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Antioxidant and antidiabetic potential of *Acanthus mollis* L. using choline chloride-based deep eutectic solvents

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Abstract

In recent years, the study of medicinal plants' therapeutic properties has increased due to their effects and biological activities. The Acanthaceae family consists of 242 genera and 3947 species mainly distributed across tropical and subtropical zones. This family is known to contain a wide range of bioactive compounds, such as, alkaloids, triterpenes, steroids glycosides, polyphenols, and polysaccharides. Herein, the antioxidant and anti-diabetic effects of *Acanthus mollis* (AM) extracted by green alternative technology; ultrasound assisted-deep eutectic solvents extraction (UAE-DES) were determined. The data obtained, showed that AM seeds had the highest Total Phenolic Content, antioxidant activity and anti-diabetic activity compared to AM seeds shell. The presence of antioxidants with anti-diabetic properties in AM could potentially serve as a foundation for innovative drug formulations capable of addressing several diseases. However, it is strongly advised that studies, including toxicity assessments, be conducted with a view towards pharmaceutical applications.

KEYWORDS

Type-2 diabetes, diabetes mellitus, *Acanthus mollis*, total phenolic content, anti-diabetic action, antioxidant activity, bioactive compounds

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1. INTRODUCTION

Diabetes mellitus, or type-2 diabetes is a metabolic disease characterized by an increase in blood sugar concentration (hyperglycemia) and impaired glucose metabolism, either as a result of reduced insulin secretion or reduced sensitivity of the body's cells to insulin. Oxidative stress can occur with the overproduction of Reactive Oxygen Species (ROS) and/or reduced antioxidant capacity. Oxidative stress causes the loss of function

and structure of healthy cells, DNA and other important macromolecules. These effects implicated in causing chronic diseases such as stroke, cardiovascular damage, or diabetes [1,2,3]. Reactive species (RS) is a broad term encompassing several types of reactive elements, including reactive oxygen species, reactive nitrogen species, sulfur reactive species and many other species primarily responsible for oxidizing molecules within living cells. In order to prevent the interaction between radicals and biological molecules, antioxidants should be located in proximity to the site's radicals are generated, enabling them to compete with radicals for biological substrates.

Antioxidants are molecules capable of inhibiting the harmful effects of free radicals. Recently, the interest in the medicinal properties of plants has given rise to a new class of therapeutic solutions known as "nutraceuticals" Coined by De Felice in 1989, this term refers to any food or food component that offers medicinal or health benefits, encompassing preventive and curative properties. The use of these substances in both food products and dietary supplements is considered as safe, as affirmed by the US Food and Drug Administration (FDA), and the Scientific Committee (SC) of the European Food Safety Authority (EFSA).

The name *Acanthus* comes from the Greek word "acantha", which means thorn or thistle, and refers to the spiny leaves of some species [4,5]. According to the Flora of China, these herbaceous or shrubby plants typically feature leaves positioned opposite each other, devoid of stipules, or they are gathered in basal rosettes. Their flowers are hermaphrodite, zygomorphic to nearly radially symmetric, and are predominantly arranged in terminal or axillary spikes and racemes or racemes [5]. The Acanthaceae family consists of 3947 species grouped in 242 genera which are located in the Mediterranean basin [4]. The family is known to contain many classes of bioactive molecules, encompassing alkaloids, triterpenes steroids glycosides, polyphenols, and polysaccharides among its many classes of molecules [6].

The extensive traditional medicinal use of *Acanthus mollis* (AM) has long advocated its effectiveness in addressing inflammation-related issues. Historically, it has been employed as a poultice, gargle and decoction. Because of its soothing and anti-inflammatory properties, AM poultices have been applied to alleviate headaches and various conditions, including wounds, burns, psoriasis, bruises, and swollen feet [7,8]. The anti-inflammatory effects of decoctions made from AM offer relief for gastrointestinal discomfort, urogenital illness and even tumors [9]. Besides, extract from AM leaves in ethanol and petroleum ether

have demonstrated antioxidant activity and a notable inhibition of nitric oxide (NO) production [10]. Herein, the in vitro antioxidant and anti-diabetic potential of AM through a green alternative technology, known as, ultrasound assisted-deep eutectic solvents extraction (UAE-DES) was determined.

2. MATERIAL AND METHODS

2.1. Apparatus

Balance (AS220/CI2), water bath (Mammert GmbH), Microplate reader (Synergy-HTX, Biotek), UV-Visible spectrophotometer (Ultrospec 2100 PRO), refrigerated centrifuge (SIGMA 2-16 PK), Ultrasound (Tierratech LT-100 PRO, 100W).

