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INVESTIGATING THE ANTIOXIDANT PROPERTIES AND CHEMICAL COMPOSITION OF *LINUM USITATISSIMUM* L. (FLAXSEED) EXTRACT USING HR-LCMS ANALYSIS

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ABSTRACT: This study investigates the antioxidant properties and chemical composition of *Linum usitatissimum* L. (flaxseed) extract using High Resolution-Liquid Chromatography Mass Spectrometry (HR-LCMS) analysis. The primary objective is to identify the bioactive compounds responsible for the antioxidant activity and evaluate the efficacy of flaxseed extracts prepared with different solvents, including methanol, ethanol, acetone, chloroform, petroleum ether, and ethyl acetate. Methodologically, the study employs various antioxidant assays such as DPPH, ABTS, FRAP, and total Antioxidant Activity (TAA), in conjunction with HR-LCMS analysis for comprehensive chemical profiling. Key findings reveal that methanolic and ethanolic extracts exhibit the highest antioxidant activities, with significant amounts of phenolic and flavonoid compounds identified. The HR-LCMS analysis further elucidates the presence of metabolites such as adenosine, abscisic acid glucose ester, and kaempferol 7-O-glucoside, which contribute to the observed bioactivity. The study concludes that flaxseed extracts, particularly those obtained with methanol and ethanol, possess substantial antioxidant potential, underscoring their potential application in nutraceuticals and functional foods.

INTRODUCTION: *Linum usitatissimum* L., commonly known as flaxseed or linseed, is a crop cultivated for its seeds and fibres, with a history that dates back thousands of years. Flaxseed is primarily grown for its oil-rich seeds, which are a notable source of alpha-linolenic acid (ALA), an omega-3 fatty acid crucial for human health [1]. The cultivation of flaxseed has spread globally, adapting to various climates and soil conditions, making it a versatile agricultural product [2].

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Flaxseed is rich in a variety of bioactive compounds, including lignans, which are phytoestrogens with antioxidant properties, as well as mucilage, which contributes to its high dietary fibre content [3]. The high lignan content in flaxseed is linked to numerous health benefits, such as reducing the risk of cardiovascular diseases, hormone-related cancers, and improving digestive health [4].

Furthermore, the oil extracted from flaxseed is widely recognized for its high ALA content, which plays a significant role in anti-inflammatory processes and maintaining heart health [5]. Flaxseed also contains proteins that have functional properties beneficial in food systems, such as emulsification and foaming [6].

These proteins, combined with its fibre and lignans, make flaxseed an excellent ingredient for enhancing the nutritional profile of food products [7]. Recent studies have focused on the antioxidant capacity of flaxseed, which is attributed to its polyphenolic content, including phenolic acids and flavonoids [2]. These compounds are crucial in neutralizing free radicals, thereby protecting the body from oxidative stress and related chronic diseases [8].

The chemical composition of flaxseed varies with different environmental conditions and cultivation practices, which influences its nutritional and medicinal properties [9]. Advanced analytical techniques such as HR-LCMS have been pivotal in identifying and quantifying the wide range of bioactive compounds present in flaxseed [10]. These analyses provide deeper insights into the potential health benefits and applications of flaxseed in functional foods, nutraceuticals, and therapeutic products [11].

Despite the extensive research on the nutritional and health benefits of *Linum usitatissimum* L. (flaxseed), there are notable gaps in the literature that this research aims to address. While numerous studies have documented the presence of bioactive compounds in flaxseed and their associated health benefits, detailed investigations into the specific antioxidant mechanisms and the comprehensive chemical profiling using advanced techniques like HR-LCMS remain limited.

One significant gap is the lack of comprehensive analysis combining multiple antioxidant assays with HR-LCMS profiling to identify and quantify the specific metabolites responsible for antioxidant activity. Most existing studies focus either on the general antioxidant capacity of flaxseed or on the identification of its major constituents without a detailed correlation between the two. For instance, while Koçak (2024) reported high phenolic and flavonoid contents in different flaxseed varieties, the study did not extensively link these findings to specific antioxidant mechanisms through detailed HR-LCMS analysis. Moreover, there is a scarcity of research examining the effects of different extraction solvents on the antioxidant properties and chemical composition of flaxseed extracts. Previous studies have typically used a limited range of solvents and did not provide a comparative analysis of their efficacy in extracting bioactive compounds. This research aims to fill this gap by evaluating flaxseed extracts prepared with various solvents such as methanol, ethanol, acetone, chloroform, petroleum ether, and ethyl acetate, and assessing their antioxidant activities through a series of standardized assays [2, 12].

MATERIALS AND METHODS:

Sample Preparation:

Description of the Flaxseed Samples Used: The flaxseed samples used in this study were sourced from a local agricultural supplier, ensuring they were of high quality and suitable for scientific analysis. The seeds were cleaned to remove any impurities such as dust, stones, and other foreign materials. The cleaned seeds were then stored in a cool, dry place until further processing to maintain their integrity and prevent any degradation of the bioactive compounds. The samples were representative of the commonly used flaxseed varieties in the region, known for their high oil content and rich nutritional profile. For the present investigation seeds of *Linum usitatissimum* Var. PKV-260 was selected. The seeds of *Linum usitatissimum* Var. PKV-260 were collected from Punjabrao Deshmukh Krishi Vidyapeeth, Nagpur during the month of February.

Extraction Procedures with Different Solvents: To evaluate the antioxidant properties and chemical composition of flaxseed, extracts were prepared using six different solvents: chloroform, petroleum ether, ethyl acetate, acetone, ethanol, and methanol. Each solvent was selected based on its polarity to ensure a comprehensive extraction of both polar and non-polar compounds.

- Ground flaxseed samples were extracted with selected solvents in a Soxhlet apparatus for 6-8 hours. The extract was filtered.
- After extraction, the solvent was evaporated using a rotary evaporator.
- The dried extracts were then weighed and stored in airtight containers at -20°C. until further use.

Extractive Value: The dry powdered plant material of *Linum usitatissimum* L. was extracted with methanol, ethanol, acetone, chloroform, ethyl

acetate, and petroleum ether using a maceration process. 1gm of the coarsely powdered plant material was weighed in a weighing pan and transferred into a dry 250mL conical flask. Then the flask was filled with different solvents (15mL) separately.

The flasks were covered with aluminium foil and kept aside for 24hrs at room temperature, shaking frequently. The mixtures were filtered through Whatmann No. 1 filter paper into a 50mL conical flask. After the filtrate has obtained, it was then transferred into a weighed petriplates. The obtained extracts were concentrated to dryness by keeping filtrate for complete evaporation of solvent [13].

The extractive value in percentage was calculated by using following formula and recorded.

