

MODULATORY EFFECTS OF *CHUKRASIA TABULARIS* A. JUSS. BARK ON *IN VITRO* GENERATED OXIDATIVE STRESS

RAJBIR KAUR^a, BIKRAM SINGH^b AND SAROJ ARORA^{a*}

^aDepartment of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar-143005, Punjab, India, ^bNatural Plant Product Division, Institute of Himalayan Bioresource Technology (IHBT), Palampur, Post Box No.6- 176061, Himachal Pradesh, India. Email: dr.sarojarora@gmail.com

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ABSTRACT

Introduction: *Chukrasia tabularis* A. Juss. (Meliaceae), commonly known as Chickcrassy is an important medicinal tree of Indian Peninsula and is used for its antimalarial, antipyretic properties since ancient times.

Objectives: The present study was designed to explore the modulatory effect of methanol extract of *Chukrasia tabularis* bark and its different fractions (hexane, ethyl acetate and butanol) on the radicals generated in different *in vitro* models.

Methods: The methanol extract was prepared using maceration method and resultant extract was fractionated as per the polarity. The *in vitro* methods used to study antioxidant potential include DPPH, ABTS, molybdate ion reduction, reducing power, hydroxyl radical scavenging i.e. deoxyribose degradation and DNA nicking, superoxide anion radical scavenging and ferrous ion chelation assays. The total phenol and flavonoid content of these fractions was also determined.

Results: It was found that methanol extract and its fractions exhibited strong hydrogen donating and hydroxyl radical scavenging ability in all the assays but did not show metal chelation ability.

Conclusion: These findings led to the conclusion that methanol extract and its fractions acted as chain breaking antioxidant rather than acting as preventive antioxidants. It was observed that the antioxidant potential of extract and fractions of *C. tabularis* bark in different assays was linearly correlated to its phenol and flavonoid content.

Keywords: Chickcrassy; Flavonoids; Hydrogen donation; Hydroxyl radicals; Phenols

INTRODUCTION

The biologists of entire world share a common vision about structural and functional similarity of human body from the past 40,000 years. But, there is apprehension about the dramatic change in the life style, nutritional status and environment of human beings, during this period, which serve as a hallmark for their survival. The ultimate result of these factors is the concomitant fall in the intrinsic antioxidative defence system that impart oxidative stress due to enhanced production of free radicals in the form of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. These free radicals, when present in balanced conditions, serve as signalling and regulatory molecules at physiological levels [2]. It has been anticipated over the past three decades that overproduction of free radicals causes metabolic and morphological changes by damaging the macromolecules and thus, play an important role in the etiology of many human diseases including cancer, atherosclerosis, stroke, diabetes, rheumatoid arthritis, hypertension, immune-inflammatory diseases, ischemia/reperfusion injury, neurodegeneration, aging and acquired immunodeficiency syndrome (AIDS) etc.[3-6].

The use of antirisk factors to unfetter the organisms from the spiteful effects of hazardous chemicals that act as mutagens, participate in free radical generation or impart electrophile toxicity was recommended by De Flora *et al.* (1992) [7]. These defensive chemicals are mainly secondary metabolites synthesized by plants. Among a variety of bioactive compounds belonging to different chemical groups, polyphenols including flavonoids are an important class of natural products having multiple polar functionality [8,9]. The phenolic compounds, as antioxidative agents, protect the cells from oxidative and electrophilic damage by causing hindrance in ROS and RNS generating process due to their peculiar redox properties depending on the structure [10,11]. The interest in this natural-product-based drug discovery system has increased in recent years due to the availability of combinatorial synthesis, high throughput screening and related approaches used in formulation preparations and drug designing [12-14]. Although, a number of

plant species, enriched with diverse array of phytochemicals, from all over the world, have been explored for their antioxidant properties yet the vast majority of plants have still not been adequately evaluated. In the present investigation, modulatory effect of methanol extract of *Chukrasia tabularis* bark and its different fractions on different oxidative stress inducing agents in a variety of *in vitro* assays including hydrogen donating, free radical scavenging and metal ion chelation ability have been studied.

Chukrasia tabularis A. Juss. (Meliaceae) commonly known as chittagong wood, chickcrassy, Burma almondwood, Lal devdari etc. is one such plant whose reference is also found in traditional healing system of India. This plant accumulates a variety of secondary metabolites including phenolic compounds, terpenes, limonoids and steroids [15]. An extract of the leaves has been reported to exhibit a high antimalarial activity, antifungal and antibacterial activities [16-18]. The bark of *C. tabularis* has been used in Ayurvedic system of medicine as an astringent and anti-diarrheal drug [19,20].

MATERIALS AND METHODS

Chemicals

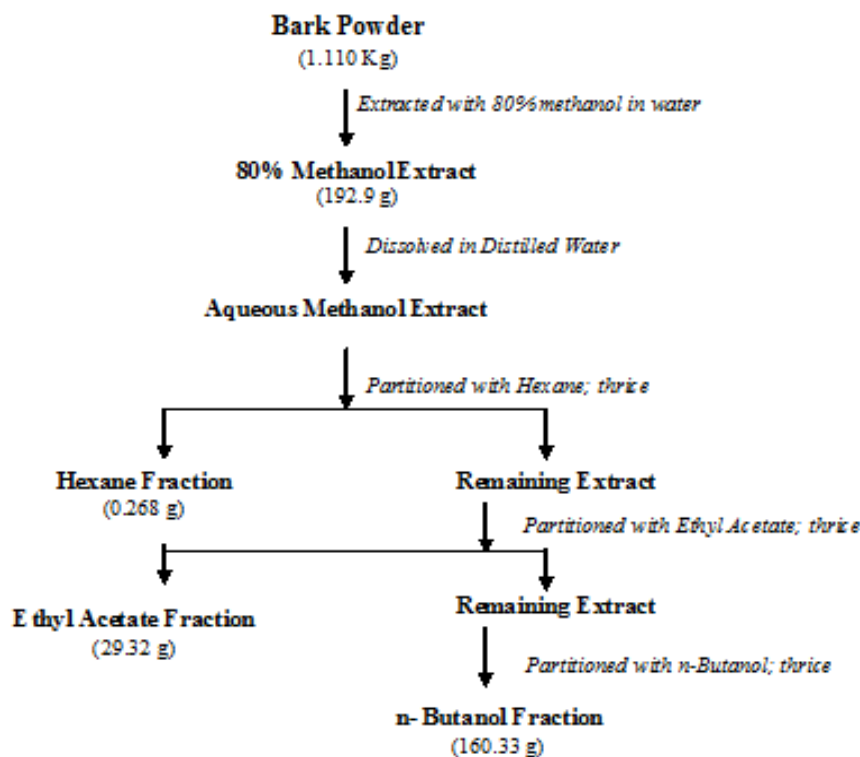
DPPH (2, 2'-diphenyl-1-picryl hydrazyl), TBA (2-thiobarbituric Acid), Ethidium bromide, ABTS (2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), potassium persulfate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2-deoxyribose was obtained from Lancaster Synthesis Inc. USA. Supercoiled plasmid pBR 322 DNA and agarose was obtained from Genei, Bangalore. Bromophenol blue, EDTA, L-ascorbic acid and Tris (hydroxymethyl) aminomethane, EDTA (Ethylenediamine tetraacetic acid), L-ascorbic acid, Folin-Ciocalteu Reagent, Sodium dihydrogen orthophosphate (NaH₂PO₄·2H₂O), di-Sodium hydrogen phosphate (Na₂HPO₄), potassium ferricyanide, trichloroacetic acid, sodium carbonate, ferric chloride, sodium nitrite, ferrozine, aluminium chloride, hydrogen peroxide, ammonium molybdate, sodium phosphate, ferrous chloride, Phenazine methosulfate, Nitroblue tetrazolium chloride, reduced Nicotinamide Adenine

