International Scholars Journals

International Journal of Pharmacy and Pharmacology ISSN: 2326-7267 Vol. 4 (3), pp. 001-010, March, 2015. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Effect of fractionation on antiradical efficacy of ethyl acetate extract of *Terminalia chebula* Retz.

Harpreet Walia[1], Rajbir Singh[1], Subodh Kumar[2] and Saroj Arora[1]*

[1]Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar-143005, Punjab, India.
[2]Department of Chemistry, Guru Nanak Dev University, Amritsar-143005, Punjab, India.

Accepted 12 October, 2014

There is currently an upsurge of interest in phytochemicals as new sources of natural antioxidants. The aim is to use them in foods and pharmaceutical preparations in order to replace synthetic antioxidants, which are being restricted due to their potential health risks and toxicity. The relative levels of antioxidant activity and the total phenolic content of ethyl acetate extract/fractions of *Terminalia chebula* have been determined using DPPH, Deoxyribose, Reducing power, Chelating power, Lipid peroxidation, DNA nicking assay and the Folin-Ciocalteu colorimetric method. There was a positive linear correlation between antioxidant activity and total phenolic content of ethyl acetate extract/fractions. Thus, it was concluded that phenolic compounds were the predominant antioxidant components in the fruits of *T. chebula*. The extract was prepared by maceration method at room temperature and further fractionated with ethyl acetate and water. It was observed that the free radical scavenging activity of fractions was comparatively more as compared to their crude extract and ethyl acetate fraction showed the maximum effect in all assays. The percent inhibition with ethyl acetate fraction (EAF) of ethyl acetate extract was observed to be 71.5, 90.0, 75.1, 74.3 and 77.6% in DPPH, site specific and non-site specific deoxyribose, chelating power and lipid peroxidation assays, respectively at maximum concentration tested.

Key words: *Terminalia chebula,* oxidative stress, phytochemicals, antioxidants, lipid peroxidation.

INTRODUCTION

In physiological system, redox regulation is a vital process for homeostasis and presently studies are focused to understand its detailed mechanism. Normally a balance in the level of ROS/RNS by endogenous and exogenous antioxidant defense system is maintained but the excessive production of ROS/RNS is deleterious to tissues as there is consensus of opinion that free radicals induce oxidative damage to biomolecules (Halliwell and Gutteridge, 1989). This damage causes atherosclerosis, aging, cancer and several other diseases (Aruoma, 1998). Moreover, free radicals are known to take part in lipid peroxidation in foods, which is responsible for rancid odours and flavours, which decrease the nutritional quality.

Nowadays, the scientists have casted some toxicological doubts on synthetic antioxidants due to their adverse side effects and people are more concerned about food safety and quality. Thus, attention is now increasingly paid to the development and utilization of more effective and non-toxic antioxidants of natural origin (Kusirisin et al., 2009; Servili et al., 2009).

In the recent years, food scientists and nutrition specialists agree that antioxidant enriched fruits and vegetables, consumed daily contribute to reducing risks of certain diseases including cancer and cardio and cerebro-vascular diseases (Liu et al., 2000; Guorong et al., 2009). They can scavenge radicals by inhibiting initiation and breaking chain propagation or suppressing formation of free radicals by binding to the metal ions, reducing hydrogen peroxide and quenching superoxide and singlet oxygen. So they are supposed to play an important role in the prevention of these diseases.

Terminalia chebula a native plant in India and Southeast

*Corresponding author. E-mail: jrosh1@rediffmail.com.

Abbreviations: CE, crude extract; **SP,** supernatant; **PP,** precipitates; **EAF,** ethyl acetate fraction; **WF,** water fraction.

Asia is extensively cultivated in Taiwan is rich in polyphenolic compounds. According to Indian Mythology, this plant originated from the drops of ambrosa (Amrita), which fell on the earth when Indra was drinking it (Srikanthmurthy, 2001). The fruits of *T. chebula* are known as black myroblan is being used for the treatment of different types of diseases and disorders since antiquity. The plant has been studied for its promising antimicrobial, antioxidant and antimutagenic properties (Saleem et al., 2002; Chen et al., 2003). It was also reported that oral administration of the extracts from *T. chebula* reduced the blood glucose level in normal and in alloxan-diabetic rats (Sabu and Kuttan, 2002). Keeping in view the immense importance of the plant, the present study was planned to evaluate the antioxidant activity of ethyl acetate extract/fractions of fruits of *T. chebula*.

MATERIALS AND METHODS

Chemicals

Deoxyribose was purchased from Lancaster. Thiobarbituric acid was procured from Sigma Aldrich USA. Other chemicals like ferrozine, FC reagent, potassium ferricyanide, ferric chloride, EDTA, hydrogen peroxide, L-ascorbic acid, Sodium hydroxide, BHA, trichloroacetic acid and other solvents were procured from CDH and were of analytical grade.

Extraction/fractionation procedure

The fruits of *T. chebula* were purchased locally from the market. It was washed with tap water, dried in oven at 40°C and ground to a fine powder. To 1000 g of fruit powder 1500 ml of ethyl acetate was added. The supernatant was collected, filtered by using Whatman sheet no.1 and evaporated through rotary evaporator to have the dry crude ethyl acetate extract (CE). CE was further partitioned by dissolving in methanol and after some time the precipitates (PP) were formed. Both PP and SP were separated and dried at room temperature separately. The dried SP was further fractioned in water and then in ethyl acetate resulted in formation of two layers namely ethyl acetate fraction (EAF) and water fraction (WF). These layers were separated and dried at room temperature separately (Flowchart 1).

