



**ANTIOXIDANT ACTIVITY AND PHYTOCHEMICAL
ANALYSIS OF FLAXSEEDS (*LINUM USITATISSIMUM L*)**

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ABSTRACT:

Flaxseed is a rich source of different types of phenolics such as lignans, phenolic acids, flavonoids, phenylpropanoids and tannins. The present study was carried out to assess the proximate composition, phytochemical screening, total phenolic, total flavonoids and antioxidant activities of acetone 70%, methanol 70%, ethanol 70% and water extracts of *Linum usitatissimum L*. The antioxidant activity of extracts was determined by free radical scavenging activity using DPPH method. The median inhibitory concentration (IC_{50}) was calculated according to DPPH method. The IC_{50} value of acetone extract of flaxseeds is 90.76 while methanol extract of flax seed is 55.74, ethanol extract of flaxseeds is 65.21 and water extract of flaxseeds is 97.40. The results showed that methanol extracts are more effective than ethanol, acetone and water extracts of seeds. Thus, flaxseeds methanol extract components were identified using Gas Chromatography- Mass Spectrometry (GC/MS).

Keywords: *Flaxseeds, Linum usitatissimum L, DPPH, antioxidant activity.*

1. INTRODUCTION:

Flaxseeds, scientifically known as *Linum usitatissimum L*. belong to Linaceae family (Ganorkar and Jain, 2013), is an annual or biannual plant, one of the most useful crops, that has been cultivated as a commercial plant in over thirty countries all over the world (Gabiana, 2005). It has been

cultivated for oil and fiber (Madhusudhan, 2009). Flaxseed is being cultivated in more than 50 countries; the majority of them are in the northern hemisphere. Canada is the main flax producer, followed by China, United States and India (Rubilar *et al.*, 2010). Flaxseed is rich in fat, protein and dietary fiber.

Chemical analysis of flaxseed averaged 30 to 40% oil, 20 to 25% protein, 20 to 28% total dietary fibre, 4 to 8% moisture and 3 to 4% ash. The oil contains vitamins A, B, D and E, minerals and amino acids, by virtue of the presence of physiologically active food components that may provide health benefits beyond basic nutrition. Day by day, incorporation of flaxseed in food and in food products has been increasing due to its high content of essential omega-3 fatty acid, alpha-linolenic acid (ALA), dietary fiber and natural phenolic antioxidants. Flaxseed is emerging as one of the key sources of phytochemicals (Shahzad *et al.*, 2006). These phytochemicals (phenolic acids, cinnamic acids, flavonoids and lignins) are antioxidants and affect the cell growth and viability. Flaxseed is an essential source of high quality protein and soluble fiber and has considerable potential as a source of phenolic compounds (Amin and Thakur, 2014).

Plant lignans are the biologically important class of phenolic compounds, the levels of lignans in food vary widely; the richest source is flaxseed, the prevailing lignan in the flaxseed is secoisolariciresinol diglucoside (SDG). There are few studies on the stability of lignans through food process showed that SDG levels remained unchanged during the manufacture of breads and cookies that contained flaxseed (Cardoso *et al.*, 2012)

One of the components dietary fibers reduces serum cholesterol and flattens the blood glucose profile,

similar to guar gum, oat gum and other viscous fibers (Jenkins 1995).

The presence of flavonoids in linseed oxidative cell damage, suggesting antiseptic, anticancer, anti-inflammatory effect and mild hypersensitive properties (Pruthi, 2007). Therefore, the aim of this study was to prepare flaxseeds extract solvent extraction methods, identifying various photochemical compounds present in the extract, performing GC-MS analysis of the compound

2. MATERIALS AND METHODS

2.1. Samples:

Flaxseeds (Sakha 3) were obtained from the Research Center of Sakha, after drying in shade; these seeds were ground into fine powder using an electric grinding machine (Model MX 491N National).

