

Full Length Research Paper

Comparative antioxidant analysis of hexane extracts of *Terminalia chebula* Retz. prepared by maceration and sequential extraction method

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Accepted 26 February, 2010

The aim of this work is to compare the antioxidant efficacy and the phenolic content of two hexane extracts viz. 'Hex 1' and 'Hex 2' of fruits of *Terminalia chebula* prepared by maceration and sequential method respectively. The extracts were tested for their relative levels of antioxidant activity and the total phenolic content using DPPH, deoxyribose, reducing power, chelating power, lipid peroxidation and Folin-Ciocalteu method. Furthermore, the UV-VIS spectrum of extracts and the correlation between total phenolic content were examined in order to give an orientation to the search of phytochemicals responsible for their activity. From the results, it was concluded that phenolic compounds were predominant in the 'Hex 2' prepared by sequential extraction method. The antioxidative potential of 'Hex 2' was also far superior to the 'Hex 1' prepared by maceration method. Such study would contribute to further knowledge relating to the extraction of plant materials by different methods.

Key words: *Terminalia chebula*, extraction method, antioxidant activity, phenolic compounds.

INTRODUCTION

Oxidation processes are intrinsic in the energy management of all living organisms and are therefore kept under strict control by several cellular mechanisms (Halliwell and Gutteridge, 2007). However, the onslaught of free radicals and the unbalanced mechanisms of antioxidant defense result in the inception of various diseases (Sinha et al., 2009). Oxidation is also responsible for the rancidity of unpreserved food rich in unsaturated fatty acids. Antioxidants are the substances which can prevent or control oxidation. The natural antioxidants are suggested as a superior alternative for the synthetic ones such as BHA (butylated hydroxyanisole) or BHT (butylated hydroxytoluene) as scientists have casted some toxicological doubts on them due to their adverse side effects (Li et al., 2008).

Though the plant kingdom offers a wide range of natural antioxidants, but still there is lack of adequate knowledge about the practical usefulness of most of

them. In the group of natural plant metabolites, phenolics are commonly found in various fruits, vegetables and herbs and they have been shown to provide a defense against oxidative stress from oxidizing agents and free radicals (Matkowski, 2006). It is implicated that polyphenols exert their activities by many pathways such as free radical scavenging, oxygen radical absorbance and chelating of the metal ions (Cook and Samman, 1996; Virgili et al., 1998; Kahkonen et al., 1999). In addition, besides its properties, they are used in modern medicines due to a general "Back to natural trend" (Castaldo and Capasso, 2002).

One of the most important medicinal plants, which are widely used in the traditional system of medicine is *Terminalia chebula*, which is also known as black myroblan. It acts as a laxative, purgative and helps in removing toxins and fats from the body resulting in their reduced absorption. It is a powerful adaptogenic agent and also a part of wonder drug "Triphla" which is composite mixture of *T. chebula*, *Terminalia bellerica* and *Embllica officinalis*, a traditional medicine used for the treatment of many chronic diseases such as aging, heart

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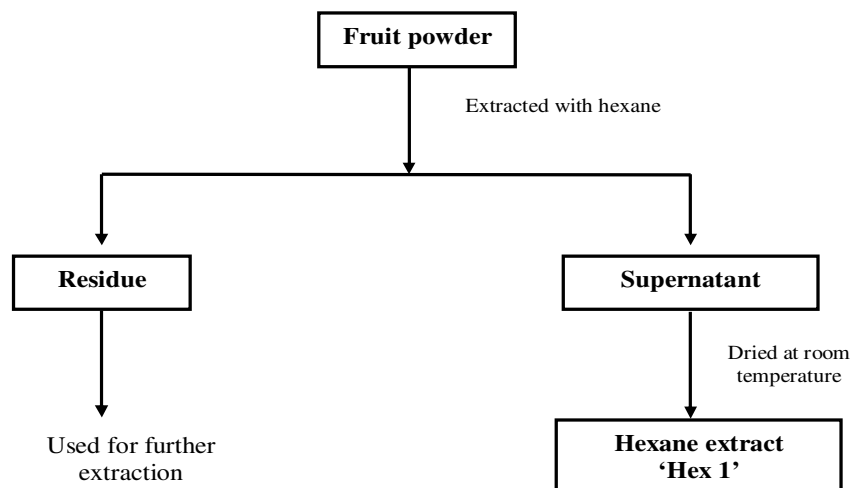


