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Isolation and Purification of Secoisolariciresinoldiglucoside oligomers (Lignan) from Flax seed and its evaluation of antioxidant activity

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Abstract: *The present study aimed to extract and purify the compound of Secoisolariciresinoldiglucoside oligomers (lignan) from flax seed (Linumusitatissimum) and its antioxidant activity. The Lignan was extracted by solvents which gave the best results were ethanol : 1,4 dioxane (1:1, v:v).SDG release after alkaline hydrolysisby using a methanolicNaOH , 20 mM, pH=8 at 50 ºC,followed by using following chromatographic techniques: Liquid-liquid, Sephadex LH-20 column chromatography, thin layer chromatographic (TLC), high performance liquid chromatographic (HPLC) and Fourier Transform Infra-Red(FTIR) . The EC50 values of Pure lignan extract (9 µg/ml) was shown possess DPPH radical scavenging activity compared to reference substances BHT and vitamin C (EC50= 3 and 4.2 µg/ml) respectively, and this was higher than partial pure lignan component (EC50= 25.5 µg/ml).The total phenolic content of the pure lignanwas higher than partial pure lignan which gave 22.312 and 14.85 □ g/ml respectively.*
Key words*: Lignan, flax seed, antioxidant activity, Sephadex LH-20, total phenolic*

I. Introduction

Lignans are diphenolic compounds of higher plants formed by the coupling of two coniferyl alcohol residues that are present in the plant cell wall (Westcott and Muir, 2003). Lignans are a group of polyphenolic compounds in plants that share structural similarities with estrogen and thus have been classified as phytoestrogens. there are two main types of lignans found in flaxseed, secoisolariciresinoldiglycoside and matairesinol, which are contained primarily in the seed coat (Sicilia *et al*., 2003).

The level of SDG in flaxseed, 1–4% (w/w) (Eliasson*et al*., 2003), is 60–700 times higher than that in other edible plant parts (Ford *et al.,* 2001).Variation in flaxseed lignan concentrations depend on the variety, location, and crop year (Westcott and Muir,1996b)**.**Secoisolariciresinol (SECO) amount found in foods. Whole seed and ground flax typically contain between 0.7% and 1.9% SDG, which is approximately 77 to 209 mg SDG/tbsp of whole seed or 56 to 152 mg SDG/tbsp of ground flaxseed (Morris, 2004).

They lignan are part of large structures such as dimers, trimers, or higher oligomers. The lignans from flaxseeds are linked within an oligomeric structure called the lignan macromolecule (Struijs*et al*. 2007), in which it is covalently bound via ester linkages to 3- hydroxy-3-methyl glutaryl (HMG), A straight chain oligomeric structure composed of 5 SDG residues interconnected by 4 HMGA residues (molecular weight of about 4000 Da)was also reported (Kamal- Eldin*et al*. 2001).The aims of this study to isolation and purified lignan from flax seed and its evaluationof antioxidant activity.

II. Materials and Methods

Flaxseeds were collected from the local market, identified as (*Linumusitatissimum L*.) by the botanist Prof. Dr. Ali Hussein AL-Musawi in the College of Science / Baghdad University.Firstly, cleaning flax from derbies which include other plants seeds, some parts of vegetarian of flaxseed and dust, Secondly grinding flaxseeds properly by a grinder machine eventually obtained on a homogenized powder that was ready for extraction.This stage involved defatting of flax oils by using Soxhelt apparatus according to (AACC, 1984). Extraction of Crude Lignan by usedthe method which was described by Rickard *et al,*(1996), involves taking 25 g of defatted powder treats with a mixture of Dioxan and Ethanol alcohol (1:1),(v:v),respectively, with a ratio (1:8),(w:v),(powder: solvent),sample put on magnetic stir for 4 hrs., then filtrated and the solvent was evaporated by rotary evaporator at 40 ^{0}C to obtained crud lignan.

Separation of Lignan:The process of separation Alkaline hydrolysis of SDG oligomers according to (Li *et al*. ,2008 and Yuan *et al*..,2008) by using an alkaline hydrolysis solution (a methanolicNaOH , 20 mM,pH=8) at 50 ºC for hydrolyzing SDG oligomers.The mixture was filtered by whatman filter paper no.1 then the supernatant was concentrated with a rotary evaporator within 45 ^{0}C. Eventually, a thick sticky texture material ,pH was corrected into 3.0 through adding drops of sulfuric acid 2 molar then the sample was stored in 4 ^{0}C.

