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Effect of fractionation on *In vitro* antiradical efficacy of acetone extract of *Terminalia chebula* Retz.

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The chemical diversity of antioxidants in complex matrices makes it difficult to separate and quantify them in natural form. Therefore, it is enviable to establish methods that can measure the total antioxidant capacity of extracts. In the present study, the different assays, especially most widely used: deoxyribose, reducing, chelating power, lipid peroxidation and DNA nicking assays have been used to assess the antioxidant capacity of acetone extract/fractions of *Terminalia chebula*. The extract was prepared by maceration method and further fractionated with ethyl acetate and water. It was observed that the radical scavenging activity of fractions was comparatively more as compared to crude extract, and ethyl acetate fraction showed maximum effect in all assays. The percent inhibition with ethyl acetate fraction of acetone extract was observed to be 79.2, 85.9, 90.1 and 88.9% in chelating power, lipid peroxidation, site specific and non-site specific deoxyribose scavenging assays, respectively at maximum concentration tested. The results of present work indicate that ethyl acetate fraction (EAF) might be the potential antioxidant for application in food products.

Key words: *Terminalia chebula,* antioxidants, lipid peroxidation assay, DNA nicking assay, reducing power assay.

INTRODUCTION

Ample generation of reducing oxygen species (ROS) proceeds to a variety of pathophysiological disorders such as arthritis, diabetes, inflammation, cancer, arteriosclerosis, ischemia-reperfusion injury, liver disease, diabetes mellitus, inflammation, renal failure, aging and genotoxicity (Kourounakis et al., 1999; Gulcin et al., 2002; Tanea, 2011; Zapico and Ubelaker, 2013). Compounds that can scavenge free radicals are effective in ameliorating the progression of these related diseases are called antioxidants. Phenolics or polyphenols have received considerable attention because of their physiological functions, including antioxidant, antimutagenic and antimutagenic and antitumour agents (Saliva et al., 1991; Kono et al., 1995). They can undergo auto-oxidation to produce hydrogen peroxide in the presence of metals and are capable of modulating certain cellular enzyme activities (Huang and Ferraro, 1992).

Phenolic compounds are ubiquitous in plants and have been associated with the sensory and nutritional quality of fresh and processed plant foods (Stoclet et al., 2004; Proestos and Komaitis, 2013). During the last few years, researchers and food manufacturers are increasingly interested in these compounds which may be exploited for the development of functional foods or in the

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chemoprevention. Fortification of foods with materials rich in phenolic compounds has been shown to impart anti-mutagenic, antiinflammatory and antioxidant properties which may be exploited for the development of health foods (Friedman, 1997). These justify the overwhelming interest in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants (Parr and Bolwell, 2000). It has generally been recognized that traditional oriental medicines have unique therapeutic roles in the prevention and treatment of many human diseases related to excess free radicals. In addition, there is considerable evidence that polyphenols isolated from medicinal plants are potential therapeutic agents (Castillo et al., 1989; Robak and Marcinkiewicz, 1995; Inoue and Jackson, 1999; Packer et al., 1999; Middleton et al., 2000; Rodrigo et al., 2011).

Terminalia chebula a native plant in India and Southeast Asia is extensively cultivated in Taiwan, and 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 are being used for the treatment of different types of diseases and disorders since antiquity. The plant has been studied for its antioxidant, antimicrobial and antimutagenic properties (Saleem et al., 2002; Chen et al., 2003; Bag et al., 2013). It is 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; Akhand et al., 2013). Keeping in view the immense importance of the plant, the present study was planned to evaluate the antioxidant activity of acetone extract/fractions of fruits of *T. chebula*.

MATERIALS AND METHODS

Chemicals

Deoxyribose was purchased from Lancaster. Thiobarbituric acid was purchased from Sigma Aldrich USA. Other chemicals like ferrozine, Folin-Ciocalteu (FC) reagent, potassium ferricyanide, ferric chloride, ethylenediaminetetraacetic acid (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. These were 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 acetone was added. The supernatant was collected, filtered by using Whatman sheet no.1 and evaporated through rotary evaporator to have the dry crude acetone extract. This dry crude acetone extract was further fractionated. For the fractionation, the crude acetone extract (AI) was redissolved in acetone and after some time the precipitates were formed. The precipitates (AP) and supernatant (AII) were separated and dried at room temperature separately. The dried supernatant (AII) was dissolved first in water and then in ethyl acetate, resulted in formation of two layers: ethyl acetate fraction (EAF) and water fraction (WF). These layers were separated and dried at room temperature (Flow chart 1).

