

Comparison of phytochemical and antioxidant properties of extracts from flaxseed (*Linum usitatissimum*) using different solvents

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Abstract

The aim of the present study was to evaluate the phytochemical constituents and free radicals scavenging activities of flaxseed (*Linum usitatissimum*) solvents extract. For phytochemical analysis of extracts, preliminary screening methods of phytoconstituents were followed in different solvents. In addition, total phenolic and flavonoids compounds were measured. *In vitro* antioxidant activity was determined by 2,2-diphenyl-2-picryl-hydrazyl (DPPH), superoxide radical scavenging activity and ferric reducing power tests. Phytochemical analysis provides evidence that both solvents extract of *Linum usitatissimum* contains an important amounts of bioactive compounds. Furthermore, using methanolic and butanolic were able to extract a higher amounts of total phenolic compounds (47.01 ± 5.40 and 43.33 ± 2.77 μg gallic acid equivalents/g of extract, respectively) and flavonoids (30.89 ± 0.09 and 29.55 ± 0.15 μg Quercetin equivalents/g of extract, respectively). *In vitro* antioxidant activities of petroleum ether, benzene, ethyl acetate, methanolic and butanolic extracts of flaxseed were evaluated. Butanolic extract exhibited a higher DPPH radical scavenging activity, whereas petroleum ether and ethyl acetate extracts showed an important hydrogen peroxides scavenging activity. Also, an increase of ferric reducing antioxidant power (FRAP) was noted with methanolic extract. The results of the current study showed that flaxseed contain important amount of phenolic compounds and higher antioxidant activities rendering them as a potential source for development of nutraceuticals.

Keywords

Linum usitatissimum,
Phenolics,
Flavonoids,
DPPH,
Antioxidant activity

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Introduction

Oxidative stress occurs when redox homeostasis within the cell is altered. This imbalance may be due to either an overproduction of reactive oxygen species (ROS) or deficiency of an antioxidant system (Rosenfeldt *et al.*, 2013). ROS have been implicated in more than 100 degenerative diseases including cardiovascular, diabetes, cancer, atherosclerosis, neurodegenerative disorders and arthritis (Saminathan *et al.*, 2013; Adebayo *et al.*, 2014). Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated (Yamuna *et al.*, 2013). In this respect, polyphenolic compounds, like flavonoids and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity (Faller and Fialho, 2010; Burgos *et al.*, 2013; Chiang *et al.*, 2013).

Presently, the possible toxicity of synthetic antioxidants has been criticized. It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit,

tea and herbs may contribute to shift the balance toward an adequate antioxidant status (Shen *et al.*, 2012). Consequently, interest in natural antioxidant, especially of plant origin, has greatly increased in recent years (Jayaprakash *et al.*, 2000).

Linum usitatissimum (Linn.), commonly known as flaxseed or linseed, belongs to the family Linaceae. It has been consumed as a food ingredient and presently has a high demand in food industries (Arvindkumar *et al.*, 2012). Flaxseed plays a major role in the field of diet and disease research due to its potential health benefits associated with linolenic acid (57%) and a major lignan, namely ecoisolariciresinol diglucoside (SDG) (Milder *et al.*, 2005).

There are numbers of studies indicating the potential of flaxseed as antidiabetic (Prasad *et al.*, 2000) and cardioprotective activity (Zanwar *et al.*, 2011), but very few studies evaluating antioxidant potential of *L. usitatissimum* and its association with ROS production. Hence, present study is carried out to compare the phytochemical and antioxidant properties of extracts from flaxseed using different solvents.

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Materials and Methods

Chemicals and reagents

All the chemicals, reagents and standards including Folin-Ciocalteu's phenol reagent, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, gallic acid were obtained from Sigma-Aldrich (St. Louis, Mo, USA), whereas, potassium dihydrogen phosphate, ferrous chloride, sulphuric acid and all other chemicals/reagents and solvents used in this study were of analytical reagent grade and purchased from Merck (Darmstadt, Germany).

Plant material and sample preparation

Linum usitatissimum was collected in Southern of Algeria (Adrar), between February and March 2015, identified taxonomically and authenticated by the Botanical Research Institute of Oran 1 University. For the present study purpose, the dried plant material was made into fine powder of 40 mesh size using the pulverizer. The coarse powder (100 g) of linseed was extracted successively with petroleum ether, benzene, ethyl acetate, methanol and butanol, each 250 mL in a Soxhlet apparatus for 24 hours (Tiwari *et al.*, 2011). All the extracts were filtered through Whatman No.41 filter paper. Then the extracts were concentrated in a rotavapor (Buchi Labortechnik AG, Postfach, Switzerland). The concentrated extracts were used for the estimation of total phenolics and flavonoids and *in vitro* antioxidant activity.

