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Studies on Nutrient and Phytochemical composition and Assessment of *in vitro* Antioxidant and Lipase activity of two commonly used edible seeds

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Abstract

Plants are a valuable source of bioactive compounds that possess notable pharmacological and nutritional benefits. Among these compounds, phytochemicals like phenolics, flavonoids, alkaloids, and terpenoids are recognized for their varied biological effects. Seeds tend to accumulate high levels of phytochemicals, which may offer protection against free radical damage and function as natural antioxidants. Antioxidants have the ability to stabilize or neutralize free radicals before they can harm cells, thus lowering the risk of degenerative diseases such as cardiovascular issues, atherosclerosis, and other related health problems through sufficient antioxidant consumption. This research focuses on both the qualitative and quantitative evaluation of phytochemical components, the assessment of antioxidant capacity, and the measurement of lipase activity in selected plant seeds or extract samples. The main aim of this study was to discover and measure the different biologically active substances found in two types of edible seeds: sunflower (*Helianthus annuus*) and chia (*Salvia hispanica*). Furthermore, we investigated their antioxidant characteristics, as these seeds are known to be abundant in antioxidants. Additionally, the study examines lipase activity, an essential enzymatic function implicated in lipid metabolism, relevant to both physiological and industrial contexts. By combining phytochemical analysis, antioxidant assessment, and lipase activity testing, this research intends to investigate the therapeutic and nutraceutical potential of the selected plant materials, thereby contributing to their prospective applications in health-promoting formulations and the management of metabolic disorders.

Keywords: Antioxidant, Secondary metabolites, Free radicals, Reactive oxygen species (ROS), Enzymatic assay, DPPH assay

Introduction

Plants demonstrate an impressive ability to biosynthesize a multitude of diverse bioactive compounds. Approximately 20% of known plants have been used in pharmaceutical studies, impacting the healthcare system in positive ways such as treating serious illness and disease (Naczk & Shahidi 2006). For instance, vitamins A, C, E, and phenolic compounds such as flavonoids, tannins, and lignins, found in fruits, all act as antioxidants (Suffredini et al. 2004). The consumption of phenolic compounds has been linked with several health benefits, a result of their medicinal properties and high nutritional value (Valko et al. 2006; Sytar et al. 2018). Plants have long been recognised as a prolific source of bioactive compounds that possess significant medicinal and nutritional value. The therapeutic potential of various plant parts—such as seeds, leaves, roots, and fruits—is largely attributed to the presence of diverse phytochemicals, which are naturally occurring secondary metabolites. (Bhandari, P. R. 2012). Lipase enzymes have become more and more prominent in the enzyme biotechnology scenario due to their versatility for hydrolysis and synthesis, their catalytic reactions often being chemo-selective, region-selective or enantio-selective. Lipases are used in many sectors such as the food, pharmaceutical, fine chemical, oil chemical, biodiesel and industrial detergent industries (Freire and Castilho 2008, Alonso et al. 2005). The participation of lipases in the worldwide enzyme industry market has grown significantly, and it is believed that, in the future, they will acquire importance comparable to that of the peptidases, which currently represent 25 to 40% of industrial enzyme sales (Hasan et al. 2006).