2.2. Proximate Analyses:

The chemical composition (moisture, proteins, lipids, ash and fiber) of triplicate samples were determined according to the standard methods of AOAC (1990). The protein content of each sample was calculated by using conversion factor 6.25. The carbohydrates were estimated by difference.

2.3. Preparation of seed extracts:

The extraction was carried out using four different solvents, separately i.e. ethanol: water (70:30, v/v), acetone: water (70:30, v/v), methanol: water (70:30, v/v) and water of the seeds powder, 100 g was soaked in 2 liters of solvent for 5 days, with shaking twice daily. After 5 days,

supernatant was separated through filtering using porous cloth. The remaining plant material was again soaked in another 2 liters of solvents for 5 days and supernatants were combined and all sample extracts were evaporated to dryness (modified of Ahmad *et al.*, 2012).

2.4. Preliminary Phytochemical screening:

Qualitative phytochemical analysis:

Preliminary phytochemical screenings of various extracts was carried out as per the standard textual procedure (Harborne, 1973).

Steroids: An aliquot of the seed extract (1ml) was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids.

Terpenoids: An aliquot of the seed extract (2ml) was added to 2ml of acetic anhydride and concentrated H₂SO₄. The formations of blue green ring indicate the presence of terpenoids.

Tannins: An aliquot of the seed extract (2ml) was added to few drops of 1% lead acetate, and the yellowish precipitate indicated the presence of tannins

Saponins: An aliquot of the seed extract (5ml) was mixed with 20ml of distilled water and then agitated in a graduated cylinder for 15 minutes. Formation of foam indicates the presence of Saponins.

Anthocyanins: An aliquot of the seed extract (2ml) was added to 2ml of 2 N HCl and ammonia. The appearance of pink-red which turns to blue-violet indicates the presence of anthocyanins.

Glycosides: Concentrate H₂SO₄ Test: 2ml. glacial acetic acid, one drop of 5% FeCl₃ and conc. H₂SO₄ were added into 5ml extract, the appearance of brown ring indicates the presence of glycosides.

Emodins: Two ml of NH₄OH and 3 ml of Benzene were added to the extract. Appearance of red colour indicates the presence of emodins.

Alkaloids: a Mayer's test: To the acidic solution, Mayer's reagent (Potassium mercuric iodide solution) was added. Cream coloured precipitate indicates the presence of alkaloids.

Phenols: Half ml of FeCl₃ (w/v) solution was added into 2 ml of test solution, formation of an intense color indicates the presence of phenols.

Flavonoids: An aliquot of the seed extract (2-3ml) and few drops of sodium hydroxide solution were added into a test tube. Formation of intense yellow colour that became colourless on addition of few drops of dilute HCl indicates the presence of flavonoids.

2.5. Quantitative analysis of phytochemicals:

2.5.1. Determination of Total polyphenols content:

Total polyphenols (TP) were determined using the Folin-Ciocalteu reagent, according to Maurya and Singh (2010). The calibration curve was made with standard of solution of gallic acid in the range of 0.01- 0.05 mg ml⁻¹ and measures were carried out at 760nm

using a UV-Vis spectrophotometer. All analyses were performed in triplicate. Gallic acid was employed as a calibration standard and results were expressed as milligrams of equivalent gallic acid dry extract.

2.5.2. Determination of total flavonoids content:

The flavonoids content of each extract was measured based on methods described by Ebrahimzadeh *et al.* (2008). Briefly, 0.5ml of sample (5g/L) was mixed with 1.5ml of methanol and then 0.1 ml of 10% potassium acetate and 2.8 ml of distilled water. The mixture was incubated at room temperature for 30 min. The absorbance was measured by a spectrophotometer at 415 nm. The results were expressed as milligrams quercetin equivalents (QE) per gram of extract (mg QE/g extract). The standard curve was prepared by quercetin in different concentrations (5-50 mg/L).