Determination of total phenolic content

The total phenolic content of the extract was determined using Folin-Ciocalteu method (Yu et al., 2002). To 100 l of extract/ fractions was added 900 l of water. To this 500 l of FC reagent was added. This was followed by the addition of 1.5 ml of 20% sodium carbonate. The mixture was shaken thoroughly and allowed to stand for 2 h. The volume of mixture was made up to 10 ml with distilled water and absorbance was observed at 765 nm. The phenolic content was calculated as gallic acid (mg/g) equivalents.

Spectroscopic analysis of extract

The crude ethyl acetate extract (CE) of *T. chebula* was analyzed by UV spectroscopy by dissolving in spectroscopic grade methanol in the concentration of 1 mg/10 ml and spectrum was recorded on UV-visible spectrophotometer (Shimadzu-1601).

Antioxidant testing

The ethyl acetate extract (CE), supernatant (SP), precipitates (PP) and two fractions that is, ethyl acetate fraction (EAF) and water fraction (WF) were tested for their antioxidant potential by using following *in vitro* assays.

DPPH assay

The H-donor activity of the ethyl acetate extract/fractions was measured by 1, 1 diphenyl - 2 - picrylhydrazyl (DPPH) method given by Blois (1958). The reaction mixture contained 200 µl of different extract/fractions concentrations (50 – 300 µg/ml) and 2 ml of DPPH (0.1 mM in methonolic solution). The reaction mixture was observed at 517 nm against a blank, which did not contain extract. The percentage inhibition was calculated as:

$$\% \text{ Inhibition} = [(B_0 - B_1) / B_0] \times 100$$

Where, B_0 is the absorbance of control, B_1 is the absorbance of reaction mixture.

Deoxyribose assay

This method was used to measure the hydroxyl radical scavenging activity of extract/fractions (Halliwell et al., 1987). This assay was performed in two ways that is, non-site specific and site-specific. In Non-site specific deoxyribose assay 0.1 ml of EDTA, 0.01 ml of $FeCl_3$, 0.1 ml of H_2O_2, 0.36 ml of deoxyribose, 1 ml of extract/fractions concentrations (10 - 100 µg/ml), 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid were added in sequence. The mixture was incubated at 37°C for 1 h. 1 ml of the incubated mixture was mixed with 1 ml of 10% trichloroacetic acid and 1 ml of thiobarbituric acid (0.025 M NaOH) and heated for 1 h on water bath at 80°C and pink chromogen developed, which was measured at 532 nm. In site-specific deoxyribose assay EDTA was replaced with phosphate buffer.
The percentage age inhibition was calculated as:

$$\% \text{ Inhibition} = [(B_0 - B_1) / B_0] \times 100$$

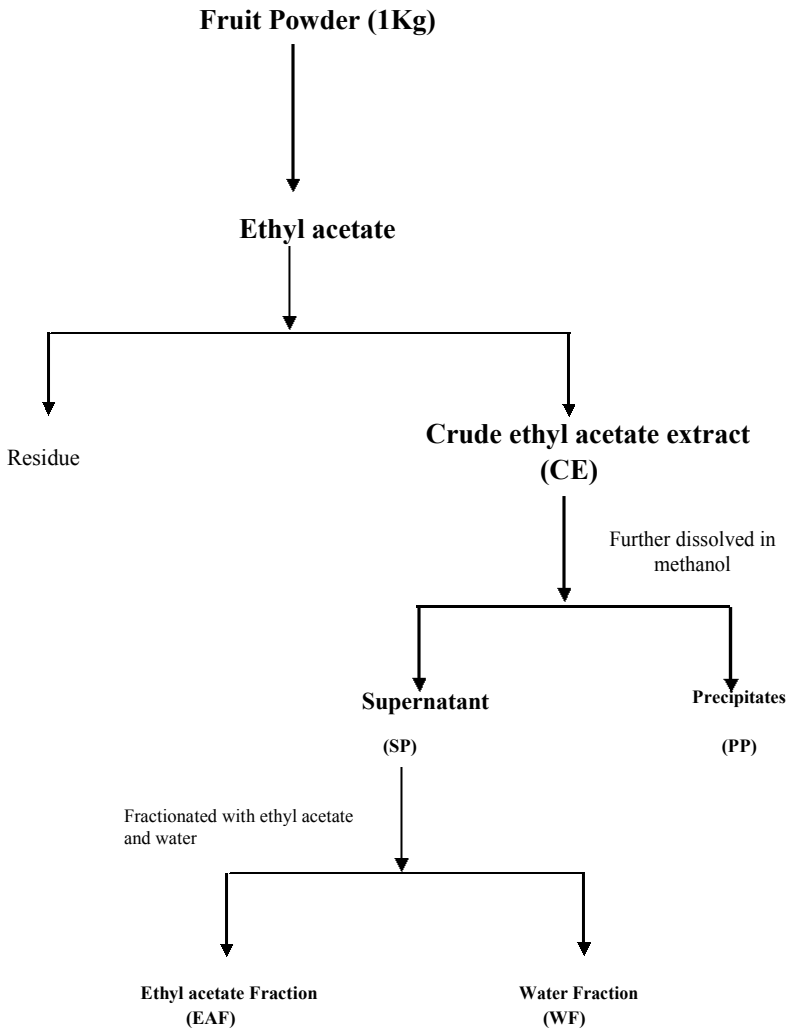
Where, B_0 is the absorbance of control, B_1 is the absorbance of reaction mixture.

