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Sustainable grape seed oil processing: Green solvent extraction and byproduct valorisation

Christian Cravotto [a], Vincent Rapinel [a], Binh Nguyen-Thanh [b], Raúl Bonet-García [b], Mickaël Bartier [c], Ombéline Claux [a], Laurence Jacques [c], Silvia Tabasso [d,*], Enrique Barrajón-Catalán [b], Anne-Sylvie Fabiano-Tixier [a]

[a] Avignon Université, INRAE, UMR SQPOV, Avignon F-84000, France
[b] Institute of Research, Development and Innovation in Biotechnology of Elche (IDiBE), Miguel Hernández University (UMH), Elche 03202, Spain
[c] Pennakem Europa (EcoXtract ®), 224 avenue de la Dordogne, Dunkerque F-59640, France
[d] Department of Drug Science and Technology, University of Turin, Via P. Giuria 9, Turin 10125, Italy

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ABSTRACT

The extraction of grape seed oil (GSO) for food and cosmetic applications was performed with 2-methyloxolane (2-MeOx) as a green solvent. The oil extraction yield, the tocopherol and tocotrienol content, the fatty acid and polyphenol profiles of the oils were analysed and compared with hexane. The entire extraction process, including the oil chemical refining, was further investigated on a pilot scale (15-litre extractor). Oil refining had a negative effect on the micronutrient content. The content of sterols, tocols and polyphenols was reduced by 19.78 %, 47.6 % and 99 % respectively. High amounts of polyphenols were recovered in refining by-products, with approximately 80 % found in gums and 10 % in soap-stock. The polyphenols contained in the crude oil and gums demonstrated effective intracellular ROS inhibition in HaCaT keratinocytes and BJ fibroblasts, as well as an anti-melanogenic effect on B16-F10 murine melanoma cells. Consequently, these products are proposed as valuable cosmetic ingredients for treating hyperpigmentation disorders. In conclusion, 2-MeOx is an excellent alternative to hexane for GSO extraction, offering high extraction efficiency, a safer toxicological profile, and the production of oil and byproducts with promising food and cosmetic applications.

1. Introduction

Vitis vinifera L. is a climbing shrub with deep, branching roots that has been cultivated for thousands of years to produce grapes. In the last 50 years, the global yield of grape production per hectare of land has more than doubled, from 5.5 t per hectare (52.0 Mt per year) in 1966–10.9 t per hectare (77.4 Mt per year) in 2016 (Alston and Sambucci, 2019). Grapes are mainly used for juice and wine production, while the rest is sold as table fruit, either fresh or dried. Wine production generates about 20 % (w/w) of grape waste, usually referred to as grape pomace or marc. Grape seeds (GSs) make up about 25 % of the grape pomace, while the remaining part consists of pulps, stems and skins (Duba and Fiori, 2015).

GSs contain around 8 −20 % oil that is found in the endosperm of the seed. In addition, GSs are composed of about 35 % fiber, 29 % extractable compounds, 7 % water and 3 % minerals (Matthäus, 2008). Grape seed oil (GSO) is abundant in bioactive compounds, including polyphenols, phytosterols, and tocopherols, which are known for their health-promoting properties, making it a valuable ingredient for food and pharmaceutical applications (Yang et al., 2021). GSO is also used in cosmetic products, where its hydrophobic and antioxidant properties make it very beneficial for skin care (Wada et al., 2018).

GSO is primarily produced in Italy, France, and Spain; however, its demand has grown throughout the rest of Europe (Maier et al., 2009). The oil yield is influenced by several factors such as GSs variety, environmental conditions, extraction techniques, solvent type, and

Abbreviations: GSO, grape seed oil; GSs, grape seeds; 2-MeOx, 2-Methyloxolane; BHT, butylhydroxytoluene; DM, dry matter; FAMEs, fatty acid methyl esters; SCE-CO_2, supercritical carbon dioxide extraction; TPC, total phenolic content; DPPH, 2,2-Diphenyl-1-Picrylhydrazyl; ROS, reactive oxygen species; PV, peroxide value.

* Corresponding author.
E-mail addresses: christian.cravotto@alumni.univ-avignon.fr (C. Cravotto), vincent.rapinel@gmail.com (V. Rapinel), binh.nguyen@goumh.umh.es (B. Nguyen-Thanh), rbonet@umh.es (R. Bonet-García), mickael.bartier@ecoxtract.com (M. Bartier), oclaux.papers@gmail.com (O. Claux), laurence.jacques@ecoxtract.com (L. Jacques), silvia.tabasso@unito.it (S. Tabasso), e.barrajon@umh.es (E. Barrajón-Catalán), anne-sylvie.fabiano@univ-avignon.fr (A.-S. Fabiano-Tixier).

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operational parameters (Garavaglia et al., 2016). Furthermore, producing high-quality GSO requires a rapid drying of GSs after juice pressing to prevent spoilage and the development of undesirable odour compounds (Matthäus, 2008).

For seeds with relatively high oil content (above 20 %), industrial extraction typically involves both pre-pressing and solvent extraction using hexane. In contrast, seeds with lower oil content, like GSs, are usually extracted directly with hexane (Uitterhaegen and Evon, 2017). Recently, mechanical pressing methods, particularly cold pressing have gained more attention (Yang et al., 2021). However, the oil yield is much lower than with solvent extraction, as only 60 −80 % of the oil is recovered (Tasan et al., 2011).

Currently, solvent extraction with hexane is the most popular method for separating oil due to its high efficiency (residual oil content < 2 %) and ability to process large volumes (Pérez et al., 2019). After solvent extraction, the oil is usually refined to remove unpleasant compounds. The resulting oil is odourless and tasteless and can be used for many culinary purposes. However, *n*-hexane is classified as an aspiration hazard, toxic to reproduction, and harmful to organs and skin (Registration Dossier, 2022). Due to its toxicity, alternative solvents are urgently needed (Cravotto et al., 2022a). Additionally, its cost fluctuates with petroleum prices, and it is not permitted for organic products production (food and cosmetics) (Carré, 2021; Regulation - 2018/848, 2024).

Some innovative techniques have been tested for GSO extraction in small-scale trials. These include supercritical carbon dioxide extraction (SCE-CO_2) (Ben Mohamed et al., 2016), aqueous ethanol extraction (Du et al., 2019), the combination of screw pressing and SCE-CO_2 (Rombaut et al., 2015), or the use of cell wall-degrading enzymes prior to oil extraction by SCE-CO_2 (Passos et al., 2009). These extraction methods have been shown to produce high quality and safety extracts as no organic solvents are used. However, it is unlikely that these techniques can fully replace the use of petrochemical solvents on an industrial scale, as their scale-up costs are high (De Jesus and Filho, 2020), and extraction is usually less efficient (Mwaurah et al., 2020) or selective for oil compared to hexane. Therefore, it is crucial to explore alternative methods suitable for industrial use, such as 2-methyloxolane (2-MeOx).

2-MeOx is a bio-based solvent derived from levulinic acid or furfural, produced from lignocellulosic biomass conversion. Its technical properties are comparable to those of hexane, so that its industrial use is possible with minimal modifications to existing extraction plants (Sicaire et al., 2014; Rapinel et al., 2020). On January 2023, 2-MeOx was approved for use in food and feed production in Europe (Directive 2009/32/EC) (Commission Directive EU, 2023). 2-MeOx offers a safer toxicological profile and a more sustainable alternative to traditional solvents (Slater et al., 2016).