Figure 1. Hexane extract of fruits of *T. chebula* prepared by maceration method.

ailments and hepatic diseases (Kaur et al., 2005; Srikumar et al., 2005). The various extracts of *T. chebula* have been reported to show broad-spectrum activities like antimutagenic, antifungal, antibacterial etc. (Kaur et al., 1998; Jagtap and Karkera, 1999; Malckzadeh et al., 2001; Suguna et al., 2002; Sandip, 2003; Vonshak et al., 2003; Bonjar, 2004). Keeping in view the immense importance of the plant, the present study was planned to evaluate the antioxidant activity of two different hexane extracts of fruits of *T. chebula*.

MATERIALS AND METHODS

Chemicals

Deoxyribose was purchased from Lancaster, USA. Thiobarbituric acid was purchased from Sigma Aldrich USA. Other chemicals like ferrozine, FC (Folin-Ciocalteu) reagent, potassium ferricyanide, ferric chloride, EDTA, hydrogen peroxide, L-ascorbic acid, sodium hydroxide, BHA (butylated hydroxyanisole), trichloroacetic acid and other solvents were procured from CDH (Central Drug House, India) and were of analytical grade.

Extraction procedure

The fruits of the *T. chebula* were purchased locally from market and were authenticated by comparing them with the samples already available in the herbarium of Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar (Voucher No. 5980). The fruits were washed, dried and finally ground to fine powders. The two hexane extracts were prepared by maceration and sequential extraction methods.

Maceration method

1 kg of dried fruit powder of *T. chebula* was suspended in hexane (1500 ml) and the mixture was stirred for 24 h. The suspended solid

was filtered through Whatman No.1 filter paper and the filtrate was collected. This procedure was performed thrice to get three filtrates of hexane and residue. Hexane filtrate were combined and dried at room temperature (35°C) in petri plates. The green coloured gummy solid left behind was named as 'Hex 1' for all further applications (Figure 1).

Sequential method

1 kg of fruit powder of *T. chebula* was suspended in methanol (1500 ml) and the mixture was kept for 24 h at room temperature. The residue was filtered through Whatman No.1 filter paper and filtrate was collected. The filtrate was dried at room temperature (35°C) by putting in petri plates to get methanol extract. 100 g of methanol extract was dissolved in 20% aqueous methanol and put into the separatory funnel. The hexane was added in separatory funnel and mixed properly. After some time the two layers were formed which were separated to get hexane filtrate and 20% aqueous methanol stock. The three filtrates of hexane were collected and dried at room temperature (35°C) in petri plates to get hexane extract named as 'Hex 2' (Figure 2).

Chemical analysis

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 was added 900 µl of water. To this 500 µl 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 extracts

The 'Hex 1' and 'Hex 2' extracts of *T. chebula* were analyzed by UV spectroscopy. For this the solution of 'Hex 1' and 'Hex 2' was prepared in spectroscopic grade methanol in the concentration of 1

