Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Der Pharmacia Lettre, 2014, 6 (3):1-8
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

In-vitro antioxidant potential of *Lawsonia inermis* Linnaeus (Seeds)

G. D. Chaudhary[1]* and A. N. Kalia[2]

[1]Rayat and Bahra Institute of Pharmacy, Kharar, Mohali, Punjab, India
[2]Department of Pharmacognosy, I. S. F. College of Pharmacy, Moga, Punjab, India

---

ABSTRACT

Antioxidant activities of the ethanol (90%) extract of seeds of Lawsonia inermis L., as well as chloroform, ethyl acetate and water fractions extracted from the 90% ethanol extract were examined by a DPPH free radical scavenging and ferric reducing power (FRPA) as Non-site specific assays and lipid peroxidation (TBARS) as site specific assay. The 90% ethanol extract and its fractions along with the reference samples, gallic acid and rutin were further analysed to determine their total phenolic content by Folin-Ciocalteu's method and total flavonoids content by Aluminium chloride method. In Non-site specific assays showed significant scavenging activity for the ethanol (90%) extract and its other fractions. Site-specific lipid peroxidation also confirms the peroxyl radical scavenging capacity of ethyl acetate fraction of ethanol extract and results were compared with standard antioxidant (Butyl hydroxy toluene). In general, the ethyl acetate fraction of the ethanol extract showed significant (P < 0.05) activity in all systems, such results might be attributed to the prominent antioxidant effect. The antioxidant activities of all the tested samples were concentration dependent. Based on the results obtained, we can conclude that the L. inermis seeds extract and its fractions may be valuable natural antioxidant sources and are potentially applicable in both medicine and the healthy food industry.

Keywords: Antioxidant activity; DPPH; FRPA; Phenolic compounds; TBARS

INTRODUCTION

Oxygen, an element indispensable for life, can, under certain circumstances, adversely affect the human body. Oxidation is essential in many living organisms for the production of energy to fuel biological processes. However, the uncontrolled production of oxygen derived free radicals is involved in the onset of many diseases such as atherosclerosis, rheumatoid arthritis, and cancer as well as in degenerative processes associated with aging [1].

The imbalance of oxidants and antioxidants of the body leads to an oxidative stress resulting in destruction of unsaturated lipids, DNA, proteins and other essential molecules. Increasing evidence suggests that oxidative damage to cell components has a relevant pathophysiological role in several types of human diseases [2]. Most of the potentially harmful effects of oxygen are due to the formation of reactive oxygen species (ROS). The uncontrolled production of ROS and the unbalanced mechanism of antioxidant protection result in the onset of many diseases and accelerate ageing. Free radicals are been reported to cause red blood cell lysis in patients with blood pathologies such as thalassemia [3]. The erythrocytes are highly susceptible to oxidative damage due to the high polyunsaturated fatty acid content of their membrane and the high cellular concentration of oxygen and haemoglobin, all of which are powerful promoters of oxidative processes [4]. Exposure of erythrocytes to free radicals leads to a number of membrane changes including lipid peroxidation [5], reduction in deformability [6], changes in cell morphology [7], protein cross-linking and fragmentation [8]. These are the most common configuration damage leading to lysis of red blood cells.

L. inermis L. (Lythraceae) commonly known as 'Mhendi' and 'Henna' is a shrub commonly used as a hedge plant. It is indigenous to North Africa, South-West Asia and India; it is now widely cultivated throughout the tropics as an ornamental and dye plant [9, 10]. Besides its use in cosmetics and as a hair dye, the henna is also used as a prophylactic in skin disorders [11] and inflammation [12], as immunostimulant [13], hepatoprotective [14], memory enhancer [15], hypoglycaemic [16], cytotoxic [17], tuberculostatic [18], bactericidal [19], fungicidal [20] and molluscicidal [21].

