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ORIGINAL PAPER

Recovery of *Pistacia lentiscus* edible oil by using 2-methyloxolane as an eco-friendly and sustainable solvent

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Abstract

The extraction of oil from oilseeds is conventionnaly performed using n-hexane. However, due to its toxicity and drawbacks, there is a growing need to explore greener solvents. In this study, 2-methyloxolane (2-MeOx) was selected to evaluate its potential as a replacement for n-hexane in extracting lipophilic compounds from *Pistacia lentiscus* seeds. Hansen solubility parameters were used to predict the solubility of target compounds in the selected solvents. Experimental analyses were focused on the extraction yield, fatty acid composition, sterol and tocopherol contents. As n-hexane, ours results indicated that 2-MeOx yielded similar performance in terms of extraction yield, fatty acid composition, sterol and tocopherol contents. Additionally, 2-MeOx extract demonstrated a high polyphenol content and exhibited strong antioxidant potential. Furthermore, it was shown that green extracted oil at low concentration had no cytotoxicity against macrophages. Moreover, the oil exhibited anti-inflammatory activity and suppressed nitric oxide (NO) secretion considerably on LPS-stimulated macrophages with IC_{50} value of 33.04 µg/mL.

Keywords *Pistacia lentiscus* · Edible oil · 2-MeOx · Hansen solubility parameters · Bioactivities

Introduction

Organic solvents are employed for millions of purposes, prompting us to consider their potential toxicity, especially in chemical, agricultural, and pharmaceutical applications. In fact, due to their toxicity and volatility, petrochemical solvents present substantial risks to both the environment and human health [1]. Currently, n-hexane, a lipophilic organic solvent, remains as the primary choice for extracting vegetable oils, with an annual consumption of around 1500 kilotons [2]. Its popularity arises from several advantages, including low cost, easy evaporation for removal, a convenient boiling point that minimizes losses during extraction and heat consumption during recovery, as well as its stability and high lipid selectivity [3]. However, despite these advantages, n-hexane presents numerous drawbacks. It is classified as a neurotoxic substance, considered a reproductive toxicant (category 2), and posess a threat to the aquatic environment (category 2) under the European Directives and the Registration, Evaluation, Authorization, and Restriction of Chemicals regulation [2–4].

As a result, there is a growing interest in utilizing safer and alternatives solvents, such as water, ionic liquids, and green solvents derived from natural and renewable sources. In fact, several researches have explored the potential of bio-based solvents to replace n-hexane in the extraction of oil from plant sources, including ethanol, isopropanol, cyclopenthylmethylether, ethyl lactate and dimethylcarbonate [5–8]. Among these alternatives, 2-MeOx a cyclic ether derived from carbohydrates found in abundant lignocellulosic biomass (representing the most abundant biomass resource on Earth) [2], emerges as a particularly promising bio-based solvent, showing significant potential to replace n-hexane in lipid extraction from various plant materials [3–9]. This solvent is environmentally benign, readily biodegrading into carbon dioxide and water [2]. Furthermore, in March 2022, the European Food Safety Authority

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approved the use of 2-MeOx as a food grade extraction solvent [10]. The utilization of 2-MeOx in edible oil extraction holds the promise of introducing a reliable industrial alternative to bolster the world's food supply [2]. Thus, 2-MeOx could be a preferable option for companies looking to improve the cleanliness of their production processes, potentially reducing costs associated with solvent losses and waste disposal compared to traditional petrochemical solvents [2].

In this study, we have explored the potential of this solvent to replace n-hexane in the extraction of vegetable oil from *P. lentiscus* seeds. According to Trabelsi et al. [12], these seeds yielded between 1.83% to 42.54% of vegetable oil depending on their maturity level. One of the most distinguishing characteristics of this oil lies in its richness in unsaturated fatty acids, particularly oleic and linoleic acids [5]. These unsaturated fatty acids, known for their health-promoting properties, hold immense potential. They have been linked to various medicinal benefits, including anti-inflammatory [5], antioxidant [12], antifungal, antibacterial [13], antidiabetic and neuroprotective properties [14]. Furthermore, they can be harnessed to develop functional foods that cater to an increasingly health-conscious consumer base [15].

The significance of this study stems from the need to optimize the extraction process of *P. lentiscus* seed oil, ensuring that it preserves its valuable phytonutrients while addressing environmental and health concerns related to the use of traditional solvents like n-hexane. By exploring safer and environmentally friendly alternatives, such as 2-MeOx, we aim to unlock the full potential of *P. lentiscus* seed oil for both health-enhancing and sustainable food application.

