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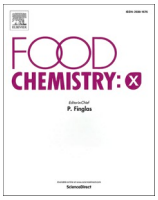

Ultrasound assisted deep eutectic solvent-based extraction of Montepulciano d' Abruzzo grape seeds for the recovery of the grape seed oil and its biological evaluation

Lorenza Marinaccio [a], Giulia Gentile [a], Gokhan Zengin [b], Stefano Pieretti [c], Azzurra Stefanucci [a,d,*], Angelo Cichelli [e], Adriano Mollica [a,d]

[a] Department of Pharmacy, G. d'Annunzio University of Chieti-Pescara, 66100 Chieti, Italy
[b] Department of Biology, Science Faculty, Selcuk University, Konya 42130, Turkey
[c] National Centre for Drug Research and Evaluation, Istituto Superiore Di Sanità, 00161 Rome, Italy
[d] SCM Nutraceutici Universitari Srl, Strada degli Oliveti 73, 66100 Chieti, Italy
[e] Department of Medical, Oral and Biotechnological Sciences, "G. D'Annunzio" University of Chieti-Pescara, 65100 Chieti, Italy

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ABSTRACT

Grape seeds are by-products of vinification process. In this work, a green ultrasound-assisted extraction of grape seeds oil was performed by using the natural volatile deep eutectic solvent (NADES) menthol: thymol 1:1. The obtained oil was compared to that deriving from UAE with *n*-hexane in terms of fatty acids composition and biological activities. The content of linoleic acid was low for the DES extracted oil; the content of linolenic acid increased from 0.53 % to 5.18 %. The grape seeds oil extracted with DES showed the best total phenolic (18.65 mg GAE/g) and flavonoid (0.73 mg RE/g) contents and the highest results in FRAP, CUPRAC, MCA and Phosphomolybdenum assays. The oil extracted by DES also showed a higher amylase inhibition (0.57 mmol ACAE/g) than *n*-hexane extract (0.47 mmol ACAE/g). Finally, the anti-inflammatory activity was assessed *in vivo* through tree different assays, suggesting that their different fatty acids composition could be partially responsible for the significant anti-inflammatory effect of the grape seed oil extracted by NADES.

1. Introduction

Grape (*Vitis Vinifera*), belonging to Vitaceae family, is highly consumed all over the world (Gupta et al., 2020). It originates in Southern Europe and Western Asia, but, nowadays, is cultivated in numerous areas of the world (Aghbali et al., 2013; Zarev et al., 2023). It's estimated that approximately the 80 % of grapes are addressed to the winemaking process (Zhu et al., 2015). The wine sector contributes for about the 0.3 % to the global carbon footprint of human activities per year (Rugani et al., 2013). The importance of the reduction of greenhouse gas emissions to fight the climate change is one of the challenges proposed by the Agenda 2030 that is focused on the sustainable development. This program born in 2015, comprises 17 Sustainable Development Goals and a total of 169 targets involving three aspects: economic, social and environmental.

Scientific research has a crucial role in the promotion of sustainable processes and technologies to valorise industry by-products as potential sources of beneficial compounds in order to reduce the environmental impact of wine industry. The grape seeds contained in the pomace, a by-product obtained after the production of wine or juice, are recycled to produce grape seed oil (Lutterodt et al., 2011). Grape seeds represent approximately the 20 % of grape weight, and among the 40–60 % of the dry material (Matthäus, 2008). The amount of the oil contained in the seeds is mostly influenced by the variety of the grapes and usually range from 10 % to 16 % of dry weight. (Gupta et al., 2020). Grape seed oil gained interest as a potential functional food and cosmetic ingredient containing both hydrophilic compounds, *e.g.* phenolics, and lipophilic compounds, *e.g.* unsaturated fatty acids (85–90 %), phytosterols and vitamin E (Carmona-Jiménez et al., 2022; Garavaglia et al., 2016; Gupta et al., 2020; Karaman et al., 2015; Shinagawa et al., 2015).

Grape seed oil is particularly rich in linoleic acid (Tangolar et al., 2009). The introduction of polyunsaturated fatty acids, *e.g.* linoleic and linolenic acids, is essential for the physiological process due to the absence of human enzymes involved in their biosynthesis (Hanganu

* Corresponding author at: Department of Pharmacy, G. d'Annunzio University of Chieti-Pescara, 66100 Chieti, Italy.
E-mail address: a.stefanucci@unich.it (A. Stefanucci).

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et al., 2012). The composition of this oil prompted the investigation of its potential benefits on human health, for instance antioxidant, antimicrobial, antitumor, anti-inflammatory activities, alleviation of metabolic syndrome and management of cardiovascular diseases (N. M. Dabetic et al., 2020; Garavaglia et al., 2016; Martin et al., 2020; Ustun Argon et al., 2020; Yang et al., 2021; Zarev et al., 2023).

