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Original Research

Assessment of Antioxidant Activities of Flaxseed (*Linum usitatisimum* L.) and Fennel Seed (*Foeniculum vulgare* Mill.) Extracts

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Abstract

Flaxseed and fennel seeds are rich sources of different types of phenolics such as lignans, phenolic acids, flavonoids, phenylpropanoids, and tannins. The present study was carried out to develop and optimize a technique for isolation and quantification of secoisolariciresinol diglucoside (SDG) from flaxseed and Anethole from fennel seeds and to assess the antioxidant activities of flaxseed (*Linum usitatisimum L.*) and fennel seeds (*Foeniculum vulgare Mill.*) extracts. The percentage of extract in fennel seeds and flaxseed was 5.72 and 6.31 %, respectively. Using HPLC, the predominant constituent in fennel seed was determined to be anethole (11.30±0.25 mg/g) and in flaxseed, it was secoisolariciresinol diglucoside (SDG) (8.35±0.52 mg/g) compared to the respective standards solution. Using SPSS, the obtained data from each parameter were statistically analyzed. There are numerous biological and pharmacological effects of trans-anethole. For antioxidant activity, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) showed higher results with acetone (80%), with fennel seed and flaxseed is 7.28±0.17 and 8.29±0.23 mM TE/g. while FTC method it was maximum for distilled water extract of fennel seed (48.35±0.19%) and Flaxseed (65.09±0.72%) and by β-carotene

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bleaching method, water extract of fennel seed and flaxseed also showed maximum antioxidant activity i.e. 83.06±0.02% and 86.23±0.38%. Additionally, it is used as a masking agent in food products as a flavoring agent. According to the findings of this study, fennel seeds and flaxseed contain bioactive compounds that may assist in the treatment of a number of chronic diseases.

Keywords: antioxidants, anethole, HPLC & GC-MS, health benefits, secoisolariciresinol diglucoside

Introduction

Antioxidants act as radical scavengers, inhibit lipid peroxidation and other free radical-mediated processes, and may protect the body from oxidative injury caused by free radical reactions [1, 2]. Due to their perceived carcinogenic potential and safety issues, the use of synthetic antioxidants in foods should be discouraged [3]. It has been discovered that phytochemicals such as phenolic compounds, terpenoids, and alkaloids are excellent reducing agents [4]. Plant-derived antioxidants, such as flavonoids, phenolic acids, and tocopherols, are increasingly utilized in food and medicine for both prevention and treatment [5]. People have ingested plants as a source of food, shelter, clothing, medicine, cosmetics, and for seeking relief from life's adversities; in an ongoing effort to improve their quality of life [6]. It is believed that these natural substances may help prevent cancer and have other health benefits due to their antioxidant properties [7, 8]. Herbal spices contain bioactive compounds that are typically added to food items to enhance flavor and extend shelf life [9, 10]. In their essential oils, extracts, and bioactive components, it is well-known that many spices and botanicals have antioxidant and antimicrobial properties [11, 12]. Fennel is a well-known umbelliferous plant with annual, biennial, and perennial growth. The seeds, stem, leaves, and other parts of the plant are all palatable. 11% of fennel seeds are oil, 10% are protein, 8% are water, and 40% are carbohydrates [13]. Among other minerals, it contains calcium, phosphorus, iron, sodium, and potassium. Fennel also includes thiamine, riboflavin, and niacin. Estragole, trans-anethole (TA), phellandrene, and fenchone are among the essential oils found in its seed, but t-anethole dominates the extract (60-70%) [14, 15]. It has anticancer, antidementia, antiplatelet, antihirsutism, hepatoprotective, and anti-hyperlipidemic properties [16, 17]. Thus, fennel seed extracts are utilized frequently in the pharmaceutical industry [18, 19]. The preponderance of flaxseed-growing countries are located in the northern hemisphere [20]. The average flaxseed chemical analysis revealed 30 to 40% lipid, 20 to 25% protein, 20 to 28% total dietary fibre, 4 to 8% moisture, and 3 to 4% ash. Due to the presence of physiologically active dietary components, the oil contains vitamins A, B, D, and E, minerals, and amino acids. which may offer health benefits beyond those associated with fundamental nutrition [21]. Due to its rich omega-3 fatty acid, alpha-linolenic acid (ALA), dietary fiber, and antioxidant content, flaxseed is increasingly used in food items [22]. These phytochemicals function as antioxidants and affect cell growth and viability [23-25]. Secoisolariciresinol diglucoside (SDG) is the most abundant lignan in flaxseed [26]. Previous flaxseed studies indicate that secoisolariciresinol diglucoside (SDG) is one of the essential dietary lignans. Therefore, the purpose of this study was to isolate and quantify anethole and secoisolariciresinol diglucoside (SDG) present in the fennel and flaxseed extract, characterize the compounds via HPLC and antioxidant activities of fennel seeds and flaxseed with different solvents.