Reducing power assay

This method is used to estimate the relative reducing activity of extract/fractions (Oyaizu, 1986) 1 ml of extract/fractions (50 – 300 µg/ml) was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. 2.5 ml of 10% tricholroacetic acid was then added to the mixture and centrifuged at 3000 rpm for 10 min. 1 ml of aliquot of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%) and absorbance was measured at 700 nm. Increase in absorbance was interpreted as increased reducing activity.

Lipid peroxidation assay

In this method, TBA reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm (Halliwell et al., 1989). Normal albino rats of the Wistar strain were used for the preparation of liver homogenate. The perfused liver was isolated and 10% (w/v) homogenate was prepared using a homogenizer at 0 - 4°C with 0.15 M KCl. The



Flow chart 1. Extraction/fractionation procedure.

homogenate was centrifuged at 3000 rpm for 15 min and clear cell-free supernatant was used for the study of *in vitro* lipid peroxidation. Different concentrations of extract/fractions mixed with 1 ml of 0.15 M KCl and 0.5 ml of rat liver homogenates were added to the test tubes. Peroxidation was initiated by adding 100 µl of 0.2 mM ferric chloride. After incubation at 37°C for 30 min, the reaction was stopped by adding 2 ml of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid (TCA) and 0.38% thiobarbituric acid (TBA) . The reaction mixture was heated at 80°C for 60 min. The samples were cooled, centrifuged and the absorbance of the supernatants was measured at 532 nm. The percentage inhibition was calculated as:

% Inhibition = $[(B_0-B_1) / B_0] \times 100$

Where, B_0 is the absorbance of control, B_1 is the absorbance of reaction mixture.

Chelating power assay

In this assay, 1 ml of extract/fractions with different concentrations was mixed with 3.5 ml of methanol and then the mixture was mixed with ferrous chloride (2 mM, 0.1 ml) and ferrozine (1 mM, 0.2 ml) for 10 min at room temperature. The absorbance was measured at 562 nm against a blank in which the extract was not added (Dinis et al., 1994). The percentage inhibition was calculated as:

% Inhibition = $[(B_0-B_1) / B_0] \times 100$

Where, B_0 is the absorbance of control, B_1 is the absorbance of reaction mixture.

DNA nicking assay

A DNA nicking assay was performed using supercoiled pBR322 plasmid DNA (Lee et al., 2002). Plasmid DNA (0.5 µg) was added to Fenton's reagents (30 mM H_2O_2 , 50 µM ascorbic acid and 80 µM $FeCl_3$) containing concentration of the extract/fractions and the final volume of the mixture was brought up to 20 µ l. The mixture was then incubated for 30 min at 37°C and the DNA was analyzed on a 1% agarose gel followed by ethidium bromide staining.

Statistical analysis

All the experiments were done in triplicates. The values are given as mean ± S.E.

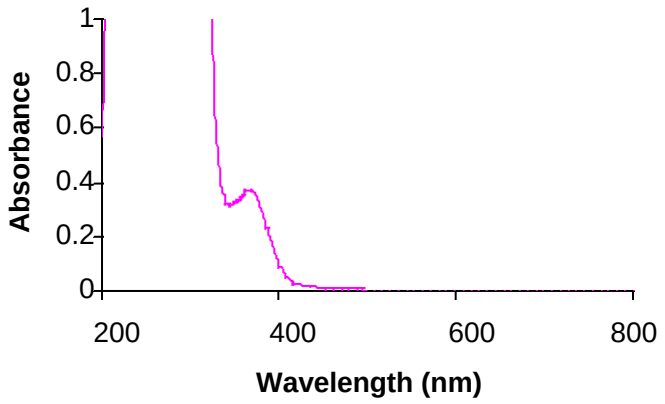


Figure 1. The UV-visible spectra of crude ethyl acetate extract (CE) of *Terminalia chebula.*

RESULTS AND DISCUSSION

Evaluation of the total antioxidant capacity of extracts cannot be performed accurately by any single method due to the complex nature of phytochemicals (Singh et al., 2007). Because multiple reaction characteristics and mechanisms would be involved so, no single assay could accurately reflect all antioxidants in a mixed or complex system. Many methods have been proposed to evaluate the antioxidant potential of natural sources of antioxidants. Reliable *in vitro* and *in vivo* methods to screen for antioxidant activity are therefore needed and many rapid tests are available, called 'direct' and 'indirect' methods (Huang et al., 2005; Prior et al., 2005; Roginsky et al., 2005).

Any method that does not involve a substrate to measure antioxidant activity are called indirect methods, which are generally used to measure the capacity of a molecule to reduce a stable artificial free radical (by hydrogen or electron transfer), or a transition metal (simply by electron transfer), 2,2-Diphenyl-1- picrylhydrazyl (Brand-Williams et al., 1995) and ferric reducing antioxidant power assays (Benzie and Strain, 1996) as well as cyclic voltammetry (Chevion et al., 2000; Kilmartin et al., 2001) are used in some of these indirect antioxidant determination methods. Conversely, direct evaluation methods involve an oxidizable substrate.