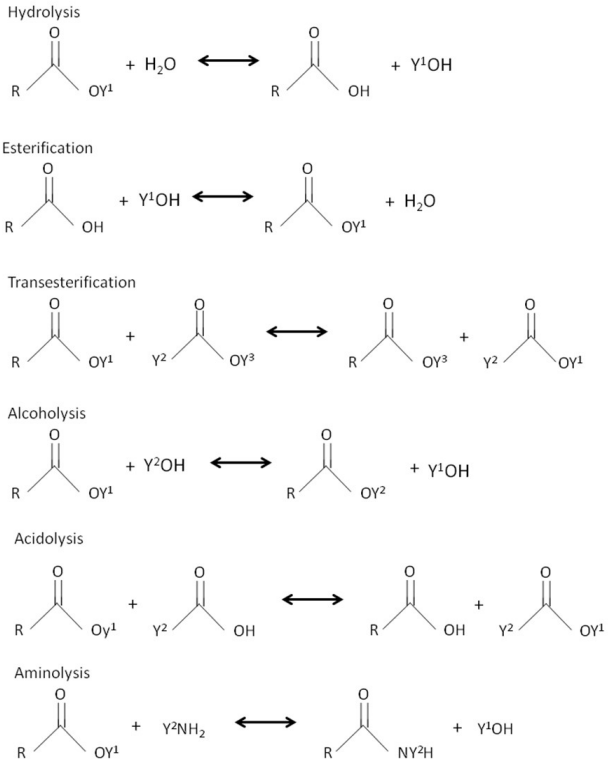

Figure 1: Reactions catalyzed by lipases. (Paques and Macedo, 2006)

Lipases are currently important as biocatalysts. A great number of articles have been published, especially concerning synthesis reactions, emphasizing the importance of lipases. The search for new lipases must be continuous and interesting seed sources must be well explored, since they may present different biochemical properties with respect to the reactions of hydrolysis and synthesis. There are a huge number of new seed sources for possible lipase exploitation.

Materials and Methods

The main aim of this research was to identify and quantify the different biologically active compounds from two edible seeds - pumpkin (*Cucurbita maxima*) and chia (*Salvia hispanica*); additionally, we have checked their antioxidant properties as the seeds are reported to be very good source of antioxidants.

Description of seeds:

Pumpkin seed – seeds are collected from a healthy pumpkin (*Cucurbita maxima*) fruit. Pumkin seeds are rich in unsaturated fatty acids, protein, vitamins and minerals that reduce risk factors of chronic diseases and cancer. The morphological description of the seed is as follows

- Shape: Flat and oval, with one axis of symmetry.
- Colour: The outer husk is white, and the kernel is light green.
- Size: Average length is 16.91 mm, width is 8.67 mm, and thickness is 3.00 mm.
- Kernel: The kernel, which is edible, can be hulled or unpulled.

Chia seed – Fresh chia seeds are collected from the market. Chia seeds are the edible seeds of a flowering plant from the mint family. The seeds are used as anti-diabetic, antihypertensive, heart disease and skin diseases.The morphological description of chia (*Salvia hispanica*) seed are as follows,

- Shape: Chia seeds are generally small, flattened ovoids.
- Size: They are about 2 mm long, 1 to 1.5 mm wide, and less than 1 mm thick.
- Colour: The colour of chia seeds can vary, with common colours including black, Gray, and black-spotted, with some varieties being white.
- Texture: When soaked in water, chia seeds absorb liquid and develop a mucilaginous coating, creating a gel-like texture.
- Weight: Chia seeds typically weigh around 1.3 mg per seed.

Preparation of seed powder:

Seeds were initially gathered and thoroughly rinsed with distilled water to eliminate any surface contaminants, such as dust, soil, or microbial residues. Following the cleaning process, the seeds were allowed to air dry to remove any excess moisture. The dried seeds were subsequently ground into a fine powder using a mechanical grinder.

To create the methanolic extract, a specified amount of the seed powder was soaked in methanol (usually 70–100%) at a suitable ratio (for example, 1:10 w/v) and left at room temperature or agitated for 24–72 hours. After this incubation period, the mixture was filtered through muslin cloth or Whatman No.1 filter paper to separate the solid residues.

The resulting filtrate was then centrifuged at a high speed (approximately 4000 rpm for 10–15 minutes) to eliminate any remaining particulate matter. The clear supernatant containing the methanolic extract was carefully collected and stored in airtight amber-colored bottles at 4°C or -20°C until needed for further use.

Qualitative Phytochemical Screening:

Phytochemical analysis of the plant extract was conducted to determine its chemical compone nts, primarily alkaloids, phenolics, flavonoids, and tannins. Initial qualitative phytochemical assessments of ethanol, aqueous, and acetone

extracts from the seeds of Pumpkin, Chia, Flax, and Sunflower were conducted following standardized procedures (Trease and Evans, 1983, Sofowora, 1993; Kokate, 1994; Harborne, 1998. Njoku and Obi, 2009; Basumatary, 2016; Sidhu and Sharma, 2016)

Test for Alkaloids: -

Reagents- Dragendorff's reagent. (Sidhu and Sharma, 2016)

1ml filtered seed extract were taken in the test tube. Then few drops of Dragendorff" s reagent was added to the extract and a white precipitation indicated the presence of alkaloids.