Grape seed oil can be extracted in a plethora of different methods. It is usually obtained by cold pressing method or Soxhlet method using organic solvents. Given the importance of environmental sustainability, other green alternatives have been investigated including supercritical fluid extraction using carbon dioxide or ultrasound-assisted extraction (UAE) (Al Juhaimi & Özcan, 2018; Bravi et al., 2007; Da Porto et al., 2013; Ustun Argon et al., 2020; Zarev et al., 2023).

Natural Volatile Deep eutectic solvents (NADES) can be exploited as green solvents along with the application of green techniques for the extraction of natural compounds avoiding the production of toxic effluents (Picot-Allain et al., 2021). DESs are composed of an hydrogen bond acceptor (HBA) and an hydrogen bond donor (HBD) and their use is advantageous because of low volatility and cost, ease of preparation, biodegradability, non-flammability and potential reusability (Chen et al., 2023; El Achkar et al., 2021; Zhang et al., 2012). In 2003, Abbot and co-workers firstly reported the production of a deep eutectic solvent combining choline chloride (HBA) and urea (HBD) (Abbott et al., 2003; Abbott et al., 2004). Different research articles have been published on the extraction of biologically active compounds from grape seeds using deep eutectic solvents, some examples are reported below. Dabetić et al. exploited NADES solutions with 30 % (*v*/v) of water, specifically choline chloride:citric acid 2:1 and choline chloride:glucose 1:1, for the extraction of phenolic compounds. In particular, the overall results showed that DES choline chloride:citric acid exhibited an elevated extraction efficiency (Dabetić et al., 2020). The extraction using DES choline chloride/citric acid was further compared to ethanol extraction (N. Dabetic et al., 2022). Kavas et al. investigated the extraction efficiency of different carboxylic acid-based DES performing a homogenizer assisted extraction (HAE). As hydrogen bond donors were used glycerol and ethylene glycol combined in different molar ratio with formic acid, acetic acid and propionic acid used as hydrogen bond acceptors (Kavas et al., 2024). Sun et al. selected the NADES composed of choline chloride and citric acid 1:2 for the extraction of polyphenols from Cabernet Sauvignon seeds (Sun et al., 2024). For the extraction and separation of grape seed polysaccharides, Chen et al. selected a pH-switchable DES composed of dodecanoic acid: octanoic acid 1:1 by using three-phase partitioning (TPP) system (Chen et al., 2023). Because of their green nature, the high stabilization and solubilization these solvents have increased in demand in a shorter time compared to that of conventional organic solvents. However, the viscosity and variations of components used, still remain a critical aspect. Unlike the extraction procedures for the recovery of hydrophilic compounds from grape seeds using DESs as green solvents, the application of hydrophobic DESs to isolate lipophilic compounds from grape seeds is relatively unexplored. To fill this gap in literature, in this work we combined the UAE technique to the extraction of grape seeds oil using a hydrophobic volatile NADES menthol: thymol, as green solvent. Our research group previously adopted the same DES for the successful extraction of lycopene from tomato skins waste (Marinaccio et al., 2024). In general, various hydrophobic DES are reported in literature for the extraction of lipophilic compounds. For instance, three hydrophobic DESs composed of oleic acid and terpenes (DL-menthol, geraniol, and thymol) were exploited for the extraction of the carotenoid astaxanthin belonging to xanthophylls, from the microalgae *Haematococcus pluvialis* (Pitacco et al., 2022); another study screened different NADESs for the recovery of hydrophobic metabolites in the Spirulina (*A. platensis*) (Wils et al., 2021), however to the best of our knowledge this is the first research work describing the extraction of grape seed oil using hydrophobic DES menthol:thymol 1:1 by UAE. These results were compared to those obtained by UAE/*n*-hexane extraction, in terms of fatty acids composition, antioxidant activity *in vitro* and anti-inflammatory activity *in vivo*.

2. Materials and methods

2.1. Materials and instruments

Grape pomace of Montepulciano d' Abruzzo was gently furnished by Tenuta del Priore, Collecorvino (PE), Italy, after the production of the local wine Montepulciano d'abruzzo. *n*-hexane (99.0 %) was purchased from Sigma-Aldrich, USA (now Millipore Sigma) along with, menthol (99.0 %) and thymol (98.5 %). The instruments used for the sample preparation and extraction were: Büchi Lyovapor L-200 apparatus, analytical balance VWR International Ltd. (Model PBA224I-1S) Lab-scale ultrasound sonicator (Cole- Parmer, Illinois, USA) (20 kHz, 400 W and 70 % amplitude), Eppendorf centrifuge 5702, rotavapor Büchi Vac V-513, vacuum vortex evaporator (Buchler Instrument).

2.2. Sample preparation

Grape pomace containing grape seeds and peels were lyophilized for 24 h. After lyophilization, the seeds were carefully collected and triturated in mortar. The sample was conserved at −20 °C until its usage.

2.3. DES preparation

The hydrophobic deep eutectic solvent menthol-thymol was prepared mixing menthol and thymol in equimolar ratio (1,1). The two compounds were stirred at room temperature for 1 h till to reach the final volume of 5 mL.