Antioxidants are widely used as food additives to provide protection against oxidative degradation of foods by free radicals [22]. In recent decades, there has been great interest in screening essential oils and various plant extracts for natural antioxidants because of their good antioxidant properties. Many kinds of antioxidative components that contain polyphenolic compounds, chlorophylls, carotenoids, tocopherol derivatives, lignan, and related isoprenoids have been isolated from different kinds of plants, such as oilseeds, cereal crop, vegetables, leaves, roots, spices, herbs, and seaweeds, for use as antioxidants. Though a large number of plants worldwide show strong antioxidant activities [23], the antioxidant properties of seeds have not been elucidated before. Therefore, the objective of the present study was to investigate the total phenolic content and antioxidant properties of its ethanol extract and its fractions of *L. inermis* L. seed by DPPH, FRPA as non-site specific assays and TBARS as Site specific assay. The present study would offer basic data on the natural antioxidant potential of seeds for the food or pharmaceutical industries, and also provides scientific reference for the large scale usage and exploitation of the plant as a resource.

Total Phenolic Compounds Content

The total phenolic compounds contents in seeds were determined using the method described previously [24]. Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants. It works by measuring the amount of substance being tested needed to inhibit the oxidation of the reagent. The sample extract dilution was oxidized with Folin-Ciocalteu's reagent and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue colour was measured at 765 nm after 30 min.

Total Flavonoids Content

The aluminium chloride colorimetric method was modified from the procedure reported by Woisky and Salatino [25]. Aluminium chloride forms acid stable complexes with the C_4 keto group and either the C_3 or C_5 hydroxyl group of flavones and flavonols. In addition, aluminium chloride forms acid labile complexes with the ortho dihydroxyl groups in the A or B ring of flavonoids.

MATERIALS AND METHODS

2.1 Plant material

The seeds of the plant were collected in the month of June from regional areas of Salem, Tamilnadu and authenticated by Dr. H.S. Singh, Scientist F & Head, Raw Material Herbarium & Museum, NISCAIR, New Delhi, India (Voucher specimen - NISCAIR/RHMD/Consult/-2008-09/1175/207). The seeds were shade dried, coarsely powdered and stored in an air tight container till use.

2.2 Extraction

Seeds powder was defatted with *n*-hexane. The dried solvent freed marc was extracted by cold maceration with ethanol (90%) till exhausted completely. The ethanol extract so obtained was freed of solvent under vacuum and further fractionated with chloroform, ethyl acetate and water by using sonication. All the extract and its fractions were preserved in a refrigerator till further use.

2.3.1 Determination of total phenolic content by colorimetric method

Preparation of standard: Gallic acid was used to make the calibration curve. 10 mg of gallic acid was dissolved in 100 ml of 50% methanol (100 µg/ml) and then further diluted to 1, 2, 4, 6, 8 and 10 µg/ml. 1 ml aliquot of each dilution was taken in a test tube and diluted with 10 ml of distilled water. Then, 1.5 ml Folin Ciocalteu's reagent was added and allowed to incubate at room temperature for 5 min. 4 ml of 20% (w/w) Na_2CO_3 were added, adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room temperature. Absorbance of the standard was measured at 765 nm. Distilled water was taken as a blank.

Preparation of sample: 1 g of sample (seed powder) was added to 15 ml of methanol (50%) and extracted for three times by maceration of 2 h. Then filtered and make up the volume with methanol (50%) in volumetric flask up to 50 ml. 1 ml aliquot of the sample was taken in a test tube and diluted with 10 ml of distilled water. Then, 1.5 ml Folin Ciocalteu's reagent was added and allowed to incubate at room temperature for 5 min. 4 ml of 20% (w/w) Na_2CO_3 were added, adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room

temperature. Absorbance of the sample was measured at 765 nm. Three parallel determinations were recorded. Quantification was done on the basis of a standard curve of gallic acid. Results were expressed as mg gallic acid equivalents (GAE) and percentage w/w [24].

