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In Vitro Antioxidant and Anticancer potential of Bark of *Costus pictus* D.DON

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ABSTRACT

Objective: To evaluate the antioxidant and anticancer potential of different fractions of bark of *Costus pictus* using various in vitro antioxidant assay systems. **Methods:** In this study, assay like DPPH radical, superoxide anion radical scavenging activity, nitric oxide scavenging activity, hydrogen peroxide scavenging activity, metal chelating activity and reducing power were used. The concentrations of total phenolic and flavonoids were also calculated for the extracts. **Result:** The present study elucidated for the first time the antioxidant property of bark of *C. pictus*. This study suggested that, among the three fractions, the chloroform fraction possesses high antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress related disorders. Moreover, all fractions possess potent anticancer properties against colon cancer cells of HT29 and lung carcinoma cells of A549. **Conclusions:** It can be concluded that the extract of the bark of *C. pictus* has potential natural antioxidant and this can be used in food industries. There are few reports on the antioxidant capacity of bark of *C. pictus* and the mechanism of different fractions of bark of *C. pictus* as antioxidative agents is still not fully understood. Hence further research is underway to analyse and isolate the active compounds responsible for the antioxidant and anticancer activity of different fractions of the bark of *C. pictus*.

1. Introduction

In a developing country like India, where disease management is a big issue the knowledge of plant derived antioxidants could reduce the cost of health care. Antioxidants are used to protect human beings from the ill-effects of oxidative stress that is exerted by enhanced production of reactive oxygen species (ROS) as a result of exposure to pollutants. The body has several mechanisms to counteract oxidative stress by producing antioxidants either naturally generated in situ (endogenous antioxidants) or externally supplied through the foods (exogenous antioxidants) [1].

Free radicals or reactive oxygen species (ROS) such as singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynitrite can damage the body by cellular or oxidative stress. This leads to the development of diseases like diabetes, cirrhosis, cancer and cardiovascular [2]. Free radicals generated in the body can be removed by its own natural antioxidant defense systems that include superoxide dismutase, glutathione peroxidase, catalase etc. However, endogenous antioxidant defenses are not completely efficient. Therefore, dietary antioxidants are required to lessen the overall effect of antioxidant stress due to excessive free radicals occurring in our system [3].

Significant antioxidant properties have been recorded with phytochemicals that are necessary for the curing of diseases like cancer [4]. The efficacy of plant extract as an antioxidant is long been well established and many more plants or plant extracts are under way. Plants contain a rich source of free radical scavenging molecules such as phenols, flavonoids, vitamins, terpenoids that hold promising antioxidant properties [5]. Many dietary polyphenolic constituents derived from the plants or plant extracts exhibited comparatively high antioxidant properties than the standard antioxidants, vitamins E or C by in vitro. These results clearly showed that plant-derived compounds may have significant protective effects in vivo [6]. Interestingly, the natural antioxidant compounds tend to be safer and have less or no side effects. In addition, they

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also possess antiviral, anti-inflammatory, anti-cancer, and hepatoprotective properties [7]. Therefore, the evaluation of antioxidant activity of various plant extracts is considered as an important step in the identification of their ability to scavenge the free radicals.

Antioxidants play an important role in the alleviation of diabetes due to oxidative stress [8]. Hence, an ideal antidiabetic plant should have good antioxidant properties. Although the antidiabetic properties of *C. pictus* are well known, this plant is not extensively explored for its antioxidant activity. Moreover, all the investigations reported so far have mainly focused on the rhizome of this plant and the bark remains unexplored. In some plants, for instance, Cinchona, it is reported that the bark has high therapeutic value than other parts. Based on the literature survey and available data, the bark of *C. pictus* was selected in this present study to evaluate the antioxidant activity and anticancer property of different fractions of bark of *C. pictus*.

2. Materials and Methods

2.1. Extraction of antioxidant from C. pictus

Coctus pictus Don was collected in and around the Botanical garden, University of Madras, Chennai, Tamil Nadu during the months of December 2011 and January 2012. Fresh stem was collected, bark was peeled out and shade dried for about a week. After that the dried bark was pulverized into powder. A 50 g shade dried powder of bark was then soaked in methanol (1:4, w/v) and kept for 10 days under room temperature with intermittent shaking. The extracts were filtered through handmade filter paper, concentrated using rotary evaporator at 40 ℃ and the yield was 3.2 g. This concentrated crude extract was further dissolved in 70% methanol and partition with Chloroform using separating funnel (three times). Each partitioned extract was filtered with Whatman No.1 filter paper and concentrated using rotary evaporator at 40 ℃ and the yield was calculated. 70% methanol fraction was again concentrated using rotary evaporator at 40 ℃ and this concentrated fraction was soluble in 100% methanol. The soluble and insoluble fractions were collected as well as its yield was also calculated. The antioxidant potential of this fraction was further analyzed.

