ULTRASOUND ASSISTED DETERMINATION OF TOTAL PHENOLIC AND FLAVONOID CONTENT OF MEDICINAL PLANT FOXNUT (EURAYLE FEROX)

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ABSTRACT

The foxnut known as makhana, is primarily found and cultivated in tropical and subtropical regions. In the current study, the antioxidant parameters like total phenolic content, DPPH radicals, and total flavonoids were determined from roasted and unroasted foxnut seed extract under ultrasound assisted extractions. The solvent concentrations of methanol 100%, 80% methanol (80 to 20 v/v methanol to water ratio), 100% ethanol, 80% ethanol (80 to 20 v/v ethanol to water ratio), decoction, and distilled water were employed. These reactive groups present in the seed extractions of *Euryale ferox* specimens have been evaluated by using an effective analytical techniques. In numerous solvents, the whole flavonoids found in the plants of roasted and unroasted foxnut seeds reached from 9.02 to 16.23 GAE (mg/100g) and from 9.54 to 16.52 CE (mg/100g).The amount of total phenolic content extracted from roasted and un roasted samples ranged from 9.51 to 16.18 GAE(mg/100g). The DPPH radical scavenging action was shown by extracts with IC_{50} values from 0.075 to 0.312 mg/mL of roasted foxnut and 0.051 to 0.162 mg/mL of unroasted extracts. According to the findings, there was 58.07-85.29% and 76.95 to 96.31% of linoleic acid oxidation inhibition in the seeds of roasted and unroasted seeds, respectively. The extracts recovered using aqueous ethanol (ethanol: water, 80:20) showed the highest levels of antioxidant activity, whereas the extracts prepared using distilled water showed the lowest values.

Keywords: Antioxidant activity, DPPH, foxnut seeds, phenolic compounds, flavonoid compounds, solvent extraction

INTRODUCTION

Fox nuts have a long history of medicinal use, as seen by the ancient Chinese and Indian medical texts. Fox nuts have particular therapeutic qualities due to their antioxidant and phenolic contents (Lee, Duan, & Mattson, 2002). Important factors that determine the antioxidant activity and general nutritional value of food items include phenolic and flavonoid molecules. They have been shown to be effective in treating human diseases in numerous investigations (Miller et al., 2016). Nuts are the third-most phytochemical-containing food item, after fruits and spices. When compared to other nutrients, the antioxidant qualities displayed by phenolic compounds are incredibly strong (Biswas, Beura, & Sagar, 2016). The primary benefit of foxnut (makhana) is its antioxidant activity, which is linked to conditions like diabetic nephropathy and proteinuria inhibition. When evaluated with TEAC, DPPH, and CAT (Catalase) SOD (Superoxide Dismutase) activity, foxnut (*Euryale ferox Salisb*) extracts show increased level of radical scavenging behavior, decreasing power and being a powerful antioxidant because it reduces lipid peroxidation. Additionally, it boosts the activity of specific antioxidant enzymes, protects against H_2O_2-driven apoptosis, and enhances cell viability. Foxnut is a notable natural antioxidant source.The ability to be utilized as nutritional foods and dietary additives, as well as for the treatment of diabetes and hyperlipidemia. They contain the flavonoid, kaempferol. Flavonoids have the ability to reduce inflammation and the effects of free radicals (Tehseen et al., 2020).

Higher levels of polyphenols with stronger antioxidant activity can be present in roasted fox nuts. Roasting has an impact on the TPC (Total Phenolic Content) and TFC (Total Flavonoid Content) of makhana. Hence, roasting boosts antioxidant activity (Mohamed Ahmed et al., 2020). When roasted fox nuts are comparing to other nuts they have the similar antioxidant activity, they show a greater capacity to remove DPPH radicals, as opposed to cooked pistachios, cashews, and almonds (Ghazzawi & Al-Ismail, 2017). In conclusion, different nuts have various amounts of total phenols and flavonoids, however roasted fox nuts have more antioxidant extent than the majority of the nuts that are commonly ingested (i.e., cashews, pistachios, and pistachios). Foods' nutritional and bioactive qualities are impacted by roasting (Dzoyem & Eloff, 2015). Numerous variables, including condition of storage, cultivars, location, plant and