Phytochemical screening

Phytochemical screening was done using standard procedures as previously described by (Tiwari *et al.*, 2011). Each extract was screened for the following phytoconstituents: alkaloids, saponins, terpenoids, flavonoids, glycosides, proteins, carbohydrate, and phenolic compounds.

Determination of total phenolics

The total phenol content was determined by Folin-Ciocalteu reagent method (McDonald *et al.*, 2001). 0.5 mL of extract (1mg/mL) and 0.1 mL of Folin-Ciocalteu reagent (0.5N) were mixed and incubated at room temperature for 15 min. 2.5 mL of 20%(w/v) saturated sodium carbonate was added, incubated for 30 min at room temperature and absorbance was measured at 760 nm. The total phenol content was expressed in terms of Gallic acid equivalent ($\mu\text{g/g}$) (Chanda and Dave, 2009).

Determination of total flavonoids

The total flavonoid content was determined by Aluminum chloride method. 1mL of sample (1mg/

mL) was mixed with 0.5mL of 1.2% aluminium chloride and 0.5mL of potassium acetate (120 mol/L). In this mixture, 1mL of methanol was added to make 3mL volume. This mixture was vortexed, incubated at room temperature for 30min and absorbance was measured spectrophotometrically at 415nm. The total flavonoid content was expressed in terms of quercetin equivalent ($\mu\text{g/g}$) (Singleton and Rossi, 1965).

DPPH free radical scavenging activity

The free radical scavenging activity of all the extracts was evaluated by 2,2-diphenyl-2-picrylhydrazyl (DPPH) according to Shenet *al.* (2010). Briefly, 0.1 mmol/L solution of DPPH in methanol was prepared, and 1 mL of this solution was added to 3 mL of extracts at different concentration (50,100, 200, 400 and 800 $\mu\text{g/mL}$). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using spectrophotometer (Genesys 10S UV-VIS Thermo Electron Corporation, USA). Ascorbic acid was used as standard. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity.

The capability to scavenging the DPPH radical was calculated by using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

Where, AControl indicates the absorbance of solution containing only the DPPH reagent whereas, ASample is the absorbance of the sample reaction.

Superoxide radical scavenging activity

The superoxide anion scavenging activity was measured as described by Srinivasan *et al.* (2007). The superoxide anion radicals were generated in 3.0 mL of Tris, hydrochloride buffer (16 mmol/L, PH8.0), containing 0.5 mL of nitroblue-tetrazolium chloride (0.3 mmol/L), 0.5 mL nicotinamide adenine dinucleotide (0.936 mmol/L) solution, 1.0 mL extract of different concentration (50,100, 200, 400 and 800 $\mu\text{g/mL}$), and 0.5 mL Tris, hydrochloride buffer (16 mmol/L, PH8.0). The reaction was started by adding 0.5 mL phenazine methosulfate solution (0.12 mmol/L) to the mixture, incubated at 25°C for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid.

The percentage inhibition was calculated by using the following equation:

$$\text{Superoxide radical scavenging activity (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

Where, AControl is the absorbance of the control reaction, and ASample is the absorbance of the sample reaction.

Reducing power assay

The reducing power of the extracts was determined by the method of Kumar and Hemalatha (2011). 1 mL of solution containing 50,100, 200, 400 and 800 µg/mL of the extracts was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 mL, 1.0%). The mixture was incubated at 50°C for 20 minutes. Then 5 mL of 10% trichloroacetic acid was added and centrifuged at 1000xg (10 min at 5°C) in a refrigerated centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance is measured at 700 nm. The experiment was performed thrice and results were averaged.

Statistical analysis

All the experiments were carried out in triplicate. The results are expressed as mean values and standard error of the mean. The one-way analysis of variance (ANOVA) and Tukey multiple comparisons were carried out to test any significant difference between the means. All statistical tests were performed using STATISTICA (Version 6.0, Statsoft, USA) at a 5% significance level.

