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EXPLORING ANTIOXIDANT ACTIVITY AND DNA PROTECTION POTENTIAL OF C*HONEMORPHA FRAGRANS*

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Keywords:

C. fragrans, DNA protection, Antioxidant activity, DPPH, FRAP, H_2O_2 Scavenging Capacity.

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ABSTRACT: *Chonemorpha fragrans* (Moon) Alston is a liana belongs to family Apocynaceae. Due to its ethanobotanical importance, it is used in traditional medicinal systems as a part of various medicinal preparations. In the work presented here the plant has been explored for its antioxidant potential and DNA protection ability. The antioxidant potential of *C. fragrans* was determined using DPPH, FRAP, Phosphomolybdanu and H_2O_2 Scavenging Capacity. Phenol and flavonoid content was measured followed by Correlation analysis between antioxidant activities and phenols and flavonoid content. In addition, DNA protection ability of extracts was evaluated using double stranded plasmid DNA. *C. fragrans* extracts have shown significant antioxidant potential. Phenol content showed high correlation with Phosphomolybdenum reduction potential where as flavonoid content with Phosphomolybdenum as well as H_2O_2 radical scavenging potential. All the extracts exhibited an ability to protect the DNA against devastating effects of Fentons reagent except methanol roots, stem bark, *in vitro* and callus extracts. The extracts have shown significant antioxidant activity and Phenol, flavonoid content was found to contribute towards antioxidant potential of *C. fragrans.* The extracts also showed the DNA protection potential pointing toward its pharmaceutical importance.

INTRODUCTION: *Chonemorpha fragrans* (Moon) Alston, a liana belonging to family Apocynaceae is known to produce commercially important anticancer compound camptothecin[1]. Use of *C. fragrans* in traditional medicinal system, states the ethnobotanical importance of this plant. The medicinal value of a plant lies in some chemical substances that produce a definite physiological action on the human body. In the work presented here, the plant has been explored using pharmacological investigations like antioxidant potential and DNA protective ability.

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Free radicals and other reactive oxygen species (ROS) are produced as by products in human body during biochemical and physiological processes. To eliminate these free radicals, human body has developed many enzymatic and nonenzymatic mechanisms but this is not sufficient in severe oxidative stress conditions.

Overproduction of such free radicals can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA) leading to many chronic diseases, such as cancer, diabetes, atherosclerosis, aging and other degenerative diseases in humans[2]. The most studied system to fight against such oxidative stress is to combat their level in the body by supplying with greater amount of natural antioxidants. Such intake of natural antioxidants is connected with the prevention of cancer and cardiovascular diseases[3]. Medicinal plants contain a wide variety of free

radical scavenging molecules with high antioxidant activity[4]. Natural antioxidants, particularly polyphenolics are safe as well as bioactive. The intake of these natural antioxidants reduces risks of cancer, cardiovascular disease, diabetes and other diseases associated with ageing[5, 6] as many of these antioxidant compounds are associated with anti-antitumor, antimutagenic, antibacterial, inflammatory or antiviral activities to some extent[2]. Use of natural antioxidants, for inactivating free radicals is receiving a lot of attention, not only for their scavenging properties, but also because they are natural, non-synthetic products. These natural product drugs play a leading role in pharmaceutical business as they are the most productive leads for development of drugs particularly anticancer agents and anti infective agents. Almost half of the drugs approved since 1994 are based on natural products[7, 8] due to which there is a growing interest in developing the products that contain mixtures of natural compounds from traditionally used medicines[9].

Isolation and characterization of natural compound and then screening it for biological activity is extremely time-consuming and expensive. Many times superior activity is a result of pharmacodynamic synergism or pharmacokinetic influences[10]. In addition, when only one activity is considered in pharmacological screens, it is not possible to detect other potentially useful activities. The most efficient alternative to this method is activity guided fractionation of plant extracts discovering novel drugs. In the current work we have used sequential extracts of *C. fragrans* for evaluation of its antioxidant potential. Use of sequential extracts can precisely give an idea for further isolation of unknown and important compounds other than CPT. Keeping these findings in mind we have tried to explore plant *C. fragrans*, which is widely used in Ayurveda in the treatment of different ailments like gynaecological disorders[11], skin diseases and inflammations[12], fever and stomach disorder[13] and still unexplored with respect to its biological potential.

