EVALUATION OF ANTIOXIDANT ACTIVITY OF SEED EXTRACTS OF *CUCURBITA PEPO, OCIMUM BASILICUM, TRACHYSPERMUM AMMI* AND *LINUM USITATISSIMUM* – INDIVIDUAL AND SYNERGISTIC EFFECT

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Abstract

Antioxidants have become academically intriguing molecules due to their numerous benefits, such as anti-aging and anti-inflammatory properties. They play a significant role in combating excess free radicals and oxidative stress. Oxidative stress is the imbalance between the free radicals and antioxidants produced by the body, that causes cell and tissue damage. Synthetic antioxidants are added to foods in food technology to enhance their nutritional value and to eliminate potential concerns like cardiovascular diseases and cancer. In recent years, research on the antioxidant properties of natural foods and their components is gaining momentum. The present study aimed at screening of phytochemicals like terpenoids, flavonoids, phenolics and evaluation of antioxidant potential in seed extracts of *Cucurbita pepo* (pumpkin), *Ocimum basilicum* (Basil), *Trachyspermum ammi* (Ajwain) and *Linum usitatissimum* (flax) in three different solvents. The detailed study analyzed the combined antioxidant potential of the selected 4 seeds in comparison with individual seeds in methanol, water and ethyl acetate solvents separately. Antioxidant effect was studied by DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (Ferric Reducing Antioxidant Power) assays. The DPPH assay results were expressed in terms of percentage inhibition of free radicals. Gallic acid was used as a standard. Cumulative percentage inhibition was found to be greater than individual seed extracts in all 3 solvents. IC 50 values were used as metrics to express antioxidant potential. The IC50 values were significantly lesser for combined seed extracts than individual seed extracts. Among the three solvents, the cumulative antioxidant potential is greater in methanolic extract (IC50 value 0.069mg/ml) than aqueous (IC50 value 0.518 mg/ml) and ethyl acetate extracts (IC50 value 0.082 mg/ml). Results of FRAP assay were directly correlated to absorbance. With increase in concentration of the seed extracts, absorbance had shown steady increase reflecting the correlation between concentration vs antioxidant potential. The results prove the significance of antioxidant power of combined seed extracts and further investigation may lead to the use of these combined super seeds as natural antioxidants in providing health benefits and preventing chronic diseases.

Key words: Combined antioxidant effect, DPPH, FRAP, flavonoids, polyphenolics, free radicals, oxidative stress, percentage inhibition, IC50.

INTRODUCTION

The kingdom of plants is renowned for its abundance of antioxidant chemicals. They have demonstrated potential as food additives and supplements that prevent oxidative stress-related disorders as well as food additives that inhibit oxidation. The plant kingdom's bioactive components may successfully inhibit low density lipoprotein (LDL) oxidation and stop

atherosclerosis by slowing and delaying its advancement to an advanced stage [1]. Antioxidants may easily scavenge free radicals [2] and can be obtained from plant kingdom. Phytochemicals like flavonoids and phenolic acids are the most varied sources of natural antioxidants and are generally safe to use as dietary supplements [1, 3, 4]. Numerous studies have demonstrated the potential of flavonoids and phenols to shield the body from the damaging effects of free radicals [5, 6].

The pumpkin seed is a good source of fatty acids like palmitic, stearic, oleic, and linoleic acids in addition to minerals like potassium, phosphorus and magnesium [7]. Research studies had shown that pumpkin seed oil, a highly dichromatic viscous oil, has potent antioxidant properties (8) and is a remarkable defence against high blood pressure and cancer.

The essential oil of ajwain seeds is mainly constituted of thymol and carvacrol [7] which are the principle components of its flavor. Other major components of the ajwain essential oil include γ-terpinene, ρ- cymene, β- pinene, myrcene, limonene, and camphene [9].

Basil oils had been reported to contain various quantities of linalool, camphor, methyl chavicol, methyl cinnamate, and eugenol [10]. Alpha linolenic acid is the main constituent of flax seed oil, which is known for its cardio protection and anti-inflammatory properties [11] The present study aimed at qualitative analysis of phytoconstituents & quantitative evaluation of antioxidant potential in seed extracts of *Cucurbita pepo* (S1), *Ocimum basilicum* (S2), *Trachyspermum ammi* (S3) *and Linum usitatissimum* (S4) in methanol, water and ethyl acetate solvents, individually and in 1:1:1:1 combination of all four seed extracts(synergistic) by DPPH and FRAP assays.

MATERIALS & METHODS

Preparation of Seed extract

Seeds obtained from National Seeds Corporation Ltd., were washed and dried. Finely powdered seeds soaked in water, methanol and ethyl acetate solvents separately for 24 hours, were homogenised using a hot plate with a magnetic stirrer at 40°C and centrifuged. The concentration of the seed extracts was 10%. The extract is roto-vapourised and the dried powders were labelled as S1, S2, S3 and S4 respectively and stored in air tight containers for use.