manufacturing procedure like roasting, bleaching and irradiation, etc., have an influence on the phytochemicals in nuts (Bolling, Chen, McKay, & Blumberg, 2011). These phytochemicals and anti-oxidants found in plant grid are extracted utilizing ultrasound-assisted extraction (USAE) which is an intriguing technique for acquiring highly valuable molecules that may raise the value of some food byproducts. The two biggest advantages will be the utilization of mild temperatures, which is advantageous for chemicals that are heat sensitive, and a more efficient extraction, which will save energy. A quantity of process variables should be taken into consideration for the USAE to be used successfully. These include the frequency, the extraction temperature, the solvent-sample interaction, the reactor properties, and the applied ultrasonic power (Hafeez et al., 2022).The initial few minutes are usually the most profitable, and during this time a high extraction rate is typically attained (Isleroglu & Turker, 2022).

One method being utilized to enhance these phytonutrients in food is roasting. Nuts are processed using it extensively to increase their nutritional value and general acceptance. Because they have a mushy feel, fox nuts must be roasted before eating. The phenolic and antioxidant profile of *Euryale ferox*; a wonder food must be maintained or improved during processing in order to get the full benefits. When kernels are roasted, they undergo browning reactions such as the Maillard reaction or non-enzymatic browning. Changes in the phenolic and antioxidant profiles, as well as color and texture, are caused by these reactions (Somporn, Kamtuo, Theerakulpisut, & Siriamornpun, 2011).

MATERIAL AND METHODS

Ultrasound Assisted extraction technique is applied for extraction of useful phytochemicals from seeds. The ultrasound assisted extraction works on the principle that ultrasound waves generate cavitations bubbles in the extraction solvent. When these bubbles collapse, they create intense local pressure and temperature, which disrupt the plant cell walls and enhance the solvent's penetration into the plant material. This facilitates the release of intracellular compounds into the solvent. The roasted and unroasted seeds fox nut *(Euryale ferox)* seeds were collected from local markete at Faisalabad, Pakistan. The seeds were kept at room temperature in sealed wrapper (Jain, Singh, & Kanjilal, 2010). The seeds were pulverized

into powder form utilizing a grinding mill. 5 gram powdered material was taken to add 10mL solvent (100% methanol) in to a flask. Flask is heated to 50–70˚C and subjected to ultra sonicator for two to three hours in order to extract the seed contents. The heated solution was filtered using Whatman filter paper to separate the solid residue ftom extract. Similar process was adopted with 80% methanol (80% to 20% methanol to water ratio), 100% ethanol, 80% ethanol (80% to 20% ethanol to water ratio), decoction, and distilled water. The rotatory evaporator was used to concentrate the extracted material at 45 ^{0}C. The concentrated material was stored at 4°C (Wang et al., 2012).

DETERMINATION OF EXTRACTION YIELD

The extraction yield from aqueous ethanol, aqueous methanol, pure ethanol, 100% methanol, decoction and distilled water was determined. The detailed results are discussed in next chapter.

DETERMINATION OF TOTAL PHENOLIC CONTENT (TPC)

The investigation of phenol concentration in foxnut seeds samples involved the utilization of the Folin-Ciocalteu reagent method. Two specific components (phosphomolybdate and phosphotungstate) were incorporated in this analytical approach. The Folin-Ciocalteu reagent consists of phosphomolybdate and phosphotungstate. Two specific components were incorporated in this analytical approach. Distilled water, the methanol extract, and Folin-Ciocalteu phenol reagent was added in a container. The mixture was allowed to incubate for about 6 minutes. sodium carbonate and distilled water was added to the mixture. The absorbance at 760 nm was measured through spectrophotometer. A calibration curve of absorbance values of standard solution (gallic acid) against different concentration was drawn. The calibration curve was further used to determine the concentration of phenolic compounds in the plant extract (Khan et al., 2021).