Spectroscopic analysis of extract/fractions

The acetone extract/fractions of *T. chebula* were analyzed by ^{1}H NMR and ultra violet (UV) spectroscopy. For nuclear magnetic resonance (NMR) spectroscopy, 1 mg acetone crude extract (AI) was dissolved in spectroscopic grade dimethyl suphoxide (DMSO), filtered and transferred to NMR tubes. The tubes were spun at 20 to 30 Hz about its vertical axis and interpretation was done. For UV analysis, the solution of acetone extract/fractions was prepared in spectroscopic grade methanol in the concentration of 1 mg/10 ml and a spectrum was recorded on UV-visible spectrophotometer (Shimadzu-1601).

Antioxidant testing

The acetone crude extract (AI), supernatant (AII), precipitates (AP), and two fractions that is, ethyl acetate fraction (EAF) and water fraction (WF) were tested for their antioxidant potential by using the following *in vitro* assays.

Deoxyribose assay

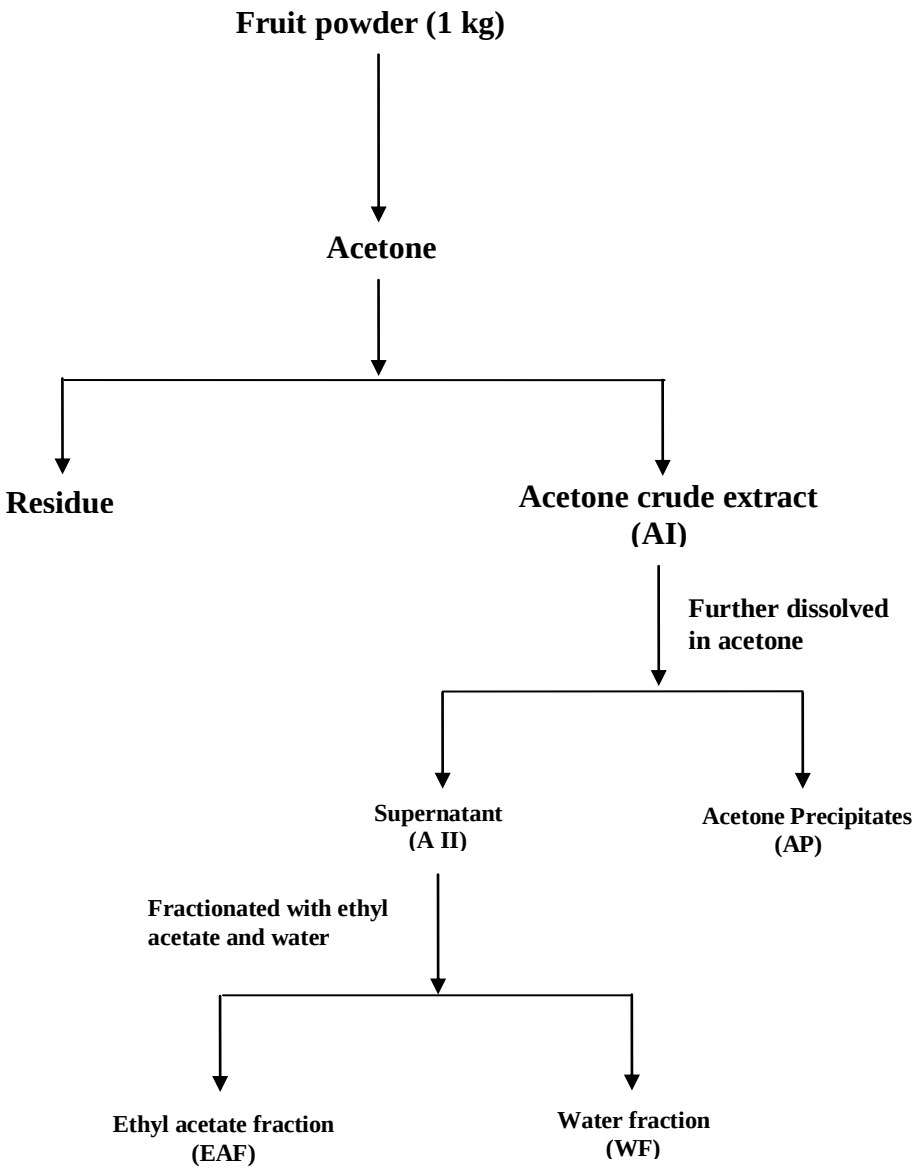
This method was used to measure the hydroxyl radicals scavenging activity of extracts (Halliwell et al., 1987). This assay was performed by two ways that is, non-site specific and site-specific. In non-site specific deoxyribose assay, 0.1 ml of EDTA, 0.01 ml of $FeCl_3$, 0.1 ml of H_2O_2, 0.36 ml of deoxyribose, 1 ml of extract concentrations (10 to 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 one hour 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.