2.2. Partition of methanol extract

Concentrated crude extract was further dissolved in water and partition with Chloroform for three times. Each partitioned extract was filtered with Whatman No.1 filter paper and concentrated using rotary evaporator at 40 ℃ and yield was calculated. The water soluble fraction was lyophilized and dried powder was dissolved in methanol. Certrifugation was carried out to separate methanol soluble fraction from the methanol insoluble fraction. The chloroform fraction of crude extract showed highest antioxidant and anticancer activity in vitro. Hence, this crude extract was chosen to analyze the metabolite profile using TLC and bioautogram. The antioxidants were identified by GC-MS.

2.3. Estimation of total phenol

The total phenolic and flavonoid contents of each active fraction were measured according to the methods described by Kumaran and Karunakaran (2006) [9]. The total phenolic content of the plant extracts were determined using FCR. This method depends on the reduction of FCR by phenols to a mixture of blue oxides which have a maximal absorption in the region of 750 nm. About 100 μL of plant extracts (100 μg/mL) and also 100 μL of gallic acid (100 μg/mL) were mixed with 500 μL of the FCR and 1.5mL of 20% sodium carbonate. The mixture was shaken thoroughly and made up to 10mL using distilled water. The mixture was allowed to stand for 2 h. Then the absorbance at 765 nm was determined against a blank which contain all reagents without the samples or the gallic acid at the same conditions. All determinations were carried out in duplicates. The total phenolic content is expressed as the number of equivalents of gallic acid (GAE).

2.4. Estimation of total flavonoids

The flavonoid content was determined by aluminium chloride method using quercetin as a reference compound. This method was carried out based on the formation of a complex flavonoid-aluminum having the absorption maximum at 415 nm. About 100 μL of plant extracts in methanol (10mg/mL) was mixed with 100 μL of 20% aluminium trichloride in methanol and a drop of acetic acid, and then diluted with methanol to 5 mL. The absorption at 415 nm was read after 40 min. Blank samples were prepared from 100 μL of plant extracts and a drop of acetic acid, and then diluted to 5mL with methanol.

The absorption of standard quercetin solution (0.5mg/mL) in methanol was measured under the same conditions. All determinations were carried out in duplicates. The amount of flavonoids in plant extracts in quercetin equivalents was calculated by the following formula:

$X = (A - mo) / (Ao - m)$

where X is the flavonoid content, mg/mg plant extract in quercetin, A is the absorption of plant extract solution, Ao is the absorption of standard quercetin solution, m is the weight of plant extract, mg and mo is the weight of quercetin in the solution, mg.

2.5. Thin layer and paper chromatography

Phytochemical screening for the presence of secondary metabolites was performed using TLC analyses (pre-coated aluminium silica gel plates, GF254, Merck) with different eluting systems. The optimized solvent system is hexane: ethyl acetate: methanol (3:2:1). The separation metabolites visualized in short, long UV light and visible region.

2.6. TLC bioautography analysis

Chloroform fraction was tested for their antioxidant

activities against DPPH$^{\bullet}$ using a TLC bioautography method by Zhang et al., 2009[10]. Briefly, an aliquot of all fractions (4 mg/mL, 8 μL) were directly deposited (as bands) onto a TLC plate. The TLC plate was then developed into a pre-saturated solvent tank with optimised solvent system as developing reagent. The developing distance from application position was 6 cm. The developed TLC plate was removed from the solvents, and allowed to air-dry for 30 min, followed by dipped with a 2.54 mM DPPH$^{\bullet}$ methanolic solution for derivatization. Bands with the DPPH$^{\bullet}$ scavenging activity were observed as white yellow bands on a purple background.

2.6. Antioxidant studies on plant extract

All the collected fractions were analyzed for its antioxidant ability by the following methods such as free radical scavenging, nitric oxide scavenging, superoxide scavenging, metal chelating, hydrogen peroxides scavenging and total reducing power.

2.6.1. Free radical scavenging activity

The scavenging effects of different fractions were determined by the method of Yan and Chen (1995)[11]. Briefly, 2.0 mL of 0.16 mM DPPH solution (in methanol) was added to the test tube containing different fractions in different concentrations such as 50, 100, 150, 200 and 250 μg/mL. The mixture was vortexed for 1 min. and kept at room temperature for 30 min. in the dark. The absorbance of all the sample solutions was measured at 517 nm with ascorbic acid as the standard. The scavenging effect (%) was calculated by using the following formula,

