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Biochemical assay to evaluate phytoconstituents and free radical scavenging activity of sunflower (*Helianthus annus* L.)

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Abstract
Aim: In the present study, HSFH-848 variety of sunflower was studied for bioactive composition of phenolic extracts of seed oil and methanolic extract of defatted seed cake.
Study Design: Phytochemical Analysis of sunflower
Place and Duration of Study: Chemistry Lab, Medicinal & Aromatic Plants Section, Old IATTE Building, CCS Haryana Agricultural University, Hisar; between July 2012 to July 2014.
Methodology: Free fatty acid, peroxide value, total unsaturated fatty acid content, iodine value, Saponification value and unsaponifiable matter in the oil were determined. Free radical scavenging activity is determined by DPPH method.
Result: The yield of seed oil, extracts of whole seed and the seed cake were 34.0±0.1%, 4.9±0.0% and 7.4±0.2% respectively. Free fatty acid, peroxide value, total unsaturated fatty acid content and iodine value in the oil were 1.3±0.1%, 1.7±0.1meq kg^{-1}, 90.2% and 120±0.5 g/100g respectively. Saponification value and unsaponifiable matter were 160 ±0. 1 mg/g KOH, 1.6±0.1% in sunflower oil respectively.
Conclusion: Because of free radical scavenging activity, high unsaturated fatty acids sunflower oil and defatted seed cake can be used as food as well feed.

Keywords: *Helianthus annus*, oil, free fatty acids, antioxidants

1. Introduction
Vegetable oil rich in antioxidant are of much interest in the food industry as they retard oxidative degeneration of lipids and hence improve the quality as well as nutritional value of food. In the cooked oils, Sunflower (*Helianthus annuus* L.) oil is the one that contain high amount of unsaturated fatty acids. This is from one of the species of the genus of 67 Helianthus species and it is an herbaceous plant that originated in North America. Sunflowers are such a special plant, in that every part of the plant can be used. The seeds, flowers, leaf, stem, and root all have several uses. The sunflower was introduced, for the first time in India in 1969 as a drought tolerant oil seed crop [1]. Sunflower (*Helianthus annuus*L.) is one of the most important annual crops in the world that is grown for edible oil. The phytochemical properties of sunflower oil has been studied earlier [2]. It is used as an ingredient in feed production because of its high protein content. However, the primary use of sunflower seed is not for edible protein. Rather it is for the oil because certain attributes of sunflower seed oil are particularly attractive to the food industries. It is a rich source of vitamins, especially vitamin E. Sunflower oil is a good source of essential fatty acids needed in body as presence of high level of unsaturated fatty acids (linoleic and oleic acids) and low level of saturated fatty acids (myristic, palmitic and stearic acids). Reported values of acid detergent fiber and lignin of defatted sunflower meal (with hull) varying from 27 to 32% and from 9 to 13.6%, respectively [3]. As we are well acquainted that free radicals has been essential part of aerobic life and transform various physiological functions [4]. Their excessive production may interrupt the body's antioxidant system which might lead to "oxidative stress". These circumstances contribute to a variety of diseases like heart diseases, carcinogenic diseases and many neurodenegerative disorders [5]. Even though the progress of some synthetic antioxidants in the past few years has flourished, they are not yet extensively used as curative agents due to their feasible toxicity. As a result of which the growth of natural antioxidant has now drawn the attention of scientific community towards of plant based material like leaf, flower, oil, roots. Therefore, the present study was aimed at the study of chemical composition and antioxidant potential of seed oil and methanol extract of defatted seed cake of sunflower.

2. Material and Methods

2.1 Seed Material and chemicals

The Sunflower seeds of variety HSFH-848 were procured from the oil seed section, Department of Genetics and Plant Breeding, CCSHAU, Hisar. The seeds were ground into fine powdered form after cleaning. The commercially available chemicals of highest purity from Merk and Ranbaxy, Qualigens were used for a range of investigational procedures.

Oil, iodine value, saponification value, unsaponifiable matter, peroxide value and free fatty acid were determined according to official methods [6-8].