Material and Methods

Flaxseed and fennel seeds were purchased at a local market in Lahore, Pakistan. Herbs were dehydrated in an IM-30 heated air furnace (Irmec, Germany) at 35 degrees Celsius and then stored at -4 degrees Celsius in polyethylene receptacles. The research was conducted at The University Institute of Diet and Nutritional Sciences (UIDNS) of The University of Lahore in Lahore, Pakistan.

Chemical Reagents

Trolox, 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS), and gallic acid were obtained from Sigma-Aldrich Inc. All solvents used for HPLC were of HPLC quality, while all other reagents were of analytical quality. The standard secoisolariciresinol (>95% purity) was provided by Research Lab (Lahore University for Women, Pakistan), while anethole was acquired from Sigma-Aldrich (Italy). The suppliers of Other Chemicals were Romil (Cambridge, United Kingdom) and LabScan (Ireland), respectively.

Standard Solution

The standard solutions were prepared by dissolving Anethole and SDG standards in HPLC-grade methanol to yield a 1000 g/ml solution, which was then stored at 4ºC [27].

Defatted Flaxseed Powder and Defatted Fennel Seed Powder Preparation

The seed samples were ground into a fine powder using a commercial blender (TSK-949, WestPoint, France). The material that passed through 80-mesh sieve

was used for extraction purposes Flaxseed and fennel powder was defatted by treating (10 g each) of powder in 60 mL n-hexane for 1 h. The resulting fat-free powder was entirely air-dried [28, 29] and refrigerated until future usage.

Magnetic Stirrer-Assisted Extraction

(MSAE) Method

Flaxseed powder and fennel powder (500 mg each) were combined separately with 20 mL distilled water and 25 mL 1 M aqueous NaOH and swirled at 400 rpm for 1 h at 60ºC using a magnetic stirrer. The hydrolysate was centrifuged for 10 min at 4000 rpm after being neutralised to pH 7 with 1 M HCl. The supernatant was recentrifuged at 11 000 g for 5 min to produce a clear liquid phase. The extract (0.6 mL) was dissolved in absolute ethanol (0.9 mL) which gives an ethanol concentration of 60% and left to rest at room temperature for at least 10 min in order to precipitate and separate proteins and polysaccharides. Further, it is centrifuged at 11 000 g for 5 min, and the supernatant is then filtered through a 0.45 µm PVDF syringe filter. After extract collection, subject it to a few hours of rotary evaporation. The extract is now prepared for HPLC analysis [27].

