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Original Article

PHYSIOCHEMICAL EVALUATION AND *IN VITRO* ANTIOXIDANT ACTIVITY OF FEW WONDER SEEDS

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ABSTRACT

Objective: This study was an attempt to compare and investigate the physiochemical nature and antioxidant potential of few wonder seeds namely flaxseed, chia, sesame, pomegranate, dill, kalonji, pumpkin and watermelon seeds.

Methods: The oil samples and methanolic extract were obtained by extraction of ground seed samples using soxhlet apparatus with n-hexane and methanol respectively. Oil samples were analysed for various physiochemical parameters like specific gravity, refractive index, iodine value, acid value and saponification values. Methanolic extracts were analysed for their antioxidant potential using different methods like TPC, DPPH, FRAP, Ferric-Ferrozine and β-Carotene assay. The fixed oil samples were converted into fatty acid methyl esters (FAMEs) and analysed over gas chromatograph for their composition.

Results: Pumpkin seeds showed highest phenolic content and highest DPPH radical scavenging activity. Chia seeds showed highest ferric reducing capacity for FRAP and ferric-ferrozine assay. All seed sample showed higher β-carotene bleaching than synthetic antioxidant BHA.

Conclusion: These wonder seeds investigated have a high potential to combat stress-related diseases owing to good antioxidant potential and presence of polyunsaturated fatty acids.

Keywords: Antioxidant assay, GC, Fixed Oil, FAMEs, DPPH, FRAP, Seeds

INTRODUCTION

On considering the unending debate of vegetarian diet not being able to bridge the deficiency gap of protein, few wonder seeds have come as a boon for all who want to stick to the vegetarian diet. There is an astounding amount of minerals and nutrients present in these small natural capsules. They are the better plant sources of niacin, folic acid, protein, iron, zinc and provide more fibre per ounce than nuts [1, 2].

Flax seeds contain lignans which is a major class of phytoestrogens. Phytoestrogens are an estrogen-like chemical that possesses antioxidant properties and have the ability to scavenge free radicals [3]. The primary source of ALA (alpha-linolenic acid) is flaxseeds which reduce the risk of cardiovascular disease by protecting the blood vessels against inflammation [2, 4]. Pumpkin seeds contain vitamin-E and MUFA such as oleic acid which is helpful in reducing bad LDL-cholesterol and increasing good HDL-cholesterol thereby reducing the risk of cholesterol [5]. Two important essential amino acids lysine and arginine are present in watermelon seeds [6]. While lysine helps in absorption of calcium which helps in the formation of collagen and all connective tissues in the body, arginine on other hand improves the cardiovascular system and sexual health. Chia seeds are rich in antioxidants.

Some of the antioxidants are chlorogenic acid, caffeic acid, myricetin, quercetin, kaempferol flavonol glycosides, and Omega-3 (alpha-linolenic acid) and Omega-6 fatty acids. Chia seeds consist of fibre which helps in maintaining blood sugar level in the body [7, 8]. Pomegranate seeds contain phytochemicals such as polyphenols which act as antioxidants. Some of the polyphenols are-ellagitannins, flavonoids, tannins, quercetin and anthocyanins. Anthocyanins have antimicrobial, anti-inflammatory and antiviral properties [9, 10]. Dill seeds contain monoterpenes-carvone, limonene and anethofuran, flavonoids kaempferol and vicenin [11]. Dill oil is chemoprotective in nature as it contains enzyme glutathione-S-tranferase which helps to neutralize carcinogens. The Kalonji seeds mainly constitute of alanine, arginine, ascorbic acid, asparagine, campesterol, carvone, cymene, cystine, dehydroascorbic-acid, eicosadienoic-acid, glucose, glutamic acid, glycine, iron, isoleucine, leucine, linolenic-acid, lipase, lysine, methionine, myristic-acid, nigellin, nigellone, oleic-acid, palmitic-acid, phenylalanine, phytosterols, potassium, β-sitosterol, alpha-spinasterol, stearic-acid, stigmasterol, tannin, threonine, thymol hydroquinone, thymoquinone, tryptophan, tyrosine, limonene and linoleic acid. It is useful in the prevention of cancer as it contains an antioxidant which prevents the formation of carcinogens by neutralizing them [12, 13].