Scavenging activity (%) = (1− Asample/ Acontrol) x 100

2.6.2. Nitric oxide radical-scavenging assay

The nitric oxide (NO) radical-scavenging activity of different fractions was assayed by the method of Sumanont et al., 2001[12], with slight modification. The nitric oxide radical was generated by the sodium nitroprusside (SNP) and assayed by the Griess reagent. A total of 950 μL of sample solution in distilled water at different concentrations was mixed with 50 μL of 100 mM SNP in 100 mM phosphate buffer (pH 7.4). Distilled water was used as the control and ascorbic acid was used for the comparison. The mixture was incubated at 20 ℃ for 2.5 h. The reaction mixture was diluted with 2 mL of Griess reagent [1 mL of 1.2 M sulfanilamide and 1 mL of 80 mM N-(1-naphthyl)-ethylenediamine dihydrochloride (NED)] at room temperature for 10 min. The absorbance was measured at 540 nm by a spectrophotometer. The NO radical-scavenging activity (%) was calculated as follows:

Scavenging activity (%) = $(1- A_{sample}/A_{control}) \times 100$

2.6.3. Ferrous metal-ion-chelating ability

The chelating of ferrous ions by different fractions was assayed by the method of Dinis et al., 1994[13], with slight modification. A total of 1 mL of sample solution in distilled water at different concentrations was mixed with 0.1 mL of FeCl2 (0.2 mM) for 30 s, and then the mixture was reacted with 0.2 mL of ferrozine (5 mM) for 10 min. Distilled water was used as the control, and EDTA was used for comparison. The absorbance of the solution was measured at 562 nm by a spectrophotometer (Hitachi). The ferrous ion-chelating ability (%) was calculated as follows:

Chelating ability (%) = (1− Asample/ Acontrol) × 100

2.6.4. Hydrogen peroxide scavenging assay

The hydrogen peroxide (H_2O_2) scavenging activity of different fractions was assayed by the method of Yen and Chung (1999) [14] with slight modification. A total of 1mL of sample solution in distilled water at different concentrations was mixed with 400 μL of H_2O_2 solution (5 mM) and incubated for 20 min. at room temperature. Distilled water was used as the control, and ascorbic acid was used for comparison. The mixture was supplemented with 600 μL of HRPase-phenol red solution (300 μg/mL HRPase and 4.5 mM phenol red in 100 mM phosphate buffer). After 10 min., the absorbance was measured at 610 nm by a spectrophotometer. The hydrogen peroxide scavenging activity (%) was calculated as follows:

Scavenging activity (%) = $(1- A_{sample}/A_{control})$ x 100

2.6.5. Reducing power

Reducing power of different fractions was determined by the method prescribed by Oyaizu (1986)[15]. Briefly, 1.0 mL of methanol containing different concentrations of sample was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1%). Reaction mixture was incubated at 50 ℃ for 20 min. After incubation, 2.5 mL of Trichloroacetic acid (10%) was added and centrifuged (650 g) for 10 min. From the upper layer, 2.5 mL solution was mixed with 2.5 mL distilled water and 0.5 mL FeCl3 (0.1%). Absorbance of all the sample solutions was measured at 700 nm using a DU 40 Spectrophotometer (Beckman, USA). Increased absorbance indicated an increased reducing power.

2.7. Anticancer studies on plant extract

2.7.1. Cell culture maintenance

Cell lines used in this study such as HT29 (colon cancer) and A549 (lung carcinoma) cell lines were purchased from the National Centre for Cell Sciences (NCCS), Pune, India. The above cell lines were maintained in Dulbecco's Modified Eagles medium supplemented with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L Sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM of Sodium pyruvate. Penicillin and Streptomycin (100 U/100 ℃g) was adjusted to 1 mL/litre. Cells were maintained at 370C with 5% CO_2 atmosphere.

2.7.2. Cell viability

Cell viability was assessed by trypan blue dye exclusion

test as reported by Chakraborty et al. (2004) [16]. The number of stained and unstained cells was counted using a haemocytometer (Improved Neubauer Brightline, USA).

2.7.3. Cytotoxicity of different fractions of Costus pictus

Both the cell lines (HT29 and A549) were selected for cytotoxicity. The cells with logarithmic growth phase were seeded in 96 well micro plates (Nunc,USA) and allowed it for adhere for about 48 h. Approximately 1x 104 cells were used for cell cytotoxicity of different fractions like . After 48 h, the confluence of monolayer was selected for the treatment of different fractions of *C. pictus*. A series of different dilutions like 50 μg to 250 μg concentrations were prepared in phosphate buffered saline. A stock concentration like 1 mg /mL was also prepared to make the working solutions. From this concentration, the working dilutions were prepared.

2.7.4. Evaluation of IC50 and MTT

The Inhibitory Concentration (IC50) value was evaluated through MTT [3-(4, 5Dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium Bromide] assay. The fractions treated cells were selected; the dead cells were pipetted out from all the fractions treated wells carefully. After removing the treated dead cells, all the wells were treated with 5 mg/mL stock MTT solution and incubated at 37 ℃ for 4-6 h. After incubation, formations of the purple colour formazone crystals at the bottom of the wells were observed. The crystals were, then, dissolved with 500 μL of concentrated DMSO (Sigma, USA). The resultant purple color was read at 620 nm in ELISA multiwell microtiter plate reader (Thermo Multiskan EX, USA). OD value was subjected to sort out percentage of viability by using the following formula Thangam et.al., 2012 [17].

$$\text{Percentage of viability} = \frac{A_{620}\ (\text{different fractions of } C.\ pictus)}{A_{620}\ (\text{untreated})} \times 100$$

2.8. Statistical Analysis of Data

The results were expressed using One-way Analysis of Variance ANOVA) and Turkey's Multiple Comparison Test was done to evaluate the significance of difference of means of various treatment groups, using SPSS statistical package (version: 17). The values are presented as Mean± S.D.

