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Antioxidant Potential of Seed Extracts of *Cucurbita pepo, Ocimum basillicum, Trachyspermum ammi* and *Linum usitatissimum* - Individual and Synergistic Effect

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ABSTRACT

The plant kingdom is known for its rich antioxidant compounds. They have shown promise not just as food additives that prevent oxidation but also as supplements that prevent diseases brought on by oxidative stress. Antioxidants are rich in phytochemicals like flavonoids, carotenoids, polyphenolics and can readily scavenge free radicals. The present study aimed at screening phytoconstituents like terpenoids, alkaloids, saponins, tannins, flavonoids, phenolics and antioxidant potential in seed extracts of *Cucurbita pepo* (pumpkin), *Ocimum basillicum* (Basil), *Trachyspermum ammi* (Ajwain) *and Linum usitatissimum* (flax) in three different solvents. The extensive study investigated the synergistic antioxidant potential of methanolic, aqueous, and ethyl acetate extracts of the selected 4 seeds in comparison with individual seed extracts. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6 sulfonic acid assay) studied the antioxidant effect. The variance in IC 50 values between solvents reveals the polarity of the solvents, as well as the polarity of the extracted phytochemicals which may affect how much phytochemical extraction is accomplished. ABTS assay measures radical scavenging capacity in terms of absorbance. The result findings were expressed as percentage inhibition in three different solvents – methanolic extracts ranging from 48.35±0.55 to 93.24±0.35; aqueous extract 49.27±0.06 to 89.63±0.05; ethyl acetate extract 52.32±0.67 to 95.66±0.04. The results established that synergistic IC_{50} values are significantly lesser indicating higher antioxidant potential than individual seed extracts in all three chosen solvents. The benefits of these seed extracts' synergistic antioxidant properties can be investigated in further detail, for their potential applications as natural preservatives in cooking oils and supplements in food products to enhance immunity and prevent chronic diseases caused by oxidative stress.

Keywords: *Synergistic effect, ABTS, Scavenging capacity, Flavonoids, Carotenoids, Free radicals, Oxidative stress.*

1. INTRODUCTION

An imbalance between the body's antioxidants and free radicals causes oxidative stress, which damages cells. Antioxidants are compounds that can slow down or stop the substrate's oxidation even when present in small amounts of the oxidizable substrate, thus helping in preventing oxidative stress in humans. The main characteristic of antioxidants is their ability to trap and stabilize free radicals (1). Plants are abundant sources of polyphenolics and possess antioxidant properties. Phytochemicals such as phenolic acids and flavonoids are the most diverse sources of natural antioxidants, commonly present in plant species, and are generally safe for use as dietary supplements (2 - 4). The plant's presence of terpenes, flavonoids, Saponins, tannins, and cardiac glycosides is also responsible for good antioxidant activity (5). Numerous studies have shown a strong relationship between various plants' phenolic levels and their antioxidant activity (6,7,8). Low-density lipoprotein (LDL) oxidation has been implicated in the development of cardiovascular illnesses as well as the onset and advancement of atherosclerosis. The bioactive ingredients derived from plant kingdoms may effectively block LDL oxidation and prevent atherosclerosis by reducing and postponing the progression to an advanced stage (9).

In present days, researchers are concentrating on the extraction and assessment of these phytochemicals to investigate their mode of action as scavengers of free radicals. The present study aimed at screening of phytoconstituents & quantitative analysis of antioxidant potential in seed extracts of *Cucurbita pepo* (S1), *Ocimum basillicum* (S2), *Trachyspermum ammi* (S3) *and Linum usitatissimum* (S4) in methanol, water and ethyl acetate solvents individually and synergistically.

Cucurbita pepo (Pumpkin) seeds are highly nutritious and packed with powerful antioxidants and their rich nutrient content may provide health benefits, such as improved energy and immune function (10). Pumpkin seeds were once used as an anthelmintic in traditional medicine in China to expel tapeworm parasites, such as *Taenia solium* (tapeworm) (11).

Ocimum basillicum commonly known as basil seeds is used in many Asian dishes such as desserts and drinks ('falooda') due to its nutritional value. Cinnamate, citronellol, geraniol, linalool, pinene, and terpineol are some of the oils that one can find across all species of basil (12). Potent antioxidants in basil seeds strengthen the immune system, protect cellular structure, and DNA, and delay the effects of skin aging. Basil may help prevent fat build-up in the liver and keep the liver healthy (13, 14).