Seeds are high in fibre, vitamin E and monounsaturated fats that can help keep our heart healthy and our body disease free. These wonder seeds are known for their ω-3 contents, but their antioxidant potential has not been studied by earlier co-workers. This study is an attempt to compare and establish their physiochemical profile and evaluate their nutritional value for their use as better food adjunct.

MATERIALS AND METHODS

Methanol used was of HPLC grade. Distilled water used was twice deionized before use. Ascorbic acid, N,N-diphenyl picrylhydrazyl radical (DPPH), ferrozine, linoleic acid, β-carotene, ferric chloride, gallic acid, 2,4,6-tripyridyl-s-triazine (TPTZ) and trolox were procured from Sigma-Aldrich. Sodium acetate, glacial acetic acid, sodium nitrate, folin-ciocalteau reagent, sodium hydroxide, aluminium chloride, butylated hydroxyl anisole (BHA), the hydrochloric acid used was of analytical grade. UV-Visible spectrophotometer (Shimadzu-UV-1800); Laminar Airflow (Khera Co.); Systronics digital pH meter 335; Ultrasonic cleaner (Life-core equipment Pvt. Ltd). Gas Chromatograph (RASTECH-2800), Abbe's refractometer and rotary vacuum evaporator (Make: Khera).

Preparation of seed samples

The flaxseed, chia seed, sesame seed, kalonji seed, pomegranate and dill seeds were procured from local market. Procured market samples were identified by agricultural scientist and voucher specimens submitted. Pumpkin and watermelon seeds were procured from PUSA institute, Delhi. The seeds were grounded with the help of food processor. All the samples were first defatted using

n-hexane and then extracted by methanol. The extracts were distilled under reduced pressure using a rotatory evaporator to obtain fixed oil and methanolic extracts respectively. All extractions were done using soxhlet apparatus.

Fig. 1: Different seed samples used for analysis

Physical and chemical parameters of fixed oil

The physical and chemical parameters of fixed oils were determined using different methods as mentioned by A. O. A. C [14] which include: Specific Gravity, Refractive index, Acid value, Saponification value and Iodine value. Refractive index was determined by Abbe's refractometer.

Assays to determine antioxidant potential

Total phenolic content

Total Phenolic Content was measured by Folin-Ciocalteau method [15, 16]. Under this assay, the metal oxides gets reduced by polyphenolic antioxidant present in seed extracts to get a deep blue coloured solution absorbance. 50 µl of methanolic extract was taken in a test tube. To it 5 ml of folin's reagent (1:9 reagent to water dilution) and 4 ml of Na_2CO_3 (1 M) was added to it after 5 min. the samples were kept undisturbed for an hour at 25°C. Absorbance was measured at 765 nm through UV-visible spectrophotometer. Standard curve was prepared by measuring absorbance of different concentrations of ascorbic acid.

DPPH assay

This method was developed by Brand and William [17]. This is used to evaluate the ability of a specific antioxidant to bleach the violet colour of DPPH solution depending on hydrogen donating power of the antioxidant solution to reduce DPPH radical. 0.6 mM stock solution of DPPH was prepared in methanol. For sample analysis 25 µl of methanol extract of each sample was added to the mixture containing 2 ml methanol and 1 ml DPPH solution. Solutions were kept for 1 hour in dark then absorbance was measured at 515 nm. The activity of samples was compared to that of 1 mg/ml concentration solutions of trolox and ascorbic acid.