Reducing power assay

This method is used to estimate the relative reducing activity of extracts (Oyaizu, 1986). 1 ml of extract/fractions (50 to 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 3,000 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, thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm (Halliwell and Guttridge, 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 to 4°C with 0.15 M KCl. The homogenate was centrifuged at 3,000 rpm for 15 min, and clear cell-free supernatant



Flow chart 1. Extraction/fractionation procedure.

was used for the study of *in vitro* lipid peroxidation. Different concentrations of extracts 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), 0.38% TBA and 0.5% butylated hydroxytoluene (BHT). The reaction mixture was heated at 80°C for 60 min. The samples were cooled and centrifuged, and the absorbance of the supernatants was measured at 532 nm.

Chelating power assay

In this assay, 1 ml of extract 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).

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 extracts/fractions and the final volume of the mixture was brought up to 20 µl. 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.

Determination of total phenolic content

The total phenolic content of the extract was determined using Folin-Ciocalteu method (Yu et al., 2002). To 100 µl of extract/ fraction was added 900 µl of water. To this, 500 µl of FC reagent was

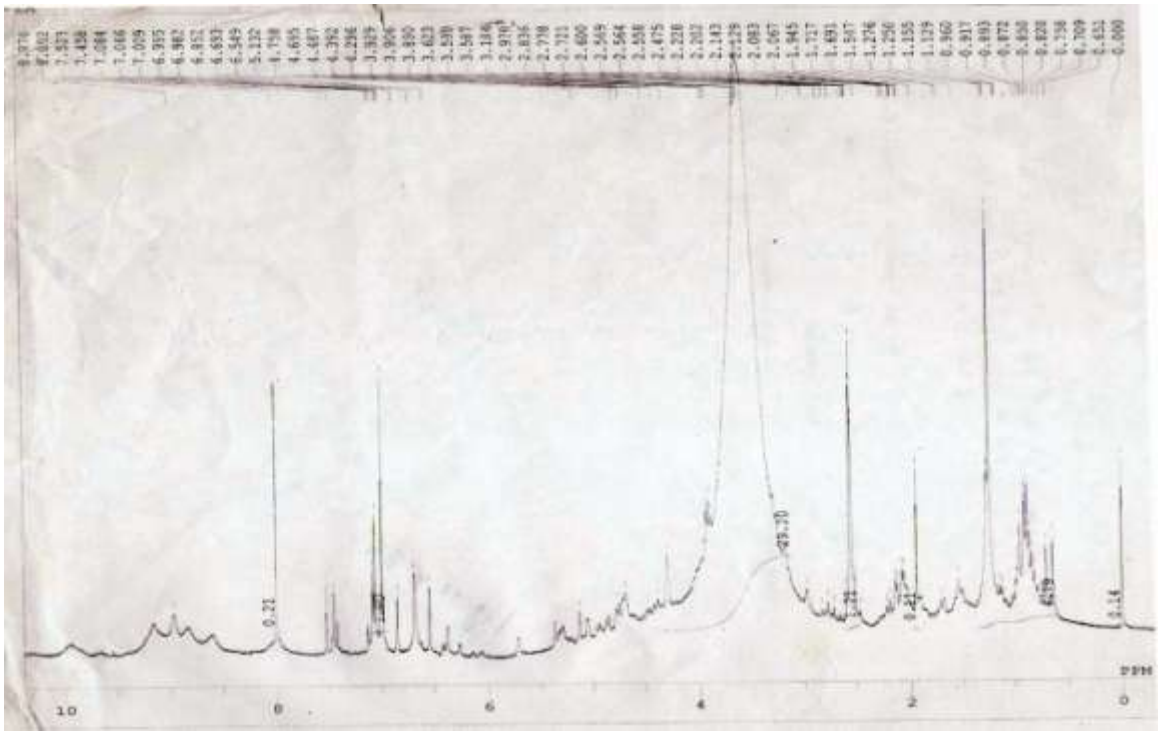


Figure 1. ^{1}H NMR spectrum of crude acetone extract (AI).

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.