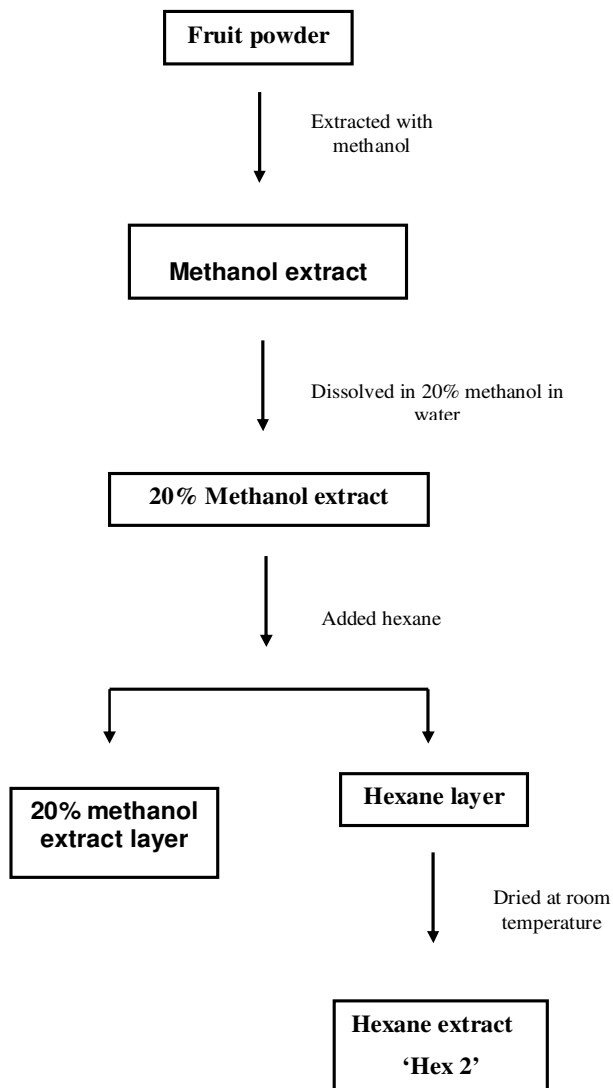


Figure 2. Hexane extract of fruits of *T. chebula* prepared by sequential method.

mg/10 ml, diluted four times and a spectrum was recorded on UV-Visible spectrophotometer (Shimadzu-1601). The results were compared with standard that is gallic acid

Antioxidant testing

DPPH assay

The Hydrogen donating activity of the 'Hex 1' and 'Hex 2' extracts was measured by DPPH method (Blois, 1958). The reaction mixture contained 200 μ l of different extracts concentrations and 2 ml of DPPH. The reaction mixture was observed at 517 nm against a blank. The % 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.

Deoxyribose assay

Deoxyribose assay was done by following the two different methodologies Viz. non-site specific and site-specific (Halliwell et al., 1987). Stock solution of EDTA (1 mM), FeCl_3 (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10mM) were prepared in distilled water. In non- site specific 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 extracts concentrations (10 - 100 μ g/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 assay EDTA was replaced with phosphate buffer. The % 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

The reducing activity of the 'Hex 1' and 'Hex 2' extracts was determined according to the method of Oyaizu (1986). 1 ml of extract/fractions (50 - 300 μ g/ml) was prepared in distilled water and mixed with 2.5 ml of phosphate buffer (0.2M, 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% trichloroacetic 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.

Chelating activity

The chelating activity of extracts was measured as given by Dinis et al. (1994) with little modifications. 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. The % 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

Lipid peroxidation assay

Lipid peroxidation assay was done according to the method given by Halliwell and Gutteridge (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 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 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 μ l of 0.2 mM ferric chloride. After incubation at 37°C for 30 min, the reaction was

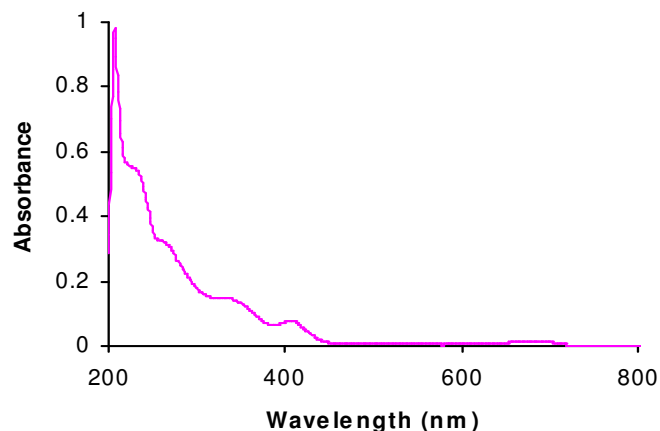


Figure 3. The UV-visible spectra of 'Hex 1' acquired by maceration method.

