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Functional Lipids and Bioactive Compounds from Oil Rich Indigenous Seeds

Azza. S. Naik, S. S. Lele

***Abstract*—**Indian subcontinent has a plethora of traditional medicine systems that provide promising solutions to lifestyle disorders in an 'all natural way'. Spices and oilseeds hold prominence in Indian cuisine hence the focus of the current study was to evaluate the bioactive molecules from *Linum usitatissinum* (LU), *Lepidium sativum* (LS), *Nigella sativa* (NS) and *Guizotia abyssinica* (GA) seeds. The seeds were characterized for functional lipids like omega-3 fatty acid, antioxidant capacity, phenolic compounds, dietary fiber and anti-nutritional factors. Analysis of the seeds revealed LU and LS to be a rich source of α-linolenic acid (41.85 ± 0.33%, 26.71 ± 0.63%), an omega 3 fatty acid (using GCMS). While studying antioxidant potential NS seeds demonstrated highest antioxidant ability (61.68 ± 0.21 TEAC/ 100 gm DW) due to the presence of phenolics and terpenes as assayed by the Mass spectral analysis. When screened for anti-nutritional factor cyanogenic glycoside, LS seeds showed content as high as 1674 ± 54 mg HCN / kg. GA is a probable good source of a stable vegetable oil (SFA: PUFA 1:2.3). The seeds showed diversified bioactive profile and hence further studies to use different bio molecules in tandem for the development of a possible 'nutraceutical cocktail' have been initiated**..**

***Keywords*—**antioxidants, bioactives, functional lipids and oilseeds

I. Introduction

THE worldwide consumption of vegetable oils has grown at a rate of 5% pa from 1975 to 2011. Increase in oil usage can be accounted to a boost in both, food consumption and industrial consumption. Though there are several plants that produce seeds rich in oil, hardly a dozen of these varieties have been utilized commercially as a source of edible oil [1].The tropical climate of the Indian subcontinent favours the growth of various herbs and spices, also flavour enhancement through the addition of spices has been an inseparable part of Indian cuisine. Studies involving the analysis of dietary patterns around world showed that populations that have a higher daily intake of spices show a lower occurrence of cancer [2]. Focusing on the benefits of incorporating spices in our diet coupled with the need to utilize the locally grown seeds, there is an urgent need to develop methods for utilization of these seeds in nutritional products.

S.S. Lele is with the Institute of Chemical Technology, Food Engineering and Technology Department, Matunga, Mumbai- 400 019, India (phone: 3361 1111, 3361 2222 Ext.2501 (D) 2410 0136 fax: 2414 5614; e-mail: dr.smita.lele@gmail.com).

Phytochemicals are biologically active compounds present in natural foods including fruits, vegetables, grains, nuts, and seeds that have the potential to prevent or delay the onset of chronic diseases, such as various cancers and cardiovascular disease [3]. Traditionally plant seed derived oil has been primarily used as a medium of cooking, a lubricant, and also utilized in pharmaceuticals and other industries [4]. However, lately the focus has been on identifying functional ingredients in oilseeds that can find therapeutic applications.

On one hand novel food targets need to be screened for their antioxidant activity that finds application against several disorders and on the other they need to be analyzed for anti-nutritional constituents that manifest their toxicities on consumption. Intrinsic antioxidants like phenolic compounds present in plants are a self mechanism for countering oxidative stress and help humans by providing similar protection against reactive species [5]. Whereas, anti-nutritional factors are a result of an evolutionary mechanism in plants that prevents them from being eaten. In humans they are known to reduce bio-availability of nutrients thus restricting the usage of such plants for human nutrition [6].

The current study revolves around four locally grown seeds i.e *Linum usitatissinum* (LU), *Lepidium sativum*(LS), *Nigella sativa*(NS) and *Guizotia abyssinica*(GA). LU (flaxseed) has generated a lot of interest by means of its phyto-constituents like fiber, omega 3 rich oil and phytoestrogens. The processed food market has been captured by a range of functional foods utilizing flax fiber and oil as active ingredients [7].

