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Proximate Analysis and Estimation of Total Phenolics, Flavonoids, Ascorbic Acid in Methanolic Extracts of Plant Seeds - *Thymus vulgaris, Salvia hispanica, Nigella sativa, Anethum graveolens*

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ABSTRACT

Plant kingdom offers many herbs that are rich in micronutrients and secondary metabolites which have the potential to improve health by preventing and treating illnesses. These nutrients when consumed in the right amounts, helps to postpone aging and increase longevity. A large number of them are sold as dietary supplements. They are becoming more and more popular these days because of their nutritional worth as well as their medicinal purposes. Ginseng, Echinacea, green tea, glucosamine, omega 3, lutein, folic acid, and cod liver oil are a few well-known nutraceuticals. The usage of edible seeds and their oil has grown in popularity because they are rich in dietary fiber, proteins, vitamins, minerals, vital fatty acids, antioxidants, phenolic compounds, secondary metabolites, and carbs. In the present study four seeds namely *Thymus vulgaris*, *Salvia hispanica*, *Nigella sativa*, *Anethum graveolens* are analyzed for their phytochemicals and their nutrient content are estimated by standard methods. The results revealed that the selected seeds possess significant amount of crude fibers, proteins and secondary metabolites and thus can be used as functional foods to improve general health.

Keywords: *Nutraceuticals, Chronic diseases, Folic acid, Secondary metabolites, Antioxidants, Therapeutic property*

1. INTRODUCTION

Natural substances that are promoted as nutritional supplements and have therapeutic or health benefits are referred to as nutraceuticals. A recent development in the food and pharmaceutical sectors is nutraceuticals that is gaining a lot of attention because of its potential (1). Animal or plant-based foods can be used to make nutraceuticals, and worldwide research studies centered on their mode of action, safety, and clinical data. These are therapeutic agents that do not propose themselves as an alternative to drugs but instead can be helpful to prevent a cluster of conditions that could occur together (metabolic syndrome), e.g., type 2 diabetes, stroke, heart disease, and cardiovascular disease (2). Plant-based diets have been followed by various populations throughout human history to prevent early aging and to improve. The nutraceutical sector currently uses a number of phytochemical groups, including polyphenols (anthocyanins, proanthocyanidins, flavanones, isoflavones, resveratrol, and ellagic acid). Recent studies indicate that certain nutraceuticals have potential for both the prevention and treatment of various diseases, including allergies, Alzheimer's disease, cancer, heart and eye conditions, obesity, diabetes, and Parkinson's disease. They also show promise in the control of inflammation and immune system function (3, 4). The goal of the current study is to assess the seeds' nutritional worth. The seed chosen were *Thymus vulgaris* (S1), *Salvia hispanica* (S2), *Nigella sativa* (S3), *Anethum graveolens* (S4).

Many aromatic plants with notable industrial value, including rosemary, sage, mint, marjoram, oregano, and thyme, are part of the Lamiaceae family. Thyme, recognized for its pleasant fragrance, is a perennial shrub that includes more than 350 aromatic species, each with distinct botanical traits and a wide range of chemical diversity (5, 6). *Thymus vulgaris* is a flowering herb belonging to the Lamiaceae family, widely recognized as thyme, which originates from Southern Europe and is found all over the world. Growing all across the world, *T. vulgaris* is an annual grass-like plant. Its expectorant, antitussive, antibroncholytic, antispasmodic, antihelminthic, carminative, and diuretic qualities see widespread application in traditional medicine. Along with being a well-known source of flavoring, it is also utilized as a culinary herb (7).