This method involves (liquid/ liquid) separation according to Westcott and Muir, (1996a).There were two separating solvent systems which were differed in their polarity these systems include: Ethyl Acetate: distilled water ratio (1:7) using separating funnel. Two layers were formed and take aqueous layer. This process repeated twice and the aqueous layer was concentrated with the rotary evaporator at 45 ^{0}C, nearly drying.
SDG extract was separated using Sephadex LH-20 column chromatography (Pharmacia Co.). The column (1.7x 49 cm)mobilized by add the homogenized gels with ethanol solution concentration 99.9%. The SDG extract was subjected to Sephadex LH-20 column chromatography and eluted with distil water and the flow rate regulated to be 1 mL/min. Fractions containing SDG were pooled, concentrated to the require volume.
The pure lignan was identified by (HPLC) according to (Westcott and Muir, 1998), using ODS- reverse phase column and an elution system under the following conditions as shown in table (1).

Table (1) Conditions on HPLC of lignan quantification.

Column	ODS.
Column length	25cm.
Flow rate	1ml/min.
Wave length	280nm
Mobile phase	acetic acid 1% , methanol 30% ,water 69%
Retention time	The time is obtained following the experiment.

FTIR Assay

The functional groups in lignan structure were detected to be compared with the standard chart. lignan contains many functional groups: aromatic benzene ring , aromatic hydroxyl groups and the C=C double bond but derivatives have more than these functional groups.
In order to obtain an indication of the antioxidant activity of lignan.

Five mL of a freshly prepared 0.004 % DPPH solution 4 mg/100 mL in methanol was mixed with 50 µL of different concentration of pure and partial pure lignan 5 , 10 , 15 , 25 , 35 and 50 mg/ml and the absorbance of each dilution, after 30 minutes, was measured at 517 nm. Butylatedhydroxytoluene (BHT) and vitamin C was the antioxidant used as positive control. (Huang *et al,* 2005).All tests were performed in triplicate and the methanol was used as blank solution. The percentage DPPH reduction (or DPPH radical scavenging capacity) was calculated as:
% Reduction = (Abs DPPH – Abs Dil.)/Abs DPPH x 100
Whereby:Abs DPPH = average absorption of the DPPH solution
Abs Dil. = average absorption of the three absorption values of each dilution.
With the obtained values, a graphic was made using Microsoft Excel. The EC_{50} of each extract (concentration of extract or compound at which 50% of DPPH is reduced) was taken from the graphic.

Determination of total phenolic contents

The amount of total phenolics in pure and partial pure lignan was determined with the Folin- Ciocalteu reagent. Gallic acid was used as a standard (Figure 1) and the total phenolics were expressed as mg/g gallic acid equivalents (Lim *et al*, 2006) Concentration of 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol.Concentration of 10, 25 and 50 mg/ml of pure and partial lignan were also prepared and 0.5 ml of each sample were introduced into test tubes and mixed with 2.5 ml of a 10 fold dilute Folin- Ciocalteu reagent and 2 ml of 7.5 % sodium carbonate. The absorbance was at read at 760 nm spectrometrically. All determination was performed in triplicate. Thus total phenolic content can be determin(Savitree *et al*, 2004 and Pourmorad *et al*, 2006).