Results and Discussion

Phytochemical constituents

Plants extracts for medicinal usage contain a variety of types of bioactive compounds. These compounds consist of multi-component mixtures, their separation and determination still creates problems. The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. Furthermore, different solvent systems are available to extract the bioactive compound from natural products. Extraction efficiency is affected by the chemical nature of phytochemicals, the extraction method used, sample particle size, the solvent used,

as well as the presence of interfering substances. Under the same extraction time and temperature, solvent and composition of sample are known as the most important parameters.

The amounts of the crude extracts obtained from flaxseeds using different extraction solvents are presented in Table 1. The preliminary phytochemical screening of different extract of *L. usitatissimum* revealed several polar and non-polar chemical constituents. The phytochemical analysis of *L. usitatissimum* extracts showed the presence of flavonoids, phenolic compounds, glycosides, alkaloids, terpenoids, proteins and carbohydrate by simple qualitative phytochemical screening methods.

All the extracts produced negative results for saponins and the methanolic and butanolic extracts show positive results for flavonoids. Plant food contains a variety of biological activities, non-nutritive compounds known as phytochemicals, which impart health benefits such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property (Narasinga, 2003).

In our investigation, the total phenolic compound content and antioxidant activity of flaxseed were studied since this plant is widely consumed by the native population (either as food or/and as medicine) (Chirinos *et al.*, 2013). Furthermore, the health benefits of flaxseed are mainly attributed to biologically active components such as α -linolenic acid, lignans, unique proteins, phenolic acids and flavonoids (Kitts *et al.*, 1999; Tarpila *et al.*, 2005; Hosseinian *et al.*, 2006). Phytochemical analysis of flaxseed by Tawheed and Monika (2014) showed the presence of flavonoids, terpenoids, tannins and phenols.

Table 1. Phytochemical analysis of *L. usitatissimum* (linn) extracts

Chemical constituents	Phytochemical Test	Methanol extract	Butanol extract	Petroleum ether extract	Ethyl acetat extract	Benzene extract
1 Flavonoids	Alkaline test	+	+	-	-	-
2 Phenolic compounds	Ferric chloride test	+	+	+	+	+
3 Glycosides	Keller-Kiliani test	+	+	+	+	+
4 Terpenoids	Salkowki test	+	+	+	+	+
	Mayer's test	+	+	+	+	+
5 Alkaloids	Wagner's test	+	+	-	+	-
	Dragendorff's test	+	+	+	+	-
6 Carbohydrate	Molish test	+	+	+	+	+
7 Saponins	Frothing test	-	-	-	-	-
8 Proteins	Millon's test	+	+	+	+	+

(+) implies presence; (-) implies absence

Table 2. Total phenolic and total flavonoid contents of *L. usitatissimum* extracts

<i>L. usitatissimum</i> extracts	TPC($\mu\text{g GAE/g}$) ^a	TFC $\mu\text{g QE/g}$ ^b	DPPH	Superoxide
			IC ₅₀ ($\mu\text{g/mL}$)	
Petroleum ether	30.22 \pm 1.17 ^b	-	345.06 \pm 13.11 ^a	287.18 \pm 5.11 ^b
Ethyl acetate	23.41 \pm 2.24 ^c	-	333.20 \pm 10.12 ^a	419.03 \pm 20.15 ^a
Butanol	43.33 \pm 2.77 ^a	29.55 \pm 0.15 ^a	203.73 \pm 14.13 ^b	241.90 \pm 19.55 ^c
Benzene	14.41 \pm 1.33 ^d	-	351.69 \pm 12.44 ^a	376.88 \pm 25.55 ^a
Methanol	47.01 \pm 5.40 ^a	30.89 \pm 0.09 ^a	220.05 \pm 14.22 ^b	262.89 \pm 9.22 ^c
Ascorbic acid	-	-	179.84 \pm 24.55 ^{b,c}	222.13 \pm 16.11 ^c

Each value is expressed as the means \pm SE (n=3). For each treatment the means within the column by different letters are significantly different at $P < 0.05$.

^aExpressed as μg of gallic acid equivalents (GAE); ^bexpressed as μg of quercetin equivalents (QE).

TPC: Total phenolics

TFC: Total flavonoids

Total phenolic and total flavonoid compounds

The flaxseed extracts obtained with methanol and butanol contained a higher amount of total phenolic compounds 47.01 \pm 5.40 and 43.33 \pm 2.77 μg gallic acid equivalents/g of extract respectively, while the lower level were observed with the benzene extract (14.41 \pm 1.33 μg gallic acid equivalents/g of extract).