DETERMINATION OF TOTAL FLAVONOIDS

The methanol extract of foxnut was mixed with an aluminum chloride solution in flask. The mixture was allowed to stand for 60 minutes at room temperature. The absorbance of the resulting color was measured at 420 nm using a spectrophotometer. The concentration of TF was subsequently determined through catechin standardization curve within the series of 5-100 ppm. Additionally, amount of the total flavonoids was denoted as carbon efficiency per dried matter. Each set of experiments underwent three separate analyses. The total flavonoid content in the

extract as milligrams of catechin was expressed as equivalent per gram of the sample (Fatima et al., 2015)

Determination of Anti-oxidant Activity through In-Vitro Assays

The anti-oxidant activity of foxnut seed extract was determined using DPPH and lenolic acid model. For DPPH radical assay, a solution consisting of 6 ml of methanol was merged with DPPH at a concentration range of 0.005%. The methanol concentration ranged from 0.10 to 5.0 mg/mL. Subsequently, the resulting mixture was subjected to calcination at ambient temperature for the duration of approximately 20 minutes. The optical density (OD) value was then measured at 518 nm wavelength(Clarke, Ting, Wiart, & Fry, 2013).

$$I\%=[A_b \div A_s]\times 100$$

The sample absorbance, A_s, and the blank absorbance, A_b, are represented in the equation above.

$$\text{linoleic acid peroxidation inhibition } \%=100-\left[\left(\frac{\text{Abs.increase of sample at 360 h}}{\text{Abs. increase of control at 446 h}}\right)-100\right]$$

DETERMINATION OF IC_{50} VALUES FOR INHIBITORY CONCENTRATION

Estimating the IC_{50} concentration is essential for evaluating the inhibitory potential of a methanol extract. The IC_{50} value represents the concentration needed to achieve 50% inhibition of DPPH radical activity. In this study, the IC_{50} value for the seed extract was determined using a simple linear equation derived from the correlation between increasing extract concentrations and their corresponding inhibition of DPPH radicals. The IC_{50} value was expressed in milligrams per milliliter (mg/mL). Additionally, a comparative analysis was conducted by comparing the obtained IC_{50} values for the extract with those of synthetic antioxidants, such as butylated hydroxytoluene (BHT) and ascorbic acid (AA). This comparison provides context for the effectiveness of the natural extract relative to established synthetic antioxidants.

STATISTICAL ANALYSIS

The results were presented as the average ± standard deviation from three separate analyses. Duncan's multiple range tests revealed significant differences between treatment means at $p \leq 0.05$. A two-way ANOVA was performed to assess the impact of variables on biochemical parameters. All statistical analyses were conducted using SPSS software, Version 16.

RESULTS AND DISCUSSION

Extraction Yield

Ethanolic solution (water: ethanol, 20:80), 100% ethanol, decoction, absolute methanol, and deionized water may all be used to measure the (g/100g) of various extracts produced from certain foxnut seeds (Table 1). Roasted Foxnut seeds yielded fluctuating volumes of anti-oxidative extract throughout ranges of 7.54–16.58 g/100g of dry content, respectively. The highest produce, obtained from 80% ethanol, was 40%, while the lowest yield, obtained from deionized water made from foxnut seeds, was 25%. Similarly, the yield of different extraction from the unroasted seeds varied significantly and ranged from 7.98 to 14.91 g/100 g of dry substance, respectively. The greatest yield (38%) came from ethanolic solution, while the least produce (27%) came from deionized water produced from foxnut seeds (Song et al., 2011).

The liquid-liquid extraction capabilities were used in the following order: aqueous ethanol, aqueous methanol, pure ethanol, 100% methanol, decoction, and water from distillation, based on the findings obtained from all chosen samples. Alcohol with 80% ethanol content produced the maximum seed extraction yield, demonstrating the solvent's greater ability to recover antioxidant components. Based on the components used and the solvent system used, the extract yield varies significantly ($p < 0.05$) according to the available data (Carvalho Moreira, 2021).

Table 1: *Obtained extract (g/100g of solids) from Euryale ferox roasted and unroasted kernels*

Sr. No.	Extracts	Roasted kernels	Unroasted kernels
1.	100% Methanol	10.05 ± 0.12	11.76 ± 0.19
2.	80% Methanol	14.62 ± 0.22	13.04 ± 0.21
3.	100% Ethanol	12.04 ± 0.20	11.56 ± 0.29
4.	80% Ethanol	16.58 ± 0.19	14.91 ± 0.23
5.	Decoction	9.04 ± 0.25	9.50 ± 0.26
6.	Deionized water	7.54 ± 0.23	7.98 ± 0.24

The data illustrates the mean ± standard deviation from three distinct experiments different superscript characters within the single column indicate statistically important changes ($p<0.05$) in the mean values of the various removing solvents.