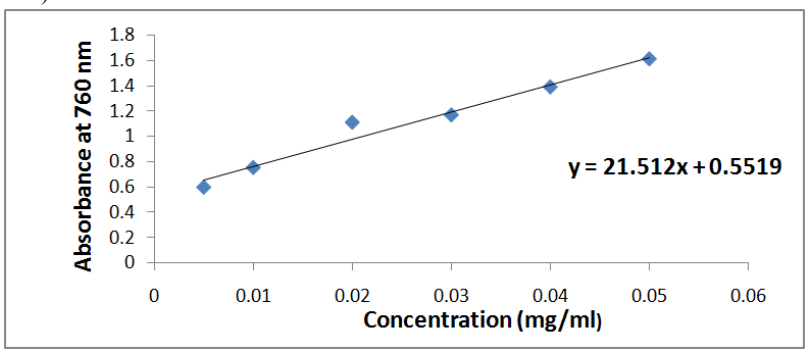

Figure (1): Standard curve of Gallic acid

III. Results and Discussion

The solvents which gave the best results were high purity methanol or 95% ethanol:1,4dioxane (1:1, v:v). Westcott and Muir (1996a) patented an alcohol-based method for isolating flaxseed lignan in greater than 90% purity. The polymeric powder obtained by ethanol :dioxane extraction of defatted flaxseed flour is found to release hydroxy methyl glutaric acid , 4-O-β-D-glucopyranosylcoumaric acid. (Westcott and Muir, 1996a). Also It had been suggested that p-coumaric acid and ferulic acid glucosides were linked directly via ester linkage of their carboxyl groups to the glucosyl moiety of SDG as a terminal unit in SDG oligomers (Struijs*et al*.,2008) and were easily released by alkaline hydrolysis (Johnsson*et al*.,2002; Ford *et al*.,2001).

The step followed by extraction is hydrolysis. Alkaline hydrolysis is the main techniques used to prepare samples for chromatographic analysis of lignans (Li *et al*., 2008). SDG release after alkaline hydrolysis. This step is achieved by two ways:The first way takes a long time to be performed, 48 hours while the second way take a shorter time around 7 hours and it is found that the direct extraction by NaOH resulted in a higher yield than that obtained by hydrolysis of alcoholic extracts due to the inefficient extraction of oligomers from flaxseed matrix with alcohol. (Yuan *et al*., 2008).

The addition of NaOH was necessary to break the ester linkages of SDG oligomers to release SDG, Sodium hydroxide is a base of choice for several inventions (Cui and Han, 2003).

previous study shows that alkaline hydrolysis resulted in the production of methyl esters of *p*-coumaric acid and ferulic acid glucosides, which are stable in methanol; when SDG oligomers were dissolved in water and hydrolyzed by adding an aqueous NaOH solution, alkaline hydrolysis resulted in the immediate production of *p*-coumaric acid and ferulic acid glucosides; when SDG oligomers were dissolved in aqueous methanol solutions (i.e., 70% aqueous methanol solution) (Li*et al*., 2008). Lignan can also be readily obtained from an aqueous ethanol (85%) extract of flaxseed meal (Empie and Gugger, 2002). Temperatures above room temperature (50 to 100ºC) are typically used for base hydrolysis. A higher temperature is needed to separate SDG from larger molecular weight compounds such as protein and starch residues which are coagulated and precipitated by the heat (Dobbins and Wiley, 2004). The pH ranges from 10 to 13 although 11.8 to 12.5 is the preferred range. After hydrolysis, the pH of the solution needs to be acidified in order to prevent the ionization of any functional groups in the aliphatic and aromatic part of the SDG molecule (Hosseinian and Beta, 2009).

On other hand, primary detection about lignan was carried out with its simplified formula by using thin layer chromatography through two solvent systems (ethyl acetate: ethanol) (1:19), which were prepared for this purpose .The detection in these methods gave the same result characterized by appearing of a dark spot on the silica gel thin layer according to Harbone (1973), where the Rf. value of lignan was 0.45.(Figure 1),this result agree with Al-Jumaily*et al*., (2012) and Okhti (2005) they found that Rf. value of lignan was 0.45.

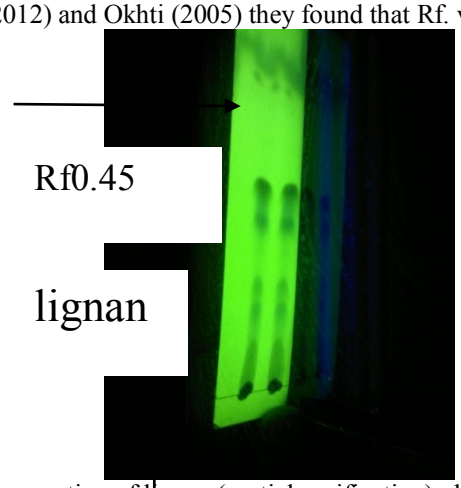

Figure (1):Partition separation of lignan (partial purification). dark spots and lines of sample loading represent lignan, with Rf. 0.45.

Separation by Partition (partial purification) liquid/liquid

Separation of lignan from another compound was carried out by many steps, which include (liq/liq) separation. This process depends on the difference in degree of polarity among compounds which play a key role in the degree of dissolving these structures in two differed solvents in its polarity. Other compounds are considered weaker than lignan (lignan has weak polarity) (Westcott and Muir, 1998). Therefore, lignan dissolves in water while other compounds are dissolving in ethyl acetate when using (ethyl acetate: water).