High-Performance Liquid Chromatography (HPLC) for Quantification of SDG and Anethole

Fennel seed and flaxseed extracts were quantified and isolated to ascertain the composition and concentration of compounds abundantly present in the extracts [27, 30]. For this purpose, high-performance liquid chromatographic analysis was used; "it was performed using a Shimadzu model LC-10A (Shimadzu, LC-10A, Japan) outfitted with a solvent delivery system, guard cartridge column, photodiode array detector, and integrator. Shimpack RP-C18 column with a particle size of 5 m, an inner diameter of 4.6 mm, and a length of 250 mm. SPD 10M AVP detector array and C-R7A integrator with class-10A software real-time analyzer. The mobile phase was a linear gradient of 1% (v/v) acetic acid in water (solvent A) and methanol (solvent B). The following linear gradient profile was performed at a flow rate of 1 mL min^{-1} for 55 minutes. A) 100% at 0 minutes, A) 40% B) 60% at 44 minutes, and A) 100% and B) 0% at 55 minutes. The solvent (mobile phase) was allowed to run at the initial gradient for three to five minutes before the next sample was injected." The column thermostat was set to 40ºC, and organic compounds that migrated were detected using absorbance at 280 nm [30, 31]. Compounds abundantly present in fennel seed and flaxseed extracts were isolated and quantified [32, 33].

Antioxidant Activity of Fennel Seed and Flaxseed

ABTS Free-Radical-Scavenging Activity Analysis

For determining the ABTS (2,20 -Azino-bis(3-ethylbenzthiazole-6-sulfonic acid) radical-scavenging capacity of Fennel seed and Flaxseed extracts, the method developed by Deng et al. [34] a little modification by [35]. "5 mL of 2.45 mM potassium persulfate solution was dissolved in 5 mL of 7 mM ABTS solution to produce ABTS radicals. The mixture was transferred to an amber container lined with aluminum foil and left in the dark for 16 hours until a stable oxidation state was reached. At 734 nm, the absorbance of the reaction mixture was adjusted to a value of 0.700. In order to accomplish this, the prepared mélange was further diluted with ethanol solution (1:89, vol/vol). After adding 1 mL of ABTS solution to 10 mL of Fennel seed and Flaxseed extracts and mixing vigorously for 10 seconds with a vortex mixer, the absorbance was measured at 734 nm using a UV-visible spectrophotometer 30 minutes later. The antioxidant capacity was measured against the standard trolox." The values derived from the calibration curve were reported in nM Trolox per gram of sample extract.

Ferric Thiocyanate (FTC) Method

The FTC method was used to evaluate the extract's antioxidant activity [36]. The developed hue was measured with a spectrophotometer at 532 nm. "Four milligrams of sample extract are weighed and then combined with four milliliters of absolute ethanol, four milliliters of 2.52% linolenic in absolute ethanol, four milliliters of 0.05 M phosphate buffer (pH 7) and three milliliters of distilled water. The mixture solution is mixed in a screw-capped tube and then placed in a 40ºC oven for approximately 10 minutes. Taking 0.1 mL of the solution, 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate are added. Exactly 3 minutes after adding 0.1 mL of 0.02 M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance is measured at 532 nm. As standards, gallic acid, L-ascorbic acid, and -tocopherol were utilized" [37]. The formula for inhibition percentage:

$$\% \text{ Inhibition} = 100 - [(A1 / A0) \times 100]$$

"Whereby A0 is the absorbance of the control reaction and A1, is the absorbance in the presence of the sample extract"

β-Carotene Bleaching Method

The β-carotene bleaching method was used to evaluate the antioxidant activity of the extract [38]. A Spectronic 20 spectrophotometer was used to measure the absorbance at 470 nm. Using an equation,

the antioxidant activity (AA) was expressed as a percentage of inhibition relative to the control.

$$AA(\%) = 100\left(1 - \frac{(A_0 - A_t)}{(A_0^0 - A_t^0)}\right)$$

"where A_0 and A_0 0 are the absorbance values measured at zero time of incubation for the test sample and control respectively and At and At 0 are the corresponding values at the end of the reaction time."

Statistical Analysis

The data were presented using the mean standard deviation (SD) format. To determine the significance of group differences, one-way analysis of variance (ANOVA) was utilized. Multiple comparison analyses utilizing the least significant difference (LSD) with P values less than 0.05 (SPSS 17) were also conducted [39].