Unlike hexane, 2-MeOx has higher water solubility. In typical industrial plants, after solvent distillation, steam stripping ensures efficient solvent removal from the oil. The solvent and steam are then condensed and recovered in a decanter, where liquid/liquid separation occurs. With 2-MeOx, the organic phase is recovered in a water-saturated form (2-MeOx/H_2O 95.5/4.5 % at 55 °C), requiring an additional distillation step to obtain dry 2-MeOx. However, studies suggest that 2-MeOx 4.5 % water can be directly used for oil extraction with efficiency comparable to the dry solvent (Claux et al., 2021a; Cravotto et al., 2022b).

This study aimed to evaluate the performance of 2-MeOx for GSO extraction at both laboratory scale and in a semi-continuous pilot system, using a 15-litre extractor. Moreover, the impact of chemical refining on GSO properties was assessed, focusing on micronutrient content, including sterols, tocols, and polyphenols. By-products such as gums and soap stock were also analysed for their polyphenol content. The biological activity of gums extracted with 2-MeOx was further evaluated in various skin cell models to investigate their potential for cosmetic applications.

2. Materials and methods

2.1. Raw material preparation

GSs were given from Belenergia group (Bari, Italy) and stored at room temperature (20 °C) until further analysis. The seeds were crushed using a grain-flaker (Korn-quetsche, Eschenfelder, Germany). The proximate composition of GSs was determined using standard protocols. The proximate values of the flaked GSs were crude protein (AOCS official methods Ac 4–91 (AOCS Official Method Ac 4–91, 2009)): 9.73 ± 0.37 %; crude lipid (ISO 659 (ISO 659, 2023)): 13.90 ± 0.06 %; ash content (AOCS official method Ba 5a-49 (AOCS Official Method Ba 5a-49, 2022)): 1.90 ± 0.08 %; moisture: 10.04 ± 0.13 %.

2.2. Standards and reagents

2-Methyloxolane (ACS grade, ≥99 %), hexane (technical grade) and all analytical standards were purchased from VWR international (Darmstadt, Germany). Analytical standards: 37 fatty acid methyl esters (FAMEs) mix, procyanidin B2, caffeic acid, gallic acid, catechin, syringic acid, p-coumaric acid, ethyl gallate.

2.3. Laboratory scale extractions

Before extraction, 2-MeOx (“butylhydroxytoluene”, BHT stabilised) was distilled to remove BHT. 2-MeOx 4.5 % water was prepared by adding water to distilled 2-MeOx. GSs were extracted for 8 hours using a Soxhlet extractor (Büchi, Flawil, Switzerland) following ISO 659 (ISO 659, 2023). Yield was determined gravimetrically, and extractions were performed in triplicate. Crude oils and defatted samples for analysis were obtained through a 2-hour Soxhlet extraction to preserve product quality and more accurately reflect industrial conditions. Solvents were evaporated under reduced pressure using a rotavapor (R-300 Büchi, Flawil, Switzerland), and crude oils were cooled under nitrogen and stored at −20°C. Defatted solids were desolventized at 50 °C in a ventilated Biosec dehydrator (Tauro Essiccatori, Italy) and stored at −20°C before analysis.

2.4. Pilot scale extraction

A pilot extractor with a capacity of around 15 L was used to extract enough oil (approx. 1 kg) for subsequent chemical refining. Similarly to the lab-scale procedure, the solvent was distilled prior to extraction using a Rotavapor R-220 Pro (Büchi, Flawil, Switzerland) to remove BHT. Extractions were performed in a double jacket bottom-filter extractor (Legallais, France) without stirring, as shown in Fig. 1. A total of 9 kg of matrix was extracted, divided into two batches of 4.5 kg each. First, 4.5 kg of flaked GSs were immersed in the reactor with 2-MeOx at a ratio of 1:2 w/w (S/L) and extracted by maceration at 60 °C for 60 minutes. At the end of the extraction, the miscella was removed from the reactor and filtered through Buchner. The solvent was then evaporated in a Rotavapor R-220 Pro (Büchi, Flawil, Switzerland) at 60 °C and 150 mbar and then in a Rotavapor R-300 (Büchi, Flawil, Switzerland) at 60 °C and 20 mbar. The matrix was extracted again with the solvent distilled during the first extraction, following the same protocol. The reactor was then loaded with 4.5 kg of fresh matrix and the same protocol was applied, again using the solvent distilled in the previous extraction. For each batch, the extraction yield was calculated gravimetrically and expressed as the percentage of crude oil (total mass from two successive extractions) relative to the dry GSs mass used. All crude oils were then mixed and stored at −20°C until analysis and refining.

2.5. Oil chemical refining

Approximately 1 kg of crude oil was chemically refined according to

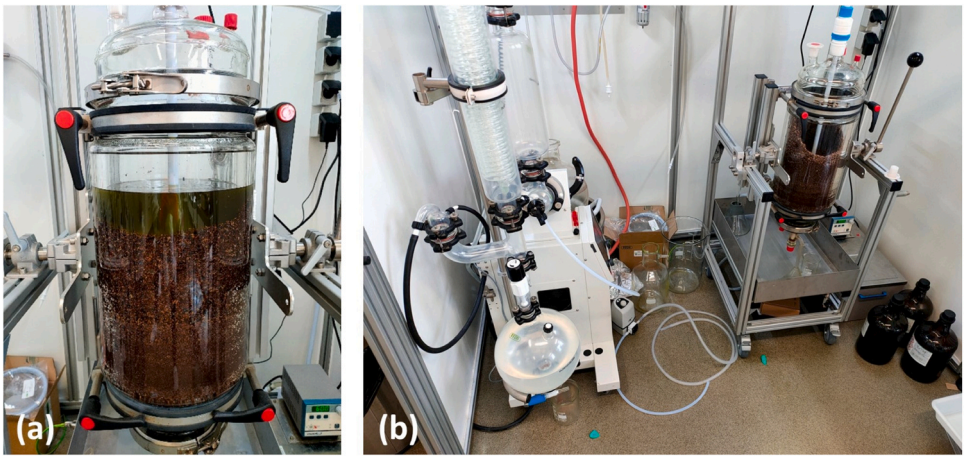

Fig. 1. (a) Double jacket pilot scale reactor; (b) Extraction system with a membrane pump for connection between reactor and rotavapor for solvent recovery.

a protocol consistent with standard practises in the oilseed industry (see supplementary information) (Gharby, 2022).

2.6. Crude extracts analysis

2.6.1. Fatty acid profile

FAMEs were prepared from GSs crude oils by acid-catalysed transmethylation. Analyses were performed using an Agilent (Japan) 7820 A gas chromatograph coupled to a flame ionisation detector (GC-FID), as described by Breil et al. (2016). The instrument was equipped with a BD -EN14103 capillary column (30 m × 320 µm × 0.25 µm). The fatty acids were identified using the retention time and the standards used for calibration.

2.6.2. Unsaponifiable compounds

Sterols were determined according to ISO 12228 (International Organization for Standardization, 2014), while tocopherols and tocotrienols were analysed following ISO 9936 (International Organization for Standardization, 2016). These analyses were performed by the ITERG analytical laboratory (Canéjan, France).

2.6.3. Quality parameters

The peroxide value (PV) of crude oils was measured using the AOCS standard method Cd 8–53 (American Oil Chemists Society AOCS, 2009). The oxidative stability of crude GSO was evaluated using the Rancimat method, following the official standard NF EN 14112 (AFNOR, 2016).

2.6.4. Total phenolic content (TPC) and 2,2-diphenylpicrylhydrazyl assay (DPPH)

TPC and DPPH antioxidant activity were determined as described in our previous work (Cravotto et al., 2022b). One gram of oil was diluted in *n*-hexane and phenolic compounds were extracted with ethanol/-water (60:40, v/v). After shaking and centrifugation, the lower phase was collected, and the upper layer was re-extracted twice. The results were expressed as gallic acid equivalents (GAE) and trolox equivalents (TE), respectively.