Dinucleotide, gallic acid and rutin were of analytical grade.

Preparation of extract

Chukrasia tabularis bark was collected from Guru Nanak Dev University Campus (Near Department of Botanical and Environmental Sciences), Amritsar (India) in the month of September. Botanical identification was made from Herbarium of Department of Botanical and Environmental Sciences, GNDU, Amritsar, where a voucher specimen (Accession No. 6422/2236 dated 7th April, 2006) was deposited.

The powdered bark was extracted with 80% methanol and the methanol extract so formed was fractionated using different solvents viz. hexane, ethyl acetate, n-Butanol (**Flowchart 1**). The supernatant was filtered using Whatman No. 1 sheet, pooled and concentrated using vacuum rotary evaporator (Strike 202, Stereo glass, Italy). The concentrated solutions were then lyophilized to get the dry form of respective fractions. The methanol extract (MEB), hexane fraction (HFB), ethyl acetate fraction (EAFB) and n- Butanol fraction (BFB) was then analyzed for their antioxidant potential using *in vitro* antioxidant testing assays.



Flowchart 1: Extraction procedure for bark of *Chukrasia tabularis*

Determination of Total Phenol Content

The total phenol content of methanol extract and its fractions *C. tabularis* was quantified spectrophotometrically as per the procedure given by Yu *et al.* (2002) [21]. The absorbance of blue coloured mixture, formed due to the reaction of Folin Ciocalteu reagent (1:1) and 20% sodium carbonate solution with extract and fraction solution, was measured at 765nm. The amount of total phenol was calculated as mg Gallic Acid Equivalents (GAE) / 100mg dry weight of extract from calibration curve of gallic acid standard solution. For the gallic acid, the curve absorbance versus concentration is described by the equation $y = 0.0016x - 0.0102$ ($R^2 = 0.9952$). Here, y = absorbance and x = concentration.

Determination of Flavonoid content

The total flavonoid content of the methanol extract of *C. tabularis* bark and its different fractions was determined using colorimetric assay given by Kim *et al.* (2003) [22]. The mixture consisting of extract solution, sodium nitrite and aluminium chloride was incubated for 5 minutes followed by addition of sodium hydroxide solution. The mixture was then thoroughly vortexed and the absorbance of pink coloured solution was determined at 510nm. The amount of total flavonoid content was calculated as mg Rutin Equivalents/100mg dry weight of extract from calibration curve of rutin standard solution. For the rutin, the equation $y = 0.0008x - 0.0019$ ($R^2 = 0.9976$) represents the absorbance versus concentration curve. Here, y = absorbance and x = concentration.

Antioxidative assays

The free radical scavenging effect of methanol extract and fractions of *C. tabularis* bark was analyzed by employing the following *in vitro* antioxidant models. These antioxidant models are based on the reduction of end point either by scavenging free radicals generated in reaction mixture or by electron donation.

DPPH assay

The measurement of hydrogen donating capability of extract was assessed using DPPH (2, 2' diphenyl-1-picryl hydrazyl) radical as substrate, following the method described by Blois (1958) [23]. In this assay, 0.2 ml of extract solution was added to 3ml of 0.1mM methanolic DPPH solution and absorbance was read at 517 nm. The decrease in absorbance at ambient temperature was correlated with the scavenging action of the test compound and compared with gallic acid (used as standard phenolic compound). The radical scavenging activity was calculated using equation $(1-AS/AC) \times 100$; AC = Absorbance of Control, AS = Absorbance of Sample solution.

ABTS radical cation scavenging assay

ABTS radical cation decolorization assay given by Re *et al.* (1999) was used to determine hydrogen donating potential of methanol extract and fractions of *C. tabularis* bark [24]. The test is suitable to determine the total antioxidant capacity of both lipophilic and hydrophilic compounds because of the solubility of ABTS^{•+} in both organic and aqueous media, stability in a wide range of pH and excellent spectral

properties. The method comprises of the generation of ABTS^{•+} by mixing two reagents (7mM of ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and 140mM of Potassium Persulfate) in a proportion to make the final concentration of Potassium Persulfate to 2.45mM in mixture. The reaction mixture was kept at 30°C for 12-16 hours in dark. After 16 hours, the reaction mixture was diluted with ethanol or PBS (pH = 7.4) to obtain the absorbance of 0.700 ± 0.020 at 734nm. The ABTS^{•+} scavenging ability was determined by mixing the 1.9ml of ABTS^{•+} solution with 0.1ml of extract or fraction solution and the absorbance was measured spectrophotometrically for 0 to 6 minutes at 734nm. The absorbance of an ABTS^{•+} blank solution was measured in each assay to correct for radical decay. All tests were performed in triplicate. The percentage inhibition of absorbance at 734nm was calculated using the formula: Inhibition (%) = $(1 - AS/AC) \times 100$; AC = Absorbance of Control, AS = Absorbance of Sample solution.

Reducing power assay

The method given by Oyaizu (1986), was used to measure the reducing activity of methanol extracts and fractions [25]. 1 ml of extract of different concentrations was mixed with 2.5 ml of phosphate buffer (200mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 minutes. A volume of 2.5 ml of 10% TCA was then added to the mixture and centrifuged at 3000 rpm for 10 minutes. 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of FeCl₃ (0.1%) and the absorbance was measured spectrophotometrically at 700nm. The absorbance of prussian blue coloured complex, formed as end product, was measured spectrophotometrically at 700nm. Increase in absorbance of the reaction mixture was interpreted as increase in reducing ability of the extract and the results were compared with gallic acid (positive control). The percentage reduction of the sample as compared to standard i.e. gallic acid was calculated by using the formula $[1 - (AS/AC)] \times 100$; AC = Absorbance of standard at maximum concentration tested and AS = Absorbance of sample.