In this communication biological evaluation of this species is carried out by antioxidant assays and DNA protection assay. The total antioxidant activity of crude as well as sequential extracts of root, bark, leaf, *in-vitro* shoots and callus from *C. fragrans* was evaluated by various antioxidant assays, including free DPPH, H_2O_2 radical scavenging activity, Phosphomolybdanum, FRAP as well as hydroxyl radical induced DNA strand scission. Total phenols and flavonoids were estimated from the extracts as most of the therapeutic properties of medicinal plants are attributed to their biologically active phenolic and flavonoid compounds, which are also considered to be powerful antioxidants[14, 15]. Phenolics as efficient free radical scavengers can potentially interact with biological systems and play a role in preventing oxidative stress induced diseases[16]. A correlation between radical scavenging capacities of extracts with total phenolic, flavonoid content and compound content was observed.

MATERIALS AND METHODS:

Preparation of plant extracts of *C. fragrans*

The plant parts of *C. fragrans* (i.e leaves, bark, roots, callus and *in vitro* shoots) were shade-dried and ground into fine powder using grinder. The extracts were prepared according to the method of Kedari and Malpathak., (2013)[17]. All the extracts were evaporated to dryness redissolved in HPLC grade methanol. All obtained fractions were dissolved as 100mg/ml of 0.01% dimethyl sulfoxide (DMSO) and diluted to yield various final working concentrations. These extracts were filtered using a 0.45µm cellulose nitrate membrane and stored at -20ºC till further analysis was carried out.

Determination of antioxidant activity

1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

Radical scavenging activity of plant extracts against stable DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate, Sigma-Aldrich Chemie, Steinheim, Germany) was determined spectrophotometrically according to the method of Fry *et al*. (2013)[18]. In this assay, free radical scavenging activity was determined by measuring the bleaching of purple coloured methanol solution of DPPH radical. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep violet to light yellow) were measured at 515nm on a double beam UV-VIS spectrophotometer. Ascorbic acid was used as standard. In the

experiment, the inhibition percent was calculated from the following equation:

% Inhibition = $((A_C-A_S)/A_C)$ x 100

Where A_C is the absorbance of the control and A_S is the absorbance in the presence of the sample of *C. fragrans* extracts or standards.
Data presented as mean of 3 experiments. Different letters with in a column for a particular treatment represent significance at $P < 0.05$.

Hydrogen Peroxide Scavenging Capacity
The ability of the *C. fragrans* extracts to scavenge hydrogen peroxide was determined according to the method of Ngonda (2013)[19]. The absorbance was measured at 230 nm using double beam UV-VIS spectrophotometer. The percentage of hydrogen peroxide scavenging of both *C. fragrans* extracts and standard compound (ascorbic acid) were calculated using following equation:

% Scavenged (H_2O_2) = $((A_C-A_S)/A_C)$ x 100

Where A_C = absorbance of the control
A_S = absorbance in the presence of the sample of *C. fragrans* extracts or standards.

Data presented as mean of 3 experiments. Different letters with in a column for a particular treatment represent significance at $P < 0.05$.

Ferric Reducing Antioxidant Potential (FRAP)
The FRAP assay was performed according to Mensah (2013)[20]. Readings of the coloured product (ferrous tripyridyltriazine complex) were taken at 593nm. The standard curve was plotted using $FeSO_4$. Results were expressed in µg of Fe (II)/g dry mass and compared with that of ascorbic acid. Data presented as mean ± standard deviation of 3 experiments. Different letters with in a column for a particular treatment represent significance at $P < 0.05$.

Phosphomolybdenum Reduction assay
Phosphomolybdenum reduction potential of extracts was determined spectrophotometrically according to the method of Parimelazhagan *et al*. (2011)[21]. The absorbance of the solution was measured at 695 nm. Activity was expressed in terms of µg/gm DW±SD equivalent of ascorbic acid. Data presented as mean ±standard deviation of 3 experiments. Different letters with in a column for a particular treatment represent significance at $P < 0.05$.