10.05g/100 g and 11.76g/100 g of solids are produced by synthesizing an antioxidant extract from *Euryale ferox* roasted and unroasted seeds in absolute methanol (Entry 1, Table 1). The antioxidant concentrate from foxnut seeds yields 14.62 g/100 g and 13.04 g/100 g of solids in 80% methanol, respectively (Entry 2, Table 1). As stated in Entry 3, Table 1, the antioxidant extract of foxnut seeds, roasted and unroasted, in 100% ethanol is 12.04 g/100 g and 11.56 g/100 g of solids, respectively. Moreover, 16.58 g/100 g and 14.91 g/100 g of solids, respectively, are the antioxidant extracts obtained from roasted and unroasted foxnut seeds in 80% ethanol (Entry 4, Table 1). As shown in Entry 5, Table 1, the antioxidant extract obtained by decoctioning foxnut seeds is 9.04 g/100 g and 9.50 g/100 g of solids, respectively. Finally, antioxidant concentrate made using foxnut seeds in deionized water had a concentration of 7.54 g/100 g and 7.98 g/100 g of dry material, respectively (Entry 6, Table 1) (Król, Amarowicz, & Weidner, 2015).

Ethanol, at an alcohol concentration of 80%, produced the optimal production of seed extracts, demonstrating the superior antioxidant recovery capabilities of this solvent. Ethanolic solution yielded the highest output, while the deionized water made from foxnut seeds produced the resulted in the lowest (Kedrina-Okutan et al., 2018).

TOTAL PHENOLIC CONTENT (TPC)

The total phenolics content in extracts of both roasted and unroasted seeds is presented in Table 1. The quantity of total phenolics extracted from the seeds of both roasted and unroasted samples varied from 9.51 to 16.18 GAE (mg/100g) and 8.01 to 16.33 GAE (mg/100g) across various diluents. The highest concentrations of TPC were detected in the roasted seeds of foxnut at 16.18 mg/100g in an ethanolic solution extract, while unroasted seeds exhibited the highest TPC values at 16.33 mg/100g in an ethanolicsolutionextract.The number of phenolic compounds recovered from foxnut seeds that had been roasted and those that hadn't using different solvents showed significant differences ($p < 0.05$). The disparity in the dissolving capabilities of various extraction solvents towards native floral antioxidants may explain the variation in Total Phenolic Compound (TPC) levels. The subsequent order delineates the hierarchy of solvents in extracting TPC: Ethanol in aqueous solution >Deionized water> Infusion > Ethanol in its purest form. Due to its remarkable efficacy in extraction and minimal toxicological effects, ethanol is frequently

utilized for the extraction of antioxidant compounds from botanical materials.(Zhang et al., 2021).

Table 2:*Total polyphenol concentration (GAE) in extracted roasted and unroasted Euryale ferox seeds (mg/100g of dry sample)*

Sr. No.	Extracts	Total phenolic content	
		Roasted seeds	Unroasted seeds
1	100% Methanol	10.98 ± 0.03	9.45 ± 0.02
2	80% Methanol	13.47 ± 0.01	14.13 ± 0.01
3	100% Ethanol	11.32 ± 0.02	11.01 ± 0.02
4	80% Ethanol	16.18 ± 0.01	16.33 ± 0.01
5	Decoction	10.01 ± 0.02	8.95 ± 0.01
6	Deionized water	9.51 ± 0.02	8.01 ± 0.01

The three independent tests' means and standard deviations make up the data. The mean value of the different extracted solvents varied considerably ($p<0.05$) depending on the unique superscript symbols in the similar column.

Both nuts roasted and unroasted in 100% methanol generate antioxidant extract levels of 10.98 g/100 g and 9.45 mg/100 g of solids, respectively (Entry 1, Table 2). For roasted and unroasted nuts, the antioxidant extract in 80% methanol is 13.47 mg/100 g and 14.13 mg/100 g of solids, subsequently (Entry 2, Table 2). The antioxidant extract is produced at 11.32 mg/100 g and 11.01 mg/100 g of solids, respectively, by submerging roasted and unroasted almonds in 100% ethanol (Entry 3, Table 2). The antioxidant extracts from the roasted and unroasted seeds yield values of 16.18 mg/100 g and 16.33 mg/100 g of solids, respectively, in 80% ethanol (Entry 4, Table 2). The decoction yields an antioxidant extract of 10.01 mg/100 g from roasted seeds and 8.95 mg/100 g from unroasted seeds, respectively (Entry 5, Table 2). According to Entry 6, Table 2, the concentrations of antioxidant extracts from roasted and unroasted nuts in deionized water are 9.51 mg/100 g and 8.01 mg/100 g, respectively (Swigonska et al., 2014).