Molybdate ion reduction assay

The tendency of plant extract and fractions to reduce molybdate ion was determined following the method of Prieto *et al.* (1999) [26]. In this method, 0.3 ml of sample solution (100 µg/ml) was mixed with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate) and the reaction mixture was incubated at 95°C for 90 minutes. The mixture was cooled to room temperature and the absorbance of coloured solution was measured at 695 nm against a blank. The standard curve was obtained using 20 - 200µg/ml concentrations of ascorbic acid. The regression equation obtained for ascorbic acid was $y = 0.004x + 0.067$ (R² = 0.990); Here, y = absorbance obtained at 695 nm and x = concentration of ascorbic acid used. The reduction ability was expressed as mg Ascorbic Acid Equivalents (AAE) / 100 mg dry weight of extract or fractions as calculated from the standard curve obtained for ascorbic acid.

Hydroxyl radical scavenging assay

Non-site and site-specific hydroxyl radical scavenging activity of extract and fractions was measured by the method given by Halliwell *et al.* (1987) and Arouma *et al.* (1987) with slight modifications [27,28]. In non-site specific assay, the Haber Weiss reaction mixture (EDTA, FeCl₃, H₂O₂ and ascorbic acid) was mixed with deoxyribose, which acts as a substrate, and extract solution (5-100µg/ml) whereas in site-specific assay, the EDTA was replaced, by same amount of Phosphate Buffer (50mM) solution. The reaction was triggered by adding ascorbic acid (1mM) which served as a reducing agent by reducing Fe³⁺ to Fe²⁺ ions and subsequent incubation of the mixture for 1 h at 37°C. To 1 ml solution of above mixture, TBA in 25 mM NaOH (1 ml, 0.5%) and TCA (1 ml, 10% w/v aqueous solution) were added. The mixture was heated for 90 minutes on water bath at 80°C and the amount of pink chromogen produced was spectrophotometrically measured at 532 nm. The inhibitory effect on the hydroxyl radicals was calculated as: % Hydroxyl radical scavenging capacity = $(1 - AS/AC) \times 100$; AC = Absorbance of Control, AS = Absorbance of Sample solution.

Superoxide anion scavenging assay

For assessing the superoxide anion scavenging ability of extract and fractions of *C. tabularis*, method described by Nishikimi *et al.* (1972) was followed with slight modifications [29]. The superoxide anions were generated non-enzymatically in a PMS-NADH system comprising of phenazine methosulphate and reduced nicotinamide adenine dinucleotide, and assayed by development of blue coloured formazan dye upon reduction of nitro blue tetrazolium. Briefly, 1ml of plant extract or fractions of different concentrations (20-200µg/ml) was mixed with 156µM NADH (1ml), 60µM NBT (1ml) and 468µM phenazine methosulphate (1ml) in phosphate buffer (pH = 8.3). The reaction was initiated with the addition of PMS. The reaction mixture was incubated at 25°C for 10 minutes. The absorbance of coloured complex was measured at 560nm and the inhibition percentage was calculated using the formula $(1 - AS/AC) \times 100$; AC = Absorbance of Control, AS = Absorbance of Sample solution.

Ferrous ion chelation assay

The potential of plant extract and fractions to chelate Fe²⁺ was determined as per the method given by Dinis *et al.* (1994), with slight modifications [30]. The method comprised of mixing of different concentrations of extract (1 ml, 20-200 µg / ml) with 3.7 ml of methanol and 2 mM ferrous chloride (0.1 ml). The reaction was initiated with the addition of ferrozine (5 mM) and the mixture was kept at room temperature for 10 minutes. The absorbance of solution was determined at 562 nm against a blank. EDTA was used as positive control. The inhibition percentage was calculated using the formula $(1 - (AS / AC)) \times 100$; AS = absorbance of sample and AC = absorbance of control. In control, the solvent used for making concentration replaced the sample.

DNA nicking assay

The ability of extract and fractions of *C. tabularis* bark to protect super coiled pBR 322 from devastating effects of hydroxyl radicals generated by Fenton's reagent was assessed by the DNA nicking assay described by Lee *et al.* (2002) with slight modifications [31]. The reaction mixture contained 0.3µl of plasmid DNA, 10µl Fenton's reagent (30 mM H₂O₂, 50µM ascorbic acid, and 80 µM FeCl₃) followed by the addition of extract (250µg/ml) and the final volume of the mixture was brought up to 20 µl using distilled water. The mixture was then incubated for 30 min at 37°C. The DNA was analyzed on 1% agarose gel (in 1X TBE Buffer) followed by ethidium bromide staining. Rutin was used as a positive control.

Densitometric analysis was also performed to analyse the DNA damage quantitatively using Quantity one® software version 4.5.2 of BIO-RAD and the amount of DNA in each Form i.e. supercoiled (Form I), open circular (Form II) and double stranded nicked and linear (Form III) was calculated.

Statistical Analysis

Each experiment was performed at least three times and results were presented as the Inhibition (%) ± SE. Regression analysis were performed along with multiple comparisons by one-way analysis of variance (ANOVA). Data were considered statistically significant at $p \leq 0.05$. IC₅₀ (the concentration of sample (µg/ml) required to scavenge 50% free radical) was also calculated from the regression equation.

RESULTS

Total Phenol and Flavonoid content

From **Table 1**, it is clear, that among extract and fractions of bark, ethyl acetate fraction contained 78.75 mg Gallic Acid Equivalents/100mg (dry weight of extract) of polyphenols followed by n-Butanol fraction (72.5 mg/100mg) and 80% methanol extract (69.375 mg/100mg). The hexane fraction of bark showed least amount of phenol content i.e. 8.73 ± 0.001 mg GAE/100mg dry weight of fraction. It is also clear from the **Table 1** that the total flavonoid content of methanol extract and fractions of *C. tabularis* bark was comparatively more than their total phenol content with crude extract i.e. MEB having higher flavonoid content of 94.375 mg RE / 100mg followed by EAFB (81.875 mg/100mg) > BFB (63.125 mg/100mg) > HFB (0.0067 mg /100mg).

Table 1: Total Phenol, flavonoid content and molybdate ion reduction ability of methanol extract and fractions of *C. tabularis* bark.