Determination of total phenol content
Total phenolic content was determined according to the method given by Khan *et al*. (2013)[22]. The absorbance was measured at 730nm using double beam UV-VIS spectrophotometer. The concentration of phenolic content was expressed as tannic acid (Himedia) equivalents (TAE) in milligrams per gram sample. Data presented as mean ± standard deviation of 3 experiments. Different letters with in a column for a particular treatment represent significance at $P < 0.05$.

Determination of flavonoid content
Flavonoid content in the extract was determined by a colorimetric method (Maleš *et al*. 2013)[23]. The absorbance was measured at 510nm using double beam UV-VIS spectrophotometer. Quercetin (Himedia) was used as standard. The total flavonoid content was then expressed in terms of mg Quercitin equivalents QE/gm of dry sample. Data presented as mean ± standard deviation of 3 experiments. Different letters with in a column for a particular treatment represent significance at $P < 0.05$.

Correlation analysis between antioxidant activities
The correlation coefficients between antioxidant activity and total phenolic, flavonoid content of *C. fragrans* extracts were calculated using correlation analysis in SPSS (Table 7).

DNA protection assay
The ability of different plant extracts to protect supercoiled pBR322 DNA from hydroxyl radicals generated by Fenton's reagent was assessed by the DNA nicking assay as described by Golla and Bhimathati, (2014)[24]. In this case, monoterpene indol alkaloid Camptothecin was used as positive control. DNA samples were analyzed using 1.2% agarose gel in TAE buffer (1.5-2V/cm). The agarose gel was stained with ethidium bromide (0.5µg/ml deionized distilled water) for 20min and visualised on Gel Doc XR system (Bio-Rad, USA).

RESULTS AND DISCUSSION:

1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay:

DPPH assay has been extensively used for screening plant extracts because of its sensitivity to detect active ingredients at low concentrations[25]. The proton radical scavenging action is one of the various mechanisms for measuring antioxidant activity. DPPH is a compound, which possess a proton and shows a maximum absorption at 517nm. When DPPH encounter proton radical scavengers its purple colour fades rapidly. This assay determines the scavenging of stable radical species of DPPH by antioxidants.

TABLE 1 DPPH RADICAL SCAVENGING ACTIVITY OF CRUDE *IN-VIVO* AND *IN-VITRO* EXTRACTS OF *C. FRAGRANS*

Solvent → fractions↓	Methanol	Ethyl acetate	Chloroform	Hexane
	% radical scavenging activity			
Roots	**91.58**	65.62	10.16	8.73
Bark	**89.21**	80.79	9.35	23.26
Leaf	43.01	41.19	27.83	3.35
***In vitro* shoots**	6.45	6	4.41	3.73
Callus	6.76	5.33	4.85	3.65

The highest activity was observed in root methanolic extract 91.58% **(**EC_{50} of 15μg/ml**)** followed by bark extracts of methanol 80.79% (EC_{50} of 24μg/ml) as shown in the table. The activity was higher than standard ascorbic acid which showed 38.21% (EC_{50} of 25μg/ml) scavenging potential. Callus extracts of hexane 3.65% (EC_{50} of 12µg/ml) and *in-vitro* shoot extracts of hexane 3.73% (EC_{50} of 31μg/ml) proved to have lowest % DPPH radical scavenging.

The results suggested that the methanol crude extracts have highest potential to scavenge DPPH free radicals than the sequential extracts. The antioxidant activity of *C. fragrans* extracts increased along with increase in the solvent polarity. The weakest antioxidant activities were found in hexane extracts except for the bark extract. From results we also concluded that the *in vitro* extracts (crude as well as sequential) have poor %DPPH radical scavenging activity than *in vivo* extracts.

Hydrogen Peroxide Scavenging Capacity

H_2O_2 is highly important because of its ability of penetrating biological membranes. Hydrogen peroxide itself is not very reactive with most biologically important molecules, but it is an intracellular precursor of hydroxyl radicals due to which it is very toxic to cells. H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (•OH) that can initiate lipid peroxidation and cause DNA damage[26]. Thus, the removal of H_2O_2 is very important for antioxidant defence in cell. Scavenging of H_2O_2 is a measure of scavenging the antioxidant activity of the extract.

Out of all extracts root extracts of ethyl acetate (59.56%) showed highest and shoot extracts of ethyl acetate (1.00%) showed lowest H_2O_2 scavenging activity (Table 2). The H_2O_2 scavenging potential of all the extract is less than standard ascorbic acid % inhibition (69.92%) suggesting that all the extracts possessed low H_2O_2 scavenging capacity.