Phytochemical analysis

Qualitative analysis was carried out for the identification of phytochemicals in the seed extracts using standard procedures [12-14].

Antioxidant Activity

The DPPH and FRAP assays were employed to determine the antioxidant potential of methanolic, aqueous and ethyl acetate extracts of the seed samples - *Cucurbita pepo* (S1), *Ocimum basilicum* (S2), *Trachyspermum ammi* (S3) *and Linum usitatissimum* (S4) - individually and in equal ratio combination of all four seeds. To determine the combined effect, equal amount of the dried seed samples of all four seeds were mixed thoroughly and extracted in the three solvents separately.

DPPH assay: The reducing ability of antioxidants towards DPPH radical was evaluated by observing the decrease in absorbance at 515–528 nm [15] When a DPPH solution was mixed with an antioxidant, its colour turned from purple to yellow due to the transfer of $H^{\bullet}$ from the antioxidant to $DPPH^{\bullet}$.

$$DPPH^{\bullet} + ArOH \rightarrow DPPH\text{-}H + ArO^{\bullet}$$

The results were expressed as IC50 values or as % scavenging of DPPH$^{\bullet}$ for all the samples both individually and synergistically. Different concentrations (100 - 800 µg) of the samples were taken in the test tubes and the volume was made up to 1.0 mL with methanol. To all the tubes, 3mL of DPPH solution (whose absorbance was pre-set to 1) was added and kept in dark condition for 15 minutes. After incubation, the absorbance was read at 517nm spectrophotometrically with the solvent as a blank. Gallic acid was used as a standard. All the tests were performed in triplicates [16].

$$\text{Percentage inhibition (\%)} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

FRAP

The ferric reducing capacity of extracts was investigated by using the potassium ferricyanide-ferric chloride method [17, 18]. 0.2 mL of each of the extracts at different concentrations were mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6), and 2.5 mL of potassium ferricyanide (1%) and incubated at 50^{0}C for 20 min, to reduce ferricyanide into ferrocyanide. 2.5 mL of 10% (w/v) trichloroacetic acid was added to arrest the reaction. Samples were centrifugated at 3000 rpm for 10 minutes. 2.5 mL of the supernatant layer was treated with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%) for measuring the absorbance at 700 nm. All the tests were performed in triplicates. Gallic acid was used as a reference compound.

RESULTS AND DISCUSSION

Qualitative and quantitative analysis of phytochemicals

Phytochemical screening indicates that all seed extracts contain carbohydrates, alkaloids, saponins, terpenoids, flavonoids and phenolics. The methanolic extracts of all the seeds were found to contain significant proportion of alkaloids, flavonoids and phenolics when compared to ethyl acetate and aqueous extracts [19].

Antioxidant activity - DPPH assay

Free radical scavenging capacity of the extracts from different samples *Cucurbita pepo* (S1), *Ocimum basilicum* (S2), *Trachyspermum ammi* (S3) and *Linum usitatissimum* (S4) were estimated using the stable DPPH radical. The percentage inhibition of the individual and the combined extracts, in all three solvents at concentrations ranging from 100 to 800 µg were found to increase with increase in concentration of the seed extracts and all the extracts shown a notable capacity to scavenge free radicals. Cumulative percentage inhibition was found to be more pronounced than individual seed extracts in all three solvents. The combined action of a broad spectrum of phytochemicals from all the seeds may be the reason for the higher synergistic % inhibition than that of the individual seed extracts at all concentrations. **(Fig 1 a to c).** Significant increase in antioxidant activity was observed in methanol and aqueous extracts of ajwain and flax seeds from 400 to 800 µg concentration. Cumulative antioxidant effect of methanolic, aqueous and ethyl acetate extracts showed **(Table 1, Fig 1d)** a steady increase with increase in concentration of seed extracts (100-800 µg). Cumulative % inhibition ranges from 50.13 ± 0.0577 to 88.47 ± 0.0577 in methanolic extract; from 28.13 ± 0.3512 to 61.57 ± 0.0208 in aqueous extract and from 50.12 ± 0.2082 to 78.63 ± 0.2082 in ethyl acetate extract.

