 ml of Folin Ciocalteu's reagent was mixed and shaken. After 5 minutes, 1.5 ml of 20 % sodium carbonate was added and the volume was made up to 10 ml with distilled water and was allowed to incubated for 2 hours at room temperature which resulted in Intense blue color. After incubation, absorbance was measured at 750 nm spectrophotometer using UV- visible Jasco V- 630 instrument. The experiments were performed in triplicates. The standard calibration curve of gallic acid was plotted to get the R^2 value and linear equation of gallic acid. The data for total phenolic contents of the extract were expressed as mg of gallic acid equivalent weight (GAE)/ 100 g of dry mass[12,13].

Determination of total flavonoid content

Flavonoid content was measured by the aluminum chloride colorimetric assay. 1ml of extract and 1ml of standard solution of quercetin (20, 40, 60, 80, and 100 mg/l) was taken in the test tubes and 4 ml of distilled water and 0.3 ml 5% sodium nitrite solution was added. After 5 minutes, 0.3 ml 10% aluminium chloride was added followed by the addition of 2 ml 1 M NaOH was added and the total volume was made up to 10 ml with distilled water and mixed well which resulted in orange yellowish colour. The absorbance was measured at 510 nm with distilled water as blank. The experiments were performed in triplicates. The standard calibration curve of quercetin was plotted to get the R^2 value and linear equation of quercetin. The data for total phenolic contents of the extract were expressed as mg of quercetin equivalent weight (QE)/ 100 g of dry mass[14,16].

Assessment of antioxidant activity

The antioxidant potential of hexane, ethyl acetate and ethanol leaf extract of *Cissampelos pareira* L var.hirsuta was determined with reference to DPPH assay and Hydroxyl (H_2O_2) radical scavenging activity assay

1, 1 diphenyl 2, picryl hydrazyl (DPPH) free radical scavenging assay.

The scavenging activity of the extracts was estimated by using DPPH as free radical scavenging assay[17]. Solution of DPPH (0.1 mM) in methanol was prepared by dissolving 1.9 mg of DPPH in methanol and volume was made up to 100 ml with methanol. The solution was kept in darkness for 30 min to complete the reaction.1 ml of DPPH solution was added to 1 ml of different (20, 40, 60, 80 and 100 mg/ml) concentrations of extract and allowed to stand at room temperature for 30 min. The mixture was measured spectrophotometrically (UV-1800, UV-VIS spectrophotometer, Shimadzu) at 517 nm. The free radical scavenging activity was calculated as using following formula,

$$\% \text{ inhibition} = (A0 - A1) / A0 \times 100$$

Where A0 is the absorbance of the control and A1 is the absorbance of the sample of test sample. A standard of ascorbic acid was run using same concentrations as that of extract[17,18]. The antioxidant activity of the sample was expressed as IC_{50} value, was defined as concentration (in mg/ml) of sample that inhibits the formation of DPPH radicals by 50%[19].

Hydrogen peroxide (H_2O_2) radical scavenging activity assay

The ability of the extract sample to scavenge H_2O_2 was determined by Hydrogen peroxide (H_2O_2) radical scavenging activity assay[20]. Solution of 0.2 M potassium dihydrogen phosphate and 0.2 M sodium hydroxide solutions were prepared as per the Indian Pharmacopoeia 1996 standards. 50 ml potassium dihydrogen phosphate solution was placed in a 200 ml volumetric flask and 39.1 ml of 0.2Msodium hydroxide solution was added and finally volume was made up to 200 ml with distilled water to prepare phosphate buffer (pH-7.4). 50 ml of phosphate buffer solution was added to equal amount of hydrogen peroxide to generate the free radicals and solution was kept aside at room temperature for 5min to complete the reaction. Extracts (1 ml) in distilled water were added to 0.6 ml hydrogen peroxide solution and the absorbance was measured at 230 nm in a spectrophotometer (UV-1800, UV-VIS spectrophotometer,Shimadzu) against a blank solution containing phosphate buffer solution without hydrogen peroxide. Concentrations selected for extract were ranging from

20 to 100 mg/ml. The percentage of scavenging of H_2O_2 of extract was measured. The ability to scavenge the H_2O_2 radical was calculated using the following equation:

$$H_2O_2 \text{ scavenging activity (\%)} = (A0 - A1) / A0 \times 100$$

Where A0 is the absorbance of the control and A1 is the absorbance in the presence of extract sample. A standard of ascorbic acid was run using same concentrations as that of extract. The antioxidant activity of the sample was expressed as IC_{50} value, was defined as concentration (in mg/ml) of sample that inhibits the formation of H_2O_2 radicals by 50%.