Results and Discussion

In this investigation, the HPLC technique guaranteed the accuracy of the chemical analysis results. Validation of the HPLC method was therefore performed in this study. Five separate standard solutions of SDG ranging from 0.0625 to 1.0 mg/mL^{-1} were used to generate the linearity curve. For each SDG solution, the linearity curve's correlation coefficient (r = 0.9995) was determined. The peaks of SDG in the samples were identified and quantified by comparing them to standard solutions of SDG. By injecting 10 µl of standard solutions, a linear curve for standard SDG was generated, and the amount of SDG was calculated using a linear regression equation. By routinely administering standard solutions of SDG and calculating the relative standard deviation as a percentage, the repeatability of the method was determined. The calibration curve of standard SDG is depicted in Fig. 2. The standard SDG calibration curve for the concentration range of 0.0625 to 1.0 mg/ mL^{-1} is displayed below. The linear equation was y = 3933.9 x + 90.625, and the R2 value was 0.9995. The SDG was retained for 38.27 minutes. As shown in Table 2, the SDG concentration in the samples was calculated to be 8.35±0.52 mg/g after scanning at 240-600 nm. Similar findings were reported by Meagher et al. [29] who demonstrated that 8.44 mg/g of SDG was extracted from flaxseed [40]. For Anethole, the exact same procedure was undertaken. The linearity curve was generated using five standard solutions of anethole

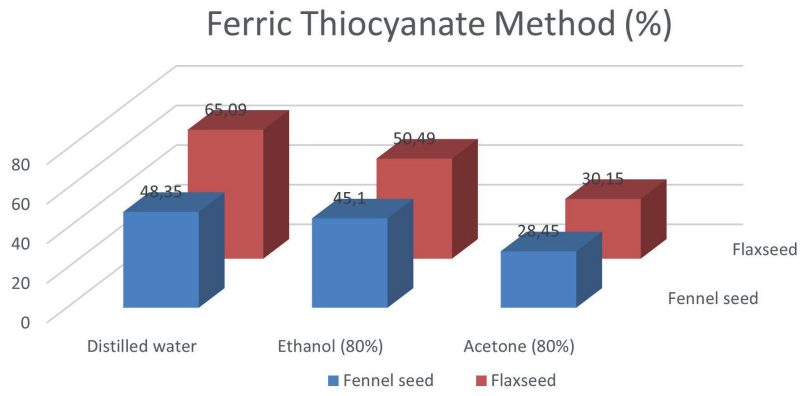


Fig. 1. Antioxidant activity of fennel and flaxseed solvent extracts by Ferric thiocyanate method (%).

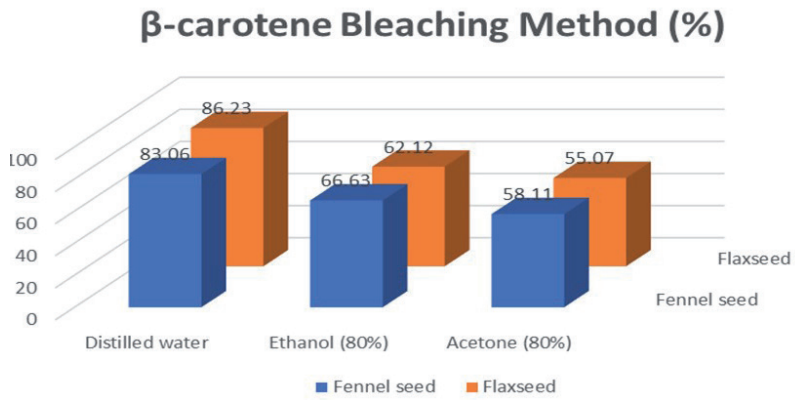


Fig. 2. Antioxidant activity of fennel and flaxseed solvent extracts by β-carotene bleaching method (%).