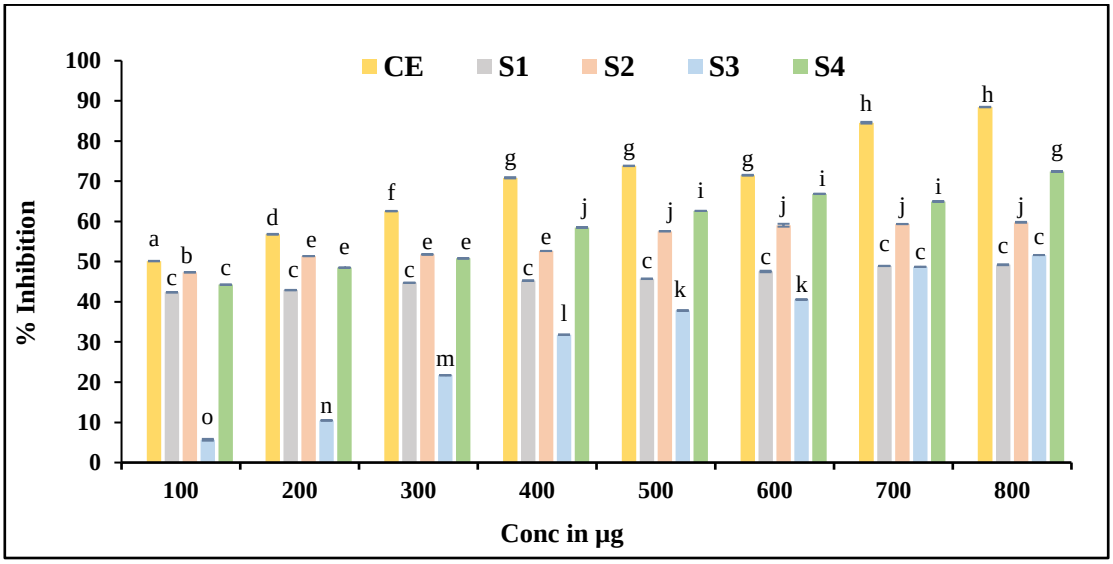

Fig 1a: DPPH assay - Comparative study of mean % Inhibition (n=3) at different concentrations of methanolic extracts; CE (S1+S2+S3+S4)-cumulative effect, S1-*Cucurbita pepo*, S2-*Ocimum basilicum*, S3-*Trachyspermum ammi*, S4-*Linum usitatissimum*

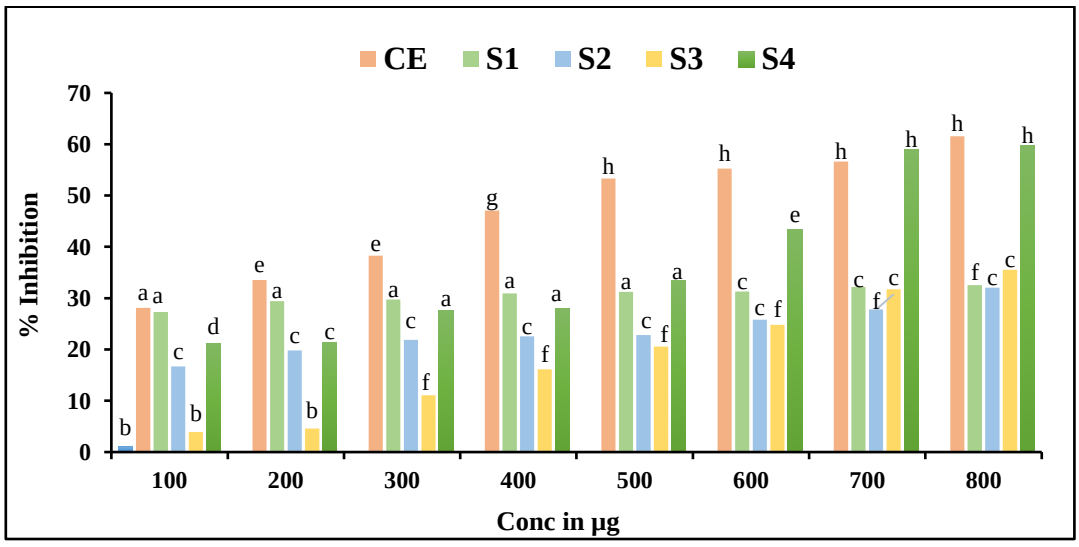

Fig 1b: DPPH assay - Comparative study of mean % Inhibition (n=3) at different concentrations of aqueous extracts; CE-(S1+S2+S3+S4)-cumulative effect, S1-*Cucurbita pepo*, S2-*Ocimum basilicum*, S3-*Trachyspermum ammi*, S4 - *Linum usitatissimum*