2.3.2 Determination of total flavonoids content by colorimetric method

Preparation of standard: Rutin was used to make the calibration curve. 10 mg of rutin was dissolved in 100 ml of 80% methanol (100 µg/ml) and then further diluted to 10, 20, 40, 60, 80 and 100 µg/ml. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of 95% methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with Shimadzu UV-Visible spectrophotometer. The amount of 10% aluminium chloride was substituted by the same amount of distilled water in blank.

Preparation of sample: About 1 g of ethanol extract and its ethyl acetate fraction were dissolved in 25 ml of 80% methanol. Similarly, 0.5 ml of ethanol extract and its ethyl acetate fraction were reacted with aluminium chloride for determination of flavonoids content as described above. Three parallel determinations were recorded. Quantification was done on the basis of a standard curve of rutin. Results were expressed as mg rutin equivalents (RE) and percentage w/w [25].

2.4 Screening for antioxidant activity

2.4.1 Chemicals and reagents

1, 1-Diphenyl-2-picryl hydrazyl (DPPH) and Butyl hydroxyl toluene (BHT) were obtained from Sigma–Aldrich Co., St. Louis, USA. Rutin was obtained from Acros Organics, NJ, USA. Ascorbic acid was from SD Fine Chemicals Ltd., Mumbai, India. All chemicals used were of analytical grade.

2.4.2 Preparation of Sample and standard solutions

A. DPPH (Non-site specific)

Preparation of sample dilution: 50 mg of each of ethanol extract and its fractions were weighed separately and dissolved in 100 ml of methanol get 500 µg/ml concentration. Lower concentrations (10, 20, 40, 80, 160 µg/ml) of these solutions were prepared by diluting serially with methanol.

Preparation of standard dilution: 10 mg of each of ascorbic acid and rutin were weighed separately and dissolved in 100 ml of methanol get 100 µg/ml concentration. Lower concentrations (5, 10, 15, 20, 25 µg/ml and 2, 4, 6, 8, 10 µg/ml respectively) of these solutions were prepared by diluting serially with methanol.

B. FRPA (Non-site specific)

Preparation of sample dilution: 50 mg of each of ethanol extract and its fractions were weighed separately and dissolved in 100 ml of methanol get 500 µg/ml concentration. Lower concentrations (25, 50, 100, 200, 400 µg/ml) of these solutions were prepared by diluting serially with methanol.

Preparation of standard dilution: 50 mg of ascorbic acid was weighed and dissolved in 100 ml of methanol get 500 µg/ml concentration. Lower concentrations (25, 50, 100, 200, 400 µg/ml) of the solution were prepared by diluting serially with methanol.

C. Lipid peroxidation (TBARS) (Site specific)

Preparation of sample dilution: 50 mg of each of ethanol extract and its ethyl acetate fraction were weighed separately and dissolved in 100 ml of methanol get 500 µg/ml concentration. Lower concentrations (25, 50, 100, 200, 400 µg/ml) of these solutions were prepared by diluting serially with methanol.

Preparation of standard dilution: 50 mg of Butyl hydroxyl toluene (BHT) was weighed and dissolved in 100 ml of methanol get 500 µg/ml concentration. Lower concentrations (25, 50, 100, 200, 400 µg/ml) of the solution were prepared by diluting serially with methanol.

2.4.3 Free radical scavenging assays (Non-site-specific)

2.4.3.1 DPPH radical scavenging activity

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts. The 0.1 mM solution of DPPH in methanol (22.2 mg in 1000 ml) was freshly prepared. Different concentrations of extract were added at an equal volume to methanolic solution of DPPH. After 30 min. at room temperature, the absorbance was recorded at 517 nm. Ascorbic acid and rutin were used as standard controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals. Radical scavenging activity was calculated by the following formula

% Free radical scavenging power = Abs. C - (Abs. S – Abs. B) / Abs. C × 100

IC50 value was determined from the plotted graph of scavenging activity against the different concentrations of L. inermis ethanol extract and its fractions, which is defined as the total antioxidant necessary to decrease the initial DPPH radical concentration by 50%. The measurements were carried out three times and their scavenging effect was calculated based on the percentage of DPPH scavenged [26, 27].