Statistical Analysis

Results obtained in the present study were expressed as mean ± SD and were analyzed using One-way ANOVA at 5% significant level. Further a multiple comparison test was conducted to compare the significant differences amongst the parameters by using SPSS 16.0 (SPSS Inc., Chicago, USA). Pearson's correlation was used to determine the correlation of data between DPPH free radical-scavenging activity and H_2O_2 free radical-scavenging activity on total phenol and flavonoid content. P values <0.05 were considered to be statistically significant.

RESULT AND DISCUSSION

Determination of total phenolic content:

The total phenolic content in the examined plant leaf extracts using the Folin-Ciocalteu's method using gallic acid as standard. The reagent was formed from a mixture of phosphotungstic acid and phosphomolybdic acid which after oxidation of the phenols, was reduced to a mixture of blue oxides of tungsten and molybdenum. The blue coloration produced has a maximum absorption in the region of 750 nm and proportional to the total quantity of phenolic compounds originally present. The gallic acid solution of concentration (10-100 ppm) conformed to Beer's Law at 750 nm with a regression co-efficient (R^2) = 0.997.The plot has a slope (m) = 0.012 and intercept = 0.069. The equation of standard curve is y = 0.012x + 0.069 (Fig:). The values obtained for the concentration of total phenols are expressed as mg of GA/g of extract (Table: 1). The total phenolic contents in the examined extracts ranged from 36.19 ± 1.73 to 62.3±1.27 mg GA/g. The highest concentration of phenols was measured in ethyl acetate extract. Ethanol and hexane extracts contains considerably smaller concentration of phenols.

Determination of total flavonoid content:

The total flavonoid content in the examined plant leaf extracts was measured with the aluminium chloride colorimetric assay using quercetin as standard. Aluminium chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavonols. In addition it also forms liable complexes with ortho dihydroxide groups in A/B rings of flavonoids. The quercetin solution of concentration (10-100 ppm) conformed to Beer's Law at 510 nm with a regression co-efficient (R^2) = 0.997. The plot has a slope (m) = 0.014 and intercept = 0.038. The equation of standard curve is y = 0.014x + 0.038 (Fig.1a). The values obtained for the concentration of total flavonoids are expressed as mg of quercetin/g of extract (Table:1). The total flavonoid contents in the examined extracts ranged from 12.52 ± 1.09 to 26.80±1.09 mg quercetin/g. The highest concentration of flavonoids was measured in ethyl acetate extract followed by ethanol, and hexane extract contains considerably smaller concentration of flavonoid.

DPPH free radical scavenging assay

DPPH is a stable free radical which shows maximum ultraviolet and visible (UV-Vis) absorbance at 517 nm. It gets reduced in presence of antioxidant present in the sample which is considered as a measure of their antioxidant activity. The results clearly demonstrates the *in vitro* antioxidant activity of ethyl acetate, ethanol and hexane leaf extract of *Cissampelos pareira*. The result revealed good antioxidant activity and was comparable with that of standard ascorbic acid. The higher concentration of the ethyl acetate extract exhibit more antioxidant activity when comparing to lower concentrations. The data suggests that the extract contains compounds that are effectively utilized as a wide spectrum of antioxidant agent. The highest

scavenging activity was noticed in the ethyl acetate extract at 100 µg/ml (76.66 ± 0.67%). Low activity was noted in ethanol extract at 100 µg/ml concentration (42.16 ± 1.88%). While other concentrations showed moderate scavenging activity. The standard ascorbic acid showed maximum activity at 100 µg/ml concentration (87.97 ± 1.66%). The ability of samples to scavenge DPPH radical was measured on the basis of their concentrations providing 50% inhibition (IC_{50}). The results of one way ANOVA test and post hoc test indicates significant difference of mean percentage scavenging between different concentrations of tested extract. The IC_{50} values of ethyl acetate extract (65.81) and ascorbic acid (51.06) were obtained using the linear regression equation. The IC_{50} values of ethanol extract (122.44) and hexane extract (115.61) were obtained using the linear regression equation. The radical scavenging ability, IC_{50} value of extracts and ascorbic acid were presented in Table:2, Fig: 3.