TABLE 2 HYDROGEN RADICAL SCAVENGING ACTIVITY OF *IN-VIVO* AND *IN-VITRO* EXTRACTS OF *C.FRAGRANS*

Solvent → fractions↓	Methanol	Ethyl acetate	Chloroform	Hexane
	% H_2O_2 scavenging activity			
Roots	30.91	**59.56**	27.81	21.44
Bark	7.99	13.33	28.73	7.28
Leaf	8.34	20.12	6.28	13.33
***In vitro* shoots**	13.13	1.00	23.63	7.49
Callus	48.51	31.9	22.05	8.66

Hydrogen peroxide scavenging potential of extracts can be attributed to the presence of phenolic groups which could donate electrons to hydrogen peroxide, there by neutralizing it into water[27]. In *C. fragrans*, ethyl acetate root extracts have shown highest H_2O_2

scavenging activity indicating that out of all the extracts the root extracts could be useful in effectively scavenge H_2O_2 preventing lipid peroxidation which cause DNA damage.

Similar to this, o**ut of all the sequential extracts of *Torilis leptophylla*** L. ethyl acetate extracts showed highest hydrogen peroxide scavenging activity where as alcoholic and aqueous extracts showed lowest hydrogen peroxide scavenging activity[27]. Hydrophilic antioxidants are also reported to be more effective in less polar media whereas lipophilic antioxidants are more effective in relatively more polar[28, 29].

Ferric Reducing Antioxidant Potential (FRAP)

In FRAP assay, non-enzymatic antioxidants react with prooxidants and inactive them. In this redox reaction, antioxidants act as 'reductants'. In this context, the antioxidant power can be referred as 'reducing ability'. In this FRAP assay, an easily reducible oxidant, Fe III is used in excess. Thus there is a reduction of Fe III-TPTZ complex by antioxidant[30, 31]. Increasing absorbance indicates an increase in reductive ability. The FRAP values of the studied fractions of *C. fragrans* are presented in **Table 3.**

TABLE 3 FERRIC REDUCING ANTIOXIDANT POTENTIAL (FRAP) OF *IN-VIVO* AND *IN-VITRO* EXTRACTS OF *C. FRAGRANS*

Solvent → fractions↓	Methanol	Ethyl acetate	Chloroform	Hexane
	µg Fe (II)/g DW			
Roots	11.36±0.051^{B}	11.82±0.052^{R}	12.38±0.051^{K}	3.41±0.056^{T}
Bark	3.86±0.051^{C}	4.43±0.016^{P}	7.54±0.097^{E}	8±0.092^{PQ}
Leaf	8.56±0.077^{O}	5.95±0.051^{M}	6.41±0.021^{J}	6.97±0.05^{S}
In -vitro shoots	16.82±0.051^{I}	17.28±0.011^{L}	17.84±0.081^{N}	9.86±0.031^{H}
Callus	10.32±0.048^{A}	10.88±0.092^{D}	19.47±0.065^{F}	19.92±0.088^{G}

All the extracts demonstrated significant antioxidant capacity with FRAP test. Stronger reducing power was observed in *in vitro* extracts like callus extracts of hexane (19.92±0.088µg Fe (II)/g DW) and callus extracts of chloroform (19.92±0.088µg Fe (II)/g DW). This was three to four times higher than control (ascorbic acid) which showed 5.24 ±0.07µg Fe (II)/g reducing power. Lowest reducing power was observed in the *in vivo* extract, that is methanolic bark extract (3.86±0.051µg Fe (II)/g DW) and roots extracts of hexane (3.41±0.056µg Fe (II)/g DW).

All these assays also suggested that the solvent used for extraction does affect the antioxidant potential of an extract. Accordinng to Iqbal, (2012)[32] also, extracting solvents affect the efficiency of antioxidant determination assays.

