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EFFECT OF SOLVENTS EXTRACTION ON TOTAL PHENOLICS AND ANTIOXIDANT ACTIVITY OF EXTRACTS FROM FLAXSEED (*LINUM USITATISSIMUM* L.)

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ABSTRACT

Background. Plant origin food ingredients are the main source of very potent antioxidants. Tocopherols, the main oilseeds natural antioxidants are very potent and when implemented into cell membranes are able to scavenge large number of free radicals. Among plant antioxidants are mainly phenolics, large and diversified group of chemical compounds with different radical scavenging potential.
Material and methods. Defatted flaxseed meals were extracted with pure alcohols and its mixture with water. Acquired extracts were analysed for the content of phenolics and flavonoids using colorimetric procedures. Antioxidative capacity was assessed by utilizing: DPPH stable free radicals; inhibition of linoleic acid oxidation and reducing power of components.
Results. Investigation was conducted on two different batches of flaxseed, assessing antioxidant capacity of compounds extracted with different polarity solvents and extracts were tested for antioxidant activity with different methods. The highest yield of extraction was achieved with 80% methanol but the extract did not contain the highest amount of phenolics and flavonoids. When 80% ethanol was used for extraction the highest amount of flavonoids was detected and also the best antioxidant capacity.
Conclusions. The results clearly showed that utilization of polar solvent enable extraction of significant amounts of phenolics and flavonoids. Those components were the most potent antioxidants present in these extracts. Content of these compounds correlated well with results from applied methods for antioxidant assessment.

Key words: flaxseeds, solvent extraction, antioxidant, total phenolics, total flavonoids

INTRODUCTION

Currently, the use of antioxidative phytochemicals such as plant polyphenols, vitamin C, phenolic acids and flavonoids in foods is gaining popularity due to their anticarcinogenic activity, potential health benefits including the prevention and lowering risk of development of cancer, heart and neurodegenerative disorders [Siddhuraju and Becker 2003, Aaby et al. 2004, Iqbal et al. 2005, Choi et al. 2007, Fan et al. 2007, Liu and Yao 2007]. Several of natural antioxidants are believed to play an important role in ameliorating oxidation process by quenching free radicals, chelating catalytic metals and scavenging oxygen

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in foods and biological systems [Kim 2005, Choi et al. 2007, Siddhuraju and Becker 2007].

Plants are valued as the best source of natural antioxidants [Shahidi 1997, Pezzuto and Park 2002, Wang et al. 2003, Rababah et al. 2004]. Among the plant sources, several oilseeds also contain considerable amounts of antioxidant other than tocopherols and as such might provide protection to vegetable oil and food lipids. These include olive, sesame seed, palm, cocoa, rice bran, grape seed, oat, cottonseed, flaxseed, rapeseed, and soybean fruits and seeds [Shahidi 1997, Iqbal et al. 2005, Devi et al. 2007, Elizabeth et al. 2007].

Flax (*Linum usitatissimum* L.), a member of the family Linaceae, is an economically important oilseed crop containing usually about 40% of oil in the seeds. Flaxseed, besides its traditional oleochemical uses, is now gaining recognition as a functional food ingredient for human nutrition [Oomah 2001, Lei et al. 2003]. Consumption of flaxseed has been demonstrated to have multitude positive health benefits including decreasing rate of tumor growth, reducing serum cholesterol level and decreasing incidence of breast, prostrate, and colon cancers [Muir and Westcott 2003, Hemmings et al. 2004, Hosseinian et al. 2006, Choo et al. 2007]. 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, Westcott and Paton 2001, Tarpila et al. 2005, Hosseinian et al. 2006].