The rationale behind this study was to address the following queries:

1. Can the same metabolites recovered using n-hexane, a conventional lipophilic solvent, also be extracted using 2-MeOx as a bio-based alternative?
2. Would substituting n-hexane with 2-MeOx for vegetable oil extraction lead to the alteration of qualitative and quantitative oil composition ?
3. Does the extract obtained with 2-MeOx demonstrate cell toxicity and exhibit beneficial biological activities?

To answer these questions we used two approaches: an in silico approach based on Hansen solubility parameters and an experimental approach based on chromatographic analyzes and in vitro tests.

Materials and methods

Hansen solubility parameters (HSPs)

Predicted solubilities of some selected metabolites usually found in *P. lentiscus* seeds oil were determined in n-hexane and 2-MeOx, using the thorey of Hansen solubility parameters (HSPs). This computational approach developed by Charles Hansen [16] have been widely utilized as a real decision-making tool based on the total (Hildebrand) solubility parameters to explain the dissolution behaviour [17–20]. It provides an efficient and convenient way to describe solvent–solute interactions based on the principle of "*like dissolves like*" [21]. The Hansen model states that the total cohesive energy density is equals to the sum of the energies required to overcome dispersion forces (δ_d^2), polar forces from dipole moments (δ_p^2), and hydrogen bonding (exchange of electrons, proton donor/acceptor) between molecules (δ_h^2), as expressed in Eq. 1:

$$\delta total^2 = \delta d^2 + \delta p^2 + \delta h^2 \quad (1)$$

where δ_{total} is defined as Hansen total solubility parameter, which now consists of three HSP: δ_d the dispersive term, δ_p the polar term, and δ_h the hydrogen bonding term.

To optimize HSP solvents, we have calculated a composite affinity parameter known as the relative energy difference number (RED). This parameter serves to determine the solubility between the solvent and solute. The RED number represent the ratio between R_{solv} and R_{spher} as given by the following equation:

$$RED = R_{solv}/R_{spher} \quad (2)$$

where R_{spher} is the radius of a Hansen solubility sphere and R_{solv} is the distance of a solvent from the center of the Hansen solubility sphere, given by Eq. 3:

$$R_{solv} = (4(\delta_{dSolu} - \delta_{dSolv})^2 + (\delta_{pSolu} - \delta_{pSolv})^2 + (\delta_{hSolu} - \delta_{hSolv})^2)^{1/2} \quad (3)$$

where "*Solu*" refers to the solute and "*Solv*" refers to the solvent. Generally, this parameter follows the classical principle of "*like dissolves like*": the smaller R_a value, the higher the expected affinity between solute and solvent. This implies that a suitable solvent has a RED number smaller than one and exhibit favorable properties for dissolution, whereas unsuitable solvent has a RED number superior than one.

The JChemPaint version 3.3 (GitHub Pages, San Francisco, CA, USA) software was used to convert the chemical structures of the solvents and solutes to their simplified molecular input line entry syntax (SMILES) notations. The SMILES notations were subsequently employed in the HSP calculation using the Yamamoto-molecular break method. This method effectively breaks down the SMILES into

corresponding functional groups and estimates their Hansen Solubility Parameters (HSPs). This method has been embedded in the HSPiP software in order to facilitate the calculation of the HSP of target metabolites and tested solvents (n-hexane, 2-MeOx). These solubility parameters were further modeled using two dimensional HSP sphere to enhance the visualization of the solute–solvent system interactions. This representation was adopted due to the low sensitivity of δ_d towards solvents [20–22].

Plant material, chemical and reagent

Pistacia lentiscus seeds were collected from trees growing in Tabarka region in North of Tunisia. All solvents were of analytical grade and purchased from VWR international (Darmstadt, Germany). The green solvent 2-methyloxolane and standards used for chromatography analyses was acquired from Sigma-Aldrich Co, St. Louis (MO, USA). All other chemical reagents were obtained from Alfa Aesar Co. (Ward Hill, MA) or Fluka (Buchs, Switzerland) and were used as received.

Lipid extraction

P. lentiscus seeds samples were air-dried in shadow at room temperature and were then finely ground to fine powder just before extraction. Oils were extracted, using two solvents (n-hexane conventionally used for lipid extraction and 2-methyloxolane as green alternative solvent) by means of a conventional soxhlet extractor during 8 h. The ratio of solvent to solid was 1:10 (w/v). After extraction, solvent was removed from the miscella under reduced pressure using a rotary vacuum evaporator (R-300, Buchi, Switzerland). The crude lipid extracts were aliquoted in an amber vial and stored at 4 °C until further analyses. All the extractions were conducted in triplicate.