Ancient Aztec and Maya tribes in Mesoamerica used *Salvia hispanica* L. in addition to corn, beans, and amaranth to make food and folk remedies. After beans, it was the second most significant crop in pre-Columbian societies. Chia was utilized for religious ceremonies, nourishment, and cosmetics in Aztec cultures. White and purple blooms are produced by *Salvia hispanica L*., which is primarily grown for its seeds. In general, chia seeds are oval-shaped, tiny, and less than 1 mm thick. They are 2 mm long and 1 to 1.5 mm wide (8, 9, 10, 11). Researchers have found that the year of production, nutrients, climate, geographical location and other factors can all impact the chemical makeup and nutritional values. For example, the altitude of the plant and climatic change may affect the fatty acid composition; the higher the altitude and the colder the area, the more ω-3 unsaturated fatty acid is present (10)]. Scientists currently refer to chia seeds as the 21st century's "golden seed." (12).

The species *Nigella sativa L*. is found throughout the nation and is naturally dispersed. It is widely grown throughout different parts of Iran. It is thought that the seeds possess anticancer, antiparasitic, laxative, carminative, and galactagogue qualities (13 - 15). Early herbal experts regarded *Nigella sativa* as "The herb from heaven" and have referred to it as a magical plant. [16]. In traditional medicine, *Nigella sativa* seeds were used to treat a variety of conditions, such as asthma, infertility, and other gastrointestinal issues. Furthermore, *N. sativa* seed oil is used to treat eczema, orchitis, swollen joints, nasal ulcers, and abscesses. It is also useful in conjunction with honey to treat bronchospasms, chest congestion, and asthmatic issues (17).

The annual herb *Anethum graveolens* has leaves that are pinnately split. The spherical stem of the plant is capable of growing to a height of 150 cm, and two to five branches emerge from its base and accompany the main stem. The flowers have a yellow hue. Dill, or *Anethum graveolens* L., is thought to be indigenous to either South-east Europe or South-west Asia (18). Anethum is indigenous to the Mediterranean, southern USSR, and Central Asia, and has been used as a condiment and medicine since the time of the Egyptians (19). Rajasthan, Gujarat, J&K, Orissa, Madhya Pradesh, and Punjab are among the Indian states where it can be found (20). After maturing, the seeds turn light brown and release a fragrant scent. In Unani medicine, it is employed to treat digestive issues, colic, and diarrhea (21). More than 56 ayurvedic remedies make use of *Anethum graveolens* L. to treat conditions like "vata," "kapha," ulcers, stomachaches, eye disorders, and uterine difficulties (22).

2. MATERIALS AND METHODS

Preparation of Seed Extract

Seeds were procured from National Seeds Corporation Ltd., Seeds were dried and powdered. 10% of the seed extract was made using three different solvents namely water, methanol and ethyl acetate. Samples of powdered seeds were soaked using different solvents for 3 days. The contents were homogenised on a hot plate at 40oC using magnetic stirrer and at 4000 rpm, the crude extract was centrifuged for 15 minutes. The liquid supernatant was kept for later use in airtight containers. Qualitative analysis was conducted with these extracts using conventional protocol to determine the presence of phytochemicals.

Proximate Analyses

Each of the four seed samples was examined for ash, moisture, protein, fiber, fat and utilizable carbohydrate by the methods of AOAC (23, 24).

Estimation of ash Content

Dry and clean crucibles were weighed accurately. Approximately 2 g of the sample was taken in a crucible and weighed accurately. The crucibles were dried in the hot air oven at 600^{o}C for 15 min till charred. After cooling, few drops of strong nitric acid were added to the sample and kept in the muffle furnace till a pure white ash was found. Then it was cooled and weighed accurately. From the weights, the amount of ash content was determined as %.

$$\text{Ash (\%)} = \frac{(m_3 - m_1)}{(m_2 - m_1)} \times 100$$

Where, m_1 is the mass of the crucible (g), m_2 is sampled mass with crucible (g) and m_3 is final mass of sample with crucible (g).

Estimation of Moisture Content

An empty clean and dry china dish was weighed accurately (W_1). Approximately 2g of the sample (finely ground powder) was added to the china dish and weighed accurately (W_2).