Isolation of antioxidants from a plant material can be carried out using different techniques and solvents because of diversity of chemical nature of these compounds and often unique distribution of these compounds in the plant matrix [Antolovich et al. 2000, Sultana et al. 2009]. Solvent extraction is the most frequently used technique for recovery of the plant antioxidants, however, the yields and antioxidant efficacy of the resulting extracts is strongly affected by a polarity of the solvent as well as the chemical nature of the isolated compounds [Sultana et al. 2009, Shabbir et al. 2011]. Polar solvents such as methanol, ethanol, either and their aqueous mixtures, are mostly recommended for the extraction of phenolics from a plant matrix. For example, in several studies pure and aqueous mixtures of methanol and ethanol have been used to extract compounds with antioxidant properties from different plant materials including fruits, vegetable, grains, medicinal plants and agro-wastes [Bonoli et al. 2004, Chatha et al. 2006, Sultana et al. 2009, Shabbir et al. 2011].

In the view of functional foods and nutraceutical attributes of flaxseed, there is much focus on investigating the phytochemicals and antioxidant properties of this crop components. Kitts et al. [1999] established low antioxidant activity of flaxseed lignans such as secoisolariciresinol diglycoside (SDG) and its mammalian lignans including enterodiol (ED) and enterolactone (EL). Whereas, Hosseinian et al. [2006] studied the antioxidant capacity of flaxseed lignans in two model systems and suggested that flaxseed lignans may be an effective alternative to some synthetic antioxidants to protect fats from developing rancidity. Likewise, Kraushofer and Sontag [2002] established that flaxseed have a considerable amount of phenolic compounds.

It is important to establish appropriate methods to effectively isolate diverse flaxseed antioxidant components. The ultimate objective of the present study was to evaluate the efficacy of four solvent systems: pure methanol, pure ethanol, 80% methanol and 80% ethanol towards isolating these components of flaxseed and also to assess their antioxidant effectiveness.

MATERIAL AND METHODS

Flaxseeds

Samples of brown seeded flaxseed (*Linum usitatissimum* L.) were obtained from a local store in Lethbridge, Alberta, Canada.

Chemicals and reagents

Folin-Ciocalteu's phenol regent (2 N), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, butylated hydroxytoluene (BHT), β-carotene, catechin, gallic acid and Tween 40 were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals/reagents and solvents used in this study were of analytical reagent grade and purchased from Merck (Darmstadt, Germany).

Defatting and extraction of flaxseed

Cleaned flaxseeds were ground using a coffee grinder. The ground material was extracted twice with

hexane, 50 g with 500 mL hexane, at room temperature using a magnetic stirrer set at 600 rpm.

The defatted meal was left at room temperature in the fume hood for 12 hrs to completely evaporate solvent and 20 g of meal individually extracted with 200 mL of: 100% and 80% methanol; 100% and 80% ethanol. Duplicate extraction was done by 12 h continuous mixing with magnetic stirrer set at 750 rpm at ambient temperature. After extraction, the supernatant and sediment were separated by filtration using filter paper (Whatman No. 1). Extracts were concentrated to dryness under vacuum at 45°C, using a rotary evaporator (BUCHI Rotavapor, Switzerland). The extracts were weighed to calculate the yield and were dissolved in small volume of initial solvent and stored at –18°C, until tested.

Determination of total phenolics (TP)

The estimation of total phenolic contents (TPC) in extracts was done following Folin-Ciocalteu procedure applying Skerget et al. [2005] modification. Briefly, to 0.5 mL of diluted extract, 20 mg/10 mL, 2.5 mL of Folin-Ciocalteu reagent and 2 mL of Na_2CO_3 (75 g/L) were added. The mixture was incubated for 5 min at 50°C, cooled to room temperature and absorbance measured at 760 nm using distilled water as blank. The amounts of TPC were calculated using gallic acid for calibration within the range of 10-100 ug/g. Gallic acid is commonly used as reference when total phenolics are assessed by Folin-Ciocalteu method. The results were expressed as mg of gallic acid equivalents per 100 g of dry matter (mg GAE/100 g DM).