S. No.	Extract /Fractions	Total Phenol Content mg GAE /100mg \pm SE	Total Flavonoid Content mg RE /100mg \pm SE	MRA mg AAE/100mg \pm SE
1.	MEB	79.38 \pm 3E-04	94.375 \pm 9E-04	82.42 \pm 23.47
2.	HFB	08.73 \pm 0.001	0.0067 \pm 0.002	nd
3.	EAFB	78.75 \pm 9E-04	81.875 \pm 3E-04	99.25 \pm 6.292
4.	BFB	72.50 \pm 1E-04	63.125 \pm 3E-04	89.67 \pm 13.64

Here, MEB = Methanol extract of bark, HFB = Hexane Fraction of Bark, EAFB = Ethyl acetate fraction of bark, BFB = butanol fraction of bark, GAE = Gallic acid equivalents, RE = Rutin equivalents, AAE = Ascorbic acid equivalents MRA = Molybdate ion reduction assay and nd= not detected

DPPH assay

In DPPH assay, the hydrogen donating ability of extracts was observed by the conversion of DPPH radical (purple coloured solution) to non radical form (yellow coloured solution) by reduction. The DPPH radical scavenging ability of methanol extract and fractions of *C. tabularis* bark were compared with gallic acid (a known phenol), and it was found that ethyl acetate fraction (EAFB) showed highest DPPH radical scavenging activity of 86.24% at 140 μ g/ml followed by BFB (80.39%), MEB (77.80%) and HFB (1.863%) at the same concentration. The hexane fraction showed negligible activity in this assay (**Figure 1A**). It is clear from **Figure 1A** that EAFB and MEB, having IC₅₀ value of 60.498 μ g/ml and 64.531 μ g/ml respectively, have lower hydrogen donating ability than that of gallic acid (8.499 μ g/ml).

ABTS assay

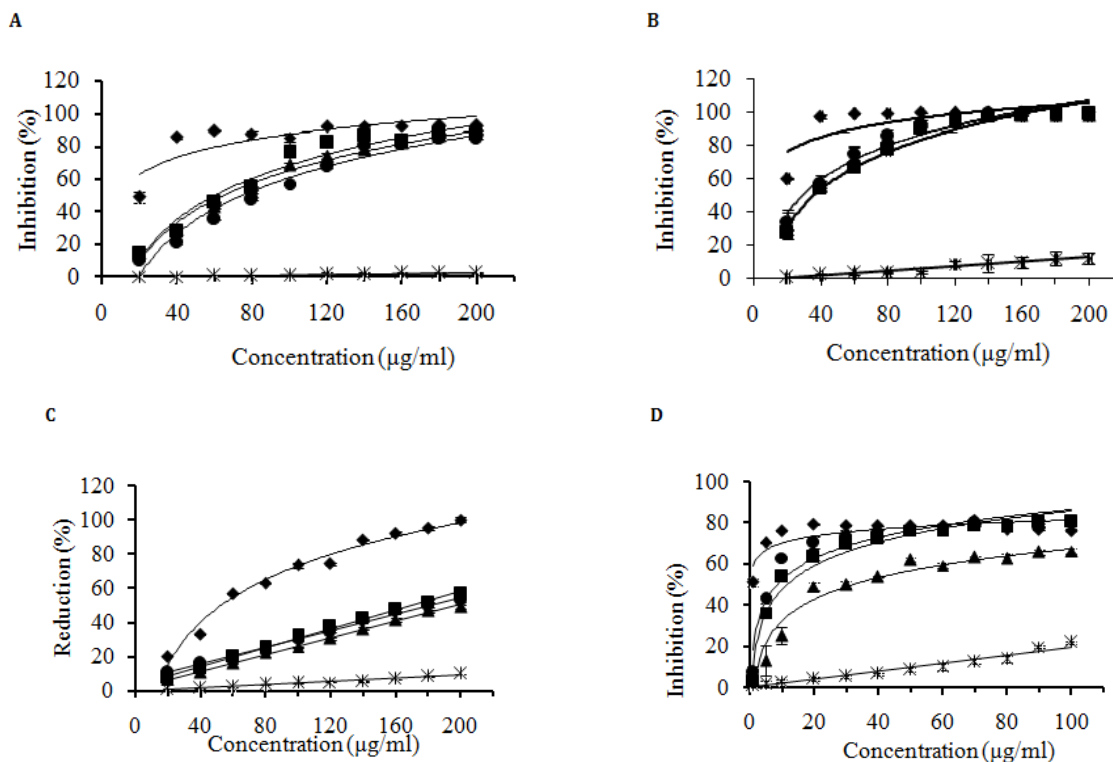
ABTS^{•+}, formed as a result of reaction between ABTS and potassium persulfate, is converted into colourless entities as a result of reduction caused by hydrogen donating ability of phenolic compounds. ABTS^{•+} scavenging ability of methanol extract of *C. tabularis* bark and its fractions is shown in **Figure 1B**. It was found that BFB and MEB exhibited almost similar inhibitory activity of 85.54% and 85.40% at 80 μ g/ml whereas EAFB scavenged 77.22% radicals at the same concentration. The HFB exhibited lowest activity among different extract and fractions of *C. tabularis* bark with 12.07% inhibition at 200 μ g/ml. The comparison of IC₅₀ values of extract and fractions with gallic acid, used as a standard phenolic compounds, revealed that MEB, BFB and EAFB exhibited IC₅₀ value (29.87 μ g/ml, 28.77 μ g/ml, 35.42 μ g/ml respectively) higher than gallic acid (2.569 μ g/ml).

Reducing Power assay

The ability of phenolic rich extract and fractions to reduce Fe (III) to Fe (II) is considered as an important indicator to measure their electron donating potential. Due to this property, the reducing power assay serves as an important tool to study antioxidant potential. The results of reducing power in terms of reduction (%) were calculated by comparing the absorbance of different fractions with that of highest concentration of gallic acid. **Figure 1C** shows the ability of extract and fractions of *C. tabularis* bark to reduce Fe (III) and that to in a linear concentration-dependent manner. Ethyl acetate fraction of bark (EAFB) showed the maximum reducing power among different fractions of methanol extract (MEB). EAFB exhibited the reduction percentage of 56.77%, in terms of gallic acid, at the maximum concentration tested (200 μ g/ml) whereas MEB has 49.33% reduction ability at the same concentration. The BFB has reduction ability of 52.80% in terms of gallic acid. The HFB was least effective among different fractions as it has shown a reduction potential of 9.777% at the maximum concentration tested.

Molybdate ion reduction assay

In molybdate ion reduction assay, the tendency of extract and fractions to reduce molybdate ions in phosphomolybdenum complex was determined and results was expressed in terms of number of Ascorbic Acid Equivalents (AAE) in mg / 100mg dry weight of extract or fractions as calculated from the standard curve obtained for ascorbic acid ($y = 0.004x + 0.067$). It is clear from **Table 1**, that the ethyl acetate fraction (EAFB) has got maximum reduction ability fraction followed by n-Butanol fraction and Methanol extract.