Our results are in agreement with other reports demonstrated that methanolic solvent was more effective isolating phenolic compounds from different plant materials (Shabbir *et al.*, 2011). In addition, our data shows that flavonoid content of *L. usitatissimum* methanolic and butanolic extracts were 30.89 \pm 0.09 and 29.55 \pm 0.15 μg Quercetin equivalents/g of extract, respectively (Table 2).

Phenolic compounds are omnipresent secondary metabolites in plants and have wide range of therapeutic uses. The scavenging ability of phenolics is mainly due to the presence of hydroxyl groups. Flavonoids are a group of polyphenolic compounds which exhibit several biological effects such as anti-inflammatory, hepatoprotective, anti-ulcer, anti-allergic, anti-viral, anti-cancer activities. They also exhibit enzymes such as aldose reductase and xanthine oxidase. They are able to scavenge the reactive oxygen species because of their phenolic hydroxyl groups and are potent antioxidants (Bhadauria *et al.*, 2012).

DPPH radical scavenging activity

Petroleum ether, benzene, ethyl acetate, methanol and butanol extracts of *L. usitatissimum* exhibited a concentration dependent DPPH radical scavenging activity (Figure 1). At 800 $\mu\text{g/mL}$, DPPH radical scavenging activity of butanol and methanol extracts of *L. usitatissimum* were 96.2% and 93.1%, respectively. At this concentration, all the extracts exhibited a higher scavenging activity. The scavenging ability was in the order: butanol >

methanol > ethyl acetate > petroleum ether > benzene.

DPPH (2,2-diphenyl-2-picrylhydrazyl) analysis is one of the best-known, accurate, and frequently employed methods for evaluating antioxidant activity. It is a stable free radical which determines the ability of pure substances or crude extracts for trapping the unpaired electron species by donating hydrogen atoms or electrons, and producing in consequence the radical disappearance and the formation of less reactive species derived from the antioxidant (Parekh *et al.*, 2007).

In the present study the effect of different extracts of *L. usitatissimum* on DPPH scavenging was increasing with the increase in the concentration of the extracts from 50-800 $\mu\text{g/mL}$ and it is thought to be due to their hydrogen donating ability. It has been observed that the extracts prepared in high polar solvents exhibited strong activities which indicates that varieties of polyphenols, and flavanoids trapped may play important role in these activities. The present findings are in agreement with the report of Teppen *et al.*, 2005; Battu *et al.*, 2011 for *Salvia tomentosa* and *Euphorbia heyneana*, respectively.

Superoxide radical scavenging activity

As shown in Figure 1, it points out that, at 800 $\mu\text{g/mL}$ petroleum ether, butanol and methanol extracts showed a higher superoxide radical scavenging activities (87.1, 96.24 and 93.5%, respectively). All the concentrations of ethyl acetate showed a lower activity. The superoxide radical scavenging activity decreased in these order: butanol > methanol > petroleum ether > benzene > ethyl acetate.

Superoxide dismutase catalyses the dismutation of the highly reactive superoxide anion to oxygen and hydrogen peroxide. These species are produced by a number of enzyme systems in auto-oxidation reactions and by non-enzymatic electron transfers that reduce molecular oxygen. It can also decrease