2.7. Chromatographic analysis

Samples were analysed using an Acquity UPLC I-class system (Waters, USA) equipped an Acquity UPLC® HSS T3 (2.1 ×100 mm, 1.8 µm) column (Waters, USA) coupled with a Synapt G2Si mass spectrometer (Waters, USA). The flow rate was 0.4 mL/min, with the column temperature set at 45°C. Mobile phases were water with 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B). The sample manager was maintained at 10 °C, and 2.5 µL of each sample was injected. The gradient began with a 1.50 min isocratic period at 99:1 (A), followed by a linear gradient to 1:99 (A) over 15.00 min, held for 2.25 min, and returned to the starting condition in 0.25 min, with a 1.00 min equilibration at 99:1 (A).

MS data were collected using negative electrospray ionization (ESI) in Sensitivity mode over a mass range of 50–1200 Da. The ionization source conditions were: capillary voltage 2.8 kV, sample cone voltage 20 V, source temperature 120 °C, desolvation temperature 450 °C, desolvation gas flow 650 L/h, and cone gas flow 50 L/h. Data were acquired in continuum mode with a scan time of 0.08 sec using MSE (low energy: 4 eV, high energy: ramp from 15 to 30 eV). Leucine Enkephalin (1 ng/µL in acetonitrile/water with 0.1 % formic acid, 1:1 v/v) was infused into the MS as a lock-mass via the lock-spray at 10 µL/min. Solutions of analytical standards (procyanidin B2, caffeic acid, gallic acid, catechin, syringic acid, *p*-coumaric acid, ethyl gallate) were used for identification and quantification.

2.8. Cell lines and culture maintenance

Human immortalized keratinocyte HaCaT cells were obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany), and the B16-F10 Murine melanocyte cell line was obtained from the American Type Culture Collection (ATCC, USA). Both cell lines were cultured with DMEM high glucose, 1 nm sodium pyruvate, 4 mM L-glutamine, and 1.5 g/L sodium bicarbonate $NaHCO_3$ (Gibco, USA), with 10 % FBS (Fetal Bovine Serum - Global Life Science, Austria) and 1 % antibiotics (100 U/mL Penicillin and 0.1 mg/mL Streptomycin – Gibco, USA) in a humidified atmosphere with 95 % air/5 % CO_2 at 37 °C. Cells were subcultured every 3 −5 days according to the supplier's recommendation.

BJ Fibroblast Cells line from ATCC (USA) was used to study cytotoxicity and their response to UV irradiation. The cells were cultured in ATCC-formulated Eagle's Minimum Essential Medium (EMEM) with 2 mM L-glutamine, 1 mM sodium pyruvate, and 1.5 g/L $NaHCO_3$ (ATCC, USA) with 10 % FBS and 1 % antibiotic cocktail in a humidified atmosphere with 5 % CO_2 at 37 °C.

Methodologies for cellular tests (viability, melanin content, intracellular antioxidant, photo-protection) are detailed in the supplementary information (Section S2).

2.9. Statistical analysis

Data were analysed using one-way ANOVA. Where applicable, multiple comparisons of means were performed using Tukey's HSD test at a 5 % significance level. Results are presented as mean ± standard deviation.

Table 1
Extraction yield and fatty acids profile of GSO.

	Laboratory scale extractions[a]			2-MeOx pilot scale extraction[b]	
	Hexane	Dry 2-MeOx	2-MeOx 4.5 %	Crude oil	Refined oil
Extraction yield (g/100 g DM)	15.45 ± 0.06b	15.68 ± 0.75b	18.88 ± 0.64a	13.34 ± 0.3c	
Fatty acids profile (relative %)					
C16:0	7.93 ± 0.12	7.94 ± 0.03	7.78 ± 0.12	7.88 ± 0.05	7.81 ± 0.04
C16:1	0.25 ± 0.01	0.25 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.21 ± 0.01
C18:0	3.92 ± 0.20	3.67 ± 0.18	3.62 ± 0.03	3.73 ± 0.13	3.91 ± 0.07
C18:1 (*n*-9)	19.14 ± 0.06	18.37 ± 0.31	18.25 ± 0.05	18.24 ± 0.16	18.80 ± 0.16
C18:2 (*n*−6)	67.88 ± 0.38	68.70 ± 0.54	69.11 ± 0.24	68.96 ± 0.37	68.48 ± 0.29
C18:3 (*n*-3)	0.46 ± 0.03	0.60 ± 0.03	0.58 ± 0.01	0.50 ± 0.01	0.39 ± 0.01
C20:0	0.17 ± 0.02	0.20 ± 0.01	0.18 ± 0.01	0.20 ± 0.01	0.17 ± 0.01
C20:1 (*n*−9)	0.19 ± 0.01	0.20 ± 0.02	0.21 ± 0.01	0.20 ± 0.01	0.22 ± 0.01
C22:0	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.03 ± 0.01
Σ SFAs	12.08 ± 0.35	11.87 ± 0.23	11.65 ± 0.17	11.87 ± 0.21	11.92 ± 0.12
Σ MUFAs	19.58 ± 0.08	18.82 ± 0.33	18.70 ± 0.06	18.68 ± 0.17	19.23 ± 0.17
Σ PUFAs	68.33 ± 0.41	69.31 ± 0.56	69.68 ± 0.24	69.45 ± 0.37	68.86 ± 0.30

[a] Soxhlet extraction;
[b] Maceration (2 consecutive extractions; n = 2); DM, dry matrix; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, poly-unsaturated fatty acids. Means in the same row with different superscript letters are significantly different; a–c, $p < 0.05$. Mean ± standard deviation of determinations (n = 3).

3. Results and discussion

3.1. Solvent performance comparison

The crude oils obtained by Soxhlet extraction using hexane, dry 2-MeOx, and 2-MeOx with 4.5 % water, as well as by pilot-scale maceration with 2-MeOx, were characterized in terms of yield, chemical composition, and micronutrient content. The extraction yield was similar or higher with dry 2-MeOx (15.68 g/100 g DM) and 2-MeOx 4.5 % water (18.88 g/100 g DM) compared to hexane (15.45 g/100 g DM) at the laboratory scale. This is probably due to the extraction of additional polar compounds, such as polyphenols, enabled by the higher polarity of 2-MeOx. Similar findings have been reported in previous studies (Brondani Teixeira Ribas et al., 2024; Claux et al., 2021b), and higher oil yields compared to hexane have been observed in both plant (Cravotto et al., 2022b; Bettaieb Rebey et al., 2019) and animal matrices (Ravi et al., 2019).

At the pilot scale, 86.3 % of the total oil was obtained from 9 kg of matrix by two consecutive maceration cycles, considering hexane extraction as the 100 % benchmark. Sicaire et al. (2015) obtained similar results by using a 6-litre percolation batch system for rapeseed cake extraction, where three 30-minute washes (L/S ratio of 1.5 kg dry solvent/kg rapeseed, 55°C) with 2-MeOx yielded 95 % of the total oil. Additionally, after five cycles, the residual oil content in the meal was lower with 2-MeOx than with hexane (0.8 % vs. 1.8 %, respectively). It is therefore likely that in this study, nearly all residual GSO would have been recovered with an additional extraction cycle.

Extraction yield and fatty acids profile of GSOs are shown in Table 1. GSO belongs to the vegetable oils with a high content of unsaturated fatty acids, which account for about 90 % of the total fatty acids. The fatty acid profile of the oils extracted with hexane and 2-MeOx was similar, with a high content of linoleic acid (~68 %) and a lower content of oleic acid (~18 %), stearic acid (~4) and saturated fatty acids (~12 %) (Table 1). The refining process did not result in any substantial changes. The data were consistent with previously published results (Matthäus, 2008; Wada et al., 2018; Zhao et al., 2017) that showed a similar trend for fatty acids.