2.2. Chemical products

The α -amylase bacterial enzyme purchased from Fisher Chemical, while 3-5-Dinitrosalicylic acid (C₇H₄N₂O₇) at a purity of 97% obtained from Thermo Scientific. Choline chloride (C₅H₁₄ClNO) (>98.0%) and Acetic acid (C₂H₃O₂) (>98.0%) were purchased from Sigma Aldrich. Monopotassium phosphate (KH₂PO₄) (>99%), Potassium dihydrogen phosphate (K₂HPO₄) (>99%) were purchased from Chembiotin. Folin-Ciocalteu reagent, sodium carbonate (Na₂CO₃), 4,6-tripyridyl-s-triazine (TPTZ) (> 98%), sodium acetate (C₂H₃NaO₂), Ferric chloride (FeCl₃) (>97%) were purchased from Sigma Aldrich, Dinitrosalicylic acid (DNS) C₇H₄N₂O₇ (97%) was purchased from Thermo Scientific.

2.3. Sample preparation

In October 2022, AM plant material harvested from a The Global Positioning Systems (GPS) location of 37,675 ° N, 21,441° E in Greece. The harvested plant material was dried in the dark at room temperature, grounded into a fine powder and sieved to obtain particles < 63 μ m. To extract the seeds and the seeds shell of AM, 50 mg of each mixed with 1 ml of Choline Chloride-Acetic acid DES for 24h at room temperature. The use of UAE-DES is considered an effective approach for the extracting phenolic compounds, offering a safer alternative to toxic organic solvents while also reducing the time and energy requirements associated with conventional methods. After extraction, the mixture was centrifuged for 15 min at 3000 rpm, and the resulting supernatant was collected and used for subsequent assays [11].

2.4. Total phenolic content colorimetric assay

Colorimetric methods were initially employed to assess the total phenolic content (TPC) in extracts of AM seeds and seed shells, and this determination was accomplished using the Folin-Ciocalteu method [12]. The reaction mixture consists of 1 mL of Folin-Ciocalteu reagent (1N), 800 μ L of sodium carbonate and 200 μ L of extract solution. The absorbance of the resultant blue complex at 760 nm converted phenolic quantities by referencing a calibration curve based on gallic acid standards. These quantities expressed as grams (g) of gallic acid equivalent/mg of dry extract.

2.5. Antioxidant activity

This assay measures the change in absorbance at 593 nm resulting from the transformation of colorless Fe³⁺-tripirydyltriazine into a blue Fe²⁺-tripirydyltriazine complex, due to the action of electron-donating antioxidants. Briefly, 1 ml of AM extract was mixed with 3 mL of the FRAP reagent which consists of 10 mL sodium acetate buffer (300 mM, pH: 3.6), 1 mL of TPTZ: 4,6-tripirydyl-triazine solution (10 mM) and 1 mL of ferric chloride (20 mM). The reaction mixture incubated at 37° C for 30 minutes [13,14]. Aqueous solutions of ferrous sulfate heptahydrate (FeSO₄.7H₂O) were the standard solutions. The FRAP activity was compared with the standard curve of FeSO₄.7H₂O and values were expressed as μ M Fe²⁺ of plant extract.

2.6. Anti-diabetic assay

The α -amylase inhibitory assay conducted in accordance with the method outlined by Yu et al., 2013 with minor modifications. 10 μ L of sample solution was premixed with 200 μ L of α -amylase (1 unit/ mL), followed by an incubation for 3 minutes. Subsequently, 100 μ L of starch solution (1% in 20 mM phosphate buffer) added into the reaction mixture. Following this, 100 μ L of dinitrosalicylic acid (DNS) added, and the mixture heated in a water bath until a yellowish-orange color developed. After cooling, the absorbance at 540 nm was measured using a spectrophotometer [15]. Acarbose was used as the positive control.

The inhibition percentage calculated as follow:

$$\text{Inhibition (\%)} = 1 - (A_0/A_1) \times 100.$$

Where: A1 represents the control (acarbose), A0 corresponds to the blank solution (solvent alone) and As, A1 denotes the sample.

2.7. LC(ESI)-MS analysis

For the profiling of bioactive metabolites in both AM samples, LC-ESI (Shimadzu, Kyoto, Japan) analysis employed. The analysis was conducted using a reverse-phase C-18 column (150 x 4.6 mm, 5 μ m), under gradient conditions at a temperature of 45°C, with a flow rate of 0.8 mL/min. The mobile phase was composed of 2 solvents, solvent A comprising 50% acetonitrile + 50% H₂O, while solvent B was a mixture of 95% H₂O, 5% MeOH and 0.2% Acetic acid. This method was applied in both positive and negative detection modes, employing a linear gradient (0-20min 20% B, 20-40 min 55% B, 40-55min 100 %B, 55-65min, 100%B, 66-75min 10%B). The reported results are expressed as mean value \pm standard deviation (SD) based on more than three measurements [16,17].