Determination of total flavonoids (TF)

The amounts of total flavonoids in flaxseed extracts were determined by procedure described Sultana et al. [2007]. One milliliter of extract containing 0.1 g/mL of dry matter was taken in a 10 mL volumetric flask, and were added sequentially: 5 mL of distilled water, 0.3 mL of 5% $NaNO_2$ and after 5 min 0.6 mL of 10% $AlCl_3$ followed after another 5 min by 2 mL of 1 M NaOH and volume made up with distilled water. The solution was mixed and absorbance recorded at 510 nm. TF amounts were expressed as catechin equivalents (CE) per dry matter, this standard is easily accessible and the most common flavonoids present in plant material. All samples were analysed thrice and results averaged.

ANTIOXIDANT ACTIVITY

DPPH radical-scavenging capacity. Free radical-scavenging activity of flaxseed extracts was determined spectrophotometrically, following procedure described by Miliauskas et al. [2004]. Extract were diluted to the concentration of 10 mg of dry extract in 10 mL of methanol. Three milliliters of freshly prepared DPPH in methanol (6×10^{-5} M) were mixed with 77 µL of extract and the mixture incubated in dark for 15 min at room temperature followed by absorbance measured at 515 nm. Butylated hydroxytoluene (BHT) was used as control. Radical-scavenging activity was calculated by the following formula:

$$\text{DPPH radical scavenging (\%)} = [(A_B - A_E)/A_B] \times 100$$

where:

A_B – absorbance of blank sample at t = 0 min,
A_E – absorbance of tested extract solution after 15 minutes incubation.

Inhibition of linoleic acid peroxidation. The antioxidant activity of the flaxseed extracts was determined by measuring the inhibition of linoleic acid peroxidation according to a method described by Kikuzaki and Nakatani [1993]. Sample of 250 µg of flaxseed extract in 0.5 mL of absolute ethanol were placed in a screw capped tube and mixed with 0.5 mL of 2.5% linoleic acid in absolute ethanol, 1 mL of 0.05 M phosphate buffer (pH = 7), and 0.5 mL of distilled water. The reaction mixture was incubated at 40°C and aliquots of 0.1 mL were sampled at every 12 h during incubation. State of oxidation was measured by sequentially adding 75% ethanol (9.7 mL), ammonium thiocyanate (0.1 mL, 30%) and ferrous chloride (0.1 mL, 0.02 M in 3.5% HCl). The mixture was incubated for 3 min, and absorbance measured at 500 nm until it reached the maximum. Butylated hydroxytoluene was used as positive control. The antioxidant activity was calculated as percentage of inhibition relative to the control using the following equation:

$$\text{AA (\%)} = 100 - [(\text{sample absorbance at 48 h} - \text{sample absorbance at 0 h})/(\text{control absorbance at 48 h} - \text{control absorbance at 0 h}) \times 100]$$

Reducing power assay. The reducing power of the extracts was determined according to the method

described by Ardestani and Yazdanparast [2007]. Equivalent volume of flaxseed extracts and BHT, the latter used as reference, were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide solution (1%, w/v). The mixture was incubated in a water bath at 50°C for 20 min. Then, 2.5 mL of trichloroacetic acid (TCA) solution (10%, w/v) was added and the resulting mixture centrifuged at 2500×g for 15 min. The upper layer was (2.5 mL) mixed with 2.5 mL of distilled water and 0.5 mL of a ferric chloride solution (0.1%, w/v) and absorbance measured at 700 nm. Reducing power is expressed as absorbance per specific amount of extract as presented in Figure 1.

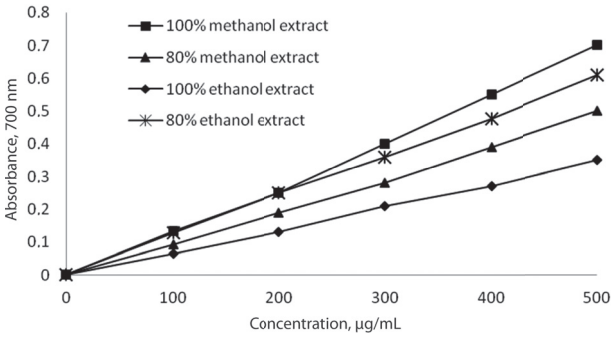

Fig. 1. Effects of extracting solvents on reducing power of flaxseed extracts, expressed as absorbance at 700 nm

Statistical analysis

All experiments were run as independent duplicates for two different flaxseed samples. Experimental results were expressed as means ± standard deviation (SD) of three parallel measurements SD ($n = 2 \times 3 = 6$). Data were analysed by one-way analysis of variance (ANOVA) using Minitab 2000 Version 13.2 statistical software (Minitab Inc. Pennsylvania, USA). A probability value of $p \leq 0.05$ was considered to denote statistically significant differences.