Extraction yield was calculated according to the following expression:

$$Extraction\ yield(\%) = \frac{\text{Mass of crude oil (g)}}{\text{Mass of dry sample}} * 100 \qquad (4)$$

Crude oil analysis

Saponifiable compounds

Neutral lipids The identification and relative quantification of neutral glycerides in *P. lentiscus* oils were carried out using high-performance thin-layer chromatography (HPTLC). Standard solutions of palmitic acid (free fatty acid, FFA), DL-α-palmitin (monoacyl glyceride, MAG), glyceryl 1,3-dipalmitate (diacyl glyceride, DAG), and glyceryl tripalmitate (triacyl glyceride, TAG) were prepared in chloroform (0.2 mg/mL), along with crude oil sample solutions (1–15 mg/mL). Silica gel 60 F254 HPTLC plates (20 × 10 cm) were prewashed by elution with isopropanol and dried at 110 °C for 20 min. The Automatic TLC Sampler 5 (ATS 5, CAMAG, Switzerland) was utilized to apply the samples and standards as 6 mm bands on the plates. Subsequently, the plates were developed in an automatic developing chamber (ADC2, CAMAG) using a mixture of n-hexane/diethyl ether/glacial acetic acid (65:35:2, v/v/v) as the mobile phase. The eluent was allowed to rise to a height of 8.5 cm from the origin. For derivatization, each plate was dipped for 6 s in a primuline dye reagent (0.005% (m/v) in acetone/water, (4:1, v/v) and left to dry for 10 min. Pictures of the plates were captured using the TLC visualizer (CAMAG) under UV 366 nm illumination. Finally, the plates were scanned in fluorescence mode at UV 366/ > 400 nm (mercury lamp) using the TLC Scanner 3 (CAMAG).

Fatty acids. Fatty acid methyl esters (FAMEs) were prepared from *P. lentiscus* oil samples through acid-catalyzed transmethylation. Initially, 1 mL of 5% (v/v) methanolic sulfuric acid solution was added to determined quantity of oil in a glass tube. Then, as an internal standard, 500 µL of triheptadecanoin (C17:0; TAG) in n-hexane (2 mg/mL) was added. The mixture was heated in a heating block at 85 °C for 90 min. After cooling to room temperature, 1.5 mL of 0.9% (m/v) NaCl solution and 1 mL of n-hexane were added to the tube, and the mixture was vortexed for 30 s. Two µL of the top organic layer was injected in split mode (split ratio 1:20) at 250 °C onto an Agilent (Japan) 7820A gas chromatography system coupled with flame ionization detector (GC-FID). The instrument was equipped with a BD-EN14103 capillary column (30 m × 320 µm × 0.25 µm). The oven temperature was initially set at 50 °C for 1 min and then increased at a constant rate of 20 °C/min up to 180 °C, followed by an increase of 2 °C/min up to 230 °C. The temperature was maintained at 230 °C for 10 min. FAMEs were identified by comparing their retention times with FAME standards using Agilent EZChrom Elite software.

Unsaponifiable compounds

Tocopherol and sterol determination The tocopherol composition of *P. lentiscus* seeds oil was determined using HPLC according to the ISO 9936 standard [23] and the separation and quantification of sterols was performed according to ISO 12228–1 standard [24].

Total phenolic evaluation Total phenolic content (TPC) of different extracts were assayed using the Folin-Ciocalteu reagent as described by Chaabani et al. [25] with some modifications. Briefly, 125 µL of each diluted extract was

added to 500 µL of distilled water and 125 µL of the Folin–Ciocalteu reagent. After six minutes, the final volume was brought up to 3 mL by adding 1250 µL of sodium carbonate (7%) followed by the addition of 1000 µL of distilled water. After incubation for 90 min in the dark and at room temperature (20 °C), the absorbance was measured at 760 nm using a UV–vis spectrophotometer (Biochrom, Libra S22, UK). The calibration curve was performed with gallic acid (concentrations ranging from 0 to 400 µg/mL) and total phenolic content was expressed as milligram of gallic acid equivalents (GAE) per gram of dry residue (mg GAE/g DR). Measurements were performed in triplicate.

DPPH radical scavenging assay

The 1.1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) radical scavenging capacity of the extracts were estimated based on the method described by Hanato et al. [26]. 250 µL of (0.2 mM) DPPH-methanolic solution was added to 1 mL of each extracts at various concentrations. The obtained mixtures were shaken vigorously and left standing in the dark for 30 min at room temperature, then the absorbance was measured spectrophotometrically at 517 nm. All samples were analyzed in triplicates and the final results were expressed as IC_{50} (µg/mL), which represent the concentration of sample required to scavenge 50% of $DPPH^{\cdot}$ free radicals. The anti-radical capacity (percent inhibition) was determined according to the following equation:

$$Percentinhibition(\%) = \frac{(\text{Acontrol} - \text{Asample})}{\text{Acontrol}} * 100$$

where $A_{control}$ is the absorbance of the control at 30 min, and A_{Sample} is the absorbance of the sample at 30 min.

