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Total phenolic content, flavonoid content and *in vitro* antioxidant activities of *Cressa cretica* Linn.

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ABSTRACT

The total phenolic content (TPC) and total flavonoid content (TFC) of ethylacetate fraction (Fr-Et) and methanol fraction (Fr-Me) of an Indian ayurvedic plant Cressa cretica Linn were measured using Folin–Ciocalteau and aluminum chloride colorimetric methods, respectively. The in vitro antioxidant activities were also investigated by using in vitro antioxidant models including 2,2´-azinobis–(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$), 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO), hydroxyl radical (OH^-), super oxide (SO) and reducing power (Fe^{3+} to Fe^{2+} transformation) assay. The TPC were 7.081±1.033 and 12.833±0.24 mg gallic acid equivalents (GAE)/g extract, while TFC were 6.664±0.985, 11.979±1.049µg quercetin equivalents (QRT)/g extract sample for (Fr-Me and (Fr-Et), respectively. Analysis of the free radical scavenging activities of the fractions revealed a concentration-dependent antiradical activity resulting from reduction of $ABTS^+$, DPPH, NO, OH^- and SO radicals to non-radical form. The scavenging activity of ascorbic acid, a known antioxidant used as positive control, was however higher and scavenging potential was in the order: ascorbic acid > Fr-Et > Fr-Me. The reducing power of ascorbic acid, Fr-Et, Fr-Me increased gradually with increasing concentration. The order of the reduction potential was ascorbic acid > Fr-Et > Fr-Me. These results obtained in the present study indicate that C.cretica fractions can be a potential source of natural antioxidant with strong antiradical capacity.

Key words: *Cressa cretica*; total phenolic content; total flavonoid content; antiradical activity; reducing power.

INTRODUCTION

Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS), which are formed under normal physiological conditions but become deleterious when not being eliminated by the endogenous systems. In fact, oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant

systems. ROS are major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro* and create oxidative stress which results in numerous diseases and disorders [1-2] such as cancer [3], cardiovascular disease and asthma[4], Alzheimer's disease [5], parkinsons disease [6], ulcerative colitis [7], arthrerosclerosis[8]. Oxygen derived free radicals such as superoxide anions, hydroxyl radicals and hydrogen peroxide are cytotoxic and give rise to tissue injuries [9]. In addition, oxidative stress causes inadvertent enzyme activation and oxidative damage to cellular system [10].

Cells are equipped with different kinds of mechanisms to fight against ROS and to maintain the redox homeostasis of cell. For example, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) play important roles in scavenging the free radicals and preventing cell injury [11]. Molecules such as ascorbic acid and α-tocopherol inhibit lipid peroxydation in cell. When the mechanism of antioxidant protection becomes unbalanced in human body, antioxidant supplement may be used to help reduce oxidative damage.

Medicinal plants are an important source of antioxidants[12]. Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of certain diseases such as cancer, heart diseases and stroke[13]. The secondary metabolites like phenolics and flavonoids from plants have been reported to be potent free radical scavengers. They are found in all parts of plants such as leaves, fruits, seeds, roots and bark [14]. There are many synthetic antioxidants in use. It is reported, however, they have several side effects [15], such as risk of liver damage and carcinogenesis in laboratory animals [16 - 18]. There is therefore a need for more effective, less toxic and cost effective antioxidants. Medicinal plants appear to have these desired comparative advantages, hence the growinginterest in natural antioxidants from plants.

Cressa cretica L. (Convolulaceae), popularly known as 'Rudanti' in Hindi and is a widely grown halophytic plant. Different parts of the plant have been claimed to be valuable in a wide spectrum of diseases [19- 21]. In earlier studies *Cressa cretica* Linn flowers exhibited cytotoxic and anti-inflammatory activity in vitro [22]. *Cressa cretica* is reported to be antibilous, antituberculosis, and expectorant [23].The whole aerial part is proved to have be anti-tussive potential [24]. Shahat *et al.*, 2004 [25] yielded five flavonoids (quercetin, quercetin-3-O-glucoside, kaempferol- 3-O-rhamnoglucoside, and rutin) from the aerial parts of *Cressa cretica*. It is also reported the fruits of *Cressa cretica* is a potential source of edible oil. The oil of *C.cretica* was free from any undesirable components and could safely be recommended for human consumption. In addition the antiviral activity from the plant was reported [26]. It is already reported that the areal parts of the plant contains scopoletin, isoflavone glycoside coumaranochrome glycoside [27]. Syringaresinol glucoside and dicaffeoyl quinic acid were also isolated [25].