2.4. Extraction of grape seeds oil

2.4.1. Extraction using n-hexane

Approximately 1.25 g of the sample were mixed with 5 mL of *n*-hexane in an ultrasound-assisted bath for 20 min at 36 °C. After centrifugation at 4400 rpm for 20 min, the supernatant was separated and evaporated by rotary evaporation. The grape seed oil extracted was further dried in high vacuum for 4 h. A final weight of 125.4 mg was obtained. A 10.2 % of yield was obtained. The final yield of grape seed oil was expressed as:

$$\frac{\text{Weight of the obtained oil}}{\text{Dry weight of the seeds}} \times 100$$

2.4.2. Extraction using DES menthol-thymol

Approximately 1.25 g of the sample was added to 5 mL of DES menthol-thymol in an ultrasound-assisted bath as above. After centrifugation (20 min, 4400 rpm) and separation of supernatant, an azeotropic mixture with 2 mL of distilled water was prepared. The deep eutectic solvent menthol-thymol was removed by evaporation using a Vacuum Vortex Evaporator at 70 °C for approximately 20 h. Lyophilisation allowed to obtain 110 mg of grape seeds oil. The final yield of grape seed oil was of 8.8 %, expressed as:

$$\frac{\text{Weight of the obtained oil}}{\text{Dry weight of the seeds}} \times 100.$$

2.5. Chemical characterization by GC

The fatty acids in the oil were esterified into methyl esters according to the ISO-5509 method (ISO, 2000). The obtained fatty acids methyl esters were analyzed on a HP (Hewlett Packard) Agilent 6890 N model gas chromatograph (GC), equipped with a flame ionization detector (FID) and fitted to a HP-88 capillary column (100 m, 0.25 mm i.d., and 0.2 μm). The analytical conditions were as described in our previous paper (Demirci Kayiran et al., 2019).

2.6. In vitro assays

2.6.1. Total phenolics and flavonoids determination

Total phenolics and flavonoids were quantified according to the procedures outlined by (Slinkard & Singleton, 1977). Gallic acid (GA) and rutin (R) were used as reference standards in the studies, with results expressed as gallic acid equivalents (GAE) and rutin equivalents (RE). The experimental details are presented in the supplemental materials.

2.6.2. Antioxidant assays

In accordance with the methodologies detailed in our prior publication (Grochowski et al., 2017), various antioxidant tests were carried out. The outcomes were represented as milligrams of Trolox equivalents (TE) per gram for the DPPH, ABTS radical scavenging, CUPRAC, and FRAP tests. In millimoles of TE per gram of extract, the phosphomolybdenum (PBD) test examined antioxidant potential, and in milligrams of disodium edetate equivalents (EDTAE) per gram of oil, the metal chelating activity (MCA) was determined. The experimental details are presented in the supplemental materials.

2.6.3. Enzyme inhibition assays

In accordance with the established protocols (Grochowski et al., 2017), experiments on enzyme inhibition were performed on the samples. Acarbose equivalents (ACAE) per gram of extract was used to measure the activities that inhibit amylase and glucosidase, while milligrams of galanthamine equivalents (GALAE) per gram of oil was used to examine the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The amount of tyrosinase inhibition for each gram of oil was measured in milligrams of kojic acid equivalents (KAE). The experimental details are presented in the supplemental materials.

2.7. In vivo assays

2.7.1. Animals

Male CD-1 mice (Harlan, Italy) of 3–4 weeks (25 g) were used for all the experiments. Mice were housed in colony cages, under standard conditions of light, temperature, and relative humidity for at least 1 week before starting experimental sessions. All experiments were performed according to Legislative Decree 27/92 and approved by the local ethics committee (Approval number 198/2013-B).

2.7.2. Zymosan-induced edema formation

Mice received an s.c. administration (20 μL/paw) of zymosan A (3.0 % *w/v* in saline) into the dorsal surface of the right hind paw. Paw volume was measured before the injections and at the peak time 4 h thereafter using a hydroplethysmometer apparatus (Ugo Basile, Italy). The increase in paw volume was then evaluated as the percentage difference between the paw volume at 4 h and the basal paw volume (Pieretti et al., 2022). Extracts were dissolved in DMSO:saline (ratio 1:3 *v*/v) and were administered s.c. into the dorsal surface of the right hind paw at the dose of 100 μg/20 μL paw 15 min before zymosan.

2.7.3. Zymosan-induced thermal hyperalgesia

The plantar test (Ugo Basile, Italy) was used to measure the sensitivity to a noxious heat stimulus to assess thermal hyperalgesia after carrageenan administration (Pieretti et al., 2022). A constant radiant heat source was directed on mouse footpad until its withdrawal, foot drumming or licking. A timer starts automatically when the heat source was activated, and a photocell stops the timer when the mouse withdraws its hind paw. Animals were acclimatized to their environment for 1 h before the measurements of paw withdrawal latency (PWL), when exploratory behaviour had ceased. The heat intensity was adjusted to obtain a baseline between 10 and 15 s and a 30 s cut off was used to avoid tissue damage. A total of 3 readings were taken from each paw and averaged. Animals were first tested to determine their baseline PWL to respond; 2 h later, each animal received an s.c. injection of 20 L of 3 % zymosan into the dorsal surface of the right hind paw. The PWL (s) of each animal to the plantar test was determined again at 4 h after the zymosan injection. Mice received an s.c. injection of extracts (100 μg/20 L) into the dorsal surface of the right hind paw, 15 min before zymosan. Extracts were dissolved in DMSO:saline (ratio 1:3 *v*/v). Data are reported as the maximum possible effect (MPE) according to the following formula: (MPE) = (post-drug latency − baseline latency)/(cut-off time − baseline latency) × 100.