2.4.3.2 Ferric reducing power assay (FRPA)
1 ml of the sample (25–400 µg/ml), 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were incubated at 50ºC for 30 min and 2.5 ml of 10% trichloro acetic acid was added to the mixture and centrifuged for 10 min at 3000 rpm. About 2.5 ml of the supernatant was diluted with 2.5 ml water and is shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm. L-Ascorbic acid was used as the standard. All tests were performed in triplicate and the graph was plotted with the average of the three determinations. [28, 29]

2.4.4 Lipid peroxidation (TBARS) (Site-specific)
Normal male rats (250 g) were used for the preparation of liver homogenate. The perfused liver was isolated, and 10% (w/v) homogenate was prepared with homogenizer at 0-4°C with 0.15 M KCl. The homogenate was centrifuged at 8000 rpm for 15 min, and clear cell-free supernatant was used for the study with *in vitro* lipid peroxidation assay. Different concentrations (50-700 µg/ml) of extract/fractions dissolved in methanol and in test tubes. 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% butyl hydroxy toluene. 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 [30, 31].

The percentage inhibition of lipid peroxidation is calculated by the formula:

Inhibition of lipid peroxidation (%) = 1 – (Abs. S/Abs. B) × 100

2.7. Statistical analysis
Results were reported as means ± S.D. of three determinations. IC_{50} values were determined by interpolations. One Way ANOVA was used to evaluate differences between groups. The differences among the means were analysed by Tukey-Kramer test for multiple comparisons using computerized program at 95% ($P < 0.05$) confidence level [32].

RESULTS AND DISCUSSION

The total phenolic compound estimation showed the absorbance 2.335 at 765 nm wavelength. The calibration curve of standard gallic acid is given in **Fig 1** and the results are depicted in **Table 1**. The total flavonoids content estimation from ethanol extract and its ethyl acetate fraction of seeds showed the absorbance 3.112 and 3.068 at 415 nm wavelength. The calibration curve of standard rutin is given in **Fig 2** and the results are depicted in **Table 2**.

