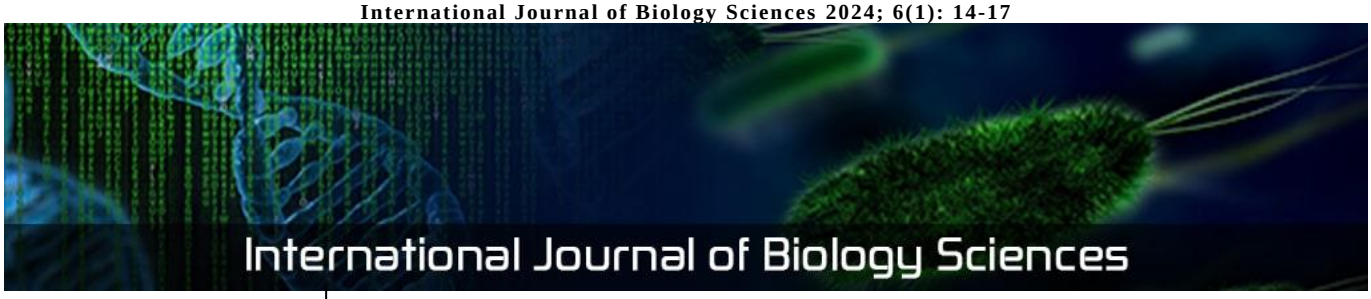

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Antioxidant activity and inhibition of elastase and tyrosinase of peptide extracts obtained from Timbe seed albumin

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Abstract
Acaciella angustissima (Timbe) is primarily used as firewood, as fodder (for goats and sheep), as a natural fuel, and as a natural tanner. Recent studies have focused on obtaining bioactive compounds, mainly phenolics, from bark and pods with potential antioxidant, antimicrobial, and antimutagenic activities. However, there have been no studies on obtaining peptides from the seeds of these species. Therefore, the objective of this work was to obtain peptide extracts with antioxidant and elastase- and tyrosinase-inhibitory properties from Timbe seeds. Albumin extracted from Timbe seeds was hydrolyzed with alcalase to generate protein hydrolysates, which were then ultrafiltered to obtain peptide extracts with a molecular weight of less than 10 kDa. The peptide extracts showed higher reducing activity against the ABTS•+ radical and a greater inhibitory effect on elastase and tyrosinase. Although albumin exhibited higher iron-reducing power, the antioxidant properties and inhibition of elastase and tyrosinase observed in both Timbe albumin and peptide extracts were similar to those reported in albumin and peptide fractions derived from other plant sources (Amaranth, white sorghum, rice, cocoa, and chia). These results demonstrate that the enzymatic hydrolysis of Timbe seed albumin is a promising process for the generation of peptide extracts with beneficial properties for skin care.

Keywords: *Acaciella angustissima*, legumes, protein hydrolysates, bioactive peptides, enzyme inhibition, skin health

1. Introduction
The skin is arguably the organ most susceptible to damage from free radicals because it has greater contact with oxygen and the environment. External factors affecting the rate of skin aging are all attributed to a single process, oxidative stress, resulting from an imbalance between the generation of free radicals and the body's antioxidant defenses [1]. Therefore, the consumption of antioxidants that can promote the prevention of oxidative damage to the skin is necessary. In addition, tyrosinase is the crucial enzyme in melanin synthesis, acting as a multifunctional oxidase that catalyzes the first two steps in mammalian melanogenesis. Hyperactivity of this enzyme causes dermatological issues such as melanoma and age spots. Hence, the search for compounds that inhibit this enzyme is of great interest for use as anti-aging agents [2]. Furthermore, skin aging is characterized by a progressive loss of tissue and changes in the extracellular matrix macromolecules of the skin, including elastin, a component of the skin's elastic fibers. With age, the quantity of elastin fibers decreases because of reduced synthesis and increased degradation, resulting in a loss of skin elasticity. Elastase is the only enzyme capable of degrading elastin [3]. Therefore, elastase inhibition prevents premature skin aging.
Over the past two decades, multiple studies have recognized various health benefits (Antioxidant, antihypertensive, antithrombotic, among others) attributed to specific protein fragments capable of modulating physiological processes, known as bioactive peptides [4]. These peptides can be obtained from plant proteins, with cereal grains (Wheat, rice, oats, rye, and corn) and some legumes such as soybean, beans, broad beans, peas, and chickpeas standing out [5]. In this context, there is scientific evidence that peptide-rich extracts exhibit antioxidant capacity and inhibit elastase and tyrosinase activities, suggesting potential anti-aging effects on the skin [6].

Therefore, it is necessary to explore new (Natural) sources of bioactive peptides.

Acaciella angustissima (Timbe) is a leguminous shrub native to Central America. This species is recognized as a significant source of secondary metabolites, including amines, alkaloids, glucosides, fatty acids, and phenolic compounds. Additionally, it is valued for its ecological contributions and various uses in traditional medicine and agriculture [25]. Some studies have reported antioxidant, antimicrobial, and antimutagenic activities of phenolic extracts obtained from Timbe pods [8, 9]. However, the presence of bioactive peptides has not been investigated.