2.7.4. Formalin test

The procedure used has been previously described (Pieretti et al., 2022). Subcutaneous (s.c.) injection of a dilute solution of formalin (1 %, 20 μL/paw) into the mice hind paw evokes nociceptive behavioral responses, such as licking or biting the injected paw, which are considered indices of pain. The nociceptive response shows a biphasic trend, consisting of an early phase occurring from 0 to 10 min after the formalin injection, due to the direct stimulation of peripheral nociceptors, followed by a late prolonged phase occurring from 10 to 40 min that reflects the response to inflammatory pain. During the test, the mouse was placed in a plexiglas observation cage (30 × 14 × 12 cm), 1 h before the formalin administration to allow it to acclimatize to its surroundings. Immediately after the formalin injection, the mouse was returned to the plexiglas observation cage, and nociceptive behaviour was continuously measured using a stopwatch for 5 min intervals for a total testing time of 40 min. The total time (s) that the animal spent licking or biting its paw during the formalin-induced early and late phase of nociception was recorded. Extracts were dissolved in DMSO:saline (ratio 1:3 v/v) and then administered s.c. into the mice hind paw at a dose of 100 μg/20 L, 15 min before the formalin.

2.8. Statistical analysis

Antioxidant and enzyme inhibitory activities are reported as mean ± SD. Statistical significance was assessed through the Student's *t*-test ($\alpha = 0.05$) using SPSS software version 14.0. For Zymosan-induced edema formation and Zymosan-induced thermal hyperalgesia assays, the statistical analysis was performed by using one-way ANOVA followed by Dunnett's multiple comparisons test. For the Formalin test, the statistical analysis was performed by using two-way ANOVA followed by Sidak's multiple comparisons test.

3. Results and discussion

3.1. Grape seeds oil extraction

The final yield of grape seeds oil obtained with DES menthol:thymol (8.8 %) was slightly lower than that of *n*-hexane extraction procedure (10.2 %), which resulted to be easier and less time-consuming. Unfortunately, the toxicity of *n*-hexane involving the peripheral nervous system and the irritation of mucous membranes in the respiratory system is well documented (Jørgensen & Cohr, 1981). These drawbacks are compensated by the benefits of using a DES. Our deep eutectic solvent mixture is composed of two natural compounds. Menthol is a cyclic monoterpene alcohol which represents the main constituent of the essential oils of *Mentha canadensis* L. and *Mentha x piperita* L., it is found in food, *e.g.* candies and chewing gum, cosmetics, shampoos (Kamatou et al., 2013; Kolassa, 2013). Thymol is a monoterpenoid phenol characterized by a peculiar odour, it appears colourless and crystalline; thymol represents the main ingredient of oil extracted from thyme, used as a food seasoning, for culinary and medical purposes (Escobar et al., 2020; Kuete, 2017). In general, NADES derives from natural primary metabolites and for this reason are considered environmentally safe and non-toxic (Mišan et al., 2019). The mixtures menthol:thymol were also used in different molar ratios for the extraction of other type of compounds, *e.g.* carboxylic acids and triterpenic acids, showing its great

versatility in the extraction of a broad range of bioactive compounds (Demmelmayer et al., 2023; Silva et al., 2020). The initial extraction costs are higher for DES than *n*-hexane, considering the price of the reagents and the electric power necessary for the evaporation instruments. Also, for further industrial applications, sustainable and economic recovery technology is required. On the other hand, this DES provides an environmental benefit being terpene a class of natural compounds considered generally safe differently to the human toxicity of organic solvents like *n*-hexane (Jørgensen & Cohr, 1981; Kamatou et al., 2013). A recent study investigated the potential sustainability from a chemical engineering point of view, of a panel of hydrophobic DESs for extractions from the water phase, determining their viscosity, the density difference between DES and water, the transfer of the DES to the water phase and the pH change. DES menthol:thymol 1:1 resulted to be relatively sustainable, satisfying all the criteria considered in the study (Van Osch et al., 2019).