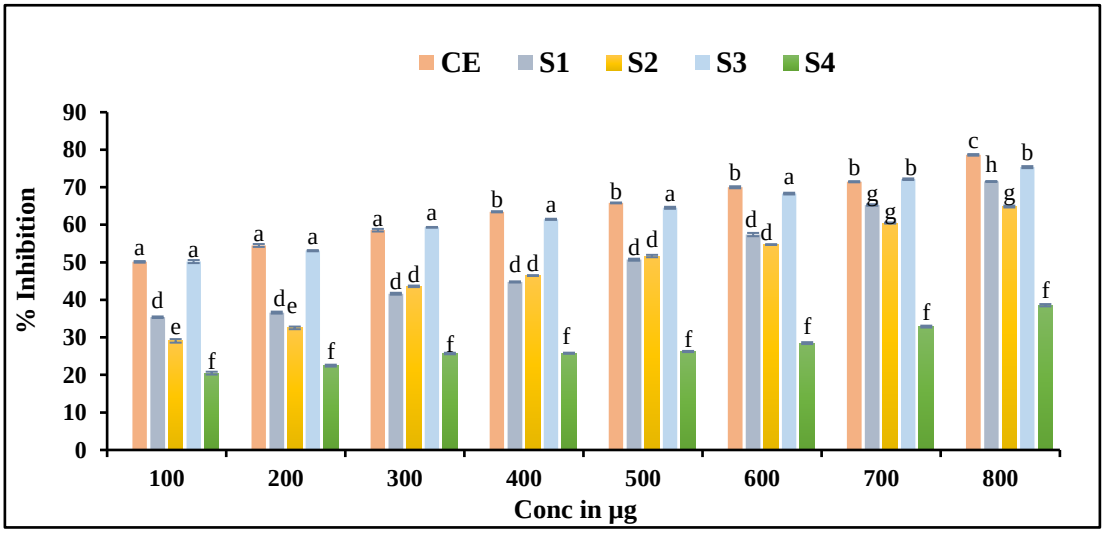

Fig 1c: DPPH assay - Comparative study of mean % Inhibition (n=3) at different concentrations of ethyl acetate extracts; CE-(S1+S2+S3+S4) - cumulative effect, S1 - *Cucurbita pepo*, S2 - *Ocimum basilicum*, S3 - *Trachyspermum ammi*, S4 - *Linum usitatissimum*

Table 1a: DPPH assay - Comparative study of % Inhibition of cumulative extract (CE) at different concentrations & in different solvents

Conc of sample in µg	Cumulative % Inhibition		
	MeOH Extract	Water Extract	EA Extract
100	50.13 ± 0.0577^{a}	28.13 ± 0.3512^{b}	50.12±0.2082^{a}
200	56.80 ± 0.1000^{c}	33.57 ± 0.0153^{b}	54.49±0.3786^{a}
300	62.57 ± 0.0577^{c}	38.27 ± 0.0153^{b}	58.56±0.3512^{c}
400	70.87 ± 0.1528^{d}	47.10 ± 0.0321^{e}	63.47±0.1528^{c}
500	73.83 ± 0.0577^{d}	53.33 ± 0.0321^{e}	65.83±0.1155^{c}
600	71.50 ± 0.1000^{d}	55.23 ± 0.0306^{e}	70.00±0.2646^{d}
700	84.57 ± 0.2082^{f}	56.63 ± 0.0321^{e}	71.47±0.1528^{d}
800	88.47 ± 0.0577^{f}	61.57 ± 0.0208^{e}	78.63±0.2082^{d}

Note: % Inhibition values of cumulative extract (CE = S1+S2+S3+S4) are given a mean ± SD. (n=3) S1-*Cucurbita pepo*, S2-*Ocimum basilicum*, S3-*Trachyspermum ammi*, S4-*Linum usitatissimum*. MeOH - methanol, EA - Ethyl acetate

The concentration of an antioxidant-containing material needed to scavenge 50% of the initial DPPH radical is known as the IC50. The half maximum inhibitory concentration (IC50) is a metric used to quantify how well a drug can inhibit a certain biological or metabolic process. More potency at scavenging DPPH radicals is indicated by a lower IC50 value, which also suggests a higher level of antioxidant activity. Cumulative antioxidant effect is found to be relatively higher than the individual seed extracts in all three solvents.

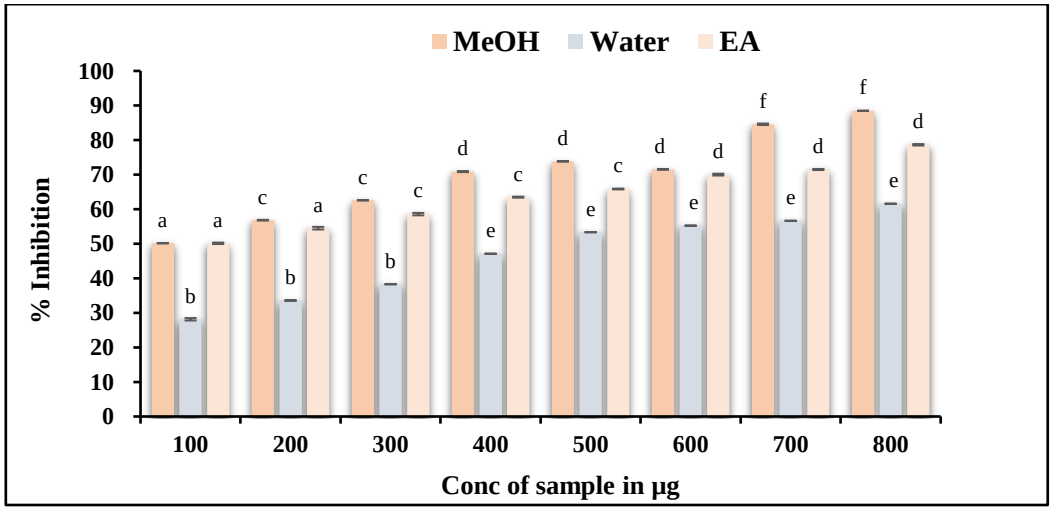

Fig 1d: DPPH assay - Comparative study of mean cumulative % Inhibition (n=3) in methanol (MeOH), aqueous and ethyl acetate (EA) extracts at different concentrations. CE (S1+S2+S3+S4) - cumulative effect, S1 – *Cucurbita pepo*, S2 - *Ocimum basilicum*, S3 - *Trachyspermum ammi*, S4 - *Linum usitatissimum*