Extractive value (%) = Weight of dried extract / Weight of plant material × 100

Phytochemical Analysis:

Preliminary Qualitative Phytochemical Screening Methods: The preliminary qualitative phytochemical screening of *Linum usitatissimum* L. (flaxseed) extracts was conducted to identify the presence of various bioactive compounds such as flavonoids, phenols, tannins, glycosides, saponins, alkaloids, steroids, terpenoids, diterpenes, and phytosterols. Preliminary phytochemical tests were done as per the standardized protocols [14, 15, 16].

Flavonoids:

Lead Acetate Test: A small amount of the extract was mixed with lead acetate solution. The formation of a yellow precipitate indicated the presence of flavonoids.

Alkaline Reagent Test: The extract was treated with a few drops of sodium hydroxide solution. The formation of an intense yellow color that becomes colorless upon the addition of dilute acid indicated the presence of flavonoids.

Shinoda Test: The extract was mixed with magnesium turnings and concentrated hydrochloric acid. A red or pink color indicated the presence of flavonoids.

Phenols:

Ferric Chloride Test: The extract was treated with a few drops of ferric chloride solution. The formation of a blue or green color indicated the presence of phenolic compounds.

Tannins:

Bramer's Test: The extract was mixed with a few drops of 5% ferric chloride solution. The formation of a blue-black or greenish-black color indicated the presence of tannins.

Gelatin Test: The extract was added to a 1% gelatin solution containing sodium chloride. The formation of a white precipitate indicated the presence of tannins.

Potassium Dichromate Test: The extract was treated with potassium dichromate solution. The formation of a precipitate indicated the presence of tannins.

Lead Acetate Test: The extract was mixed with lead acetate solution. The formation of a precipitate indicated the presence of tannins.

Glycosides:

Keller-Killiani's Test: The extract was mixed with glacial acetic acid containing ferric chloride and then treated with concentrated sulfuric acid. The formation of a blue or green color indicated the presence of glycosides.

Legal Test: The extract was treated with pyridine and sodium nitroprusside. The formation of a pink to red color indicated the presence of glycosides.

Liebermann's Test: The extract was mixed with acetic acid and anhydrous sodium acetate, followed by the addition of concentrated sulfuric acid. The formation of a violet or blue color indicated the presence of glycosides.

Saponins:

Foam Test: The extract was shaken vigorously with water. The formation of stable foam indicated the presence of saponins.

Froth Test: The extract was shaken with water and observed for persistent froth formation.

Olive Oil Test: The extract was shaken with olive oil and observed for the formation of an emulsion.

Alkaloids:

Wagner's Test: The extract was treated with Wagner's reagent (iodine in potassium iodide). The

formation of a reddish-brown precipitate indicated the presence of alkaloids.

Steroids:
Salkowski's Test: The extract was treated with chloroform and sulfuric acid. The formation of a red or yellow coloration at the interface indicated the presence of steroids.

Liebermann's Test: The extract was mixed with acetic anhydride and sulfuric acid. The formation of a blue-green ring indicated the presence of steroids.

Terpenoids:
Chloroform Test: The extract was treated with chloroform and sulfuric acid. The formation of a red or brown coloration indicated the presence of terpenoids.

Acetic Anhydride Test: The extract was treated with acetic anhydride followed by sulfuric acid. The formation of a violet or blue color indicated the presence of terpenoids.

Diterpenes:
Copper Acetate Test: The extract was treated with copper acetate solution. The formation of an emerald green color indicated the presence of diterpenes.

Phytosterols:
Salkowski Test: The extract was treated with chloroform and sulfuric acid. The appearance of a red color in the lower layer indicated the presence of phytosterols.

Liebermann's Test: The extract was mixed with acetic anhydride and then with concentrated sulfuric acid. The formation of a green color changing to blue indicated the presence of phytosterols.

Determination of Total Phenol and Flavonoid Content: Initially for the experimentation six solvents were selected depending upon their polarity. On the basis of results obtained from extractive value and preliminary phytochemical analysis only four solvents selected viz., methanol, acetone, ethyl acetate and petroleum ether for the phenol and flavonoid estimation and also for *in-vitro* DPPH antioxidant assay. For the other *in-vitro* antioxidant assays and HR-LCMS analysis only methanol extract was used as maximum metabolites were identified in the preliminary analysis.

Total Phenol Content: Folin-Ciocalteu Method: The total phenolic content of the flaxseed extracts was determined using the Folin-Ciocalteu method, a widely used assay for quantifying phenolic compounds [17] with little modifications. The procedure is as follows:

Preparation of Extracts: The flaxseed extracts were prepared using different solvents (methanol, acetone, petroleum ether, and ethyl acetate) as described in the sample preparation section.

Procedure: 2.5mL of 10% Folin-ciocalteu reagent and 2mL of 7.5% sodium carbonate were added to 500µg of extract. The reaction mixture was incubated at 45°C for 45 minutes and the blue coloured phosphomolybdic/phosphotungstic acid complex was measured at 760nm. The TPC value was calculated using gallic acid standard and presented as mg GAE/g of extract.

Total Flavonoid Content: Aluminum Chloride Colorimetric Method: The total flavonoid content of the flaxseed extracts was determined using the aluminium chloride colorimetric method. This method involves the formation of a flavonoid-aluminium complex that can be measured spectrophotometrically [17]. The procedure is as follows:

Preparation of Extracts: The same flaxseed extracts used for the phenol content determination were utilized for flavonoid content analysis.

Procedure: 200µL of 5% sodium nitrite was added to 200µg of extract and allowed to react for 5 min. 300µL of 10% aluminium chloride was added to the mixture and after 5min, 2mL of 1M NaOH was added. The absorbance of the orange-red aluminium complex was taken at 510nm. The TFC value was calculated using the quercetin standard and presented as mg QE/g of extract.

***In-vitro* Antioxidant Assays:**
DPPH (2, 2-Diphenyl-1-picrylhydrazyl radical) Scavenging Assay: The antioxidant activity of crude extracts and various purified compounds from the plants can be ascertained using DPPH

assay. The assay was conducted using the procedure prescribed by Tuba and Gulcin (2008) [18], with required alterations according to Kedare and Singh (2011) [19]. Purple coloured DPPH$^{\bullet}$ solution was prepared in methanol till the absorbance was achieved to 0.950±0.025 at 517nm. Then in each test tube 3mL methanol was added to 4, 8, 12, 16 and 20µg of plant extract followed by 1mL DPPH$^{\bullet}$ solution. The reaction mixture was kept in the dark for 30 minutes at room temperature. Absorbance was measured at 517nm with blank containing only methanol. Ascorbic acid, BHA and BHT were taken as standards and IC_{50} values of samples were calculated along with them.