Therefore, this work aimed to evaluate the antioxidant and elastase- and tyrosinase-inhibitory properties of peptide extracts produced by the enzymatic hydrolysis of Timbe seed albumin.

2. Materials and Methods

Figure 1 shows the experimental strategy for obtaining peptide extracts from Timbe seed albumin.

2.1 Plant material: Timbe pods were harvested at Amazcala, El Marques, Queretaro, Mexico during spring. Seeds were collected and air-dried for 72 h. afterwards, the seeds were subjected to grinding using an electric mill (Bosch TSM6A, Stuttgart, Germany). The flour was sieved through a 250-µm mesh and stored at 4 ºC.

2.2 Flour defatting: The obtained flour was defatted with hexane (10 mL/g) under constant stirring for 24 h at room temperature, followed by vacuum filtration. Defatted flour was placed in a fume hood for 24 h to allow solvent evaporation and stored at 4 ºC until use.

2.3 Albumin extraction: The Timbe seed albumin extraction was performed according to Voigt and Biehl [10]. Defatted flour (1:10 w:v) was mixed with Tris-HCl 10 mM (pH 7.5) containing EDTA 2 mM and sodium ascorbate 5mM. The slurry was stirred during 4 h at 4 °C and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was dialyzed in membranes of molecular weight 14 kDa cutoff for 2 days with deionized water at 4ºC, with a change every 24 h. The sample of dialysis tubes was centrifuged as indicated above and the supernatant (albumin) was stored at -20 ºC. The protein concentration was determined according to Bradford [11].

2.4 Enzymatic hydrolysis of albumin: Albumin was hydrolyzed with alcalase (protease from *Bacillus licheniformis*, Sigma-Aldrich®) at pH 7.5 and 50 ºC with an enzyme: substrate ratio of 1:10 (w:w). Hydrolysis reaction was performed for 120 min. At the end of the reaction time, the sample was heated at 95 °C for 10 min. The sample was centrifuged at 5000 rpm for 10 min at 4 °C. The obtained supernatant was denominated albumin hydrolysate.

2.5 Peptide extracts preparation: The obtained albumin hydrolysate was filtered using ultrafiltration membranes with a molecular weight cutoff of 10 kDa (Merck Millipore®). Ultrafiltered samples were obtained and denominated peptide extracts. Peptide concentration in the peptide extracts was performed by TNBS method [12] using L-Leucine as standard.

2.6 Determination of antioxidant activity: Antioxidant properties of albumin and peptide extracts was assessed through the scavenging effect on ABTS•+ radical and reducing power.

a) Scavenging effect on ABTS•+ radical: The ABTS•+ radical scavenging effect of albumin and peptide extracts was determined according to Re *et al.* [13]. The results were reported in percentage inhibition using the following equation:

$$ABTS\ radical\ scavenging\ activity\ (\%) = \left[1 - \left(A_{sample} - A_{blank}/A_{control}\right)\right] \times 100$$

Where *A* is the absorbance at 734 nm.

b) Ferric reducing antioxidant power (FRAP assay): The reducing power of albumin and peptide extracts was determined according to Wang *et al.* [14]. The results were reported in absorbance at 700 nm.

2.7 Elastase and tyrosinase inhibition

a) Elastase: Elastase inhibitory effect of albumin and peptide extracts was determined according to Royer *et al.* [15].

b) Tyrosinase: Tyrosinase inhibitory effect of albumin and peptide extracts was determined according to Ochiai *et al.* [16].

The results were reported in percentage of inhibition using the following equation:

$$Inhibition\ (\%) = [1 - (A/B)] \times 100$$

Where, A = enzyme activity with sample, and B = enzyme activity without sample.

2.8 Statistical analysis: All the data are presented as mean ± SD (n = 3). Data were analyzed by analysis of variance and Tukey´s tests for comparisons between albumin and peptide extracts using Statgraphics® (Centurion XVI).