This sample was dried for 6-12 hours at 110°C in a hot air oven until its weight remained consistent. The crucible was then left in the desiccators to cool for half an hour. It was weighed once again once it had cooled (W_3). The moisture content was computed as percentage from the weights using the formula.

$$\text{MC (\%)} = \frac{(W_2 - W_3)}{(W_2 - W_1)} \times 100$$

Where, MC = Amount of moisture in the sample (%), W_1= Mass of dish (g), W_2 = Mass of

Sample and dish before drying (g), W_3 = Mass of sample and dish after drying (g).

Estimation of total protein by Lowry's method

A modified version of Lowry's technique was used to calculate the total protein content. A 0.5 g sample was weighed, ground in a mortar and pestle with 5 ml of 0.01M sodium phosphate buffer (pH 7), and centrifuged for 15 minutes at 8000 rpm. 0.1 ml of supernatant was made up to 1 ml with distilled water and mixed with 5 ml alkaline copper reagent. After 10 minutes of standing at room temperature, 0.6 ml of the Folin-Ciocaltcau reagent (1:1 dilution) was added, and it was then incubated for 30 minutes at room temperature in the dark. The absorbance was then measured at 660 nm. The calibration curve was generated using bovine serum albumin (BSA) as the reference standard. The linear equation of a standard curve made using BSA was used to calculate the total protein content (25).

Estimation of fiber content

One gram of the sample was dissolved in fifty milliliters of 1.25% sulfuric acid in a crucible. After 15 minutes at 70°C on a hot plate, this solution was filtered, and 50 milliliters of sodium hydroxide were added to the sediment. Once again, this solution was heated to 70°C for 15 minutes on a hot plate. The estimated total fiber content was expressed in grams.

$$\text{Crude Fiber (\%)} = \frac{(W_2 - W_1) - (W_3 - W_1)}{\text{Weight of the sample}} \times 100$$

Where W_1= Mass of dish (g), W_2 = Mass of sample and dish before ashing (g), W_3 = Mass of sample and dish after ashing (g).

Crude fat

Ether extract as an estimate of crude lipid was determined using soxhlet extraction method. The solvent, then was evaporated by heating on a steam bath. The flask containing the extracted fat was dried on a steam bath to a constant weight. The percent crude fat was determined by using the following formula:

$$\% \text{ fat} = [(W_3 - W_1) / (W_2 - W_1)] \times 100 / \text{Weight of the sample}$$

Where W_3 is dried mass of fat with beaker (g), W_1 is a mass of the beaker (g), W_2 is the mass of the sample in the beaker.

Total carbohydrate (CHO) was calculated by difference after analysis of all the other items in the proximate analysis.

$$\text{CHO} = 100 - (\% \text{ moisture} + \% \text{ crude protein} + \% \text{ crude fat} + \% \text{ ash} + \% \text{crude fiber})$$

3. QUANTITATIVE ANALYSIS

Estimation of total Polyphenolics

The amount of polyphenol in the various extracts were determined by Folin-Ciocalteu reagent method with some modifications (26 - 28). One ml of seed extract was mixed with 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml of 2% Na2CO3 solution. At room temperature, the resultant mixture was incubated for 15 minutes. The absorbance of the sample was measured at 765nm. 0.1 mg/ml of gallic acid was utilized as the standard. Every test was conducted in triplicate. Based on the standard curve, the results (Table 4) were calculated and presented as gallic acid equivalent (mg/g of extracted compound).

Estimation of Flavonoids

To ascertain the flavonoid content, the aluminum chloride colorimetric method (24) was applied with slight modifications. 3 ml of methanol, 0.2 ml of 10% aluminium chloride, 0.2 ml of 1M potassium acetate, and 5.6 ml of distilled water were combined with 1 ml of seed extract (10%) and allowed to stand at room temperature for half an hour. At 420 nm, the absorbance was measured. Quercetin (1 mg/ml) was utilized as a standard. Triplicates of each test were conducted. The standard curve was used to calculate the flavonoid contents, which were then represented as quercetin equivalent (mg/g of extracted substance).