2.6. Determination of total antioxidant activity:

The antioxidant activities of the acetone (70%), methanol (70%), ethanol (70%) and water extracts were assessed by measuring free radical scavenging activity via the discoloration of these solvents of the free radical 1,1 diphenyl-2-picrylhydrazyl (DPPH) as described by Brand – Williams *et al.* (1995) as follows: Two ml of acetone (70%), methanol, ethanol (70%) and water solution of either test material at various concentrations (1-64 µg/ml) and methanol solution used as control

were added to 2 ml solution of DPPH (25mg/L) in methanol, and the reaction mixture was shaken vigorously and left in darkness for 30 min. Finally, the absorbance of the mixture was measured against pure methanol (blank) at 517 nm T80 UV/Vis spectrophotometer. The percentage of radical scavenging activity was calculated using the following formula:

$$\text{Radical scavenging (\%)} = [(A_0 - A_1 / A_0) \times 100]$$

Where: A_0 is the absorbance of control and A_1 is the absorbance of the sample extracts. The 50% inhibitory concentration value (IC_{50}) is indicated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radicals.

2.7. Identification of Flaxseeds methanolic extract using gas chromatography-mass spectrometry (GC/MS)

The GC/MS analysis was performed using a thermo Scientific, trace GC Ultra / ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30m, 0.25mm, 0.1mm film thickness) in the mass spectrum lab., National Research Center (NRC), Dokki, Giza. For GC/MS detection, an electron ionization system with ionization energy of 70 eV used, Helium gas was used as the carrier gas at a constant flow rate of 1ml / min. The injector and MS transfer line temperature was set at 280°C.

The quantification of all the identified components was investigated using a percent relative

peak area. A tentative identification of compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.

2.8. Statical analysis:

Experimental results were means \pm SD of three parallel measurements. Analysis of variance was performed by ANOVA procedures. GraphPad Prism® was used for statistical calculations GraphPad Software, San Diego, CA, USA (Motulsky, 1999).

3. RESULT AND DISCUSSION:

3.1. Chemical composition

The average values for the chemical composition flaxseeds are given in Table (1).

Table 1: Proximate analysis of flaxseeds

Component	Proximate %*
Moisture	7.02 \pm 0.28
Ash	3.77 \pm 0.21
Fats	37.2 \pm 2.41
Fiber	25.4 \pm 1.12
Protein	21.2 \pm 0.24
Carbohydrate	5.47 \pm 2.41

* Each value is expressed as the mean \pm SD (n=3)

The flaxseeds content was 25.4% fiber, 37.2% total lipid, 21.2% protein, 3.77% ash ,7.02% moisture and 5.47 % carbohydrate.

In this study the moisture in flax seed is lower than that reported by Amin and Thakur (2014) who found that the moisture content of flaxseeds

was to be 7.7% as reported by Canadian Grain Commission (2009) and 7.2% reported by Eggie (2010). While ash content in this study is agreement with Amin and Thakur (2014) who found that the ash content of flaxseeds was 3.8 and is close to the value of 3.4% and 3.3% reported by Canadian Grain Commission (2009) and Eggie (2010) respectively. In this study protein content of flax seed is low compared with Amin and Thakur (2014) who found Protein content was to be 28.86. Canadian Grain Commission (2009) and Eggie (2010) found that protein content was 22.9%, 23.1%, respectively. The value of protein was 10% as reported by Amin and Thakur (2014). The crude fat content of flaxseeds much lower than 45.2% reported by Canadian Grain Commission (2009). The crude fat content of flaxseeds was found to be much higher than 27.3% as reported by Eggie (2010). Amin and Thakur (2014) found that crude fiber content of flaxseed was 5.61. An analysis of brown Canadian flax averaged 41% fat, 20% protein, 28% total dietary fibre, 7.7% moisture and 3.4% ash, which is the mineral-rich residue left after samples are burned (Morris, 2003).

3.2. Qualitative analysis of phytochemicals:

Qualitative analysis carried out on seeds showed the presence of phytochemical constituents and the results are summarized in Table 2. It shows the presence of Phytochemicals such as steroids, terpenoids, anthocyanins, emodins, glycosides,

flavonoides and phenols present in acetone, methanolic, ethanolic and water extracts, while tannins were absence in acetone, ethanol and water. Saponins were absence in ethanol,

methanol and water extracts but present in acetone, both alkaloids and glycosides was absent in ethanol (70%) and water extract.