LS also known as 'garden cress' is a native shrub used in traditional culinary recipes that also finds application in Ayurvedic and Unani medicine. In Ayurveda it is known to work against flatulence and phlegm while in Unani medicine it is commonly used against aliments like bronchitis, rheumatism and other inflammatory diseases. Also the seeds have been reported to contain exceptionally high amount of alpha linolenic acid thus being as an excellent vegetative source of omega-3 fatty acid [8].

NS commonly known as black cumin or kalongi is a small annual herb that has been used extensively in traditional plant based medicine systems of Far east, Middle east and Asia. It is a rich source of several phytochemicals and has been used to treat bronchial asthma, rheumatism, obesity, hypertension and gastrointestinal problems and many common infections [9] .GA (Niger) is an oilseed crop mainly grown in Ethiopia and India.

Niger seeds have been used as bird and cattle feed. To a limited extent they have been used in human diets [10].

Thus the present study was carried out with an aim to investigate four indigenous cultivars for their bioactive factors like functional lipids, antioxidant capacity, phenolic compounds, dietary fiber and anti-nutritional factors.

II. Material And Methods

A. Plant Seeds

The seed sample for LU, LS, NS and GA was procured from APMC, Vashi, (India) a local market place. Fig.1 shows the external morphology of the acquired seeds. These were then dried at 55°C overnight and stored in airtight container at 4°C. Before commencing with the extraction in Soxhlet apparatus, the seeds were freshly ground and passed through a 1mm sieve.

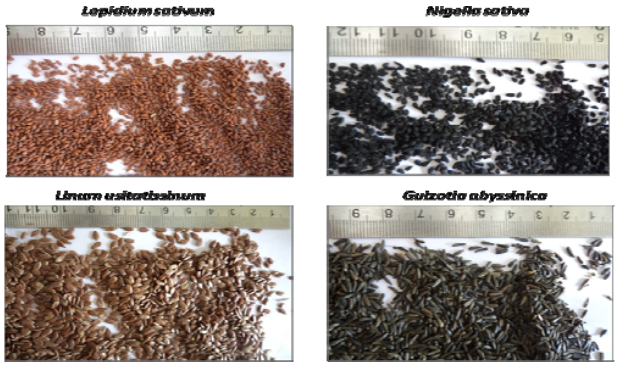

Fig. 1 Experimental sample seeds

B. Chemicals

Standard fatty acids (GC grade) were supplied by Sigma Aldrich, India. Analytical and HPLC grade solvents were obtained from SD Fine Chemicals, Mumbai, India. All other reagents were procured from Himedia, Mumbai, India. Total Dietary Fibre Analysis Kit (K-TDFR 05/12) was gifted by Megazyme International, Ireland.

C. Instrumentation

A Varian 450-GC (Gas Chromatograph) 220-MS (Mass Spectrometer) having Ion Trap Mass Analyzer was utilized for analysis of the seed extract. A highly polar column CP-Sil 88 (25 m x 0.25 mm i.d., 0.39 mm o.d) led to proper separation of individual fatty acids from the petroleum ether extract. The injector was maintained at 250° C.

Helium was used as a carrier gas and the flow was adjusted to 1ml/min. A Split ratio of 1:20 was used at the injector. The pressure of the carrier gas was 6 kg/cm^2. GCMS was operated at an ionization voltage of 70 eV and a trap temperature of 220 °C with a mass range of 40–350 atomic mass units.

D. Fat Extraction and Methyl Esterification

A Extraction of oil was carried out using Soxhlet apparatus. 5 gms of powdered seed was brought in contact with Petroleum ether and the extraction was carried out for 6 hours until complete defatting of the seeds was achieved.

The extract was then concentrated by evaporating the solvent in Rotavac (Buchi, Switzerland) and stored at -20 ^{0}C till further analysis.

Esterification was done in accordance with the method reported by Hoshi [11]. In brief the method is as follows: 0.2 ml of 20 mM cupric acetate monohydrate in methanol and 1 ml of 0.5 N HC1 in methanol were added together, and the mixture was left for 2-3 h at room temperature. The reaction was stopped with the addition of 0.4 ml of water. The lower chloroform layer was separated and concentrated by rotary evaporator at 35 °C. Finally the fatty acid methyl esters (FAME) were dissolved in hexane (1 ml), filtered through Whatman No. 1 filter paper and analyzed by GCMS.