FRAP assay

This method was developed by Benzie and Strain [18]. FRAP assay uses antioxidants as reductant in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. At low pH (3.6), reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorption at 593 nm. 100 µl of methanolic extract of each sample were mixed with 3 ml of freshly prepared straw yellow coloured working FRAP reagent (10:1:1 mixture of acetate buffer, TPTZ and $FeCl_3$). Absorbance was measured spectrophotometrically at 595 nm with water taken as reference at 0 min and then after 10 min. The samples were kept in water bath at 37 °C. Ferric reducing power was determined in terms of ascorbic acid equivalent (AAE).

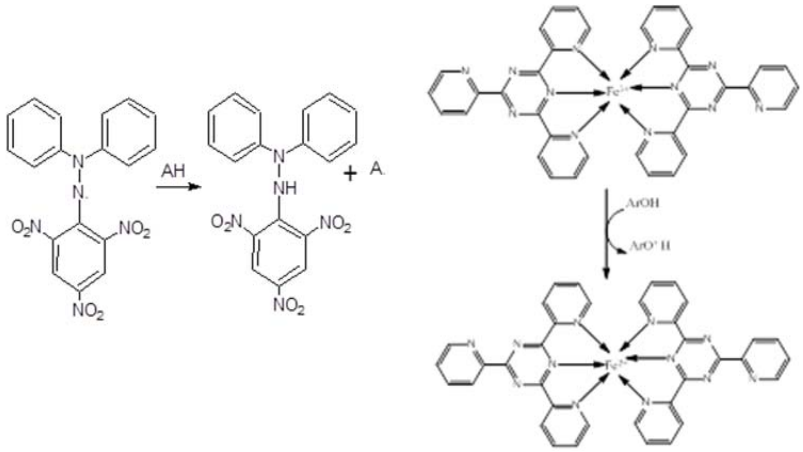

Fig. 2: DPPH radical reacting with antioxidant and Ferric-TPTZ complex

Ferrous-ferrozine assay

Ferrozine is a profoundly ferrous-settling ligand such that ferric particle in the vicinity of ferrozine effortlessly oxidizes free radicals, and itself decreased to Fe(II)-FZ, yielding a high molar absorptivity. The ferrous-ferrozine method can be used to determine the antioxidant potential against free radicals [19]. 0.1 ml of methanolic extracts, 0.4 ml of methanol, 1.5 ml of ferric-ferrozine mixture, 2 ml of acetate buffer (pH 5.5) and 0.5 ml of water was mixed together in a test tube. The mixture was allowed to stand for 30 min. and absorbance was measured at 562 nm.

β-Carotene assay

β-carotene bleaching method is used for evaluating antioxidant activity [20, 21]. The principle of this method is based on discolouration of β-carotene due to the breaking of conjugated double bond by the addition of lipid or lipid peroxyl radical (L· or LOO·) to double bond of carbon of β-carotene. When the appropriate antioxidant is added to the solution, the discoloration can be retarded by competing for the reaction between β-carotene and antioxidant with subjected radical.

$$\beta - \text{carotene} + \text{ROO.} \rightarrow \text{Bleaching}$$

$$\text{ROO.} + \text{AH} \rightarrow \text{A.} + \text{ROOH}$$

Method used was suggested by Marco [22]. 2500 µl of the beta-carotene emulsion mixture was taken in a test tube to which 350 µl of the methanolic extract was added, and the system was incubated at 50 °C for 2 h. Absorbance was measured at 470 nm. Blank solution was prepared from 350 µl of 96% methanol, linolenic acid and beta-

carotene mixture for 2 h at 50 °C temperature. The bleaching of our samples was compared with that of standard antioxidants like BHA and ascorbic acid.

Statistical analysis

Results were expressed as mean values and standard deviation of three independent determinations. Statistical analyses were determined using a statistical software program (SPSS for Windows version 11.0). The data were subjected to analysis of variance using the general linear model to determine significant differences between samples ($p<0.05$).

Gas chromatographic analysis of fixed oils

The fixed oils were extracted from seeds by n-hexane using soxhlet apparatus. The extracts obtained were concentrated using rotary vacuum evaporator to obtain the fixed oils. The fixed oil was converted into FAMEs and was run on GC system for analysis of fatty acid composition of fixed oils of seed. GC showed all the compounds present in the fixed oils and was identified by comparing with GC of known standard (Restek, fame # 13 mix). Detector used was FID. Carrier gas used was Helium with a flow rate of 1 ml/min. Oven initial temperature was 150 °C with 1-minute hold.