Phosphomolybdenum reduction assay

The antioxidant capacity of the extracts was measured spectrophotometrically through phosphomolybdenum method, based on the reduction of Mo (VI) to Mo (V) by the extracts and the subsequent formation of green phosphate/Mo (V) compounds due to antioxidant compounds present in the extracts. The absorption of green coloured complex is noted at 765nm. The present study demonstrated that in *C. fragrans* extracts the activity methanolic *in vitro* shoot extracts (0.284±0.002mmol/gm) exhibited the highest antioxidant capacity for phosphomolybdate reduction whereas chloroform shoots extract (0.003±0.0005 mmol/gm) showed lowest phosphomolybdenum reduction **(Table 4).**

TABLE 4 PHOSPHOMOLYBDENUM ANTIOXIDANT ACTIVITY OF *IN-VIVO* AND *IN- VITRO* EXTRACTS OF *C.FRAGRANS*

Solvent → fractions↓	Methanol	Ethyl acetate	Chloroform	Hexane
	% scavenging activity			
Roots	0.047±0.001^{E}	0.011±0.002^{Q}	0.014±0.005^{O}	0.063±0.003^{B}
Bark	0.06±0.001^{C}	0.017±0.002^{M}	0.039±0.0009^{G}	0.038±0.003^{H}
Leaf	0.033±0.0014^{J}	0.014±0.003^{OP}	0.021±0.003K^{L}	0.021±0.002^{K}
In -vitro shoots	0.284±0.002^{A}	0.052±0.0004^{D}	0.003±0.0005^{T}	0.042±0.002^{F}
Callus	0.036±0.0014^{HI}	0.009±0.0003^{R}	0.017±0.0009^{MN}	0.005±0.0002^{S}

Natural antioxidants can be phenolic compounds (tannins, flavonoids, phenolic acids and tocopherols), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), carotenoids as well as ascorbic acid[33]. According to Parimelazhagan *et al.* (2011)[21] the phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, tocopherols and carotenoids, suggesting the presence of these compounds in these extracts. Also the antioxidant activity of mushroom seems to be due to the presence of phenolic compounds, flavonoids and anthocyanosides[34, 35, 36, 37]. The antioxidant capacity of various solvent fractions of *T. leptophylla* was found to be maximum in hexane followed by methanol fraction. Recent studies have shown that many flavonoid and related polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants[38]. In case of *Launaea procumbens* also, the results suggested that the strong antioxidant activity of extracts might be due to the presence of phenolics compounds present in the extract[38]. Considering these findings we also estimated phenols and flavonoid content from all the extracts and its correlation was studied with above mentioned antioxidant activities.

Total phenol content

Phenols are commonly found in plant kingdom and have multiple biological properties[39, 40]. Most of the therapeutic properties of medicinal plants are attributed to the presence of phenols and flavonoids, which are considered as powerful antioxidants[14, 15]. The antioxidant activity of phenolics is mainly due to their redox properties which make them act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They also may have a metallic chelating potential[41]. The results are shown in **Table 5**.

TABLE 5 TOTAL PHENOL CONTENT OF CRUDE *IN -VIVO* AND *IN -VITRO* EXTRACTS OF *C. FRAGRANS*

Solvent → fractions↓	**Methanol** mg TAE/g	**Ethyl acetate**	**Chloroform**	**Hexane**
Roots	16.25±0.048^{C}	1.304±0.048^{NO}	4.027±0.018^{H}	1.332±0.011^{N}
Bark	35.402±0.061^{B}	1.611±0.007^{M}	7.145±0.037^{E}	3.822±0.014^{I}
Leaf	13.503±0.053^{D}	2.343±0.013^{K}	3.191±0.013^{J}	1.295±0.01^{NOP}
In- vitro shoots	54.193±0.356^{A}	4.264±0.016F^{G}	0.372±0.002^{RS}	4.331±0.007^{F}
Callus	1.989±0.006^{L}	0.451±0.001^{R}	0.962±0.004^{Q}	0.214±0.0008ST

TAEs = Tannic Acid Equivalent

Out of all the methanolic extracts, *in-vitro* shoot extracts of methanol possessed the highest amount of total phenol (54.193±0.356mg TAEs/g dry weight) followed by bark extracts of methanol (35.402±0.061 mg TAEs/g dry weight) and root extracts of methanol (16.25±0.048mg TAEs/g dry weight). In sequential extracts highest phenolic content were seen in bark extract of chloroform (7.145±0.037mg TAEs/g dry weight) and root extract of chloroform (4.027±0.018 mg TAEs/g dry weight). Lowest level of phenols was observed in callus extracts of hexane (0.214±0.0008mg TAEs/g), callus extract of ethyl acetate (0.451±0.001mg TAEs/g) and shoot extract of chloroform (0.372±0.002 mg TAEs/g).