The aim of the present work was to evaluate the total phenolics content, flavonoid content of ethyl-acetate, methanol fractions of *C.cretica* and to evaluate its *in vitro* antioxidant potential. Because different antioxidant compounds may act *in vivo* through different mechanisms, no single method can fully evaluate the total antioxidant capacity. For this reason, several complimentary test systems, including 2,2´-azinobis – (3-ethylbenzothiazoline-6-sulfonic acid),

1,1-diphenyl-2-picrylhydrazyl, nitric oxide, hydroxyl radical, super oxide and reducing power assay methods were used in this study.

MATERIALS AND METHODS

Plant material and extract preparation: Fresh aerial parts of *Cressa cretica*, were collected from Nalban island of Chilika lake, Orissa, India and was preliminarily identified by Dr. M. Brahmam, Senior Scientist, Natural product division, Institute of Mineral and Material Technology, (formerly known as Regional Research laboratory, Bhubaneswar) India and which was later on confirmed from Botanical Survey of India, Howrah, West Bengal, India (CNH/I-I/32/2010/Tech.II/237 – 3). A voucher specimen has been kept in our laboratory for future reference. The aerial parts were air-dried, pulverized to a coarse powder in a mechanical grinder, passed through a 40-mesh sieve and extracted in a soxhlet extractor with methanol. The extract was decanted, filtered with Whatman No. 1 filter paper and concentrated at reduced pressure below 40◦C through rota-vapor (Rotavapor RII, Buchi Labortechnik AG, Switzerland) to obtain dry extract (16.73%w/w). *Cressa cretica* methanolic extract (CME) was adsorbed on to the 250 g of silica gel of 60–120 mesh size and fractionated using solvents of increasing polarity such as hexane (Fr-He), ethylacetate (Fr-Et), and methanol (Fr-Me). The fractions were subjected for preliminary phytochemical screening to show the presence of steroid, alkaloid, glycoside, tannin, triterpenoid, carbohydrates reducing sugar, and fatty acids according to standard methods [28-29]. Since Fr-He is devoid of flavonoids, phenols, steroids and glycosides, so further studies were carried out with Fr-Et and Fr-Me.

Total Phenolic content (Folin-Ciocalteau assay): Total phenolic contents of Fr-Et/ Fr-Me fractions were determined using Folin–Ciocalteu assay [30]. Briefly, 100 mg of fractions were individually dissolved in 10 ml of methanol. Then, 0.1 ml of these solutions was mixed with 2.5 ml of 10-fold diluted Folin–Ciocalteau reagent, and 2.0 ml of 7.5% sodium carbonate (Na_2CO_3). After incubation at 40 ºC for 30 min, the absorbance of the reaction mixtures was measured at 760 nm by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). Gallic acid was used as a standard and TPC of the extracts were expressed in milligram gallic acid equivalents (mg GAE/g extract).

Total flavonoid content: Total flavonoid content was determined by the aluminium calorimetric method [31], using quercetin as a standard. Briefly, the test samples were individually dissolved in DMSO. Then, the sample solution (150 µl) was mixed with 150 µl of 2% $AlCl_3$. After 10 min of incubation at ambient temperature, the absorbance of the supernatant was measured at 435 nm by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). Three replicates were made for each test sample. The total flavonoid content was expressed as quercetin equivalents in microgram per gram extract (µg QRT/g extract).

In vitro antioxidant assays

ABTS assay: The antioxidant potential was measured by 2,2´-azinobis – (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay that measures the relative ability of antioxidant substances to scavenge the $ABTS^+$ cation radical generated in the aqueous phase. 3.5 ml reaction mixture contained 0.17 mM ABTS, 25 – 250 µg/ml Fr-He/ Fr-Et/ Fr-Me / ascorbic

acid and phosphate buffer (pH 7.4). The method used was based on Miller and Evans 1996 [32] modified by Lister and Wilson 2001[33]. The absorbance at 734 nm was measured using UV–vis spectrophotometer. The antioxidant capacities of samples were measured against the standard.

Determination of DPPH radical scavenging activity: The free radical scavenging activity was evaluated by the DPPH assay [34]. In its radical form, DPPH absorbs at 517nm, but upon reduction by an antioxidant or a radical species, the absorption decreases. Briefly, 1ml of 0.25mM solution of DPPH in methanol was added to 1ml of Fr-Et/ Fr-Me solution in methanol (25 – 250 µg/ml). After 20min, the absorbance was measured at 517nm. Ascorbic acid was used as a positive control. The percentage DPPH decolorisation of the sample was calculated by the equation, % of DPPH scavenging = $[(A_{control} - A_{extarct})/ A_{control}] \times 100$
Where A is the absorbance

Nitric oxide radical scavenging (NO) assay: The nitric oxide radical inhibition activity was measured [35] using Griess reagent. Briefly, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations of Fr-Et/ Fr-Me and ascorbic acid dissolved in methanol and incubated at room temperature for 150 min followed by addition of 0.5 ml of Griess reagent (1% sulfanilamide, 2 % H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride). The absorbance of the chromophore formed was read at 546 nm.