RESULTS AND DISCUSSION

Extract yields and antioxidant activity

The amounts of the crude extracts obtained from flaxseeds using different extraction solvents are presented in Table 1. The yields of antioxidative components extracted by 80% methanol varied significantly ($p < 0.05$) from other solvents used. The amount of compounds extracted ranged from 9.6 for pure ethanol to 13.0% for 80% aqueous methanol, indicating that added water, the most polar solvent used, caused further extraction of polar components. Our findings are in agreement with previous investigation by Chatha et al. [2006] who reported that maximum extract yield from rice bran was obtained when aqueous methanol solvent was used. Kasote et al. [2011] extracted defatted flaxseed meal with n-butanol but recovery of phenolics was very low. Anwar et al. [2006] reported higher yields of compounds with antioxidative properties isolated with methanol/water mixture from different plant materials including leaves of *Moringa oleifera.* Similarly, in another investigation by Sultana et al. [2007] aqueous methanol was found to be the most effective solvent extracting the most efficiently antioxidants from barks of some native trees. The variations in the extract yields from flaxseeds using different solvents might be explained by the polarity of extracted components and solvents applied [Hsu et al. 2006, Shabbir et al. 2011]. Amongst other contributing factors affecting efficiency of the extraction is also solubility of endogenous compounds present in extracted material [Siddhuraju et al. 2003, Sultana et al. 2007].

Table 1. Effects of extracting solvents on the extract yields during extraction of defatted flaxseeds

Solvent	Yield, g/100 g of dry weight
100% methanol	10.2 ±0.5^{ab}
80% methanol	13.0 ±0.4^{c}
100% ethanol	9.6 ±0.6^{a}
80% ethanol	10.9 ±0.4^{b}

Values (mean ±SD) are average of three independent experiments.
Different superscript letters within the same column indicate significant ($p < 0.05$) differences within the extracting solvents.

The amounts of total phenolic (TP) compounds, extracted from flaxseeds with the different solvent mixtures, ranged from 1360-3260 mg GAE/100 g of extract and are presented in Table 2. The flaxseed

extracts obtained with 80% aqueous ethanol contained the highest amount of TP while the lowest amounts were observed in the extracts where pure ethanol was used. Our results are in line with the report of Sultana et al. [2007] who also found that 80% aqueous ethanol showed the best effectiveness extracting phenolic components from barks of some plants. Bonoli et al. [2004] reported that maximum amount of phenolic compounds was extracted from barley flour when aqueous ethanol and acetone were applied as extractants. Our data are in agreement with studies demonstrated that aqueous methanol and aqueous ethanol extracting solvents were more effective isolating phenolic compounds from different plant materials [Siddhuraju and Becker 2003, Anwar et al. 2006, Chatha et al. 2006, Shabbir et al. 2011].

Flaxseed is reported to be a good source of phenolic components, the amount of which was typically reported at 500 mg/100 g of flaxseed, when 80% aqueous methanolic was used [Veliouglu et al. 1998]. From our experiments, we observed that the maximum of phenolic compounds was extracted from flaxseed with 80% aqueous ethanolic and achieve 300 mg/100 g of flaxseed (data not included). Comparison of our data with published literature is rather difficult because variations were observed for total phenolic contents which might be attributed to the differing varieties of flaxseed, extracting solvent, extraction temperature and technique employed [Kasote et al. 2011, Kraushofer and Sontag 2002, Lorenc-Kukula et al. 2005, Oomah et al. 1995]. It is now widely accepted that phenolics are among the phytochemicals which are abundantly found in cereals, grains, fruits and many other plant sources. These have attracted a great deal of public and scientific interest because of their multiple biological activities such as antioxidant and anticancer and other health promoting properties [Ardestani and Yazdanparast 2007, Thompson et al. 2005].