The end product is a yellow substance not sticky. This is a strong indicator that many unwanted related compounds were removed. The repeated washing increases the purity of lignan, because in the first time both solutions reached were saturated with substances, (AL-Shemary, 2004).

Purification by column chromatography

This step is achieved by using ColumnchromatographySephadex LH-20 according to Li*et al.*, (2008) in the purification of lignanfrom the flaxseed. In this study, the purification of SDG was carried out by this Columnchromatography and repeated many times to obtain sufficient amount of lignan.

On the other hand Westcott and Paton,(2001) used another technique called ultrafiltration using solvents systems, separation columns and Millipore filters with different pores size due to the purification depending on molecular weight. In this technique steps of purification were reduced.

When purification of lignanwas done by Colum chromatography, chemical examination was carried out by using chemical reagents. This was specified for sugar groups that are bound by SDG. Structurally, SDG contained two sugar moieties that closely related with SDG .Therefore, these reagents were used to detect these groups, including firstly Molish's reagent. Thus violet color appearance is an indication of SDG. While in Benedict's result, the orange color (which then changed into red sediment in the bottom of test tube) is an indication for SDG. This result is supported by Fehling's reagent where red sediment appeared. This agrees with AL-Awaad (2001) and AL-Shemary (2004), as showed in table (1).

Table (1):Chemical reagents had been used in detection about sugar moieties in SDG compound.

Reagent	Result	Colour
Molish's reagent	+	Violet
Benedict's reagent	+	Orange
Fehling's reagent	+	red sediment

Thin Layer Chromatography (TLC)

Detection about lignan was carried out by using thin layer chromatography through two solvent systems (ethyl acetate: ethanol) (1:19), which were prepared for this purpose .The detection in these methods gave the same result characterized by appearing of a dark spot only on the silica gel thin layer according to Harbone (1973), where the Rf. value of lignan was 0.45. (figure 2).

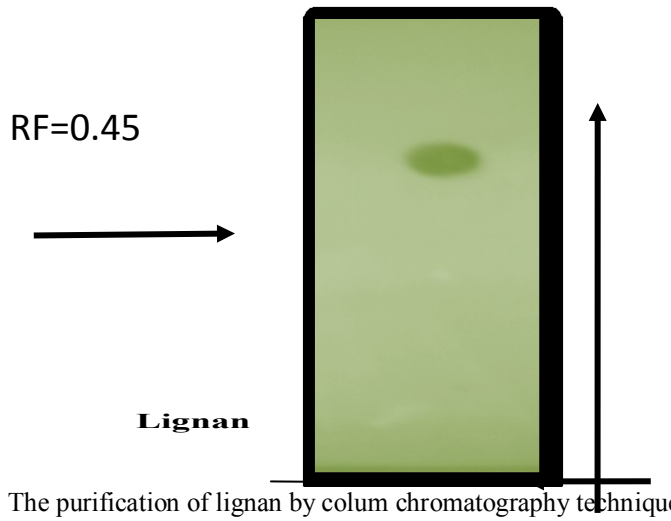

Figure (2): The purification of lignan by colum chromatography technique, Dark line which pointed purified lignan with Rf (0.45).

High-performance liquid chromatography (HPLC)

HPLC is the most used analytical technique for detection and quantification of lignan (Willfor*et al.,* 2006). HPLC method for analysinglignan in flaxseed was developed by Johnsson et al. (2000) and was later applied by others (Eliasson*et al.,* 2003; Johnsson*et al.,* 2002; Nemes and Orsat, 2010). The SDG submitted to identify by HPLC was purified by the column chromatography on a Sephadex LH-20.

Results of H.P.L.C were revealed that the one peak of SDG and the retention time is (7.008 min) as shown in figure (3).These results are in line with those of Zhang and Xu, (2007) as shown in figure (4), There is the retention time and one peak that document the SDG as already pure and the percentage of purity is more than 90%. So coloum chromatography is high efficient in purification, similar to those found by (Li *et al*.,2008).

The isolated SDG was determined by of H.P.L.C (Figure 3) and others tests that were done to detect the purity of SDG. The study also depends on the results of other researches on the same compound like (Westcott and Muir, 1998 and Al-Jumaily *et al*., 2012) .While Paul and David (1981) used (H.P.L.C and Gel filtration in the purification of lignan) from podophyllum.