3. Results

3.1. Extraction and Yield

Natural antioxidants present in the plants are closely related to their medicinal and pharmaceutical properties. Thus antioxidant capacity is a widely used parameter for assessing the bioavailability of food stuffs as medicinal plants. The antioxidant properties of plant extracts should be evaluated in a variety of model systems using several indices to ensure the effectiveness of such antioxidant materials. In this study, antioxidant activity methods and extraction systems of different polarities, i.e., chloroform, methanol and water were compared. To our knowledge, this is the first record on the antioxidant potential of bark of *C. pistus*. Solvent extraction is the most commonly used method in sample preparations from plants. The total yield values of methanolic extracts of bark of *C. pictus* under analysis are given in Fig. 1. Methanolic extraction further fractionated into methanol soluble and methanol insoluble fraction. Yield, total phenol and total flavonoids of all fractions are given Fig. 1. Chloroform fraction contains high amount of phenols (0.240 mg/g) and Methanol insoluble fraction contains high amount of flavonoids (0.188 mg/g).

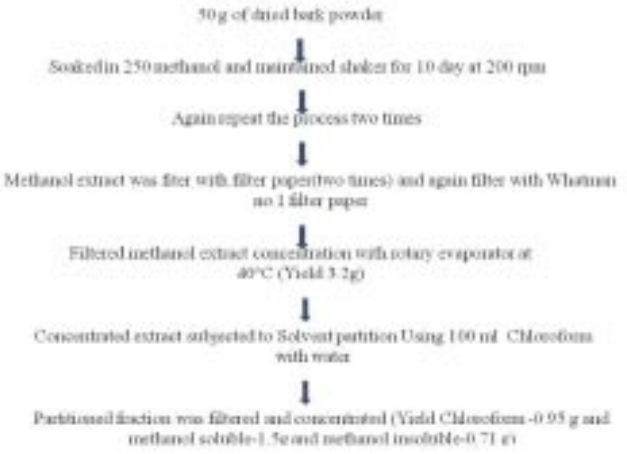


Fig. 1. Schematic representation extraction and solvent partition of bark of *C. pictus*

There are several methods for the determination of antioxidant activities. The chemical complexity of extracts, often a mixture of dozens of compounds with different functional groups, polarity and chemical behaviour, could lead to scattered results, depending on the test employed. Therefore, an approach with multiple assays for evaluating the antioxidant potential of extracts would be more informative and even necessary. In this study, mainly six methods using DPPH radical, superoxide anion radical scavenging activity, nitric oxide scavenging activity, hydrogen peroxide scavenging activity, metal chelating activity and reducing power were used. The concentrations of total phenolic and flavonoids were also calculated for the extracts (Table 1).

In an attempt to establish a potential relationship with different activities, we have determined the amount of phenolic compounds in various extracts tested. We can easily conclude that bark of *C. pistus* is rich in phenol and flavonoids, whereas chloroform fraction is rich in total phenol and poor in flavonoid and methanol soluble fraction was poor in total phenol and rich in flavonoid but methanol insoluble fraction was poor in flavonoid and phenol.

3.2. TLC bioautography and GC-MS analysis

Secondary metabolites profile was initially identified by TLC of *C. pistus* of chloroform fraction and result was shown in Fig.2. Further antioxidant was identified by TLC bioautography against DPPH• *C. pistus* of chloroform fraction and result was shown in Fig.3. Moreover, in order to obtain these active components from this plant profile of chemical compositions was analysed in GC-MS and profile shown in Fig.4. In the analysis it was observed that phenolic compounds were the major compounds of intense in the chloroform fraction.

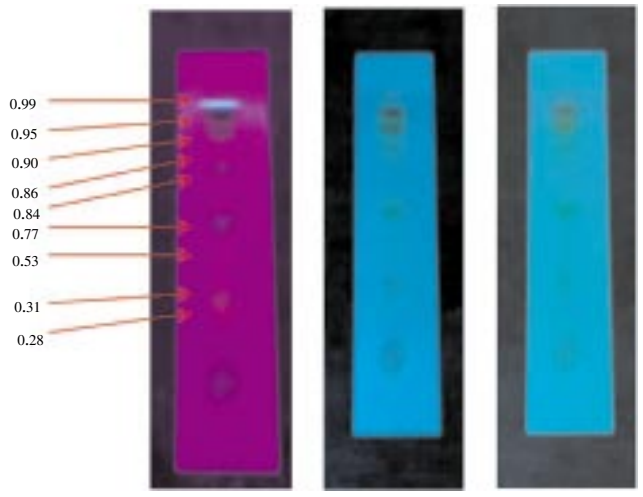


Fig. 2. TLC profile of Chloroform fraction of bark of *C. pictus* (a) UV short (b) UV Long (c) Visible light

