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RESEARCH ARTICLE

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Pistacia lentiscus L. edible oil: green extraction with bio-based solvents, metabolite profiling and *in vitro* anti-inflammatory activity[☆]

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Abstract – This study assesses, qualitatively and quantitatively, the performance of ten bio-based solvents to replace hexane for the extraction of oil from *Pistacia lentiscus* edible fruits. Solvent screening was achieved through theoretical approach using the COnductor-like Screening MOdel for Realistic Solvation (COSMO-RS) predictions and an experimental protocol based on GC-FID and HPTLC analysis. Unsupervised multivariate analyses were performed on GC-FID and HPTLC data. Experiments corroborated theoretical predictions indicating that MeTHF was the best alternative solvent to replace hexane. Moreover, the obtained extract exhibited a good anti-inflammatory activity, inhibiting by 91.9% NO release in RAW 264.7 macrophages. These findings highlighted the potential of green solvents especially MeTHF in lipid extraction and exhibited that *P. lentiscus* oil presents a potential anti-inflammatory property and great nutritional value, notably high amount of oleic and linoleic acid, thus encouraging its valorization in food, cosmetic or pharmaceutical industry.

Keywords: *Pistacia lentiscus* L / oil extraction / bio-based solvents / COSMO-RS / anti-inflammatory activity

Résumé – L'huile comestible de *Pistacia lentiscus* L.: éco-extraction par des agro-solvants, profilage métabolique et activité anti-inflammatoire *in vitro*. Cette étude consiste à évaluer, qualitativement et quantitativement, la performance de dix agro-solvants afin de remplacer l'hexane pour l'extraction de l'huile à partir des fruits comestibles de *Pistacia lentiscus*. Le criblage des solvants a été réalisé par une approche théorique en utilisant des outils de prédiction tels que COSMO-RS et une approche expérimentale basée sur les analyses CG-DIF et HPTLC. Des analyses multivariées ont été effectuées sur les données CG-DIF et HPTLC. Les résultats expérimentaux ont confirmé les prédictions théoriques indiquant que le MeTHF était le meilleur solvant alternatif pour substituer l'hexane. En outre, l'extrait obtenu présentait une bonne activité anti-inflammatoire, inhibant de 91,9 % la libération de NO dans les macrophages RAW 264.7. Ces résultats ont mis en évidence le potentiel des solvants verts, en particulier du MeTHF dans l'extraction lipidique et ont montré que l'huile de *P. lentiscus* présentait une activité anti-inflammatoire potentielle et une grande valeur nutritionnelle, notamment une teneur élevée en acide oléique et linoléique, encourageant ainsi sa valorisation dans les industries alimentaire, cosmétique ou pharmaceutique.

Mots clés : *Pistacia lentiscus* L / extraction d'huile / agro-solvants / COSMO-RS / activité anti-inflammatoire

1 Introduction

Pistacia lentiscus L. (Lentisk) belongs to the Anacardiaceae family. Lentisk fruits yield an edible oil with a high nutritional value due to its richness in monounsaturated fatty acids, omega 3 fatty acid, β-sitosterol, α-tocopherols and minerals (Mezni *et al.,* 2014). This edible oil has been used traditionally by Tunisians in their daily diet as condiment, in salads and pastries (Trabelsi *et al.,* 2012). The *Pistacia lentiscus* fruit is used as a source of medicinal oil for the treatment of many diseases such as scabies, respiratory allergies and rheumatism and also for the fabrication of antidiarrhea pills (Ben Khedir *et al.,* 2017). Recently, several studies have reported the pharmacological virtues of this fixed oil (Mezni *et al.,* 2016; Naouar *et al.,* 2016; Ben Khedir *et al.,* 2017).

Conventionally, oils and lipids are extracted using hexane as solvent. Nevertheless, this flammable solvent, derived from petroleum, has recently been categorized as reprotoxic category 2 substance under the REACH regulation (European Directives and Registration, Evaluation, Authorization and Restriction of Chemicals), as category 2 aquatic chronic toxic and listed as forbidden substances in cosmetic products (Rapinel *et al.,* 2016). Nowadays, the development of green chemistry has prompted chemists to find alternative solvents to hazardous ones (Kerton and Marriott, 2013).

Bio-based solvents are renewable resource produced from natural source such as wood, starch, fruits and vegetable oils. These green solvents have a good solvation power. Also, they are biodegradable, non-toxic and non-flammable. Indeed, many studies have shown the potential of alternative solvents for lipid extraction instead of hexane (Sicaire *et al.,* 2015; Breil *et al.,* 2016; Cascant *et al.,* 2017).