The oxidizable substrate usually consists of individual or mixed lipids, plant proteins, fluorophores, chromo-phores, DNA or fluids containing biologically active chemical species such as low- density lipoproteins and biological membranes. They are based on assessing the inhibitory effect of a potentially antioxidant substance on the oxidative degradation of a substrate in a test system subjected to natural or accelerated oxidation conditions. Some of these methods are deoxyribose assay, lipid peroxidation assay and DNA nicking assay. Keeping all this in mind, the present study was aimed to divulge the antioxidant activity of ethyl acetate extract/fractions of *T. chebula* by using various direct and indirect *in vitro* assays.

The total phenolic content in ethyl acetate extract/ fractions ranged from 168 - 235 mg/g of gallic acid. The highest total phenolic level was detected in ethyl acetate fraction (235 mg/g) . The CE, SP, PP and WF have 168, 207, 179 and 214 mg/g phenolic content respectively. The results suggest that the greater antioxidant activity of ethyl acetate fraction as compared to the crude extract at the same concentration may be due to the higher total phenolic content. UV analysis also concluded that the crude ethyl acetate extract exhibited maximum absorbance that is, $_{max}$ in region 364 nm which strongly points towards the presence of phenolic and polyphenolic compounds (Figure 1).

The hydrogen atom or electron donation abilities of the ethyl acetate extract and its fractions were measured from the bleaching of the purple-coloured methanol solution of 1, 1- diphenyl-2-picrylhydrazyl (DPPH). Figure 2 depicts the antioxidant potential of ethyl acetate extract/ fractions against DPPH radical. The ethyl acetate extract and its fractions exhibited dose response relationship up to 300 g/ml of concentration. It is clear from the figure that the ethyl acetate fraction (EAF) showed maximum inhibition against this stable radical that is, 71.5% at 500 g/ml of concentration. Among water and ethyl acetate fraction (EAF), the ethyl acetate fraction increases the percentage inhibition of crude extract (CE) from 61.2 to 71.5% at 500 g/ml of concentration. The supernatant (SP), water (WF) and precipitates (PP) showed 65.1, 68.3 and 58.2% respectively at the same concentration. Fractionation of crude extract in water and ethyl acetate also influenced the reaction time of the assay. For the crude extract the reaction time was 25 - 30 min but for fractions it was reduced to 10 - 15 min. The IC_{50} value was minimum for EAF that is, 49.2 µg/ml

Figure 3 describe the percentage inhibition of ethyl acetate extract and its fractions in deoxyribose assay using non-site specific and Figure 4 describe the percentage inhibition of ethyl acetate extract and its fractions in site-specific method. In this system, the extract/ fractions exhibited a stronger concentration dependent inhibition of deoxyribose oxidation up to 100 g/ml of concentration. The extract/fractions showed more inhibition in site specific as compared to non-site specific. This showed that the extract/fractions are not only good hydroxyl radical scavengers but they also had strong chelating activity. In both assay the ethyl acetate fraction (EAF) showed maximum inhibition of 75.1% in non-site specific and 90.0% in site-specific respectively at 100 g/ml of concentration. The order of percentage inhibition is same in both assay that is, EAF (75.1%) > WF (72.0%) > SP (70.3%) > CE (68.6%) > PP (56.2%) in non-site specific and EAF (90.0%) > WF (85.1%) > SP (80.5%) > CE (78.1) > PP (65.1%) in site-specific respectively at 100 g/ml of concentration. The ethyl acetate extract and its fractions showed more activity than gallic acid which

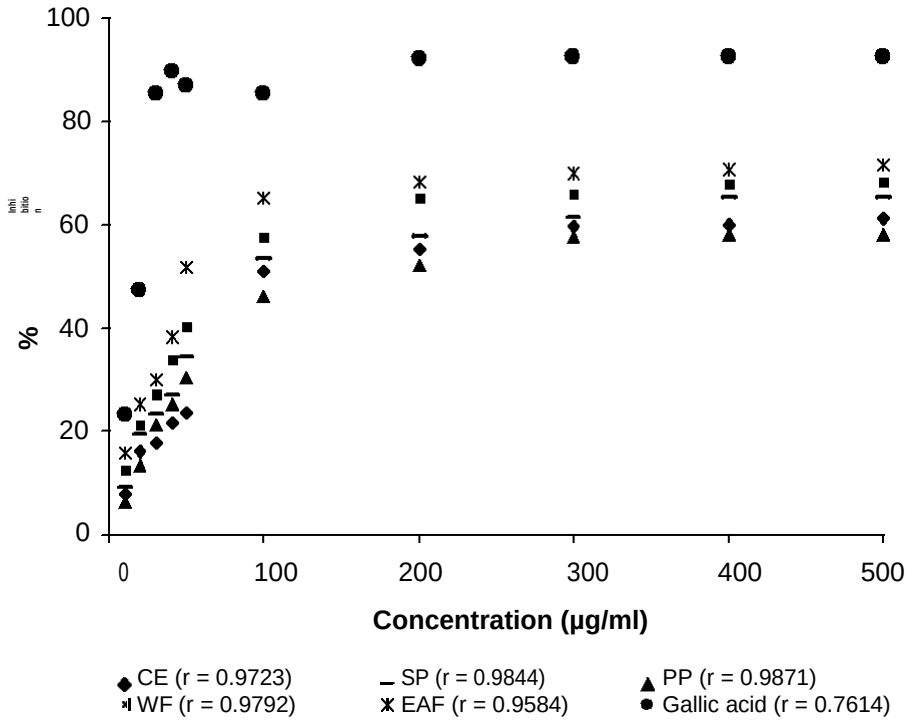


Figure 2. Effect of ethyl acetate extract/fractions of *Terminalia chebula* in DPPH assay. CE: Crude extract, SP: Supernatant, PP: Precipitates, WF: Water fraction, EAF: Ethyl acetate fraction.