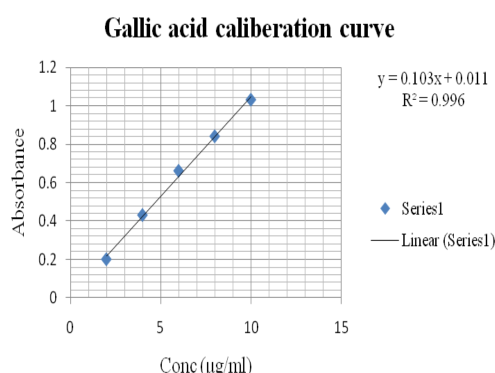


Fig 1

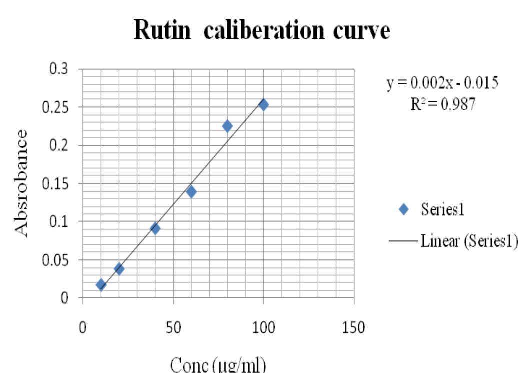


Fig 2

Table 1: Estimation of total phenolic compounds content

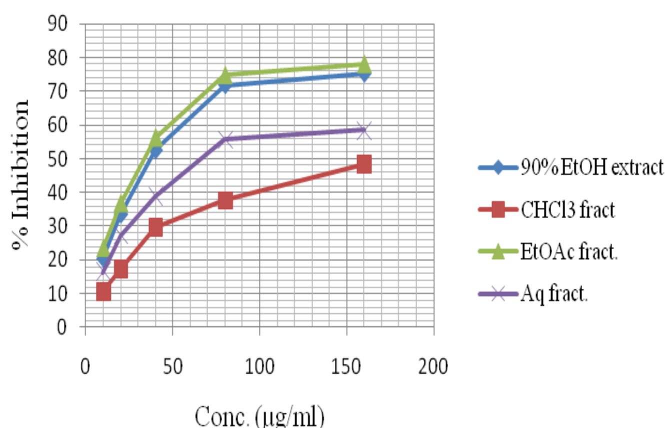
Test Sample	Total Phenolic Content (mg gallic acid equivalents) (GAE)	Total Phenolic Content (% w/w)
Seeds powder	26.87	3.35

Table 2: Estimation of total flavonoids content

Test Sample	Total Flavonoids (mg rutin equivalents)	Total Flavonoids (% w/w)
Ethanol (90%) extract	1563.5	9.771
Ethyl acetate fraction	1541.5	9.634

Free radicals are highly reactive molecules with an unpaired electron and are produced by radiation or as by-products of metabolic processes. They initiate chain reactions which lead to disintegration of cell membranes and cell compounds, including lipids, proteins and nucleic acids. Antioxidant compounds scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus reduce the level of oxidative stress and slow/prevent the development of complications associated with oxidative stress-related diseases.

3.1 DPPH method



The ethanol and its fractions of the *L. inermis* L. seeds showed promising free radical scavenging effect of DPPH in a concentration dependent manner up to a concentration of 80 µg/ml are shown in **Fig 5**. The ethyl acetate fraction of ethanol extract showed more scavenging activity than the ethanol extract. Ascorbic acid and rutin were used as the reference standards are shown in **Fig 3** and **Fig 4** respectively. The results were expressed as the dose required to cause 50% inhibition for extract (IC_{50}) and the results are depicted in **Table 3**. The present investigation has shown that the extract and its fractions of seeds of *L. inermis* L. exhibited DPPH scavenging activity, the most effective being ethyl acetate fraction of ethanol extract which exhibited significantly higher DPPH scavenging activity (IC_{50} = 33.71±0.211 µg/ml) followed by ethanol extract (IC_{50} = 37.05±0.247 µg/ml) when compared with the IC_{50} values of the standards ascorbic acid and rutin (IC_{50} = 8.84±0.05 and 3.78±0.153 µg/ml respectively).

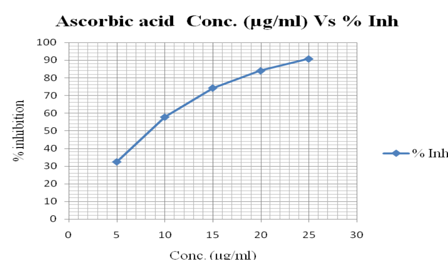


Fig 3

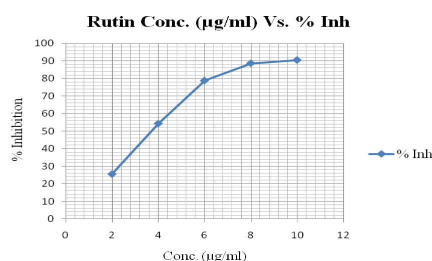


Fig 4

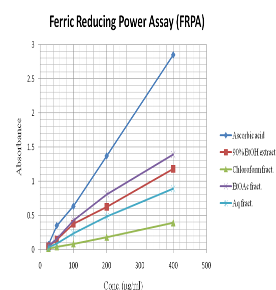


Fig 5

Table 3: 50% inhibition for extract (IC_{50}) of L. inermis L. by DPPH method

S. No.	Extract	IC50 (µg/ml)±S.D.
1.	Rutin (Standard)	3.78±0.153
2.	Ascorbic acid (Standard)	8.84±0.05
3.	Ethanol (90%) Extract	37.05± 0.247[a]
4.	Chloroform fraction	168.95±8.523
5.	Ethyl acetate fraction	33.71±0.211[a, b]
6.	Aqueous fraction	66.29±3.625