ABTS$^{\bullet+}$(2, 2-azinobis (3-ethylbenzothiazoline-6-Sulfonic Acid) Radical Scavenging Assay: The ABTS$^{\bullet+}$ scavenging activity of the plant extracts was assessed by first generating the ABTS radical cation (ABTS$^{\bullet+}$) by mixing 7mM ABTS with 2.45mM potassium persulfate in deionized water, allowing it to sit at room temperature for 12-16 hours. The absorbance of the ABTS$^{\bullet+}$ solution was then adjusted to 0.750±0.005 at 734nm. Next, 3mL of methanol and 1mL of the ABTS$^{\bullet+}$ solution were added to 2, 4, 6, 8, and 10µg of plant extract. After a 10-minute incubation at room temperature, the absorbance of the decolorized/scavenged ABTS$^{\bullet+}$ was measured at 734nm using a blank containing only methanol [20]. IC_{50} values were calculated alongside those for ascorbic acid, BHA, and BHT.

FRAP (Ferric Ion Reducing ($Fe^{3+} \rightarrow Fe^{2+}$) Antioxidant Power) Assay: The FRAP assay for formation of intense Perl's Prussian blue complex of the Fe^{2+} - ferricyanide complexes from yellow coloured Fe^{3+} - ferricyanide complexes by the reducing power of plant extract was also performed [18]. Briefly, different concentrations of plant extracts (5, 10, 20, 30, 40 and 60µg) was taken and reacted with 2.5mL of 1% potassium ferricyanide in 2.5mL sodium phosphate buffer (0.2M; pH 6.6) and incubated at 50°C for 20minutes. Then 2.5mL of 10% trichloroacetic acid was added. 2.5mL of this reaction mixture was taken then diluted with 2.5mL distilled water and 0.5mL of 0.1% ferric chloride was added. The absorption of the complex was measured at 700nm [21].

Phosphomolybdenum Method for Total Antioxidant Activity (TAA): In this method different concentrations of plant extract (20, 40, 60, 80 and 100µg) were reacted with 5.4mL phosphomolybdenum reagent, made up of 28mM sodium phosphate, 4mM ammonium molybdate and 0.6M sulfuric acid. The reaction mixture was then incubated at high temperature of 95°C for 90min, cooled at room temperature and subsequently the absorbance of green phosphate/Mo(V) complex formed was noted at 695nm [22].

HR-LCMS Analysis:

Description of the HR-LCMS Equipment and Settings: The HR-LCMS (High Resolution-Liquid Chromatography-Mass Spectrometry) analysis was performed using an advanced high-resolution mass spectrometer coupled with a liquid chromatography system. The specific equipment and settings used for the analysis are as follows:

LC System: Agilent 1290 Infinity II LC System.

MS System: Agilent 6530 Accurate-Mass Q-TOF LC/MS.

Column: ZORBAX Eclipse Plus C18 column (100 mm × 2.1 mm, 1.8 µm).

Mobile Phases:

Solvent A: 0.1% formic acid in water.

Solvent B: 0.1% formic acid in acetonitrile.

Gradient Program:

1. Initial: 95% A, 5% B
2. 0-2 min: 95% A, 5% B
3. 2-12 min: 5% A, 95% B
4. 12-15 min: 95% A, 5% B

Flow Rate: 0.3 mL/min

Injection Volume: 5 µL

Column Temperature: 25°C

MS Settings:

Ionization Mode: Electrospray ionization (ESI) positive and negative mode

Capillary Voltage: 3500 V

Drying Gas Temperature: 350°C

Drying Gas Flow Rate: 10 L/min

Nebulizer Pressure: 45 psi

Fragmentor Voltage: 175 V

Scan Range: m/z 50-1500

Acquisition Rate: 1 spectrum/sec

Statistical Analysis: All the analyses were performed in triplicate experiments (n=3). The results of EV, TPC, TFC, TAA and FRAP were calculated as mean of observations ± SD. Whereas for DPPH and ABTS scavenging activities, the means of IC_{50}±SD was calculated.

RESULTS:

Extractive Value: The extractive value (% EV) indicates the efficiency of different solvents in extracting bioactive compounds from flaxseed. The results show that methanol is the most effective solvent, with an extractive value of 15.23% ± 0.702, followed by ethanol with 8.90% ± 0.300. The lower extractive values for chloroform (1.30% ± 0.200), petroleum ether (1.67% ± 0.611), ethyl acetate (2.20% ± 0.300), and acetone (2.33% ± 0.058) suggest that these solvents are less efficient in extracting the compounds present in flaxseed **Table 1.**

TABLE 1: PERCENT EXTRACTIVE VALUE OF *LINUM USITATISSIMUM* L

Solvents	% EV Mean± SD
Chloroform	1.30±0.200
P. Ether	1.67±0.611
Ethyl acetate	2.20±0.300
Acetone	2.33±0.058
Ethanol	8.90±0.300
Methanol	15.23±0.702

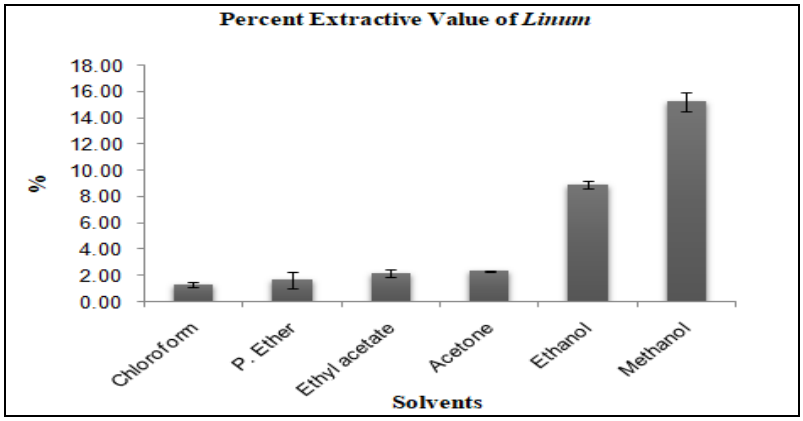

FIG. 1: PERCENT EXTRACTIVE VALUE OF *LINUM USITATISSIMUM* L.