Temperature programming of 10 °C increase to 200 °C with 2 min hold then 5 °C increase up to a final temperature of 280 °C, 5 min hold was done. Injector temperature 200 °C. Injection Volume was 1 µl. Detector used in FID.

RESULTS

Table 1 tabulates the results obtained for the various physiochemical parameters obtained for the various oil samples extracted from selected wonder seeds.

Table 1: Physical and chemical parameters of fixed oil

S. No.	Seed name	Specific gravity	Acid value (mg KOH/g)	Saponification value (mg KOH/g)	Iodine value (mg/g)	Refractive index	Nature of oil based on Iodine value
1	Flax	0.724±0.001	6.545±0.009	243.1±0.005	180.83±0.04	1.480±0.01	Drying oil
2	Pumpkin	0.584±0.004	12.518±0.009	247.5±0.02	82.92±0.03	1.470±0.01	Non-drying oil
3	Chia	0.650±0.002	7.381±0.01	242.1±0.02	155.59±0.02	1.481±0.02	Drying oil
4	Watermelon	0.700±0.016	4.999±0.008	252.0±0.025	132.00±0.16	1.475±0.01	Drying oil
5	Dill	0.946±0.010	13.331±0.008	176.7±0.098	124.59±0.025	1.490±0.02	Semi-drying oil
6	Pomegranate	0.939±0.004	5.461±0.004	243.3±0.036	172.58±0.02	1.498±0.01	Drying oil
7	Kalonji	0.935±0.004	0.340±0.003	172.5±0.015	112.32±0.015	1.473±0.03	Non-drying oil
8	Sesame	0.918±0.002	1.720±0.002	195.0±0.001	108.10±0.02	1.471±0.01	Non-drying oil

Results shown are mean of triplicate measurement±SD

Gas chromatographic analysis of fixed oils

In the GC profile of the FAMEs of the samples were correlated to the retention time (tR) of standard samples run at the time of determination under similar conditions and results obtained are illustrated in fig. 3. The major compound in flaxseed, pumpkin, chia and dill seed was linolenic acid. Flax seeds showed peaks at tR 8.01, 9.98 and 10.19 are corresponding to palmitic, linoleic and linolenic acid respectively obtained on comparison with standards. Pumpkin seed was found to contain myristic acid, palmitic acid, linoleic acid and linolenic acid corresponding to peaks obtained at tR 3.49, 7.98, 9.94 and 10.15 respectively.

Watermelon seed was found to contain myristic acid, palmitic acid, linoleic acid and linolenic acid corresponding to peaks obtained at tR 3.56, 8.03, 9.97 and 10.24 respectively. Dill seed was found to contain myristic acid, pentadecanoic acid, palmitic acid, linoleic acid, stearic acid, hexadecanoic acid and linolenic acid corresponding to peaks obtained at tR 3.86, 3.96, 4.04, 5.57, 8.01, 9.79 and 9.89 respectively. Sesame seed was found to contain stearic and oleic acid corresponding to peaks obtained at tR 9.79 and 9.87 respectively. Kalonji seed was found to contain palmitic acid and oleic acid corresponding to peaks obtained at tR 8.01 and 9.87 respectively. Chia seed was found to contain myristic acid, palmitic acid, linoleic acid and linolenic acid corresponding to peaks obtained at tR 3.55, 8.00, 9.96 and 10.18 respectively. Pomegranate seed was found to contain palmitic acid, stearic acid, oleic acid, linoleic acid, punicic acid and linolenic acid corresponding to peaks obtained at tR 8.02, 9.81, 9.89, 10.17, 11.53 and 11.66 respectively. A peak at tR 3.18-3.21 is corresponding to n-hexane which is used as a solvent for fixed oil.