3. RESULTS AND DISCUSSION

3.1. Total phenolic content

The feasibility of using sustainable deep eutectic solvents (DES) as an alternative to conventional volatile organic solvents (VOSs) in the extraction of phenolic compounds from AM seeds and seeds shell was determined. Phenolics compounds are among the most prevalent secondary metabolites found throughout the plant kingdom. They have received much attention as potential natural antioxidants due to their effective ability to scavenge free radicals. They are small molecules characterized by their structures having at least one phenol unit and can be fractionated into varieties, such as phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbens, and curcuminoids. They have also shown to have immunomodulatory [18], anti-cancer [19,20] and anti-bacterial effects [21], amongst others. In this study, TPC of Acanthus seeds and their shells were investigated using Folin-Ciocalteu assay, with the outcomes expressed as Gallic acid equivalents. DES extract of AM showed that AM seeds had the highest TPC (48.60 \pm 1.34 mg/Gdw) followed by AM shell (18.79 \pm 3.21mg/Gdw). In studies used methanolic leaf extract of *Nyctanthes arbor-tristis* L, TPC was calculated as 78.48 \pm 4.2 equivalent mg TAE/g (tannic acid equivalent) [22], 20.95 \pm 1.20 mg of GAE/ g in *Vula walu* (*Blechnum orientale*) [23], 38.31 \pm 0.58 mg GAE/g in *Gnetum gnemon*[24].

These results were also similar to those obtained by Sarikurkcu using ethanol as extraction solvent for *Acanthus spinosus* L. species [25]. Overall, the findings support that the proposed DES are able to yield extracts with similar total

phenolic content obtained with conventional solvents.

3.2. AM seeds and seed shells effectively reduce free radicals

Medicinal plants constitute a highly significant source of pharmaceutical agents and serve as the foundation of traditional medicine in several countries worldwide. The use of medicinal plants to address health issues can be viewed as an alternative to conventional medical practices. The FRAP assay was developed for the assessment of the ferric reducing capability of biological fluids and aqueous solutions containing various compounds. It involves the reduction of a ferric tripyridyltriazine (fer-TPTZ) complex to its ferrous form at low pH conditions, resulting in a distinct change in color to an intense blue shade, which can be quantified by measuring the change in absorption at 593 nm. The FRAP assay showed that both AM seeds ($646 \pm 54 \mu\text{M}$) and shell ($342 \pm 37 \mu\text{M}$) extracts had a good antioxidant activity. One study from other medicinal plants showed $287 \pm 8 \mu\text{g Fe/mg}$ antioxidant activity for *H. japonicum* crude plant extracts [26], much lower than AM seeds and shells antioxidant activity. The versatility of the FRAP assay allows its application to various types of extracts, including aqueous, alcohol and DES from different plant sources. The antioxidant capabilities of plant extracts are influenced not only by their composition but also by the specific testing conditions employed. Numerous *in vitro* methods have been developed to assess antioxidant capacity, often based on either hydrogen atom or electron transfer mechanisms. These antioxidants compete to reduce free radicals, resulting in observable color changes when the radicals are reduced. The results obtained through the FRAP assay signifies the importance of both AM seeds and seed shells in effectively reducing free radicals.

3.3. *In vitro* anti-diabetic potential of AM seeds and shells

Several traditional herbs and natural dietary supplements, historically employed for diabetes management, are gaining popularity due to increasing evidence supporting their effectiveness and safety. In fact, plants play a significant role, either indirectly or directly, in the development of many current pharmaceuticals. Interestingly, α -amylase inhibitory activity has been investigated not only in traditional herbs and spices but also in a diverse range of food extracts [15]. It has been documented that long-term type-2 diabetes is linked to several complications, including atherosclerosis,

myocardial infarction, neuropathy and nephropathy [25, 26]. These complications are associated with chronically elevated glucose levels and are believed to be linked to oxidative stress. Currently, in the realm of diabetes treatment, there is a focus on inhibiting α -amylase. Medications that act as α -amylase inhibitors are recognized as oral hypoglycemic agents because they play a crucial role of impeding the breakdown of disaccharides into monosaccharides, thereby helping to maintain normal blood sugar level [29].

Antioxidants have been prescribed to reduce long-term oxidative stress-related complications in diabetes. This study focused on assessing the anti-diabetic potential of AM seeds and shells in an *in vitro* setting. The antidiabetic activity of crude polyphenols using different concentrations (50-3.125 mg/mL) was evaluated and the results showed that AM extracts were good inhibitors of α -amylase in amount-dependent manner (from $87.16 \pm 0.69\%$ to $50.44 \pm 7.33\%$) and (from $74.98 \pm 3.39\%$ to $20.06 \pm 2.55\%$) for AM seeds and shell respectively (Figure 1). Similar to our findings *Blechnum orientale*, *Citrus limon*, *Mussaenda raiateensis* extracts, were shown to exhibit similar anti-diabetic effects [23]. The collected data showed that AM seeds exhibited the highest levels of Total Phenolic Content, antioxidant activity and anti-diabetic effects when compared to AM shells. The importance of plant-derived phenolics and natural antioxidants are warranted due to their tendency to produce adverse side effects. The presence of antioxidant substances with anti-diabetic properties in AM could serve as a foundation for innovative drug formulations capable of addressing various diseases.