Hydrogen peroxide (H_2O_2) radical scavenging activity assay

The extracts demonstrated hydrogen peroxide (H_2O_2) radical scavenging activity a in a dose dependent manner. The highest scavenging activity was noticed in the ethyl acetate extract at 100 µg/ml (82.11 ± 1.76%). Low activity was noted in hexane extract at 100 µg/ml concentration (43.26 ± 1.23%). While other concentrations showed moderate scavenging activity. The standard ascorbic acid showed maximum activity at 100 µg/ml concentration (94.37 ± 1.18%). Similar dose dependent scavenging was observed for standard, ascorbic acid. The IC_{50} values of ethyl acetate extract (46.19) and ascorbic acid (34.67) were obtained using the linear regression equation. The IC50 values of ethanol extract (92.54) and hexane extract (117.18) were obtained using the linear regression equation. The radical scavenging ability, IC_{50} of extracts and ascorbic acid were presented in Table: 3, Fig: 4.

Correlation of total phenol and flavonoid content and antioxidant assay

The total phenol contents are closely related with antioxidant activity. The results in table 4 shows that there are significant good linear positive correlation ($r=0.997$, $p<0.05$, $r^2=0.994$) between the total phenol content and H_2O_2 free radical scavenging assay. However, there was no significant correlation between total flavonoid content and antioxidant activity. This indicated that there are satisfactorily good relationship between phenolic content and antioxidant activity in *Cissampelos pareira var.*hirsuta leaf extract.

Table. 1. Total phenolic and flavonoid content in the leaf extracts of *Cissampelos pareira L*

SL.NO	Extract	mg of GA/g of extract	mg of Quercetin/g of extract
1	Hexane	36.19±1.73	12.52±1.09
2	Ethyl acetate	62.3±1.27	26.80±1.09
3	Ethanol	41.19±1.27	23.47±0.81

Table:2 % Inhibition data and IC_{50} of DPPH radical scavenging of leaf extracts *cissampelos pareira L* and ascorbic acid

SL.No	Concentration µg/ml	% of inhibition			Standard Ascorbic acid
		Ethyl acetate Extract	Ethanol extract	Hexane Extract	
1	20	15.26±0.87[a]	16.25±0.77[a]	13.46±0.83[a]	24.59±1.54
2	40	23.74±1.45[b]	21.00±1.95[b]	18.03±1.52[b]	39.90±2.04
3	60	50.88±2.08[c]	28.29±1.22[c]	32.11±1.71[c]	61.08±1.65
4	80	60.45±2.18[d]	36.79±1.63[d]	36.83±1.51[d]	71.99±1.52
5	100	76.66±0.67[e]	42.16±1.88[e]	42.58±1.43[e]	87.97±1.66
IC_{50} value		65.81	122.44	115.61	51.06

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Table:3 % Inhibition data and IC_{50} of H_2O_2 radical scavenging of leaf extracts *cissampelos pareira L* and ascorbic acid

SL.No	Concentration µg/ml	% of inhibition			Standard Ascorbic acid
		Ethyl acetate Extract	Ethanol extract	Hexane Extract	
1	20	33.15±1.61 [a]	21.01±1.36 [a]	16.11±1.65 [a]	33.29±1.84 [a]
2	40	47.09±1.64 [b]	27.31±1.09 [b]	27.99±1.68 [b]	57.35±1.11 [b]
3	60	59.28±0.92 [c]	32.08±1.57 [c]	32.09±1.57 [c]	76.19±1.08 [c]
4	80	70.23±1.25 [d]	47.40±1.62 [d]	38.38±1.21 [d]	81.41±0.71 [d]
5	100	82.11±1.76 [e]	53.44±1.32 [e]	43.26±1.23 [e]	94.37±1.18 [e]
IC_{50} value		46.19	92.54	117.18	34.67

Table 4. The correlation between antioxidant capacity of leaf extracts of *cissampelos pareira* L var. hirsuta and total phenolics and flavonoids.