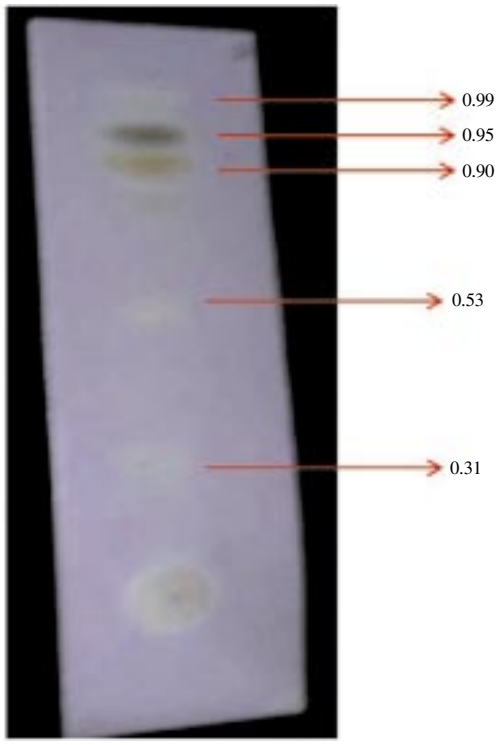
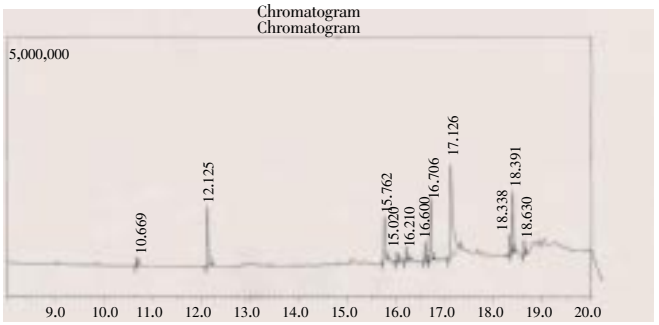


Fig. 3. TLC plate stained with 2.54 mM DPPH• solution in methanol, and visualized under visible light. Twenty microliters of the chlorofrom fraction (4 mg/mL) of bark of *C. pictus*



Peak Report TIC

PEAK	R.TIME	AREA	AREA%	NAME
1	10.669	244007	1.33	3-Methyl-2 proplonyl-benzolc acld
2	12.125	2303402	12.59	Phenol, 2,4-bis(1,1-dimethylethyl)-
3	15.762	1631513	8.92	2,6,10-TRIMETHYL,14-ETHYLENE-14-PENTADECNE
4	16.020	376898	2.06	2-HEXADECEN-1-OL,3,7,11,15-TETRAMETHYL-,[R-[R*,R&(E)]]-
5	16.210	568926	3.11	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
6	16.600	600164	3.28	7,9-DI-tert-butyl-1-oxaspllro(4,5)deca-6,9-diene-2,8-dione
7	16.706	235929	12.90	Methyl patmitate
8	17.126	6925115	37.86	n-Hexadecanolc acld
9	18.338	571157	3.12	Methyl linoleate
10	18.393	2174886	11.89	Methyl cis-7-octadecendate
11	18.630	537828	2.94	ctadecanoic acid, methyl ester
		18293725	100.00	

Fig. 4. GC-MS profile of Chloroform fraction of bark of *C. pictus*

3.3. DPPH radical scavenging activity

The DPPH radical scavenging activities of the bark samples of different fractions of *C. pistus* increased with increasing concentration (Fig. 5). The DPPH radical scavenging activities of different fractions of bark of *C. pistus* and ascorbic acid were in increasing order MIS >MS > Chloroform fraction > ascorbic acid.

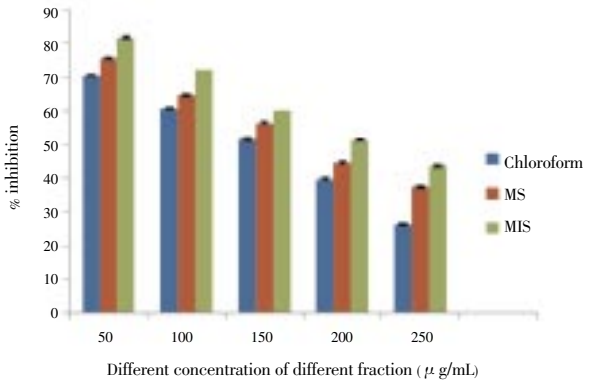


Fig. 5. Scavenging activity of different fractions of bark of *C. pictus* on DPPH radicals. Values are means± SD of three determinations.