Anti-inflammatory activity

Cell culture

The RAW 264.7 murine macrophage cells were obtained from the American Type Culture Collection (ATCC, Manassas, USA). These cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5% carbon dioxide.

Cell viability assay

Cell viability was evaluated using a Resazurin assay [27]. RAW 264.7 cells (2×10^5 cells/mL) were cultured in 24-well plates and incubated for 24 h. Subsequently, the RAW 264.7 cells were exposed to *P. lentiscus* oil samples at various concentrations (10–200 µg/mL). The extracts were first dissolved in DMSO and then diluted with the culture medium to achieve different concentrations, ensuring that the final DMSO concentration remained below 0.1% (v/v) to prevent any potential solvent toxicity. After 24 h of treatment, the fluorescence was measured using an automated 96-well Fluoroskan Ascent FlTM plate reader (Thermo-Labsystems) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

Evaluation of the anti-inflammatory activity by nitrite quantification

The anti-inflammatory activity of the *P. lentiscus* edible oil was assessed on the murine macrophage RAW 264.7 cell line by measuring nitric oxide (NO) levels using the Griess reagent. RAW 264.7 cells were seeded in 24-well plates at a density of 2×10^5 cells per well and allowed to attach for 24 h at 37 °C. After 60 min, the cells were treated with Lipopolysaccharide (LPS) at a concentration of 100 µg/mL, either alone or in the presence of different concentrations of the *P. lentiscus* seeds oil extracts (10–100 µg/mL). After 24 h of LPS stimulation, the amount of accumulated nitrite in the culture supernatant was determined using the Griess reaction [28]. The absorbance at 540 nm was measured, and the levels of nitric oxide (NO) produced by the murine macrophage-like RAW264.7 cells were determined by comparing the results with a sodium nitrite standard curve (0–50 µM).

Statistical analysis

All experiments were performed in triplicate and these values were then presented as mean values along with their standard derivations. The results are considered statistically significant at ($P<0.05$). Statistical analyses were performed using JMP 14 software (SAS Institute In., Cary, NC, USA).

Results and discussion

In silico study: solute–solvent solubility prediction using Hansen solubility parameters (HSPs)

The efficient extraction of lipid depends heavily on the dissolution behavior of lipids in the chosen solvent, making the selection of an appropriate solvent a crucial parameter for achieving complete extraction and obtaining a higher lipid yield [5].

In this work the theortical solubility of major molecules of *P. lentiscus* seed oil in 2-MeOx compared to n-hexane taken as reference was predicted through HSPs parametrs.

The selection of these molecules was based on previous study reporting that *P. lentiscus* seed oil is composed mainly by three free fatty acids (FFAs): palmitic acid (C16), oleic acid (C18:1), linoleic acid (C18:2), and six triacylglycerids (TAGs): Palmitoyl-oleyl-linoleoylglycerol (POL), Palmitoyl-dioleylglycerol (POO), Stearoyloleyl-linoleoylglycerol (SOL), Stearoyl-dilinoleoylglycerol (SLL), Oleyl-dilinoleoyl-glycerol (OLL) and Trioleylglycerol (OOO) [11–29].

Hansen solubility parameters were calculated for n-hexane and 2-MeOx and for the main components of *P. lentiscus* seed oil (Table 1). These results were used in plotting a 2D HSP map (Fig. 1). The Hansen solubility parameter difference, which represent the distance between two points, reflects the difficulty of two molecules to solubilize themselves. So, the closer the solvent dot to the target molecule in the Hansen map is, the greater the extraction efficiency of the tested solvents. Moreover, the relative energy difference (RED) numbers were calculated for the studied solvents for the extraction of FFAs and TAGs. Solvents exhibiting RED < 1 are potentially good solvents. Results are summarized in Table 2.

The RED values obtained from HSPiP calculations suggested that FFAs and TAGs solubility in 2-MeOx *P. lentiscus* lipid extract could be relatively higher than in the n-hexane extract. 2-MeOx has RED ≤ 1 for all metabolites and as illustrated in Fig. 1 is closer to fatty acids and triacylglycerids, which means that this solvent has a good potency to dissolve main components of *P. lentiscus* seed oil and can be more efficient than n-hexane (the RED values of all metabolites in 2-MeOx are inferior than in n-hexane). This hypothesis will be validated experimentally based on lipid yield and GC-FID quantification.