Table 1. The extracted percentage in both fennel seeds and flaxseed.

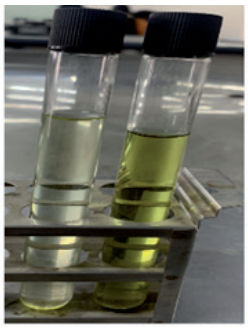
Medicinal Plants	Plant parts	Extract Concentration (%)	Color	
Fennel Seeds	Seed	5.72	Green	
Flax seed	Seed	6.31	Lite yellow	

Table 2. Quantification of major constituents of fennel seed and flaxseed

Compounds	Content (mg/g)*
Anethole	11.30±0.25
Secoisolariciresinol diglycoside	8.35±0.52

*Values are mean±SD of three independent experiments.

Table 3. Means for ABTS assay (mM TE/g) of fennel and flaxseed solvent extracts

Solvents	Fennel seed	Flaxseed
Distilled water	4.26±0.028^{c}	3.12±0.36^{c}
Ethanol (80%)	5.70±0.27^{b}	6.24±0.31^{b}
Acetone (80%)	7.28±0.17^{a}	8.29±0.23^{a}

Data is represented as Mean±SD (n = 3). Means with different superscript letters are significantly different at p≤.05.

ranging in concentration from 0.0625 to 1.0 mg mL^{-1}. The linearity curve correlation coefficient (r = 0.9999) was determined for all anethole solutions. Anethole peaks in the samples were identified and quantified using anethole standard solutions as a comparison. By administering 10 µl of standard solutions, the linear curve for standard Anethole was generated, and the quantity of Anethole was calculated using the linear regression equation. By routinely administering standard solutions of anethole and calculating the relative standard deviation as a percentage, the repeatability of the method was determined. The calibration curve for anethole standard over the concentration range of 0.0625 to 1.0 mg/mL^{-1}. The retention duration for Anethole was 41.21 minutes. As shown in table no. 2, anethole was also scanned at 240-600 nm, and its concentration in the samples was calculated to be 11,300.25 mg/g. Krizman et al. also reported similar findings, showing that fennel seed extracts of Anethole contain 14.44 mg/g [41].

Flaxseed extract were identified. Secoisolariciresinol diglucoside (SDG) (2R,3R)-2,3-Bis[(4-hydroxy-3-methoxyphenyl)methyl]butane-1,4-diyl di--D-glucopyranoside, phylloquinone, sucrose, palmitic acid, and Palmidrol are also present in flaxseed [33, 40]. Due to its potent antiproliferative, antioxidant, antiestrogenic, and/or antiangiogenic properties, secoisolariciresinol diglucoside has been demonstrated to prevent certain cancers, including breast, lung, and colon cancers [42]. Palmitic acid is abundant in flaxseed. Palmitic acid supports metabolic, epidermal, and anti-inflammatory health. A high ratio of Palmatic Acid to other healthy lipids may raise cardiovascular disease risk [43]. Fennel seeds also contain primary components like "Trans-Anethole, Limonene, fenchone, Estragole, and Oleic acid". Trans-anethole levels are higher in fennel seed extract, which is consistent with recent study by Odeh & Allaf [27]. Additionally, fennel seeds are abundant in trans-anethole, which may help to improve cardiovascular health, stimulate estradiol production, and fortify the respiratory system. Moreover, chemopreventive, anti-diabetic, immunomodulatory, neuroprotective, antimicrobial, and antithrombotic properties have been attributed to it [44].