Hydrohyl radical scavenging (OH) assay: Hydroxyl radical scavenging activity was determined based on the ability to compete with deoxyribose for hydroxyl radicals [36]. Hydroxyl radicals produced by the reduction of H_2O_2 by iron, in presence of ascorbic acid degrade deoxyribose to form products, which on heating with 2-thiobarbituric acid (TBA) form a pink colored chromogen. Briefly, the reaction mixture, of a final volume of 1.0ml, containing 0.4 ml of 20mM sodium phosphate buffer (pH 7.4) , 0.1 ml of 25 – 250 µg/ml of Fr-He/ Fr-Et/ Fr-Me, 0.1 ml of 60 mM deoxyribose, 0.1 ml of 10 mM H_2O_2, 0.1 ml of 1 mM ferric chloride, 0.1 ml of 1 mM EDTA and 0.1 ml of 2 mM ascorbic acid, was incubated at 37º C for 1h. The reaction was terminated by the addition of 1 ml of 17 mM TBA and 1 ml of 17 mM trichloroacitic acid(TCA). The mixture was boiled for 15 min, cooled in ice, and the absorbance measured at 532 nm. Ascorbic acid was used as a positive control. Distilled water in place of test fractions or ascorbic acid was used as control and the sample solution without deoxyribose as sample blank.

Superoxide radical scavenging (SO) assay: The super oxide anion scavenging activity was determined by the method of Nishimiki *et al.* 1972 [37]. SO anion derived from dissolved oxygen by a phenazine methosulfate(PMS)/NADH coupling reaction reduces nitroblue tetrazolium (NBT), which forms a violet colored complex. A decrease in color after addition of the antioxidant is a measure of its superoxide scavenging activity. To the reaction mixture containing phosphate buffer (100 mM, pH 7.4), NBT (1mM) solution, NADH (1mM) and Fr-Et/ Fr-Me (25 – 250 µg/ml) in methanol, 1ml of 1mM PMS was added. After incubation at 25ºC for 5 min, the absorbance was measured at 560 nm against a blank. Ascorbic acid was used as a positive control.

Ferric reducing antioxidant power (FRAP) assay: The reductive potential was determined based on the chemical reaction of Fe^{3+} to Fe^{2+} [38]. To 100 – 500 µg/ml Fr-Et/ Fr-Me and

ascorbic acid standard in 1 ml of methanol, 2.5 ml each of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1% w/v) was added and the mixture incubated at 50 ºC for 20 min, followed by addition of 2.5 ml of TCA (10% w/v). The mixture was centrifuged for 10 min at 1000g, the upper layer (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1% w/v), and the absorbance was measured at 700 nm.

Statistical analysis: The data are expressed as Mean ± Standard Deviation (S.D.). All data were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test with equal sample size. The difference was considered significant when *p* value < 0.05.

RESULTS

Phytochemical screening: The results of the preliminary phytochemical screening of the Fr-He revealed the presence of fatty acids and fixed oils. Fr-Et showed the positive results for steroids, terpenes, flavonoids ,tannins, glycosides, carbohydrates etc and Fr-Me revealed the presence of tannins, flavonoids, phenols.

Total phenolic content and total flavonoid content: The content of phenolics compounds in the different fractions were determined through a linear gallic acid standard curve (y = 0.019x + 0.149, r^2 = 0.996). The total phenolic content of the fractions varied form 7.081± 1.033 to 12.833± 0.24 mg GAE /g extract. Fr-Et found to have more phenolic content than Fr-Me. The total phenolics in Fr-Me was 7.081 mg GAE/g and in Fr-Et it was 12.833 mg GAE/g. Total phenolics content of the fractions is arranged in the following ascending order: Fr-Me < Fr-Et ($p<0.01$) (Table 1). In this study, the total flavonoid content (TFC) of the fractions was evaluated by aluminum colorimetric assay. Quercetin (QRT) was used as a standard and the total flavonoid content of *C.cretica* fractions were expressed in microgram of quercetin equivalents per gram of extract (µg QRT/g extract) (y=0.006x + 0.152, r^2 = 0.987). The data presented in Table 1 indicates that the highest flavonoid content of 11.979µg QRT/g extract was observed in the Fr-Et and comparatively lower content was observed in Fr-Me (6.664µg QRT/g extract) ($p<0.001$). TFC of the fractions is arranged in the following sequence: Fr-Et> Fr-Me ($p<0.01$).