3.2. Micronutrient composition and oxidative stability

Among total lipids, neutral lipids represent the major fraction of GSO; Ohnishi et al. (1990) reported a relative proportion of neutral lipids of 97 %, followed by glycolipids at 2 % and phospholipids at 1 % (Ohnishi et al., 1990; De Marchi et al., 2012). Moreover, phytosterols are naturally occurring compounds in vegetable oils that are structurally similar to cholesterol and are not synthesised by humans. Besides their well-known role in reducing cholesterol absorption, phytosterols have beneficial effects on inflammatory markers and oxidative stress (Escolà-Gil, 2019).

In this study, the oils extracted with 2-MeOx (both dry and 4.5 % water) at laboratory and pilot scales had slightly lower total sterol content compared to those extracted with hexane (Table 2). A similar finding was reported by Bettaieb Rebey et al. (2019), that reported a higher sterols content in fennel and anise oil extracted with hexane compared to 2-MeOx (Bettaieb Rebey et al., 2019). Additionally, SCE-CO_2 yielded the highest sterol content. The higher sterol recovery with hexane is likely due to the completely apolar nature of this solvent.

All extracts exhibited a similar sterol profile, with β-sitosterol (71.4 −72.3 %) being the most abundant, followed by stigmasterol (9.7 −10.5 %), campesterol (8.5 −8.8 %), and sitostanol (3.5 −4.0 %). These findings align with previously reported literature (Yang et al., 2021).

Refining is the final step in vegetable oil production, aimed at improving oil quality by removing unwanted components such as free

Table 2
Sterols content and profiles of GSO.

	Laboratory scale extractions			2-MeOx pilot scale extraction	
	Hexane	Dry 2-MeOx	2-MeOx 4.5 %	Crude oil	Refined oil
Sterols profile (relative %)					
Cholesterol	0.2	0.2	0.5	0.2	0.2
Brassicasterol	0.2	< 0.1	< 0.1	< 0.1	< 0.1
24-Methyl-cholesterol	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Campesterol	8.8	8.6	8.5	8.6	8.6
Campestanol	0.3	0.3	0.3	0.3	0.4
Stigmasterol	10.4	10.2	9.9	10.5	9.7
δ−7-Campesterol	0.1	0.1	0.1	0.1	< 0.1
D5,23-Stigmastadienol	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Clerosterol	1.0	0.8	0.9	0.9	0.8
β-Sistosterol	71.4	71.9	72.3	71.8	71.4
Sitostanol	3.5	3.7	3.6	3.6	4.0
δ−5-Avenasterol	1.6	1.5	1.3	1.5	1.9
δ−5,24-Stigmastadienol	0.6	0.6	0.6	0.5	0.9
δ−7-Stigmastenol	1.5	1.5	1.3	1.4	1.7
δ−7-Avenasterol	0.5	0.6	0.5	0.6	0.5
Total (mg/kg oil)	5413	4935	5103	4797	3848

fatty acids (FFA), aldehydes, ketones, pigments, and minor compounds (Kreps et al., 2014). Water degumming has little effect on sterol content, while the major reduction occurs during neutralization, due to oil alkalization and high temperatures. Additionally, free sterols are partially distilled out during deodorization, with the extent of reduction depending on the deodorization temperature (Pan et al., 2020; Verleyen et al., 2002). In this study, the refining process resulted in a 19.78 % reduction in total sterol content, without altering the sterol profile. This decrease is consistent with previous studies, which reported a reduction of up to 29 % in total sterol content in refined GSO (Yang et al., 2021) and decreases of 29 −51 %, 20 %, and 19 −38 % in rapeseed, soybean, and sunflower oils, respectively (Régis et al., 2016).

Vitamin E, a group of lipophilic antioxidants in vegetable oils, consists of tocopherols and tocotrienols (tocols), which exhibit strong antioxidant activity by scavenging alkoxyl and peroxyl radicals (Gliszczyńska-Świgło et al., 2007). The tocols content in GSO is influenced by extraction and processing methods, grape variety, and factors like agricultural practices and growing conditions (Yang et al., 2021). According to the Codex Alimentarius, GSO tocols content ranges from 240−410 mg/kg (Related Information Documents, 2022), with α-tocopherol, α-tocotrienol, and γ-tocotrienol making up about 80 % of the total tocols (Ben Mohamed et al., 2016).

In this study, the total tocols content was higher in oils extracted with 2-MeOx compared to hexane, although the difference was not statistically significant ($p < 0.05$) (Table 3). However, this result differs from previous findings on olive pomace oil, where hexane yielded higher tocols content (Cravotto et al., 2022b).

In the crude oils extracted with 2-MeOx (both dry and 4.5 % water), α-tocopherol (40.5 −43.0 %), γ-tocotrienol (29.6 −32.4 %), α-tocotrienol (18.9 −19.1 %) and γ-tocopherol (7.5 −7.7 %) were the major compounds. The oil extracted with hexane showed a lower relative α-tocopherol content (35.9 %) and a higher relative γ-tocotrienol content (36.9 %).

It is known that the tocols content decreases in both chemical and physical refining processes, with the extent of loss varying by refining step, increasing in the following order: winterisation < degumming < bleaching < deodorisation (Kreps et al., 2014). In this study, refining led to a 47.6 % reduction in total tocols content. Similar findings have been reported in other oils, with tocols content decreasing by 23.5 % in primrose oil (Pan et al., 2020), 8.86 % in hazelnut oil (Durmaz and Gökmen, 2019), 15 −51 % in rapeseed oil, 0 −82 % in soybean oil, 32 −38 % in sunflower oil and up to 59 % in GSO (Yang et al., 2021; Régis et al., 2016). Wada et al. (2018) also reported a decrease in tocols of 27.7–54.1 % after refining muscadine GSO obtained with different extraction methods (Soxhlet with hexane, mechanical expression, and enzyme-assisted aqueous extraction). The crude oils obtained by Soxhlet extraction with hexane had a tocols concentrations of 441.1 −905.5 mg/kg oil (Wada et al., 2018).

Peroxide value (PV) and oxidative stability are important indicators of oil stability over time. Crude oils typically have higher PVs than refined oils, and about twice the oxidative stability, mainly due to their higher antioxidant content (tocols and polyphenols), which decreases during refining (Kreps et al., 2014). On a laboratory scale, PV values (showed in Table 3) were similar for crude oils extracted with hexane and dry 2-MeOx, while nearly half as low for oils extracted with 2-MeOx 4.5 % water. This is consistent with literature on unrefined oils extracted with hexane from air-dried GSs, which reported a PV of 35.0 meq/kg oil (Wada et al., 2018). It had already been shown that the presence of 4.5 % water reduces the amount of peroxides in the extracted oil (Claux et al., 2021a). During the pilot test, refinement drastically lowered the PV to 2.15 meq/kg oil. Furthermore, refined GSO was within the limits of the Codex standards: peroxide value up to 10 mEq/kg oil and an acid value up to 0.6 mg KOH/g oil (Section 2, 2023).

The oxidative stability determined by the Rancimat method at 110 °C was six times higher for oil extracted with dry 2-MeOx and seven times higher with 2-MeOx 4.5 % water than for oil extracted with hexane (Table 3). This increased stability correlated positively with higher tocols (Table 3) and polyphenol (Table 4) content. The crude oil extracted in the pilot test showed the highest oxidative stability, as it had the highest concentrations of polyphenols, tocopherols, and antioxidant activity. After refining, the strong reduction of antioxidant compounds led to a consequent reduction of oxidative stability to 7.2 h of induction period. Hashemi et al. (2017) similarly found that the refining process significantly reduces GSO stability under accelerated oxidation conditions.