E. Analysis of FAME by GCMS

Different temperature programs were used for analysis of the four seeds (Table 1). LU [12] and LS [13] were analyzed using reported temperature programs. The LU protocol was subsequently modified and applied to the remaining two seeds.

TABLE I
Temperature Program for Analysis of Fatty Acids using CP-Sil 88 Column

Seed	Temperature program			
	Temperature (° C)	Rate (°C/min)	Hold time (mins)	Total time (mins)
Nigella sativa & *Guizotia abyssinica*	60	-	4	4.0
	200	10	5.0	23.0
	225	2.0	0.0	35.5
Lepidium sativum	142	-	-	-
	184	6.0	3.0	10
	225	6.0	0.0	16.83
Linum usitatissinum	80	-	1	1
	140	30	-	3
	225	5	10	30

F. Extraction of Phenolic Compounds

The extraction was undertaken by means of the method reported by Demiray [14] which involved extracting 0.5 g of dry, ground, defatted sample using 20 mL of methanol under shaking condition at 180 rpm for 1h at 27 °C. The extraction mixture was centrifuged at 8000 rpm for 5 min at 40°C followed by filtration. The supernatant was recovered and used for the determination of total phenolic content and antioxidant activity. All experiments were carried out in triplicate.

G. Determination of total phenolic content (TPC)

Total phenolic content was determined using the Folin-Ciocalteu method [15] with some modifications. An aliquot of 100 µl of an extract was mixed with 2.5 ml of Folin–Ciocalteu phenol reagent (10X dilution) and allowed to react for 5 min.

This was followed by addition of 2.5 ml of saturated Na_2CO_3 solution and an incubation period of 1 h before the absorbance of the reaction mixture was read at 725 nm by UV-Vis spectrophotometer (Hitachi U-2001; Japan). Total phenolic content was standardised against gallic acid (R^2 = 0.984) and expressed as milligrams of gallic acid equivalents (GAE) per grams.

H. Analysis of Bioactives in ME (methanolic extract) of Nigella Sativa

The superior antioxidant activity of *Nigella Sativa* was investigated by analyzing the bioactive compounds present in the methanol fraction obtained for estimating TPC. The analysis was carried out on the GCMS system installed with the HP-5 column, utilizing the procedure reported for the methanolic extract of *Alpinia officinarum* [16].

I. Evaluation of Antioxidant Activity

The free radical-scavenging activity was determined by the ABTS radical cation decolorization assay as described by Demiray [14] with slight modification. The ABTS radical cations ($ABTS^+$) were produced by reacting 7 µM stock solution of ABTS with 2.45 µM potassium persulphate (final concentration) followed by keeping the mixture in the dark at room temperature for 12–16 h before use. The ABTS solution was diluted to an absorbance of 0.7 ± 0.06 at 734 nm (UV-visible spectrophotometer, U-2001). 20 µl of the methanolic extract was mixed with 2 ml of ABTS reagent and the absorbance was taken 6 minutes after the initial mixing of extract at734 nm. Standard curve was prepared by using trolox and the $ABTS^+$. Scavenging capacity of the extract was compared with that of the standard. The trolox equivalent antioxidant capacity (TEAC) of an extract translates to the concentration of trolox solution that has the same antioxidant capacity as the extract. Antioxidant potential was expressed as µM of trolox equivalent antioxidant activity per g dry weight of plant sample (R^2= 0.998). All determinations were performed in triplicate.

J. Dietary Fibre Analysis

Total dietary fibre was quantified along with its soluble and insoluble fractions using an enzyme kit for Total Dietary Fibre, K-TDFR 05/12 (Megazyme, Ireland).