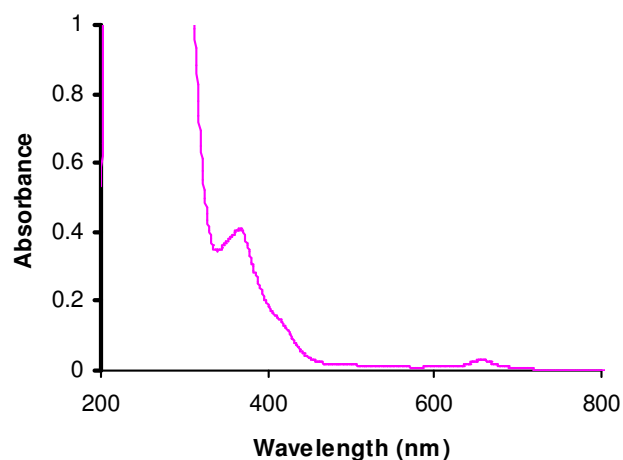


Figure 4. The UV-visible spectra of 'Hex 2' acquired by sequential method.

stopped by adding 2 ml of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid (TCA), 0.38% thiobarbituric acid (TBA) and 0.5% butylated hydroxytoluene (BHT). The reaction mixtures were heated at 80°C for 60 min. The samples were cooled and centrifuged, and the absorbance of the supernatants was measured at 532 nm. An identical experiment was performed to determine the amount of lipid peroxidation obtained in the presence of inducing agents without any extract. The % 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

Statistical analysis

The data presented as Mean \pm S.E. of three independent experiments.

RESULTS AND DISCUSSION

Phenolic compounds have been extensively investigated in the past 30 years. They have one or more aromatic rings bearing hydroxyl groups that are potentially able to act as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers (Rice-Evans et al., 1996; Mattei et al., 1998). In order to determine the presence of phenolic content the Folin-Ciocalteu method was used, which showed that the hexane extract prepared by maceration method has low phenolic content as compared to hexane extract prepared by sequential method. The phenolic content of 'Hex 1' extract prepared by maceration method was 10 mg/g of GAE, while it was 145 mg/g of GAE in 'Hex 2' extract prepared by sequential method. To further confirm the presence of phenolic content, the UV analysis of 'Hex 1' and 'Hex 2' extract was also done which again confirmed the presence of phenolic compounds in them.

The UV spectrum of the 'Hex 1' extract gives absorbance at λ_{max} of 402 nm along with end absorption around 200 nm (Figure 3). The features showed the presence of low phenolic content in this extract. In general, the small structureless absorption in the range 250 - 400 nm indicated the presence of mixture of phenolics and fatty acids compounds. The UV spectrum of 'Hex 2' extract revealed the absorption around λ_{max} 363 nm, which indicated the presence of polyphenolic compounds (Figure 4). Phenolic compounds exhibited two major absorption bands in the ultraviolet/visible region that is a first band in the range between 320 and 380 nm and a second band in the 250 - 285 nm range (Matthaus, 2002). So, it can be concluded that the 'Hex 2' extract contain phenolic compounds as a major phytochemical. The hydrogen donating ability of 'Hex 1' and 'Hex 2' extracts was measured and compared by the bleaching of the purple coloured methanol solution of 1, 1-Diphenyl-2-picrylhydrazyl (Jao and Ko, 2002).