3.2. Fatty acids composition of grape seeds oil

The fatty acid composition of the oil samples was analyzed using the GC-FID technique and the results are summarized in Table 1. The fatty acid composition depends on the extraction solvents used. In both oils, the main fatty acid was C 18:2 ω6 (linoleic acid) and its content in the oil extracted with deep eutectic solvent was almost 17 % lower than in the oil extracted with *n*-hexane. Consistent with our results, several researchers reported that linoleic acid was the dominant fatty acid in the composition of grape oils (Caicedo-Paz et al., 2024; Leon-Bejarano et al., 2024; Ojha et al., 2024; Tangolar et al., 2009). After linoleic acid, C 18:1 ω9 was the second dominant fatty acid in the grape oils with a value of 15.49–20.29 %. C 16:0 (palmitic acid) and C 18:0 (stearic acid) were the main fatty acids in the grape oils tested. Based on these results, the polyunsaturated fatty acid content (61.35 %–67.53 %) was higher than the saturated (12.05 %–22.26 %) and monounsaturated fatty acid content (16.40 %–20.43 %). The content of linoleic acid is low in the DES extract, while the content of C 18:3 ω3 (linolenic acid) was significantly increased (from 0.53 % to 5.18 %). In light of these data and considering the toxic properties of *n*-hexane, this DES represents a safe alternative to the use of hydrocarburic solvents for the recovery of MUFA and PUFA from grape seeds.

3.3. Total phenolic and flavonoid content

The importance of phenolic compounds as co-adjuvant of diverse therapies for the treatments of human diseases (Singh & Yadav, 2022), prompted the development of several extraction methods to allow their isolation (Hikmawanti et al., 2021). In this sense, we investigated the effect of DES extraction on the recovery of phenolic compounds from grape seeds. The results are shown in Table 2. The grape seed oil extracted by DES menthol: thymol in UAE shows higher total phenolics and favonoids content than that obtained by *n*-hexane within the same technique. According to this, several authors reported that the use of DES increases the yield of phenolic compounds (Alchera et al., 2024; Bragagnolo et al., 2024; Grisales-Mejía et al., 2024), in particular menthol:thymol mixture can improve the osmotic gradient forcing the extraction of phenolic compounds (Bragagnolo et al., 2024). Moreover diverse total phenolic contents in grape oil (Grinvald et al., 2024; Joujou et al., 2024; Mohamed Ahmed et al., 2024) can be due to different parameters such as the extraction solvents and techniques, temperature alongside geographical and climatic conditions. The reason why the content of total phenolics and flavonoids in DES extract is higher than that of *n*-hexane extract, could be an improved solubilization of the most hydrophobic compounds belonging to these classes of molecules in menthol:thymol mixture. The factors responsible of their ability to extract and dissolve polyphenol compounds are the relatively low entropy of NADES systems and the formation of hydrogen bonds. Indeed, thymol:menthol mixtures at different molar ratio have been previously investigated for the dissolution and removal of quercetin from aqueous medium showing their high efficiency in quercetin extraction from contaminated water (Bergua et al., 2022).

Table 1
Fatty acid composition of the grape oils. Values reported are means ±S.D. SFA: saturated fatty acids. MUFA: monounsaturated fatty acids. PUFA: polyunsaturated fatty acids. Different letters indicate significant differences in the oils ($p < 0.05$, by student *t*-test).

Fatty acids	Grape seeds oil-*n*-hexane	Grape seeds oil-DES
C 14:0	0.05 ± 0.01^{b}	1.42 ± 0.59^{a}
C 16:0	7.69 ± 0.03^{b}	14.47 ± 0.88^{a}
C 17:0	0.08 ± 0.01^{b}	0.90 ± 0.05^{a}
C 18:0	4.23 ± 0.06^{b}	5.48 ± 0.30^{a}
ΣSFA	12.05 ± 0.02^{b}	22.26 ± 0.03^{a}
C 16:1 ω7	0.14 ± 0.03^{b}	0.91 ± 0.13^{a}
C 18:1 ω9	20.29 ± 0.13^{a}	15.49 ± 0.87^{b}
ΣMUFA	20.43 ± 0.11^{a}	16.40 ± 1.00^{b}
C 18:2 ω6	67.01 ± 0.09^{a}	56.17 ± 0.60^{b}
C 18:3 ω3	0.53 ± 0.01^{b}	5.18 ± 0.42^{a}
ΣPUFA	67.53 ± 0.08^{a}	61.35 ± 1.03^{b}

Table 2
Total phenolic and flavonoid results are expressed as the media of 3 parallel experiments ± standard deviation (SD). TPC: total phenolic content; TFC: total flavonoid content; GAE: gallic acid equivalents; RE: rutin equivalents. Different letters indicate significant differences in the oils ($p < 0.05$, by student *t*-test).

Samples	TPC (mg GAE/g)	TFC (mg RE/g)
Grape seeds oil-*n*-hexane	15.62 ± 0.62^{b}	0.13 ± 0.01^{b}
Grape seeds oil-DES	18.65 ± 0.74^{a}	0.73 ± 0.02^{a}

3.4. Antioxidant activity

Antioxidant compounds are excellent weapons for defending against free radical attacks (Chaudhary et al., 2023), in fact the high antioxidant capacity is associated with health-promoting effects. We investigated the antioxidant capacity of grape oils extracted with *n*-hexane and deep eutectic solvent using various tests including radical scavenging, reducing power, metal chelation and phosphomolybdenum.