In this study, the effectiveness of ten bio-based solvents to substitute hexane for the extraction of lipids from Lentisk berries was evaluated through a computational approach, using COSMO-RS software, and an experimental approach based on GC-FID and HPTLC analysis. Then, anti-inflammatory activity of the best alternative solvent was assessed *in vitro* on murine macrophages.

2 Materials and methods

2.1 Reagents and solvents

Among hydrophobic solvents, ten green alternatives were selected from different chemical families. These are represented by two alcohols (ethanol [EtOH] and isopropanol [IPA]), three esters (ethyl acetate [EtOAc]), ethyl lactate and dimethyl carbonate [DMC]), three terpenes (*p*-cymene, α-pinene and limonene) and two ethers (methyltetrahydrofuran [MeTHF] and cyclopentyl methyl ether [CPME]). Hexane, taken as reference in this study, EtOH, IPA, MeTHF (purity 99.5%), DMC (purity 99%), EtOAc (purity 99.5%) and limonene were purchased from VWR International (Darmstadt, Germany). *p*-Cymene was sourced from Acros (Hampton, NH, USA). CPME (reagent plus 99.5%) was obtained from Sigma Aldrich (Sigma Co., St. Louis, MO, USA). Ethyl lactate and α-pinene were supplied from Merck (Darmstadt, Germany). All solvents used in extraction were of technical grade.

Hexane of HPLC grade, methanol, chloroform, propan-2-ol, diethyl ether, methyl acetate, acetic acid, sulfuric acid, potassium chloride, sodium chloride and water were of analytical grade and were purchased from VWR International (Darmstadt, Germany). Primuline and acetone were of analytical grade and were provided by Sigma Aldrich. Fatty acid, neutral and polar lipid standards were acquired from Sigma Aldrich (Paris, France). HPTLC plates (20 × 10 cm, silica gel 60 F254) were obtained from Merck (Darmstadt, Germany).

2.2 Plant material

Lentisk fruits were collected from Tabarka in North of Tunisia in September 2016 located at the following geographical coordinates obtained by GPS, (36°5783,6' N and 08°5736,2' E, altitude: 20 m). Fruits were air-dried in the dark at room temperature and were ground using MF 10 basic microfine grinder drive, IKA® (Rotar speed 6500 rpm, MF-sieves of 2 mm of diameter).

2.3 Lipid extraction

Oils were isolated from Lentisk fruit by a Soxhlet extractor. Thirty gram of ground dried fruit were weighted inside a 30 × 100 mm cellulose thimble (Macherey-Nagel, Düren, Germany) and loaded into the extraction chamber of a 125 mL Soxhlet extractor fitted with a condenser, which was extracted with 300 mL of solvent placed in a 500 mL distillation flask. Samples were extracted under reflux during 8 h with the selected solvents (hexane, EtOH, IPA, EtOAc, ethyl lactate, DMC, *p*-cymene, α-pinene, limonene, MeTHF and CPME). All extractions were conducted in triplicate. Subsequently, solvents were evaporated under reduced pressure, using a rotary evaporator at 45 °C except ethyl lactate, *p*-cymene, α-pinene and limonene extracts. These solvents have a high boiling point (154, 177, 155 and 176 °C, respectively) and their elimination are not possible by conventional evaporation under reduced pressure. So, to facilitate their evaporation, the principle of hetero-azeotropic distillation was applied. Thus, 50% (V/V) of water were added to *p*-cymene, α-pinene and limonene extracts and 70% (V/V) of water were added to ethyl lactate extracts. Then, the mixtures were evaporated as described before. The dried residues were weighted and oils were aliquoted in vials and stored at 4 °C until analysis. Oil content was determined according to NF V 03-908 and was expressed as a percentage of the total weight of lipids obtained after extraction relative to the dry weight of Lentisk.

2.4 Computational methods: COSMO-RS simulations

COSMO-RS is a computational prediction model that combines a quantum chemical with thermodynamic statistics to determine and predict the chemical potential of molecules in a liquid without experimental data (Klamt, 2003). It is a powerful method used as a decision-making tool for solvent screening (Sicaire *et al.,* 2015; Rapinel *et al.,* 2016; Ravi *et al.,* 2018).

COSMO-RS simulation consists on two-step. First, the microscopic step is where simulation is performed in a virtual

conductor environment for the molecules. In this environment, molecule induced polarization charge density "σ" on the surface (σ-surface). Thus, the solute molecule is converted to its energetically optimal state, *via* the quantum calculation self-consistency algorithm triple zeta valence polarized basis set (TZVP), with respect to its geometry and electron density.