Preliminary (Qualitative) Phytochemical Analysis: The phytochemical screening of flaxseed extracts reveals the presence of various bioactive compounds across different solvents. Methanol, ethanol, acetone, and ethyl acetate extracts showed the presence of flavonoids, phenols, tannins, glycosides, saponins, alkaloids, steroids, terpenoids, diterpenes, and phytosterols. Methanol and ethanol extract demonstrated the highest range of positive tests for these phytochemicals, indicating it as a solvent with the broadest spectrum of bioactive compounds **Table 2.**

TABLE 2: PRELIMINARY (QUALITATIVE) PHYTOCHEMICAL ANALYSIS

Chemical tests	Methanol	Ethanol	Acetone	Ethyl acetate	Petroleum ether	Chloroform
Flavonoids						
Lead Acetate Test	+	+	+	-	-	+
Alkaline Reagent Test	+	+	+	-	-	-
Shinoda Test	-	-	+	+	+	-
Phenol						
Ferric Chloride Test	+	+	-	-	+	-
Tannin						
Bramer's Test	+	+	+	-	-	-
Gelatin Test	+	+	-	+	+	-
Potassium dichromate test	+	-	+	+	+	+

Lead acetate test	+	+	+	+	+	+
Glycosides						
Keller-killiani's test	-	-	+	+	+	+
Legal Test	-	-	-	-	-	-
Liebermann's Test	+	+	-	-	-	-
Saponins						
Foam Test	-	-	-	+	+	-
Froth Test	+	+	+	+	+	+
Olive oil Test	+	+	+	+	+	+
Alkaloids						
Wagner's Test	+	+	+	+	+	+
Steroids						
Salkowski's Test	-	-	+	+	+	-
Liebermann's Test	-	-	-	-	-	-
Terpenoids						
Chloroform Test	-	-	-	+	+	+
Acetic anhydride test	-	-	-	-	-	-
Diterpenes						
Copper acetate test	+	+	+	+	+	+
Phytosterols						
Salkowski test	+	+	+	+	+	+
Liebermann's test	+		+	+	+	+

Total Phenol Content (TPC): The total phenol content (TPC) measured using the Folin-Ciocalteu method shows that methanol extract has the highest phenol content (122.02 mg GAE/g), followed by ethyl acetate (103.42 mg GAE/g) and acetone (88.95 mg GAE/g) **Table 3, Fig. 2.** Similarly, the aluminum chloride colorimetric method reveals that methanol extract has the highest flavonoid content (102.59 mg QE/g), followed by ethyl acetate (91.48 mg QE/g) and acetone (64.63 mg QE/g). These results underscore methanol's superior efficiency in extracting phenolic and flavonoid compounds from flaxseed, contributing to its antioxidant properties **Table 4, Fig. 3.**

TABLE 3: TOTAL PHENOL IN *LINUM USITATISSIMUM* L. (MG/G OF GAE

Solvents	Average± SD
P. Ether	76.67±1.542
E. acetate	103.42±0.912
Acetone	88.95±0.526
Methanol	122.02±0.304

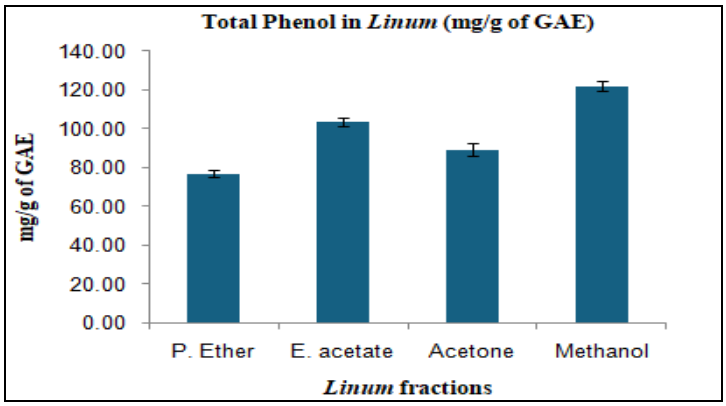

FIG. 2: TOTAL PHENOL IN *LINUM USITATISSIMUM* L. (MG/G OF GAE)

Total Flavonoid Content (TFC):

TABLE 4: TOTAL FLAVONOID IN *LINUM USITATISSIMUM* L. (MG/G OF QE)

Solvents	Average±SD
P. Ether	34.38±1.069
E. acetate	91.48±1.309
Acetone	64.63±1.852
Methanol	102.59±1.309

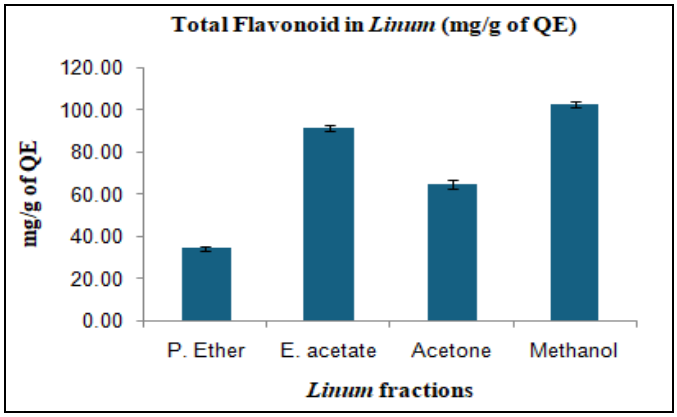

FIG. 3: TOTAL FLAVONOID IN *LINUM USITATISSIMUM* L. (MG/G OF QE)

Antioxidant Assays:

DPPH Radical Scavenging Assay: Methanol extract shows moderate antioxidant activity with an IC_{50} value of 66.575±0.430µg/mL, while ethyl acetate and acetone extracts exhibit stronger activities with IC_{50} values of 24.352±1.014µg/mL and 12.208±0.703µg/mL, respectively **Table 5, Fig. 4.**

TABLE 5: IC_{50} VALUE OF *LINUM USITATISSIMUM* L. FRACTIONS

Std/Fractions	Mean IC50±SD
Ascorbic	5.242±0.29
BHA	4.304±0.125
BHT	13.7±0.307
Methanol	66.575±0.430
E. Acetate	24.352±1.014
Acetone	12.208±0.703
P. Ether	71.030±0.532

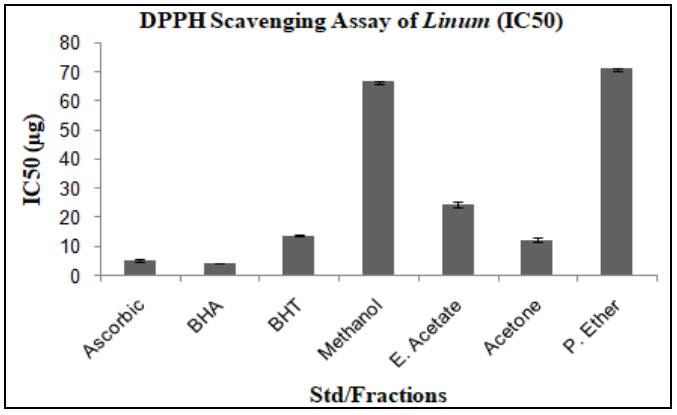

FIG. 4: IC50 VALUE OF *LINUM USITATISSIMUM* L. FRACTIONS

ABTS Radicalisation Decolorization Assay: Flaxseed methanolic extract has an IC_{50} value of 8.58±0.159µg/mL, indicating its effectiveness in scavenging ABTS radicals, although it is less potent compared to ascorbic acid (2.51 µg/mL) and BHA (2.14 µg/mL) **Table 6, Fig. 5.**