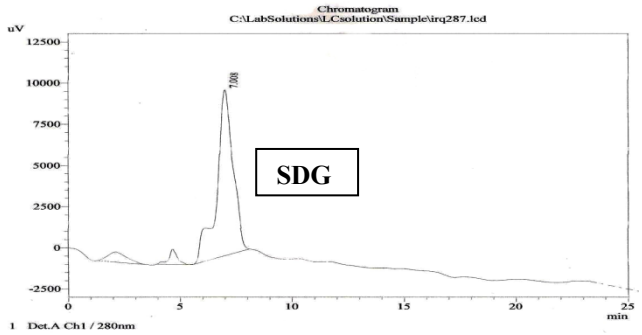

Figure (3): H.P.L.C. quantification of purified lignan SDG, retention time (7.008 min) and the absorbance of lignan .

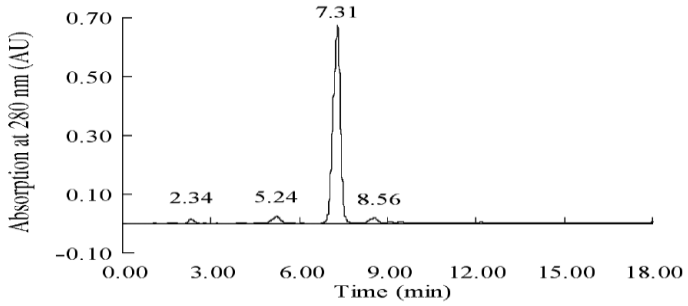

Figure (4): HPLC chromatogram of purified SDG (Zhang and Xu 2007).

Fourier Transform Infra-Red (FTIR) for pure lignan

Infrared (IR) spectroscopy was applied for the detection and analysis of any purified compound besides the chemical tests. Since I.R light can be reflected from materials, the loading of even small amounts of any product is needed to enhance the very small spectral signal to be recorded (Black and Bale, 2004)

Fourier Transform Infra-Red (FTIR) spectrophotometers were used for recording spectra in the region 4000 cm^{-1} to 670 cm^{-1} (2.5µm to 15 µm) or in some cases down to 200 cm^{-1} (50 µm). Fourier transform spectrophotometers used polychromatic radiation and calculate the spectrum in the frequency domain from the original data by Fourier transformation (British Pharmacopeia, 2004).Lignan has many functional groups Phenolic– OH group stretching at wave length 3437.15, Aromatic C=C at wave length 1639.49 and Aliphatic C–O at wave length 1103.28 (Table 2). Figure (5) shows the infrared spectra for the extracted pure lignan.

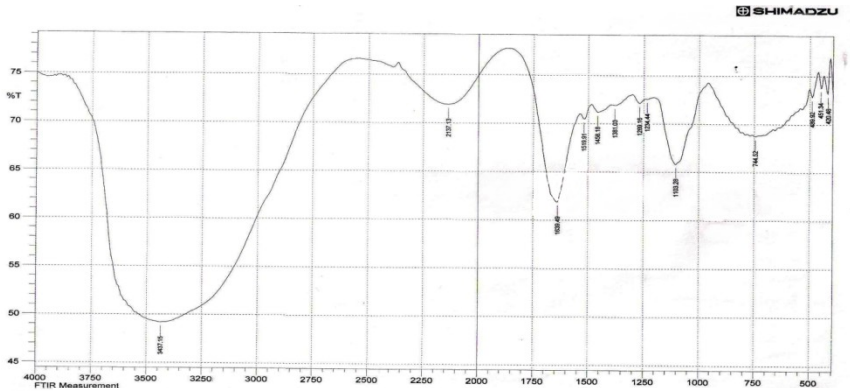

Figure (5): The infrared spectrum for lignan

Table (2): The IR frequencies region for the functional groups of the standard lignan and the extracted pure lignan

The Functional Group	I.R Frequencies Standard groups (cm^{-1})	I.R. Frequencies of Extracted pure Lignan
Phenolic–OH group stretching	3650-2500	3437.15
Aromatic C=C	1680-1620	1639.49
Aliphatic C–O	1300-1000	1103.28

The Evaluation Antioxidant activity of lignan was carried out as follows:
Figure (6) illustrates the concentration of DPPH radical due to the scavenging ability of the extract and standards. BHA and vitamin C were used as references. The EC50 values of Pure lignan extract (9 µg/ml) was shown possess DPPH radical scavenging activity compared to reference substances BHT and vitamin C (EC50= 3 and 4.2 µg/ml) respectively, and this was higher than partial pure lignan component (EC50= 25.5 µg/ml).