Table 1: Total phenolics content and total flavonoid content of *C. cretica* extracts (n=3)

Sl. No.	Samples (extracts)	Total Phenolic content (mg GAE/g extract)	Total flavonoid content (µg QRT/g extract)
1	Fr-Me	7.081±1.033	6.664± 0.985
2	Fr-Et	12.833±0.24 a***	11.979± 1.049 a***

*Values are expressed as mean ± standard deviation (S.D.) from triplicate determination; a: Fr-Et compared to Fr-Me; *** $p<0.001$.*

***In vitro* antioxidant assay:** Analysis of the free radical scavenging activities of the fractions revealed a concentration-dependent antiradical activity resulting from reduction of $ABTS^+$ (fig. 1), DPPH (fig. 2), NO (fig. 3), OH^- (fig. 4) and SO (fig. 5) radicals to non-radical form. The scavenging activity of ascorbic acid, a known antioxidant used as positive control, was however higher and scavenging potential was in the order: ascorbic acid > Fr-Me > Fr-Et Fig. 6 presents the reduction potential of Fr-Et and Fr-Me.

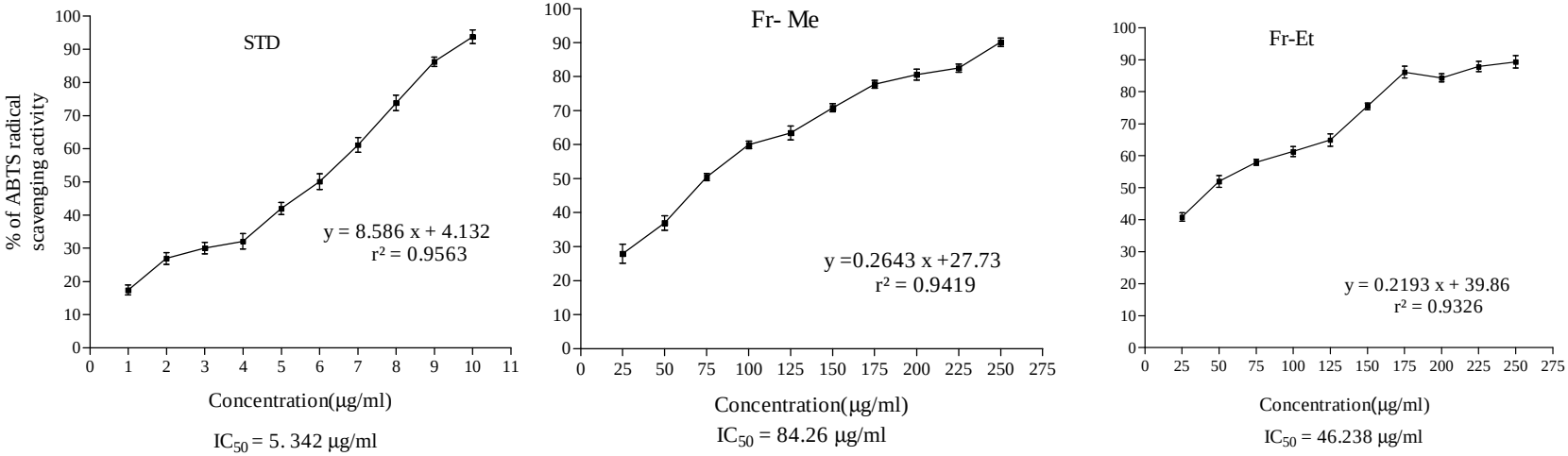

Fig. 1: Inhibition of ABTS radical by STD (Ascorbic acid), Fr-Me and Fr-Et; Data are represented as mean ± S.D. of two independent experiments each.