The maximum tested dose in this assay was 500 $\mu\text{g/ml}$ and it was observed that 'Hex 1' extract exhibited 7.1% inhibition and 'Hex 2' extract exhibited 67% inhibition (Figure 5). The reaction time of 'Hex 1' extract was 25 - 30 min and for 'Hex 2' extract 15 - 20 min which showed a efficient hydrogen donating ability of 'Hex 2' than 'Hex 1'. Lee et al. (2005) investigated the antioxidant activity of aqueous extract of fruits of *T. chebula* by employing DPPH and reducing power assays. The results showed that the extract is good hydrogen donor and reducing agent, which show activity in time and dose dependent manner. Naik et al. (2004) also studied the antioxidant activity of aqueous extract of *T. chebula* by using stopped-flow kinetic spectrometer and gamma-radiation-induced lipid peroxidation in rat liver microsomes. Based on the results it was concluded that the aqueous extract of *T. chebula* acts as a potent antioxidant and since it is able to protect cellular organelles from the radiation-induced damage, it may be considered as a probable

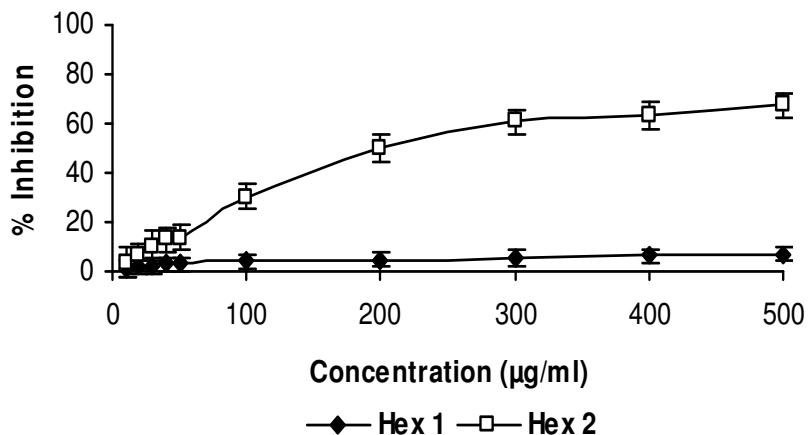


Figure 5. Effect of different concentrations of 'Hex 1' and 'Hex 2' extract of *T. chebula* in DPPH assay.

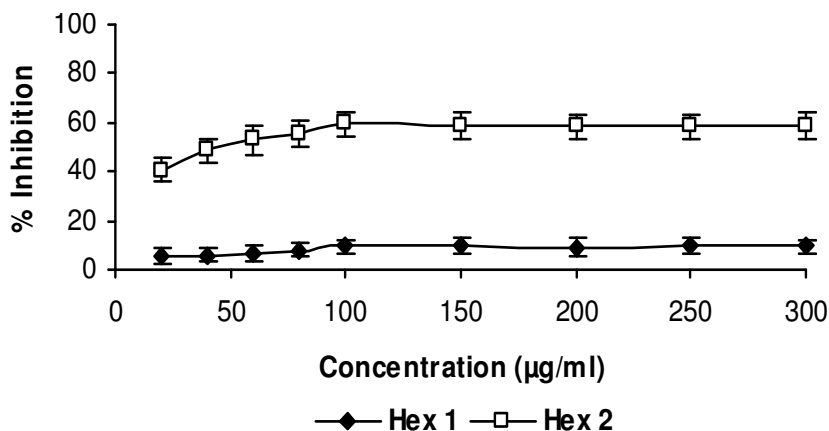


Figure 6. Effect of different concentrations of 'Hex 1' and 'Hex 2' extract of *T. chebula* in non-site specific deoxyribose assay.

radioprotector.