3.3. Phenolic compounds profile and effect of the refining process

The total phenolic content of GSO is influenced by factors such as grape variety, post-harvest treatments, seed storage conditions, oil extraction methods, and the refining process (Yang et al., 2021). Increasing evidence supports the beneficial effects of plant polyphenols on human health. Polyphenols' health-promoting properties are closely linked to their antioxidant potential, contributing to neuroprotection, anti-inflammatory, anti-allergic, anti-atherogenic, anti-thrombotic, and anti-mutagenic effects. They also help reduce morbidity and slow the progression of cardiovascular, neurodegenerative, and cancer diseases (Gorzynik-Debicka et al., 2018).

Due to the hydrophilic nature of phenolic compounds, they are not efficiently recovered during GSO extraction. Therefore, large amounts of these compounds remain in the seed cake (Lutterodt et al., 2011). The literature reports different total phenolic content (TPC) of GSO with

Table 3
Tocopherol and tocotrienol profile, peroxide value, and oxidative stability of GSO.

	Laboratory scale extractions			2-MeOx pilot scale extraction	
	Hexane	**Dry 2-MeOx**	**2-MeOx 4.5 %**	**Crude oil**	**Refined oil**
Tocopherol and tocotrienol content (mg/kg oil)					
α-Tocopherol acetate	< 5	< 5	< 5	< 5	< 5
α-Tocopherol	174	210	245	273	143
β-Tocopherol	< 2	3	3	2	< 2
γ-Tocopherol	40	39	44	40	24
δ-Tocopherol	< 2	< 2	< 2	< 2	< 2
α-Tocotrienol	91	99	108	133	61
β-Tocotrienol	< 2	< 2	< 2	< 2	< 2
γ-Tocotrienol	179	168	169	178	98
δ-Tocotrienol	2	< 2	< 2	< 2	3
Total	485 ± 73a,b	518 ± 78a,b	570 ± 85a	626 ± 94a	328 ± 49b
Vitamine E activity (mg α-TE[a]/kg oil)	179	215	251	278	146
PV (meq/kg oil)	33.27 ± 0.24b	35.07 ± 0.01a	14.64 ± 0.21d	24.88 ± 0.73c	2.15 ± 0.09e
Oxidative stability (h)	5.1 ± 1.5c	29.1 ± 4.4b	35.5 ± 5.3b	73.5 ± 11.0a	7.2 ± 1.5c

[a] α-Tocopherol equivalent; PV, Peroxide value. Means in the same row with different superscript letters are significantly different; a–e, $p < 0.05$. Mean ± standard deviation of determinations (n = 3).

Table 4
Total phenolic content, antioxidant activity, and concentration of major phenolic compounds.

	Laboratory scale extractions			2-MeOx pilot scale extraction		
	Hexane	Dry 2-MeOx	2-MeOx 4.5 %	Crude oil	Refined oil	Gums[a]
Total phenolic content (mg GAE/kg oil)	188.6 ± 13.4c	5066.5 ± 176.0b	5480.0 ± 651.5b	5591.7 ± 137.2b	72.9 ± 7.2c	20072.0 ± 213.9a
Antioxidant activity (mg TE/kg oil)	20.7 ± 8.3c	11208.9 ± 296.1b	10697.8 ± 960.2b	12473.1 ± 1882.6b	16.7 ± 2.1c	41757.4 ± 2825.8a
Gallic acid (mg/kg oil)	DT	2890.0 ± 159.3	2061.1 ± 33.7	4066.0 ± 72.3	ND	8085.8 ± 338.4
Catechin (mg/kg oil)	< 0.1	176.5 ± 3.3	223.8 ± 10.6	311.8 ± 7.2	ND	160.9 ± 7.0
Epicatechin (mg/kg oil)	< 0.1	91.4 ± 1.2	133.0 ± 6.3	158.5 ± 8.0	DT	64. 3 ± 3.4
Syringic acid (mg/kg oil)	< 0.1	65.2 ± 3.0	71.5 ± 4.5	79.4 ± 1.3	ND	153.0 ± 13.1
p-Coumaric acid (mg/kg oil)	DT	62.4 ± 1.1	75.1 ± 1.5	68.4 ± 0.6	DT	91. 1 ± 2.9
Procyanidin B2 (mg/kg oil)	< 0.1	12.0 ± 0.5	76.6 ± 11.1	27.8 ± 1.8	< 0.1	12.1 ± 0.6
Caffeic acid (mg/kg oil)	ND	18.7 ± 0.3	16.8 ± 1.0	23.8 ± 0.4	ND	47.3 ± 1.0

[a] Solid obtained after water degumming (mg/kg dry solid); ND, not detected; DT, detected with a concentration below the limit of quantification. Means in the same row with different superscript letters are significantly different; a–c, $p < 0.05$. Mean ± standard deviation of determinations (n = 3).

various extraction methods: hexane (56 −358 mg GAE/kg oil) (Rombaut et al., 2015; Zhao et al., 2017; Harbeoui et al., 2018), SCE-CO_2 (28 −350 mg GAE/kg oil) (Ben Mohamed et al., 2016; Rombaut et al., 2014; Wen et al., 2016), mechanical pressing (8 −153 mg GAE/kg oil) (Rombaut et al., 2015, 2014; Bjelica et al., 2019; Bail et al., 2008) and a combination of SCE-CO_2 and mechanical pressing (253 mg GAE/kg oil) (Rombaut et al., 2015).

In this study, TPC increased with the extraction solvent, as follows: hexane (189 mg GAE/kg oil), dry 2-MeOx (5066 mg GAE/kg oil), and 2-MeOx 4.5 % water (5480 mg GAE/kg oil) (Table 4). No significant differences ($p < 0.05$) were observed between oils extracted with 2-MeOx in the laboratory and on a pilot scale. The high phenolic content in extracts obtained using 2-MeOx was consistent with previous findings on olive pomace (Cravotto et al., 2022b). Moreover, the antioxidant activity correlated strongly with TPC, as shown by a linear correlation ($R^2 = 0.974$) between TPC and DPPH.

Fine et al. (2016) studied the impact of crushing and refining processes on minor compounds in sunflower, rapeseed, and soybean oils, finding that phenolic compounds are almost entirely removed during refining, with a 93–98 % loss. Due to their hydrophilic nature, phenolics are mainly reduced during the early refining stages, such as water degumming and neutralization, which involve water use (Fine et al., 2016). Degumming, the first stage of oil refining, uses water to aggregate and remove phospholipids, resulting in a precipitate composed mainly of water, phospholipids, and some triglycerides (Wada et al., 2018). In our study, after chemical refining, the TPC was strongly reduced by more than 98 % (72.9 mg GAE/kg oil) compared to crude oil.

The TPC values of the oil after each refining step are presented in Fig. 2. Water degumming resulted in the most significant removal of phenolic compounds, with an 80.7 % reduction in TPC, followed by neutralization, which caused an additional 9.7 % decrease. As expected, the refining by-products (gums and soap-stock) had high TPC values.

The main phenolic compounds in GSs are gallic acid, catechin, epicatechin, and various procyanidins (Maier et al., 2009). Zhao et al. (2017) identified gallic acid, epicatechin, epicatechin gallate and pentagalloylglucose as the major phenolic compounds in 48 muscadine GSO varieties (Zhao et al., 2017).

In this study, UPLC-MS was used to identify and quantify key phenolic compounds. Gallic acid, catechin, and epicatechin were the most abundant phenolics in crude oils extracted with 2-MeOx. Furthermore, the high concentration of these compounds in the gums confirmed that a substantial portion of phenolics is removed during water degumming, which negatively impacts the oil's oxidative stability (Zacchi and Eggers, 2008).