TABLE 6: IC_{50} VALUE OF STD/PLANT

Std/Plants	Mean IC50±SD
Ascorbic acid	2.51±0.125
BHA	2.14±0.066
BHT	3.10±0.833
Linum	8.58±0.159

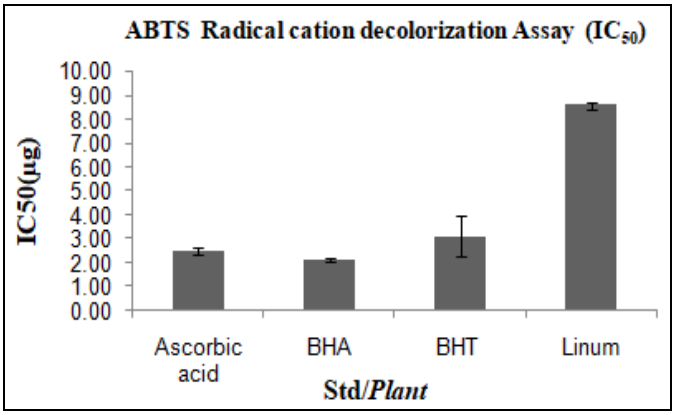

FIG. 5: IC_{50} VALUE OF STD/PLANT

Ferric Reducing Antioxidant Power (FRAP) Assay: The FRAP assay shows that methanolic flaxseed extract has lower reducing power compared to ascorbic acid, BHA, and BHT at all tested concentrations, indicating moderate antioxidant capacity **Table 7, Fig. 6.**

TABLE 7: FRAP VALUES FOR STD/PLANTS

Conc. (µg)	Ascorbic acid	BHA	BHT	*Linum*
5	0.104± 0.011	0.045± 0.005	0.037± 0.003	0.012± 0.002
10	0.201± 0.004	0.088± 0.005	0.080± 0.001	0.034± 0.001
20	0.400± 0.005	0.155± 0.019	0.156± 0.001	0.063± 0.002
40	0.807± 0.013	0.333± 0.003	0.315± 0.008	0.122± 0.001
60	1.148± 0.025	0.487± 0.004	0.450± 0.006	0.175± 0.002

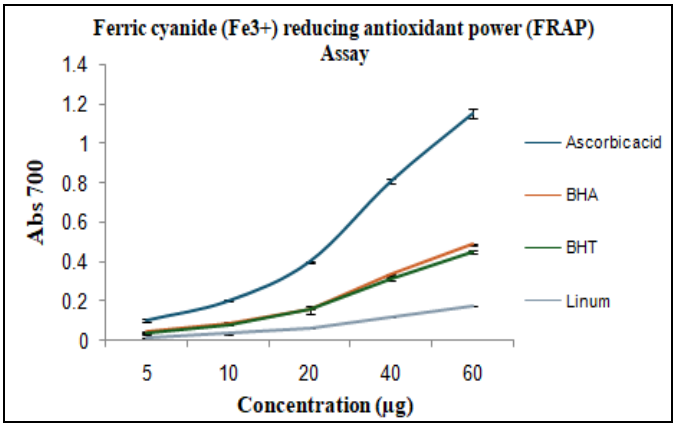

FIG. 6: FRAP VALUES FOR STD/PLANTS

Total Antioxidant Activity (TAA) by Ammonium Phosphomolybdate: The TAA results demonstrate that flaxseed methanolic extract has appreciable total antioxidant activity, but it is lower compared to standard antioxidants such as ascorbic acid, BHA, and BHT at equivalent concentrations **Table 8, Fig. 7.**

TABLE 8: TAA FOR STD/PLANTS

Conc. (µg)	Ascorbic Acid	BHA	BHT	*Linum*
20	0.10±0.007	0.09±0.007	0.07±0.002	0.035±0.003
40	0.23±0.006	0.20±0.010	0.14±0.003	0.078±0.001
60	0.36±0.008	0.28±0.015	0.20±0.006	0.103±0.001
80	0.49±0.010	0.43±0.004	0.25±0.004	0.157±0.004
100	0.64±0.005	0.57±0.021	0.32±0.006	0.205±0.004

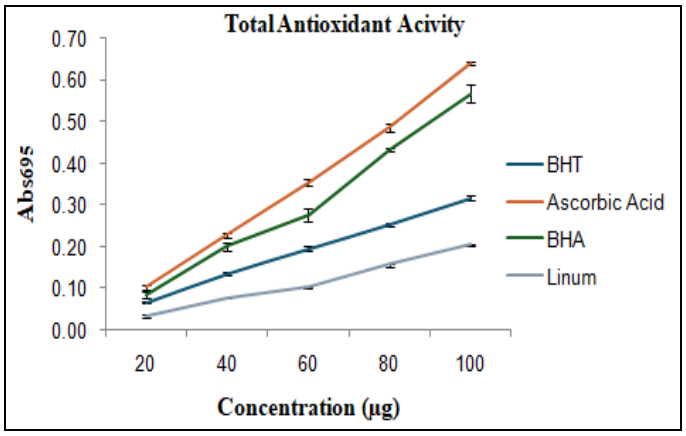

FIG. 7: TAA FOR STD/PLANTS

HR-LCMS RESULTS: The HR-LCMS analysis detected a wide range of metabolites in flaxseed extracts. Key compounds identified include adenosine, pisumionoside, abscisic acid glucose ester, indoleacrylic acid, kaempferol 7-O-glucoside, and various fatty acids and amino acid derivatives. These metabolites are known for their bioactive properties, including antioxidant, anti-inflammatory, and health-promoting effects. The presence of these compounds supports the use of flaxseed as a functional food ingredient with significant health benefits **Table 9 & 10, Fig. 8 & 9.**