2.2 Oil

Ground samples (100g) of seed were pre-weighed in a thimble and set for soxhlet extraction using petroleum ether. The heating rate was adjusted to give a condensation rate of 2-3 drops/sec. and extracted for 16 hrs. Removed the thimble and retained petroleum ether. The excess of petroleum ether was evaporated from the solvent flask on a hot water bath and dried the flasks in a desiccator and weighed.

$$\text{Oil content in sample (\% dry wt. basis)} = \frac{(b-a)\times 100}{Wt.of\ sample\ (g)}$$

2.3 Iodine Value

Two grams of oil sample was taken in a 500 ml glass stoppered conical flask containing 10 ml of $CHCl_3$. The flask was swirled until the sample entirely dissolved. Iodine monochloride (25 ml) was added to it. Kept the flask in dark for nearly one hour. Then 15 ml of KI solution and 100 ml of water were added and shook vigorously. Thereafter, titration was carried out with standard sodium thiosulphate solution. Two blanks were made in the same style by omitting the sample. If 'B' is volume of standard sodium thiosulphate solution (ml) used for blank and 'S' is volume of standard sodium thiosulphate solution (ml) used for sample, then Iodine number is calculated by:

$$\text{Iodine number } \left(\frac{g}{100}\right) = \frac{(B-S)(\text{Normality})(12.7)}{\text{Weight of sample}}$$

2.4 Saponification Value

Oil sample (2g) was taken into the flask in which 25 mL of 0.5 N alcoholic KOH solutions was added. Approximately for 60 min. reflux condenser was heated in boiling water. During heating the flask was swirled regularly. After that a drop of 1% phenolphthalein was added and titrated with 0.5 N HCl. Operations were performed with blank as well. Volume of 0.5 N HCI used for sample and blank was denoted by 'A' and 'B'.

$$\text{Saponification value} = \frac{(B-A)\times 28.05}{\text{Wt.of oil(g)}}$$

2.5 Saponification and removal of unsaponifiable Material

Each replicate of seed oil (5g) was refluxed individually with 50 mL of 2 M potassium hydroxide in 95% ethanol for 1 hr. The mixture was allowed to cool, diluted with 50 mL of water and extracted with 50 mL of diethyl ether for 3 times. The combined ether extracts were washed thrice with distilled water (100 mL) and after washing extract were allowed to stand overnight. The ether was isolated under vacuum at 40ºC and the residue dried out by evaporation with acetone to obtain the unsaponifiable matter.

2.6 Peroxide Value

In a conical flask the 5g oil was poured. In the flask 30 mL of acetic acid and $CHCl_3$ mixture was added. Saturated KI solution (0.5 mL) was added to it and allowed to stand for 1 min. After that about 450 mL of water was added to the flask and then titrated against standard 0.01 N sodium thiosulphate to liberate all iodine free $CHCl_3$ layer until the blue colour just vanished. Omitting oil similarly blank was also titrated and final peroxide value was calculated by:

$$\text{Peroxide value} = \frac{\text{Volume of Na2S2O3}\times\text{Normality}\times 1000}{\text{Wt.of oil(g)}} \text{ meq / kg oil}$$

2.7 Fatty Acid Spectrum

A suitable amount of oil sample was taken in a test tube and 0.5 ml of 0.5 N sodium methoxide was added and covered with aluminium foil and then immersed in a water bath at 65ºC and shook vigorously for 2-3 min. The mixture became homogenous indicating the complete esterification of the oil sample. The test tube was removed from the water bath and cooled to room temperature. One ml of carbon disulphide was added and shaken for 1 - 2 min. Approximately 100 mg of activated charcoal was added mixed uniformly and filtered through Whatman No. 1 filter paper. The filtrate constituted all the methyl esters of fatty acids.

2.8 Fractionation of Methyl Esters by GLC

Methyl esters of fatty acids were separated using Chemito 8610 HT Gas chromatograph equipped with a BPX70 and FID, 0.25mL fused silica column was used. The carrier gas was hydrogen and injection was operated in the split mode by split ratio of 50:1. Injector and detector temperature were 270 ºC and 280 ºC respectively. The oven temperature was held at 70 ºC for 1 min and then programmed at 30 ºC min^{-1} to 170 ºC followed by further programming at 30 ºC min^{-1} to 200 ºC and held at this temperature for 6 min. Data was analyzed with, Chemito 5000 Integrator (Tashniwal Instruments, India Ltd.).