Test for Phenolic: -

Reagent- 5% aqueous ferric chloride ($FeCl_3$). (Pasto and Johnson, 1979.)

1ml of seed extract was taken in the test tube. Then 1 ml of water was also added in the test tube and then few drops of 5% $FeCl_3$ was added. Formation of black-blue colour of solution showed positive results.

Test for Flavonoid: -

Reagent- Alkaline reagent test (1ml of extract+2ml of 20% NaOH solution+ few drops dil. HCL). (Singh and Kumar, 2017)

1ml of seed extract was taken in the test tube. Then 2ml of 20% NaOH solution was added and the solution appeared yellowish colour. After that added dil. HCL was added drop wise until the solution became colourless. Colourless solution indicates presence of flavonoids.

Test for Tannin: -

Reagent- 10% Alcoholic Ferric chloride ($FeCl_3$). (Uma et. al. 2017)

1ml of seed extract was taken in the test tube. Then few drops of 10% $FeCl_3$ was added to the extract. Brownish-black colour of the solution indicates the presence of tannins.

Antioxidant activity by DPPH method:

The radical scavenging ability of various seeds was assessed using the DPPH method. This method evaluates the capacity of antioxidants to neutralize DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals. DPPH is a stable free radical that absorbs light at a wavelength of 517 nm, giving it a purple appearance. When an antioxidant interacts with DPPH, it either donates a hydrogen atom or an electron, thus transforming DPPH into a colorless or pale-yellow form. This change in color is measured spectrophotometrically, facilitating the quantification of the antioxidant's effectiveness.

Preparation of stock solution: -

To make DPPH solution, 0.4 gm of DPPH was dissolved in 100 ml ethanol and deep violet colour formed. The DPPH solution was then preserved in a conical flask by covering parafilm or aluminium foil to keep away from light.

Preparation of test solution: -

The portion of test solution to different fractions were diluted to obtain a dilution series of concentration of extracts ranging from 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, and 1.0 mg/ml. and ascorbic acid solutions were prepared as slandered in same range of concentrations.

Method: -

A volume of 2 ml of DPPH solution was mixed with 1 ml of the extract at various concentrations. The reaction mixtures were created in low light conditions and shaken thoroughly. After allowing to incubate in darkness at room temperature for 30 minutes, the absorbance was measured at 520 nm with a spectrophotometer. The control sample included all the components except for the extract.

Scavenging activity (%) =

[(Absorbance of sample – Absorbance of control) / Absorbance of control] × 100

Determination of Lipase activity of different edible seeds:

Preparation of seeds-

Pumpkin (*Cucurbita maxima*), and **Chia** (*Savia hispanica*) seeds were purchased from the local market.

To evaluate lipase activity in seeds, a detailed and biochemically sound protocol is employed to guarantee optimal recovery of enzymatically active components. First, mature and uncontaminated seeds are gathered, thoroughly rinsed with distilled water to remove surface impurities, and then air- or oven-dried at low temperatures to avoid moisture interference. The dried seeds are finely ground using a precooled mortar and pestle to prevent thermal denaturation of the proteins. A specific amount of seed powder is then mixed with an ice-cold extraction buffer—generally phosphate buffer (pH ~7.2)—enriched with non-ionic detergents (such as Triton X-100), chelating agents like EDTA, and optionally protease inhibitors to stabilize the enzyme and inhibit proteolysis. The homogenate is centrifuged at high speed (10,000–12,000 × g, 4°C, for 10–15 minutes), and the supernatant obtained, which contains crude lipase, is collected for the enzymatic assay. Lipase activity is measured either colorimetrically or through titrimetric methods using triglyceride emulsions (like olive oil), where liberated free fatty acids are quantified via titration. Throughout this process, strict temperature control is maintained to preserve enzymatic activity and ensure reproducibility. (Winkler, U. K., & Stuckmann, M. 1979)