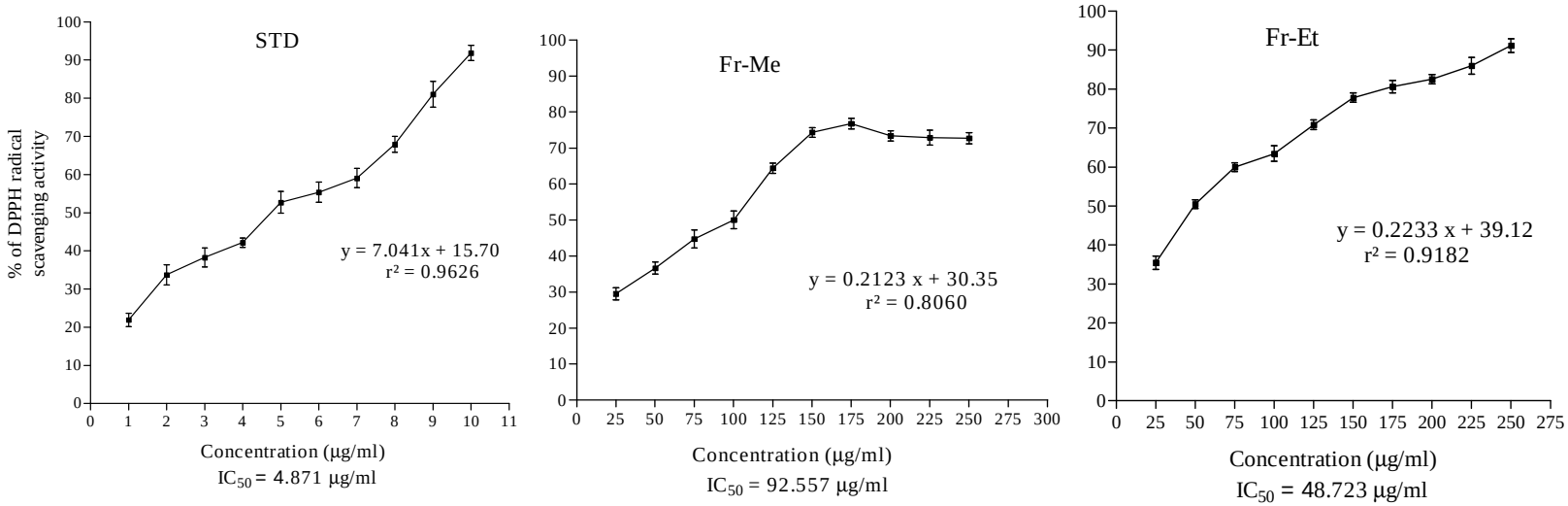

Fig. 2: Inhibition of DPPH radical by STD (Ascorbic acid), Fr-Me and Fr-Et; Data are represented as mean ± S.D. of two independent experiments each.

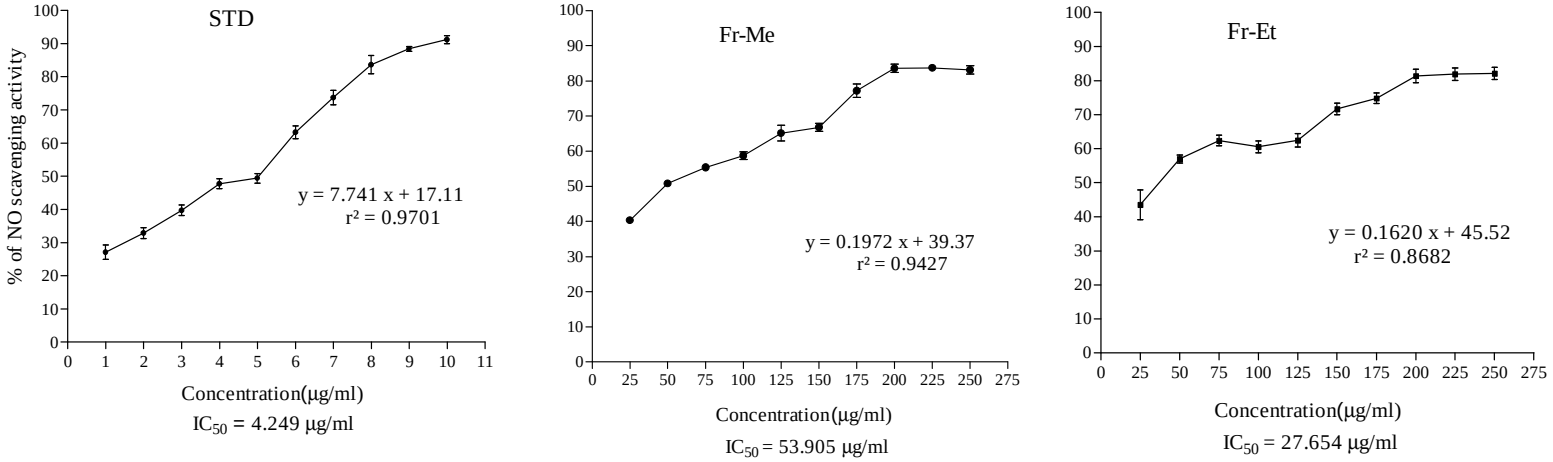

Fig. 3: Inhibition of nitric oxide radical by STD (Ascorbic acid), Fr-Me and Fr-Et; Data are represented as mean ± S.D. of two independent experiments each.