2.9 Determination of Free fatty acids

Free fatty acids were determined as per modified literature method [9]. Fifty mL of denatured alcohol was added to 1g of oil sample. Few drops of phenolphthalein were added and the contents were titrated against 0.1 N sodium hydroxide till a permanent light pink color appeared which persisted for at least 1 min. The percentage of free fatty acids was calculated by using the following formula:

$$\text{Free fatty acids (in terms of oleic acid)} = \frac{100\times 282\times\text{Volume NaOH used}}{\text{Wt.of oil}\times 10\times 1000}$$

2.10 Determination of Mineral Contents

The sample was digested by wet oxidation. In a conical flask 0.2 g of each extract was mixed with 5 mL nitric acid and 1mL of perchloric acid and kept it overnight at room temperature. After that, digestion was done on low temperature at 70-80 ºC and then at higher temperature. Digest the sample until the volume of the solution reduced to about 1mL. Made the final volume upto 10 mL using distilled water and analyzed by using an atomic absorption spectrometer [10].

2.11 Determination of total phenolic content

Determination of total phenolic content was done by Folin-

Ciocalteu reagent using gallic acid as standard [11]. To a 50 ml volumetric flask 1.0 ml extract, 1.0 ml Folin–ciocalteau reagent and 2.0 ml of Na_2CO_3 (20%w/v) were added and mixed and final volume was made to 50 ml. After 8 minutes, the mixture was centrifuged for 10 minutes at 6000 rpm. Then the absorbance of supernatant solution was measured against a blank prepared similarly with the same solvent but omitting the extract 730 nm using Shimadzu UV-Vis spectrophotometer (UV-2600). A blank was also prepared by following same aforementioned procedure without asample. After multiplication with the dilution factor, the concentration of phenolic content was expressed as equivalent to milligrams of gallic acid per gram of extract (mg $GAEg^{-1}$) by using the standard plot.

2.12 Determination of Flavonoid Content

The aluminium chloride colorimetric assay, was used [12]. Briefly, one mL of extracts or obtained solution of catechin (0.02, 0.04, 0.06, 0.08 and 0.01 mg mL^{-1}) was added to test tubes that contained 4mL of double distilled water. To the mixture 0.3mL 5% $NaNO_2$was added. 0.3mL 10% $AlCl_3$ was added after 5 minute. Immediately, 2mL of 1M NaOH was added and with double distilled water, the total volume was made upto 10mL. The solution was mixed thoroughly and the absorbance of both the samples, standard and blank was read at 510 nm using Shimadzu UV-Vis spectrophotometer (UV-2600).

2.13 Determination of Total Tocopherol

Using aliquots of 10, 15, 20 and 25 mg of a solution of $\propto$ −tocopherol, volume was made to 8mL with ethanol. Added 1mL of 2,2̕ -dipyridyl reagent and mixed. 1mL the ferric chloride reagent was added and shook the mixture for 10 seconds. The absorbance of the mixture was read at 520 nm against ethanol as a blank. Then the standard curve was drawn. The above same procedure was followed by using 10, 20, 30, 40 mg sample solutions [13]. The content of $\propto$ −tocopherol in the extract was calculated by using regression equation of the standard curve. During color develop solutions were protected from sunlight.

2.14 Deremination of Total carotenoids

Determination of total carotenoids was done by dissolving 0.5 g oil sample in cyclohexane (2.5% w/v) and the absorbance was read at 417 nm [14].

$$\text{mg carotene/kg oil} = \frac{(\text{absorbance at 417 nm}) \times \text{sample in volume in ml}}{0.204 \times (\text{sample weight in g})}$$

2.15 2, 2'-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Method

The antioxidant activity of the extracts was evaluated according to DPPH free radical scavenging method [15]. 2, 2'-Diphenyl-1-picrylhydrazyl is a stable free radical that shows a maximum absorption at 517 nm in methanol. When DPPH encounters proton donating substances such as a radical species and an antioxidant, the absorbance at 517 nm disappears because of the scavenging of radical. The radical scavenging effect of each fraction was measured on the basis of this principle. Briefly 0.03, 0.06, 0.09, 0.12, 0.15 mg of methanol extract of sunflower were added to 1mL of DPPH radical (DPPH: 0.025 gL^{-1} in methanol) final volume was made with 10 mL in methanol and mixed by vortex for 5 minutes. At a regular interval the absorbance of the sample was measured at 517 nm till a steady state is reached (40 min) by using the Shimadzu UV-Vis spectrophotometer (UV-2600). Similarly, a control sample was also prepared. The antioxidant activity was expressed as the% of decline of the absorbance after 2 hrs relative to the control, corresponding to the percentage of DPPH that was scavenged.