RESULTS AND DISCUSSION

The NMR spectrum of crude acetone extract (AI) of *T. chebula* showed signals spreading from 0.5 to 10 ppm. These pointed to the presence of multiple components in the extract. The signals between 0.5 to 3.0 δ pointed to the fatty esters or terpenoides. The presence of signals between 3.5 to 5.0 δ clearly showed the presence of glycosides. The number of signals between 6.0 to 8.0 ppm referred to polyphenolics, which may be present as glycosides. The large number of exchangeable and inter/intramolecularly H- bonded protons were also observed between 8.5 to 10.0 δ (Figure 1).

The acetone crude extract (AI) constituted 23.5% of fruit powder and was dark brown in colour. The UV analysis showed the presence of peak at λ_{max} = 362 nm in crude acetone extract which points towards the presence of polyhenols. The AII, AP, WF and EAF showed maximum absorbance λ_{max} at 363, 362, 363 and 368 nm, respectively, strappingly signifying the presence of glycosides of phenolic nature (Figure 2).

The results of acetone extract/fractions are depicted in Figures 3 to 8. It was observed that the fractions that is, water and ethyl acetate were more effective as compared to the crude acetone extract. The amount of total phenolics in extract/fractions ranged from 340 to 780 mg/g of gallic acid. The total phenolic content was maximum in ethyl acetate fraction (EAF) that is, 780 mg/g of gallic acid, which signify its highest antioxidant activity. The crude acetone extract (AI), supernatant (AII), acetone precipitates (AP), and water fraction (WF) had 437, 557, 340 and 64 7mg per gram phenolic content, respectively.

To date, the $^{\cdot}$OH is one of the most reactive free radical species known with damaging effects to almost every biological molecule found in living cells. It can be generated *in vivo* in the presence of both superoxide radicals and transition metals, such as iron or copper via the Haber-Weiss reaction (Castro and Freeman, 2001). In order to substantiate the free radical scavenging capacity of acetone extract/fractions in an *in vitro* Fenton-type assay system: non-site-specific (Fe^{2+} + H_2O_2 + EDTA) and site specific (Fe^{2+}+H_2O_2) was used, in which $^{\cdot}$OH radicals are generated in free solution that attack the deoxyribose substrate and fragmenting it into thiobarbituric acid reactive substances (TBARS). Figures 3 and 4 depicts the activity of acetone extract/fractions in non-site specific and site-specific deoxyribose assay, respectively. It was observed that the extract/fraction showed a dose response relationship up to 100 µg/ml.

Furthermore, a comparatively high activity was noticed in site-specific assay than in non-site specific assay