If we compare phenolic content in *in-vivo* and *in -vitro* sequential extracts, *in-vivo* extracts showed greater phenolic content than *in -vitro* extracts (as seen in **Table 6)**. Whereas *in-vitro* extracts of methanol are higher in phenolic content than *in -vivo* methanol crude extracts. This also suggested that *in-vitro* extracts of methanol and *in-vivo* sequential extracts can show better antioxidant due to high phenolic content.

Flavonoid content

Flavonoids are important in the plant for normal growth development and defence against infection and injury. Several studies have reported that plant flavonoids showing antioxidant activity *in-vitro* also function as antioxidants *in-vivo*[42,43]. Flavonoids present in the extract may possess antioxidant activity through their scavenging or chelating process which contains hydroxyl functional group as suggested by Hendra *et al.* (2011)[44].

They have wide range of biological activities like antiviral, anti inflammatory, antitumor, antimicrobial, estrogenic, antiestrogenic and antioxidant, mutagenic and antimutagenic because of which they are emerging as neutraceuticals in pharmaceutical industries[45]. Relatively highest

amounts of flavonoids were present in methanolic bark (1746.66±7.36 mg QEs/g dry weight), followed by leaves extract of methanol (1060.21±3.75 mg QEs/g dry weight) and bark extract of chloroform (967.52±6.54 mg QEs/g dry weight) of *C. fragrans*. Lowest flavonoids were detected in callus extracts of hexane (3.78±0.1 mg QEs/g dry weight), callus extracts of chloroform (11.62±1.3QE mg QEs/g dry weight), roots extracts of ethyl acetate (20.47±3.07Pmg QEs/g dry weight) **(Table 6).**

TABLE 6 TOTAL FLAVONOID CONTENT IN CRUDE *IN-VIVO* AND *IN-VITRO* EXTRACTS OF *C. FRAGRANS*

Solvent → fractions↓	Methanol mg QEs/g DW	Ethyl acetate	Chloroform	Hexane
Roots	293.39±2.76^{E}	20.47±3.07P^{Q}	75.29±5.05^{L}	66.78±3.43^{LM}
Bark	**1746.66±7.36^{A}**	171.7±2.02^{J}	967.52±6.54^{C}	219.9±4.24^{H}
Leaf	**1060.21±3.75^{B}**	216.279±4.53^{HI}	273.94±4.25^{F}	85.37±4.01^{K}
***In vitro* shoots**	547.71±2.85^{D}	255.88±5.44^{G}	27.82±0.62^{OP}	64.71±2.38^{MN}
Callus	32.39±1.95^{O}	5.372±0.09^{RS}	11.62±1.3^{QR}	3.78±0.1^{RST}

QEs = Quercetin Equivalents

The amount of flavonoids varied significantly in *C. fragrans* extracts. Comparison of all the extracts showed greater flavonoid content in the *in-vivo* extracts (methanolic as well as sequential) as compared to *in-vitro* extracts. These results were totally in contrast with the MTT cell proliferation assay results, suggesting that flavonoids are not responsible for the antiproliferative effect of *C. fragrans.* The *in-vivo* plant parts used for extraction (stem and roots) were woody parts and have shown higher flavonoid content as compared to *in-vitro* extracts.

Correlation analysis between antioxidant activities

The results obtained from above mentioned study indicated that *C. fragrans* methanolic roots and bark extracts, root extract of ethyl acetate, callus extracts of chloroform and methanolic shoot extract have significant antioxidant activity against various antioxidant systems *in-vitro* (DPPH, H_2O_2 radical scavenging, FRAP). From these antioxidant results various antioxidant activities of *C. fragrans* extracts can be correlated with the mechanisms like hydrogen donating ability, lipid peroxidation inhibitor and their effectiveness as scavengers of hydrogen peroxide and free radicals. Thus the correlation analysis carried out between phenol, flavonoid and antioxidant activities have shown that there was a high correlation between phenolic content and Phosphomolybdenum, H_2O_2 radical scavenging assay as seen from **Table 5**.