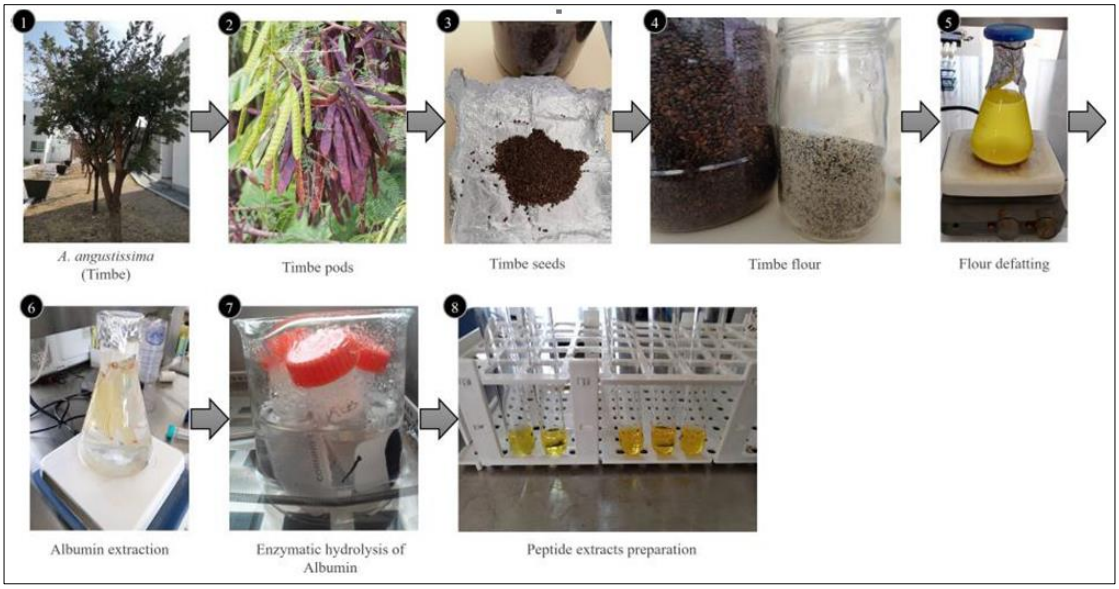

Fig 1: Steps for the production of peptide extracts from Timbe seed

3. Results

The fat content (ether extract) in Timbe seeds was 7.22 g/100 g of flour; likewise, in the defatted flour, a significantly lower (p<0.05) fat content of 0.1 g/100 g of flour was obtained compared with the initial content in the seed, indicating the efficiency of the hexane defatting process. The albumin extraction yield from defatted Timbe flour was 2.2 g/100 g, and the peptide concentration in the extracts obtained from the enzymatic hydrolysis of albumin was 460±10 µg/mL.

Table 1 displays various bioactivities (antioxidant and elastase and tyrosinase inhibition) obtained from both peptide extracts and the protein (albumin), showing distinct behavior for each determination. Peptide extracts exhibited higher reducing activity against the ABTS•+ radical and greater inhibitory activity against elastase and tyrosinase. However, albumin showed a higher iron- reducing power.

Table 1: Antioxidant activity and inhibition of elastase and tyrosinase of albumin and peptide extracts obtained from Timbe seed

Compound			**Bioactivity**	
ABTS•+ radical scavenging activity (%)		FRAP (Abs. λ=700 nm)	Elastase inhibition (%)	Tyrosinase inhibition (%)
Albumin (protein)	45.76±0.14b	0.234±0.007a	17.08±2.11b	ND
Peptide extracts	58.85±0.45a	0.076±0.006b	68.68±2.26a	41.95±0.40

Results are shown as mean ± SD (n = 3).

Means with different superscript (in the same column) are significantly different (p<0.05). The concentration of albumin and peptide extracts in the assays was 450 µg/mL., Abs. = Absorbance at a wavelength (λ) of 700 nm, ND = Not detected

4. Discussion

Initially, the defatting process of Timbe flour allowed obtaining a suitable raw material for protein (albumin) extraction. To the best of our knowledge, there are no reports of albumin content in Timbe seeds; however, the albumin extraction yield was similar to that reported in amaranth grain [17] and cocoa seed [18]. It is worth mentioning that the extracted protein yield depends on various factors, including the extraction method, type and concentration of solvent used, the use of reducing agents, and seed variety.

This study is the first to produce peptide extracts from Timbe seeds. In this regard, the peptide concentration obtained falls within the range reported for peptide extracts derived from cocoa seeds [17] and soybeans [19], as well as white sorghum grain [20]. Ultrafiltration has been widely used for peptide separation (within a specific molecular weight range) and has been applied to plant- derived protein hydrolysates to separate peptides between 1 and 10 kDa [21].

It is noteworthy that both albumin and peptide extracts exhibited antioxidant properties through electron transfer and/or hydrogen atom transfer mechanisms. The FRAP assay has been widely employed in studies as a standard method for evaluating antioxidant activity, where an increase in absorbance values at 700 nm indicates an increase in the reducing power of the antioxidant [19].

Similarly, methods for evaluating free radical scavenging represent a chemically valid parameter for measuring the antioxidant effect of peptides [18]. The values of ABTS•+ radical reducing activity and FRAP obtained in both albumin and peptide extracts were similar or higher than those reported in albumin and peptides from amaranth grain [22] and cocoa seed [18]. Thus, peptide extracts from Timbe seeds could counteract damage caused by free radicals, thus preventing oxidative stress. Finally, Timbe seed peptide extracts showed high inhibitory activities against elastase and tyrosinase, similar to those reported in peptide fractions derived from rice bran albumin [23] and chia seed [24]. This indicates that peptide extracts can prevent the appearance of wrinkles and loss of skin elasticity as well as prevent age spots, hyperpigmentation, and melanoma formation [20, 24].

5. Conclusion

Timbe seeds can serve as a raw material for the generation of peptide extracts with antioxidant activity and elastase- and tyrosinase-inhibitory capacity. This work provides other use for Timbe seeds and lays the bases for future research

related on the production and characterization of peptides for potential applications in the design of skincare products.

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