Moreover, during neutralisation, excess base can cause the

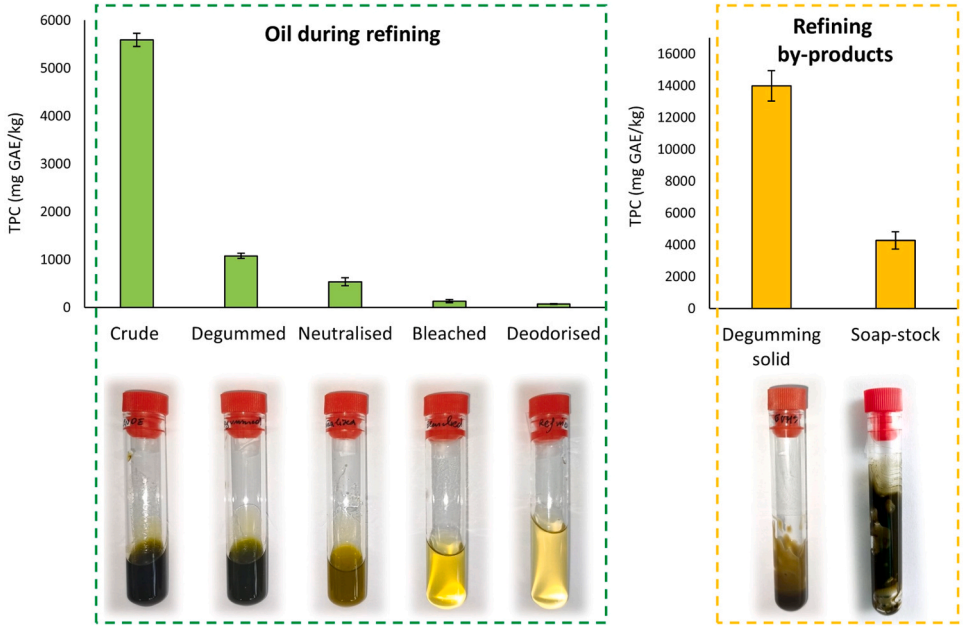

Fig. 2. Total phenolic content of GSO during refining and in refining by-products.

dissociation of the phenolic hydroxyl group into sodium phenolate, increasing the molecule's hydrophilicity. These hydrophilic compounds dissolve in the alkaline solution and are removed from the oil along with soap stock (Kreps et al., 2014). The recovery of bioactive compounds from soap stock is a growing area of interest, adding value to the edible oil processing sector (Chen et al., 2014).

Overall, gums and soap stock obtained during the refining of oil extracted with 2-MeOx are rich in phenolic compounds, offering potential as novel cosmetic ingredients. These by-products could provide significant value to otherwise low-value materials. For this reason, biological activities of polyphenols derived from crude oil and gums in various skin cell models are discussed in the next section.

3.4. Cellular studies

Previous results demonstrated that crude oil and gums samples exhibited the highest TPC values. In this section, their biological activities were evaluated in various skin cell models to determine their potential as novel cosmetic ingredients. Polyphenols were recovered by simple ethanol/water (60:40) extraction, and the resulting extract was directly used for cellular assays. Following an initial cytotoxicity assessment for each cellular model (see Supplementary Information, Section S3, Figs. S1–3), their effects on melanogenesis regulation, oxidative stress mitigation, and photoprotection were investigated.

3.4.1. Melanin content assay

Melanin, an important pigment in mammals, is responsible for the coloration of skin, eyes, and hair, and provides protection against ultraviolet radiation. However, excessive melanin production can lead to skin disorders like melasma, post inflammatory pigmentation, and solar lentigo, which become prominent with aging (Sato and Toriyama, 2009).

While various treatments for melasma exist, researchers are looking for more effective therapies with fewer side effects. In response to growing consumer demand for natural remedies, the cosmetics industry has increasingly focused on plant-based cosmetics with skin-lightening properties (Maddaleno et al., 2021). Phytochemicals such as flavonoids, coumarins, tannins and terpenes have shown skin lightening effects, in addition to their skin antioxidant and anti-inflammatory properties, by inhibiting tyrosinase activity and suppressing the uptake and distribution of melanosomes (Karadeniz et al., 2023).

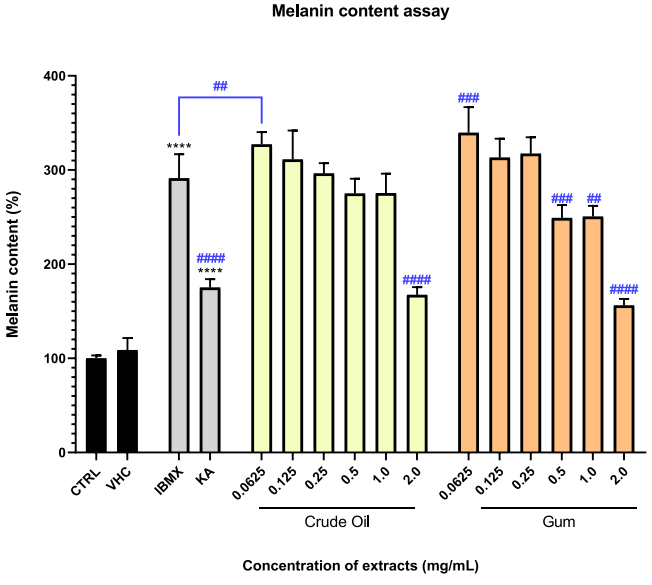

Fig. 3. Melanin content assay with B16-F10 Melanocytes treated with Crude Oil and Gums extracts, normalized with Hoechst reading. IBMX (200 μM) is used as a negative control, and IBMX with Kojic Acid (300 μM) is used as the positive control. Data is presented as the mean of four replicates ± SD. * ($p < 0032$) ** ($p < 0002$), *** ($p < 0{,}0002$), and * ** * ($p < 0{,}0001$) indicate statistically significant differences compared to the control containing only medium. # ($p < 0032$) ## ($p < 0002$), ### ($p < 0{,}0002$), and #### ($p < 0{,}0001$) indicate statistically significant differences compared to negative control with 200 μM IBMX in culture medium. VHC and Ctrl indicate vehicle control sample and untreated samples respectively.

For these reasons, the hydroalcoholic extracts of the crude oil obtained with 2-MeOx and the gums were tested for their anti-melanogenic properties.

A dose-dependent effect was observed with crude oil and gums extracts, which significantly reduced melanin content at their highest tested concentrations (Fig. 3). Notably, at these concentrations (2 mg/mL), the extracts demonstrated efficacy comparable to that of kojic acid (300 μM), highlighting their potential for application in skin-lightening products. These anti-melanogenic effects are likely attributable to the high polyphenol content (mainly gallic acid, catechin and epicatechin) in the crude oil and gums (Orhan and Deniz, 2021).

In a previous study, catechins and gallic acid demonstrated significant inhibitory effects on melanogenesis by directly inhibiting tyrosinase activity and down-regulating tyrosinase expression (Sato and Toriyama, 2009). Additionally, No et al. (1999) found that flavan-3-ols with a gallic acid moiety at the 3 position strongly inhibited tyrosinase activity (No et al., 1999). In conclusion, gums and crude oil may serve as promising sources of bioactive compounds for the effective treatment of hyperpigmentation disorders.

3.4.2. Oxidative stress mitigation

Two different cell lines, HaCaT keratinocytes and BJ fibroblasts, were tested in this assay. HaCaT keratinocytes, found in the epidermis, are physiologically affected by both UVA and UVB radiation, while BJ fibroblasts, located in the dermis, are predominantly affected by UVA. Both UVA (3.0 J/cm^2) and UVB (0.120 J/cm^2) exposure led to a significant increase in intracellular reactive oxygen species (ROS) in both cell lines (Fig. 4). The magnitude of ROS increase varied depending on the cell type and UV exposure, but a consistent 20–50 % rise in intracellular ROS was observed in all experiments ($p < 0.001$).