The second step used the statistical thermodynamic calculation. In this stage, the polarization charge density was used for the quantification of the interaction energy of the pair-wise interacting surface segments regarding electrostatics and hydrogen bonding. The polarization charge was converted into a surface composition function (σ-profile) that gives information of the molecular polarity distribution. Thus, the σ-surface and the σ-profile allowed the characterization of a reference molecule (minimum energy, stable molecule) as a solute in a perfect conductor. The obtained σ-profile was then used to calculate the chemical potential of the surface segment (σ-potential) using COSMOthermX program (version C30 release 17.02). The affinity of a solvent and the interaction between solutes and solvent in mixed state can be predicted using the σ-profile and the σ-potential. The solutes were selected after exhaustive literature review. The relative solubility of target free fatty acids (palmitic, oleic and linoleic acids) and TAGs (palmitoyl-oleyl-linoleoylglycerol [POL], palmitoyl-dioleylglycerol [POO], dioleyl-linoleoyl-glcerol [OOL] and trioleylglycerol [OOO]) in different solvents (hexane, EtOH, IPA, DMC, EtOAc, CPME, MeTHF, limonene, α-pinene, *p*-cymene and ethyl lactate) was calculated with COSMOtherm software (C30 1401, Cosmo-thermX14, COSMOlogic GmbH &Co. KG) as follows:

$$log_{10}(x_j) = log_{10}\left[\frac{\exp(\mu_j^{\text{pure}} - \mu_j^{\text{solvent}} - \Delta G_{j,\text{fusion}})}{RT}\right], \quad (1)$$

μ_j^{pure}: chemical potential of pure compound *j* (joule/mol); μ_j^{solvent}: chemical potential of *j* at infinite dilution (joule/mol); $\Delta G_{j,\text{fusion}}$: free energy of fusion of *j* (joule/mol); x_j: solubility of *j* (g/g solvent).

Relative solubility is calculated in infinite dilution. The logarithm of the best solubility is set to 0 and all other solvents are ranked relatively to the best or reference solvent.

2.5 Qualitative and quantitative analyses of lipids

2.5.1 High performance thin layer chromatography (HPTLC)

Polar and neutral lipids of Lentisk oil were detected by charring and quantified with a CAMAG 3 TLC densitometer (CAMAG, Muttenz, Switzerland) through two different HPTLC developments. Classes of lipids were identified in comparison with known polar and neutral lipid standards. Plates were conditioned with a mixture of chloroform/methanol (2:1, v/v) and activated at 110 °C for 60 min on the TLC Plate Heater (CAMAG, Muttenz, Switzerland). The oil samples were spotted onto 20 × 10 cm HPTLC plates (Silica gel 60 F254; Merck KGaA, Darmstadt, Germany) by means of an ATS 5 automatic TLC sampler (CAMAG, Switzerland). Plates were developed in an ADC2 automatic developing chamber (CAMAG). The separation of neutral

lipids was performed with hexane/diethyl ether/glacial acetic acid in a ratio of 65:35:2 (v/v/v) to a height of 85 mm. For polar lipids, the eluent system was a mixture of methylacetate/isopropanol/chloroform/methanol/KCl (0.25% solution) in a ratio of 25:25:25:10:9 (v/v/v/v/v) running to a height of 85 mm. The dried plate was dipped for 6 s in a reagent (10 mg of primuline, 160 mL of acetone and 40 mL of water) and then scanned using a TLC Scanner 3 with a WinCATs software (CAMAG). Results are expressed as percentage of lipid classes in total Lentisk fruit oil (LFO).

2.5.2 Fatty acid methyl ester (FAME) preparation and gas chromatography analysis

Before the injection of samples in GC-FID, fatty acid methyl esters (FAMEs) were prepared from the extracted oil by means of acid-catalyzed trans-methylation as reported by Morrison and Smith (1964). One millimeter of methanolic sulfuric acid (5% v/v) was mixed with a determined quantity of extracted oil and 400 μL of Triheptadecanoin (C17:0 TAG) used as internal standard. The mixture was heated at 85 °C for 90 min. After cooling, 1 mL of hexane and 1.5 mL of sodium chloride (0.9%) solution were added to the mixture and shaken vigorously during 30 s. Two microliter of the top organic layer was injected in split mode (split ratio: 1:20) at 250 °C onto a 7820 A gas chromatography system (Agilent technologies, USA) equipped with flame ionization detector and a BD-EN14103 capillary column (30 m × 320 μm × 0.25 μm). Helium was used as a carrier gas at 33 cm s^{-1}. The oven temperature was programmed as follows: firstly, temperature was maintained at 50 °C for 1 min, then increased to 220 °C at a rate of 20 °C/min and reached 280 °C at a rate of 2 °C/min. Finally, it was maintained isothermally for 10 min at 230 °C. FAMEs were identified by comparing their retention time with those of purified standards (Sigma Co., St. Louis, MO, USA) using Agilent EZChrom Elite software.