The hydroxyl radicals are most reactive free radical that can damage DNA in its proximity. Some of the dietary phytochemicals are capable of either sequestering Fe^{2+} and/or scavenging $\text{HO}\cdot$ radicals thus preventing damage to DNA. The antioxidant can donate hydrogen to the hydroxyl radical and neutralize them. In the present study it was observed that in non-site specific deoxyribose assay, the "Hex 1" extract showed 9.5% and "Hex 2" extract showed 49.2% inhibition at 300 $\mu\text{g/ml}$ of maximum concentration tested (Figure 6). In case of site-specific the "Hex 1" extract showed 21.1% and "Hex 2" extract showed 67.7% inhibition at 300 $\mu\text{g/ml}$ of concentration (Figure 7). Our results suggested that "Hex 2" extract is not only effective scavengers of $\text{HO}\cdot$ but also iron chelator, as it showed more activity in site-specific assay than in non-site specific assay. Thus the compounds, which are able to chelate iron preferentially and change the metal complex in a less redox active form (compared with EDTA-metal complex) will protect

deoxyribose against damage in the presence of ascorbate and H_2O_2 .

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Awika et al., 2003; Jeong et al., 2004). Literature reports have shown that the reducing power of bioactive compounds is associated with antioxidant activity (Duh, 1998; Yildirim et al., 2001; Siddhuraju et al., 2002; Dorman et al., 2003). For reducing ability, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of "Hex 1" and "Hex 2" extracts of *T. chebula*. In this assay, the "Hex 1" extract showed the absorbance of 0.228 absorbance and "Hex 2" showed the absorbance of 0.524 absorbance at 500 $\mu\text{g/ml}$ of concentration (Figure 8). Less activity of "Hex 1" extract may be due to the absence of phenolic compounds or co-occurrence of other compounds like fatty acids, which hindered its antioxidative activity and made these moieties non-functional. In both cases, beyond 500 $\mu\text{g/ml}$ concentration the absorbance ceased to rise. On the

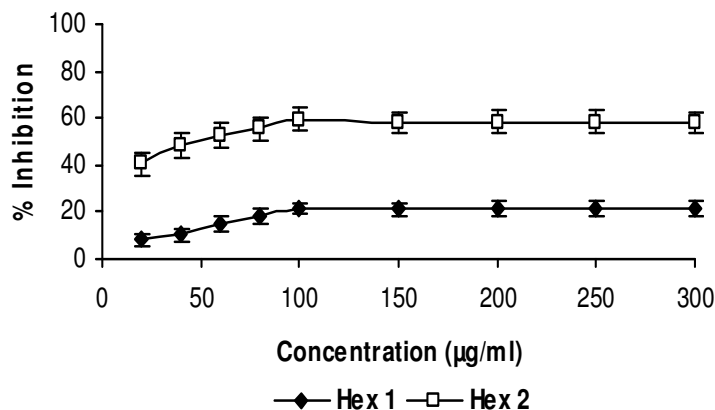


Figure 7. Effect of different concentrations of 'Hex 1' and 'Hex 2' extract of *T. chebula* in site-specific deoxyribose assay.

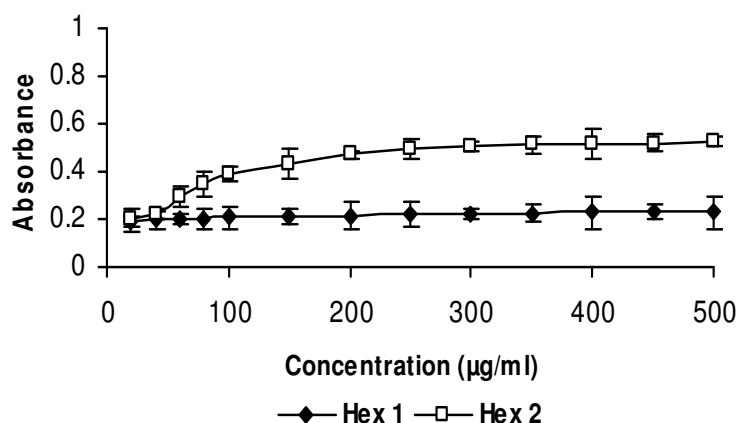


Figure 8. Effect of different concentrations of 'Hex 1' and 'Hex 2' extract of *T. chebula* in reducing power assay.