Several studies, employing computational software to theoretically evaluate the possibility of replacing n-hexane with 2-MeOx, have consistently shown that 2-MeOx performs better than n-hexane in extracting lipids from a variety of plant materials [4, 9–22].

Table 1 Hansen solubility parameters, technical and ecological properties of solvents used experimentally

	HSPs			Solvent properties					
	δd	δp	δh	B.p (°C)	Log P	Mw (g mol^{-1})	Density (g cm^{-3})	Resource	CO_2 footprint (kg kg^{-1})
Hexane	13.9	0.1	0.1	68	3.9	86.18	630.24	Petrolum	1.9
2-MeOx	15	4.7	3.9	80	0.8	86.13	86.89	Cereal crop	0.19

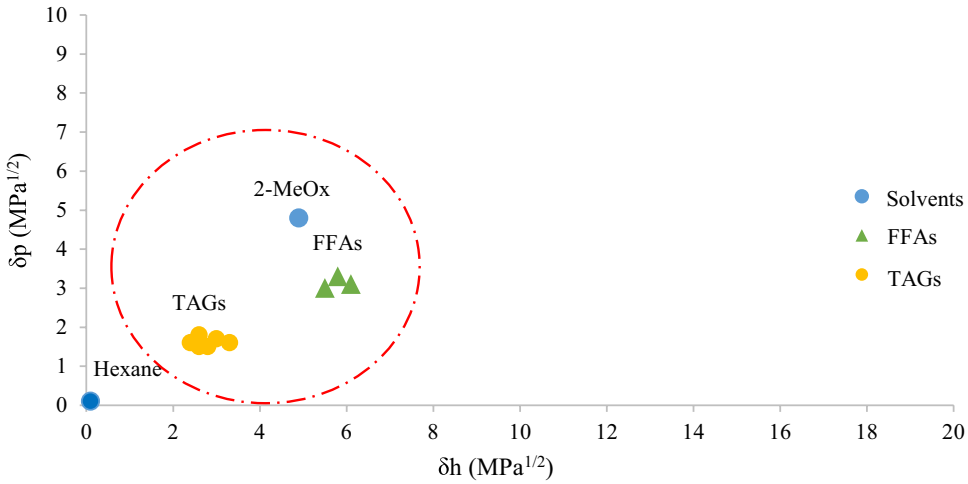

Fig. 1 2-MeOx for solubilizing free fatty acids and triacylglycerids (TAGs) via Hansen's settings

Table 2 The relative energy difference (RED) values for HSP assisted screening of alternative solvent to n-hexane for the solvation of *P. lentiscus* edible seed oil

	Fatty acids			Triglycerides					
	C16	C18:1	C18:2	POL	POO	SOL	SLL	OLL	OOO
Hexane	1.76	1.71	1.90	1.03	1.05	1.10	1.19	1.08	1.07
2-MeOx	0.45	0.47	0.54	0.95	1.00	0.98	0.90	0.91	1.00

Experimental study

Crude oil analysis: lipid yield, neutral lipid and fatty acid composition

Figure 2 illustrates that 2-MeOx and n-hexane yielded equivalent amounts of lipids, with no statistically significant difference observed between these two solvents. The predominant components in extracts obtained using 2-MeOx and n-hexane were triglycerides (TAGs) (97.75% and 97.76%, respectively), while diacylglycerides (DAGs) comprised only between 2.25% and 2.24%, respectively of the total lipid content.

After transmethylation of fatty acids, extracted oils using n-hexane and 2-MeOx were analyzed by GC-FID. Figure 3 reported the chemical composition of *P. lentiscus* seed'oil extracted by tested solvents. Notably, no significant differences were detected bettwen n-hexane and 2-MeOx. The results are consistent both quantitatively and qualitatively.

The predominant fatty acids identified in both n-hexane and 2-MeOx extracts were oleic acid (C18:1n9) (49.27–49.72%, respectively), linoleic acid (C18:2n6) (23.31–23.74%, respectively) and palmitic acid (C16) (22.15–22.41%, respectively), which represent more than 90% of total fatty acids in all extracted oils.

Oleic acid (omega 9) and linoleic acid (omega 6), classified as essential fatty acids, are renowned for their anti-inflammatory, anti-cancer properties, as well as their nutritional benefits [30–32]. In 2-MeOx lipid extract, 3 saturated fatty acids namely capric acid (C10:0), lauric acid (C12:0) and miristic acid (C14:0) were detected in a very tiny amount that not exceed 0.6% and were not detected in n-hexane samples.