2.6 Chemometric data treatment

GC-FID and HPTLC data were analyzed using SIMCA software version 14.1.0.2047 (Umetrix, Umeå, Sweden), a multivariate statistical analytical software, for unsupervised Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). For HCA analysis, similarities between the different samples were estimated by means of the Ward linkage distance algorithm. Similar samples were characterized by small distances and the opposite for dissimilar samples. The data matrix was subjected to log transformation before the multivariate analysis.

2.7 Measurement of the anti-inflammatory activity by nitrite quantification

2.7.1 Cell culture

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

Table 1. Conductor-like screening model for real solvent (COSMO-RS) assisted solvent screening: relative solubility (log10 [Xsolub]) of major compounds of *Pistacia lentiscus* fruit oil in several solvents. The logarithm, log10 (Xsolub), of the best solubility is fixed to zero and the rest of solvents are given relatively to the best one.

	FFAs			TAGs			
Solvents	FFA1	FFA2	FFA3	TAG1	TAG2	TAG3	TAG4
Hexane	−1.27	−1.34	−1.31	0	0	−0.17	−0.001
EtOH	−0.001	−0.05	0	−2.52	−2.53	−2.19	−2.55
IPA	0	0	0	−1.49	−1.51	−1.24	−1.52
EtOAc	0	0	0	0	0	0	0
Ethyl lactate	−0.22	−0.27	−0.06	−3.23	−3.24	−3.99	−3.15
DMC	−0.56	−0.51	−0.22	−1.51	−1.46	−1.21	−1.41
p-cymene	−1.03	−1.03	−0.9	0	0	0	0
α-pinene	−1.08	−1.11	−1.03	0	0	0	0
Limonene	−0.96	−0.98	−0.88	0	0	0	0
MeTHF	0	0	0	0	0	0	0
CPME	0	0	0	0	0	0	0

Solubility index   Reference or equivalent   Better than reference   Lesser than reference

FFAs: FFA1 (C16:0); FFA2 (C18:1); FFA3 (C18:2). TAGs: TAG1: OOO (C18:1 × 3); TAG2: OOL (C18:1 × 2 + C18:2); TAG3: POO (C16:0 + C18:1 × 2); TAG4: POL (C16:0 + C18:1 + C18:2).

2.7.2 Measurement of nitrite production

RAW 264.7 cells were seeded in 24-well plates at a density of 2×10^5 cells per well and were allowed to attach for 24 h at 37 °C.

Then, after 60 min, treated cells with increasing concentrations of LFO extracts, dissolved in the DMSO, were stimulated with 100 µg/mL lipopolysaccharide (LPS). After 24 h, the quantity of nitrite accumulated in the culture supernatant was determined based on the Griess reaction (Aboura *et al.,* 2017). The absorbance at 540 nm was then measured and nitric oxide (NO) levels, produced by murine macrophage-like RAW264.7 cells, were determined by comparison with a $NaNO_2$ standard curve.

3 Results and discussion

3.1 *In silico* screening

Simulations showed that all bio-based solvents have a good potency to replace hexane for the extraction of FFAs (Tab. 1). In fact, green solvents have a high solubilization power than hexane, as log10 (Xsolub) of green solvents are higher than log10 (Xsolub) of hexane. Regarding the log10 (Xsolub) of TAGs, EtOAc, *p*-cymene, α-pinene, limonene, MeTHF and CPME are equal to 0, endorsed as good solvent candidates for TAGs extraction. Nevertheless, EtOH, IPA, Ethyl lactate and DMC are theoretically not suitable for TAGs extraction compared to hexane as solubility values are below zero.

According to the *in silico* screening, MeTHF, EtOAc and CPME seem to be the most suitable alternative solvents for the extraction of LFO major metabolites.

3.2 Experimental solvent screening

To validate theoretical COSMO-RS predictions, the performance of alternative solvents to substitute hexane for the extraction of LFO was tested experimentally.