Table 2. Phytochemical screening tests for constituents of flaxseeds extracts.

Constituent	Acetone70%	Ethanol 70%	Methanol 70%	Water
Steroids	+	++	++	+
Terpenoids	+	+++	++	+++
Tannins	-	-	+	-
Saponins	+	-	-	-
Anthocyanins	+	+	+	+
Emodins	+	++	+	+
Alkaloids	+	-	+	-
Glycosides	+	-	++	-
Flavonoids	++	+	+++	+
Phenols	++	+++	++	+

(+++), (++) , (+) and (-) refer to high, moderate, low and absent amounts respectively.

The present results were in agreement with Shobha and Borhade (2013) who showed that Phytochemical analysis of flaxseed extract contain flavonoids, sterols and triterpenes, Flavonoids were found in the extract and are potent water soluble antioxidants. Amin and Thakur (2014) reported that phytochemical analysis of ethanol extract of flaxseeds contain tannins, flavonoids, terpenoids, phenols and proteins and amino acids were present while it did not contain saponins, sterols, and glycosides. Nazir *et al.* (2012) who found that the Phytochemical screening of methanol extract of flaxseeds revealed the presence of alkaloids, saponins and carbohydrates, while they gave negative reaction for tannins, glycosides, reducing sugars and steroids.

3.3. Quantitative analysis of phytochemicals:

3.3.1. Total Phenolics and Flavonoids Content:

Table (3) showed the total phenolic content of flaxseeds extract ranges from (11.5mg- 15.5 mg/ g), as Gallic acid equivalents (GAE). Water extract and acetone extract were at the lower end of total phenolic acids content (11.5 and 12mg/ g) whereas methanol extract and ethanol extract showed the highest total phenolic acids content at (13.5and 15.5mg/g) .

Plant phenolics have multiple biological functions such as antioxidant, anti-inflammatory, anti-cancer and antimicrobial activities (John and Grohmann, 2001).

Our data (Table 3) are in agreement with studies demonstrated

that 70% ethanol and 70% methanol extracting solvents were more effective isolating phenolic compounds from different plant materials (Shabbir *et al.* 2011).

Table3. Total phenolic compounds and Total flavonoids of flaxseeds extracts

Sample	Total phenolic compounds (mg/g) ^a	Total flavonoids (mg/g) ^b
Flaxseeds extract (Acetone 70%)	12±0.8165	3.75±0.50
Flaxseeds extract (Ethanol 70%)	15.5 ^{***} ±0.58	2.5*±0.58
Flaxseeds extract (Methanol 70%)	13.5 ^{**} ±0.58	5*±0.0
Flaxseeds extract (Water)	11.5 ±0.58	1.5 ^{***} ±0.58

a: mg GAE /g of dry seed extract; **b:** mg QE/g of dry seed extract. Each value is expressed as the mean.± SD (n=3). (*, **and ***) Significant and highly significant respectively at $P < 0.05$ vs flaxseeds acetone extract.

Sultana *et al.* (2007) who also found that 80% aqueous ethanol showed the best effectiveness extracting phenolic components from barks of some plants. Amin and Thakur (2014) reported that the phenolic content in ethanol extract of flaxseeds was found to be (21.52mg/g); this value is higher than our result (15.5mg/ g). Also higher values were found by Punia and Deen (2016), their results showed that the content of total phenols varied from (153.00 ± 1.41 mg GAE/g (acetone extract) to 267.00± 4.24 mg GAE/g (water extract) in flaxseed (Hisar) and 219.00± 4.23 mg GAE/g (acetone extract) to 360.00± 0.40 mg GAE/g (ethanol extract) in flaxseed (Rajmathal hills). Sue-siang *et al* (2014) reported that mixed solvent (methanol: acetone: water, 7:7:6 v/v/v)was found to be the most effective solvent system that gave the highest yield of phenolic compounds (774.33 ± 2.08 mg GAE/100g fresh weight) in flaxseed cake.