3.4. Nitric oxide anion scavenging activity

The Nitric oxide scavenging activities of the bark samples of different fractions of *C. pistus* increased with increasing concentration (Fig. 6). The DPPH radical scavenging activities of different fractions of bark of *C. pistus* and ascorbic acid were in increasing order MIS >MS > Chloroform fraction > ascorbic acid.

Table 1

IC_{50} value of different fraction of bark of *C.pictus*

Antioxidant assay	Different fraction of bark of C.pictus (IC 50 value at ~μg/ml)			Ascorbic acid (IC 50 value at μg/ml)
	Chloroform fraction	MS fraction	MIS fraction	
DPPH free radical scavenging assay	200	200	250	40
NO scavenging assay	100	200	200	22
O• scavenging assay	100	150	200	36
H2O2 scavenging assay	200	300	450	61
Fe2+ scavenging assay	400	500	500	83
HT29 cells	200	200	250	–
A549 cells	125	150	200	–

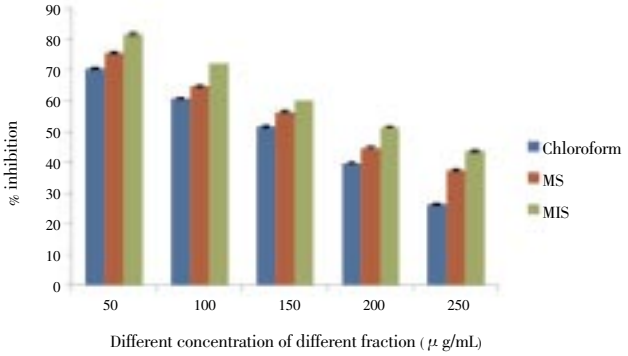


Fig. 6. Scavenging activity of different fractions of bark of C.pictus on NO radicals. Values are means± SD of three determinations.

3.5. Superoxide anion scavenging activity

In this study, the superoxide anion scavenging effects of various *C. pistus* was analysed (Fig. 7). All the bark samples of *C. pistus* exhibit excellent superoxide anion scavenging activity lower than that of ascorbic acid. Among the different fractions of bark of *C. pistus* tested, chloroform fraction exhibited the highest superoxide scavenging activity (IC50 value 100 μg/mL) in a dose dependent manner. The superoxide scavenging activities of different fractions of bark of *C. pistus* were in the order of MIS >MS > Chloroform fraction > ascorbic acid.

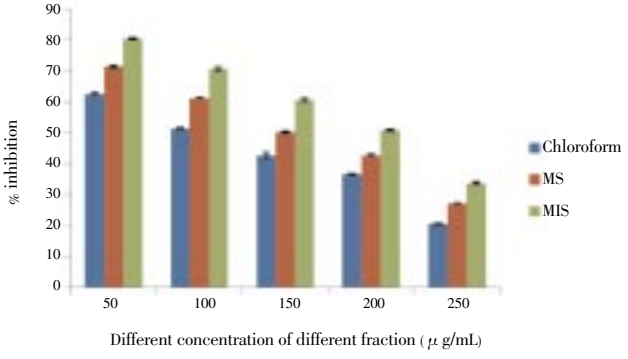


Fig. 7. Scavenging activity of different fractions of bark of *C. pictus* on superoxide radicals. Values are means± SD of three determinations

3.6. H_2O_2 scavenging activity

Fig. 8 shows that, different fractions showed fairly high and moderate scavenging capacity against H_2O_2, respectively. The scavenging activity of chloroform fraction was found to be higher than other fractions.

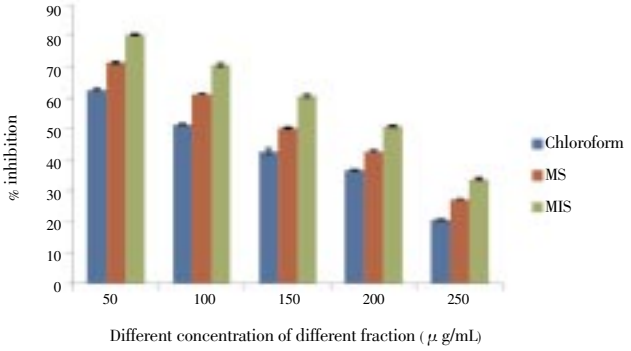


Fig. 8. Scavenging activity of different fractions of bark of *C. pictus* on hydrogen peroxide . Values are means± SD of three determinations

3.7. Metal chelating activity

In this study, chloroform fraction of bark of *C. pistus* showed a strong, concentration-dependent metal chelating activity. Its chelating ability was shown in Fig. 9 and table 1.