K. Estimation of cyanogenic glycoside content

Cyanogenic glycoside contents of sample were determined by alkaline titration method in accordance with published reports [17]. Sample added to 200 ml distilled water was distilled for 2 h to collect the resulting distillate. To the distillate, 20 ml of a 2.5% NaOH was added and the final volume was made up to 250 ml. To 100 ml of diluted distillate 8.0 ml of 6M NH_4OH solution and 2.0 ml of 5% KI was added and then titrated against 0.02M $AgNO_3$ solution using a 10 ml microburette. The end-point was characterized by a permanent turbidity against a black background. Cyanogenic glycoside content was calculated using the formula.

$$\text{Cyanogenic glycoside mg/100g} = \frac{TV \times 1.08 \times EV}{SM \times AL} 100,$$

Where;

TV	= Titre value (cm^3)
EV	= Extract vol. (cm^3)
SM	= Sample Mass (g)
TV	= Titre value (cm^3)
AL	= Aliquot (cm^3) used
N/B 1cm^3 of 0.02 N $AgNO_3$	= 1.08 mg HCN

III. Results and Discussion

A. Profiling of Functional Lipids

Based on the conventional functional food definition, functional lipids can be described as lipids that provide additional physiological and health promoting benefits apart from the regular nutrition function [18]. Fatty acids belonging to omega-3 and omega-6 family are known for their health protective effect and hence belong to this category [19]. These fatty acids are known as essential fatty acids as the human body cannot synthesize them due to the absence of the desaturase enzyme required for their synthesis. Consumption of these fatty acids through diet is therefore essential for the well being of human beings. The optimum omega-6: omega-3 ratio of 4:1- 10:1 is seldom met except in fish eating Japanese populations [20]. Thus the current study helped us to identify lesser studied omega-3 rich plant sources for the benefit of the vegetarians. Since flaxseed has proved to be a rich source of vegetative omega-3, our study incorporated it as one of the target seeds. GCMS analysis of the seeds under study, revealed that there was a wide distribution of saturated, mono-unsaturated and poly-unsaturated fatty acids as seen in the Table 2, 3, 4 and 5. Indian NS seeds showed highest amount of linoleic acid followed by stearic acid as opposed to the Iranian seeds that have been reported to contain more oleic acid than stearic acid [21]. LS seeds had α-linolenic acid as the major fatty acid though the amount at 26.71 ± 0.63 % was lower than the reported value of 30.2% [8]. In agreement with the published results [10], linoleic acid was the chief component in the petroleum ether extract of GA seeds. For assessing the functional benefits of the lipids the omega-6: omega-3 ratio was estimated from the relative abundance of each in the gas chromatogram and found to be the highest in case of NS (36:1) and lowest in LU (0.36:1) and LS (0.45:1) thereby establishing the ones with the lower ratios as functional lipids. However due to high content of PUFA's these oils are more prone to oxidation thereby limiting their usage to room temperature applications. GA oil having a composition of SFA: PUFA (saturated fatty acid: unsaturated fatty acid) in the ratio of 1:2.3 is a far more stable oil that can be used as a replacement to the conventionally used vegetative oils for deep frying purposes. Apart from the aforementioned fatty acid components, NS oil also showed a beneficial compound Linalool which has been widely reported to have anti-inflammatory activity [22]. Thus the differential composition of oils will be beneficial in creating novel bioactive recipes through blending of these oils to the required final composition.

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TABLE II
BIOACTIVE COMPOUNDS PRESENT IN THE PETROLEUM ETHER EXTRACT OF *NIGELLA SATIVA* SEEDS

Seed Sample	IUPAC Name of the bioactive and reported activity	Other Name	Chemistry	RT (min)	Bioactive Content[#] (%)	Omega-6: Omega-3 ratio
Nigella sativa	Methyl tetradecanoic acid methyl ester	Myristic acid methyl ester	Saturated fatty acid ester	15.61	0.245± 0.03	36:1
	Hexadecanoic acid methylester	Palmitic acid methyl ester	Saturated fatty acid ester	17.7	12.2± 0.16	
	Octadecanoic acid methylester	Stearic acid methyl ester	Saturated fatty acid ester	19.79	27.49± 0.28	
	Octadecadienoic acid methylester	Linoleic acid methyl ester	Unsaturated fatty acid ester, omega-6	19.23	55.1± 0.33	
	Octadecatrienoic acid methylester	α-Linolenic acid methyl ester	Unsaturated fatty acid ester, omega-3	20.26	1.62± 0.11	
	Eicosadienoic acid methylester		Unsaturated fatty acid ester, omega-6	23.66	3.34± 0.13	
	Linalool methylester (anti-inflammatory)		Terpene alcohol		<0.2%	
	Hydroxylamine		Ammonia related			
	Nonadecane		Parafinn hydrocarbon			
	Cyclohexadiene		Cycloalkene			