Trachyspermum ammi, commonly known as Ajwain seeds comprise 2.5-5% essential oil. Ajwain essential oil is a major contributor to its odor and taste. The essential oil is mainly constituted of thymol (15) and carvacrol which are the principal components of its flavour.

Flax (*Linum usitassimum*) seed oil is rich in polyunsaturated fatty acids specifically alpha-linolenic acid (16). Its high nutrient content and medicinal values make it a useful diet ingredient for improved health benefits (17). A research study suggested that daily administration of 100 mg secoisolariciresinol diglucoside (SDG) can be effective at reducing cholesterol levels in the blood and the risk of hepatic diseases in moderately hypercholesterolemic men (18).

2. MATERIALS AND METHODS

Preparation of Seed Extract

Seeds obtained from National Seeds Corporation Ltd. were dried and powdered. Seed extracts (10%) were prepared in water, methanol, and ethyl acetate separately. Samples soaked in respective solvents were homogenized on a hot plate using a magnetic stirrer at 40ºC and centrifuged. The extract is rota-vaporized to preserve the chemical composition of the extract. The dried powders were labeled as S1, S2, S3, and S4 respectively, and stored in air-tight containers for further use.

Phytochemical Analysis

Qualitative analysis was carried out for the identification of phytochemicals in the seed extracts using standard procedures (19 - 21).

Antioxidant Activity

ABTS assay was employed to determine the antioxidant potential of methanolic, aqueous, and ethyl acetate extracts of the seed samples - *Cucurbita pepo* (S1), *Ocimum basillicum* (S2), *Trachyspermum ammi* (S3) *and Linum usitatissimum* (S4) - individually and synergistically. To determine the synergistic effect, an equal amount of the dried seed samples of all four seeds were mixed thoroughly and extracted in the three solvents.

ABTS Free Radical Scavenging Assay

ABTS radical cation decolorization assay (22) was performed to determine the free radical scavenging activity of plant samples. In the presence of antioxidants, the blue ABTS radical cation is converted back to its colorless neutral form that can be monitored spectrophotometrically. The reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1), stored in the dark at room temperature for 12-16 h to produce $ABTS^{\cdot+}$ radical cation. Different concentrations (10 to 120 µg) of plant extract were added to 3.995 ml of diluted $ABTS^{\cdot+}$ solution, the absorbance was measured at 734 nm after 30 min. An appropriate solvent blank was run in each assay. Absorbance was taken in triplicate. Percentage inhibition was calculated using the formula,

$ABTS^{\cdot+}$ scavenging effect (%) = ((AB–AA)/ AB) × 100

Where AB is the absorbance of ABTS radical + methanol; AA is the absorbance of ABTS radical + sample extract/standard. Ascorbic acid was used as standard.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is the most effective instrument for determining the kinds of chemical bonds (functional groups) that are present in a substance. The chemical bond is characterized by the wavelength of light that is absorbed. It is possible to identify a molecule's chemical bonds by analyzing its infrared absorption spectra. FTIR – ALPHA-II, BRUKER spectrometer was used to obtain the FTIR spectra of methanolic extract of all the seeds. The reflectance of the extracts was measured from 4000 - 500 cm^{-1}, and the ATR crystal was always cleaned with acetone.

3. RESULTS AND DISCUSSION

Qualitative and Quantitative Analysis of Phytochemicals

Phytochemical screening indicates that all seed extracts contain carbohydrates, alkaloids, Saponins, terpenoids, flavonoids, and phenolic. The methanolic extracts of all the seeds were found to contain a significant proportion of alkaloids, flavonoids,

and phenolics when compared to ethyl acetate and aqueous extracts.