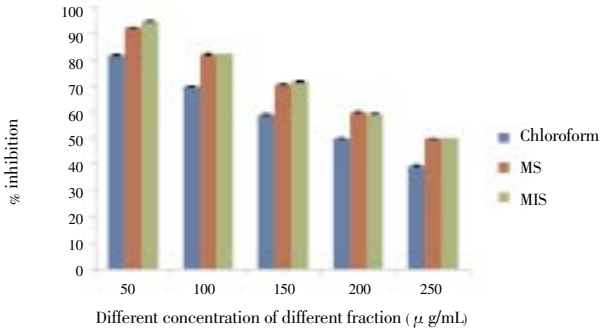


Fig. 9. Metal chelating ability of different fractions of bark of *C. pictus* . Values are means± SD of three determinations.

3.8. Reducing power

The present study, chloroform fraction of bark of *C. pistus* exhibited a stronger reducing power compared to shown in Fig. 10, but ascorbic acid has the strongest reducing power among all the different fractions of bark of *C. pistus*.

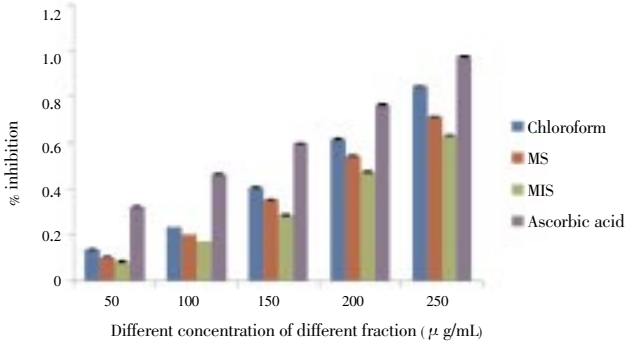


Fig. 10. Reducing ability of different fractions of bark of *C. pictus*. Values are means± SD of three determinations

3.9. Cytotoxic activity

In the present study, the cytotoxic effect of three different fractions such as chloroform, methanol soluble and methanol insoluble fraction on HT29 and A549 cells was evaluated by MTT assay. MTT assay is a well-established in vitro method for assessing cytotoxicity against cancer cell lines. Effects of different cell viability are presented in Fig. 11-12. The IC50 value was calculated from the 50% formazan formation compared with a control without administration of different fractions of bark of *C. pictus*. The MTT assay was used to detect the cell viability. The viability of HT29 and A549 cells was remarkably decreased in a dose-dependent manner after treatment with doses of different fractions such as 50-250 μg for 24 h. The cytotoxicity of different fractions according to the IC50 value was approximately 125 μg/mL, 150 μg/mL and 200 μg/mL for Chloroform, MS and MIS fraction respectively at 24 h treatment in HT29 cells. The cytotoxicity of different fractions according to the IC50 value was approximately 125 μg/mL, 150 μg/mL and 175 μg/mL for Chloroform, MS and MIS fraction respectively at 24 h treatment in A549 cells All the measurements were repeated three times Thangam et.al, 2012 [17].

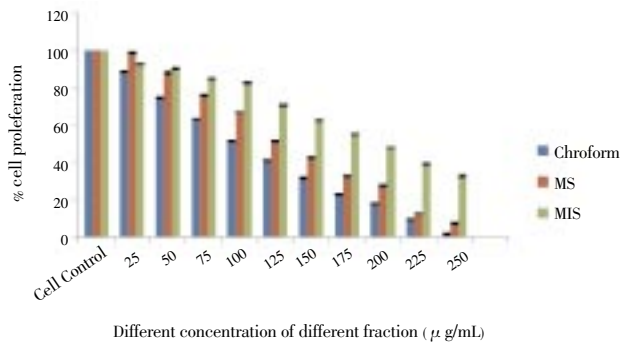


Fig. 11. Cell viability on different fractions of bark of *C. pictus* on HT29 cell in cytotoxicity assay in vitro. Each data point is the mean± standard deviation obtained from three independent experiments

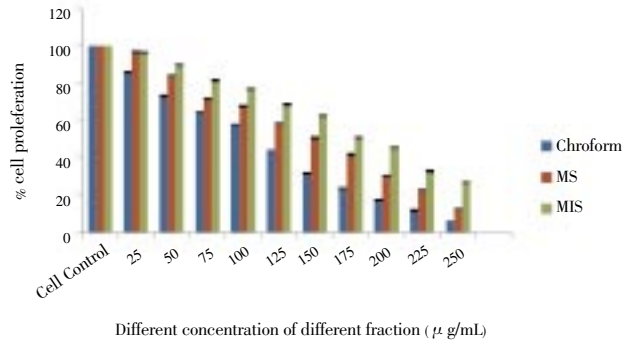


Fig. 12. Cell viability on different fractions of bark of *C. pictus* on A549 cell in cytotoxicity assay in vitro. Each data point is the mean± standard deviation obtained from three independent experiments