average of three replicates ± standard deviation

TABLE III
BIOACTIVE COMPOUNDS PRESENT IN THE PETROLEUM ETHER EXTRACT OF *GUIZOTIA ABYSSINICA* SEEDS

Seed Sample	IUPAC Name of the bioactive	Other Name	Chemistry	RT [min]	Bioactive Content[#] (%)	Omega 6: Omega 3 ratio
Guizotia abyssinica	Tetradecanoic methyl ester	Myristic acid methyl ester	Saturated fatty acid ester	15.61	1.77± 0.05	13: 1
	Phenol	-	-	16.0	0.443±0.07	
	Docosatetraenoic acid methylester	Adrenic acid methyl ester	Unsaturated fatty acid ester, omega 6	17.7	10.1± 0.11	
	Pentadecanoic acid methylester	-	Saturated fatty acid ester	19.79	28.31±0.37	
	Octadecadienoic acid methylester	Linoleic acid methyl ester	Unsaturated fatty acid ester, omega 6	19.92	54.39±0.26	
	Octadecatrienoic acid methylester	α- linolenic acid methyl ester	Unsaturated fatty acid ester, omega 3	20.26	5.0± 0.09	

average of three replicates ± standard deviation

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TABLE IV
BIOACTIVE COMPOUNDS PRESENT IN THE PETROLEUM ETHER EXTRACT OF *LEPIDIUM SATIVUM* SEEDS

Seed Sample	IUPAC Name of the bioactive	Other Name	Chemistry	RT [min]	Bioactive Content[#] [%]	Omega 6: Omega 3 ratio
Lepidium sativum	Hexadecanoic acid methylester	Palmitic acid methyl ester	Saturated fatty acid ester	7.37	9.83±0.13	0.45: 1
	Octadecenoic acid methylester	Oleic acid methyl ester	Mono unsaturated fatty acid, Omega 9	10.89	20.86±0.26	
	Octadecadienoic acid methylester	Linoleic acid methyl ester	Unsaturated fatty acid ester, omega 6	11.09	12.4± 0.43	
	Octadecanoic acid methylester	Stearic acid methyl ester	Saturated fatty acid ester	11.27	5.0± 0.03	
	Octadecatrienoic acid methylester	α–linolenic acid methyl ester	Unsaturated fatty acid ester, omega 3	11.69	26.71±0.63	
	cis 13- eicosenoic acid methylester	Paullinic acid methyl ester	Mono unsaturated fatty acid, Omega 7	14.94	18.52±0.21	
	Eicosenoic acid methylester	-	Mono unsaturated fatty acid, Omega 9	15.31	5.65± 0.07	
	Methyl eicosatrienoic acid methyl ester	-	Unsaturated fatty acid ester, omega 3	15.43	0.79± 0.11	

average of three replicates ± standard deviation

TABLE V
BIOACTIVE COMPOUNDS PRESENT IN THE PETROLEUM ETHER EXTRACT OF *LINUM USITATISSINUM* SEEDS

Seed Sample	IUPAC Name of the bioactive	Other Name	Chemistry	RT [min]	Bioactive Content[#] [%]	Omega 6: Omega 3 ratio
Linum usitatissinum	Hexadecanoic acid methylester	Palmitic acid methyl ester	Saturated fatty acid ester	11.35	7.2± 0.18	0.36:1
	Octadecenoic acid methylester	Oleic acid methyl ester	Mono unsaturated fatty acid, Omega 9	14.58	24.86±0.82	
	Octadecadienoic acid methylester	Linoleic acid methyl ester	Unsaturated fatty acid ester, omega 6	14.7	14.96±0.14	
	Heptadecanoic acid methyl ester	Margaric acid methyl ester	Saturated fatty acid ester	14.87	10.6± 0.13	
	Octadecatrienoic acid methylester	α–linolenic acid methyl ester	Unsaturated fatty acid ester, omega 3	15.2	41.85±0.33	
	Eicosenoic acid methylester	-	Mono unsaturated fatty acid, Omega 9	18.3	0.54± 0.08	