The total flavonoid contents (Table 2) in flaxseeds ethanolic extract and flaxseeds water extract (2.5 and 1.5 mg/ g) are lower than those found in flax seed methanolic extract and acetone extract (5 and 3.75 mg/ g). Sue-siang *et al* (2014) showed that the amounts of total flavonoids extracted from flaxseeds using different solvents showing considerable variations among different extracts.

3.4. Antioxidant activity

Antioxidant activity of ethanol, methanol, acetone and water extracts of seeds were determined by DPPH method, which is a stable organic free radical with an absorption maximum band around 515- 528 nm (Stankovi, 2011) and is widely used for evaluation of antioxidant potential of compounds. The results show the inhibition of flaxseed extracts (Table 4). Aqueous methanol (70%) extract exhibited maximum inhibition level of 62.10% followed by 70 % ethanol extract (48.06%), 70% aqueous

acetone extract (40.26%) and water extract (39.47%).

Table 4. Antioxidant activity of flaxseeds extracts..

Sample	%inhibition	IC ₅₀ (µg/ml)
Flaxseeds (Acetone 70%)	40.26	90.76
Flaxseeds(Methanol 70%)	62.10	55.74
Flaxseeds (Ethanol70%)	48.06	65.21
Flaxseeds(water)	39.47	97.40

The IC₅₀ values correspond to the amount of extract required to scavenge 50% of radicals present in the reaction mixture.

2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene was found to be the second main compounds (10.21 %) and hexadecanoic acid, ethyl ester (CAS) was identified as the third constituents in the flaxseeds extract (5.67). Additionally the fourth component was Phenol, 2, 6-bis (1,1-dimethylethyl)-4-methyl- (CAS) (3.0%), also there were other components less than 3.0% (Table 5).

Our result in the same line with Dharshini *et al.* (2013) who revealed the presence of compounds by forming 5 major peaks in the ethanol extract of flaxseed were squalene (45.27%), 9, 12, 15, octadecatrienoic acid,(z,z,z)-(24.6%),pyrrolidine,1-(1-oxo-7,10-hexadecadienyl)- (17.60), oleic acid (10.16%) and sucrose (9.80 %) respectively. The triterpene has also been found to have protective activity against several carcinogens. Substances related to squalene, including β- carotene, coenzyme Q10 (ubiquinone) and vitamins A, E, and K 10. The primary therapeutic use of squalene currently is as an adjunctive therapy in a variety of cancers.

Our result were in agreement with Shabbir *et al.* (2011) who found that the aqueous methanol was 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. Amongst other contributing factors affecting efficiency of the extraction is also solubility of endogenous compounds present in extracted material (Sultana *et al.*, 2007). Amin and Thakur (2014) reported that the IC₅₀ value of ethanol extract of flaxseeds is 33.718 mg/ml.

3.5. Identification of Flaxseeds methanolic extract using gas chromatography-mass spectrometry (GC/MS)

Data illustrated in Table 5 and Fig 2 revealed presence of 7 compounds as a main component were separated from flaxseed extract and identified by GC-MS spectroscopy. 9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-were identified as the major compound reached to 24.94 %,