		TPC	TFC	DPPH	H_2O_2
TPC	Pearson Correlation	1	.801	.982	.997*
	Sig. (2-tailed)		.408	.122	.047
	N	3	3	3	3
TFC	Pearson Correlation	.801	1	.673	.844
	Sig. (2-tailed)	.408		.530	.361
	N	3	3	3	3
DPPH	Pearson Correlation	.982	.673	1	.965
	Sig. (2-tailed)	.122	.530		.169
	N	3	3	3	3
H_2O_2	Pearson Correlation	.997*	.844	.965	1
	Sig. (2-tailed)	.047	.361	.169	
	N	3	3	3	3

*. Correlation is significant at the 0.05 level (2-tailed).

key:

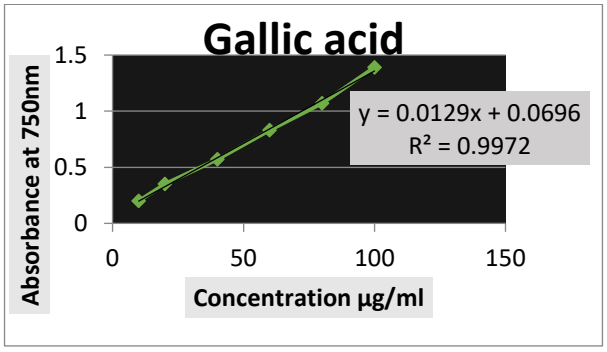
TPC - total Phenol Content

TFC – Total flavonoid content

DPPH - DPPH radical Scavenging Activity

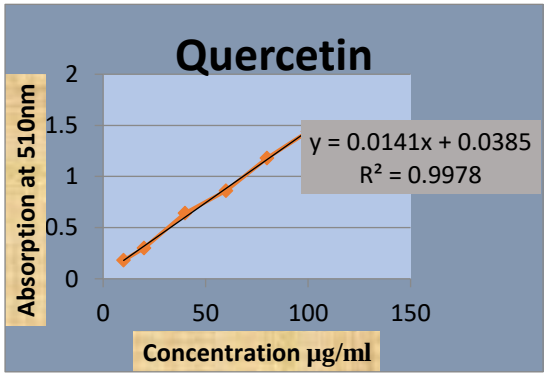
H_2O_2 - Hydrogen peroxide scavenging activity

Fig 1: Total phenolic content for gallic acid



R^2 values represented mean data set of n=3

Fig. 2: Total flavonoid content for quercetin.



R^2 values represented mean data set of n=3

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Fig 3: DPPH radical scavenging of leaf extracts of *cissampelos pareira* and ascorbic acid

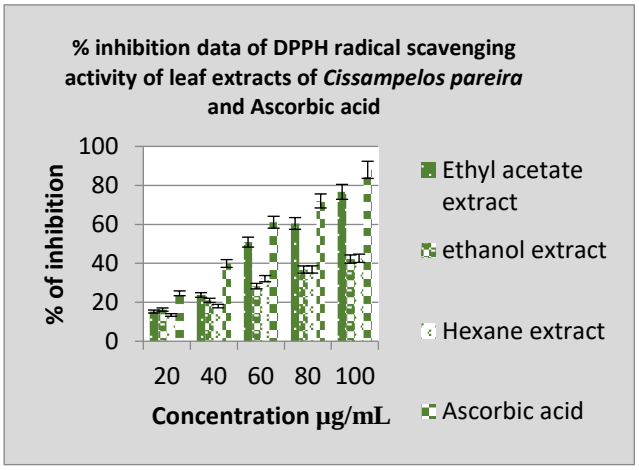
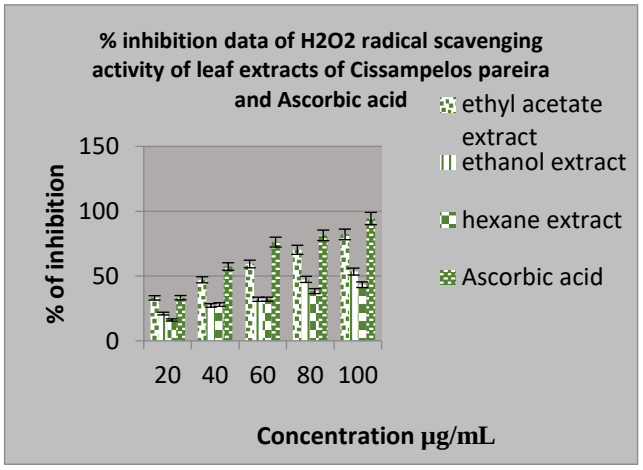


Fig:4. H_2O_2 radical scavenging of leaf extracts *cissampelos pareira* and ascorbic acid