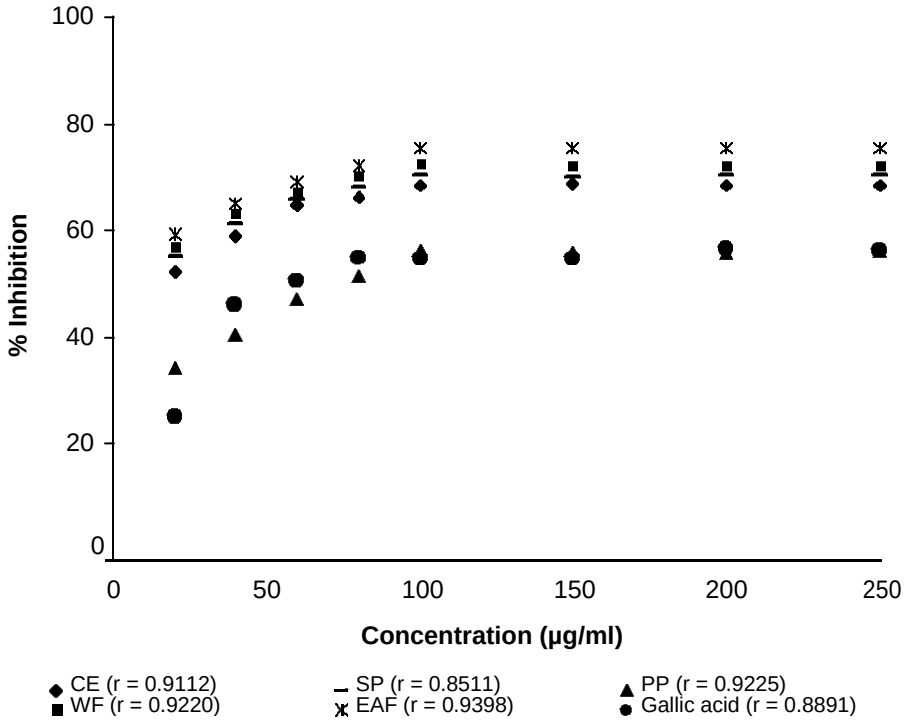


Figure 3. Effect of ethyl acetate extract/fractions of *Terminalia chebula* in Non-site specific deoxyribose assay.
CE, Crude extract; SP, Supernatant; PP, Precipitates; WF, Water fraction; EAF, Ethyl acetate fraction.

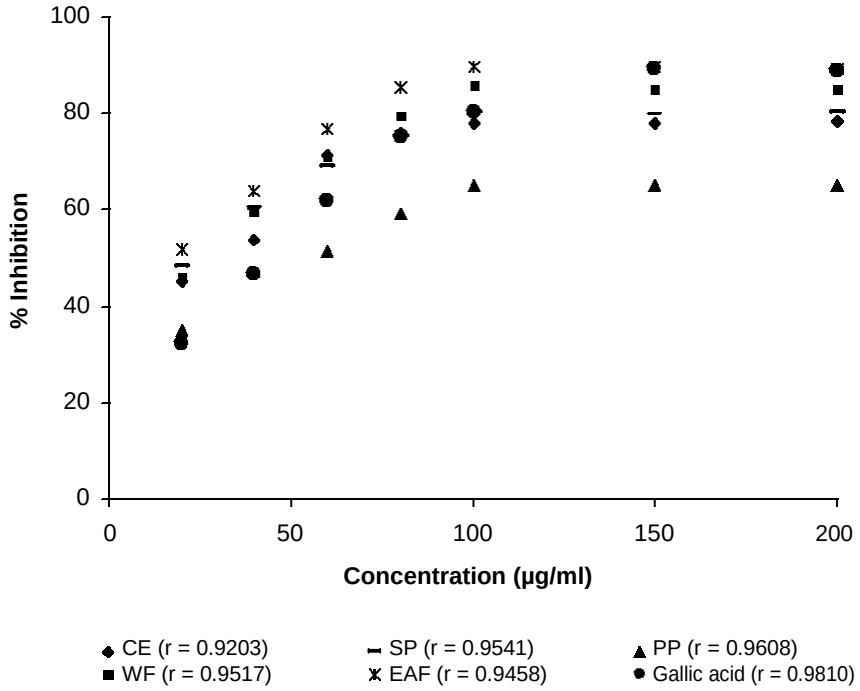


Figure 4. Effect of ethyl acetate extract/fractions of *Terminalia chebula* in Site-specific deoxyribose assay.
CE, Crude extract; SP, Supernatant; PP, Precipitates; WF, Water fraction; EAF, Ethyl acetate fraction.

was used as a standard. Furthermore, a comparatively high activity was noticed in site-specific assay than in non-site specific assay indicating the high chelating activity of the extracts/fractions. The presence of phenolic groups in extract/fractions could be responsible for $^{\cdot}OH$ radical scavenging activity.