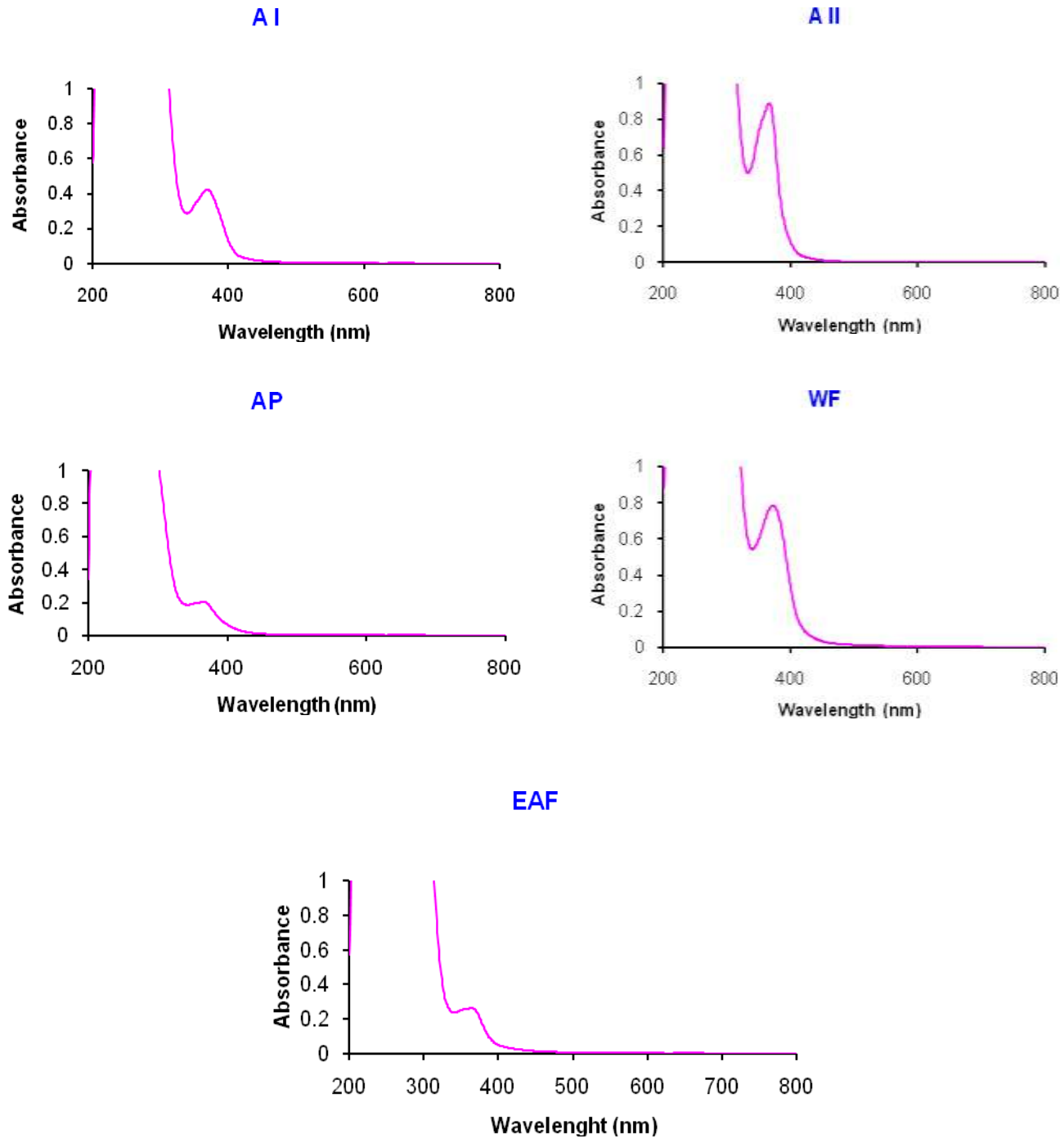


Figure 2. The UV-Visible spectra of acetone extract/fractions of *Terminalia chebula.*
A I: Crude extract, **A II**: Supernatant, **AP**: Precipitates, **WF**: Water fraction, **EAF**: Ethyl acetate fraction

indicating the high chelating activity of the extracts/fractions. The crude acetone extract (AI) showed 79.3 and 86.8% inhibition in non-site specific and site specific assay at 100 µg/ml of concentration, respectively. A maximum inhibition of 90.1 and 88.3% was shown by ethyl acetate fraction (EAF) in site specific and non-site specific assay, respectively at 100 µg/ml of concentration.

It was noticed that in both the assays, EAF showed maximum inhibition. The acetone precipitates (AP) showed the minimum inhibition that is, 62 and 60.1% in site specific and non-site specific assay, respectively. It is also clear from Figures 3 and 4 that the extract exhibited good antioxidant and chelating activity than standard antioxidant, that is gallic acid. The presence of phenolic

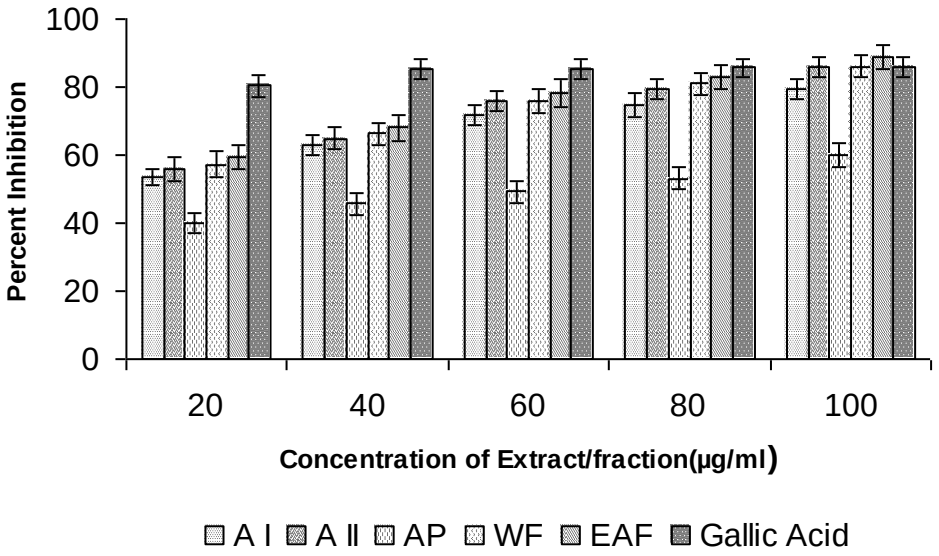


Figure 3. Histogram showing the pattern of inhibition by acetone extract/fractions in non-site specific deoxyribose assay.