average of three replicates ± standard deviation

B. Quantification of the Total Phenolic Content and antioxidant activity

Estimation of phenolic compounds and antioxidant activity is crucial in understanding the health promoting effects of the seed extract. Several researchers have found correlation between the antioxidant activity and the TPC [23]. Hence the methanolic extracts of all seeds for possible correlation of the two parameters were analyzed. The choice of solvent was based on reports highlighting the efficiency of methanol to extract maximum phenolics [24]. Fig. 2 depicts highest TPC value for GA seed (14.25 ± 0.15 mg GAE/gm DW) and NS seeds show highest antioxidant ability (61.68 ± 0.21 TEAC/100 gm DW) as assayed by the ABTS ((2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) method. The results fail to show any positive correlation between the two factors. This could be attributed to the presence of bioactive molecules other than phenolic compounds in the polar extract. Certain flavonoids that play a key role in providing antioxidant protection fail to optimally react with the $ABTS^{+}$ moiety and thus get underestimated [25]. The enhanced antioxidant acitivity seen in NS seed extract was further investigated through GCMS analysis. The methanolic extract of NS was analysed on a HP-5 column (Table 6) and subsequent spectral analysis showed the presence of molecules like thymoquinone, o-cymene, a-phellandrene and valencene. Thymoquinone [26], o-cymene [27], α-phellandrene [28], and valencene [29] are known for their anti-inflammatory and antioxidant abilities. Thus it can be concluded that the presence of the aforementioned compounds attributes to the enhanced antioxidant activity of the NS seeds.

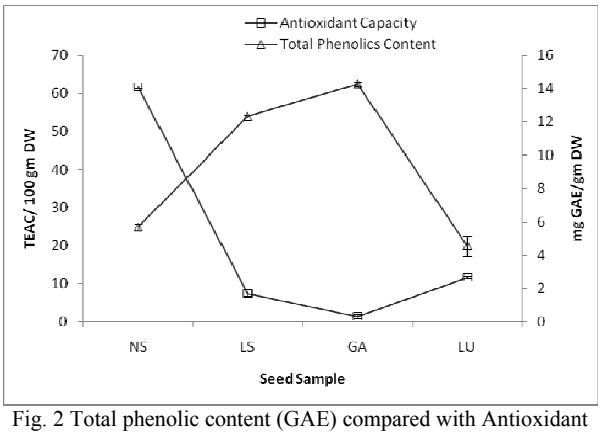


Fig. 2 Total phenolic content (GAE) compared with Antioxidant activity (TEAC) of the seeds

C. Soluble and Insoluble fractions of Total Dietary Fiber

Fiber rich diets have been known to lower the risk of many diseases like diabetes, coronary heart disease and even obesity [30]. Intracellular fiber like pectin and gum are water soluble in nature whereas fiber derived from cell wall i.e cellulose and hemicelluloses comprises the insoluble fraction [31].

Modern processing techniques such as peeling and seeding leads to reduction in the amount of fiber content in our final food product [32].

Thus there is a need to consciously incorporate fiber rich products in our diet. The functional food market has seen a boost in the sale of several whole grain and other fiber rich products. In accordance with these trends dietary fiber was sucessfully isolated; whose content varied in the different seeds as shown in Fig. 3. LU and LS seeds showed appreciably higher amount (TDF of 23.12 and 23.43 gm/100 gm) of soluble and insoluble dietary fiber as compared to the other seeds. Thus the fiber isolated from these seeds can easily be used for fortification of innovative bakery products.

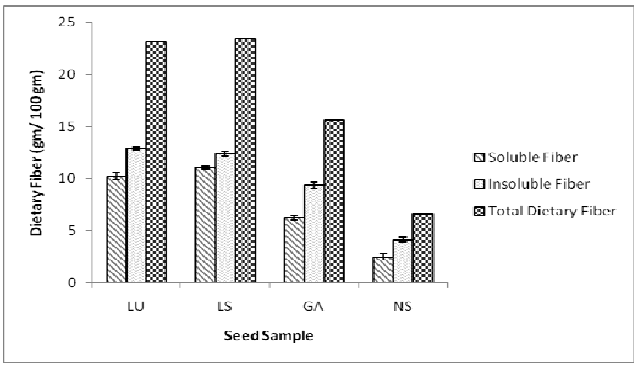
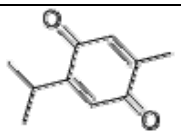
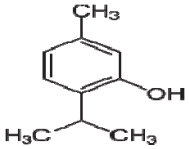
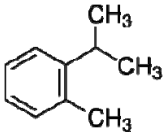
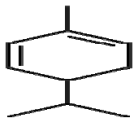
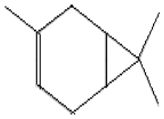
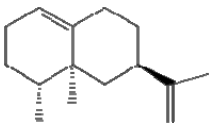