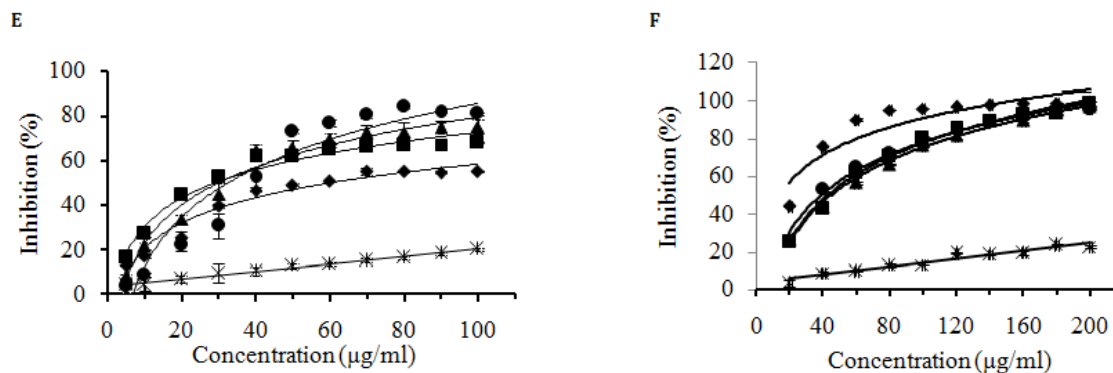


Fig. 1: Antioxidant activity of methanol extract of *Chukrasia tabularis* bark and its different fractions in different *in vitro* assays: (A) Hydrogen donation assay (B) ABTS radical cation Scavenging (C) Reducing Power assay (D) Non-site specific hydroxyl radical scavenging assay (E) Site- specific hydroxyl radical scavenging assay and (E) Superoxide anion radical scavenging assay. The results are expressed as Inhibition (%) \pm SE. Here, \blacklozenge = gallic acid, \blacktriangle = methanol extract of bark, \times = hexane fraction of bark, \blacksquare = ethyl acetate fraction of bark and \bullet = butanol fraction of bark

Hydroxyl radical scavenging assay

Figure 1D shows the hydroxyl radical scavenging ability of extract and fractions of *C. tabularis* bark in non-site specific manner. It has been found that the methanol extract of bark and its fractions showed the scavenging ability in a dose dependent manner. The results revealed that out of different fractions; BFB and EAFB was most active one with a remarkable inhibition of 79.93% and 79.01% respectively at 70 μ g/ml. MEB was also effective in scavenging \cdot OH radicals and it exhibited an inhibitory effect of 63.23% at the same concentration i.e. 70 μ g/ml. Likewise, HFB showed least activity among different extract and fractions with an inhibitory activity of 12.7% at 70 μ g/ml.

The hydroxyl radical scavenging ability of methanol extract and fractions of *C. tabularis* bark in site specific assay is shown in Figure 1E. The dose dependent inhibitory effect was observed for different extract and fractions. BFB was most effective one showing inhibitory effect of 83.81% at 80 μ g/ml concentration. MEB and EAFB also showed a remarkable inhibition of 72.53% and 66.51% respectively at the same concentration i.e. 80 μ g/ml. However, HFB showed an inhibitory effect of 16.4% at the same concentration. It was observed that all the extract and fractions of bark showed pronounced effect in non-site specific assay as compared to site-specific assay indicating that extracts act as better \cdot OH radical scavengers rather than chelating agents (Figure 1D and 1E).

Superoxide Anion Radical Scavenging Assay

The superoxide anion radical scavenging activities of methanol extract of bark of *C. tabularis* and their fractions were analyzed in comparison with known phenolic compounds gallic acid and the results are shown Figure 1F. It was found that the extract and fractions have shown superoxide anion radical scavenging potential in a dose dependent manner with EAFB and BFB showed similar inhibitory effect of 79.94% and 79.72% respectively at 100 μ g/ml concentration. The MEB exhibited an inhibitory effect of 76.06% at the same concentration. The HFB was least effective showing an inhibitory percentage of 13.19% at 100 μ g/ml concentration. One way ANOVA and Tukey's HSD post hoc test at $p \leq 0.05$ level of significance for superoxide anion scavenging ability confirmed the significance of results obtained.

Ferrous ion chelation assay

Figure 2 demonstrate the chelating ability of different extract and fractions of *C. tabularis* bark in comparison with EDTA- a known metal chelator and standard antioxidant compound i.e. gallic acid for Fe^{2+} . It is clear from the results obtained that none of the extract and fractions exhibited a remarkable metal chelating potential. It was found that MEB, HFB, EAFB and BFB exhibited ferrous ion chelating ability of 7.373% , 3.933% , 5.0% and 18.42% respectively at the highest concentration tested i.e. 200 μ g/ml.

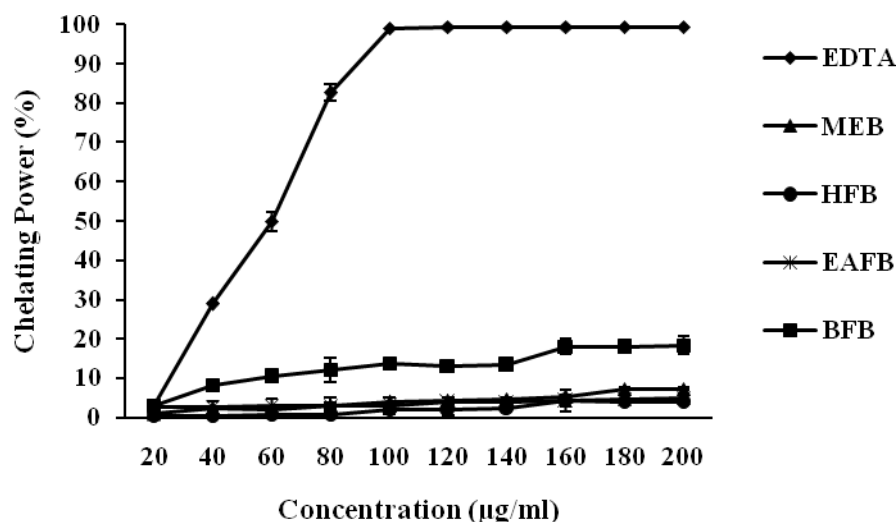


Fig. 2: Metal chelating activity for methanol extract of *Chukrasia tabularis* bark and its different fractions

DNA nicking assay

It has been found that in DNA nicking assay, when the plasmid DNA (pBR322 DNA) was dissolved in the Fenton's reaction mixture, there was a time dependent increase of single stranded and double

stranded nicked and linear forms of DNA (Form II and Form III respectively) due to hydroxyl radicals generated in reaction mixture. However, addition of various fractions of plant extract to reaction mixture reduced the hydroxyl radical mediated strand breaking and conversion of supercoiled DNA (Form I) to Form II and III DNA.