The amounts of total flavonoids (TF) extracted from flaxseeds using different solvents were ranged from 190 to 480 mg CE/100 g extract, showing considerable variations among different extracts tested in this study (Table 2). Using pure methanol we were able to extract the highest amounts of TF (480 mg CE/100 g), whereas much lower amounts were extracted with pure ethanol (190 mg CE/100 g). Similarly high level of TF was found in extracts when 80% ethanol was used (390 mg CE/100 g).

The amounts of TF extracted in this study when recalculated on flaxseeds varied from 20-60 mg CE/100 g of flaxseed. These levels, although slightly lower, are in close agreement with the values found in different Canadian flaxseed cultivars, 35-71 mg CE/100 g of flaxseed, as reported by Oomah et al. [1996]. Plants flavonoids are widely distributed in fruits, vegetables, and beverages such as wine and tea [Hollman and Arts 2000]. Due to their bioactives related to amelioration of inflammation, anticarcinogenic and anti-mutagenic

Table 2. Effects of extracting solvents on the total phenolic, total flavonoid contents, free radical scavenging capacity and percentage inhibition of linoleic acid peroxidation of flaxseeds extracts

Solvents	TPC mg GAE/100 g DM	TFC mg CE/100 g DM	DPPH % scavenging	Inhibition of oxidation %
100% methanol	2 700 ±74^{c}	480 ±20^{d}	83.6 ±1.5^{b}	77.4 ±2.0^{c}
80% methanol	2 020 ±83^{b}	350 ±20^{b}	81.3 ±1.3^{c}	63.0 ±1.5^{b}
100% ethanol	1 360 ±50^{a}	190 ±15^{a}	42.2 ±0.6^{a}	56.7 ±1.0^{a}
80% ethanol	3 260 ±110^{d}	390 ±12^{c}	87.5 ±1.0^{b}	81.7 ±1.7^{d}
BHT	–	–	94.3 ±2.7^{d}	88.2 ±2.0^{e}

Values (mean ±SD) are average of three independent experiments. Different superscript letters within the same column indicate significant ($P < 0.05$) differences within the extracting solvents.
TPC – total phenolics.
TFC – total flavonoids.

properties these compounds are under investigation for their health properties [Oomah et al. 1996, Thompson et al. 2005].

The results of radical scavenging activities using DPPH stable free radicals in Table 2 are presented. The flaxseed extracts at concentration of 25 µg/mL showed significant ($p < 0.05$) capacity towards scavenging DPPH radicals (42.2-87.5%; Table 2). The extracts produced with 80% aqueous ethanol, 80% aqueous methanol and pure methanol offered the highest scavenging properties, scavenging capacity >80%. BHT used as control and when used at the same concentration, exhibited significantly higher scavenging activity at 94% compared to flaxseed extracts. DPPH radical scavenging capacity of the tested flaxseed extracts, except when pure ethanol was used, could be explained by the presence of appreciable amounts of total phenolics and flavonoids in these extracts. DPPH, a stable organic free radical, is often employed for evaluation of the antioxidant activity of compounds. The extent of reaction mainly depends on the hydrogen donating ability of the antioxidants which is predominantly governed by their structure and degree of hydroxylation [Shahidi 1997].

The inhibition of linoleic acid oxidation was also used to assess an antioxidant activity of the flaxseed extracts. At a concentration of 250 µg extracts inhibited oxidation of linoleic acid by 56.7 to 81.7% (Table 2). Aqueous ethanol (80%) extract exhibited maximum inhibition at the level of 81.7% followed by pure methanol extract (77.4%), 80% aqueous methanol extract (63.0%) and pure ethanol extract (56.7%). BHT at the same concentration inhibited linoleic acid oxidation at 88.2%, higher than those observed for the flaxseed extracts.