Antioxidant Activity

ABTS Free Radical Scavenging Assay

ABTS assay measures radical scavenging capacity in terms of absorbance. With the increase in concentration, absorbance shows a steady decrease indicating greater antioxidant potential. Percentage inhibition was determined in all three solvents individually and synergistically for concentrations ranging from 10 to 120 µg. The cumulative antioxidant effect was found to be significantly greater than the individual seed extracts in all three solvents. The cumulative antioxidant potential is comparable in all three solvents - Percentage inhibition of methanolic extracts ranging from 48.35±0.55 to 93.24±0.35; aqueous extract 49.27±0.06 to 89.63±0.05; ethyl acetate extract 52.32±0.67 to 95.66±0.04 (Table 1a,1b,1c, 1d, Fig 1a,1b,1c, 1d). The results of the ABTS assay indicated that the synergistic antioxidant activity was relatively greater than that of the individual seed extracts. IC_{50} values are given in (Table 1e).

Table 1a: ABTS assay - Comparative study in methanolic extracts

Conc. of Sample (µg)	% Inhibition				
	S1	S2	S3	S4	CE
10	49.33±1.17^{a}	43.60±1.00^{b}	38.53±1.14^{d}	7.80±0.87^{e}	48.35±0.55^{a}
20	54.33±0.85^{a}	55.73±1.14^{b}	48.17±0.65^{a}	13.40±0.72^{e}	54.36±0.81^{a}
40	61.57±0.42^{a}	63.40±0.46^{b}	59.17±0.47^{a}	16.60±0.72^{e}	66.54±1.31^{b}
80	70.43±1.05^{b}	68.43±2.01^{b}	64.37±0.47^{a}	18.27±0.31^{e}	78.62±0.70^{b}
100	77.33±1.21^{b}	75.30±0.56^{b}	70.70±0.46^{a}	28.33±1.03^{f}	85.45±0.50^{c}
120	83.20±0.56^{b}	79.80±0.56^{b}	78.77±1.79^{a}	35.40±0.72^{f}	93.24±0.35^{c}

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

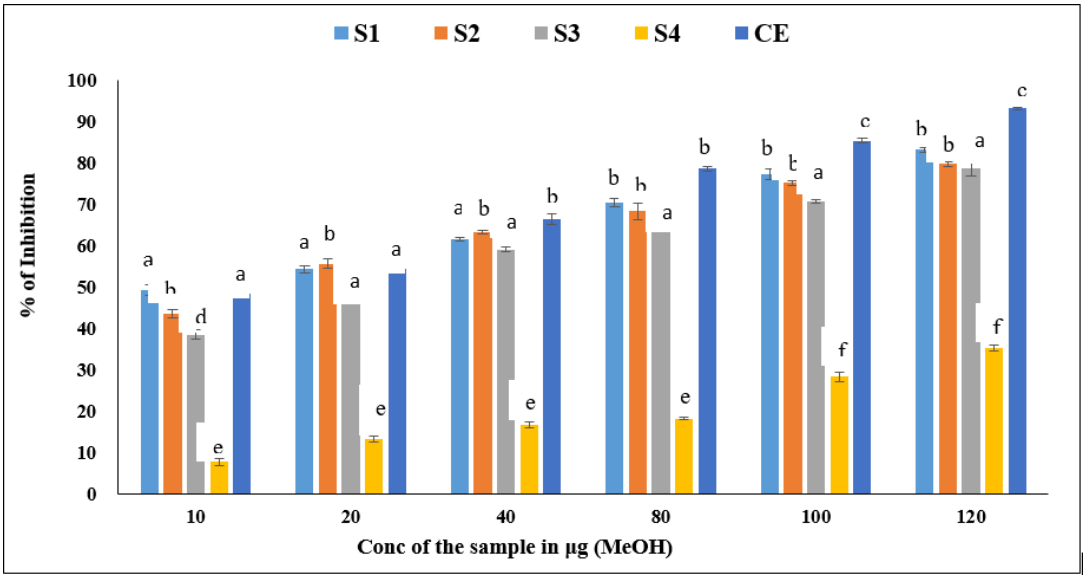

Figure 1a: ABTS assay - Comparative study of mean % Inhibition (n=3) at different concentrations of methanolic extracts; S1 - *Cucurbita pepo*, S2 - *Ocimum basilicum*, S3 - *Trachyspermum ammi*, S4 - *Linum usitatissimum*, CE (S1+S2+S3+S4) –cumulative effect