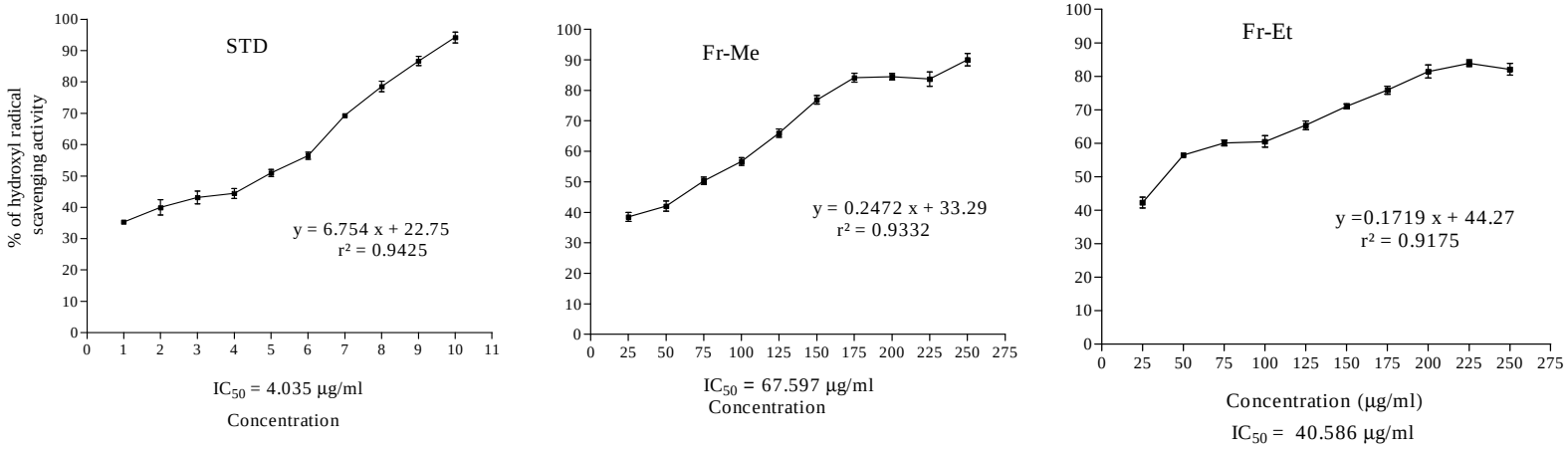

Fig. 4: Inhibition of hydroxyl radical by STD (Ascorbic acid), Fr-Me and Fr-Et; Data are represented as mean ± S.D. of two independent experiments each.