***In vitro* assays**

Total phenolic content

Total phenolic content (TPC) was presented in terms of ascorbic acid equivalent (AAE). Absorption was measured at 756 nm. Fig. 4 (i) shows the standard curve for ascorbic acid and (ii) shows the AAE concentration of phenolic in seed extracts among which pumpkin seeds showed the highest value. Order of concentration of TPC was as follows:

Pumpkin>Chia>Watermelon>Dill>Flax>Kalonji>Pomegranate

DPPH assay

Brand-Williams method was employed and absorption was measured at 516 nm. The fig. 5 depicts the % DPPH radical inhibition of various samples.

The maximum % of DPPH radical scavenging activity of the seed extracts, taking trolox and ascorbic acid as a reference, was found to be in the case of pumpkin seed extract while the least activity was found in the case of flaxseed indicating least potential of free radical scavenging activity towards DPPH.

FRAP assay

This method was based on the method of Benzie and Strain method [18]. Ferric reducing capacity was measured in terms of AAE and the standard curve for ascorbic acid obtained is given below in fig. 6 (i) and fig. 6 (ii) gives the reducing capacity of seed extracts at 0 min and after 10 min.

From the above data, it was observed that ferric reducing power of the seed extract was found to be maximum in case of chia seeds giving higher absorbance value of 1.958 after 10 min. This indicates that Chia has a greater ferric reducing power. The least absorbance was observed by pumpkin seeds with a value of 0.532 which indicates pumpkin seed extracts has the least ferric reducing capacity.

Ferric-ferrozine assay

Ferrozine is a highly ferrous-stabilizing ligand such that ferric ion in the presence of ferrozine easily oxidizes antioxidants and is itself reduced to Fe(II)-FZ, yielding a very high molar absorptivity (at 562 nm) and thus enhanced sensitivity for most antioxidants. Fig. 7 (i) gives the standard curve of ascorbic acid and (ii) shows the response of seed extracts under test conditions in terms of AAE. It shows that chia seeds have shown greater absorbance value of 1.277 which indicates that chia seeds have a greater ferric reducing activity while the least value of absorbance was observed in the case of pumpkin seeds which show least potential to reduce the ferric ions and thus has least antioxidant potential in terms of ferric reducing activity.