DPPH assay revealed the cumulative antioxidant potential in the three chosen solvents are in the order: methanol > ethyl acetate > water. **(Table 1b)** The difference in the yield of phytochemicals in the extracts may be attributed to the extent of extraction of the bioactive components which in turn was influenced by the polarity of the solvents [17]. Amongst other contributing factors affecting the efficiency of extraction is pH, temperature, method of extraction, storage conditions and also solubility of endogenous compounds present in extracted material [18].

Table 1b: IC 50 values of the seeds in methanol, aqueous & ethyl acetate extracts - DPPH assay

Sample	IC50 (in mg/ml)		
	Methanol extract	Aqueous extract	Ethyl acetate extract
S1	0.848	3.39	0.443
S2	0.184	1.84	0.49
S3	0.727	0.98	0.086
S4	0.232	0.669	1.456
CE	0.069	0.518	0.082

Note: CE (S1+S2+S3+S4) - cumulative effect, S1 - *Cucurbita pepo*, S2 - *Ocimum basilicum*, S3 - *Trachyspermum ammi*, S4 - *Linum usitatissimum*; Values are expressed in mg of GAE per ml of the seed extracts

FRAP Assay

The assay was based on the reducing power of a compound (antioxidant) to reduce potassium ferricyanide to potassium ferrocyanide. A potential antioxidant will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); which then reacts with ferric chloride to form blue coloured ferric-

ferrous complex that has an absorption maximum at 700 nm [20, 21]. In FRAP assay the absorbance is directly correlated to the reduction potential of the test sample. The cumulative reduction potential of the combined extracts is notably higher than that of individual seed extracts in all three solvents **(Table 2a to c).** The synergistic antioxidant potential of the seed extracts compared to individual seed extracts is depicted graphically. **(Fig 2a to d, Table 2a to d)** Greater the absorbance , greater the FRAP value (concentration of ferrous ions) which is directly correlated to greater reducing power of the test sample. The results reveal that cumulative ferric reducing power of the extracts follow the order: aqueous extract > methanol extract > ethyl acetate extract.

Table 2a: FRAP assay - Comparative study in methanolic extracts

	Absorbance at 700 nm				
Conc of sample in µg	**CE**	**S1**	**S2**	**S3**	**S4**
100	0.600±0.0031^{a}	0.359±0.0032^{i}	0.333±0.008^{i}	0.570±0.0031^{a}	0.455±0.002^{g}
200	0.654±0.0064^{b}	0.387±0.0068^{i}	0.367±0.003^{i}	0.591±0.0060^{a}	0.502±0.005^{g}
300	0.715±0.0020^{c}	0.430±0.0065^{i}	0.410±0.004^{i}	0.612±0.0040^{a}	0.545±0.003^{g}
400	0.749±0.0060^{d}	0.473±0.0040^{j}	0.456±0.006i	0.665±0.0060^{a}	0.595±0.003^{g}
500	0.787±0.004^{e}	0.497±0.0060^{j}	0.494±0.007^{i}	0.685±0.0104^{a}	0.649±0.003^{h}
600	0.812±0.002^{e}	0.553±0.0030^{j}	0.510±0.002^{i}	0.710±0.001^{a}	0.689±0.003^{a}
700	0.845±0.0076^{e}	0.582±0.0025^{j}	0.530±0.002^{i}	0.734±0.002^{a}	0.745±0.002^{a}
800	0.889±0.0017^{f}	0.612±0.0020^{j}	0.564±0.004^{i}	0.756±0.0006^{a}	0.789±0.004^{a}

Note: Values are given as mean absorbance ± SD (n=3); S1 - *Cucurbita pepo*, S2 - *Ocimum basilicum*, S3 – *Trachyspermum ammi*, S4 – *Linum usitatissimum*, CE (S1+S2+S3+S4) - cumulative effect.