These findings showed that the flaxseed lignans SDG and SECO exhibited strong antioxidant and protective effects in quenching the DPPH.

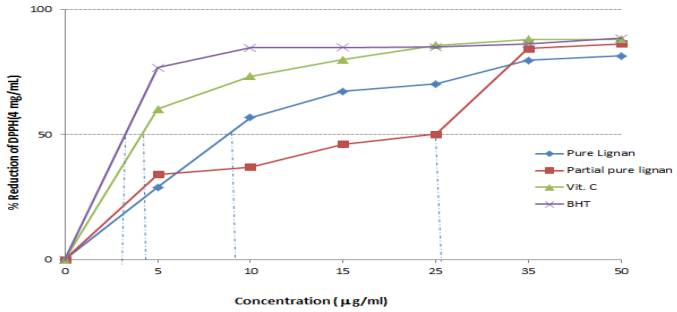

Figure (6): Percentage of DPPH reduction using lignan samples (pure and partial pure) and appropriate controls after 30 min of exposure.

Zanwar*et al*. (2010) assessed *in vitro* antioxidant activity of ethanolic extract of *L. usitatissimum*(EE-LU) by using DPPH radical scavenging, at doses 100, 200, 300, 400 and 500 µg/ml. EE-LU showed dose dependant antioxidant activity in different studied models, maximum at 500 µg/ml.Kasote, (2013) showedFlaxseed lignans SDG, SECO, ED and EL are found to be equal or somewhat more potent than BHT, vitamin E. Thus, they could have commercial potential as an alternative to these antioxidants. Flaxseed lignans could be the good choice of natural antioxidants for oil stability. Flaxseed antioxidant may have potential application in food and heath industry as food stabilizer, nutraceutical etc.

Determination of total phenolic contents

Phenolic compounds are a class of antioxidant agents which act as free radical terminators and their bioactivities may be related to their abilities to chelate metals, inhibit lipoxygenase and scavenge free radicals (Roya and Fatemeh, 2013).

The amount of total phenol was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard compound and the total phenols were expressed as □g/g gallic acid equivalent using the standard curve equation: y = 21.512 + 0.5519, where y is absorbance at 760 nm. The total phenolic content of the pure lignanwas higher than partial pure lignan show in (Table 3).

Table (3): Total Phenolic content of pure and partial pure lignan

Pure lignan (mg/ml)	**Total phenol (□g/g)**
10	12.60
25	18.687
50	22.312
Partial pure lignan (mg/ml)	**Total phenol (□g/g)**
10	10.24
25	12.44
50	14.85

The prevailing lignan in the flaxseed is secoisolariciresinoldiglucoside (SDG) (Cardoso Carraro*et al*., 2012).Plant lignans are the biologically important class of phenolic compounds. They belong to a group of phenols which are characterized by coupling of two phenylpropanoid units (Willfor*et al*., 2006).

The content of SDG varies between 6-29 g/kg in the defatted flaxseed powder (Johnsson*et al*., 2002; Eliasson*et al*., 2003; Beejmohun, 2007). Flaxseed was reported to contain 8-10 g/kg total phenolic acids, about 5 g/kg of esterified phenolic acids and 3-5 g/kg of etherified phenolic acids (Oomah*et al*., 1995). They are either in free and/or bound forms. flaxseed possesses about 0.3-0.71 g of total flavonoids per kg of flaxseed (Oomah*et al*., 1996). Flaxseed including minor amounts of phenolic acids as p-coumaric and simple phenols as vanilline (Siger et al., 2008).

SDG is most studied flaxseed phenolic compound pertaining to its *in vitro* antioxidant potential so far. Flaxseed lignan, SDG exhibited antioxidant activity by either direct radical scavenging or by inhibition of lipid peroxidation. Prasad (1997) studied hydroxyl radical scavenging potential of SDG. The ability of SDG to scavenge exogenously generated hydroxyl radical (•OH) was investigated by using ultraviolet (UV) light photolysis of H2O2, and by studying ability to prevent OH-induced lipid peroxidation in biological system.

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