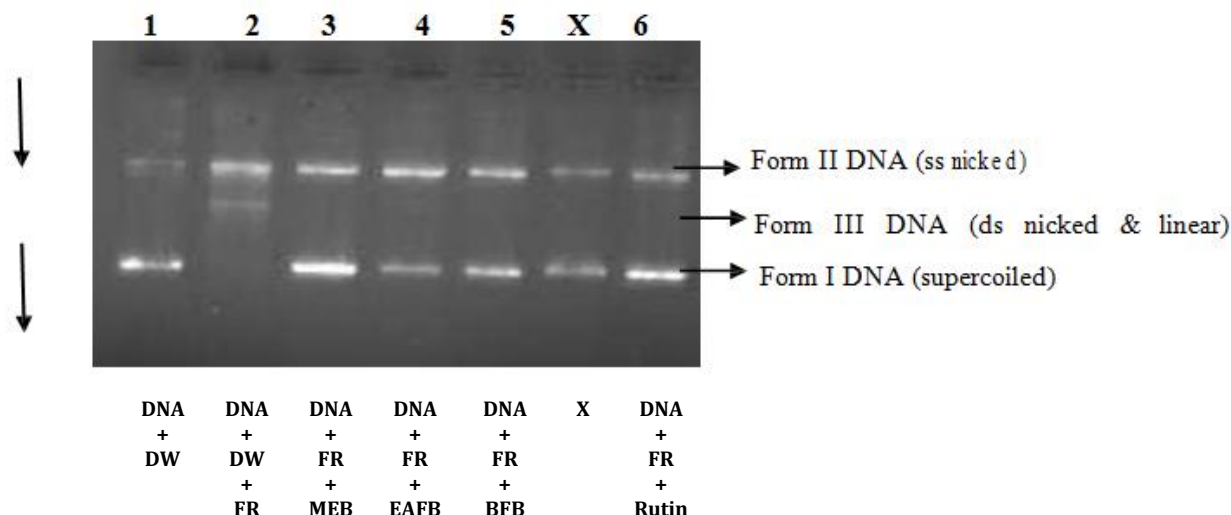


Fig. 3: DNA Nicking assay for different fractions of bark of *Chukrasia tabularis* at 250µg/ml

It is clear from Table 2 and Figure 3 that the amount of supercoiled DNA, in the presence of methanol extract and fractions solution and FR, was found to be 55.55%, 43.77%, 49.66% and 57.20% in case of MEB, EAFB, BFB and rutin respectively. This amount of integrated supercoiled

DNA was found to be similar to that of negative control i.e. without any treatment (57.92%). However, in Fenton's reagent treated DNA (positive control), Form I i.e. supercoiled DNA was completely broken-down to ss nicked i.e. Form II (56.84%) and ds nicked DNA i.e. Form III (43.16%).

Table 2: Densitometric analysis of different forms of DNA after treatment with different fractions of *Chukrasia tabularis* bark (250µg/ml)

	C	FR	MEB	EAFB	BFB	Rutin
Form I DNA	2400.6 (57.92)	0	3014.09 (55.55)	1990.23 (43.77)	2267.0 (49.66)	2664.4 (57.20)
Form II DNA	1744.45 (42.08)	2482.46 (56.84)	2411.84 (44.45)	2557.26 (56.24)	2298.36 (50.34)	1993.7 (42.80)
Form III DNA	0	1885.14 (43.16)	0	0	0	0
Total DNA	4145.13	4367.6	5425.93	4547.49	4565.41	4658.1

*within bracket amount of DNA in percent was shown.

The results of one way ANOVA and Tukey's HSD post hoc test revealed that the inhibitory effect obtained against the concentration used in different assays (DPPH, ABTS, site and non-site specific hydroxyl radical scavenging, superoxide anion radical scavenging, reducing power and metal chelation) were statistically significant at $p \leq 0.05$.

DISCUSSION

Free radicals may be considered as an important class of carcinogens as they are involved in the expansion of tumor clones and acquisition of malignant properties due to their destructive nature (Dreher and Junod, 1996) [32]. It has been postulated by Halliwell and Gutteridge (1985) that the reactive oxygen species induce biological damage by reacting with cellular macromolecules, such as, nucleic acids, proteins as well as various membrane components and thus act as direct and indirect initiators as well as promoters of mutagenesis and carcinogenesis [33]. The reactive oxygen intermediates, including superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radicals are known to mediate this macromolecular damage. These reactive species also increase the lipid peroxidation, which in turn alter the integrity of membrane bound enzymes [34-36]. On the basis of this, it has been hypothesized that antioxidant intervention might be considered as the safest approach in the prevention of processes leading to mutagenesis. However, complexity of free radical chemistry caused a major hindrance in the identification of potential antioxidant

candidate. To combat this problem, the potential antioxidant substances are tested in a variety of *in vitro* models and such an approach expand the horizon of antioxidant study. The mechanisms that contribute to the antioxidant capacity of phenols and flavonoids include free radical scavenging ability, hydrogen or electron donation ability, chelation of redox active metal ions, modulation of gene expression and interaction with the cell signaling pathways. However, in food systems antioxidants have been found to act through two mechanisms of action: preventive or chain breaking antioxidants. Chain breaking antioxidant showed their effect by donating hydrogen atoms to the radicals or by scavenging initiating radicals and thus breaking the chain reactions whereas preventive antioxidants are characterized by their tendency to chelate metal catalyst or by reducing the concentration of ROS in solution (Arouma, 2003) [37]. The free radical scavenging and antioxidant activities of phenolics are reported to depend upon the arrangement of functional groups about the nuclear structure. Both the number and configuration of H-donating hydroxyl groups are the main structural features influencing the antioxidant capacity of phenolics [38,39].

Keeping this in mind, different *in vitro* antioxidant radical systems are used in order to elucidate a full profile of antioxidant potential of extracts and fractions of *C. tabularis*. In the present study, hydrogen or electron donating capacity was determined using molybdate reduction capacity, DPPH radical scavenging, ABTS radical cation scavenging and reducing power assay. Metal chelation ability of

extract and fractions was determined by chelating assay, hydroxyl radical scavenging ability was determined using deoxyribose degradation assay and DNA nicking assay whereas O_2^- scavenging ability was estimated using superoxide anion radical scavenging assay.