Antioxidant Analysis

ABTS Free-Radical-Scavenging Activity Analysis

Analysis of variance for ABTS assay of fennel seed and flaxseed solvent extracts revealed a significant effect of different solvents and their concentrations. Acetone extracts had the highest ABTS value (7.28±0.17 mM TE/g), followed by ethanol (5.70±0.27mM TE/g) and distilled water (4.26±0.028 mM TE/g) with fennel seed [45]. As shown in Table 3, acetone extracts of flaxseed yielded the best results (8.29±0.23 mM TE/g), followed by ethanol (6.24±0.31 mM TE/g) and distilled water (3.12±0.36 mM TE/g), Similar results was fond by Waszkowiak & Gliszczyńska-Świgło [46] for flaxseed, who mentioned that ethanolic extract of flaxseed exhibited 5.06±0.28 mmol Trolox g^{-1}) ABTS radical-scavenging activity while aqueous extract showed 3.83±0.17 inhibition. Therefore, it can be concluded that fennel seed and flaxseed are abundant in phytochemicals with high antioxidant potential.

Antioxidant Activity by Ferric Thiocyanate Method

In actual food systems, lipids and water coexist in multiple phases with an emulsifier. Therefore, an antioxidant test utilizing a heterogeneous system, such as an oil-in-water emulsion, is required. Autoxidation of linoleic acid in ethanol buffer is one of the model systems for this evaluation that satisfies the aforementioned standards [36]. Evaluation was conducted using the linoleic acid emulsion system/ thiocyanate method under these conditions. During the peroxidation of linoleic acid at 37ºC in an incubator, the absorbance values increased due to the formation of ferric thiocyanate, which is the color of red blood [47]. At a concentration of 0.5 mg/mL, the antioxidant activity of flaxseed extracts in distilled water, ethanol (80%), and acetone (80%) was 65.09±0.63%, 50.49±0.72%, and 30.15±0.88%, respectively. This result aligns with a previous study conducted by Punia & Deen [48], found that ethanol to have the highest antioxidant activity, followed by acetone and distilled water. For fennel seed, the antioxidant activity exhibited by distilled water, ethanol (80%) and acetone (80%) of fennel seed were 48.35±0.19%, 45.10±0.34% and 28.45±0.11% as shown in Fig. 1. Similar results were found by [49], who also noted that distilled water and ethanol inhibit peroxidation in the linoleic acid system by 99.1% and 77.5%, respectively.

β-carotene Bleaching Method (%)

The antioxidant activity of carotenoids is based on radical adducts of carotenoids with free radicals from linoleic acid in the β-carotene bleaching method. The free radical of linoleic acid affects the highly unsaturated β-carotene models. By neutralizing the linoleate free radical and other free radicals formed in the system, the presence of various antioxidants can inhibit the extent of β-carotene bleaching [38]. Thus, the antioxidant activity of additional compounds may be measured. The result of the current study showed that distilled water showed higher antioxidant activity 66.63±0.05% followed by ethanol (80%) 66.63±0.05% and acetone (80%) 58.11±0.11% in fennel seed, Similar results were found by Oktay et al. [50]. In the same way, flaxseed showed higher antioxidant activity with water 86.23±0.38% followed by ethanol (80%) 62.12±0.15% and acetone (80%) 55.07±0.72% as presented by Fig. 2. The result of the current study is in harmony with the previous research done by Punia & Deen [48].

Conclusions

Flaxseed and fennel seeds are the most abundant and nutritious food moieties in their family and have great importance in the field of phytochemicals and nutrition. HPLC method was validated and utilized for the isolation and quantification of SDG and Anethole by comparison to established standards. The predominant constituent in fennel seed was determined to be anethole (11.30±0.25 mg/g) and secoisolariciresinol diglucoside (SDG) (8.35±0.52 mg/g) in flaxseed compared to the respective standards solution. As well as their concentrations, flaxseed and fennel seeds have been identified as significant sources of phenolic compounds known as antioxidants. Therefore, it may be concluded that flaxseed and fennel seeds are an exceptional source of disease-preventing compounds. Future research on flaxseed and fennel seed can be expanded in the interest of human welfare.

Authors Contributions

All authors contribute equally to this work.

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Conflict of Interest

The authors declare no conflict of interest.

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