Values are means ± S.D. of three determinations. IC_{50} values were determined by interpolations. One way ANOVA followed by Tukey's multiple test; a = Results significantly different from Standard, P < 0.05; b = Results significantly different from EtOH extract, P < 0.01

3.2 FRPA method

The reducing power of ethanol extract and its fractions of the *L. inermis* L. seeds are shown in **Fig 6**. Ascorbic acid was used as the reference standards. The reducing power of extract and its fractions were found to increase in concentration dependent manner up to a concentration of 400 µg/ml the values were remained lower compared to the ascorbic acid and the results are depicted in **Table 4**. The ethyl acetate fraction of ethanol extract showed more reducing power than the ethanol extract. The reducing capacity of a compound may serve as a like the antioxidant activity, the reducing power of the extract and its fractions increased with increasing the concentration. Increase in absorbance of the reaction mixture indicates the increase in the reducing power of the sample. The reducing power showed by the extract is statistically significant ($P < 0.05$). The antioxidant activity has been attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and free radical scavenging [33].

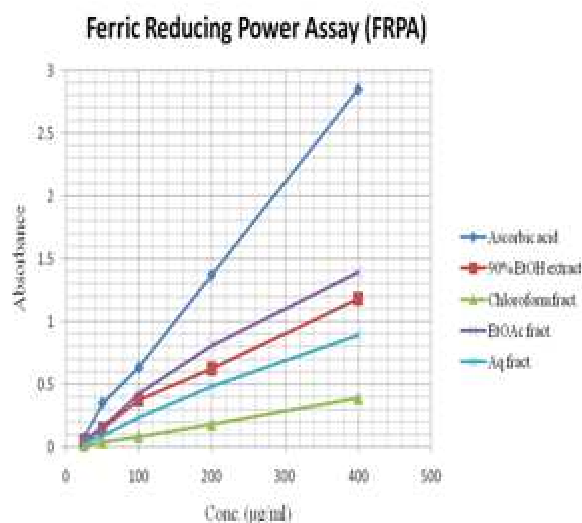


Fig 6

3.3 Lipid peroxidation (TBARS)

The results of the TBARS assay for ethanol extract and its ethyl acetate fraction are given in **Fig 7**. Both the samples showed anti lipid peroxidation activities, which are lower than that of butyl hydroxyl toluene (BHT). The percentage antioxidant activity of ethanol extract and its ethyl acetate fraction aqueous extracts and BHT increased with increasing concentration as shown in **Fig 7**. These results were also expressed as the dose required to obtain 50% antioxidant index and the results are depicted in **Table 5**.

Table 4: Total reducing power of extract and its fractions

Conc. (µg/ml)	Ascorbic acid (Standard)	EtOH extract	Chloroform fract.	EtOAc fract.	Aq fract.
25	0.078	0.046	0.016	0.057	0.035
50	0.355	0.144	0.042	0.165	0.094
100	0.638	0.377[a]	0.085	0.428[a, b]	0.236
200	1.374	0.623[a]	0.182	0.813[a, b]	0.485
400	2.852	1.182[a]	0.391	1.394[a, b]	0.895

Table 5: Lipid peroxidation inhibition (TBARS) of extract and its fraction

S. No.	Extract	IC_{50} (µg/ml)±S.D.
1.	BHT (Standard)	48.89±0.011
2.	Ethanol (90%) extract	93.00±0.124[a]
3.	Ethyl acetate fraction	87.54±0.158[a, b]