The oxidative stress induced by UVA in HaCaT keratinocytes was inhibited by both crude oil and gums extracts, with the strongest reactive oxygen species (ROS) inhibition observed at the highest concentrations tested (Fig. 4A, Table 5). Gums extract exhibited the most pronounced effect, achieving 86.7 % inhibition at 2.0 mg/mL, while crude oil reached 53.4 % inhibition at the same concentration.

For UVB-induced ROS production in HaCaT cells (Fig. 4B, Table 5), gums extract was again the most potent, showing at least 1.5 times greater efficacy than crude oil, with 99.2 % and 65.6 % inhibition, respectively, at 2.0 mg/mL.

In BJ fibroblasts, UVA exposure led to a 50 % increase in ROS levels. Both crude oil and gums extracts effectively reduced ROS levels, even in the absence of UV irradiation, and extended this effect to irradiated groups (Fig. 4C, Table 5). At concentrations above 1.0 mg/mL, the pro-oxidative effects of UVB (0.12 J/cm^2) were completely neutralised by both extracts. At 2.0 mg/mL, crude oil showed a 71.9 % inhibition of ROS, while gums achieved 53.0 %, indicating that crude oil was more effective in fibroblasts than gums extract.

These findings highlight gums extract as the most active sample in mitigating oxidative stress in skin cells, consistent with its higher total phenolic content (TPC), particularly rich in phenolic acids and catechins, which are known for their strong antioxidant properties (Table 4). The extracts also reduced basal oxidative stress under non-irradiated conditions, though this reduction was only statistically significant in certain cases.

3.4.3. Photo-protection assay

HaCaT keratinocytes and BJ fibroblasts were used in a photoprotection assay to evaluate the effects of crude oil and gums extracts

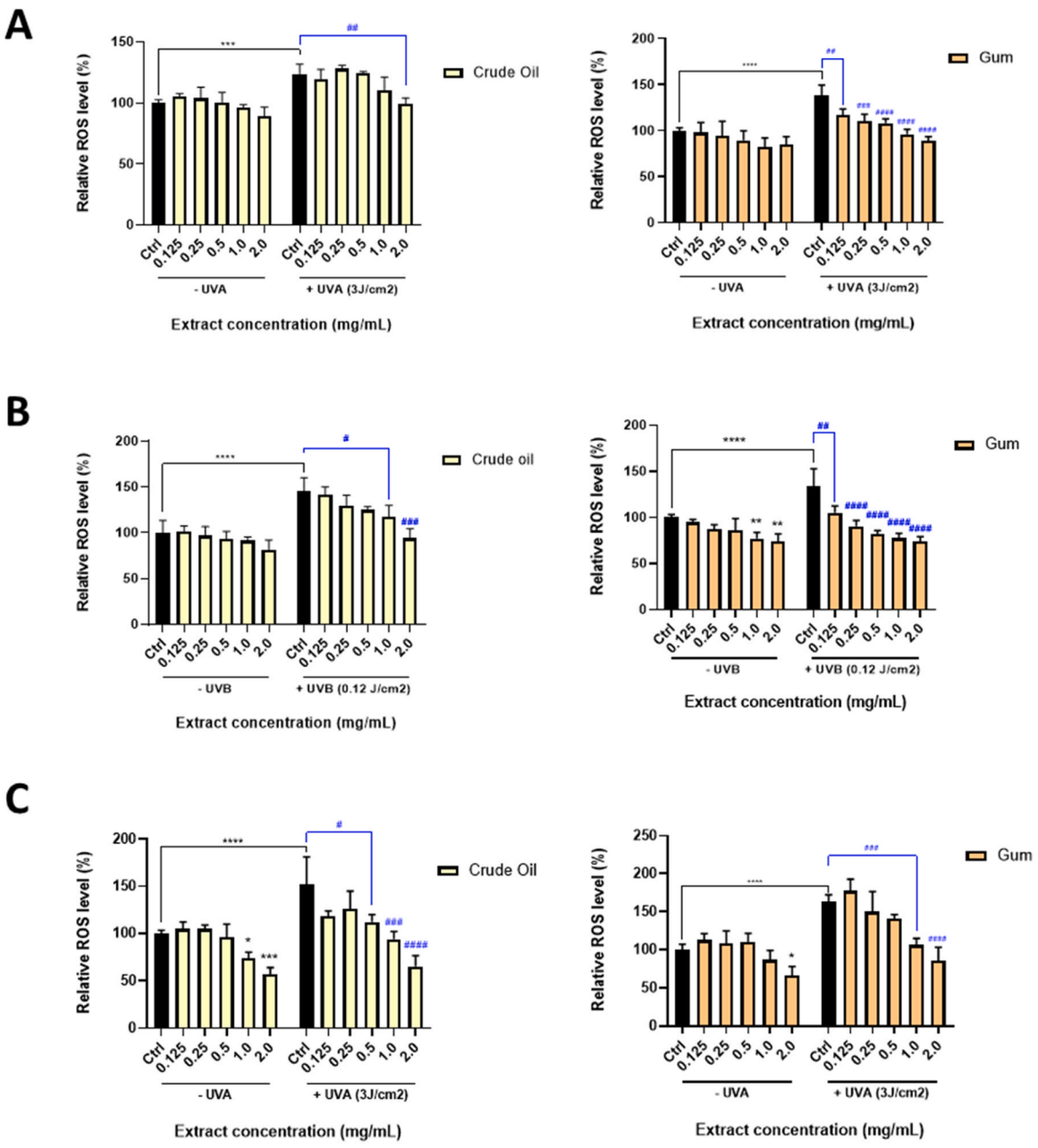

Fig. 4. Intracellular ROS level on HaCaT Keratinocytes treated with UVA (A) or UVB radiation (B). BJ fibroblast treated with UVA irradiation (C). Crude oils (yellow bars) or gums (orange bars) concentrations are showed in the bottom axis. Data is presented as the mean of four replicates ± SD. * ($p < 0032$) ** ($p < 0002$), * ** ($p < 0{,}0002$), and *** * ($p < 0{,}0001$) indicate statistically significant differences compared to non-irradiated medium control with the absence of the extract. # ($p < 0032$) ## ($p < 0002$), ### ($p < 0{,}0002$), and #### ($p < 0{,}0001$) indicate statistically significant differences compared to 3.0 J/cm^2 UVA-irradiated medium control with the absence of the extract. Ctrl indicates vehicle control sample.

under UVA (3.0 J/cm^2) and UVB (0.120 J/cm^2) irradiation (Fig. 5). Five concentrations of each extract were tested, similar to the ROS assay.

As expected, UVA (3.0 J/cm^2) irradiation did not significantly affect HaCaT cell viability (Fig. 5A). Although a slight photo-protective effect was observed upon treatment with the extracts, it was not statistically significant at any concentration for either extract.

In contrast, UVB (0.12 J/cm^2) exposure caused significant cytotoxicity, reducing cell viability by 50–75 % compared to controls ($p < 0.0001$). Crude oil did not reverse UVB-induced cytotoxicity, but the highest concentrations of gums extract showed a modest protective effect, with a 27.2 % increase in cell viability compared to the irradiated control (Fig. 5B).

Similarly, UVA exposure did not significantly affect BJ fibroblast viability, aligning with the intracellular ROS findings from UVA-irradiated HaCaT cells, indicating that UVA at 3.0 J/cm^2 is not cytotoxic to this cell type. Treatment with either extract did not result in statistically significant changes in fibroblast viability (Fig. 5C).