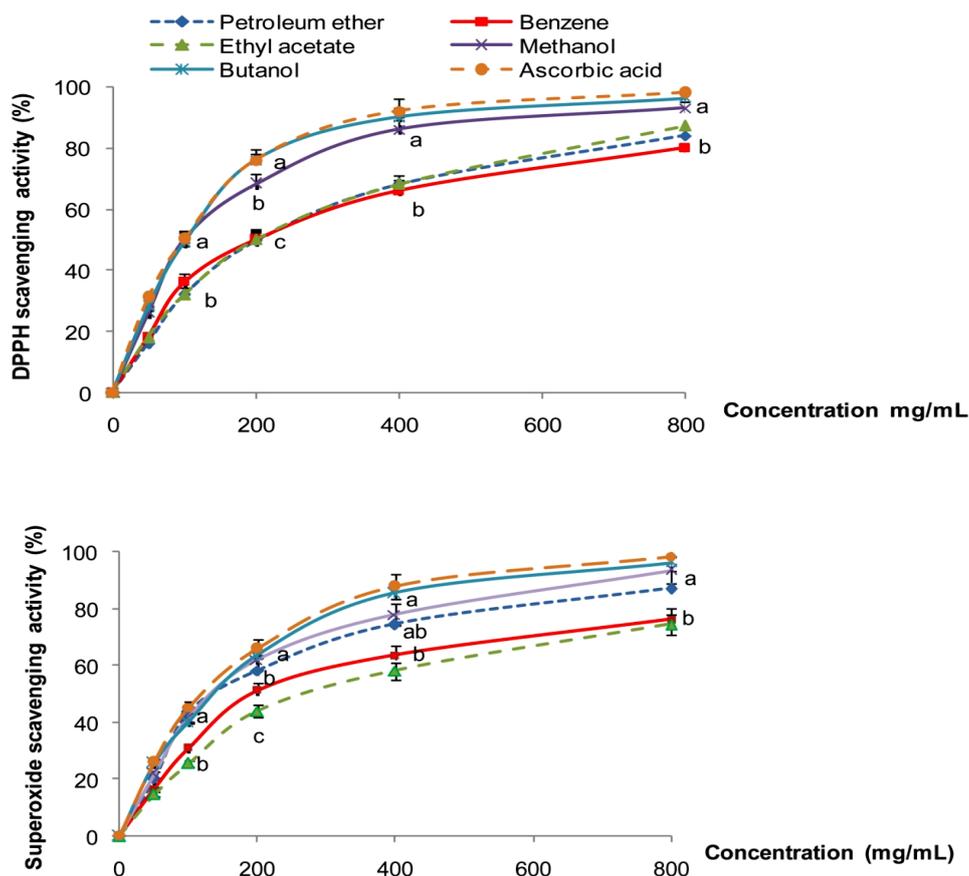


Figure 1. DPPH and superoxide scavenging activities of different extracts of *Linum usitatissimum* and standard ascorbic acid. Plots with different alphabets indicated on top are statistically different ($P < 0.05$)

certain iron complexes such as cytochromes (Sena *et al.*, 2009; Gulcin *et al.*, 2011). Superoxide anion is very harmful to cellular components (Korycka-Dahl and Richardson, 1978). They are effectively scavenged by flavonoids. Our data reveals that the different extracts of *L. usitatissimum* showed a potent superoxide scavenging activity when compared to ascorbic acid. Accordingly, it is suggested that the low to moderate ferrous ions chelating effects of these fractions would be rather beneficial to protect the cell against oxidative damage (Rajesh *et al.*, 2008). Similar trend of metal ion scavenging activity was observed in the species, *Acalypha fruticosa* (Thambiraj *et al.*, 2012), *Naregamiaalata* (Joseph *et al.*, 2010) and *Leucas ciliate* (Qureshi *et al.*, 2010).

IC_{50} values

The IC_{50} was utilized to classify antioxidant activity of a sample in comparison with the standard. The effective dose of 50% inhibition (IC_{50}) was also obtained from a plot of percentage inhibition beside extract concentration. All the experiments were run in triplicate and mean values therefore, were calculated against ascorbic acid as positive control

(Table 2). IC_{50} values of butanol and methanol extracts of *L. usitatissimum* for DPPH were found to be respectively 203.73 ± 14.13 and $220.05 \pm 14.22 \pm \mu\text{g/mL}$. Moreover, for superoxide radical scavenging IC_{50} values were 241.90 ± 19.55 and $262.89 \pm 9.22 \mu\text{g/mL}$; these results were similar to the activity of the positive control, ascorbic acid 179.84 ± 24.55 and $222.13 \pm 16.11 \mu\text{g/mL}$.

For petroleum ether flaxseed extract, IC_{50} values of DPPH and superoxide radical scavenging were 345.06 ± 13.11 and $287.18 \pm 5.11 \mu\text{g/mL}$, respectively. A higher DPPH IC_{50} values were noted with ethyl acetate ($333.20 \pm 10.12 \mu\text{g/mL}$) and benzene ($351.69 \pm 12.44 \mu\text{g/mL}$) extracts. For superoxide radical scavenging, the values were 419.03 ± 20.15 and $376.88 \pm 25.55 \mu\text{g/mL}$, respectively; these results were increased approximately by 50% as compared to the butanol extract. Moreover, a highly relationships between the results of both DPPH• and superoxide scavenging activities and total phenolic compounds (TPC) (estimated with Folin-Ciocalteu reagent) ($r = +0.89$ and $+0.92$; $p < 0.01$). Our findings are in agreement with the report of Anwar, F and Przybylski R (2012). Numerous investigations of the

antioxidant activity of plant extracts have confirmed a high correlation between the values of phenol concentration and antioxidant activity (Katalinić *et al.*, 2004; Borneo *et al.*, 2008), while other reports do not (Dasgupta and De, 2007). So, the extracts prepared in high polar solvents exhibited a strong activity which indicates that varieties of polyphenols, and flavonoids trapped may play important role in these activities.