The reducing capacity of a compound may be considered as an important indicator of its antioxidant activity [Hsu et al. 2006]. In this assay, ferric ions are reduced to ferrous ions and with it change in color of the reaction mixture from yellow to bluish green. Higher color intensity, greater the absorbance and higher reducing activity. The reducing power of the tested flaxseed extracts in the concentration range of 100-500 µg/mL (Fig. 1) increased in a concentration dependent manner ($r^2 = 0.9979$-0.9990). From the present data, it may be assumed that flaxseed extracts have electron donors capable to neutralize free radicals and converting them to stable products and terminating free radical initiated reactions.

Comparison among results of different antioxidants assays

It is now well established that plant extracts possess antioxidant activities causing breaking free radical chain, chelating catalytic metals as well as scavenging oxygen in food and biological systems [Kim 2005, Shahidi 1997]. It is important to develop relation between methods assessing properties of different antioxidant so that some comprehensive and convenient protocols may be established for overall antioxidant activity evaluation of the plant materials. Evaluation of antioxidant activity of a typical material with an assay based on one chemical reaction seems to be rather questionable, yet there is a need to employ multitude of tests to adequately assess antioxidant activity.

In the present study we used different antioxidant assays such as: measurement of inhibition of linoleic acid peroxidation, DPPH radical scavenging capacity, total phenolics, total flavonoids and reducing potential to evaluate the antioxidant activity of flaxseed extracts. Correlation between the results of different antioxidant assays in Table 3 is shown. A good correlation between TP and TF was observed ($r = 0.8021$) which could be supported by the previously reported trends by Sidduraju and Becker [2003]. The present results also revealed that phenolic compounds are effective scavengers of free radicals as is evident by a good correlation between DPPH radical scavenging activity with TP ($r = 0.7078$) and TF ($r = 0.8633$).

An excellent correlation between % inhibition of linoleic acid peroxidation and TP ($r = 0.9842$) and TF ($r = 0.8304$) and moderate with DPPH scavenging capacity assay ($r = 0.6485$) were established. Antioxidant activity of phenolic compounds is often associated with their redox properties, which allow them to act as reducing agents and results of this study supporting this notion. The correlation between reducing power and TP and TF were high ($r = 0.8608$) and ($r = 0.9849$), respectively. Furthermore, a reasonable correlation ($r = 0.7899$) was also observed between reducing power and DPPH radical scavenging activity.

These results are in accordance with the previous investigation of Sultana et al. [2007] who reported that phenolics are powerful scavengers of DPPH radicals

Table 3. Relationship between different antioxidant assays and presence of phenolic compounds described by correlation coefficient (r; n = 6)

Variables	TPC	TFC	DPPH· scavenging	% inhibition	Reducing power
Total phenolic contents	–	0.8021	0.7078	0.9843	0.8609
Total flavonoid contents	0.8021	–	0.8633	0.8304	0.9849
DPPH scavenging	0.7078	0.8633	–	0.6485	0.7899
% inhibition	0.9843	0.8304	0.6485	–	0.9016
Reducing power	0.8609	0.9849	0.7899	0.9016	–

and also act as good reducing agents. Correlation of % inhibition with reducing power was also very good at r = 0.9016. Variations, as observed in the present investigation, for correlation coefficients among different antioxidant assays support the necessity to use multitude of assays to adequately assess antioxidant efficacy of usually complex mixture of antioxidative compounds present in plants, which often act with different scavenging mechanism.

CONCLUSION

Based upon the results of the present investigation it can be concluded that 80% aqueous ethanol and pure methanol are the most effective solvents for recovering antioxidant components from flaxseed while the pure ethanol has shown the least efficacy among others. It is further recommended to optimize the antioxidant extraction efficacy of these solvents using different extraction techniques. The data presented would certainly help to ascertain the potency of the flaxseeds as viable source of natural antioxidants and thus support their uses as functional and nutraceutical food ingredient. However, further research is needed to identify individual flaxseed components forming antioxidative system and to develop their specific properties and application in food system.

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