DISCUSSION

There is an increasing evidence that different plant extracts was done according to indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress. The antioxidant activity of the leaf extracts of *Cissampelos pareira* L var. hirsuta was assessed by DPPH scavenging and H_2O_2 scavenging assay. In the present investigation, the highest DPPH scavenging activity was noticed in the ethyl acetate extract at 100 µg/ml (76.66 ± 0.67%). The DPPH radical scavenging assay is a decolonization assay that will measure the capacity of antioxidants to directly scavenge DPPH radicals by measuring its absorbance with spectrophotometer at 517 nm. In the presence of R-H (hydrogen/electron donating compound) or antioxidant, DPPH radical will be reduced to its nonradical form giving rise to the color ranging from yellow to colorless. Its absorption intensity also decreased according to the number of electron captured[21]. In the case of H^2O^2 scavenging activity, the highest activity was noticed in the ethyl acetate extract at 100 µg/ml (82.11 ± 1.76%). H_2O_2 is a weak oxidising agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly. Once inside the cell, H_2O_2 can probably react with Fe2+, and /or Cu2+ ions to form hydroxyl radical and this might be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of H_2O_2 getting accumulated. Scavenging of H^2O^2 by the plant extract could be attributed to its phenolics which donate electron to H^2O^2, thus reducing it to water. The extract was capable of scavenging H^2O^2 in a concentration dependent manner. In agreement with other researchers, Sangh Pratap *et al*[22], reported free radical scavenging potential of Methanolic extract of *Leptodenia pyrotechnica* exhibited antioxidant potential against hydrogen peroxide with IC_{50} value of 65.48µG/mL with respect to IC_{50} value of standard ascorbic acid 60.98µG/mL and against DPPH with IC 50 value of 154.69µG/mL with respect to IC_{50} value of standard ascorbic acid 121.69µG/mL represents the antioxidant potential of standard and extract.

The antioxidant activity of the extracts might be due to the presence of phenolic compounds such as phenol, flavonoids, tannins etc. In this study, the total phenolic content (TPC) was determined using the Folin-Ciocalteau method, using gallic acid as standard. The highest concentration of phenols was measured in ethyl acetate extract (62.3±1.27mg GAE/g extract**).** Plant phenols constitute the major group of compounds that act as primary antioxidant[23]. They can react with active oxygen radicals, such as hydroxyl radicals[24], superoxide anion radicals[25] and lipid peroxyl radicals and inhibit the lipid peroxidation at an early stage. This is because of their scavenging ability due to their hydroxyl groups. The total flavonoid content was measured with the aluminium chloride colorimetric assay using quercetin as standard. The highest concentration of flavonoids was measured in ethyl acetate extract (**26.80±1.09**27mg QE/g extract**).** The current investigation also suggested that content of flavonoids in the plant extracts was highly related to their antioxidant capacity. It has been confirmed that pharmacological effects of flavonoids is correlating with their antioxidant activities. The result indicates strong association between antioxidant activities and phytochemicals (phenolic and

flavonoid) suggesting that phenolic and flavonoid compounds are probably responsible for the antioxidative activities.

In consistent with the present study, Heida Nadia Zulkefli *et al*[26] , reported the antioxidant activity of stem extract of *Tinospora crispa* showed high antioxidant activity in the following order: DPPH radical scavenging, reducing power and metal chelating assay (98.8%, 0.957, 81.97%) than *Tabernaemontana corymbosa* leaves (90.04%, 0.652, 69.64%), stem (82.78%, 0.819, 36.70%) and root extracts (63.25%, 0.469, 51.56%), respectively. The high antioxidant activity in the stem extract of *Tinospora crispa* is due to the presence of apigenin and magnoflorine. The high antioxidant activity in *Tabernaemontana corymbosa* extract is due to its high phenol contents. There were significant linear positive correlation ($r=0.788$, $p<0.001$, $r2=0.621$) between the total phenolic content and DPPH free radical scavenging assay in the crude extracts of *Tinospora crispa* and *Tabernaemontana corymbosa*. Meanwhile, a significant moderate positive correlation was observed between the total phenolic content and ferric reducing power assay ($r= 0.556$, $p<0.05$, $r2= 0.309$). However, there was no significant difference in the correlation coefficient of total phenolic content and metal chelating assay.

CONCLUSION

In conclusion, the present investigation provides the evidence that the hexane, ethyl acetate and ethanol extract of *Cissampelos pareira*, which contains flavonoid and phenolic contents, shows potential antioxidant and free radical scavenging activity. These in vitro assays demonstrate that the plant extracts is an important source of natural antioxidant which might be preventive against oxidative stress.

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