Both oils were inactive toward DPPH and ABTS radicals (Table 3). Among the antioxidant mechanisms, the reducing effect depends on the electron donating ability of the extracts. If a plant extract has a high ability to donate electrons, it could be considered a powerful antioxidant. CUPRAC and FRAP assays are based on the reduction of Cu^{2+} to Cu^{+} and Fe^{3+} to Fe^{2+}, respectively (Bibi Sadeer et al., 2020). From Table 3, it can be seen that the oil obtained with DES had a higher reducing ability than the oil extract with *n*-hexane. The results obtained can be explained by the high phenolic concentration in the oil extracted with DES. The phosphomolybdenum test is one of the total antioxidant capacity tests and involves the conversion of Mo(VI) to Mo(V) by antioxidants in the acidic state. The reducing ability of the oil extracted with DES was almost twice than that of the oil extracted with *n*-hexane. Metal chelation is thought to inhibit the production of hydroxyl radicals in the Fenton reaction and is therefore considered an important antioxidant mechanism. As observed in other antioxidant assays, grape oil extracted using DES exhibited greater metal chelating activity compared to grape oil extracted with *n*-hexane. Literature reports that DES mixtures increase antioxidant effectiveness in the preparation of various plants extracts (Cabrera et al., 2024; Milošević et al., 2024; Sik et al., 2024). The enhanced antioxidant activity of grape seed oil extracted by using DES menthol: thymol is probably due to the content of phenolic and flavonoid compounds detected in the TPC and TFC assays (paragraph 3.2), but we cannot exclude the presence of other bioactive molecules such as amino acids that can give a positive result in the colorimetric and *in vitro* assays. Moreover the composition of DES could influence this

Table 3
The results of antioxidant assays are the media of three different experiments ± SD.; TE: trolox equivalents; EDTAE: EDTA equivalents. Different letters indicate significant differences in the oils ($p < 0.05$, by student *t*-test).

Samples	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	Phosphomolybdenum (mmol TE/g)	MCA (mg EDTAE/g)
Grape seeds oil-*n*-hexane	Na	na	30.51 ± 0.29[b]	16.62 ± 0.09[b]	0.57 ± 0.02[b]	13.90 ± 2.58[b]
Grape seeds oil-DES	Na	na	71.46 ± 1.81[a]	34.95 ± 0.46[a]	1.13 ± 0.01[a]	32.66 ± 0.18[a]

factor; thymol has been investigated for its antioxidant properties through different preclinical studies on cell lines and animal models (Nagoor Meeran et al., 2017). Its ability to scavenge OH radicals was also examined by Venu et al. (Venu et al., 2013). In our study the deep eutectic solvent was completely evaporated through the formation of an azeotropic mixture with distilled water in rotavapor followed by high vacuum vortex evaporator, however we cannot exclude the presence of thymol in negligible trace in the seeds oil, increasing its antioxidant effect. Overall, the grape seeds oil obtained with DES showed higher effectiveness than the oil obtained with *n*-hexane *in vitro,* thus this mixture may be considered as a powerful system to develop novel functional foods based on grape seeds oil.

3.5. Enzyme inhibition activity

Targeting enzymes involved in cellular mechanisms related to neurodegenerative diseases represents one of the therapeutic approaches for their treatment. The inhibition of some enzymes can help the management of serious health problems such as diabetes, Alzheimer's disease or obesity (De Oliveira et al., 2024; Kenakin, 2017). In this context, several compounds are commonly used as enzyme inhibitors in the production of oral drugs but most of them have unpleasant side effects. The oil samples were tested against several enzymes and results are summarized in Table 4. Data are very closed each other, moreover none of them shows activity against AChE.

The oil extracted with *n*-hexane showed greater BChE inhibition (5.64 mg GALAE/g) than the oil extracted with DES (5.13 mg GALAE/g), but this was not statistically significant ($p > 0.05$). The tyrosinase inhibitory effect of the oil extracted with DES (53.13 mg KAE/g) was higher than those obtained with *n*-hexane (50.99 mg KAE/g), but not statistically significant ($p > 0.05$). Amylase inhibition for DES extracted oil (0.57 mmol ACAE/g) was higher than that of *n*-hexane oil (0.47 mmol ACAE/g) ($p < 0.05$). This fact is embodied by the complex nature of phytochemicals and their interactions both synergetic and antagonistic.

3.6. Anti-inflammatory activity

Chronic disorders are often associated with inflammation processes difficult to manage. Thus, the consumption of nutriments with anti-inflammatory properties could be helpful in case of chronic diseases (Gupta et al., 2020). A previous study reported the ability of polyphenols contained in grape seeds extract of inhibiting the release of arachidonic acid involved in the leukotrienes and prostaglandins synthesis (Gupta et al., 2020; Santangelo et al., 2007). Another study reported that the treatment with grape seed oil from Muscat Ottonel variety (obtained after the fermentation processes as a waste) induce the decrease in paw-edema at 180 min time point after the administration of 1 % carrageenan in mice, while the assumption of grape seed oil over a period of ten days didn't result in an inflammation protective effect suggesting its acute anti-inflammatory effect (Zarev et al., 2023).