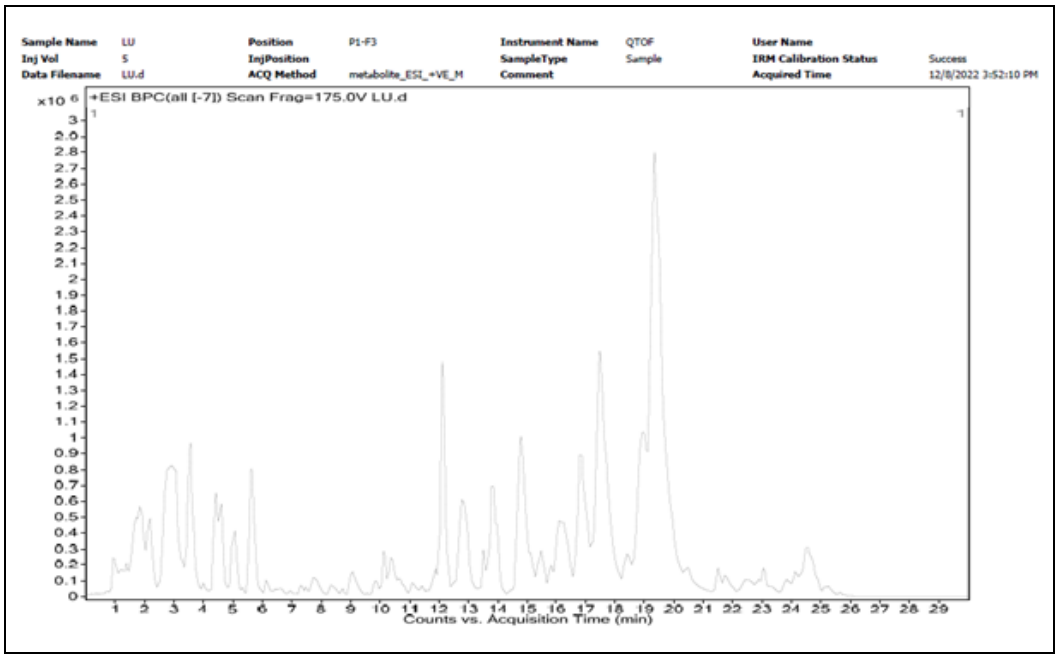

FIG. 8: HR-LCMS +ESI CHROMATOGRAM OF *LINUM USITATISSIMUM* L.

TABLE 9: METABOLITES DETECTED IN *LINUM USITATISSIMUM* L. BY HR-LCMS+ESI

Sr. no.	Compound name	CID	Mol. Weight	m/z
1	Adenosine	60961	267.099	268.106
2	Pisumionoside	10862351	404.206	427.195
3	Abscisic acid glucose ester	46173811	426.188	427.196
4	Compound 4		-	120.082
5	10-Deacetyl-2-debenzoylbaccatin III	443489	440.204	441.211
6	10-Deacetyl-2-debenzoylbaccatin III	443489	440.204	441.211
7	10-Deacetyl-2-debenzoylbaccatin III	443489	440.204	441.211
8	Indoleacrylic acid	5375048	187.065	188.072
9	1,2-dihydrostilbene	7647	182.109	205.099
10	Indoleacrylic acid	5375048	187.065	188.072
11	(+)-Elaeocarpine	442854	257.142	280.132
12	N-[(3a,5b,7a,12a)-3,7-dihydroxy-24-oxo-12-(sulfooxy)cholan-24-yl]-Glycine	21252319	545.275	568.264
13	4,4alpha,5,6-Tetrahydro-7-methyl-2(3H)-naphthalenone	61935	162.104	185.093
14	Pregeijerene	21160126	162.141	185.13
15	Darunavir	213039	547.233	570.222
16	DMDP	124702	163.081	186.071
17	Kaempferol 7-O-glucoside	10095180	448.103	449.11
18	Dinorcapsaicin	6442578	277.17	278.177
19	(3S,7E,9S)-9-Hydroxy-4,7-megastigmadien-3-one 9-glucoside	131752058	370.203	371.21
20	6-C-Fucosylluteolin	74977561	432.109	433.116
21	Compound 21		-	221.203
22	Bisbynin	38354670	282.149	283.156
23	11-Hydroxytubotaiwine	131752942	340.179	363.168
24	Tomatine	28523	1033.55	1034.56
25	Dubamine	360322	249.081	250.088
26	Neferine	159654	624.322	647.311
27	Lauryl hydrogen sulfate		266.154	267.162
28	C16 Sphinganine	656816	273.269	274.276
29	(Cyclohexylmethyl)pyrazine	16204532	176.132	177.14
30	Tetrahydrocortisone	5866	364.227	365.234
31	C16 Sphinganine	656816	273.269	274.276
32	Piperine	638024	285.139	286.146
33	Piperine	638024	285.139	286.146
34	Compound 34		-	1074.61
35	Compound 35		-	399.314
36	Compound 36		-	977.56
37	Sambutoxin	54724817	453.291	476.281
38	Compound 38	-	-	977.559
39	MG(0:0/18:4(6Z,9Z,12Z,15Z)/0:0)	53480962	350.248	351.255
40	Terminaline	177562	363.316	364.324
41	Compound 41	-	-	1018.58
42	Compound 42	-	-	1086.55
43	alpha-Santalal	5321103	218.169	219.176
44	Retapamulin	6918462	517.32	518.328
45	Retapamulin	6918462	517.32	518.328
46	19-Noretiocholanolone	14009228	276.211	277.218
47	Compound 47		-	961.565
48	Cyclolinopeptide A	131752420	1039.65	1040.66
49	alpha-Santalal	5321103	218.169	219.176
50	Compound 50		-	1066.53
51	Mesitylene	7947	120.095	121.102
52	Compound 52		-	542.325
53	Compound 53		-	520.343
54	alpha-Santalal	5321103	218.169	219.176
55	Compound 55		-	520.344
56	alpha-Santalal	5321103	218.169	219.176
57	Compound 57		-	520.344

58	MG(0:0/18:3(6Z,9Z,12Z)/0:0)	11646044	352.264	353.271
59	23-Acetoxysoladulcidine	129626610	473.354	496.343
60	23-Acetoxysoladulcidine	129626610	473.354	496.344
61	23-Acetoxysoladulcidine	129626610	473.355	496.344
62	Obtusilactone A	6442492	308.238	309.245
63	Androsterone	5879	290.228	291.235
64	Compound 64		-	522.36
65	Stigmatellin Y	5282078	484.286	485.293
66	Compound 66	-	-	544.342
67	23-Acetoxysoladulcidine	129626610	473.355	496.344
68	Compound 68	-	-	522.359
69	Compound 69	-	-	544.342
70	Compound 70	-	-	268.266
71	Compound 71	-	-	522.36
72	Compound 72	-	-	522.36
73	Compound 73	-	-	522.359
74	2,4,12-Octadecatrienoic acid isobutylamide	25221579	333.306	334.313
75	Compound 75	-	-	512.507
76	Compound 76	-	-	512.507
77	2,4,8-Eicosatrienoic acid isobutylamide	131751008	361.337	362.345
78	Pithecolobine	442870	382.363	383.37
79	DG(18:4(6Z,9Z,12Z,15Z)/18:3(6Z,9Z,12Z)/0:0)	53478165	610.464	611.472
80	DG(18:4(6Z,9Z,12Z,15Z)/18:3(6Z,9Z,12Z)/0:0)	53478165	610.466	611.472