Reducing power

The reducing power of different extracts of *L. usitatissimum* and standard ascorbic acid is illustrated in Figure 2. The extracts of *L. usitatissimum* exhibited a concentrated dependent increase in reducing power. Among all the different extracts studied, butanolic extract showed higher reducing power which is comparable to standard ascorbic acid. Our results are in agreement with those reported by Sangeetha *et al.* (2010) using *Sphaeranthus indicus* Linn. (Asteraceae).

Antioxidants may act as free radical scavengers, reducing agents, chelating agents for the transition of metals, quenchers of singlet oxygen molecules and or activators of antioxidative defense enzyme system to suppress the radical damages in biological systems (Prasad *et al.*, 2000; Zanwar *et al.*, 2011).

The reducing capacity of a compound may serve as an important indicator of its potential antioxidant activity (Ho *et al.*, 2012). The reducing properties are generally associated with the presence of reductones (Krishnamoorthy *et al.*, 2011). According to Gordan (1990), antioxidant action of reductones is based on the breaking of the free radical chain by donating

a hydrogen atom. Our results showed that butanol extract exhibited the highest reducing power than all other extracts in the higher concentration of 800 µg/mL and the value was comparable to that of the standard (Figure 2). The marked antioxidant activity of *L. usitatissimum* may be due to the presence of polyphenols which may act as reductones to convert free radicals into more stable products and terminate free radical chain reaction. Similar trend of observation on polyphenolic constituents' dose dependent reducing power activity has been reported for several other plant extracts (Zhu *et al.*, 2002; Amarowicz *et al.*, 2004).

Conclusion

The medicinal properties of plants have been the centre of attraction for researchers in recent scientific developments throughout the world, due to their potent antioxidant properties and economic viability. Based upon the results of the present investigation it can be concluded that butanol and methanol are the most effective solvents for recovering antioxidant components from flaxseed. It is further recommended to optimize the antioxidant extraction efficacy of these solvents using different extraction techniques. The medicinal properties of flaxseed extract may be due to the presence of above mentioned phytochemicals rendering them as a potential source for the isolation of compounds for development of nutraceuticals. Based on the previous results, pharmacognostical studies are suggested to confirm the antioxidant ability before going for commercialization.

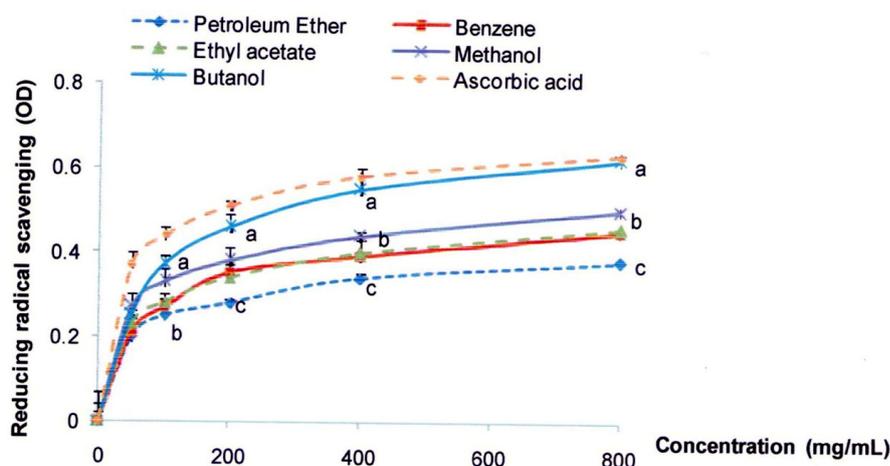


Figure 2. Reducing power of different extracts of *L. usitatissimum* and standard ascorbic acid. Plots with different alphabets indicated on top are statistically different ($P < 0.05$)

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