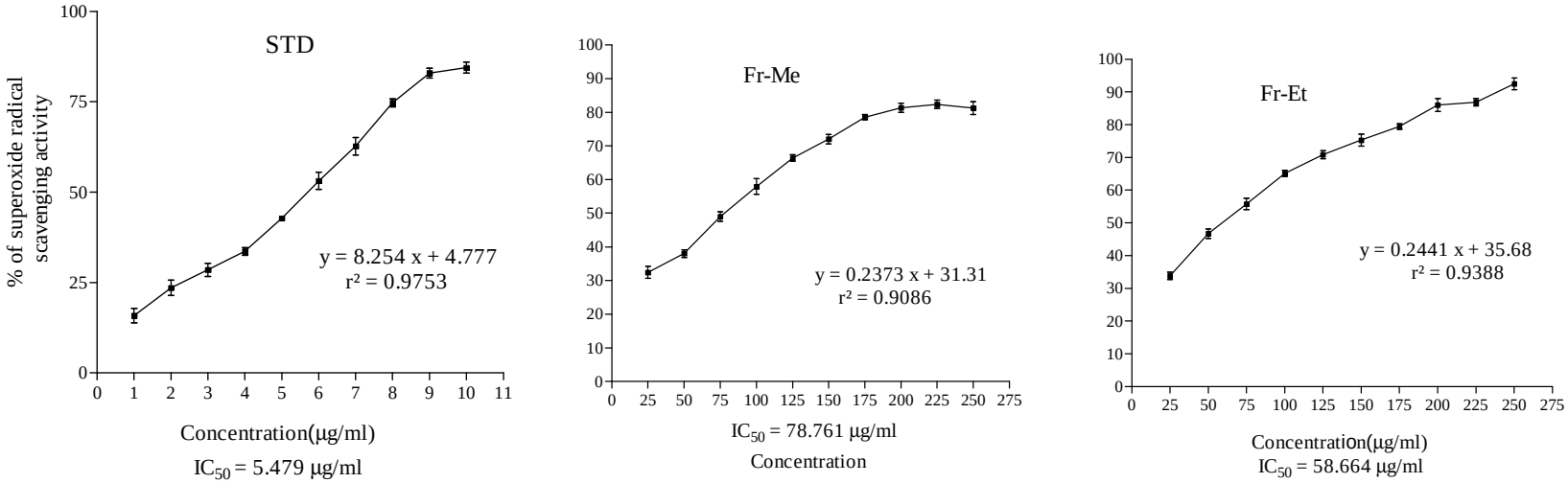

Fig. 5: Inhibition of super oxide radical by STD (Ascorbic acid), Fr-Me and Fr-Et; Data are represented as mean ± S.D. of two independent experiments each.

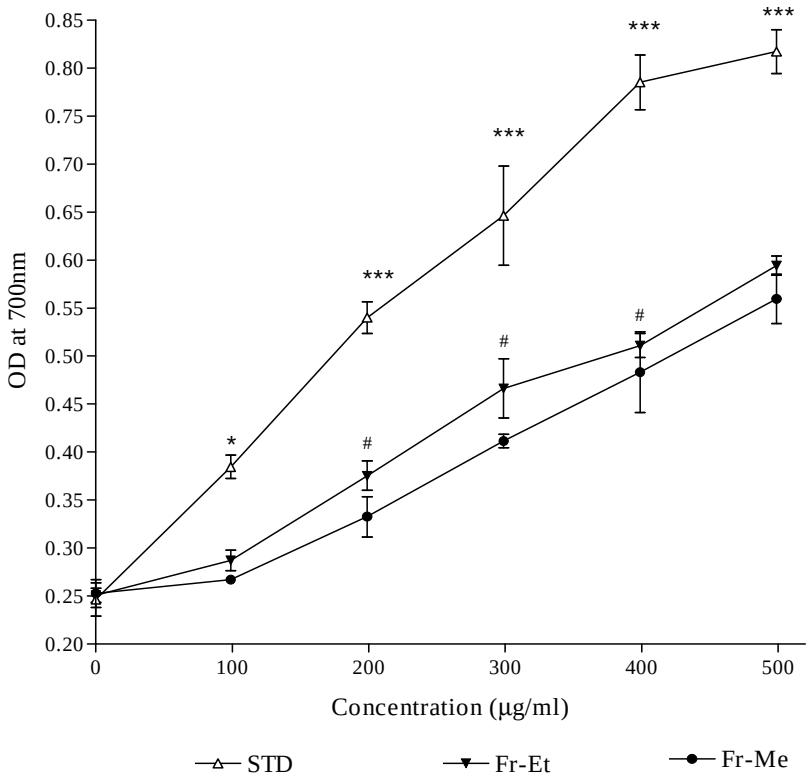

Fig. 6: The reduction potential of STD (Ascorbic acid) and Fr-Et, Fr-Me (mean ± S.D.; n = 6);
: significantly different from Fr-Et, Fr-Me (p<0.05); *: significantly different from Fr-Et, Fr-Me (p<0.001); #: Fr-Et significantly different form Fr-Me (p<0.05).*

The reducing power of ascorbic acid, Fr-Et, Fr-Me increased gradually with increasing concentration. The order of the reduction potential was ascorbic acid > Fr-Et >Fr-Me.

DISCUSSION

These results suggest that the higher levels of antioxidant activity were due to the presence of phenolic components. The same relationship was also observed between phenolics and antioxidant activity in roseship fractions [39]. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups [40]. The phenolic compounds may contribute directly to antioxidative action [41]. It is known that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when ingested up to 1 g daily from a diet rich in fruits and vegetables [42]. Phenolic compounds from plants are known to be good natural antioxidants. However, the activity of synthetic antioxidants was often observed to be higher than that of natural antioxidants [43]. Phenolic compounds, at certain concentrations, markedly slowed down the rate of conjugated diene formation. The interests of phenolics are increasing in the food industry because they retard oxidative degradation of lipids and there by improve the quality and nutritional value of food [44]. The 2, 2´ -azinobis (3-ethyl-benzothiazoline 6-sulfonate) (ABTS) formed from the reaction ABTS-e → $ABTS^+$ reacts quickly with ethanol/hydrogen donors to form colorless ABTS. The reaction is pH – independent. A decrease of the $ABTS^+$ concentration is linearly dependent on the antioxidant