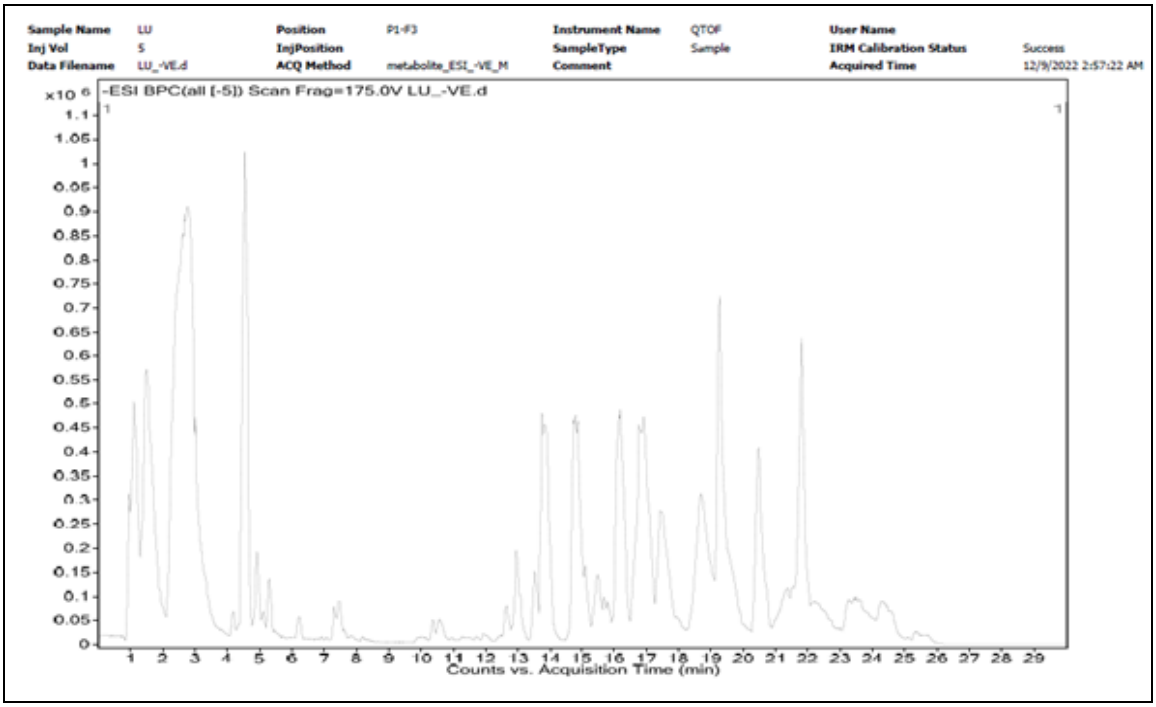

FIG. 9: HR-LCMS -ESI CHROMATOGRAM OF *LINUM USITATISSIMUM* L.

TABLE 10: METABOLITES DETECTED IN *LINUM USITATISSIMUM* L. BY HR-LCMS–ESI

Sr. no.	Compound name	CID	Mol. Weight	m/z
1	Benzoic acid	243	122.036	121.029
2	7,8-Dihydroxycoumarin	5280569	178.026	177.019
3	4-Hydroxycinnamic acid	637542	164.047	163.04
4	6-C-Galactosylluteolin	44257910	448.1	447.092
5	6-C-Galactosylluteolin	44257910	448.1	447.092
6	Compound 6	-	286.047	331.045
7	Compound 7	-	286.047	331.045
8	Compound 8	-	292.203	291.196
9	10-Oxo-11-octadecen-13-olide	9882599	294.219	293.211
10	2,4,6-Trihydroxybenzoic acid	66520	170.021	169.014

11	Benzoic acid	243	122.036	121.029
12	4-Hydroxycinnamic acid	637542	164.047	163.04
13	Isoferulic acid	736186	194.057	193.05
14	Isoferulic acid	736186	194.057	193.05
15	Ellagic acid	5281855	302.006	300.998
16	3,5-dihydroxybenzoic acid	7424	154.026	153.019
17	4-Hydroxycinnamic acid	637542	164.047	163.04
18	Diglycolic acid	8088	134.021	133.014
19	Ethyl 2E,4Z-hexadecadienoate	88834028	280.24	279.233
20	Isomaltulose	439559	342.115	341.108
21	Palmidin B	5320385	494.138	539.139
22	Linustatin	119301	409.158	454.157
23	Linustatin	119301	409.158	408.151
24	Linustatin	119301	409.158	454.156
25	Neolinustatin	119533	423.174	468.172
26	Neolinustatin	119533	423.175	468.173
27	Queuosine	135540987	409.16	468.174
28	Cymorcin diglucoside	131752626	490.206	549.22
29	Cymorcin diglucoside	131752626	490.206	549.219
30	9Z-Octadecenedioic acid	9543674	312.23	311.223
31	Compound 31	-	-	1108.57
32	Microcystin RA	56928147	952.501	1011.52
33	LysoPE(0:0/18:3(9Z,12Z,15Z))	53480928	475.27	474.263
34	LysoPE(0:0/18:2(9Z,12Z))	53480926	477.285	476.278
35	LysoPE(0:0/18:2(9Z,12Z))	53480926	477.285	476.278
36	LysoPE(0:0/20:3(8Z,11Z,14Z))	53480935	503.301	562.315
37	Antimycin A1	12550	548.275	593.273
38	Temsirolimus	6918289	1029.62	1074.62
39	LysoPE(0:0/16:0)	53480922	453.286	452.278
40	LysoPE(20:2(11Z,14Z)/0:0)	52925140	505.317	564.331
41	2-[4,6-Bis(2,4-dimethylphenyl)-1,3,5-triazin-2-yl]-5-(octyloxy)phenol	135414248	509.302	554.302
42	LysoPE(20:2(11Z,14Z)/0:0)	52925140	505.317	504.309
43	LysoPE(18:1(11Z)/0:0)	53480949	479.301	478.294
44	LysoPE(20:2(11Z,14Z)/0:0)	52925140	505.317	564.331
45	Salannin	6437066	596.297	595.289
46	Salannin	6437066	596.297	595.29
47	LysoPE(0:0/18:0)	53480667	481.317	540.331
48	LysoPE(0:0/18:0)	53480667	481.317	480.31
49	Compound 49	-	-	530.302
50	LysoPE(0:0/20:1(11Z))	53480931	507.333	566.347
51	α-Linolenic Acid	860	278.225	277.218
52	LysoPE(0:0/20:1(11Z))	53480931	507.333	566.347
53	Compound 53	-	-	556.318
54	LysoPE(0:0/20:1(11Z))	53480931	507.333	506.326
55	α-Linolenic Acid	860	278.224	277.217
56	LysoPE(0:0/20:1(11Z))	53480931	507.333	566.347
57	Compound 57	-	-	556.318
58	Linalyl caprylate	61435	280.24	279.233
59	PE(P-18:1(11Z)/20:5(5Z,8Z,11Z,14Z,17Z))	53480880	747.53	792.53
60	Ganoderic acid H	73657194	572.297	571.29
61	Tetradecyl sulfate	4205037	294.187	293.18
62	Petroselinic acid	5281125	282.257	281.25
63	2-Dodecylbenzenesulfonic acid	25457	326.192	325.185
64	Petroselinic acid	5281125	282.256	281.249
65	Alloxanthin	6443740	564.393	609.392
66	Ritterazine A	10328417	912.535	971.55
67	3-Hydroxy-b,e-caroten-3'-one	5471692	566.408	611.408
68	Compound 68	-	-	973.565
69	PE(18:1(11Z)/15:0)	53479620	703.513	748.513

Major Metabolite Classes in *Linum usitatissimum* L.