Table 1b: ABTS assay - Comparative study in aqueous extracts

Conc. of Sample (µg)	% of Inhibition				
	S1	S2	S3	S4	CE
10	46.26±0.06^{a}	47.52±0.13^{a}	41.59±0.06^{c}	42.35±0.09^{c}	49.27±0.06^{a}
20	51.46±0.03^{a}	53.66±0.13^{b}	48.64±0.03^{a}	49.85±0.02^{a}	56.49±0.08^{b}
40	59.84±0.03^{b}	61.35±0.09^{b}	57.65±0.09^{f}	55.68±0.04^{f}	66.72±0.09^{c}
80	65.43±0.02^{e}	71.65±0.10^{d}	65.83±0.05^{e}	70.54±0.04^{d}	75.69±0.06^{d}
100	71.85±0.05^{d}	80.54±0.10^{g}	73.46±0.05^{d}	75.68±0.05^{d}	84.56±0.08^{g}
120	77.36±0.03^{d}	86.56±0.28^{g}	80.34±0.04^{d}	80.56±0.08^{d}	89.63±0.05^{g}

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

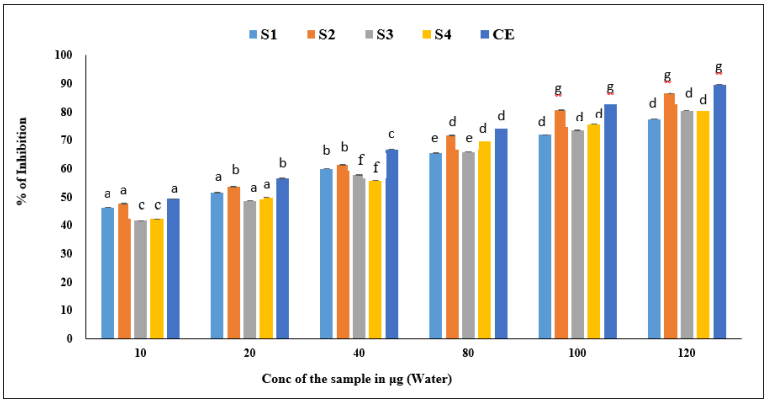

Figure 1b: ABTS assay - Comparative study of mean % Inhibition (n=3) at different concentrations of aqueous extracts; S1-*Cucurbita pepo*, S2-*Ocimum basilicum*, S3–*Trachyspermum ammi*, S4-*Linum usitatissimum*, CE (S1+S2+S3+S4) - cumulative effect.

Table 1c: ABTS assay - Comparative study in ethyl acetate extracts

	% of Inhibition				
Conc. of Sample (µg)	S1	S2	S3	S4	CE
10	49.23±0.41^{a}	47.10±0.74^{b}	49.34±0.47^{a}	45.50±0.70^{b}	52.32±0.67^{a}
20	55.35±0.50^{c}	54.36±0.65^{c}	56.64±0.82^{c}	48.20±0.66^{b}	58.14±0.04^{c}
40	62.75±0.40^{d}	63.74±0.88^{d}	63.25±0.72^{d}	58.93±0.31^{b}	65.28±0.03^{e}
80	71.57±0.68^{f}	70.21±1.05^{g}	73.54±0.18^{f}	68.43±0.49^{g}	77.40±0.05^{h}
100	76.94±0.26^{i}	78.65±0.63^{h}	79.97±0.66^{h}	78.30±0.26^{j}	87.44±0.04^{i}
120	83.21±0.63^{i}	82.27±0.71^{i}	86.34±0.53^{j}	80.53±0.31^{i}	95.66±0.04^{j}