other hand 'Hex 2' extract showed significant increase in absorption and this is possibly due to the presence of phenolic compounds as confirmed by UV spectroscopy. Variations in activity of hexane extracts of maceration and sequential methods are expected, which may be influenced by such factors as presence of polyphenolic compounds, stability of the specific antioxidant components and variations in extractability.

Iron and copper are essential transition metal elements in the human body for the activity of a large range of enzymes and for some proteins involved in cellular respiration, O₂ transport and redox reactions. But, because they are transition metals, they contain one or more unpaired electrons that enable them to participate in one-electron transfer reactions. Hence, they are powerful catalysts of autoxidation reactions, such as participation in the conversion of H₂O₂ to HO• in the Fenton reaction and in the decomposition of alkyl peroxides to the highly reactive alkoxy and hydroxyl

radicals (Lloyd et al., 1997; Hippeli and Elstner, 1999). In the present study, it was observed that the iron chelating effect of 'Hex 1' extract was 4.8% and 'Hex 2' extract was 59.5% at 250 µg/ml of concentration (Figure 9). From the results it is clear that the 'Hex 2' extract interfered with the formation of ferrous-ferrozine complex, suggesting that it have marked iron chelating activities and capture ferrous ion before the formation of ferrozine. A significant inhibitory effect of the extracts might be due to the presence of phenolics and their glycosides as indicated by UV analysis (Figure 4). Transition metal chelation to form low redox potential complexes is an important antioxidant property (Halliwell et al., 1995) and measuring chelation of iron (II) is one method for assessing this property. Since the reaction is dependent on the affinity of an antioxidant towards iron (II) in relation to ferrozine, the assay is affected by both binding constant and concentration of antioxidant and thus only strong iron antioxidant chelator is detected.

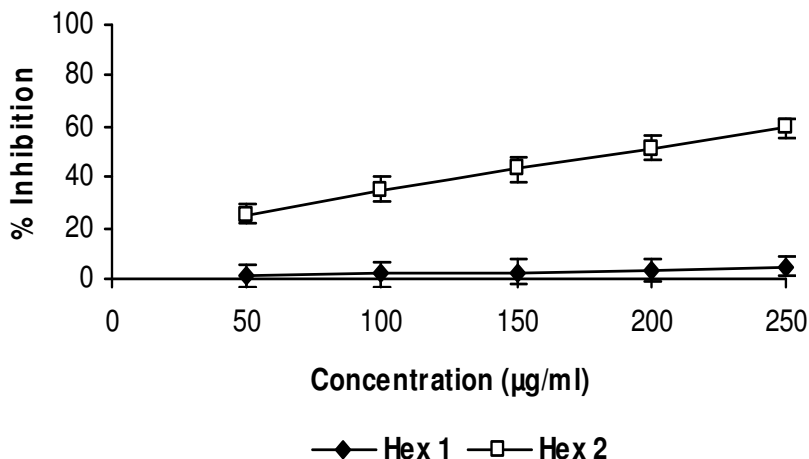


Figure 9. Effect of different concentrations of 'Hex 1' and 'Hex 2' extract of *T. chebula* in chelating power assay.