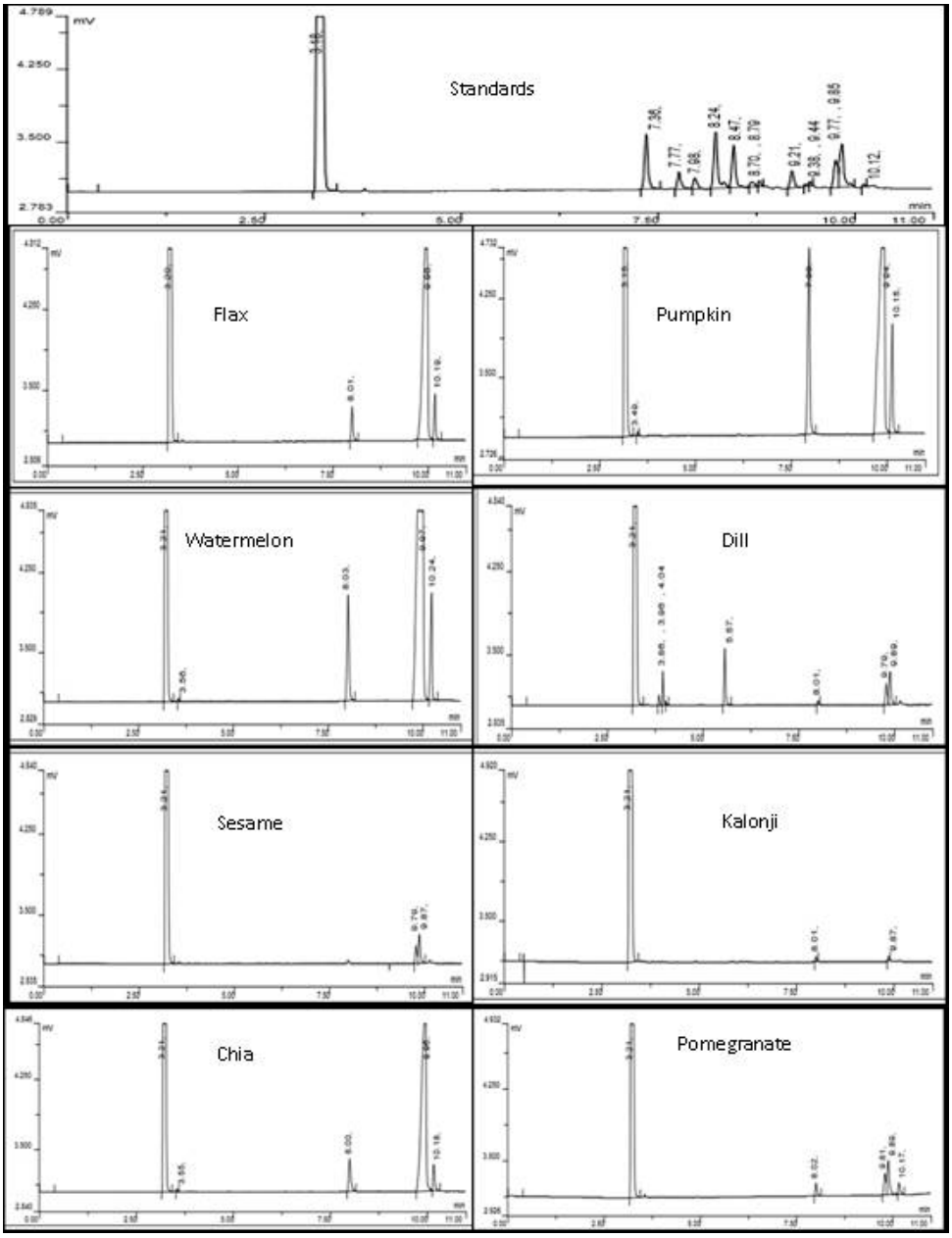

Fig. 3: Gas chromatographs of standards and FAMEs of fixed oil of seed sample

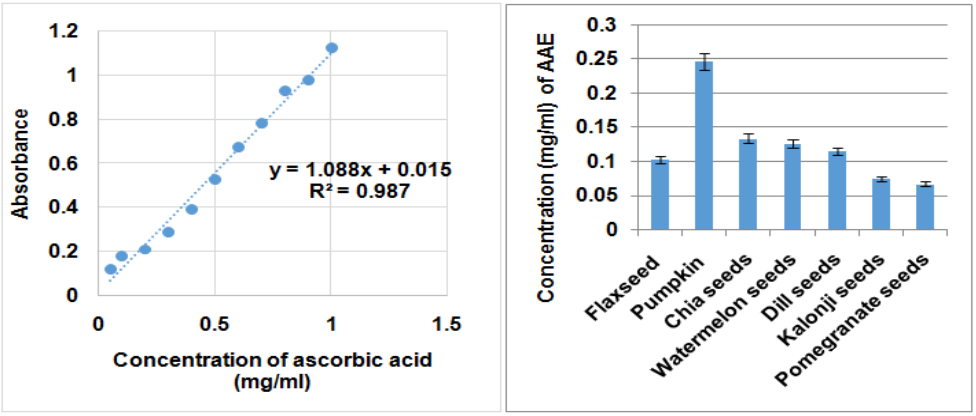

Fig. 4: (i) Standard curve of ascorbic acid at 756 nm. (ii) Bar graph showing AAE concentration of different extracts for evaluation of total phenolic content each value represents a mean±SD (n = 3)