The results indicated that the ethyl acetate extract/ fractions has more hydrogen donating ability, which may be due to the presence of polyphenolic glycosides as indicated by UV analysis which indicated the presence of phenolic compounds. Earlier, numerous workers (Halliwell et al., 1987; Pin-Der- Duh et al., 1999) have employed this system to assess the biological activity of various natural plant derived biomolecules. One reported that the molecules that can inhibit deoxyribose degradation are those that can chelate iron ions and render them inactive or poorly active in a Fenton reaction which strengthens our result obtained in iron chelating assay (Smith et al., 1992).

It is likely that the chelating effect of ethyl acetate extract/fractions on metal ions may be responsible for the inhibition of deoxyribose oxidation. In this case also the IC_{50} value was minimum for EAF, which was 15.1 and 18.7 µg/ml for non-site specific and site specific respectively.

The antioxidant activity of ethyl acetate extract/fraction was also discernible in the reducing power assay, which primarily evaluates hydrogen donating ability. Figure 5 depicts the reducing power of ethyl acetate and BHA, a known antioxidant. The reducing power of extract/ fractions showed dose response relationship up to 350 µg/ml of concentration. However, as anticipated, the reducing power of BHA was relatively more pronounced than that of ethyl acetate extract/fractions. In this the minimum absorbance was shown by precipitates (PP) that is, 0.710 and maximum by ethyl acetate fraction that is, 1.310 at 500 µg/ml of concentration. The crude extract (CE), supernatant (SP) and water fraction (WF) showed 0.911, 1.150 and 1.280 absorbance respectively at maximum concentration. Tanaka et al. (1998) and Yildirim et al. (2001), have also observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The presence of reductants (that is, antioxidants) in the extract/ fractions causes the reduction of the Fe^{3-} /ferricyanide complex to the ferrous form. Therefore, the Fe^{2-} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Our data on the reducing power of extract/fractions suggest that it is likely to contribute significantly towards the observed antioxidant effect.

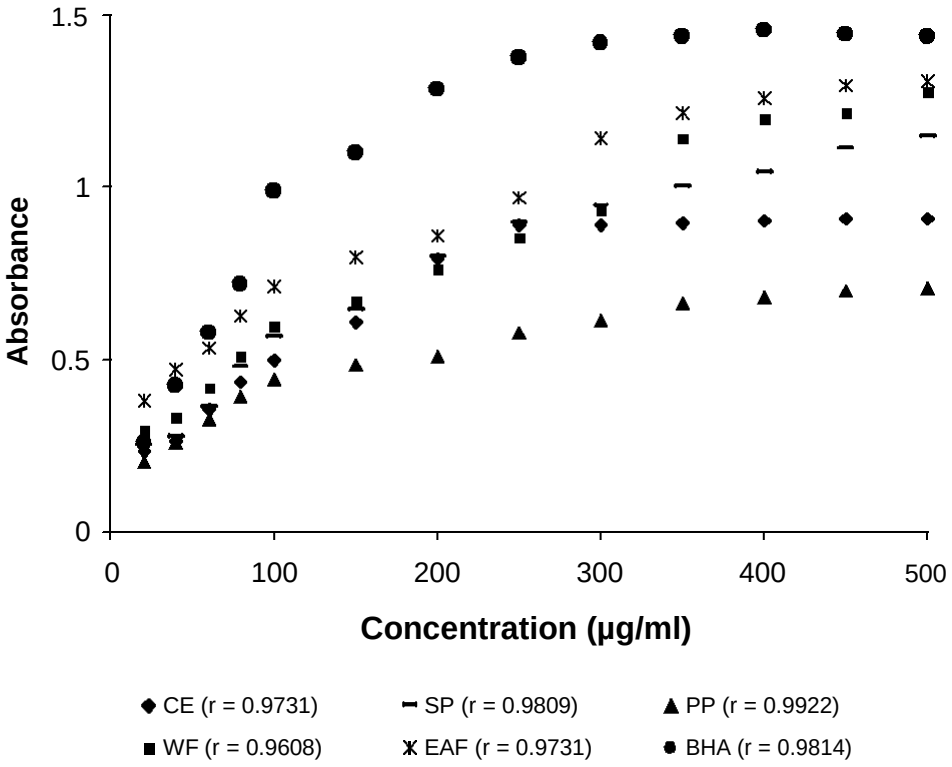


Figure 5. Effect of ethyl acetate extract/fractions of *Terminalia chebula* in reducing power assay.
CE, Crude extract; SP, Supernatant; PP, Precipitates; WF, Water fraction; EAF, Ethyl acetate fraction.

Figure 6 depicts the chelating activity of ethyl acetate extract/fractions. In this assay also, the ethyl acetate fraction (EAF) showed maximum inhibition that is, 74.3% with and crude extract (CE) showed moderate effect that is, 30.5% at 350 g/ml concentration which showed drastic change in inhibition after fractionation. The metal chelating effect of these samples decreased in the order of EAF > WF > SP > PP > CE with values. 74.3, 70.5, 64.3, 52.1, 30.5% respectively at 350 g/ml concentration.