Estimation of Ascorbic acid

The 2,4-DNPH technique (29) was used to determine the ascorbic acid content of each of the four seed samples. A calibration curve with varying concentrations, ranging from 1 to 10 µg/ml, was created using the appropriate dilution technique with ascorbic acid. Sample extract is prepared by blending 1g of sample in the blender. The sample was combined with 50 ml of a 5% metaphosphoric acid-acetic acid solution in a 250 ml conical flask. Remaining amount of 50ml of 5% metaphosphoric

acid solution was added into the flask. Solution was filtered through Whatman filter paper, and one ml of the filtrate was used to measure the amount of vitamin C. Few drops of bromine solution were added to one ml of the filtered sample solution and stirred. The surplus bromine solution was then eliminated from the sample solution by adding a few drops of thiourea solution. All of the standard ascorbic acid solutions and the sample solution were then mixed with 1 ml of 2,4-DNPH solution. Coupling reaction occurs due to 2,4 DNPH solution. All of the standards and sample solution were held at 37°C for three hours in order to complete the reaction. Five ml of H_2SO_4 were added after the solutions had cooled on an ice bath. absorbance was measured at specific wavelength.

4. RESULTS AND DISCUSSION

Following conventional protocols, a qualitative examination of the selected seeds' extract in methanol, water and ethyl acetate was carried out and the results are shown in Table 1. The findings indicated the existence of bioactive substances like carbohydrates, alkaloids, saponins, proteins, flavonoids and are tabulated in Table 2. Tannins were absent in all the seed extracts and terpenoids were absent in *Thymus vulgaris and Salvia hispanica.*

Table 1: Procedure for the phytochemical analysis (30 - 33)

Phytochemicals	Procedure	Observation	Inference
Reducing sugars	(a) (a) 2 ml of extract + Molisch reagent. Added con. H2SO4 along the sides (b) 5 drops of extract + Benedict's reagent - boiled in a water bath (c) 2 ml of extract + Fehling's solution A and B in equal volume – boiled in a water bath.	(a) Reddish Violet ring (b) Orange precipitate (c) Reddish brown Precipitate	Present
Alkaloids	2ml of extract + dil HCl. Filtered. (a) 1 ml of filtrate + 1 ml of Mayor's reagent. (b) 1 ml of filtrate + 2 ml of Wagner's reagent. (c) 1 ml of filtrate + 1 ml of Dragendroff's Reagent.	(a) white / cream precipitate (b) Reddish brown precipitate (c) Orange red precipitate	Present
Saponins	1 ml of extract + 2 ml distilled water + shook vigorously for 2 minutes	Frothing persists for 10 minutes	Present
Fixed oil	1 ml of extract + 0.2 ml KOH (0.5N) + 1 drop of phenolphthalein. Boiled in a water bath.	Disappearance of pink colour	Present
Flavonoids	1 ml of the extract + 20% of NaOH	Yellow colour turns to colourless on adding acid	Present
Steroids	1 ml of extract + glacial acetic acid. 1 ml of con. H2SO4 was added along the sides.	Appearance of greenish blue Colour	Present
Phenols	1 ml of extract + 5% neutral ferric chloride	Dark green / violet colour	Present
Proteins	1 ml of extract + 5% NaOH + 1% CuSO4 solution	Appearance of purple colour	

Terpenoids	5 ml extract + 2 ml chloroform	Reddish brown colour at interface	Present
Tannins	1 ml of extract + 20 ml of distilled water, Boiled in a water bath – filtered. 1 ml of cold filtrate + 5 ml of distilled water + few drops of 10% ferric chloride	Precipitate or any colour change	