Discussion

The systemic literature collection, pertaining to this investigation indicates that the plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical scavengers. Therefore, it is necessary to determine the total amount of phenols and flavonoids in the plant extract chosen for the study. Flavonoids are the most diverse and widespread group of natural compounds and are likely to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity. The contents of total phenols and flavonoids were estimated by the standard curves and expressed as gallic acid equivalents for total phenols and rutin equivalents for flavonoids. The extract contains more than 20% of total flavonoids and is rich in phenols. Phenolic compounds are considered to be the most important antioxidants of plant materials. They constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Antioxidant activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals. In addition, they possess ideal structural properties for free radical scavenging properties[18]

DPPH is a commercial oxidising radical, which can be reduced by antioxidants. The stable DPPH can be used to study the reaction kinetics of antioxidants, quantify and to compare the free radical scavenging capacities of different antioxidants. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [19]. Thus an antioxidant candidate which proves promising in the DPPH antioxidant assay would provide an optimistic scaffold for prospective in vivo studies. Prasad et al. (2005) and Zhao et al. (2006) reported that phenolics and flavonoids reduce the DPPH radical by their hydrogen donating ability [20,21]. The result obtained in this investigation revealed that the DPPH radical scavenging

activities of *C. pistus* might be attributed to the hydrogen donating ability.

Nitric oxide is a key signaling in the physiological and pathological conditions and when react with macromolecules may induce inflammatory. It has been reported to play an important role in various inflammatory processes such as carcinomas, muscle sclerosis, arthritis and ulcerative colitis [22]. NO scavenging effect of different fractions was shown in Fig. 6. The percentage inhibition displayed by all fractions and chloroform fractions showed a potent scavenger of nitric oxide and thus confirmed the use of this plant for the treatment of antiinflammatory diseases caused by nitric oxide formation. In this study, bark of *C. pistus* depicted a significant inhibitory effect against nitric oxide generation.
Super oxide is biologically important because it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals. The superoxide anion can be generated by illuminating a solution containing riboflavin. It was reported that the superoxide anion scavenging activity could be due to the action of a free hydroxyl group of phenolic compounds. Furthermore, flavonoid molecule with polyhydroxylated substitution on ring A or B and a free 3-hydroxyl substitution could present the superoxide scavenging activity [23].

Hydrogen peroxide is not dangerous as it is, but may well be because of its ability to form the hydroxyl radical, thereby emphasising on the importance of its elimination. Indeed, it has already been proven that dietary phenols protect mammalian and bacterial cells from cytotoxicity induced by H2O2 indicating that the observed H_2O_2 scavenging activity of our plants could be due to the presence of phenols and flavinoids [24].

Metal chelating activity was significant since it reduced the concentration of the catalysing transition metal in lipid peroxidation[1] . It has been reported that chelating agents form bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, thereby stabilising the oxidised form of the metal ion [1]. Furthermore chemical and biological diversity of aromatic and medicinal plants depend on factors such as growth habitat, climatic conditions, vegetation phase and genetic modifications [25].

Reducing power is generally associated with the presence of reductones, which exert antioxidant action by breaking the free radical chain through donating a hydrogen atom [26]. In this assay, Fe3+ /ferricyanide complex is reduced to the ferrous form by antioxidants and can be monitored by measuring the formation of navy blue colour at 700 nm [27]. The reducing power of *C. pistus* is probably due to the presence of di and monohydroxyl substitutions in the aromatic ring, which possess potent hydrogen donating abilities as described [1].

Several plant species rich in flavonoids are reported to have disease preventive and therapeutic properties. This observation is of particular importance since flavonoids are ingredients of many vegetables and fruits and the association of vegetable and fruit consumption with reduced cancer risk has been reported [28-30]. In addition, the in vitro data support findings that a mixture consisting of these sterols compounds exerted cytotoxic activity against human lung and breast cancer cells [31]. Cytotoxic activity effect of different fractions showed in Fig. 12 and 13. Cytotoxic activity recorded in the present study is in accordance with this finding, since the phytochemical evaluation indicated the presence of flavonoids in all of the plant species with promising activity. The cytotoxic activities of active plants are probably due to presence of flavonoids. From this study, it is evident that the *C. pistus* bark extract has potent in vitro cytotoxic activity against both cell lines. Further studies are also in process to evaluate the most potent fraction of the active plant.

4. Conclusion

The present study elucidated for the first time the antioxidant property of bark of *C. pictus*. This study suggested that, among the three fractions, the chloroform fraction possesses high antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress related disorders. Moreover, all fractions possess potent anticancer properties against HT29 and A549 cells. Therefore it can be concluded that the bark has a potential natural antioxidant and this can act as an effective ingredient food industries. There are few reports on the antioxidant capacity of bark of *C. pictus* and the mechanism of different fractions of bark of *C. pictus* as antioxidative agents is still not fully understood. Hence further research is underway to analyse and isolate the active compounds responsible for the antioxidant and anticancer activity from different fractions of bark of *C. pictus*.

Conflict of interest statement

We declare that we have no conflict of interest.

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