3.6.1. Zymosan-induced edema formation

Mice were pretreated with a dose of extract (100 μg) s.c. into the mice hind paw, 15 min before zymosan s.c. injection in the same way, and edema was evaluated before and 4 h after the stimulus. As shown in Fig. 1, the edema formation was significantly inhibited by grape seeds oil extracted with *n*-hexane (C2). The grape seeds oil extracted using DES menthol-thymol (C1) resulted ineffective.

3.6.2. Zymosan-induced thermal hyperalgesia

The results of these experiments are reported in Fig. 2. After zymosan administration in the mice paw, a hyperalgesic effects was recorded as a reduction in nociceptive threshold to thermal stimuli (Fig. 2). Extracts induced a reversion of the hyperalgesia induced by zymosan, with the following order to magnitude: C2 > C1.

3.6.3. Formalin test

The results of these experiments are shown in Fig. 3. In the formalin test, administration of extracts at the dose of 100 μg did not change the animals' behavioral response induced by formalin in the early phase.

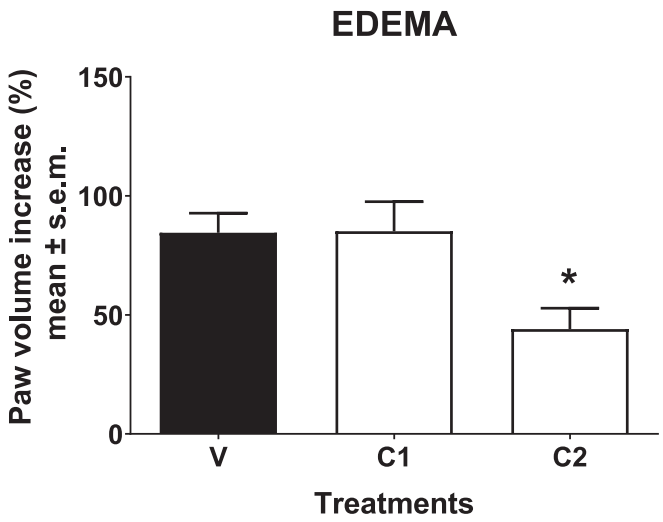

Fig. 1. Effects induced by C1 and C2 extracts administered s.c. into the mice hind paw at the dose of 100 μg/paw, 15 min before zymosan (3.0 % *w/v* in saline, 20 μL/paw) administration in the same paw. Statistical analysis was performed by using one-way ANOVA followed by Dunnett's multiple comparisons test. * is for $p < 0.05$ *vs.* V (vehicle-treated animals). $N = 6$.

Table 4
The inhibition values are the media of three experiments ± SD. GALAE: galantamine equivalents; KAE: kojic acid equivalents; ACAE: acarbose equivalents. Different letters indicate significant differences in the oils ($p < 0.05$, by student t-test).

Samples	AChE inhibition (mg GALAE/g)	BChE inhibition (mg GALAE/g)	Tyrosinase inhibition (mg KAE/g)	Amylase inhibition (mmol ACAE/g)	Glucosidase inhibition (mmol ACAE/g)
Grape seeds oil-*n*-hexane	na	5.64 ± 0.38[a]	50.99 ± 1.87[a]	0.47 ± 0.002[b]	8.09 ± 0.04[a]
Grape seeds oil-DES	na	5.13 ± 0.91[a]	53.13 ± 3.08[a]	0.57 ± 0.005[a]	7.94 ± 0.15[a]

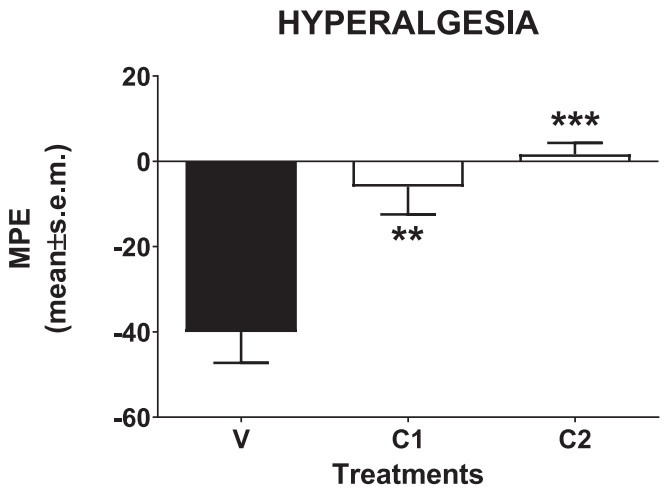

Fig. 2. Effects induced by extracts on zymosan-induced thermal hyperalgesia. Extracts were administered s.c. into the mice hind paw at the dose 100 μg, 15 min before zymosan (20 μL of 3 % zymosan) administration in the same paw. Pain threshold was measured before and 4 h after zymosan administration. Data are reported as the maximum possible effect (MPE) according to the following formula: (MPE) = (post-drug latency − baseline latency)/(cut-off time − baseline latency) × 100. Statistical analysis was performed by using one-way ANOVA followed by Dunnett's multiple comparisons test. ** is for $p < 0.01$ and *** is for $p < 0.001$ *vs.* V (vehicle-treated animals). N = 6.