Extraction and Isolation of lipase:

Lipase was extracted from the endosperm of seeds using the procedure outlined by Huang et al. (1983), with slight alterations as described by Eze et al. (2005). The seeds were stripped of their hulls, rinsed in distilled water, and ground with a pestle and mortar in a cold 150 mM Tris-HCl buffer at pH 7.5, which included 0.4M sucrose, 0.6mM EDTA, 2.0mM β-mercaptoethanol, and 1.95% (w/v) Tween 80. The homogenate underwent filtration using four layers of cheesecloth and filter paper, followed by centrifugation of the filtrate at 5000 xg for 30 minutes at 4oC. The upper layer, known as lipid bodies (fat pad), was extracted with a spatula; the supernatant (water-soluble part) and the pellet were gathered into different containers. The supernatant was labeled as crude lipase. The pellet was rinsed in buffer, and lipase activity was measured in the three fractions (Michael *et al,* 2001).

Lipase activity was assayed in each of the crude extracts isolated from the different seeds.

Partial Purification of Lipase:

For purification, the supernatant was subjected to fractionation using 90 % saturation of ammonium sulphate following the method outlined by Michael et al. (2001). The proteins that had precipitated were eliminated by centrifugation at 10,000 rpm for 15 minutes at 4 °C. The precipitate obtained was dissolved in Tris-HCl buffer (10 mM, pH 8.5), dialyzed overnight in the same buffer, and the dialyzed enzyme sample was stored at 4 °C in a refrigerator as a partially purified enzyme source for subsequent characterization and immobilization.

Estimation of Protein

The amount of protein present in crude extract and partially purified sample was estimated by the method of Lowry et al (1951) using bovine serum albumin (BSA) as a standard.

Assay of the enzyme:

The lipase activity in both crude extract and partially purified sample was assessed using the titrimetric method described by Malik et al. 2000. The activity was assessed through the titration of fatty acids released from olive oil due to the catalytic effect of lipase on NaOH. An emulsion was prepared using 180 ml of distilled water, 20 ml of olive oil, 1 g of gum-arabic, and 0.4 g of sodium benzoate.

In a standard reaction mixture, 5 ml of olive oil emulsion, 5 ml of Tris-HCl buffer (10 mM, pH 8.5), and 1 ml of enzyme sample (both crude extract and partially purified sample) were combined and incubated at 35°C for 10 minutes. Following incubation, the reaction was stopped by adding a mixture of acetone and methanol (1:1). Concurrently, a control reaction mixture was prepared without adding the enzyme sample and handled accordingly. Each reaction mixture that was terminated was titrated with 0.025 N NaOH, using 1% phenolphthalein as the indicator.

The amount ofNaOH needed to neutralize the free fatty acid in the sample was recorded for calculating enzyme activity. A single unit of lipase activity was defined as the quantity of protein necessary to release 1µmol of fatty acid from olive oil each minute under standard conditions. The specific activity and purification fold were determined from the activity (Maliks *et al*, 2000)

Lipase characterisation:

Polyacrylamide gel electrophoresis-

Enzyme purification or purity was checked on non-denaturing native PAGE using a 10% gel concentration with slight modifications to the method. Gel was stained using the Coomassie Brilliant Blue R-250 staining solution. Total enzyme protein used for PAGE was 10 µg for crude as well as partially purified lipase. (Holt and Hartman, 1994).

Results

Different Phytochemical analysis of two seed extracts (Ethanolic and Aqueous) is represented below:

The total amounts of different bio reactive compounds present in the ethanolic and aqueous extraction varied greatly in the edible seeds.