Table 2b: FRAP assay - Comparative study in aqueous extracts

	Absorbance at 700 nm				
Conc of sample in µg	**CE**	**S1**	**S2**	**S3**	**S4**
100	0.659±0.005^{a}	0.429±0.006^{b}	0.578±0.001^{c}	0.574±0.010^{c}	0.645±0.013^{a}
200	0.693±0.008^{a}	0.468±0.005^{b}	0.596±0.004^{c}	0.597±0.002^{c}	0.692±0.004^{a}
300	0.722±0.010^{a}	0.526±0.005^{b}	0.624±0.001^{c}	0.611±0.015^{c}	0.738±0.007^{a}
400	0.780±0.005^{d}	0.598±0.001^{c}	0.669±0.002^{c}	0.640±0.017^{c}	0.776±0.007^{d}
500	0.823±0.005^{e}	0.617±0.003^{c}	0.692±0.001^{j}	0.683±0.010^{j}	0.805±0.010^{d}
600	0.843±0.015^{e}	0.639±0.002^{c}	0.725±0.002^{i}	0.711±0.018^{i}	0.833±0.011^{d}
700	0.867±0.005^{e}	0.678±0.001^{c}	0.751±0.005^{h}	0.747±0.011^{h}	0.866±0.004^{e}
800	0.921±0.008^{f}	0.707±0.001^{c}	0.794±0.005^{g}	0.784±0.008^{g}	0.901±0.009^{f}

Note: Values are given as mean absorbance ± SD (n=3); S1 - *Cucurbita pepo*, S2 - *Ocimum basilicum*, S3 - *Trachyspermum ammi*, S4 - *Linum usitatissimum*, CE (S1+S2+S3+S4) - cumulative effect.

Table 2c: FRAP assay - Comparative study in ethyl acetate extracts

	Absorbance at 700 nm				
Conc of sample in µg	**CE**	**S1**	**S2**	**S3**	**S4**
100	0.532±0.004^{a}	0.322±0.010^{i}	0.346±0.009^{e}	0.256±0.011^{k}	0.520±0.010^{a}
200	0.554±0.011^{a}	0.354±0.010^{i}	0.396±0.007^{f}	0.283±0.010^{k}	0.543±0.011^{a}
300	0.628±0.016^{a}	0.418±0.015^{f}	0.434±0.011^{f}	0.314±0.011^{i}	0.614±0.013^{b}
400	0.676±0.006^{b}	0.462±0.006^{f}	0.461±0.010^{f}	0.347±0.011^{i}	0.643±0.005^{b}
500	0.711±0.014^{b}	0.490±0.009^{f}	0.495±0.012^{g}	0.372±0.012^{i}	0.668±0.010^{b}
600	0.751±0.009^{c}	0.526±0.014^{g}	0.528±0.007^{g}	0.420±0.015^{i}	0.703±0.009^{b}
700	0.802±0.013^{d}	0.562±0.009^{g}	0.546±0.008^{g}	0.441±0.011^{j}	0.740±0.013^{b}
800	0.853±0.007^{d}	0.599±0.015^{h}	0.584±0.012^{h}	0.452±0.007^{j}	0.764±0.011^{b}

Note: Values are given as mean absorbance ± SD (n=3); S1 - *Cucurbita pepo*, S2 - *Ocimum basilicum*, S3 - *Trachyspermum ammi*, S4 - *Linum usitatissimum*, CE (S1+S2+S3+S4) - cumulative effect.

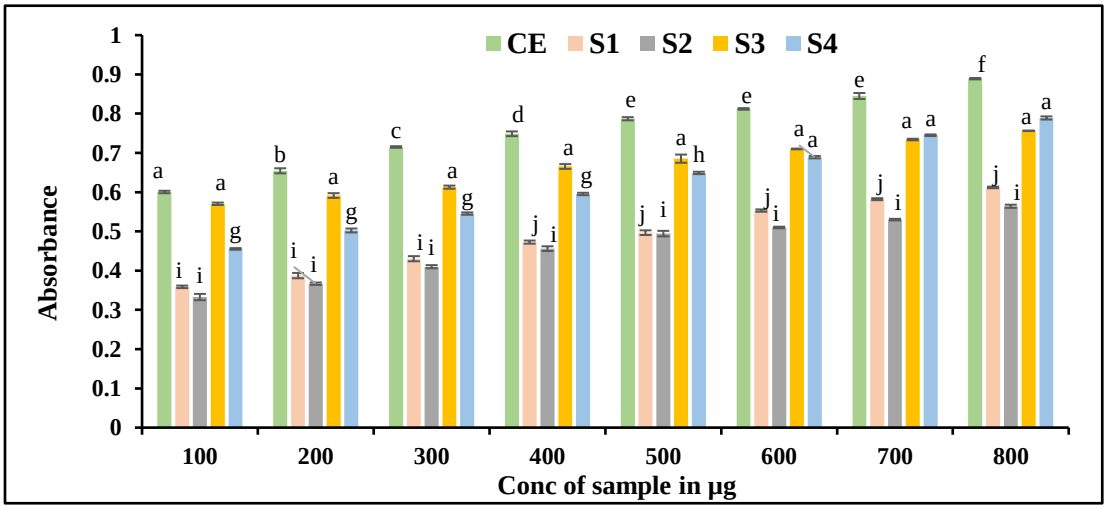

Fig 2a: FRAP assay - Comparative study in methanolic extracts. S1-*Cucurbita pepo,* S2-*Ocimum basilicum*, S3-*Trachyspermum ammi*, S4-*Linum usitatissimum*, CE (S1+S2+S3+S4)-cumulative effect.