Table 2: Phytochemical analysis of various extracts of the seed

Phyto chemicals	**Test**	***Thymus vulgaris***			***Salvia hispanica***			***Nigella Sativa***			***Anethum graveolens***		
		WE	MeE	EAE	WE	MeE	EAE	WE	MeE	EAE	WE	MeE	EAE
Carbohydrates	MoT	++	+	+	++	+	+	++	+	+	++	+	+
	BeT	+	-	-	++	++	-	+++	+	-	++	+	++
	FT	-	-	-	-	+	+	++	++	++	++	+	+
Alkaloids	MT	+	-	-	++	+	+	++	+	++	+	+	+
	WT	++	++	++	++	++	+	++	+	+	++	++	++
	DT	+	++	++	++	+	+	+	+	++	++	++	+
Saponins	FT	-	-	+	+	+	++	+	+++	+	-	+	+
Fixed Oil	ST	-	+	-	-	-	++	-	-	+	-	-	++
Flavonoids		+	+		++	+++	+	+	++	+	+	++	++
Steroids		-	+	+	-	++	-	-	+	-	+	+	+
Phenol		+	+	-	-	+	-	+	++	-	++	+++	-
Protein	BT	+	++	+	+	+++	+	+	+	-	++	++	-
Terpenoids	SaT	-	-	-	-	-	-	+	+	++	++	-	+
Tannins		-	-	-	-	-	-	-	-	-	-	-	-

Note: *MoT-Molisch test; BeT-Benedict's test; FT-Fehling's test; MT-Mayer's test; WT-Wagner's test; DT- Dragendroff's test; FT-Foam Test; ST-Sap Test; BT-Biuret Test; SaT-Salkowski Test; WE-Water extract; MeE-Methanol extract; EAE – Ethyl acetate extract.*

Nutritional analyses of the seeds (Table 3) indicated the presence of crude fiber in all the four seeds. The percentage of crude fiber in the seeds were found to be 28.75 in *Anethum, graveolens*, 17.89 in *Salvia hispanica*, 14.2 in *Thymus vulgaris* and 5.5 in *Nigella sativa.*

Table 3: Nutritional analyses of seeds

		Thymus vulgaris	***Salvia hispanica***	***Nigella sativa***	***Anethum, graveolens***
1	Ash	11.1	3.2	4.5	6.8
2	Moisture	11.0	6.1	6.12	13.43
3	Protein	10.08	15.12	22.5	18.93
4	Crude Fiber	14.2	17.89	5.8	28.75

5	Crude fat	6.11	30.23	30.9	9.40
6	Carbohydrate	47.51	29.13	30.12	22.69

Note: *Values are given as percentage*

Thymus vulgaris had the lowest protein content (10.08%) while *Nigella sativa* had the highest (22.5%). among the four seeds. *Salvia hispanica* and *Nigella sativa* were found to contain almost same proportion of crude fat. Fibers play a significant role in the physical structure effect, which involves binding food into big particles. This characteristic of the fibers may lengthen the time and effort required for mastication in the mouth and brings satiety (34 - 36).

In Folin Ciocalteu's method, the total phenolic content of the seed extracts was measured using gallic acid as a standard. The mixture of phosphotungstic acid and phosphomolybdic acid that forms the reagent is converted to a mixture of blue tungsten and molybdenum oxides following the phenols' oxidation. The blue hue that is created is proportionate to the entire amount of phenolic compounds that were initially present and exhibits a maximum absorption in the 750 nm range. The phenolic content (Table 4, Fig.1a, 1b) was found to be in the order *Salvia hispanica* > *Anethum graveolens* > *Nigella sativa* > *Thymus vulgaris.* However, *Anethum graveolens* was found to have a larger flavonoid concentration (Table 5, Fig. 2a, 2b) than the other three seeds.