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

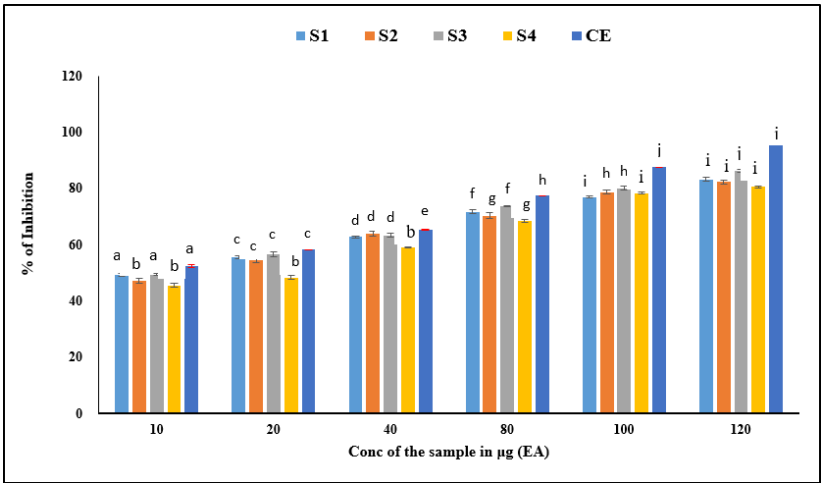

Figure 1c: ABTS assay - Comparative study of mean % Inhibition (n=3) at different concentrations of Ethyl acetate extracts; S1-*Cucurbita pepo*, S2-*Ocimum basilicum*, S3-*Trachyspermum ammi*, S4 -*Linum usitatissimum*, CE (S1+S2+S3+S4)-cumulative effect.

Table 1d: ABTS assay - Comparative study of mean cumulative % Inhibition in methanol, aqueous, and ethyl acetate extracts at different concentrations.

Conc. of Sample (µg)	Methanol	Water	EA
10	48.35±0.55[a]	49.27±0.06[b]	52.32±0.67[b]
20	54.36±0.81[a]	56.49±0.08[b]	58.14±0.04[b]
40	66.54±1.31[b]	66.72±0.09[b]	65.28±0.03[b]
80	78.62±0.70[d]	75.69±0.06[c]	77.40±0.05[c]
100	85.45±0.50[d]	84.56±0.08[c]	87.44±0.04[c]
120	93.24±0.35[d]	89.63±0.05[c]	95.66±0.04[d]

Note: Results are expressed as % Inhibition ± SD (n=3);

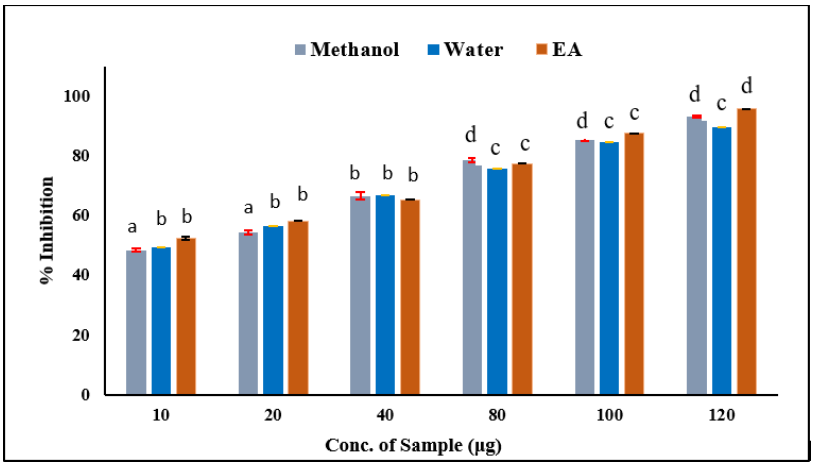

Figure 1d: ABTS assay - Comparative study of cumulative % Inhibition at different concentrations & in different solvents

Table 1e: IC_{50} values of the seeds in methanol, water & ethyl acetate extracts - ABTS assay

	IC_{50} (in mg/ml) Methanol extract	IC_{50} (in mg/ml) Water extract	IC_{50} (in mg/ml) Ethyl acetate extract
S1	0.007	0.016	0.004
S2	0.011	0.012	0.008
S3	0.030	0.027	0.004
S4	0.201	0.025	0.021
CE	0.008	0.003	0.002

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

FTIR

FTIR analysis helps in the characterization, identification, and validation of organic compounds based on absorption bands and peaks at specific wave numbers in the functional group region (4000-1600cm^{-1}) and fingerprint region (1600-650cm^{-1}). FT-IR spectrum (Fig 2) was taken for all four seed extracts in methanol and based on the characteristic peaks the functional groups present were validated.