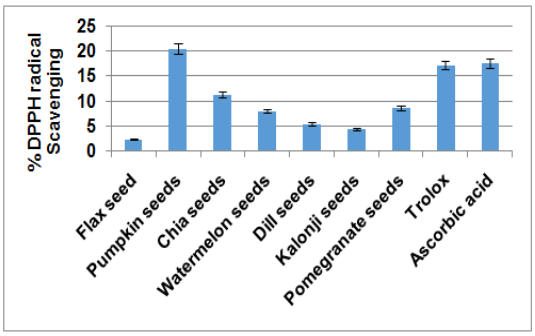

Fig. 5: Bar graph showing % DPPH radical scavenging observed after 1 hour at 516 nm. Each value represents a mean±SD (n = 3)

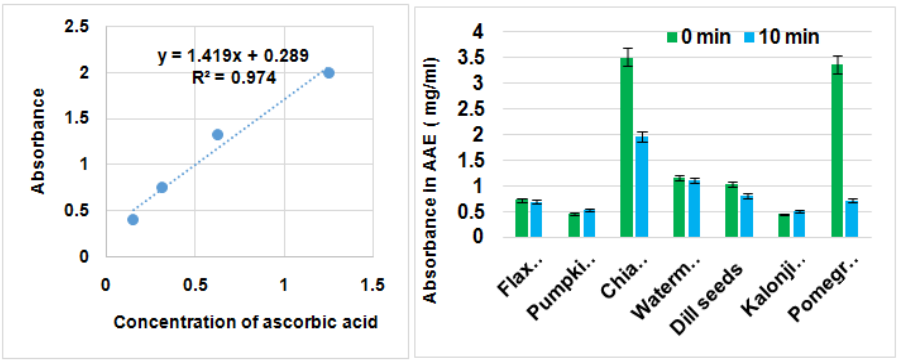

Fig. 6: (i) Ascorbic acid standardisation curve at 595 nm (ii) Bar graph showing absorbance value of seed extracts in terms of AAE. Each value represents a mean±SD (n = 3)

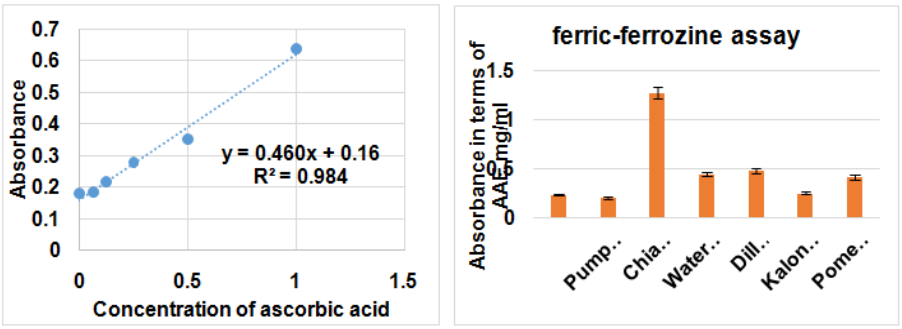

Fig. 7: (i) Standard curve of ascorbic acid at 562 nm (ii) Absorbance showing AAE ferric-ferrozine reducing assay of various seed samples. Each value represents a mean±SD (n = 3)

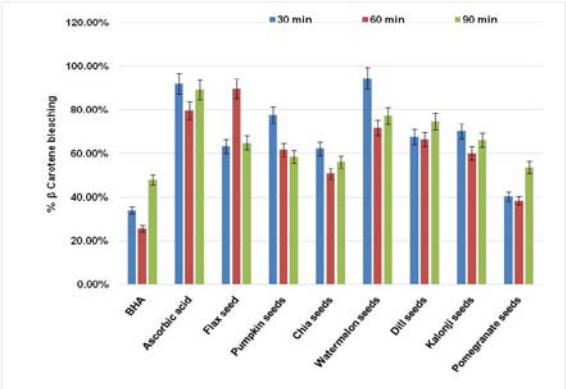

Fig. 8: Bar graph showing rate of β-carotene bleaching by samples after 30, 60and 90 min. Results shown are the average value of three replicates with mean±SD (n=3).