concentration. All fractions at tested doses (25 – 250 µg/ml) revealed good scavenging activity for $ABTS^+$ in a dose dependent manner, but the activity was higher in case of Fr-Et (IC_{50} = 46.238 µg/ml)than Fr-Me (IC_{50} = 84.260 µg/ml) (Fig. 1). The DPPH radical is considered to be a model for a lipophilic radical. A chain in lipophilic radicals was initiated by the lipid autoxidation. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [45]. The reduction capability of DPPH was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. Positive DPPH test suggests that the samples were free radical scavengers. The scavenging effect of Fr-Et and ascorbic acid on DPPH radical was compared. On the DPPH radical, Fr-Et had significant scavenging effects with increasing concentration in the range of 25–250 µg/ml and when compared with that of ascorbic acid, the scavenging effect of Fr-Me was lower. The IC_{50} values were found to be 48.723, 92.557 µg/ml for Fr-Et, Fr-Me respectively (Fig.2). A higher DPPH radical-scavenging activity is associated with a lower IC_{50} value. Nitric oxide plays an important role in various types of inflammatory processes in the body. In the present study the fractions of the *C.cretica* aerial parts were checked for its inhibitory effect on nitric oxide production. Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by the fractions. The Fr-Et at varied concentrations showed remarkable inhibitory effect of nitric oxide radical- scavenging activity compared to Fr-Me (Fig. 3). Results showed the percentage of inhibition in a dose dependent manner for all the fractions tested. The concentration of Fr-Et needed for 50% inhibition (IC_{50}) was found to be 27.654µg/ml, whereas 53.905µg/ml was needed for Fr-Me respectively. The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells [46-47]. This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity [48-49]. The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins [29]. The effect of the fractions from *C.cretica* on the inhibition of free radical-mediated deoxyribose damage were assessed by means of the Iron (II)-dependent DNA damage assay. The Fentone reaction generates hydroxyl radicals (OH) which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component. Oxygen radicals may attack DNA either at the sugar or the base, giving rise to a large number of products. Ascorbic acid was highly effective in inhibiting the oxidative DNA damage. As shown in Fig. 4, the fractions displayed potential inhibitory effect of hydroxyl radical-scavenging activity. All results showed hydroxyl radical scavenging activity in dose dependent manner. IC_{50} values were found to be 40.586, 67.597 µg/ml for Fr-Et and Fr-Me respectively (Fig. 4). The ability of the above mentioned fractions to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of active oxygen species, thus reducing the rate of the chain reaction. Ascorbic acid was used as reference standard. It is explained that high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical-scavenging activity by phenolics than their specific functional groups [49]. Super oxide is a reactive oxygen species, which can cause damage to the cells and DNA leading to various diseases. It was therefore proposed to measure the comparative interceptive ability of the antioxidant fractions to scavenge the super oxide radical. Several *in vitro* methods are available for generation of super oxide radicals [50]. In our study super oxide radicals were generated by auto-oxidation of hydroxylamine in presence of

NBT (Nitro blue tetrazolium). The reduction of NBT in presence of antioxidants was measured. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. As shown in Fig. 5, the all fractions at varied concentrations had superoxide scavenging activity. IC_{50} values of Fr-Me, Fr-Et and ascorbic acid were found to be 78.761, 58.662 and 5.479µg/ml, respectively. All of the fractions had a scavenging activity on the superoxide radicals in a dose dependent manner (25–250 µg/ml in the reaction mixture). Nonetheless, when compared to ascorbic acid, the superoxide scavenging activity of the extract was found to be low. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom [41]. The results of the ferric reducing assay indicated that Fr-Et had stronger reducing power than Fr-Me (Fig. 6). This could be due to the presence of more reactive concentration of bioactive constituents in Fr-Et than Fr-Me.

In the present study, there exists a positive correlation between the total phenolics content and the antioxidant activity which is in accordance with the earlier findings [51]. We found higher *in vitro* antioxidant activity in Fr-Et with higher polyphenols. The higher radical scavenging efficacy of Fr-Et may be due to retention of antioxidant phytochemicals in this extract.

Moreover, these results suggest that *Cressa cretica* may offer effective protection from free radicals and support that *C.cretica*, is a promising source of natural antioxidant. However, further work is required on the isolation and identification of the antioxidant components present in it since it is also a precondition for a more extensive understanding of the mechanisms involved in the antioxidant capacity.

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