Ferrous ions could stimulate lipid peroxidation by Fenton reaction and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Halliwell, 1991) . Chelating agents may serve as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ions (Gordon, 1990). Since ferrous ions were the most effective prooxidants in food system the moderate to high ferrous- ion chelating abilities of the various extract/fractions would be beneficial (Yamaguchi et al., 1988).

In order to determine whether the extracts are capable of reducing *in vitro* oxidative stress, the traditional lipid peroxidation assay that determines the production of malondialdehyde and related lipid peroxides in living system was carried out. Peroxidation is important in food deterioration and in the oxidative modification of biological molecules particularly lipids. Inhibition of lipid peroxidation by any external agent is often used to evaluate its antioxidant capacity. Figure 7 gives the level of inhibition of lipid peroxidation in terms of TBARS pro-duced in rat liver homogenate induced by ferric chloride system in the presence of extract/fractions. The maximum effect was found in EAF with IC_{50} value of 41.5 µg/ml. The order of inhibition of peroxidation was EAF (78.1%) > WF (73.1%) > SP (69.3%) > CE (63.2%) > PP (50.5%) at 100 µg/ml of concentration. The increase in inhibition can directly be correlated with the increase in correlated with the increase in polyphenolic content. The total phenolic content of the ethyl acetate fraction (235 mg/g) support that there is direct relationship between amount of phenolic compounds and antioxidant activity. The UV analysis crude ethyl acetate extract exhibited $_{max}$ at 364 which revealed the presence of polyphenolic compounds. The results obtained with the ethyl acetate extract and its fractions were compared with positive control that is, BHT.

In the DNA nicking assay antioxidative activity was assessed by measuring the degree of protection on DNA scission by ethyl acetate extract/fractions that was induced by the attack of $^{\cdot}OH$ radicals, which was shown by the agarose electrophoresis pattern. In this assay when pBR322 plasmid DNA was exposed to Fenton

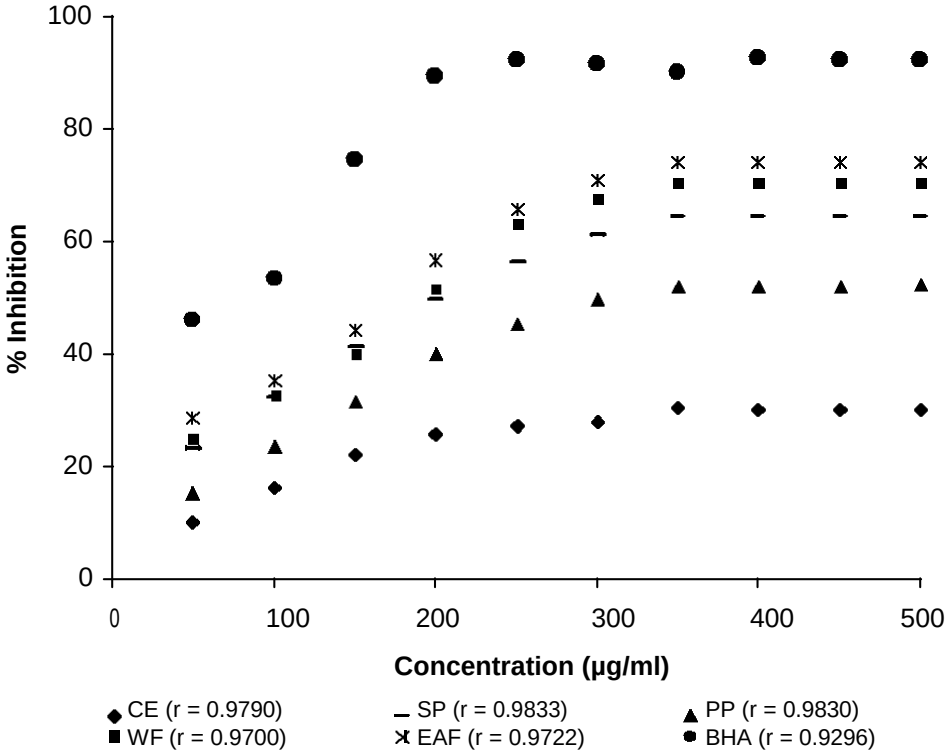


Figure 6. Effect of ethyl acetate extract/fractions of *Terminalia chebula* in chelating power assay.
CE, Crude extract; SP, Supernatant; PP, Precipitates; WF, Water fraction; EAF, Ethyl acetate fraction.