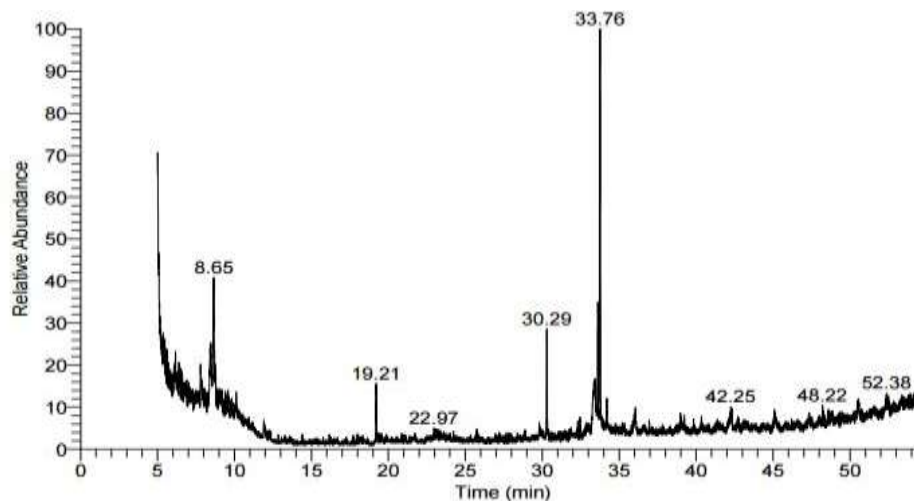


Fig.2. GC/MS chromatogram separation of flaxseed methanolic extract

Table 5: Phytochemicals of methanol extract of flaxseeds

Peak	Rt*	Conc %**	Compounds	M.W	Probability
1	6.43	1.67	2-[(Trifluoroacetoamido)ethyl] 2,2',3,6,6'-penta-O-benzyl- α -D-lactoside	963	58.79
2	6.73	0.68	1,4-Bis(3,3'',4,4''-tetra butyl-2,2':5,2''-terthio n-2-yl)-1,3-butadiyne	990	91.75
3	8.65	10.21	2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	136	6.59
4	19.21	3.0	Phenol, 2,6-bis(1,1-dimethyl ethyl)-4-methyl- (CAS)	220	33.37
5	30.30	5.67	Hexadecanoic acid, ethyl ester (CAS)	284	54.91
6	33.76	24.94	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	306	12.33
7	42.26	2.19	YGRKKRRQRRRGPKVRRLLDL/6	2599	8.03

*Retention time

**Conc % = the percent of concentrations based on peak area integration

4. CONCLUSION:

Flaxseed (*Linum usitatissimum* L.) is a multi-purpose crop and its consumption is beneficial for human health. The results of the present study

revealed that the methanol is the more efficient solvent to extract the total phenolic compounds and flavonoids from flaxseeds when compared to the selected solvents for the study. For this

flaxseeds may constitute a good source of healthy compounds, useful in the prevention of diseases.

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الملخص العربي

النشاط المضاد للأكسدة والتحليل الكيميائي النباتي لبذور الكتان (*Linum usitatissimum L*)

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بذور الكتان مصدر غني لأنواع مختلفة من الفينول مثل Lignans ، والأحماض الفينولية ، الفلافونويد ، phenylpropanoids و التانينات. وقد أجريت هذه الدراسة لتقييم التركيب الكيميائي، والفحص الكيميائي النباتي ، ومجموع المركبات الفينولية ، ومجموع الفلافونيدات والنشاط المضادة للأكسدة من مستخلص (الأسيتون 70%، الميثانول 70%، الإيثانول 70% و المستخلص المائي) من بذور الكتان . تم تقدير النشاط المضاد للأكسدة وفقا للنشاط الكاسح للجذور الحرة باستخدام طريقة DPPH، وقيمة IC₅₀ لمستخلص بذور الكتان بالأسيتون هي . 90,76 في حين مستخلص بذور الكتان بالميثانول هو 55,74 ، مستخلص بذور الكتان بالإيثانول هو 65,21 والمستخلص المائي من بذور الكتان 97,40 وفقا للنتائج لوحظ ان مستخلص الميثانول هو أكثر فعالية من مستخلص الإيثانول والأسيتون والماء من البذور . لذلك قمنا بالتعرف على محتوى مستخلص بذور الكتان بالميثانول من الفينولات والفلافونويد والتانينات باستخدام التحليل الكروماتوجرافي الغاز وتحليل طيف الكتلة.

الكلمات مفتاحية: بذور الكتان - داي فينيل بيكريل هيدرازين - النشاط المضاد للأكسدة.