In an ethanolic solution, a significant amount of alkaloids and flavonoids was detected in pumpkin seeds, whereas the aqueous solution yielded lower amounts. For chia seeds, both extraction methods revealed the presence of only phenols and flavonoids. (Tables 1 and 2)

Table-1: Preliminary Phytochemical Screening of **Pumpkin** (*Cucurbita maxima*), and **Chia** (*Savia hispanica*) seeds. (Ethanolic solution)

Seed	Solvent	Alkaloid	Phenol	Flavonoid	Tannin
Pumpkin	Ethanol	+++	-	+++	-
Chia	Ethanol	-	++	++	+

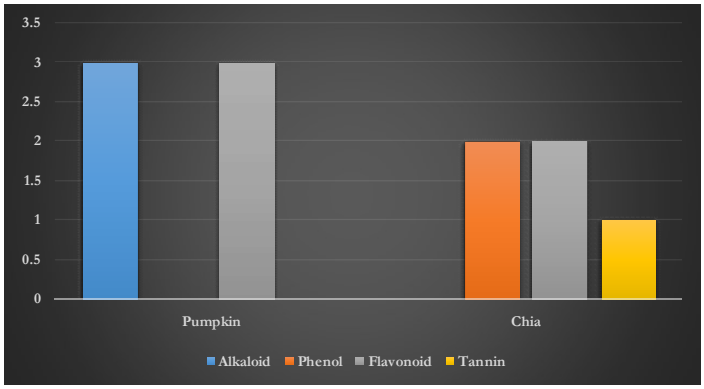

Figure-2: Graphical representation of different proportion of phytochemicals in different seeds [Ethanolic solution]

Table 2: Preliminary Phytochemical Screening of **Pumpkin** (*Cucurbita maxima*), and **Chia** (*Savia hispanica*) (Aqueous solution)

Seed	Solvent	Alkaloid	Phenol	Flavonoid	Tannin
Pumpkin	Aqueous	++	-	+	-
Chia	Aqueous	-	++	+++	-

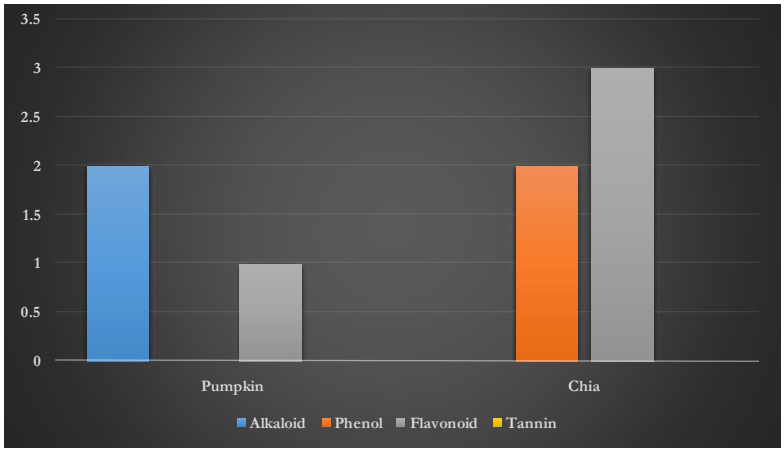

Figure-3: Graphical representation of different proportion of phytochemicals in different seeds [Aqueous solution]

Estimation of Antioxidant activity by DPPH assay in two different seeds

The antioxidant activity of different seeds was evaluated by measuring the reducing ability and free radical scavenging activity of two extracts (ethanol and aqueous) using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Ascorbic acid is used as standard control. The percentage of inhibition of the ascorbic acid in different concentrations is given in Table 3

Table-3: Percentage of inhibition of the ascorbic acid in different concentrations

Percentage of inhibition of ascorbic acid					
Concentration(µg/ml)	0.2	0.4	0.6	0.8	1.0
Percentage of inhibition	81.82	85.86	82.83	79.80	78.79