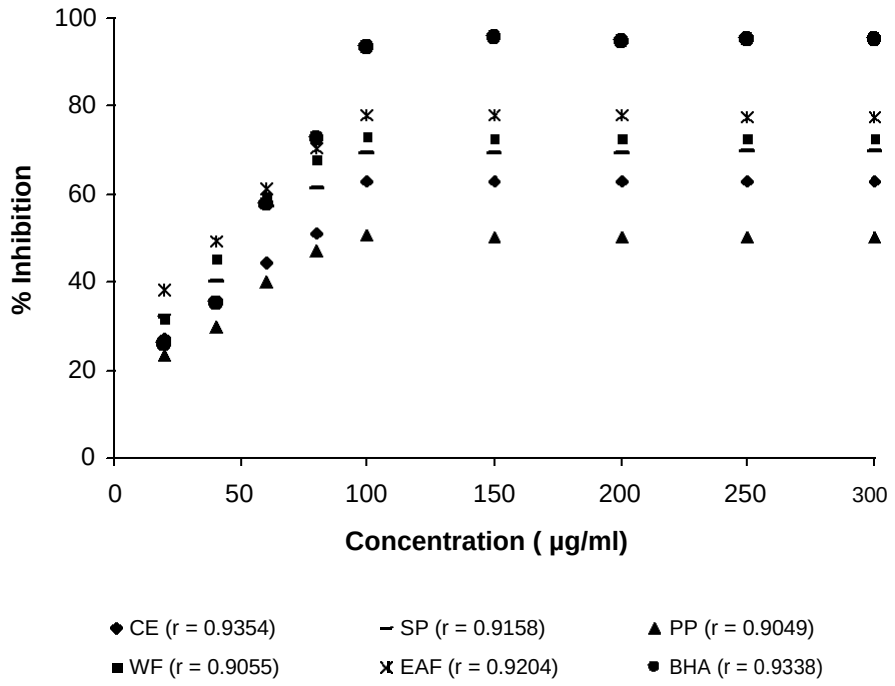


Figure 7. Effect of ethyl acetate extract/fractions of *Terminalia chebula* in Lipid peroxidation assay.
CE, Crude extract; SP, Supernatant; PP, Precipitates; WF, Water fraction; EAF, Ethyl acetate fraction.

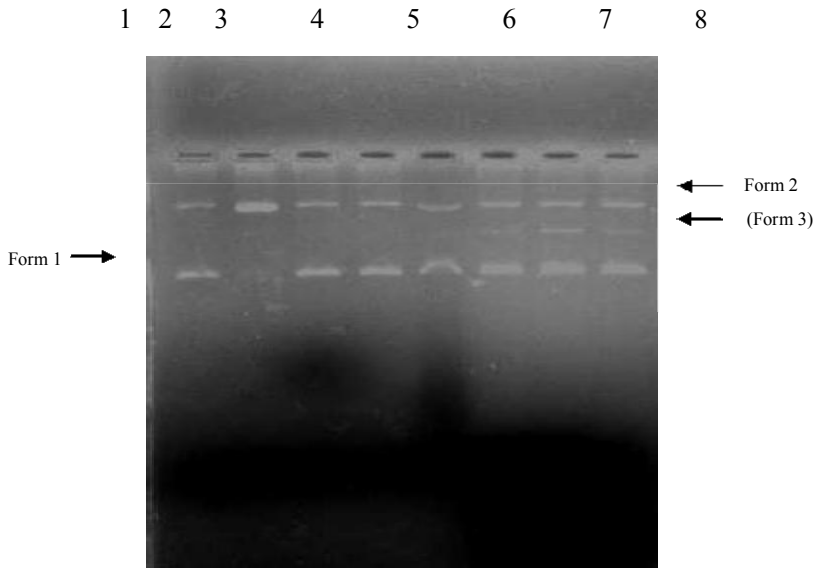


Figure 8. Protective effect of ethyl acetate extract/fractions of *Terminalia chebula* in DNA nicking assay.
Lane 1: pBR322 DNA + H_2O; Lane 2: pBR322 DNA + Fenton reagent + H_2O; Lane 3: pBR322 DNA + Fenton reagent + Rutin (500 g/ml); Lane 4: pBR322 DNA + Fenton reagent + CE (800 g/ml); Lane 5: pBR322 DNA + Fenton reagent + SP (800 g/ml); Lane 6: pBR322 DNA + Fenton reagent + PP (800 g/ml); Lane 7: pBR322 DNA + Fenton reagent + WF (800 g/ml);Lane 8: pBR322 DNA + Fenton reagent + EAF (800 g/ml).
CE, Crude extract; SP, Supernatant; PP, Precipitates; WF, Water fraction; EAF, Ethyl acetate fraction.

reaction, it caused a change in DNA band from Form I (Native plasmid DNA) to Form II (single-stranded, nicked circular plasmid DNA) or to Form III (Linear plasmid DNA). It is clear from the results that ethyl acetate extract/ fractions scavenge the $^{\cdot}OH$ radicals and protect the pBR322 plasmid DNA (Figure 8). The different concen trations were tried but at the concentration of 800 µg/ml the extract/fractions showed the reduction in form II and III and increased form I which is a normal DNA. The extract/fractions showed comparable effect to rutin. It is clear from the results that the CE (Lane 4), SP (Lane 5), PP (Lane 6), WF (Lane 7) and EAF (Lane 8) exhibited a notable protection against hydroxyl radical induced by Fenton reaction. Even the precipitates (PP) showed equivalent effect to rutin, which was used as standard.

Conclusion

On the basis of results in this study, it can be concluded that ethyl acetate fraction (EAF) of ethyl acetate extract from *T. chebula* showed strong antioxidant properties in DPPH assay, deoxyribose assay, reducing power, ferrous ions chelating activity, lipid peroxidation. Further-more, this fraction also exhibited comparatively more inhibition of $^{\cdot}OH$ radicals induced by Fe^{2+} in DNA nicking assay as compared to other extract/fraction. The results of present work indicate that ethyl acetate fraction (EAF) might be the potential antioxidant for application in food products. However further investigations of its activity *in vivo* is necessary to elaborate and explore this promise.

ACKNOWLEDGMENT

We are grateful to CSIR, New Delhi, for financial support in the form of major research project.

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