TABLE 7 CORRELATION ANALYSIS BETWEEN ANTIOXIDANT ACTIVITIES

Correlation	FRAP	DPPH% radical scavenging	Phosphomolybdenum	H_2O_2 radical scavenging
Phenol	0.030779	0.249966	0.864776	0.84397
Flavonoid	-0.35751	0.43365	0.267105	0.929963

The correlation between the phenol content and H_2O_2 radical scavenging activity was highly correlated, suggesting there presence is responsible for H_2O_2 radical scavenging activity. It also proves that phenols are efficient free radical scavengers which can potentially interact with biological systems and play a role in preventing oxidative stress. Phenols also showed positive correlation with phosphomolybdenum assay. Such positive correlation between total phenolic content of plant extracts and related antioxidant activity has also been reported by different workers[4, 16, 46]. The results suggested that phenolic and flavonoid compounds contributed significantly to the antioxidant capacity of *C. fragrans* extracts. When flavonoid content was correlated with all the antioxidant activities, it showed a highly positive correlation with H_2O_2 radical scavenging potential. This analysis suggested that phenols as well as flavonoid content had capacity for antioxidant activities of *C. fragrans*. However, flavonoid content showed poor correlations with DPPH activities and it showed negative correlation with FRAP suggesting the presence of antioxidant compounds other than flavonoids and phenols. To check the effect of this antioxidant activity at DNA

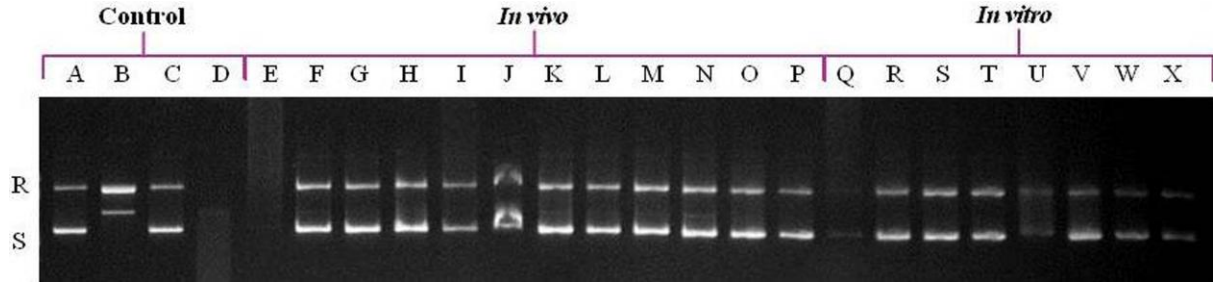
level, DNA protection assay was carried out further.

DNA protection assay

The free radical scavenging effects of all the extracts were studied on plasmid DNA damage. Addition of Fentons reagent to double stranded plasmid results in time dependent increase of single stranded (ss) and double stranded (ds) nicked and linear forms of DNA due to the attack of **.**OH radicals generated in the reaction mixture. These radicals attack on the nitrogenous bases or the deoxyriboxyl backbone of DNA. Addition of an antioxidant to this reaction mixture prevented the double stranded DNA from the hydroxyl radical mediated strand breaking and conversion of supercoiled DNA(S) to relaxed form(R) DNA. Antioxidant activity of plants are directly related to DNA protection activity, either by chelating the transition metal[47], or by inhibiting the enzymes involved in the initiation reaction of DNA break[48]. Kumar *et al*. (2010)[49] has reported such oxidative modification of DNA to contribute to aging and various diseases which include cancer and chronic inflammation.

As seen from **Fig 1** the extracts of *C. fragrans* (4µg/ml concentrations) showed inhibitory effect against **.**OH radicals mediated damage as this extract maintained the integrity of supercoiled DNA. The effects were compared with a known phenolic compound (Standard antioxidant) Quercetin. The intensity of supercoiled band was considered for as a measure of activity as compared to Quercetin.