DPPH radicals and ABTS radical cations are the two most widely used chromogens to determine the antioxidant activity of biological materials [40]. The phytochemicals present in the extracts terminate the oxidation process through hydrogen donation and convert the free radicals to stable forms. Among the extract and fractions of *C. tabularis* bark, ethyl acetate fraction showed highest DPPH radical scavenging activity of 86.24% at 140 µg/ml concentration with an IC_{50} value of 60.498 µg/ml but higher than that of gallic acid (8.499 µg/ml). MEB and BFB showed lesser activity than ethyl acetate fraction with IC_{50} value of 64.531 and 74.525 µg/ml respectively whereas hexane fraction of bark did not show any activity in this assay. It was found that methanol extract and its fraction showed effective ABTS⁺ scavenging ability with an IC_{50} value of 28.77, 29.87, 35.42 and 761.06 µg/ml in BFB, MEB, EAFB and HFB respectively. The standard compound i.e. gallic acid exhibited an IC_{50} value of 2.569 µg/ml, much lower than that of methanol extract and its different fractions.

The comparison of activity of extract and fractions of *C. tabularis* bark in DPPH and ABTS assays led to a conclusion that these fractions exhibited higher activity in ABTS⁺ scavenging assay than that of DPPH assay. The differential behavior of fractions in both these assays could be justified by the reports of Nenadis and coworkers (2004). They studied structure-activity relationships among different phenolic antioxidants including hydroxycinnamic acid derivatives, simple polyphenols, polyhydroxybenzoates and flavonoids using DPPH and ABTS⁺ scavenging assays. TEAC (Trolox Equivalent Antioxidant Capacity) values indicated that all the phenolic compounds possessing at least one hydroxyl group in the aromatic ring were considerably active toward ABTS⁺ and the antioxidant activity in this assay was not effected by number and position of hydroxyl groups. However, similar effect was not observed in DPPH assay. Nenadis *et al.* (2004) found that coumaric acid and isoferulic acids were inactive in DPPH assay but possessed significant activity towards ABTS⁺. Despite the differences in the position of hydroxyl groups, there was no difference in the activity of *o*-coumaric, *m*-coumaric, isoferulic acids and caffeic acids in ABTS scavenging assay. Furthermore, TEAC values of *p*-coumaric acids (hydroxycinnamic acid derivative), as calculated by using the ABTS⁺ assay, was found to be similar to that of rosmarinic acid (an ester of caffeic acid). However, number and position of hydroxyl groups in these phenolic antioxidants significantly altered their activity in DPPH assay [41]. Shi and coworkers (2009) reported the DPPH radical scavenging activity of ethanolic extract of *Prunus mume* and its isolated phenolic compounds (chlorogenic acid isomers). It was found that these isomers exhibited higher antioxidant activity as compared to crude ethanolic extract. The ABTS⁺ scavenging activity of extracts was found to be in agreement with that of DPPH radical scavenging activity [42].

The electron donating potential of natural compounds was determined using reducing power assay and molybdate reduction assay as in both these assays end point occurred after the reduction of substrate molecules by the antioxidants [25,26]. In the reducing power assay, reduction of Fe (III) to Fe (II) by phenolic antioxidants resulted in the formation of Prussian blue coloured complex (Ferric ferrocyanide) i.e. $Fe_4 [Fe (CN)_6]_3$ that was used as an indicator to measure their electron donating potential whereas in molybdate reduction assay, reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of green coloured phosphate/Mo(V) complex at acidic pH served as an indicator of electron donation [26,43]. It was found that MEB, EAFB and BFB of *C. tabularis* bark exhibited lower activity with an IC_{50} value of 197.746 µg/ml, 170.197 µg/ml and 180.653 µg/ml respectively in comparison to gallic acid (52.61 µg/ml). In a similar manner, in molybdate reduction assay the results revealed that the ethyl acetate fraction of *C. tabularis* bark has maximum tendency to reduce molybdate ions in terms of ascorbic acid equivalents i.e. 99.25 mg AAE/100mg dry weight of extract. The results obtained in the present study are in accordance

with the earlier reports of Kanatt *et al.* (2005) that the reducing ability of methanol extract of *Mentha* species was due to the presence of compounds acted as reductones and further terminated the radical induced chain reaction by donating electrons [44]. In a similar manner, Prasad and co-workers (2009) demonstrated the electron donating ability of different fractions of Wampee (*Clausena lansium* (Lour.) Skeels) peels using total antioxidant capacity assay and reducing power assay. It was found that different fractions of Wampee exhibited good antioxidant activity in phosphomolybdenum method. The value of antioxidant activity of ethyl acetate fraction at 100 µg/ml concentration was 2.5 ± 0.005 that was significantly higher than BHT (1.3 ± 0.005). Furthermore, total antioxidant capacity was correlated with the total phenol content of these fractions [45].

The metals including iron (Fe^{3+}/Fe^{2+}) and copper (Cu^{2+}/Cu^+) are important in the redox properties of biological systems and the compounds having abilities to chelate active species such as Fe^{2+} or Cu^+ block the primary steps involved in the free radical generation system. Furthermore, these metals are involved in the generation of hydroxyl radicals by reducing endogenous H_2O_2 . The chelating power assay determines the amount of freely available ferrous ions in the solution as Fe^{2+} ions has specificity to form purple coloured complex on interaction with Ferrozine. The formation of coloured complex is interrupted in the presence of chelating agents [46]. In the present study, it has been found that the fractions of bark did not have any chelating ability. Although, it is presumed that the metal chelation ability of phenolic compounds is related to the presence of -OH groups but anomalous behaviour might be related to the presence of such type of flavonoids or phenols that lack required structure that furthermore contributed to chelation ability. It was reported by Wang *et al.* (2008), that the chelation reaction was dependent on the affinity of an antioxidant toward Fe^{2+} in relation to ferrozine therefore, it was assumed that the binding constant and concentration of antioxidants also affected the chelating ability of compounds [47].