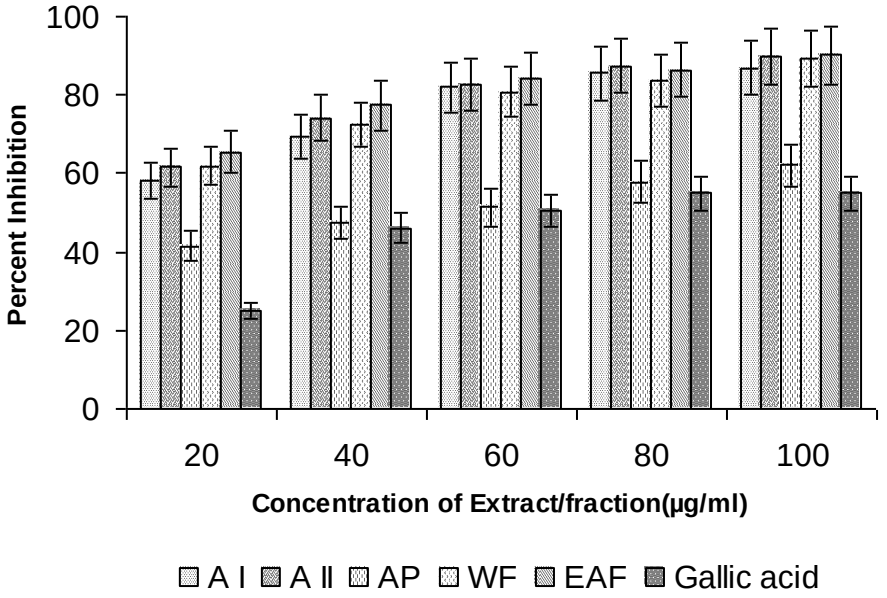


Figure 4. Histogram showing the pattern of inhibition by acetone extract/fractions in site specific deoxyribose assay.

groups in extract/fractions could be responsible for $^{\cdot}OH$ radical scavenging activity. The results indicated that the acetone extract/fractions has more hydrogen donating ability, which may be due to the presence of polyphenolic glycosides as indicated by ^{1}H NMR spectrum that showed major signals between 3.0 to 5.5 δ and 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 acetone extract/fractions on metal ions may be responsible for the inhibition of deoxyribose oxidation.

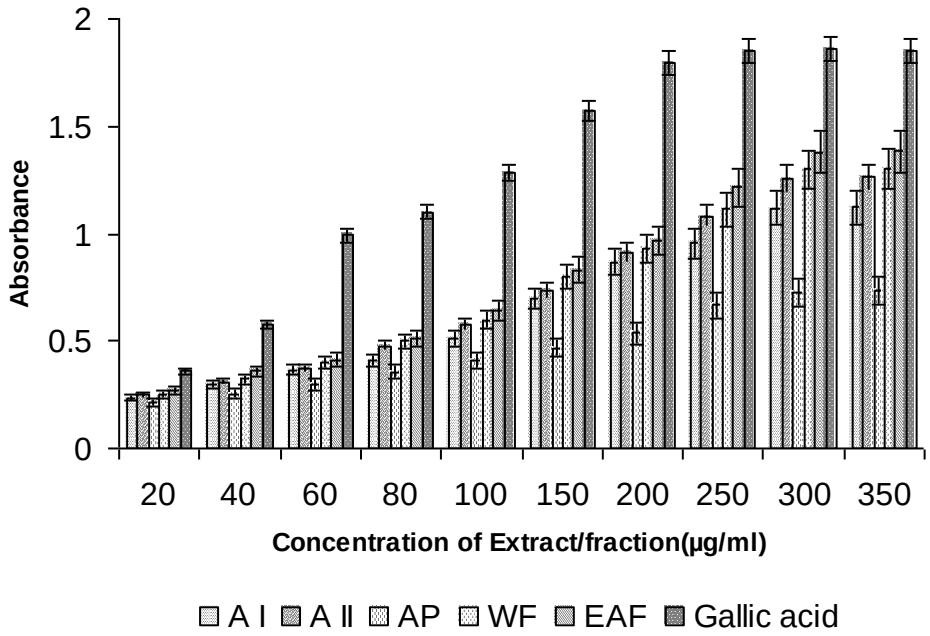


Figure 5. Histogram showing the pattern of absorbance by acetone extract/fractions in reducing power assay

Iron, a transition metal, is capable of generating free radicals from peroxides by the Fenton reaction and is implicated in many diseases (Halliwell and Gutteridge, 1990). Fe^{2+} has also been shown to produce oxyradicals and lipid peroxidation, and reduction of Fe^{2+} concentrations in the Fenton reaction would protect against oxidative damage.