FIG 1. DNA PROTECTION ASSAY:



INHIBITORY EFFECT OF *C. FRAGRANS* EXTRACTS ON DNA NICKING CAUSED BY HYDROXYL RADICALS

Lane A: Supercolied pUC 19 DNA, **B**: DNA + Fenton's reagent, **C**: DNA+ Fenton's reagent+ Camptothecin (2µg), **D**: 0.1% DMSO, Lane **E-X** : DNA + Fenton's reagent+ test compound (2ug) **E**: MeOH Root, **F**: EtOAc Root, **G**: CH_3Cl Root, **H**: C_6H_{12} Root, **I**: MeOH Bark, **J**: EtOAc Bark, **K**: CH_3Cl Bark, **L**: C_6H_{12} Bark, **M**: MeOH Lvs, **N**: EtOAc Lvs, **O**: CH_3Cl Lvs, **P**: C_6H_{12} Lvs, **Q**: MeOH *In- vitro* **R**: EtOAc *In -vitro*, **S**: CH_3Cl *In- vitro*, **T**: C_6H_{12} *In -vitro*, **U**: MeOH Callus, **V**: EtOAc Callus, **W**: CH_3Cl Callus, **X**: C_6H_{12} Callus. Position of relaxed (R) and supercoiled (S) plasmids have been shown on the leftside

All the extracts exhibited an ability to protect the DNA against devastating effects of Fentons reagent except methanol roots (Lane E), stem bark (Lane I), *in- vitro* (Lane Q) and callus (Lane U) extracts, suggesting that all the extracts have potential of protecting double stranded DNA except methanol

roots, stem bark, *in- vitro* and callus extracts. Out of all crude methanol extracts only leaf extract (Lane M) was able to show the protective activity against free radicals. This could be correlated with the compounds present in leaf extracts. One of the possible mechanisms explaining the protective effect of *C. fragrans* extracts against DNA scission could be the scavenging of reactive oxygen species by flavonoid constituents of the extracts as flavonoids have the high scavenging activities as suggested by Zhang *et al*. (2013)[50]. Out of all the sequential extracts water extract of *E. agallocha* Linn. has shown very good DNA protective activity which was considered to be related with the flavonoid content[50].

Similar to our results, literature survey revealed that some of the Indian medicinal plant extracts *viz. K. paniculata*, *S. trifoliatus*, *A. scholaris* and *F. benjamina* provided good protection against the damage caused by .OH radicals[49]. In *Anthocephalus cadamba* all the sequential extracts have showed the potential to protect the plasmid DNA (pBR322) against the attack of hydroxyl radicals generated by Fentons reagent[51]. Also, *Curcuma aromatica* and *Curcuma zedoaria*

extracts protected the DNA at the concentration of 250µg/ml[52]. These DNA protection activities indicated that the extracts can be used as a potential source of natural antioxidants or neutraceuticals with potential application to reduce oxidative stress with consequent health benefits.

The expected DNA protection activity of *C. fragrans* was thought to be due its high antioxidant activity. DNA protection activity was not reported for *C. fragrans* till date, however the good antioxidant DPPH free radical scavenging activity, FRAP reducing potential was the indication that this plant will have good DNA protection activity as well. High correlation between total phenols content with H_2O_2 radical scavenging potential, phosphomolybdenum values and flavonoid content with H_2O_2 radical scavenging potential also support the idea that phenols and flavonoids may be the principal contributor of the antioxidant power in *C. fragrans*. Similar to the report of Attaguile *et al*. (2000)[53] in *C. fragrans* also flavonoid content was responsible for broad pharmacological properties like protection against the damaging action of free radicals.

Antioxidants prevent the deleterious consequences of oxidative stress due to which there is an increasing interest in exploring protective biochemical function of natural antioxidants from medicinal herbs. Antioxidant activity of *C. fragrans* has pointed to interesting antioxidant prospect exhibited by its extracts. It also reveals the mechanism of ROS scavenging and thereby affirms its role as an antioxidant. Results support the idea that these plants can be an effective source of natural antioxidants for medicinal applications. These antioxidant effects of *C. fragrans* emphasises on the need of further investigation of their other beneficial biological properties as such antioxidants are known to prevent cancer and cardiovascular diseases. This study has provided a basis for assessing the extracts further with respect to its anticancer potential. Further research is also needed to investigate what chemical compounds are present in this medicinal plant, especially in the extracts found with high antioxidant potential. Such study might direct us towards development of anticancer leads. This is the first report of antioxidant activity in crude and sequential extracts of *C. fragrans*.

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