Table 4: Amount of polyphenolics (mg) in different seed extracts

Seed Extract	**Amount of total phenolics in mg per ml of the extract**
Thymus vulgaris (S1)	0.029 ± 0.002
Salvia hispanica (S2)	0.072 ± 0.001
Nigella sativa (S3)	0.039 ± 0.004
Anethum graveolens (S4)	0.069 ± 0.001

Note: Values are expressed as amount ± SD; n=3

Table 5: Amount of flavonoids in mg per ml expressed as quercetin equivalents in different seeds

Seed Extract	**Amount of flavonoids in mg per ml of the extract**
Thymus vulgaris (S1)	0.023 ± 0.001[a]
Salvia hispanica (S2)	0.045 ± 0.007[b]
Nigella sativa (S3)	0.086 ± 0.007[c]
Anethum graveolens (S4)	0.143 ± 0.002[b]

Note: Values are expressed as amount ± SD; n=3

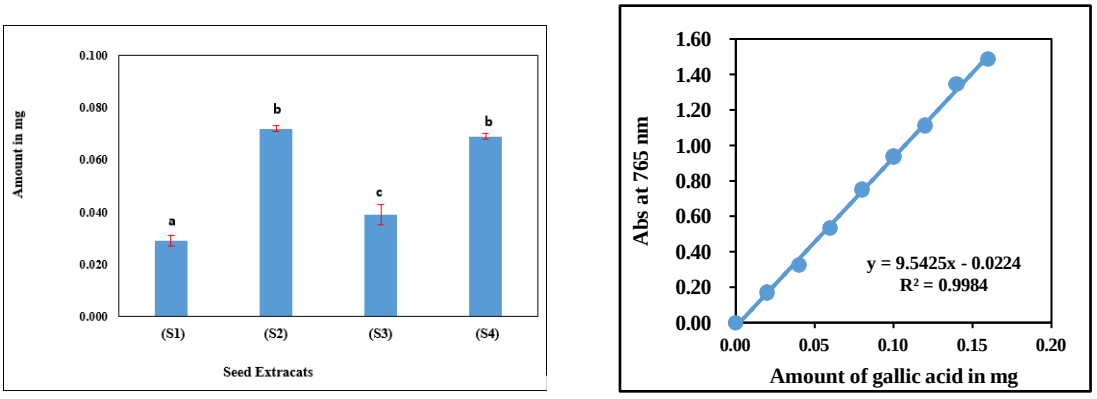

Fig. 1a: Estimation of total phenolics, (b) Amount of total phenolics expressed as gallic acid equivalents (mg per ml) of the seed extract. *Thymus vulgaris* (S1), *Salvia hispanica* (S2) *Nigella sativa* (S3) and *Anethum graveolens* (S4)

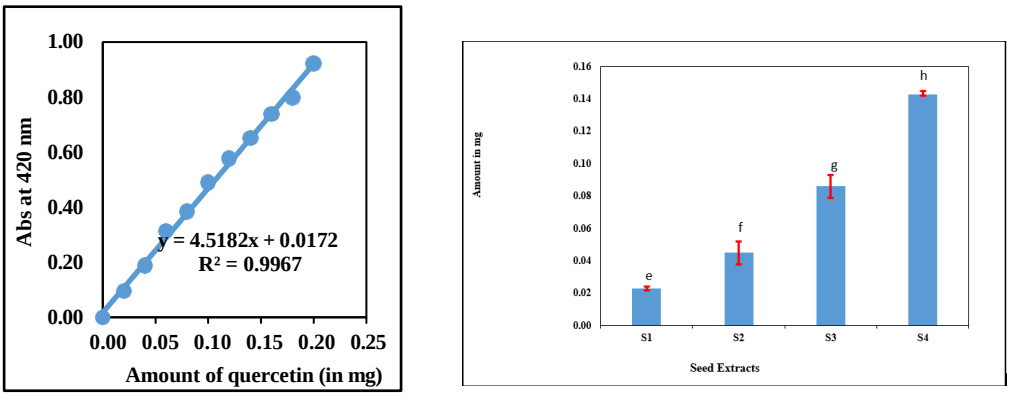

Fig 2a: Estimation of flavonoid, (2b) Amount of flavonoid expressed as quercetin equivalent (mg per ml) of the seed extract. *Thymus vulgaris* (S1), *Salvia hispanica* (S2) *Nigella sativa* (S3) and *Anethum graveolens* (S4)