Fig. 3 Content of soluble, insoluble and total dietary fiber in the studied seeds

D. Estimation of cyanogenic glycoside content

Cyanogenic glycoside is an anti-nutritional factor known to be a potent cytochrome oxidase inhibitor that inhibits aerobic respiratory system [33]. Based on the available reports of flaxseed containing this anti-nutritional factor, the seeds were analyzed for it [34].As seen from Fig.4 LS seeds showed maximum content of cyanogenic glycoside (1674 ± 54 mg HCN/Kg). Though there have been earlier reports on qualitative analysis [35], to the best of our knowledge this is the first report quantifying the amount of the anti-nutrient in the seeds. Though the oil extracted from the seeds is devoid of cyanogenic glycoside, the direct use of this seed for human consumption dictates caution. Preprocessing of seeds by soaking, roasting etc. needs to be undertaken to reduce the anti-nutritional content before using these seeds in culinary preparations.

TABLE VI
BIOACTIVE COMPOUNDS PRESENT IN THE METHANOLIC EXTRACT OF THE SEEDS

Seed Sample	Bioactive compound	Chemistry	Retention Time (mins)	Bioactive Content# (%)	Reported Activity
NS	Thymoquinone / p-Cymene-2,5-dione	 Quinone	42.64	34.67	Anti-inflammatory/ Antioxidant
	m-Thymol	 Monoterpene phenol	44.50	2.47	Anti-inflammatory
	o-Cymene	 Monoterpene	19.93	31.18	Derivative of cymene & para form-anti-inflammatory
	3-(Prop-2-enoyloxy)dodecane	Hydrocarbon derivative	2.23	12.16	NA
	α-Phellandrene	 Monoterpene	9.86	9.7	Anti-inflammatory
	S-3-Carene	 Aromatic	35.15	4.83	Flavour compound in pepper
	Valencene	 Sesquiterpene	47.97	4.97	Anti-inflammatory

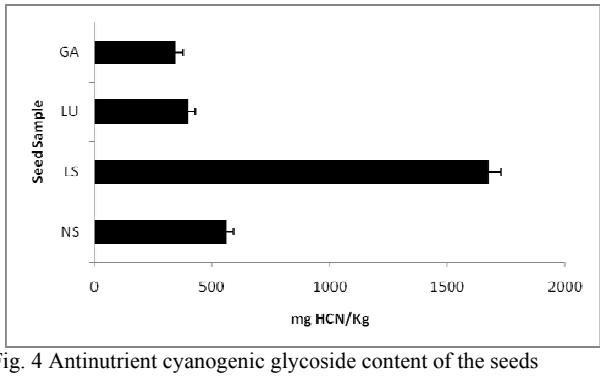


Fig. 4 Antinutrient cyanogenic glycoside content of the seeds

IV. Conclusion

The seeds of *Linum usitatissinum, Lepidium sativum, Nigella sativa* and *Guizotia abyssinica* were seen to have varied bioactive profiles making them an excellent choice for functional food and nutraceutical applications. A diet rich in LS and LU seeds will provide the much required balance of omega-6: omega-3 thereby countering the effect of otherwise consumed pro-inflammatory diet. Polar extract of NS seeds showed a strong antioxidant behavior which could be utilized in preservation of PUFA rich LS and LU oils. On the other hand GA oil could be used in gastronomic applications as an alternative to edible vegetable oil. In conclusion the studied seeds were a rich source of funational lipids, dietary fiber, antioxidant and anti-inflammatory molecules that could be isolated and further studied for potential application against inflammatory disorders.

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