2.16 Statistical analysis

Three replicates of each sample were used for statistical analysis and resulting values are expressed as mean± S.D. Correlation analyses of antioxidant activity, flavonoids and total phenolic content were carried out using Pearson correlation programme in Online Statistical Analysis (OPSTAT www.hau.ernet.in).

3. Result and Discussion

Soxhlet extraction of the sunflower seed oil with petroleum ether (60-80 ºC) gave light yellow color yield of 34±0.1%. The yield of methanolic extract of whole seed and defatted seed cake were 4.9±0.0%, 7.4±0.2%.

3.1 Chemical Properties of oil

Free fatty acid and peroxide values are considered as important measures of oil quality. The mean peroxide value, iodine value, saponification value and unsaponifiable matter were 1.7±0.1meq kg^{-1}, 120±0.5 g/100g, 160±0.1 mg/g KOH and 1.6±0.1% respectively. The peroxide value was low in oil which stands for good index of stability of oil and its vulnerability to rancidity during storage. Concentration of free fatty acid was observed 1.3±0.1%. Literature reported a little higher peroxide value i.e. 2.6±2.0 meq kg^{-1} and 0.2±0.0% free fatty acid in a sunflower oil [16]. In codex standard for named vegetable oils, the reported iodine value was 118 -141g/100g while saponification value was 188 -194% in sunflower.

3.2 Fatty Acid Composition

Oleic acid and Linoleic acid were the major unsaturated fatty acid observed as described in Table 1. Total unsaturated fatty acid content was 90.2%. Among the all fatty acids studied, oleic acid was found to be highest in oil. High levels of oleic acid are considered advantageous for human being healthiness and convey stability to oil during storage and deep fat frying.

Table 1: Relative percent composition of fatty acids in seed oil of sunflower

Fatty acid	HSFH-848
Palmitic ($C_{16:0}$)	6.6±0.1
Stearic acid ($C_{18:0}$)	1.3±0.4
Oleic acid ($C_{18:1}$)	46.5±0.4
Linoleic acid ($C_{18:2}$)	43.7±0.2
Total saturated fatty acids (%)	7.9
Total Unsaturated fatty acids (%)	90.2

Values are mean of three replicates ± standard error.

3.3 Minerals

The sunflower seeds contained considerable quantity of vital minerals. Zinc and iron were 0.2±0.3, 2.8±0.1 mg/100g in whole seed of Sunflower. The corresponding values for the same were 0.5±0.2 mg/100g and 4.5±0.1 mg/100g in defatted seed cake.

3.4 Phenolic Contents of sunflower extracts and their antioxidant activities

Phenolic compounds are extensively dispersed in oils. The

results showed that the content of total phenol, flavonoid, tocopherols, carotenoid value were 21.7±1.5 mg GAE/100g, 12.9±2.9 mg CAE/100g, 47.5±3.1 mg/100g and 10.2±3.5 mg kg^{-1} in oil extract respectively. Comparative values obtained for defatted seed cake are listed in Table 2.

Effective concentration (EC_{50}) exhibited by phenolic extract of crude seed oil was at the concentration of 0.0056±0.2 mg/ml of the extract. The corresponding maximum antioxidant activity exhibited was 72% at the concentration of 0.009 mg $mL^{-1}$of the extract. The corresponding maximum antioxidant activity exhibited by methanol extracts of defatted seed cake of hybrid HSFH-848 of Sunflower was 80% at the concentration of 0.01mg mL^{-1} of the extract (Table 3).

Table 2: Phenolic constituents of sunflower extracts with their antioxidant activities.