TOTAL FLAVONOIDS

The total amount of polyphenol concentration found in the roasted and unroasted seed extracts is displayed in Table 2. In numerous solvents, the whole flavonoids found in the plants

of roasted and unroasted foxnut seeds reached from 9.02 to 16.23 GAE (mg/100g) and from 9.54 to 16.52 CE (mg/100g).When using different solvents for roasted and unroasted *Euryale ferox* seeds, there were discernible variations in the number of flavonoids extracted ($p<0.05$).The variation in the capacity of extraction solvents to solubilize natural floral antioxidant compounds could potentially account for the observed disparity in total flavonoid content concentrations.It was discovered TFC extraction by various solvents follows the subsequent order: ethanolic solutionrefined from water > Pure ethanol > 100% methanol > Decoction. The high extraction efficiency and low toxicity of ethanol have established it as a widely favored option for extracting antioxidant compounds from plant matrices.(Liaquat, Pasha, Ahsin, & Salik, 2022).

Table 3:*Concentration of total flavonoids CE (mg/100g of dry mass) GAE (mg/100g of dry specimen) in foxnut seed extracts, both roasted and unroasted*

Sr. No.	Extracts	Total amount of flavonoids	
		Roasted seeds	Unroasted seeds
1	100% Methanol	12.05 ± 0.01	11.46 ± 0.03
2	80% Methanol	14.56 ± 0.02	13.20 ± 0.01
3	100% Ethanol	13.01 ± 0.01	12.30 ± 0.02
4	80% Ethanol	16.23 ± 0.02	16.52 ± 0.01
5	Decoction	11.01 ± 0.01	10.17 ± 0.02
6	Deionized water	9.02 ± 0.03	9.54 ± 0.01

Three distinct experiments were conducted in order to obtain the mean ± SD data. Numerous symbols within the identical column signify noteworthy ($p<0.05$) differences in means among the various extracting solvents.

Roasted and unroasted seeds of *Euryale ferox* in 100% methanol form antioxidant extract at levels of 12.05 mg/100 g and 11.46 mg/100 g of solids, respectively (Entry 1, Table 3). Roasted and unroasted nuts provide antioxidant extracts of 14.56 mg/100 g and 13.20 mg/100 g of solids, respectively, in 80% methanol (Entry 2, Table 3). When processed in 100% ethanol, roasted and unroasted kernelsoutput 13.01 mg/100 g and 12.30 mg/100 g of solids of the antioxidant extract, respectively (Entry 3, Table 3). The antioxidant extract produced by roasted and unroasted seeds in 80% ethanol is 16.23 mg/100 g and 16.52 mg/100 g of solids, respectively (Entry 4, Table 3).According to Entry 5, Table 3, the antioxidant extract created by

decoction from roasted and unroasted seeds is 11.01 mg/100 g and 10.17 mg/100 g of dry content, respectively. Antioxidant extracts from roasted and unroasted seeds had concentrations of 9.02 mg/100g and 9.54 mg/100g in deionized water, respectively (Entry 6, Table 3)(Lee, Ju, & Kim, 2002).

CONCLUSION

This investigation examined the phenolic and flavonoid content (antioxidant properties) of roasted and unroasted foxnut seed extracts. The total flavonoids found in the plants of roasted and unroasted foxnut seeds ranged from 9.02 to 16.23 GAE (mg/100g) and from 9.54 to 16.52 CE (mg/100g) respectively. The amount of total phenolic content extracted from roasted and un roasted samples ranged from 9.51 to 16.18 GAE (mg/100g) and 8.01 to 16.33 GAE (mg/100g).The DPPH radical scavenging action was shown by extracts with IC_{50} values from 0.075 to 0.312 mg/mL of roasted foxnut and 0.051 to 0.162 mg/mL of unroasted extracts. According to the findings, there was 58.07-85.29% and 76.95 to 96.31% of linoleic acid oxidation inhibition in the seeds of roasted and unroasted seeds, respectively.

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