Concerning lipid classes, HPTLC analyses of LFO obtained by green solvents and hexane as reference are illustrated in Table 2. Results showed that the major lipids extracted with both alternative and conventional solvents were TAGs (72.16–99.99%). Figure 1a exhibited the unsupervised PCA biplot representing PC2 *versus* PC1 with 54.5 and 23.6% respectively, explaining a total variance of 78.1%. MeTHF, CPME, EtOAc, DMC, *p*-cymene and hexane extracts are located near TAGs. IPA and EtOH were closer to phospholipids (Lyso, PI, PC and PE) and were closely separated from the other solvents along PC1 and PC2. This indicates that alcohols have a good solvation power of polar lipids. In fact, the presence of these phospholipids in alcohol extracts can be explained by the high polarity of these solvents compared to hexane (Breil *et al.,* 2016). On the other hand, ethyl lactate, limonene and α-pinene were separated from alcohol solvents along PC1 and from other solvents along PC2 and were closer to MAG and DAG. In fact, these solvents have a different lipid class profile than the others, characterized by a high content of DAG (19.73, 12.68 and 8.29% for ethyl lactate, limonene and α-pinene, respectively). Furthermore, a higher MAG content was detected in ethyl lactate extract (8.10%) than hexane (2.24%). These solvents have a high boiling point which could affect their efficiency and induce lipid degradation (Cascant *et al.,* 2017).

Extracted oils were then analyzed by GC-FID after transmethylation of fatty acids. LFO were mainly composed

Table 2. Lipid classes of *Pistacia lentiscus* fruit oil obtained by various solvents.

	Amounts in total extracts (%)										
	Hexane	EtOH	IPA	EtOAc	Ethyl lactate	DMC	*p*-cymene	α-pinene	Limonene	MeTHF	CPME
Neutral lipids (%)											
MAG	2.24 ±0.01	–	–	–	8.10 ±0.03	–	–	1.51 ±0.01	–	–	–
DAG	–	–	–	–	19.73 ±0.00	–	–	8.29 ±0.08	12.68 ±0.01	–	–
TAG	97.76 ±0.62	84.93 ±0.48	92.21 ±0.83	99.50 ±0.78	72.16 ±0.18	99.62 ±0.19	99.99 ±0.09	90.20 ±0.76	87.32 ±1.10	97.75 ±1.12	99.04 ±1.25
FFA	–	–	–	–	–	–	–	–	–	–	–
Polar lipids (%)											
LYSO	–	0.07 ±0.02	0.09 ±0.00	–	–	–	–	–	–	–	–
E	–	3.32 ±0.03	2.04 ±0.16	0.50 ±0.05	–	0.38 ±0.02	–	–	–	2.25 ±0.00	0.96 ±0.11
PI	–	1.27 ±0.01	–	–	–	–	–	–	–	–	–
PC	–	10.41 ±0.14	5.66 ±0.17	–	–	–	–	–	–	–	–

Data are expressed as mean ± SE of triplicate samples. MAG: monoacylglycerol. DAG: diacylglycerol. TAG: triacylglycerol. FFA: free fatty acid. LYSO: lysophosphatidilcholine. PE: phosphatdylethanolamine. PI: phosphatidylinositol. PC: phosphatydylcholine.

by oleic acid (C18:1n9) (41.8–48.37%), linoleic acid (C18:2n6) (22.21–26.1%) and palmitic acid (C16) (22.11–23.96%) (Tab. 3). These results are in agreement with previous studies (Dhifi *et al.,* 2013; Mezni *et al.,* 2014).

To assess the influence of the solvent extraction on overall metabolites, GC-FID data of LFO obtained using the different studied solvents were applied to PCA. PCA model (Fig. 1b) indicated that the two first principal components explain 55.7% of the total variance with 34.6 and 21.1% for PC1 and PC2, respectively. PCA data exhibited that *p*-cymene was closely separated from hexane, MeTHF, IPA, EtOH, ethyl lactate, EtOAc and DMC along the PC1 and from CPME and α-pinene along PC2. α-Pinene was located in the opposite side of hexane indicating a different metabolite profile. However, it can be noticed that hexane, MeTHF, CPME, ethyl lactate, limonene, EtOH, IPA, EtOAc and DMC were located in the middle of all FFAs.

Obviously, the PCA provides a multivariate overview of the tendency of clustering/separation of samples due to their metabolite profiles, but cannot show the underlying degree of similarities between clusters. So, Hierarchical Cluster Analysis (HCA) was performed on GC-FID data using Ward linkage distance method. The resulting HCA dendrogram (Fig. 2) showed that the dissolving power of tested solvents could be classified into four different clusters. α-pinene and *p*-cymene formed the two first clusters. The third cluster was composed by CPME, limonene, ethyl lactate and EtOH and the fourth contained hexane, MeTHF, IPA, EtOAc and DMC. From these results, it can be concluded that MeTHF, IPA, EtOAc and DMC extracts have similar fatty acid profiles compared to

hexane extracts. In contrast, *p*-cymene and α-pinene showed the highest dissimilarity to the other two clusters.