Parameters	Oil	Defatted seed cake	BHA (Standard)	BHT (Standard)
Total phenol (mg GAE/100g)	21.7±1.5	15.9±2.3	_	_
Total flavonoids (mg CAE/100g)	12.9 ±2.9	8.6±0.5	_	_
Total tocopherol (mg/100g)	47.5±3.1	12.4±1.3	_	_
Carotenoid content (mg/kg)	10.2±3.5	10.2±3.5		
EC_{50} mg/ml of phenolic extract	0.0056±0.2	0.0061±0.1	0.0026±0.2	0.0011±1.0
Max. activity% Conc. (mg/ml)	72 (0.009)	80 (0.01)	82 (0.009)	94 (0.009)

Values are mean of three replicates ± standard error

Table 3: Antioxidant activity (%) of sunflower extracts with comparison to BHA and BHT taken as standard

Conc. in mg/ml	Antioxidant Activity (%)			
	Oil	Defatted seed cake	BHA	BHT
0.001	12	10	19	49
0.002	17	15	42	69
0.003	28	26	57	79
0.004	34	32	69	86
0.005	45	42	74	89
0.006	51	49	78	91
0.007	59	60	80	92
0.008	67	68	82	93
0.009	72	76	82	94
0.010	72	80	82	94
0.011	-	83	-	-
0.012	-	85	-	-
0.013	-	88	-	-
0.015	-	88	-	-

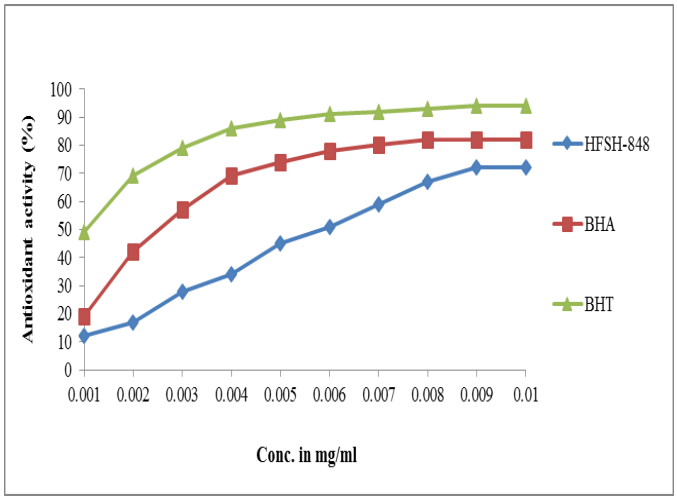

Fig 1: Antioxidant activity (%) of phenolic extracts of crude seed oil of sunflower (BHA & BHT taken as standard).

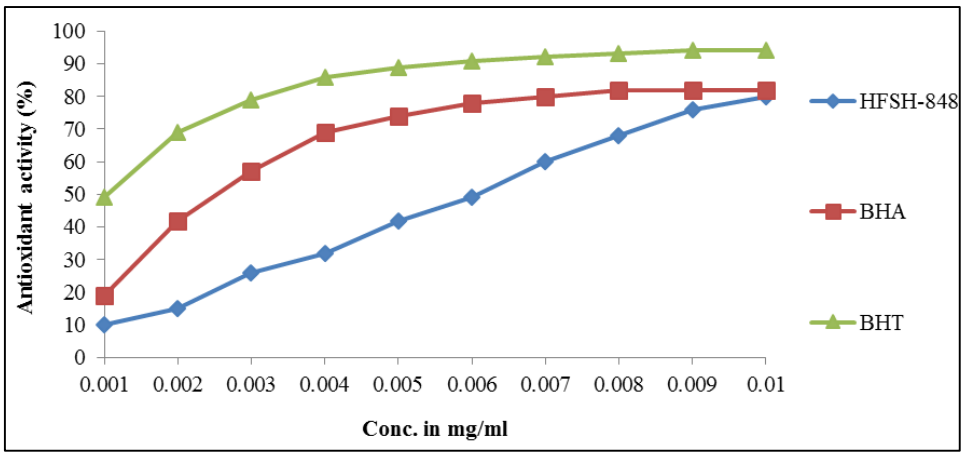

Fig 2: Antioxidant activity (%) of methanol extract of defatted seed cake of sunflower (BHA & BHT taken as standard)

3.5 Kinetic Study of Phenolics

For the reaction kinetics between DPPH and phenolic content of the oil, various absorbance values were observed with time and were noted after every 10 minutes till a plateau was reached. Further reaction was done after 50 minutes of incubation. The kinetic curve of oxidation inhibition was constructed to clarify the mechanism of antioxidant action of phenolic compounds present in the oil and defatted seed cake extract (Fig.3). For comparison the activity of synthetic antioxidant BHA and BHT were determined. Thus result showed that the antioxidants of the studied methanolic extract of sunflower seed oil may be effective in blocking the chain reactions by interaction with the peroxyl radicals.