Values are means ± S.D. of three determinations. IC_{50} values were determined by interpolations. One way ANOVA followed by Tukey's multiple test; a = Results significantly different from Standard, P < 0.05; b = Results significantly different from EtOH extract, P < 0.01

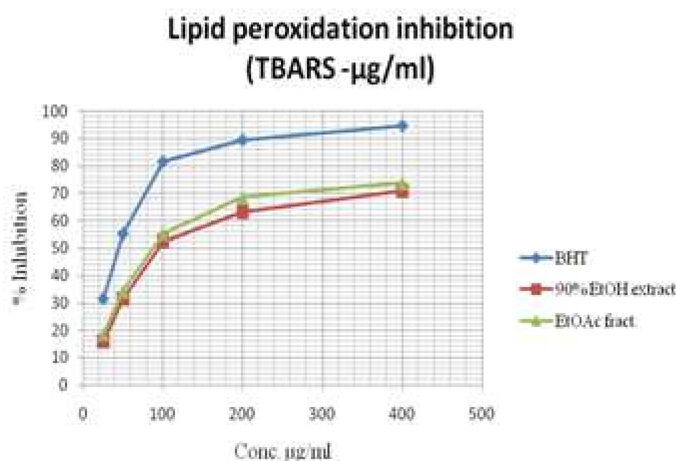


Fig 7

The extract and its ethyl acetate fraction were tested to be effective in reducing the production of TBARS in a dose-dependent manner, thus allowing calculation of the concentration that would inhibit the TBARS production by 50% (i.e. the IC_{50}). Thus the decrease in the MDA levels in the presence of increased concentration of extract and its ethyl acetate fraction indicates their role as antioxidants. TBARS assay was used to determine the anti lipid peroxidation properties of the ethanol extract and its ethyl acetate fraction. Thus ethanol extract and its ethyl acetate fraction inhibit the initiation of lipid peroxidation by scavenging the free radicals that form alkylperoxyl and alkoxyl radicals or can donate hydrogen atom to alkylperoxyl and alkoxyl radicals and thus stop chain propagation.

CONCLUSION

The ethanol extract and its fractions (chloroform, ethyl acetate and aqueous) of *L. inermis* L. seeds exhibited different levels of antioxidant activity in all the models studied. The results from various free radical scavenging systems revealed that the *L. inermis* L. had significant antioxidant activity and free radical scavenging activity, with effective scavenging activity against free radicals such as DPPH and FRPA. In addition, *L. inermis* L. inhibited peroxides in rat liver homogenate. These results clearly revealed that ethyl acetate fraction of ethanol extract of seeds of *L. inermis* L. might act as a potential antioxidant for biological systems susceptible to free radical mediated reactions and therefore it might act as hepatoprotective, reduce the risk of aging related diseases and/or promote general human health. Based on these data, free radical scavenging property may be one of the mechanisms by which this drug is useful as a traditional medicine. However, additional studies are needed to characterize the bioactive compounds responsible for the observed *in-vitro* antioxidant in *L. inermis* L. and different antioxidant mechanisms.

Acknowledgement
The authors extend their sincere thanks to I.S.F. college of Pharmacy, Moga for providing the required institutional facilities.