Yields were around 6.14 and 8.61% (Fig. 3). No significant difference between hexane, EtOH, EtOAc, ethyl lactate, DMC, limonene and MeTHF was observed. CPME, *p*-cymene and α-pinene resulted in the lowest lipid yields (6.65, 6.14 and 5.67% respectively). In contrast, IPA yields were relatively higher than hexane but this may be related to the higher extraction of phospholipids as shown in HPTLC analyses.

3.3 *In silico* screening *versus* experimental screening

The theoretical COSMO-RS results were compared to the experimental data. The COSMO-RS prediction indicated that MeTHF, CPME, and EtOAc were theoretically the most suitable solvents to replace hexane. However, taken into account global yield, lipid composition and statistical analysis, the experiments showed that among these 3 predicted alternative solvents, only MeTHF and EtOAc were the suitable solvents for hexane. Furthermore, DMC appeared to be a good candidate to replace hexane experimentally, which was not in accordance with theoretical screening. Experimentally, CPME gives the lowest lipid yields and was not a good alternative to replace hexane.

The selection of an alternative solvent has to consider parameters other than solubility, as Log *P*, boiling point, energy required for the evaporation of the solvent and toxicity index (Verpoorte *et al.,* 2008; Ernst *et al.,* 2014). Indeed, based on solubility matching, economic and energy parameters MeTHF was found to be the best alternative bio-based solvent
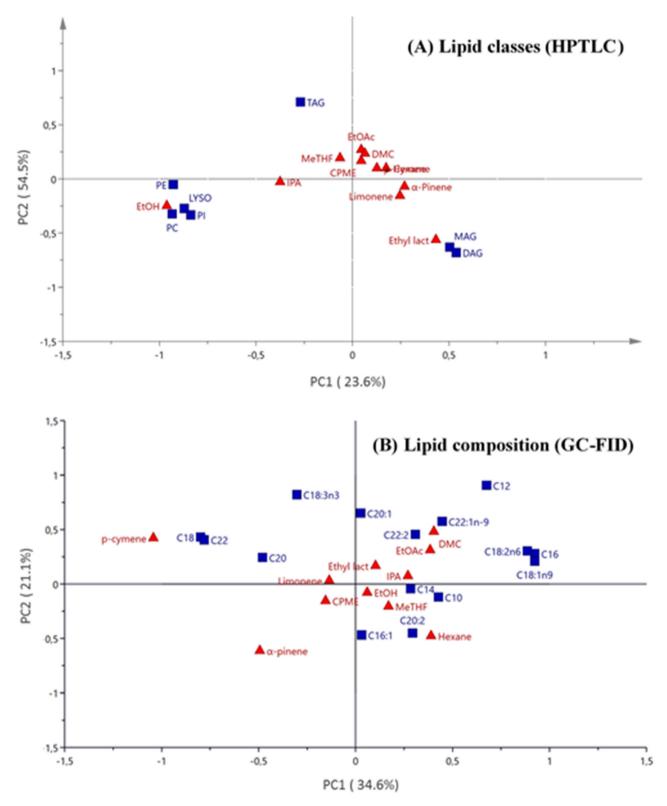
Fig. 1. Principal component analysis biplot (score and loading values) represented by PC1 *versus* PC2 for (A) lipid classes obtained by HPTLC and (B) lipid composition obtained by GC-FID, and tested solvents.

to replace hexane for the extraction of LFO. This bio-based solvent derived from renewable source has a lower toxicity (Clarke *et al.,* 2018) and its use in pharmaceutical chemical processes has been approved by Antonucci *et al.* (2011). Also, this solvent required practically the same energy to evaporate 1 kg of solvent as hexane, low boiling point and log *P* > 0.

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Table 3. Fatty acid composition of *Pistacia lentiscus* fruit oils extracted with different solvents.