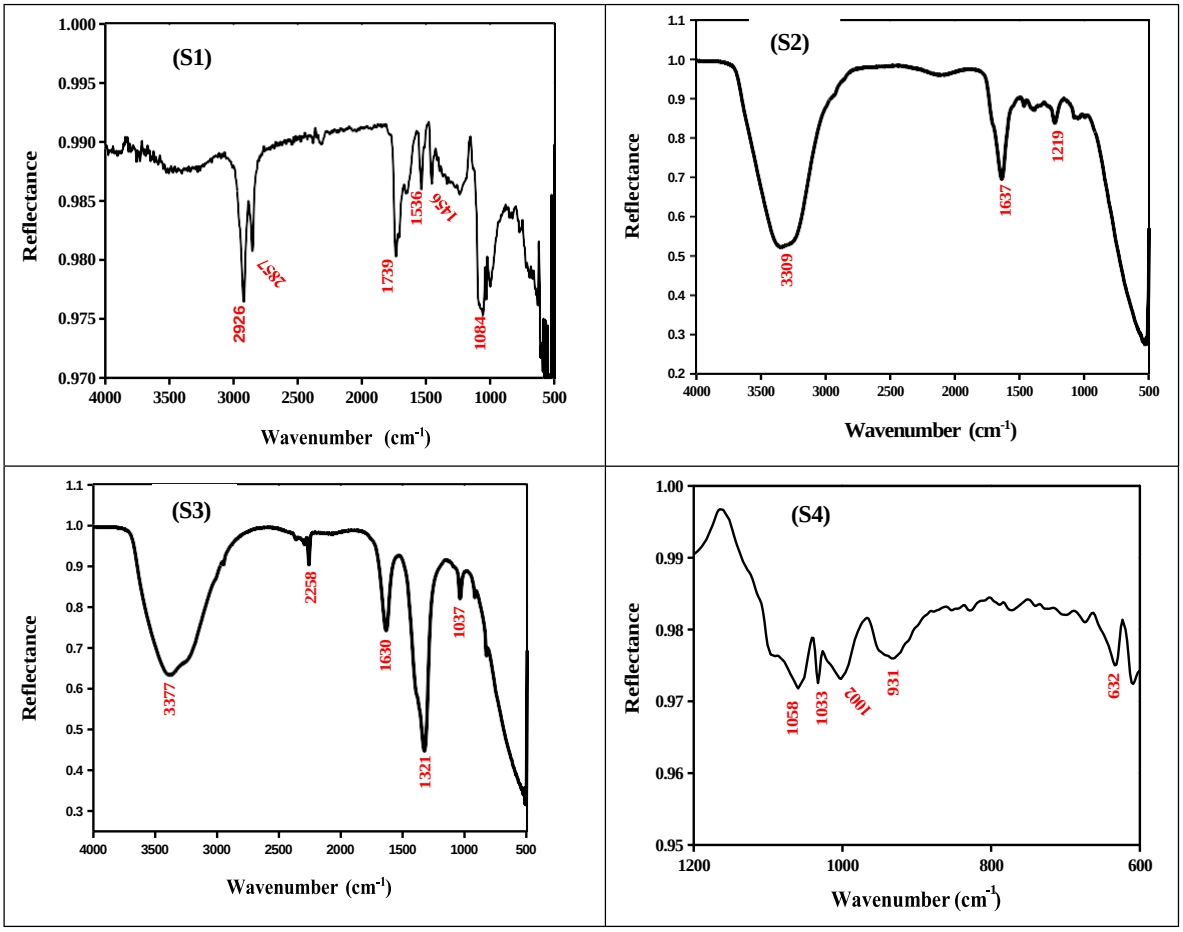

Figure 2: FT-IR spectra of methanolic extracts of all four seeds: (S1)-*Cucurbita pepo,* (S2)-*Ocimum basilicum*, (S3)-*Trachyspermum ammi*, (S4)-*Linum usitatissimum*