The antioxidant activity of acetone extract/fraction was also discernible in the reducing power assay, which primarily evaluates hydrogen donating ability. Figure 5 depicts the reducing power of acetone extract/fractions and gallic acid, a known antioxidant. The reducing power of extract/fractions showed dose relationship up to 350 µg/ml of concentration. However, as anticipated, the reducing power of gallic acid was relatively more pronounced than that of acetone extract/fractions. In this, the minimum absorbance was shown by acetone precipitates (AP), that is 0.731 and maximum by ethyl acetate fraction, that is 1.385 at 350 µg/ml of concentration. Earlier authors (Tanaka et al., 1988; 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/fraction suggest that it is likely to contribute significantly towards the observed antioxidant effect.

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 6 gives the level of inhibition of lipid peroxidation in terms of TBARS produced in rat liver mitochondria induced by ferric chloride system in the presence of extract/fraction. The order of inhibition of peroxidation was EAF (85.9%) > WF (79.5%) > AII (70.5%) > AI (68.3%) > AP (60.3%) at 100 µg/ml of concentration. The increase in inhibition can directly be correlated with the increase in polyphenolic content. The total phenolic content of the ethyl acetate fraction (780 mg/g) reveals that there is direct relationship between amount of phenolic compounds and antioxidant activity. The UV analysis of EAF of acetone extract exhibited λ_{max} at 362 which revealed the presence of polyphenolic compounds. The comparison of results with

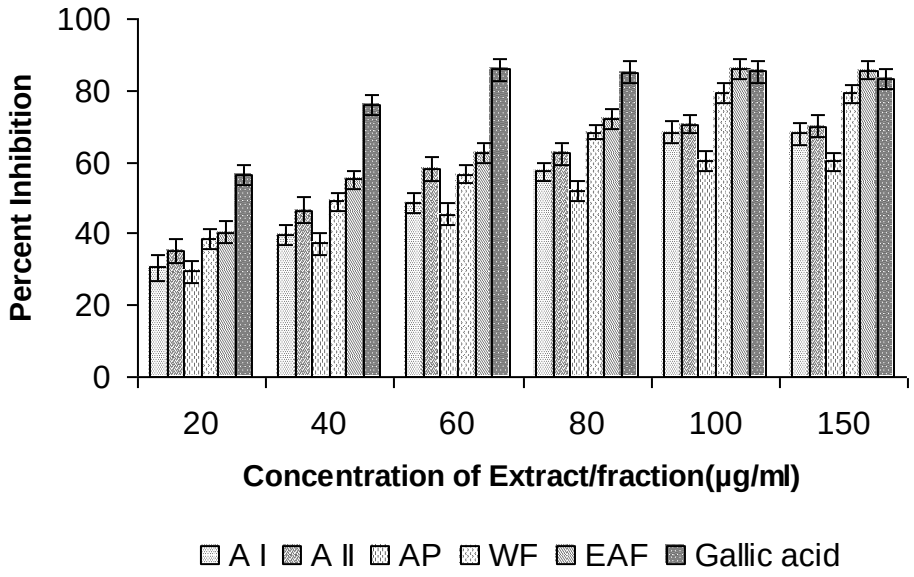


Figure 6. Histogram showing the pattern of inhibition by acetone extract/fractions in lipid peroxidation assay.

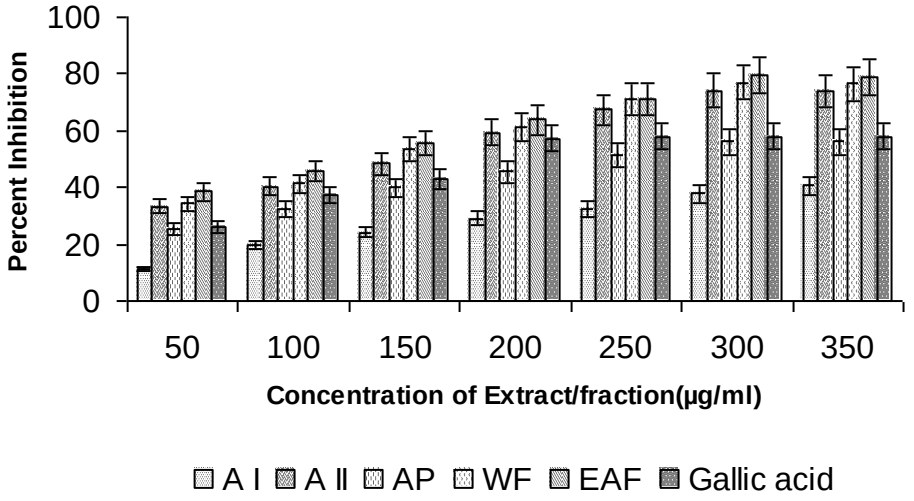


Figure 7. Histogram showing the pattern of inhibition by acetone extract/fractions in chelating power assay.

gallic acid indicated that the extract/fractions exhibited more or less the same inhibition.