These results suggest that while both extracts effectively mitigate the initial oxidative stress and ROS production caused by UV radiation, they are less effective in protecting against the longer-term cytotoxic effects of UVB radiation on cell viability. This may be due to the direct DNA damage caused by UVB, which is not observed with UVA. Future studies could explore combining the antioxidant properties of these extracts with additional UVB filters to enhance protection against UVB-induced DNA damage.

Table 5
ROS inhibition level (%) of different extracts tested on both HaCaT keratinocytes and BJ fibroblast using 3.0 J/cm^2 UVA-or 0.12 J/cm^2 UVB-irradiation dose levels respectively.

	HaCaT keratinocytes				BJ fibroblasts	
	UVA irradiated		UVB irradiated		UVA irradiated	
Concentration (mg/mL)	**Crude oil**	**Gum**	**Crude oil**	**Gum**	**Crude oil**	**Gum**
0.125	44.2 %	49.7 %	14.0 %	67.5 %	75.0 %	7.8 %
0.25	2.6 %	55.6 %	24.7 %	89.2 %	60.3 %	39.3 %
0.5	2.0 %	46.5 %	25.1 %	114.6 %	67.2 %	55.9 %
1.0	41.6 %	55.6 %	38.3 %	98.6 %	48.9 %	64.2 %
2.0	53.4 %	86.7 %	65.6 %	99.2 %	71.9 %	53.0 %

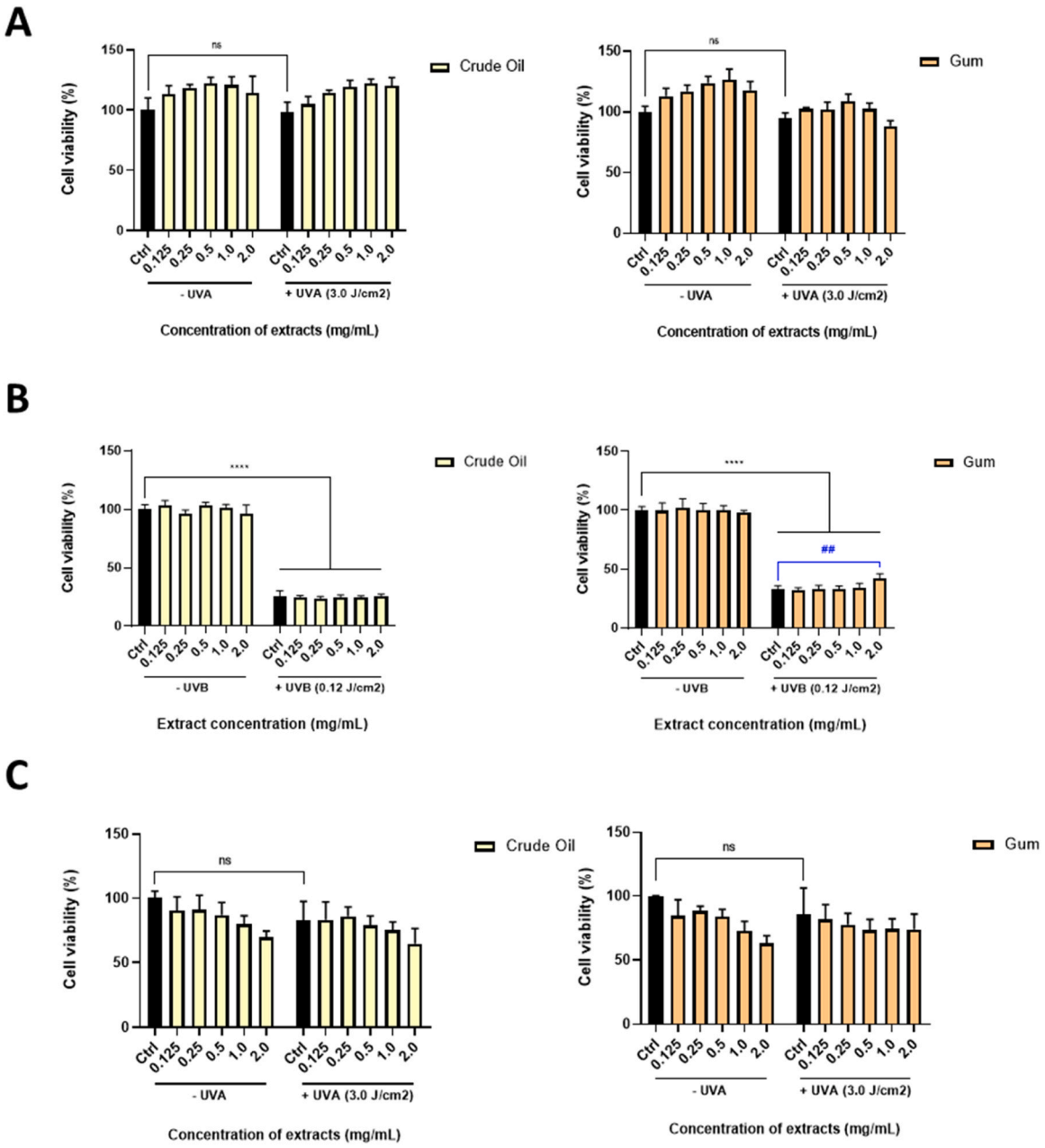

Fig. 5. Photo-protection assay on HaCaT Keratinocytes treated with UVA (A) or UVB radiation (B). BJ fibroblast treated with UVA irradiation (C). Crude oils (yellow bars) or gums (orange bars) concentrations are showed in the bottom axis. Data is presented as the mean of four replicates ± SD. There was no significant (ns) difference between non-irradiated and irradiated control. Brief incubation with extracts slightly increased HaCaT cell viability after 24 hours. Ctrl indicates vehicle control sample.

4. Conclusion

In this study, 2-MeOx achieved similar or higher GSO extraction yields compared to hexane, without affecting the fatty acid profile. The sterol, tocopherol, and tocotrienol compositions in oils extracted with 2-MeOx were consistent with literature, and 2-MeOx resulted in the extraction of high amounts of polyphenols. A pilot-scale test confirmed the scalability of the process, achieving an 86.3 % yield compared to hexane after only two maceration cycles.

However, chemical refining significantly reduced the oil's micronutrient content, decreasing sterols by 19.78 %, tocols by 47.6 %, and polyphenols by nearly 99 %. Notably, high concentrations of polyphenols were recovered in the gums and soap-stock by-products. Both the crude oil and gums polyphenols effectively inhibited intracellular ROS in HaCaT keratinocytes and BJ fibroblasts following UVA and UVB exposure. Additionally, these extracts exhibited potent skin-whitening effects, comparable to Kojic acid (300 mM), at 2.0 mg/mL.

In conclusion, 2-MeOx proves to be a valuable green solvent for producing GSO for food and cosmetic applications. During refining, approximately 80 % of the polyphenols are recovered in the gums, offering opportunities to valorise this by-product for high-value cosmetic ingredients. This process could also be adapted and applied to other plant-based materials, broadening its potential for various industrial applications.

CRediT authorship contribution statement

Mickaël Bartier: Writing – review & editing. **Raúl Bonet-García:** Validation, Investigation. **Binh Nguyen-Thanh:** Writing – original draft, Validation, Investigation. **Vincent Rapinel:** Methodology, Data curation, Conceptualization. **Christian Cravotto:** Writing – original draft, Validation, Data curation, Conceptualization. **Anne-Sylvie Fabiano-Tixier:** Writing – review & editing, Supervision, Project administration. **Enrique Barrajón-Catalán:** Writing – original draft, Validation, Conceptualization. **Silvia Tabasso:** Writing – review & editing, Supervision, Project administration. **Laurence Jacques:** Resources, Funding acquisition. **Ombéline Claux:** Writing – review & editing, Data curation.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fbp.2024.12.006.

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