The bands observed at 3309 cm^{-1} (S2) and 3377 cm^{-1} (S3) may be attributed to phenolic O–H stretching vibrations (23) of thymol and carvacrol. The absorption bands at 1739 cm^{-1} (S1) may be due to the C=O stretching vibration of polyunsaturated fatty acids (24). Bands at 1637 cm^{-1} (S2), 1630 cm^{-1} (S3) may be assigned to C=C aromatic stretching vibrations of p-cymene, thymol, γ-terpinene and β-pinene and C=O in the flavonoid structures (25). This could also be due to the C=O stretching vibration of caffeic acid and its derivatives (26). The band at 1456 cm^{-1} (S1) could be related to CH_3, CH_2, flavonoids, and aromatic rings, where the vibrations would be the bending vibration of C–H and the stretching vibration of aromatics as in vitamin E (27, 28). The C-OH stretching vibrations due to flavonoids were represented by the bands at 1084 cm^{-1} (S1), 1037 cm^{-1} (S3), and 1033, 1022 cm^{-1} (S4) (29). The bands at 1084, 1037, 1033, and 1022 cm^{-1} could be due to carbohydrates (30). The band at 1084 cm^{-1} could also be due to symmetric stretching of (C–O–C) of 1,8-Cineole (31). The peaks at 2926 cm^{-1} (S1), and 2857 cm^{-1} (S1) indicate the C–H stretching vibrations of CH_2 and CH_3 groups (32), of sugars and carbohydrates (33). The C–O stretching vibration peak is observed at 1219 cm^{-1} (S2) and it may also be due to C-OH deformation vibrations in flavonoid compounds (29). The peak at 931 cm^{-1} (S4) may be due to O–H bending deformation; 1058 cm^{-1} (S4) may be due to O–H deformation of secondary alcohols. C–C stretching vibration is indicated by the peak at 1235 cm^{-1} (S4). The fingerprint zone, which spans from 1400 to 900 cm^{-1}, is named for the numerous distinctive single bands of low intensities that are ascribed to particular functional groups like C–H, and C–O bonds (34). However, it was difficult to distinguish the various components of these samples since the chemical compositions of the seed extracts were diverse and complex, and their fingerprints substantially overlapped.

Thymol and carvacrol are the components of the essential oil found in Ajwain (7). Other major components of the Ajwain essential oil include γ- terpinene, ρ- cymene, β- pinene, myrcene, and limonene. The biologically important bioactive components present in flax seeds are cinnamic acids, phenolic acids, ALA, lignans, Secoisolariciresinol glycoside (SDG), and dietary fiber (35, 36). Basil contains two important water-soluble flavonoids -antioxidants orientin & viceninare as well as terpenoids-linalool, camphor, anisole, and methyl cinnamate (37). Tocopherols, ß-sitosterol, and delta-7-sterols constitute a large quantity of pumpkin seed oil (38). These seeds' diverse macro- and micronutrient composition, along with their broad spectrum of active biochemicals and secondary metabolites, contribute to their combined antioxidant effect, which is proven to be sufficiently strong to qualify them as functional foods enhanced with antioxidant potential.

Owing to their nutritional richness, antioxidant capacity, and medicinal qualities, these seeds are becoming more and more recognized as functional foods or super seeds (39 - 44). They can be utilized as a natural antioxidant equivalent for commercially available synthetic ones by blending these seed flours with wheat flour or maize flour, combining these seed oils with cooking oils, and adding them to salads and drinks. They can also be used in food products as sensory and taste enhancers.

Factors affecting the efficiency of the extraction of phytochemicals, the polarity of the extracted compound and its solubility in the chosen solvent, pH, temperature at which extraction is done, method of extraction, storage conditions of the extract, and also the solubility of endogenous compounds present in extracted material decide the overall antioxidant effect (45 - 50). In conclusion, the synergistic antioxidant capacity determined by ABTS assay was found to give better results in ethyl acetate extracts compared to aqueous and methanolic extracts.

4. CONCLUSION

In summary, the study results support the view that the chosen seeds are promising sources of natural antioxidants. The four chosen seeds had considerably different total phenolic contents and antioxidant capacities, but their combined effect was stronger than that of each seed alone. Based on the qualitative and quantitative analysis of phytochemicals, it was found that all seed extracts contained significant amounts of carbohydrates, alkaloids, saponins, terpenoids, flavonoids, and phenolics. The methanolic extracts, in particular, showed higher concentrations of alkaloids, flavonoids, and phenolics compared to ethyl acetate and aqueous extracts. The ABTS free radical scavenging assay demonstrated that the antioxidant potential increased with concentration, with the cumulative effect of all seed extracts in different solvents being significantly higher than ethyl acetate extracts compared to aqueous and methanolic extracts.

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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