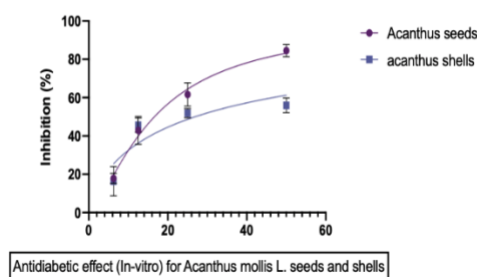


Figure 1: Anti-diabetic effects of both AM seeds and shells as determined by *in vitro* α -amylase inhibitory activity

3.4. LC(ESI)MS analysis

To clarify the structures of phenolic compounds, the antioxidant profiles of AM seeds and seed shell

were provisionally isolated, as shown in Table 1. The LC-MS analysis showed the presence of sixteen major peaks, primarily corresponding to phenolic acids [2,7,10], flavonoids [4,11] and tanins [5]. Phenolic compounds are well-known for their antioxidant properties and are commonly found in medicinal plants. The demand for natural antioxidants has driven our investigation into the biological activities and the identification of the responsible molecules, aiming to gain a better understanding of the chemical composition of AM. Interest-

ingly, the chemical profiles of AM seeds and shells were similar. As a result, the significant antioxidant and anti-diabetic activities noted in AM seeds and shells can be attributed to their abundance of secondary metabolites making them a valuable source of antioxidants with various health benefits. Similar to our findings other medicinal plants were shown to exhibit similar chemical profiles which also showed anti-oxidant and anti-diabetic effects [23,24,26,29].

Table 1.
LC-MS analysis of both AM seeds and shells

	Identified molecule	Ionization mode	M/Z	Molecular formula	Acanthus seeds	Acanthus shells
1	Pyrogalllic acid	(-)	125	C ₆ H ₆ O ₃	5.21	5.23
2	Na-Salicylate	(-)	159	C ₇ H ₅ NaO ₃	17.97	17.97
3	3,5-dimethoxy-4-hydroxy tannic acid	(-)	224.05	C ₁₀ H ₁₂ O ₅	30,20	30,16
4	Quercetin	(-)	300.5	C ₁₅ H ₁₀ O ₇	23,28	23,25
5	Rutin	(-)	609	C ₂₇ H ₃₀ O ₁₆	32,28	32,32
6	Citric Acid	(-)	191	C ₆ H ₈ O ₇	2,68	2,65
7	Tannic acid	(-)	1060.80	C ₇₆ H ₅₂ O ₄₆	26,61	26,58
8	Catechin	(-)	289	C ₁₅ H ₁₄ O ₆	13,49	13,48
9	Silymarin	(-)	481	C ₂₅ H ₂₂ O ₁₀	47,75	47,79
10	Deosmin	(-)	607	C ₂₈ H ₃₂ O ₁₅	39,30	39,50
11	Kampferol	(-)	285	C ₁₅ H ₁₀ O ₆	49,90	49,95
12	Coumaric acid	(-)	163	C ₉ H ₈ O ₃	24,26	24,26
13	Ascorbic acid	(-)	175	C ₆ H ₈ O ₆	2,56	2,55
14	Cyanidin-3-glucoside	(+)	484	C ₂₁ H ₂₁ ClO ₁₁	46,28	46,25
15	Cinnamic acid	(-)	149.06	C ₉ H ₈ O ₂	2,49	2,46
16	Gallic acid	(-)	169.17	C ₇ H ₆ O ₅	4,41	4,44

5. CONCLUSION

In recent years, the use of DES for the extraction of bioactive compounds from natural raw materials and by-products has experienced a significant surge in popularity. This surge can be attributed to the unique characteristics of these solvents and the advantages they offer in this field. Notably, the combination of choline chloride-acetic acid DES has demonstrated the ability to effectively extract total phenolic compounds, thereby enhancing their recovery from both AM seeds and seeds shells. The extracts from AM have proven to be a valuable source of phytochemicals possessing various biological activities, including antioxidant and anti-diabetic potential [30,31]. However, studies regarding the toxicity evaluation are highly advisable for this sample. Further, there is a need to deter-

mine various pharmacological properties through a range of *in vitro* and *in vivo* tests.

CONSENT TO PUBLISH

All authors of this paper are aware of the submission and agree to its publication.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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