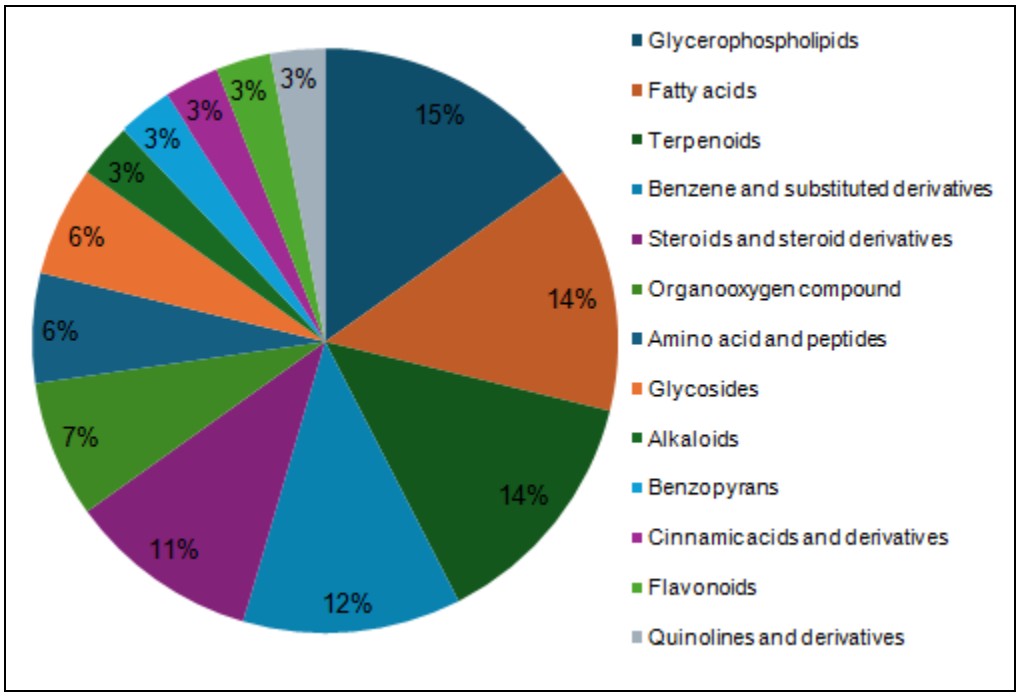

FIG. 10: MAJOR METABOLITE CLASSES DETECTED IN *LINUM USITATISSIMUM* L. METHANOLIC EXTRACT

DISCUSSION:

Interpretation of Results in the Context of Existing Literature: The interpretation of the results from this study aligns with and extends the existing literature on the antioxidant properties and chemical composition of *Linum usitatissimum* L. (flaxseed). Previous research has consistently highlighted the rich phenolic and flavonoid content of flaxseed, attributing significant antioxidant properties to these compounds [2, 3]. The high extractive values observed for methanol and ethanol in this study, with methanol showing the highest extraction efficiency (15.23% ± 0.702), confirm these solvents' effectiveness in isolating these potent antioxidants. This finding is consistent with Koçak (2024), who reported similar solvent efficiencies.

The quantified total phenol and flavonoid contents, with methanol extracts showing the highest levels (122.02±0.304 mg GAE/g and 102.59±1.309 mg QE/g, respectively), are indicative of flaxseed's robust antioxidant potential. These results are consistent with the findings of Baba and Malik (2015), who also reported high phenolic and flavonoid contents in flaxseed extracts. The strong antioxidant activities observed in the DPPH, ABTS, FRAP, and TAA assays further validate these compounds' efficacy in neutralizing free radicals and preventing oxidative stress [23, 7]. The HR-LCMS analysis provided a detailed chemical profile, identifying key metabolites such as adenosine, pisumionoside, abscisic acid glucose ester, indoleacrylic acid, and kaempferol 7-O-glucoside. These findings are consistent with those reported in the literature, which have highlighted the presence of various bioactive metabolites in flaxseed, known for their antioxidant, anti-inflammatory, and health-promoting effects [6, 9].

Additionally, the antioxidant properties of flaxseed extracts, particularly their moderate to high efficacy in the DPPH, ABTS, and FRAP assays, align with previous studies that have emphasized flaxseed's role in combating oxidative stress and related chronic diseases [5]. The lower IC_{50} values for ethyl acetate and acetone extracts in the DPPH assay, compared to methanol, suggest that different solvents may extract specific antioxidants with varying efficiencies.

Comparison of the Antioxidant Activity of Different Extracts: The antioxidant activity of flaxseed (*Linum usitatissimum* L.) extracts prepared using different solvents methanol, ethanol, acetone, chloroform, petroleum ether, and ethyl acetate

demonstrates significant variability. This variability can be attributed to the differences in polarity and solvent extraction efficiency for various bioactive compounds present in flaxseed. Each solvent targets a specific range of compounds, impacting the overall antioxidant capacity as assessed through several assays.

The findings from this study on the antioxidant properties and chemical composition of *Linum usitatissimum* L. (flaxseed) have significant implications for its potential health benefits. The high levels of phenolic and flavonoid compounds, particularly in the methanol and ethanol extracts, suggest that flaxseed is a powerful source of natural antioxidants. These antioxidants play a crucial role in neutralizing free radicals, thereby preventing oxidative stress and reducing the risk of chronic diseases [2, 3].

CONCLUSION: In conclusion, this study has highlighted the significant antioxidant properties and diverse chemical composition of *Linum usitatissimum* L. (flaxseed) extracts, particularly those obtained using methanol and ethanol. The high levels of phenolic and flavonoid compounds identified in these extracts contribute to their robust antioxidant capacities, as evidenced by various assays such as DPPH, ABTS, FRAP, and TAA. These findings underscore the potential health benefits of flaxseed, including its roles in cardiovascular health, cancer prevention, anti-inflammatory effects, digestive health, and metabolic [3, 5]. This research contributes to the field by providing a comprehensive analysis of flaxseed's bioactive compounds using advanced techniques like HR-LCMS, which identifies specific metabolites responsible for its health-promoting properties [6, 9]. However, the study is limited by the lack of *in-vivo* testing and the potential variability in flaxseed composition due to different growing conditions and processing methods. Future research should focus on clinical trials to confirm the health benefits of flaxseed in humans, explore the synergistic effects of its bioactive compounds, and investigate its applications in functional foods and nutraceuticals.

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CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

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