Ascorbic acid, also known as vitamin C, can retain the membrane-bound antioxidant α-tocopherol in its reduced state and remove harmful free radicals and other reactive oxygen species that are produced during cell metabolism and linked to a number of diseases and tissue damage (37). As a necessary micronutrient, this vitamin is mostly obtained from eating fruits and vegetables (38). The potential of ascorbic acid is to replenish vitamin E and certain other antioxidants (39). Ascorbic acid content (Table 6, Fig 3a, 3b) of *Anethum graveolens* and *Nigella sativa* are comparable and ascorbic acid content is highest in *Thymus vulgaris* among all the seeds. Similar findings were observed by many researchers in India, who studied non-spice medicinal plants and their various biological applications for treating diseases (40-46).

Table 6: Ascorbic acid content in different seed samples

Seeds	**Amount (in mg) of ascorbic acid per 100 g of seed**
Salvia hispanica	2.3 ± 0.002[a]
Thymus vulgaris	84 ± 0.001[b]
Anethum graveolens	27.8 ± 0.001[c]
Nigella sativa	26.6 ± 0.001[c]

Note: Values are expressed as amount ± SD; n=3

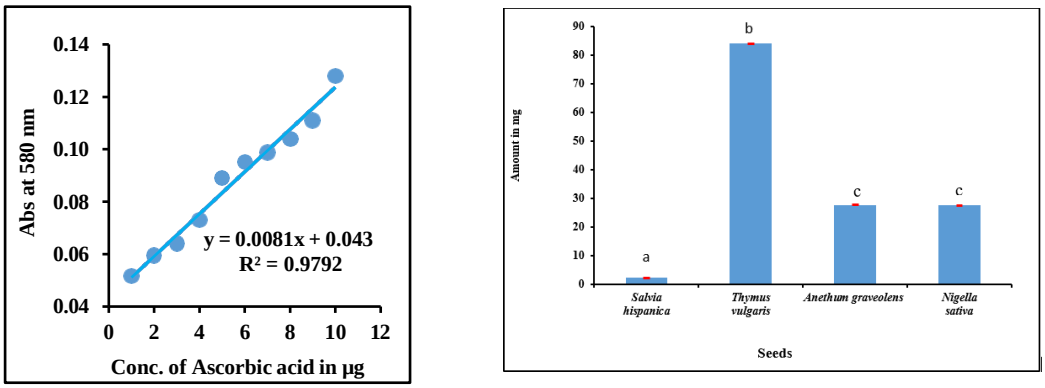

Fig 3a: Calibration curve of ascorbic, (3b) Amount (in mg) of Ascorbic acid in 100 g of the seed

5. CONCLUSION

Food security is a major issue that the entire world faces. Improved use of underutilized grains, such as thyme, chia, nigella, dill seeds, can be a useful choice to combat nutritional security because of their broad nutritious makeup. In recent years, plant-based raw materials have gained a lot of attention due to their high nutritional value. Based on the study's findings, we draw the conclusion that consumers should be aware of the advantages of consuming a variety of medicinal plant herbs,

seeds, roots etc., and select those with the highest nutritional and therapeutic value to support a balanced diet. The substantial amounts of fat, crude fiber, and proteins in these selected seeds can make them as useful foods and aid in the prevention of diseases linked to poor nutrition. Flavonoids and phenolics found in these extracts may help manage diabetes and associated consequences. Thus these seeds rich in bioactive contents should undoubtedly be used as functional food ingredients and as essential components of a balanced, healthful diet.

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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