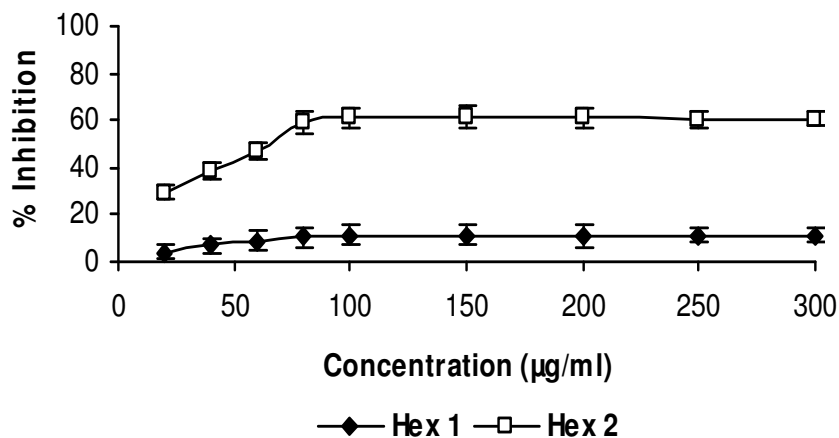


Figure 10. Effect of different concentrations of 'Hex 1' and 'Hex 2' extract of *T. chebula* in lipid peroxidation assay.

Lipid is not only a major component of biomembrane system, but also an important ingredient of foods. Lipid decomposition through peroxidation results in injury of cells and tissues in living system and decreases the safety and nutritional value in oil food products (Finkel and Holbrook, 2000; Marnett, 2002; Koleva et al., 2003). Lipid peroxidation has been broadly defined as the oxidative deterioration of polyunsaturated lipids (Kappus, 1991). Inhibition of lipid peroxidation by any external agent is often used to evaluate its antioxidant capacity. 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. As the trend shown in earlier experiments, here also the 'Hex 2' extract indicated the highest ability of 60.5% at 300 µg/ml dose and 'Hex 1'

extracts possessed 11.3% inhibition at the same dose (Figure 10). Decrease in lipid peroxidation by 'Hex 2' extract may be a result of its ability to scavenge HO• radicals by phenolic compounds present in them that were confirmed by quantitative determination and spectroscopic analysis. Cheng et al. (2003) examined the antioxidant activity of different extracts and pure compounds of *T. chebula* for anti-lipid peroxidation, anti-superoxide radical formation and free radical scavenging activities. The results showed that all tested extracts and pure compounds of *T. chebula* exhibited antioxidant activity at different magnitudes of potency. Naik et al. (2003) investigated the antioxidant activity of four aqueous extracts from different parts of medicinal plants used in Ayurveda viz., *Momardica charantia*, *Glycyrrhiza glabra*, *Acacia catechu* and *Terminalia chebula*. The antioxidant activity of these extracts was tested by

studying the inhibition of radiation induced lipid peroxidation in rat liver microsomes at different doses as estimated by thiobarbituric acid reactive substances (TBARS). Of all these extracts, *T. chebula* showed maximum inhibition in the TBARS formation and hence is considered the best antioxidant among these four extracts.

Polyphenols in 'Hex 2' extract can combine with hydroxyl radical and convert them into water molecules and stabilize them to inhibit MDA production or they chelate the active iron sites so that the Fenton reaction could not take place. Polyphenols easily transfer a hydrogen atom to lipid peroxy radicals and form the aryloxy radical, which is incapable of acting as a chain carrier or couples with another radical and thereby quenches the radical process (Pekkarinen et al., 1999; Yildirim et al., 2000; Sestili et al., 2002). So, it may be supposed that different polyphenols are responsible for antioxidative potential of 'Hex 2' extract.

Conclusion

In the light of results obtained in the present study it is suggested that 'Hex 2' extract of *T. chebula* prepared by sequential method exhibited good hydrogen donating, radical scavenging, metal chelating, reducing and antioxidant potential. It has been suggested that compounds, which possessed antioxidant activity can inhibit mutation and cancer because they can scavenge a free radicals or induce the antioxidant enzymes (Siddaiah et al., 2007). The extraction method effect the activity of the extract. From results it is clear that the sequential extraction method is better than maceration extraction method as phenolic compounds are extracted in 'Hex 2' extract and it showed good antioxidant activity than 'Hex 1'.

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