The data shows that there is no significant discrepancy in the overall proportions of each fatty acid class between the two extraction solvents. Monounsaturated fatty acids (MUFA) predominate, making up 50.83% to 51.11% of the total extracted oil, followed by saturated fatty acids (SFA) at 25.09% to 25.19%, and polyunsaturated fatty acids (PUFA) at 23.89% to 24.20%. This suggests that *P. lentiscus* oil

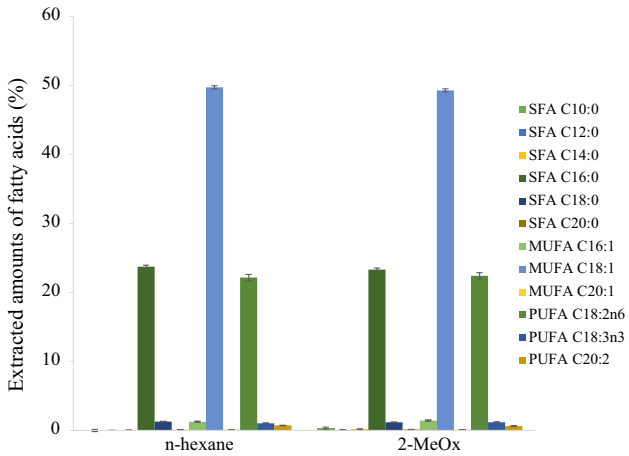

Fig. 3 Fatty acid composition of *P. lentiscus* seed oil extracted with n-hexane and 2-MeOx determined by using GC-FID. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids

can be categorized as monounsaturated oil. These findings align with previous studies [5, 11, 29, 33, 34]. Experimental results underscore the viability of 2-MeOx as a promising alternative to n-hexane for lipid extraction from *P. lentiscus* seed as predicted by HSPiP parametrs.

In fact, several studies have reported that 2-MeOx has a high solvent power and extraction efficiency for lipophilic natural products and food ingredients. This makes it a viable alternative to petroleum-based solvents. Therefore, the solubility power of 2-MeOx compared to n-hexane can be attributed to its solute–solvent interactions and structural properties and its ability to effectively extract lipophilic substances [2–22].

Unsaponifiable compounds

Unsaponifiable compounds includes lipophilic compounds such as tocopherol, sterol and carotenoid, as well as hydrophilic compounds such as phenolic compounds possessing

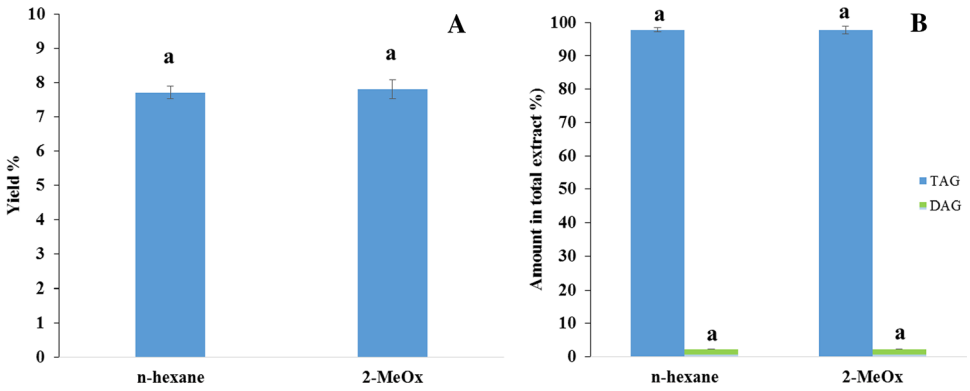

Fig. 2 Lipid yield (**A**) and lipid classes (**B**) of *P. lentiscus* seed oil obtained with n-hexane and 2-MeOx. Values with different lowercase letters in superscript were significantly different at $p < 0.05$

Table 3 *P.lentiscus* seed oil tocopherol composition

	Quantity of Tocopherol (mg/g)			
	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol
n-hexane	$20.86^{a} \pm 0.54$	$2.49^{a} \pm 0.78$	$0.37^{a} \pm 0.90$	$0.36^{a} \pm 0.29$
2-MeOx	$20.89^{a} \pm 0.12$	$2.43^{a} \pm 0.29$	$0.36^{a} \pm 0.01$	$0.37^{a} \pm 0.04$

Data were expressed as mean ± SD calculated from three detrminations. nd: not detected. Values with different lowercase letters in superscript were significantly different at $p < 0.05$

food value and antioxidative properties considered as index of edible oils quality.

Tocopoherol and sterol detetrmination

Many research studies have emphasized the relationship between the properties of vegetable oils and the quantities of tocopherols they contain. As a result, the ability to accurately measure the concentrations of these natural antioxidants holds significant importance [35].