β-carotene assay

The β-carotene assay basically indicates the potential of antioxidants towards neutralizing free radical and thereby suppressing the bleaching of β-carotene because of free radicals of linoleic acid generated due to oxidative degradation process. The highest value of radical scavenging activity was observed in the case of watermelon seed extract while the least value was observed in the case of pomegranate seed extract as indicated below in fig. 8.

DISCUSSION

The acid value was found to be highest in the case of pumpkin and dill seed oils indicating the presence of free fatty acids. Iodine value was found to be maximum in case of flaxseed and pomegranate oil which showed a high degree of unsaturation. Saponification number which is the index of average molecular weight of fatty acids showed higher values for flax, pumpkin, chia, watermelon and pomegranate seeds. The lyophilized methanolic extracts upon analysis of total

phenolic content (folin-ciocalteau assay), radical scavenging activity (DPPH assay and β-carotene assay) and ferric reducing activity (FRAP assay and ferric ferrozine assay) of different seed sample showed different responses. The TPC and DPPH scavenging activity were found to be highest for pumpkin seed surpassing ascorbic acid and trolox under the test conditions. High antiradical activity may be due to high polyphenol content as phenolics act as a good radical stabiliser. In the β-carotene assay, the response was best by watermelon seed extract while in the case of ferric reducing activity best-reducing capacity was shown by chia seeds. The GCMS of FAMEs of fixed oils of oil seeds revealed the presence of polyunsaturated fatty acids like linoleic, linolenic and punicic acids in oil samples. Punicic acid is an omega-5-fatty acid that has six times greater antioxidant potential as compared to conjugated linoleic acid (CLA). It is therefore also called a super CLA. Polyunsaturated fatty acids lower the risk of cardiovascular diseases. Dietary intake of polyunsaturated fatty acids has been shown in preliminary studies to decrease the risk of developing amyotrophic lateral sclerosis [23, 24]. The importance of the ratio of omega-6/omega-3 essential fatty acids as established by comparative studies shows an Omega-6: Omega-3 ratio under 4:1 may contribute to health [25]. These seeds contain vitamins, minerals, and other nutrients necessary to create new life. They are the better plant sources of niacin, folic acid, protein, iron, zinc and fiber. These seeds can act as quick healthy snacks that are low in calories. Researchers have found that certain seeds are super foods, capable not only of packing a nutritional punch, but also lowering blood pressure, easing arthritis, and keeping cholesterol in check. Flaxseed represents a valuable source of phenolic antioxidant. However, *in vitro* and *in vivo* antioxidant efficacies of all the flaxseed phenolics have not been thoroughly documented. Kasote has discussed flaxseed phenolics together with their antioxidant potential [26]. In work carried out by Marineli *et al*., chia seed presented a good source of protein (25.32 g/100 g), oil (30.22 g/100 g) and total dietary fiber (37.50 g/100 g), with predominant insoluble fiber (35.07 g/100 g). The main fatty acids, ranked order of abundance, were α-linolenic acid>linoleic acid>palmitic acid ~ oleic acid>stearic acid [27]. In another study, Mutahar *et al.* have evaluated antioxidant activity of pomegranate peel extract in methanol showed higher yield and phenolic content than water or ether extract [28]. Antioxidant potential was determined by DPPH assay. Our work gives a comparative view of all these seeds moreover we have used β carotene assay that takes into account nonpolar antioxidants also.

CONCLUSION

The physicochemical analysis of the selected seeds or nutrition packed natural capsules and *in vitro* analysis of their methanolic extracts revealed their immense potential as antioxidants. These mineral packets are capable of fulfilling the daily nutrient requirement to maintain good health. These seeds are rich in all omega 3, 6 and 9 fatty acids and are extremely beneficial for heart, skin, hair, brain and bones. Their regular intake helps in reducing bad cholesterol & oxidative stress levels, while improving the digestive health and overall sense of well-being. A roasted seeds combo is an ideal source of energy and micronutrients for weight watchers.

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CONFLICT OF INTERESTS

Declared none

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