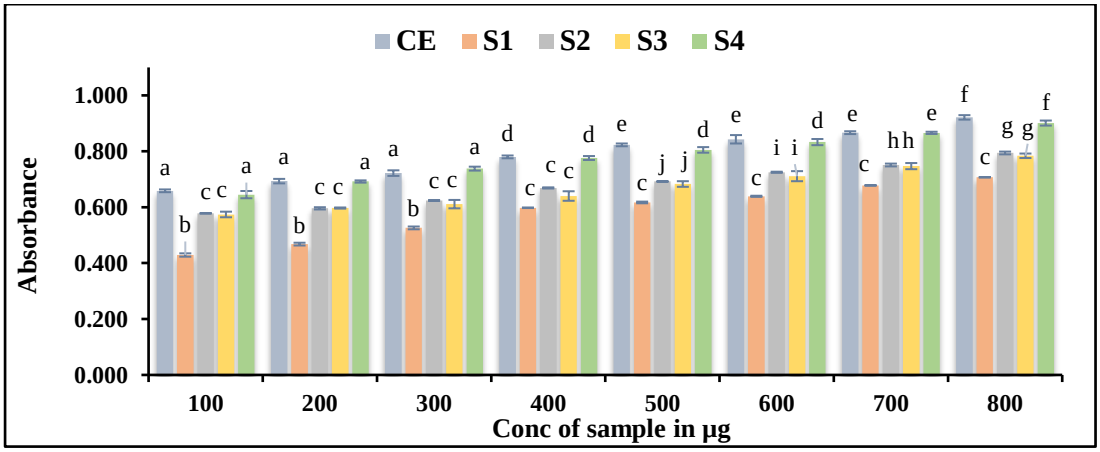

Fig 2b: FRAP assay - Comparative study in aqueous extracts. S1-*Cucurbita pepo*, S2-*Ocimum basilicum*, S3-*Trachyspermum ammi*, S4-*Linum usitatissimum*, CE (S1+S2+S3+S4)-cumulative effect.

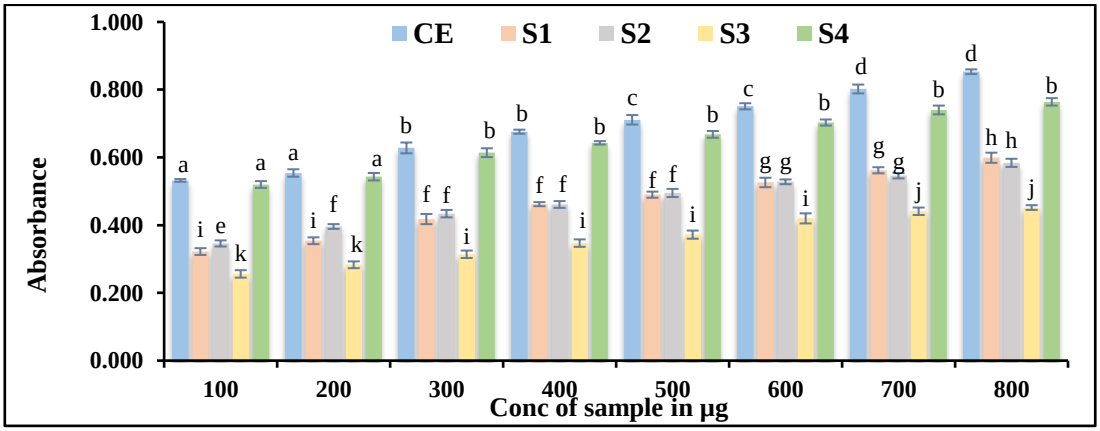

Fig 2c: FRAP assay - Comparative study in ethyl acetate extracts. S1-*Cucurbita pepo*, S2-*Ocimum basilicum*, S3-*Trachyspermum ammi*, S4-*Linum usitatissimum*, CE (S1+S2+S3+S4) -cumulative effect.

Table 2d: FRAP assay - Comparative study of antioxidant power of cumulative extracts in methanol, aqueous and ethyl acetate extracts at different concentrations

	Absorbance at 700nm		
Conc of sample in µg	**Methanol**	**Water**	**Ethyl Acetate**
100	0.600 ± 0.0031^{a}	0.659 ± 0.005^{b}	0.532 ± 0.004^{c}
200	0.654 ± 0.0064^{b}	0.693 ± 0.008^{b}	0.554 ± 0.011^{c}
300	0.715 ± 0.0020^{b}	0.722 ± 0.010^{b}	0.628 ± 0.016^{d}
400	0.749 ± 0.0060^{b}	0.780 ± 0.005^{b}	0.676 ± 0.006^{d}
500	0.787 ± 0.004^{b}	0.823 ± 0.005^{e}	0.711 ± 0.014^{d}
600	0.812 ± 0.002^{b}	0.843 ± 0.015^{e}	0.751 ± 0.009^{f}
700	0.845 ± 0.0076^{e}	0.867 ± 0.005^{e}	0.802 ± 0.013^{f}
800	0.889 ± 0.0017^{e}	0.921 ± 0.008^{g}	0.853 ± 0.007^{h}

Note: Increase in absorbance values of cumulative extract (CE = S1+S2+S3+S4) are given as mean ± SD. (n=3) S1-*Cucurbita pepo*, S2-*Ocimum basilicum*, S3-*Trachyspermum ammi*, S4-*Linum usitatissimum*, CE (S1+S2+S3+S4)-cumulative effect.