					Extracted amounts of fatty acids (%)						
	Hexane	EtOH	IPA	EtOAc	Ethyl lactate	DMC	*p*-cymene	α-pinene	Limonene	MeTHF	CPME
Boiling point (°C)	69	78	82.8	77.1	154	90	177	155	176	80	106
C8:0	–	–	–	–	–	–	–	–	5.00±0.77	–	–
C10:0	–	0.2±0.00	0.21±0.15	0.16±0.12	–	–	–	–	–	0.32±0.03	0.12±0.04
C12:0	–	0.08±0.01	0.08±0.00	0.13±0.00	–	–	–	–	–	0.08±0.02	0.08±0.01
C14:0	–	0.13±0.00	0.18±0.00	0.2±0.00	–	0.18±0.01	0.15±0.06	–	0.46±0.19	0.16±0.00	0.21±0.01
C16:0	23.96±0.58	23.21±0.05	23.19±0.02	23.59±0.09	23.63±0.00	23.4±0.08	22.11±0.24	23.3±0.32	22.39±0.48	23.09±0.09	23.36±0.10
C16:1	1.34±0.17	1.6±0.12	1.53±0.00	1.54±0.02	1.29±0.00	1.52±0.00	1.43±0.08	3.8±0.41	1.96±0.09	1.7±0.01	1.48±0.02
C18:0	1.15±0.02	1.13±0.00	1.07±0.08	1.26±0.01	1.2±0.00	1.23±0.00	7.38±0.36	1.93±0.52	1.87±0.01	0.96±0.00	1.25±0.01
C18:1n9	48.37±0.46	46.11±0.00	45.32±0.15	45.95±0.11	45.13±0.08	46.02±0.13	41.96±0.30	43.89±0.65	41.8±0.88	47.48±0.41	45.55±0.49
C18:2n6	23.31±0.53	25.2±0.00	26.03±0.00	24.62±0.14	26.1±0.01	24.88±0.15	22.21±0.34	24.43±0.19	23.47±0.29	24.12±0.24	25.44±0.38
C18:3n3	1.57±0.10	2.01±0.07	2.11±0.09	1.95±0.08	1.93±0.06	2.02±0.04	3.34±0.25	2.2±0.16	2.05±0.29	1.86±0.08	2.11±0.12
C20:0	0.01±0.00	0.13±0.01	0.17±0.10	0.09±0.03	0.2±0.07	0.11±0.05	0.29±0.16	0.43±0.08	0.61±0.02	0.15±0.01	0.08±0.04
C20:1	0.11±0.05	0.2±0.03	0.12±0.00	0.25±0.03	0.52±0.00	0.27±0.01	0.34±0.10	–	0.38±0.20	0.09±0.01	0.3±0.03
C20:2	0.19±0.28	–	–	–	–	–	–	–	–	–	–
C22:0	–	–	–	–	–	–	0.77±0.10	–	–	–	–
C22:1n–9	–	–	–	0.26±0.03	–	0.30±0.02	–	–	–	–	–
C22:2	–	–	–	–	–	0.07±0.02	–	–	–	–	–
ΣSFA	25.12	24.88	24.9	25.43	25.03	24.92	30.71	25.66	30.33	24.76	25.11
ΣMUFA	49.82	47.91	46.97	48.00	46.95	48.11	43.73	47.7	44.15	49.26	47.33
ΣPUFA	25.06	27.2	28.14	26.56	28.03	26.97	25.55	26.64	25.52	25.98	27.56

Data are expressed as mean ± SE of triplicate samples. ΣSFA: total saturated fatty acids. ΣMUFA: total monounsaturated fatty acids. ΣPUFA: total polyunsaturated fatty acids.
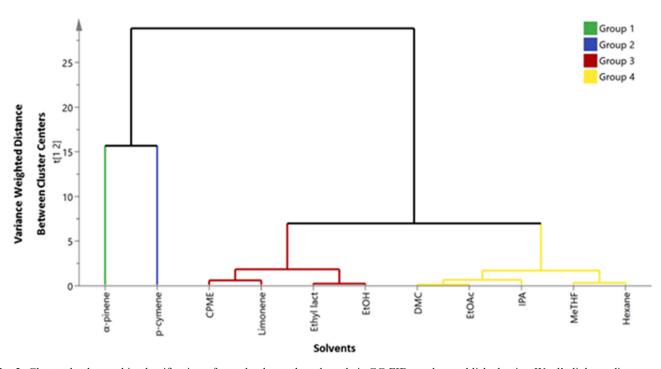
Fig. 2. Cluster dendrographic classification of tested solvents based on their GC-FID results established using Ward's linkage distance.
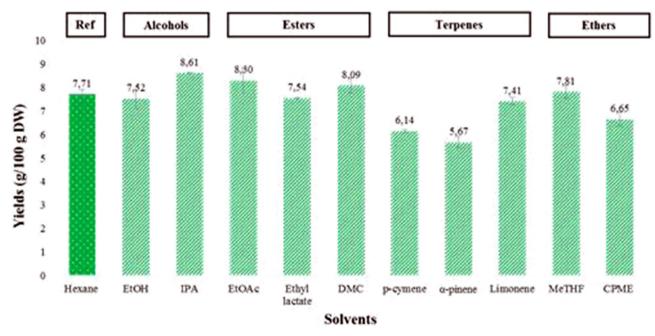

Fig. 3. Comparison of lipid extraction yields obtained by bio-based solvents compared to the reference.