3.6 Descriptive Statistics

Through the correlation analysis for phytochemical contents, it was found that total phenols showed highly synergistic effect with EC_{50} value obtained in both the extracts of

sunflower. But rest of the phytoconstituents do not showed high significant relations due to several factors. As per earlier reports, presence of various phenolic substances complex in the extract was responsible for synergistic effect whereas negative correlation existed when phenolic compounds are correlated in different modes in various assay systems [17]. Also, the antioxidant activity of fractions may not only be due to the presence of phenolic compounds but also related to the presence of some individual active components in the extracts. The unclear relationship between the antioxidant activity and total phenolic content may be explained by the fact that the total phenolic content does not incorporate all the antioxidants. In addition, the synergism between the antioxidants in the mixture makes the antioxidant activity not only dependent on the concentration but also on the structure and interaction between the antioxidants.

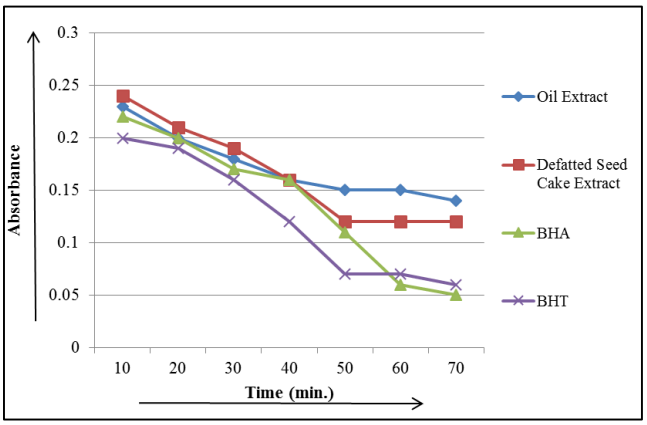

Fig 3: Reaction kinetics between DPPH and phenolics of oil sample of hybrid HSFH-848 of Sunflower (BHA & BHT have taken as standard).

Table 4: Pearson correlation matrix for oil extract

	EC_{50}	Phenolics	Flavonoids	Tocopherol	Carotenoids
EC_{50}	1.000	0.955^{**}	0.896^{NS}	0.797^{NS}	0.765^{NS}
Phenolics	0.955^{**}	1.000	0.991^{**}	0.784^{NS}	0.837^{NS}
Flavonoids	0.896^{NS}	0.991^{**}	1.000	0.993^{**}	0.975^{*}
Tocopherol	0.797^{NS}	0.784^{NS}	0.993^{**}	1.000	0.798^{NS}
Carotenoids	0.765^{NS}	0.837^{NS}	0.975^{*}	0.798^{NS}	1.000

* Significant at 5%;** Significant at 1%

Table 5: Pearson correlation matrix for defatted seed cake extract

	EC_{50}	Phenolics	Flavonoids	Tocopherol	Carotenoids
EC_{50}	1.000	0.970^{*}	0.967^{*}	0.799^{NS}	0.887^{NS}
Phenolics	0.970^{*}	1.000	0.778^{NS}	0.527^{NS}	0.830^{NS}
Flavonoids	0.967^{*}	0.778^{NS}	1.000	0.987^{*}	0.803^{NS}
Tocopherol	0.799^{NS}	0.527^{NS}	0.987^{*}	1.000	0.606^{NS}
Carotenoids	0.887^{NS}	0.830^{NS}	0.803^{NS}	0.606^{NS}	1.000

* Significant at 5%; ** Significant at 1%

4. Conclusion

Nutritionists are interested in natural antioxidant, which protect the body against damage by reactive oxygen species. As per our study, the result provides information about nutrition and health impact of sunflower oil, which serves as nutritional sources of natural antioxidants for oxidative stress based disease prevention and health promotion. Presence of phenol, flavonoids and tocopherol in sunflower oil makes it a potent antioxidant for nutraceutical use.

5. Acknowledgement

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6. Competing Interests

Authors have declared that there is no conflict of interest in this manuscript.

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