Table 3 provides the quantities of tocopherols in both n-hexane and 2-MeOx extracts of *P. lentiscus* oil. All the extracts exhibited similar total and individual tocopherols contents. Results showed that α-tocopherol is the predominant tocopherol, representing more than 90% of the total tocopherols. β and γ-tocopherol together make up only about 9% of the total tocopherols. δ-tocopherol remains undetectable in both n-hexane and 2-MeOx extracts. This results aligns with findings reported by Dhifi et al. [29]. In this context, Claux et al. [9] noted that substituting n-hexane with 2-MeOx for soybean oil extraction has no impact on the tocopherol composition.

Furthermore, the same trend was observed for sterol composition (Table 4). Thus, the total and individual sterol content of *P. lentiscus* oil was similar for both n-hexane and 2-MeOx (Table 4). Among the sterols, β-sitosterol was found to be the major components of *P. lentiscus* oil (representing 85.94 mg/g in n-hexane and 86.87 mg/g in 2-MeOx extracts), followed by stigmasterol and sitostanol. Our finding is in accordance with previous research reported by Mezni et al. [32] on *P. lentiscus* seed oil. This later, reported that the major sterol identified in *P. lentiscus* seed oil is β-sitosterol, which makes up more than 54% of the total sterols. Claux et al. [9] also reported that the use of 2-MeOx in replacing n-hexane for the extraction of soybean oil does not affect the sterol composition.

These sterols are naturally occurring plant compounds and can have various potential health benefits. They are often studied for their potential role in reducing cholesterol levels and their antioxidant properties [36].

Table 4 *P. lentiscus* seed oil sterol composition

	Quantity of sterol (mg/g)	
	n-hexane	2-MeOx
Cholesterol	nd	nd
β-sitosterol	$85.94^{a} \pm 0.2$	$86.87^{a} \pm 3.2$
Ergosterol	$0.63^{a} \pm 0.14$	$0.59^{a} \pm 0.06$
Sitosterol	$3.03^{a} \pm 0.5$	$2.93^{a} \pm 0.14$
Stigmasterol	$14.78^{a} \pm 0.39$	$14.91^{a} \pm 0.34$
Δ-5, 23, stigmastadienol	$1.45^{a} \pm 0.01$	$1.07^{a} \pm 0.07$
Sitostanol	$6.15^{a} \pm 0.05$	$5.96^{a} \pm 0.01$
Δ-5, 24, stigmastadienol	$1.90^{a} \pm 0.53$	$1.85^{a} \pm 0.46$
Δ-7, avenasterol	$1.18^{a} \pm 0.03$	$0.94^{a} \pm 0.02$
Uvaol	$3.87^{a} \pm 0.1$	$3.69^{a} \pm 0.01$
Total	119.13	118.71

Data were expressed as mean ± SD calculated from three determinations. nd: not detected

Values with different lowercase letters in superscript were significantly different at $p < 0.05$

Polyphenol content and antioxidant potential

As demonstrated previously, 2-MeOx displays similar potential for extracting lipophilic metabolites (fatty acids, tocopherols and sterols) from *P. lentiscus* seed. Therefore, the antioxidant properties of only the eco-extract (2-MeOx) of *P. lentiscus* were exclusively assessed.

In the present study, the antioxidant potential of 2-MeOx lipid extract, was investigated. Therefore, 2-MeOx extract of *P. lentiscus* oil exhibited a high antioxidant potential to scavenge DPPH radical ($IC_{50} = 16.89$ µg/mL). The notable antioxidant potential of this oil can be credited to its high content of tocopherol and sterols [12]. Likwise, the presence of natural hydrophilic antioxidants, specifically phenolic compounds enhanced this activity [15]. These compounds possess numerous hydroxyl groups in their structure, which enable them to effectively counteract and neutralize free radicals. The phenolic concentration in oil plays a significant role in determining its functionality and overall quality. Phenolic compounds can have an impact on the oil's flavor profile and serve as protective agents for the fatty acids, guarding them against oxidation [37]. Moreover, they can actively contribute to enhancing the antioxidant properties alongside other natural antioxidants inherent in the oil. In this specific investigation, the total phenolic content extracted with 2-MeOx was quantified at 870.5 mg GAE/kg of oil. This finding aligns with the literature, it is evident that *P. lentiscus* oil contains a higher polyphenol content compared to virgin argan oil, which typically ranges from 6.07 to 152.04 mg GAE/kg of oil [38] and surpasses the polyphenol content of various other edible vegetable oils, such as extra virgin olive oils (170–210 mg GAE/kg),