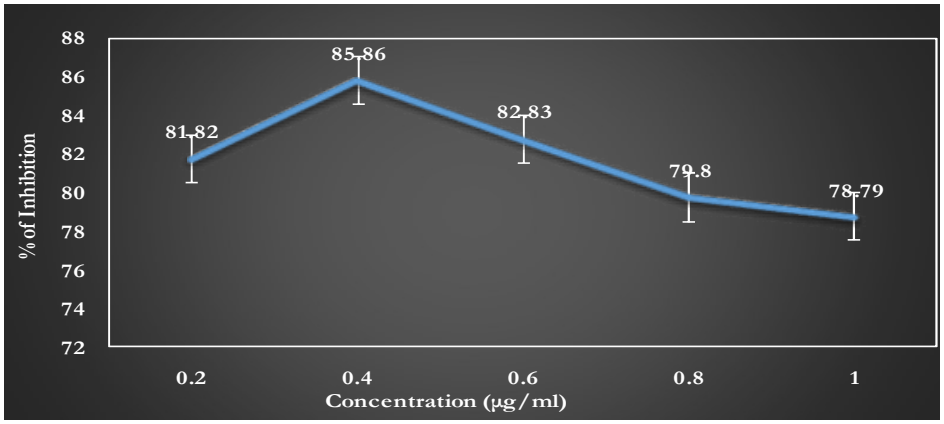

Figure 4: Graphical representation of the percentage of inhibition of ascorbic acid

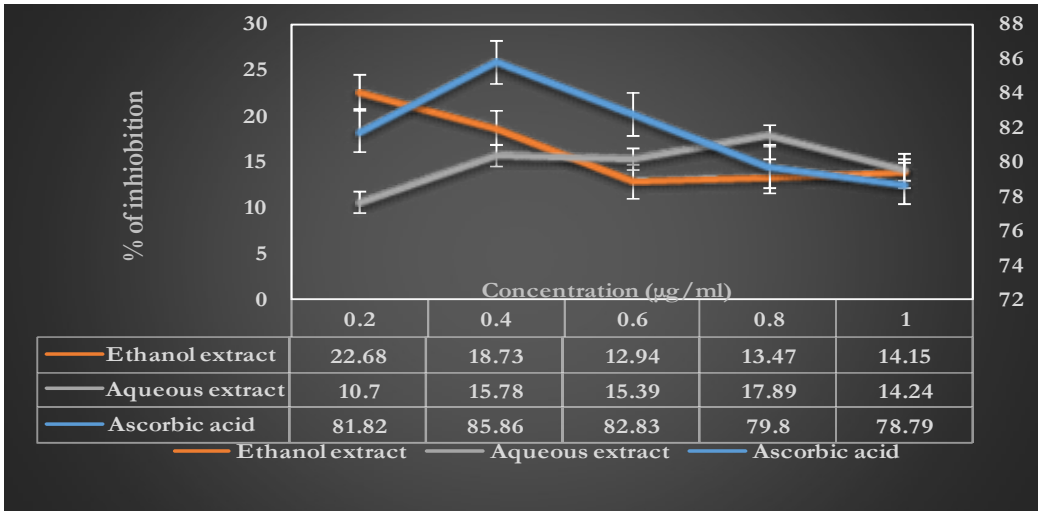

Figure-5: Graphical representation of the percentage of inhibition of Pumpkin seed (Ethanolic & Aqueous) extracts.

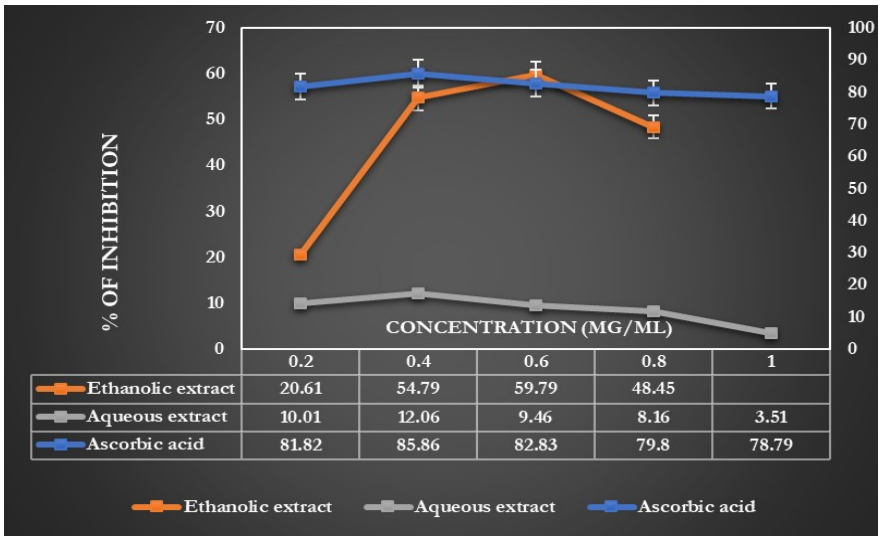

Figure 6: Graphical representation of the percentage of inhibition of Chia seed (Ethanolic & Aqueous) extracts