Figure 7 depicts the chelating activity of acetone extracts/fractions. The maximum inhibition was shown by EAF, which was 79.2% and minimum with acetone crude (AI) that is, 40.7% at 350 µg/ml of concentration. The extract/fractions exhibited more chelating activity as compared to standard (gallic acid). 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 pro-oxidants in food system, the moderate to high ferrous-ion chelating abilities of the various extract/fractions would be beneficial (Yamaguchi et al., 1988).

In the DNA nicking assay, antioxidative activity was assessed by measuring the degree of protection on DNA scission by acetone extract/fractions that was induced by the attack of $^{\cdot}OH$ radicals, which was shown by the agarose

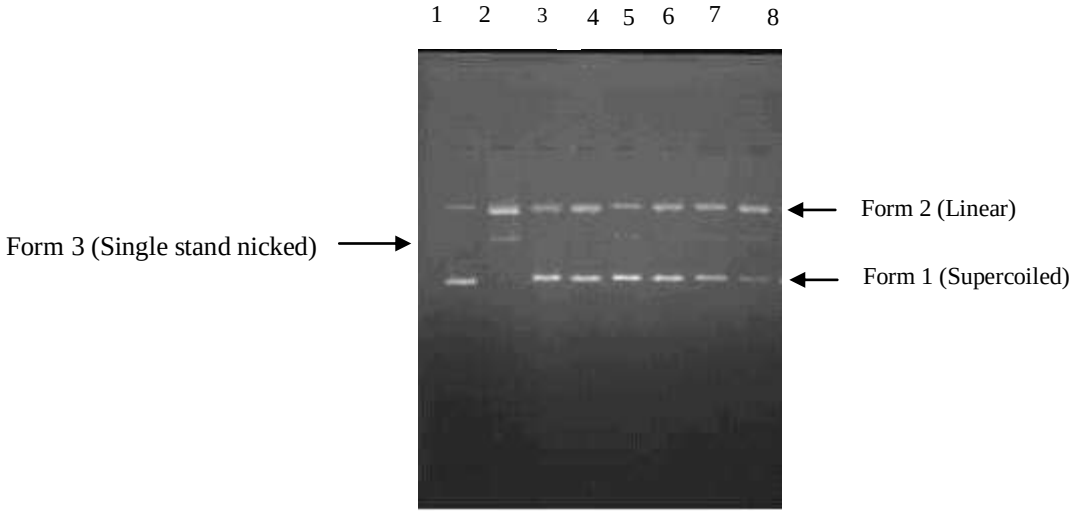


Figure 8. Inhibitory effects of acetone extract/fractions of *Terminalia chebula* at 250 µg/ml concentration on pBR322 DNA nicking caused by hydroxyl radicals. Lane 1, Native DNA; lane 2, DNA + Fenton reagent; lane 3, DNA + Fenton reagent +Gallic acid; lane 4, DNA + Fenton reagent + A I; lane 5, DNA + Fenton reagent + A II; lane 6, DNA + Fenton reagent + EAF; lane 7, DNA + Fenton reagent + WF; lane 8, DNA + Fenton reagent + AP.

agarose electrophoresis pattern. In this assay, when pBR322 plasmid DNA was exposed to Fenton 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). The extract/fractions scavenge the $^{\cdot}OH$ radicals and protect the pBR322 plasmid DNA. Different concentrations were tried but at the concentration of 250 µg/ml, the extract/fractions showed the reduction in Form II and III, and increased in Form I which is a normal DNA. The extract/fractions showed comparable effect to gallic acid (Figure 8). The ethyl acetate fraction showed best result among all the extracts/fractions and precipitates showed the minimum effect.

Conclusion

On the basis of this study, it can be concluded that EAF of acetone extract from *T. chebula* showed strong antioxidant properties in deoxyribose assay, reducing power, ferrous ions chelating activity, lipid peroxidation. Furthermore, ethyl acetate 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 EAF might be the potential antioxidant for application in food products.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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