REFERENCES

[1] B Halliwell; JMC Gutteridge. *The Lancet*, **1984**, 323, 1396-1397.
[2] BN Ames; MK Shigenaga; TM Hagen. *Proc. Natl. Acad. Sci*, **1993**, 90, 7915-7922.
[3] JL Vives-Corrons; A Miguel-Garcia; MA Pujades; A Miguel-Sosa; S Cambiazzo; M Linares. *Eur. J. Haematol*, **1995**, 55, 327-331.
[4] MR Clemens; M Ruess; Z Bursa; HD Waller. *Free Radic Res. Commun*, **1987**, 3, 265-271.
[5] JF Koster; RG Slee. *Biochem. Biophys. Acta*, **1983**, 752, 233-239.
[6] M Kurata; M Suzuki; K Haruta; K Takeda. *Comp. Biochem. Physiol*, **1994**, 107(A), 7-12.
[7] E Shinar; EA Rachmilewitz; A Shifter; E Rahamim; P Saltman. *Biochem. Biophys. Acta*, **1989**, 1014, 66-72.
[8] MC Vissser; A Stern; F Kuypers; J Van den Berg; CC Winterbourn. *Free Radic. Biol. Med*, **1994**, 16, 703-712.
[9] BN Sastri. The Wealth of India: Raw Materials, 1st ed., CSIR Publication, New Delhi, **1962**, 6(L-M), pp.47-50.
[10] AK Gupta. Quality standards of Indian medicinal plants, 1st ed., ICMR Publication, New Delhi, **2003**, pp. 123-129.
[11] V Natarajan; S Mahendraraja; T Menon. *Biomed*, **2000**, 20(4), 243-245.
[12] BH Alia; AK Bashir; MOM Tanira. *Pharmacol*, **1995**, 51, 356-363.
[13] BR Mikhaeil; FA Badria; GT Maatooq; MMA Amer. *Zeitschrift fuer Naturforschung Section C Journal of Biosciences*, **2004**, 59, 468-476.
[14] S Ahmed; A Rahman; A Alam; M Saleem; M Athar; S Sultana. *J. Ethnopharmacol*, **2000**, 69, 157-164.
[15] MR Iyer; SC Pal; VS Kasture; SB Kasture. *Indian J Pharmacol*, **1998**, 30(3), 181-185.
[16] I Syamsudin; H Winarno. *Res. J. Pharmacol*, **2008**, 2(2), 20-23.
[17] S Endrini; A Rahmat; P Ismail; YH Taufiq-Yap. *J. Med. Sci*, **2007**, 7(7), 1098-1102.
[18] VK Sharma. *Tubercle*, **1990**, 71(4), 293-296.
[19] NAA Ali; WD Julich; C Kusnick; U Lindequist. *J. Ethnopharmacol*, **2001**, 74(2), 173-179.
[20] MN Anwar; J Begum; M Yusuf; JU Chowdhury JU; S Khan; M Nural. *Bangladesh J Microbiol*, **2007**, 24(1), 76-78.
[21] A Singh; DK Singh. *Indian J Exp Biol*, **2001**, 39, 263-268.
[22] I Gulcin; M Oktay; OI Kufrevioglu; A Aslan. *J. Ethnopharmacol*, **2002**, 79, 325-329.
[23] M Milos; T Kulisic; M Jukic. *Food Chem*, **2006**, 94, 550-557.
[24] VL Singleton; R Orthofer; RRM Lamuela. *Methods in Enzymology*, **1999**, 299, 152–178.
[25] R Woisky; A Salatino. *J. Apic. Res*, **1998**, 37, 99-105.
[26] MS Blois. *Nature*, **1958**, 29,1199-1200.
[27] R Singh; N Singh; BS Saini; HS Rao. *Indian J. Pharmacol*, **2008**, 40(4), 147-151.
[28] M Oyaizu. *Japanese J. Nut*, **1986**, 44, 307-315.
[29] CR Tenpe; U Aman; B Amol; PG Yeole. *Phcog. Mag*, **2008**, 4(15), 124-129.
[30] B Halliwell; JMC Gutteridge. In free radicals in biology and medicine. 2nd ed., Japan Scientific Societies Press, Tokyo, **1989**, pp. 152-165.
[31] S Arora; R Singh. *African J. Biotechnol*, **2009**, 8(7),1270-1272.
[32] H Hosseinzadeh; SMT Mansouri; B Naghizadeh. *Iranian Biomed. J*, **2005**, 9(4),181-185.
[33] SK Sharma; VK Gupta. *Phcog. Mag*, **2008**, 4(13)(Supp 1), 70-74.