The DPPH scavenging activity of four different seeds was studied, and it was found that ethanolic extracts show more antioxidant potential for Chia seeds than that of pumpkin seeds.

Purification of lipase:

Pumpkin seed, and Chia seed are a rich source of lipase. It has been reported that lipase activity could be elevated in germinated seeds as compared to non-germinated seeds. In this study, an attempt was made to purify and optimization of lipase from the aforementioned four seeds.

For the extract of pumpkin seeds, partial purification was achieved through fractionation using 90% saturation of ammonium sulphate. The ammonium sulphate present in the fractionated sample was eliminated by dialysis. As shown in Table 3, lipase was partially purified with a 1.17-fold increase and a specific activity of 0.89 after applying 90% saturation of ammonium sulphate. Our findings indicate that the combination of 90% ammonium sulphate saturation and dialysis effectively resulted in a 2.54-fold purification of lipase.

Table 4 Purification table: Pumpkin seed

Purification steps	Vol. (mL)	Conc of protein (mg/mL)=a	Total Protein (mg)	Enzyme activity (µm min^{-1}) =b	Total activity	Specific activity (µm min^{-1} /mg prot)=b/a	Purification fold
Crude extract	200	88.8	17760	67.67	13534	0.76	1
Ammonium sulphate fraction	20	81.7	1634	72.67	1453.4	0.89	1.17
Dialysis fraction	13	68.2	886.6	132.1	1717.3	1.94	2.54

For the Chia seed, the extract underwent a process of partial purification through fractionation using 90% ammonium sulphate saturation. Dialysis was performed to eliminate the presence of ammonium sulphate from the fractionated sample. Table 4 indicates that lipase was partially purified with a 1.32-fold increase and a specific activity of 0.99 by employing 90% ammonium sulphate saturation. Our findings demonstrated that utilizing 90% ammonium sulphate saturation combined with dialysis is effective for achieving a 2.29-fold purification of lipase.

Table 5 Purification table: Chia seed

Purification steps	Vol. (mL)	Conc of protein (mg/mL)=a	Total Protein (mg)	Enzyme activity (µm min^{-1})=b	Total activity	Specific activity (µm min^{-1} /mg prot)=b/a	Purification fold
Crude extract	200	94.6	18920	71.25	14250	0.75	1
Ammonium sulphate fraction	20	84.9	1698	84.25	1685	0.99	1.32
Dialysis fraction	13	74.5	968.5	139.5	1813.5	1.87	2.49

From the result of this study it was concluded that, lipase enzyme could be partially purified in single step from crude extract of germinated sunflower seed by using ammonium sulphate precipitation. From different ammonium sulphate precipitation, 90% saturation of ammonium sulphate in crude extract is an effective for lipase purification.

Conclusion

The health community has increasingly focused on the importance of natural antioxidants in the prevention and treatment of diseases. This study identified substantial amounts of biologically active compounds in various plant seeds that demonstrate strong antioxidant properties. Despite their significant potential, knowledge regarding the composition of phenolics, flavonoids, alkaloids, and tannins in chia and pumpkin seeds remains limited. The soaked seeds of these plants were discovered to be excellent sources of phytochemicals, which could serve as natural components in the food industry. The seed extracts demonstrated notable in vitro antioxidant activities. This finding is backed by high concentrations of dietary fiber, total phenolics, flavonoids, and flavanols. Currently, lipases are regarded as essential biocatalysts. In the authors' laboratory, lipases derived from chia and pumpkin seeds have been utilized for various reactions, yielding very promising outcomes. Sources of seed lipases could offer a more economical and quicker method to produce these important biocatalysts.

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