Hydroxyl radicals play an important role in the initiation of lipid peroxidation causing oxidative stress mediated cell injury in aerobic organisms [48]. Regarding mechanism of action, Gutteridge (1987) suggested that transition metals including iron have varying binding affinities for different molecules (act as ligands) with highest affinity for proteins such as transferrin and lactoferrin or low affinity for albumin, carbohydrate and phosphate esters. It was found that in case of reaction of low-affinity ligand bound metal ion with H_2O_2 , the damage to the ligand occurred at specific site as -OH would be formed at the site of metal binding. The incorporation of scavenger molecules that have higher binding affinity for metal ions as compared to ligands could help to avoid such site specific damage as in such a situation -OH radical's production could be directed elsewhere [49]. In the present study, damage exerted by -OH with their production in solution (non-site specific) or at particular site was determined using deoxyribose as detector molecule in deoxyribose degradation assay. It was found that methanol extract of *C. tabularis* bark and its fractions showed pronounced effects in the presence of EDTA that suggested their greater potential to scavenge -OH present in the free solution and thus protected the degradation of deoxyribose (detector molecule) to thiobarbituric acid reactive material. The ethyl acetate fraction of bark that exhibited highest activity in site-specific assay has lowest IC_{50} value of 29.338 µg/ml whereas butanol fraction with IC_{50} value of 8.665 µg/ml was more efficient in non-site specific assay. The results of DNA nicking assay was also in confirmation with the observation made in hydroxyl radical scavenging in deoxyribose degradation assay, as the addition of extracts to the pBR322 DNA containing Fenton's reaction minimizes the formation of Form II (single stranded nicked DNA) and Form III (double stranded nicked and linear DNA) and thus maintain the Form I (supercoiled) DNA by a hydrogen abstraction mechanism. Superoxide anion radicals (O_2^-) are cytotoxic when present at high concentration due to their potential to react with biological membranes. In the present study, these radicals are generated non-enzymatically from the dissolved oxygen by Phenazine Methosulphate/NADH coupling reaction that further resulted in the reduction of nitroblue tetrazolium to blue colored adduct (diformazan) that was measured

spectrophotometrically at 560nm. It was found that methanol extract of *C. tabularis* bark and fractions resulted in significant reduction in the absorbance of blue colored formazan that indicated the consumption of superoxide anion radicals in the reaction mixture. It has been found that BFB was more effective in scavenging O_2^- radicals followed by EAFB, MEB and HFB respectively with an IC_{50} value of 39.283, 43.470, 45.332 and 439.221 $\mu\text{g/ml}$ respectively.

In case of extracts and fractions of *C. tabularis* bark, methanol

extract and its fractions showed maximum effectiveness that might be attributed to the presence of phenols and flavonoids as determined by using Folin-Ciocalteu reagent and colorimetric assay. The column chromatography of ethyl acetate fraction led to the isolation of compounds of steroidal nature (β -sitosterol and β -sitosterol β -d- glucoside). The HPLC analysis of methanol extract of bark showed the presence of catechin, quercetin and rutin (Table 3). The protective effect of different fractions of bark might be due to synergistic interaction of phenolic and steroidal compounds present in them.

Table 3: Chemical constituents of *Chukrasia tabularis* isolated from bark and leaves and identified using chromatographic, NMR and HPLC techniques (Kaur et al., 2011)

Plant Part	Chemical Constituents
Bark of <i>C. tabularis</i>	β - sitosterol, β - sitosterol- β - d- glucoside, Catechin, Quercetin, Rutin

In HPLC analysis, besides peaks corresponding to standards, other peaks were also present which showed the presence of other phenolic compounds.

A correlation analysis was done to explore the relationships amongst the different antioxidant variables used to explore the antioxidant efficacy of extracts and fractions of *C. tabularis* bark (Table 4).

Table 4: Linear correlation coefficient (r) between polyphenolic composition and antioxidant capacity in bark.

	MRA	DPPH	ABTS	NSS	SS	RP	MC	SOD
TPC	0.984**	0.986**	0.995***	0.954**	0.964**	0.967**	0.351	0.992***
TFC	0.926*	0.973**	0.954**	0.864	0.903*	0.894	0.168	0.938*

* represents significance at $p \leq 0.1$ ** represents significance at $p \leq 0.05$ *** represents significance at $p \leq 0.01$ **** represents significance at $p \leq 0.001$ Here, TPC = Total Phenol Content, TFC = Total Flavonoid Content, MRA = Molybdate ion Reduction, DPPH = DPPH assay, NSS = Non-site specific hydroxyl radical scavenging, SS = site specific hydroxyl radical scavenging, RP = reducing power and MC = Metal Chelation

From Table 4, it is clear that all the antioxidant assays were highly correlated with the total phenol content except for metal chelation, in case of bark. However, only DPPH exhibited strong correlation with the total flavonoid content and has significant correlation coefficient of 0.9731 at $p \leq 0.05$. Amongst the methods

used for quantifying antioxidant activities, it is clear from Table 5, that the methods used to determine free radical scavenging through hydrogen donation i.e. DPPH and assays involving electron donation (MRA and RP) were significantly correlated with each other.

Table 5: Linear correlation coefficient (r) among the different methods for quantifying the antioxidant capacities in bark.

	MRA	DPPH	ABTS	NSS	SS	RP	MC	SOD
MRA	1							
DPPH	0.978**	1						
ABTS	0.982**	0.967**	1					
NSS	0.989**	0.939*	0.962**	1				
SS	0.953**	0.911*	0.985**	0.949*	1			
RP	0.997***	0.964**	0.967**	0.996***	0.939*	1		
MC	0.408	0.225	0.438	0.514	0.575	0.434	1	
SOD	0.994***	0.970**	0.996***	0.982**	0.979**	0.985**	0.451	1

* represents significance at $p \leq 0.1$ ** represents significance at $p \leq 0.05$ *** represents significance at $p \leq 0.01$ **** represents significance at $p \leq 0.001$ Here, MRA = Molybdate ion Reduction, DPPH = DPPH assay, NSS = Non-site specific hydroxyl radical scavenging, SS = site specific hydroxyl radical scavenging, RP = reducing power and MC = Metal Chelation

It has been found that strong correlation existed between different methods (MRA, DPPH, NSS, SS and RP) while metal chelation exhibited weaker correlation with other methods. In addition to this, it was also found that site specific and non-site specific hydroxyl radical scavenging ability also lacks significant correlation (0.9495). However, a strong correlation was found to exist between MRA and DPPH ($r = 0.9787$), MRA and RP (0.9968) at $p \leq 0.05$. Therefore, on the basis of values of linear correlation coefficients, it is well documented that the antioxidant potential of extract and fractions of *C. tabularis* bark in different assays was linearly correlated to its phenolic content. The observation

is further substantiated by the similar reports in literature [50-53].

CONCLUSION

It might be concluded from the present study that methanol extract and fractions of *C. tabularis* bark act as potent chain breaking antioxidants due to their ability to modulate the effect of radicals generated in different *in vitro* models either by hydrogen donation or by scavenging free radicals. The antioxidant potential of these extract and fractions is found to be correlated to the

presence of phenols and flavonoids in them that due to their particular structure and arrangement of functional groups contribute to the bioactivities.

However, the most appropriate use of extract and fractions of *C. tabularis* bark will ultimately depend on understanding their mechanism of action at molecular, cellular, tissue, and organ level as well as in whole animals, therefore these extracts and fractions will be further tested for their activity at all levels.

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