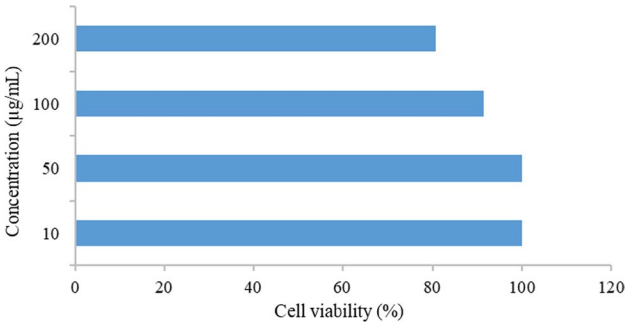

Fig. 4 Cell viability (%) evaluation using resazurin assay for *P. lentiscus* oil obtained with 2-MeOx as green alternative solvent to n-hexane on RAW 264.7 cells. The percent cell viability was determined by comparison to the untreated control

soybean oil (60–80 mg/kg), sunflower oil (3–4 mg/kg), and corn oil (less than 1 mg/kg) [39].

Anti-inflammatory activity

Cytotoxic effect of 2-MeOx extract on macrophage cells

The cytotoxic effect of the *Pistacia* oil obtained with 2-MeOx on macrophage cells was studied after 24 h of cell treatment with different concentrations. Results in Fig. 4 showed that at 10, 50 and 100 µg/mL extracts caused no reduction in cell viability for RAW 264.7 macrophages. Indeed cell viability was equal to 100% for 10 and 50 µg/mL and decreased slightly to 91.42% at 100 µg/mL. A few cells proliferation inhibition was detected at higher concentration of 200 µg/mL. These results allowed us to set the concentration range of 10 to 100 µg/mL for oil in order to assess their anti-inflammatory activity to avoid an impact on cell viability.

Effect of green extracted oil on NO production

In our study, the assessment of anti-inflammatory activity is based on the ability of our oil to inhibit the production of nitrites induced by LPS. Indeed, the stimulation of macrophages during the inflammatory response leads to overproduction of several pro-inflammatory mediators, including nitric oxide (NO) via inducible nitric oxide synthase (iNOS). NO overproduction can induce tissue damage through cytokine-mediated processes [40]. It can also lead to cytotoxicity vasodilation and edema [41]. Thus, NO is a reliable marker for screening new anti-inflammatory treatments in vitro.

Table 5 showed clearly that *P. lentiscus* oil obtained with 2-MeOx contributed significantly to the inhibition of nitrite production in a dose-dependent manner as the inhibitory effect increases with increasing oil concentration.

Table 5 Effect of *P. lentiscus* seeds oil obtained with 2-MeOx as alternative solvent to n-hexane on NO production inhibition (%) in LPS-stimulated RAW 264.7 macrophages (mean ± SD, n = 3 replicates)

2-MeOx	
Concentration (µg/ml)	NO production inhibition (%)
10	5.61 ± 0.99
50	75.67 ± 0.57
100	91.88 ± 0.17
L-NAME (25 µg/ml)	35.25 ± 0.31

At 50 µg/mL oil inhibited NO production in activated macrophages by 75% while at higher dose of 100 µg/mL, NO inhibition reached 91%. The IC_{50} of *P. lentiscus* oil was low of 33.04 µg/mL.

The potent anti-inflammatory properties of *P. lentiscus seed* oil may be attributed to the influence of certain active components, primarily phenolics, tocopherols, and sterols. Furthermore, sterols could potentially provide protection against certain mediators involved in the progression of inflammatory damage. Specifically, β-sitosterol, which present the major sterol on *Pistacia* oil, has been observed to diminish the release of nitric oxide (NO) in activated macrophages, which is associated with a decrease in the activity of inducible nitric oxide synthase (iNOS) [42]. Additionally, α-tocopherol, a major component in the tocopherol fraction of *Pistacia* oil, has been identified as both an antioxidant and an anti-inflammatory agent [43].

Conclusion

The potential of 2-MeOx as an alternative for n-hexane in the extraction of *P. lentiscus* edible oil was demonstrated in this study. Thus, experimental results corrobarted with theoretical HSP simulations. Experimentally, 2-MeOx showed comparable lipid yield to the reference n-hexane. No significant differences in fatty acid acid composition, tocopherol and sterol content was observed. TAGs constituted the predominant lipid class in *Pistacia* edible oil (> 97%). This oil was caracterised by its richness in oleic and linoleic acids as well as secondary metabolites such as sterols, tocopherols and total polyphenols. These metabolites may contribute to its important antioxidant and anti-inflammatory properties. As a result 2-MeOx can be considered as an environmentally and economically viable alternative to conventional petroleum-based solvents for the extraction of lipophilic foodstuff and natural products.

Declarations

Conflict of interest No potential conflict of interest was reported by the authors.

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