Nevertheless, DMC and EtOAc required more energy to evaporate 1 kg of solvent (0.194 KW/h, 0.217 kW/h) compared to hexane (0.121 KW/h).

3.4 *In vitro* anti-inflammatory activity

During the inflammatory process, macrophage produces mediators, such as nitric oxide (NO) by inducible NO synthase (iNOS). Thus, NO is recognized as a mediator and regulator in acute inflammatory responses. An excessive production of NO induces a variety of inflammation and carcinogenesis aspects. In the present study, the anti-inflammatory activity of MeTHF lipid extract, which was found to be the best alternative solvents to substitute hexane for the extraction of LFO, was investigated using LPS-activated RAW 264.7 macrophages. Firstly, the cytotoxicity of MeTHF lipid extract was evaluated.
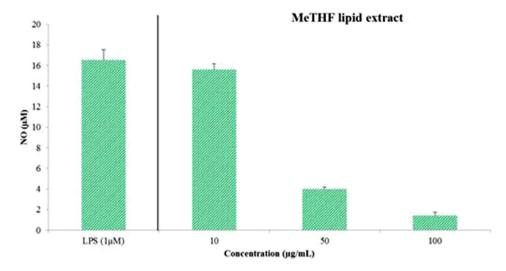
Fig. 4. Effect of *Pistacia lentiscus* oil obtained with MeTHF on NO overproduction in LPS-stimulated RAW264.7 macrophages. Each value represents the mean ± SD of triplicate.

RAW 267.4 cells were treated with increasing concentrations of MeTHF lipid extract (10–200 μg/mL). MeTHF lipid extracts did not reveal any significant cytotoxicity against RAW 267.4 macrophage cells until 100 μg/mL (data not shown). In fact, our experiments were carried out at the nontoxic concentrations of 10, 50 and 100 μg/mL. Then, the effect of MeTHF lipid extracts on nitrite secretion was investigated. As shown in Figure 4, MeTHF extract decreased NO release in a dose-dependent manner. In fact, NO levels in RAW 267.4 cells decreased from 15.6 μM (10 μg/mL) to 1.4 μM (100 μg/mL), while NO production in LPS-activated cells, not treated with LFO, was higher (16.5 μM) than treated cells. These results suggest the important anti-inflammatory activity of MeTHF lipid extract. In fact, MeTHF lipid extract was mainly composed by oleic and linoleic acid (47.48 and 24.12%, respectively). These two unsaturated fatty acids have anti-inflammatory properties playing a major role in the recruitment of inflammatory cells at the inflammation site (Ben Khedir *et al.*, 2017).

4 Conclusion

The potential of alternative green solvents to replace hexane for lipid extraction from *Pistacia lentiscus* L. berries was sought and the anti-inflammatory activity of LFO obtained with the most suitable alternative solvent was evaluated herein. Some discrepancies between theoretical and experimental screening were noticed. For COSMO-RS simulation MeTHF, EtOAC and CPME were the best suitable solvents for hexane. Nevertheless, experiments highlighted that MeTHF was the best alternative solvent compared to hexane qualitatively and quantitatively. Our data approved the potential of alternative solvents especially MeTHF to substitute the toxic hexane, conventionally used for lipid extraction.

Results also highlighted the great nutritional value of LFO regarding the high amount of oleic and linoleic acid which may protect against some metabolic disorders, cardiovascular and cancer diseases (Trabelsi *et al.,* 2012) and demonstrated its strong anti-inflammatory activity in RAW 264.7 macrophages.

These results would encourage the use of LFO in food industry as edible oil and also its valorization in pharmaceutical and cosmetics manufactory.

Conflicts of interest. The authors declare that they have no conflicts of interest in relation to this article.

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Cite this article as: Chaabani E, Abert Vian M, Dakhlaoui S, Bourgou S, Chemat F, Ksouri R. 2019. *Pistacia lentiscus* L. edible oil: green extraction with bio-based solvents, metabolite profiling and *in vitro* anti-inflammatory activity. *OCL* 26: 25.