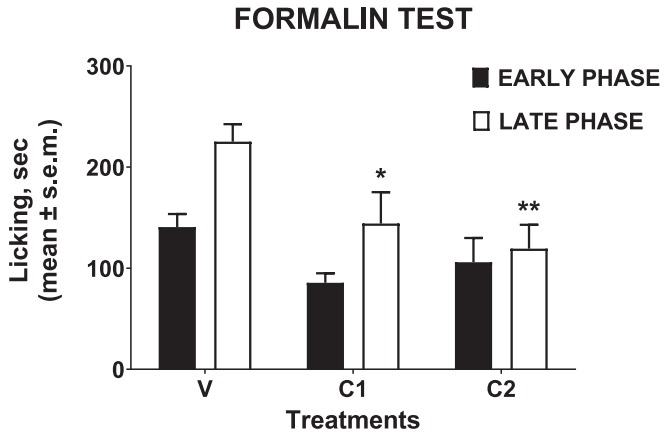

Fig. 3. Effects induced by extracts administered s.c. into the mice hind paw at the dose 100 μg, 15 min before the formalin (1 %, 20 μL/paw). Statistical analysis was performed by using two-way ANOVA followed by Šídák's multiple comparisons test * is for $p < 0.05$, ** is for $p < 0.01$ *vs.* V (vehicle-treated animals). N = 6.

Extracts induced a significant reduction in the licking time induced by the aldehyde in the late phase of the test, with the same order of magnitude as observed in the hyperalgesia experiments namely C2 > C1.

The difference in terms of anti-inflammatory activity between the grape seed oil extracted with DES and *n*-hexane could be related to the different fatty acid composition. As shown in Table 1, polyunsaturated fatty acids represented the major percentage of fatty acids present in both grape seeds' oils. The main fatty acid in both oils was linoleic acid, but its content in grape seeds oil extracted with DES was almost 17 % lower than in the oil obtained with *n*-hexane. Considering the anti-inflammatory properties of linoleic acid (Saiki et al., 2017; Stuyvesant & Jolley, 1967), this difference in its content could be the reason why the anti-inflammatory effect of the DES extract is lower than *n*-hexane extract. The omega-6/omega-3 (n-6/n-3) ratio in grape seeds oil extracted with NADES is lower than that of grape seeds oil obtained by *n*-hexane, indicating a high anti-inflammatory activity for the first extract. In fact, omega-3 possesses potent immunomodulatory activity acting on intracellular signaling pathways, transcription factor activity and gene expression, while omega-6 inhibits the anti-inflammatory and inflammation-resolving effect of omega-3 fatty acids. Also, low levels of linolenic acid may be desired in edible oils, considering that high levels of this fatty acid can produce oxidized non-beneficial products.

4. Conclusion

To summarise, this research project aimed to evaluate the advantages of applying a green extraction approach using UAE combined to DES menthol: thymol as alternative to *n*-hexane for the extraction of grape seeds oil. The fatty acid composition of the grape seeds' oils was different, showing that the content of linoleic acid decreased by using DES, on the contrary the content of C18:3 ω3 (linolenic acid) was significantly increased from 0.53 % to 5.18 %, which is a desirable feature for the determination of its anti-inflammatory activity. In terms of total phenolic and flavonoids content, the grape seed oil obtained by DES extraction shows the best results. Its high phenolic content may explain the major reducing ability in FRAP and CUPRAC assays, Phosphomolybdenum and MCA assays compared with the oil extracted with *n*-hexane. Regarding enzyme inhibition activity, the oil extracted with DES was more effective than the oil extracted with *n*-hexane only for amylase inhibition. The anti-inflammatory assays showed that, while the only effective extract in inhibiting zymosan-induced edema formation was that in *n*-hexane, both grape seeds' oils induce a reversion of the hyperalgesia, also reducing the licking time in the late phase of the formalin test. In conclusion, the use of a completely green extraction technique leads to an improvement of some features of grape seeds oil extract, *e.g.* its phenolic and flavonoids content, antioxidant and amylase inhibition; moreover the anti-inflammatory activity was maintained. This preliminary work opens new perspective in the use of hydrophobic deep eutectic solvents for the recovery of lipophilic compounds from food matrices. Despite the long evaporation time of thymol: menthol system, this mixture should be further considered as a more sustainable and alternative solvent to counteract the negative environmental impact of organic ones.

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CRediT authorship contribution statement

Lorenza Marinaccio: Writing – original draft, Data curation, Conceptualization. **Giulia Gentile:** Methodology, Conceptualization. **Gokhan Zengin:** Formal analysis, Data curation. **Stefano Pieretti:** Validation, Supervision. **Azzurra Stefanucci:** Writing – review & editing, Writing – original draft, Supervision. **Angelo Cichelli:** Writing – review & editing, Funding acquisition. **Adriano Mollica:** Writing – review & editing, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102273.

Data availability

Data will be made available on request.

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