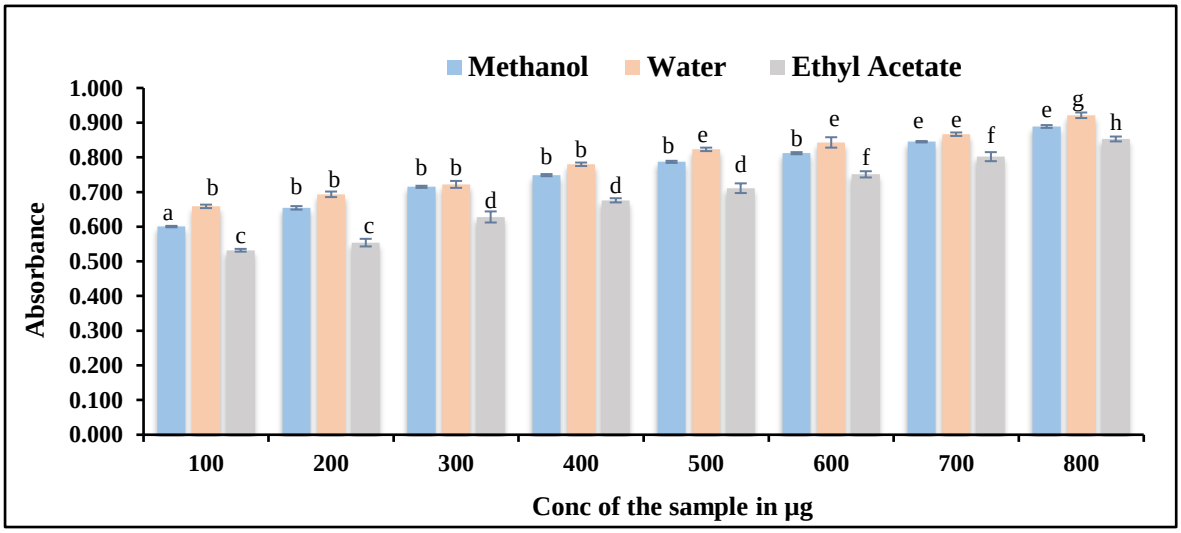

Fig 2d: FRAP assay – Comparative study of antioxidant power of cumulative extracts in methanol, aqueous and ethyl acetate at different concentrations. S1-*Cucurbita pepo*, S2-*Ocimum basilicum*, S3-*Trachyspermum ammi*, S4-*Linum usitatissimum*, CE (S1+S2+S3+S4)-cumulative effect

Conclusion

The study's findings, taken together, lend credence to the idea that the selected seeds are promising natural antioxidant sources. Despite having significantly varying total phenolic contents and antioxidant capabilities, the four selected seeds worked better together than they did separately. Owing to their high nutritional content, antioxidant capacity, and therapeutic properties, these seeds are increasingly being acknowledged as super seeds or functional foods [22-25]. These seed flours can be used as natural antioxidants by mixing them with maize or wheat flour. As taste and sensory enhancers, they can also be added to food products.

Cinnamic acids, phenolic acids, alpha linolenic acid (ALA), lignans, Secoisolariciresinol diglycoside (SDG) and dietary fiber are the biologically important bioactive components present in flax seeds [26, 27]. Water-soluble flavonoid -antioxidants orientin & viceninare as well as terpenoids-linalool, camphor, anisole and methyl cinnamate contribute to the antioxidant property of basil seeds [28]. Tocopherols, ß-sitosterol, and delta-7-sterols constitute a large quantity of pumpkin seed oil [29]. These seeds' diverse macro- and micronutrient composition, along with their wide range of active biochemicals and secondary metabolites, all contribute to their combined antioxidant effect, which has been demonstrated to be strong enough to qualify them as functional foods with enhanced antioxidant potential.

Factors including the extracted compound's polarity and solubility in the selected solvent, pH, extraction technique, extraction storage conditions, and the solubility of endogenous compounds in the extracted material all affect the total antioxidant effect [18].

The assays revealed the higher antioxidant effect of the combined seed extracts in comparison to the individual seed extracts. In conclusion the synergistic antioxidant effect in DPPH assay was found to give better results in methanolic and ethyl acetate extracts when compared to aqueous extract whereas in FRAP assay the aqueous extracts showed notably higher reduction power. Further research may pave way for use of these super seeds as functional foods to improve general well- being and immunity of humankind.

Ethical statements

Not applicable

Conflict of Interest

The authors disclosed no conflicts of interest. The paper's writing and content are entirely the authors' responsibility.

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