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Unsaponifiable fraction isolated from grape (*Vitis vinifera* L.) seed oil attenuates oxidative and inflammatory responses in human primary monocytes

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Running title: Anti-oxidant and anti-inflammatory activities of GSO

Abbreviations

CCR: C-C chemokine receptor, **GSO:** grape seed oil, **GSOUF:** grape seed oil unsaponifiable fraction, **IL:** interleukin, **LPS:** lipopolysaccharide, **NO:** nitric oxide, **PBMC:** peripheral blood mononuclear cell, **ROS:** reactive specie oxygen, **TLR:** toll like receptor, **TNF:** tumor necrosis factor, **UF:** unsaponifiable fraction

ABSTRACT

Grape (*Vitis vinifera* L.) seed has a well-known potential for production of oil as a byproduct of winemaking and is a rich source of bioactive compounds. Herein, we report that unsaponifiable fraction (UF) isolated from grape seed oil (GSO) possesses anti-oxidative and anti-inflammatory properties in human primary monocytes. UF isolated from GSO was phytochemically characterized by GC-MS and HPLC. Freshly human monocytes were used to analyse the effects of GSOUF (10-100 μg/mL) on oxidative and inflammatory responses using FACS analysis, RT-qPCR, and ELISA procedures. GSOUF skewed the monocyte plasticity towards the anti-inflammatory non-classical $CD14^{+}CD16^{++}$ monocytes and reduced the inflammatory competence of LPS-treated human primary monocytes diminishing TNF-α, IL-1β, and IL-6 gene expression and secretion. In addition, GSOUF showed a strong reactive oxygen species (ROS)-scavenging activity, reducing significantly nitrite levels with a significant decrease on *Nos2* gene expression. Our results suggest that UF isolated from GSO has significant potential for management of inflammatory and oxidative conditions and offer novel benefits derived from the consumption of GSO in the prevention of inflammatory-related diseases.

Keywords: Unsaponifiable, grape seed oil, monocyte, sterols, inflammation.

INTRODUCTION

Grapes, the berries of *Vitis vinifera* L. spp *sativa*, are widely cultivated in climate zones all over the world for various utilizations since ancient times.[1] During wine production, approximately 25% (w/w) of the grape results in byproduct, which is comprised of skins and seeds.[2] For instance, grape seed has a high antioxidant potential; its beneficial effects include the modulation of antioxidant enzyme expression, protection against oxidative damage in cells, anti-atherosclerotic and anti-inflammatory effects, and protection against some cancer types, in both humans and animal models.[1,3,4] Recently, grape seed has shown a potential for production of oil, up to 15%, as a byproduct of winemaking.[5] A straightforward calculation indicates that the 2015 world grape seed oil (GSO) production would have been ≈261 tons of oil, with worth about $US 1.58-2.08 million. GSO is very high in linoleic acid (58–78%, 18:2n-6) followed by oleic acid (3–15%, 18:1n-9) and minor amounts of saturated fatty acids (10%).[4] Additionally, this oil is reported to contain minor components such as phenolic compounds.[6] The beneficial effects of GSO are thought to be due to its polyphenolic and vitamin E content. In contrast, little effort has been expended to characterize the unsaponifiable fraction (UF) of GSO. The UF, about 1.5–2% of the oils, is an important source of interesting minor compounds.[7,8] As part of ongoing investigations on bioactive secondary plant metabolites in medicinal and food plants, our aim of the present study was to conduct a detailed analysis to establish the anti-oxidant and anti-inflammatory effects for this valuable oilseed crop.

The production of reactive oxygen species (ROS), the down-regulation of antioxidant response genes, and the secretion of pro-inflammatory mediators work as an inflammatory beacon for leukocytes, which contribute to all stages of atherosclerosis and other inflammatory disorders, therefore representing an important therapeutic targets.[9] Human monocytes are classified into three subsets; $CD14^{++}CD16^{-}$ (claasical monocytes), intermediate $CD14^{++}CD16^{+}$ (intermediate monocytes), and $CD14^{+}CD16^{++}$ (non-classical monocytes).[10] So far, classical monocytes represent the major fraction (about 85% of total monocytes) and highly express CCR2, they are professional phagocytes giving rise to M1 macrophages, which generate ROS and secrete cytokines (TNF-α, IL-1β, and IL-6) in response to LPS during infection or inflammation.[11] Intermediate monocytes display highest levels of CCR5, TLR4, CD163, and HLA-DR during activation and also secrete pro-inflammatory cytokines.[12] Non-classical monocytes are less granular and smaller in size, with lower expression of CCR2 than classical or intermediate subsets.[12] These monocytes rich in CD16 are functionally involved in tissue repairing, patrolling, and wound healing, and have the tendency to be polarized into M2 macrophages with an anti-inflammatory phenotype in response to a variety of stimuli, including IL-4.[13] The aim of the present study was to investigate the effects of GSOUF on human primary monocytes activation as hallmarks of oxidative and inflammatory disorders.

MATERIALS AND METHODS

Isolation and chemical characterization of unsaponifiable fraction from grape seed oil

The UF was isolated from GSO (1 Kg, Naturgreen, Murcia, Spain) following conventional procedures and its components were analysed following the IUPAC method and described in Montserrat-de la Paz *et al*.[7] In brief, GSO was saponified at 80ºC by refluxing with 50 ml of 2 N potassium hydroxide solution in ethanol (Panreac, Barcelona, Spain), boiling gently until the solution became clear and then for additional 20 min. Heating was stopped by addition of 50 ml distillate water through the top of the condenser and the solution was swirled. After cooling to 30-35ºC, the solution was rinsed several times with water and UF was extracted with diethyl ether (Panreac) used as solvent for evaporation by distillation on a rotary evaporator (B-480 model, Büchi Labortechnik, Essen Germany) at 30ºC under vacuum. Then the wash water was removed, and the organic sample was dried with anhydrous sodium sulphate, filtered, taken to dryness and the residue was weighed. The yield in all samples was between 2.2% and 2.4%.

Quantitative analysis of UF aliphatic alcohols, sterols, and triterpenic alcohols were performed according to the European Regulation EEC/2568/91 for olive oil. α-Cholestanol and 1-eicosanol (Sigma–Aldrich, Madrid, Spain) were added as internal standards. UF was extracted, as mentioned above, and the bands corresponding to the sterols, triterpenic alcohol, and aliphatic and terpenic alcohols fractions were separated, by thin-layer chromatography, on a basic silica gel plate. The sterols recovered from the plate were transformed into trimethylsilyl ethers and the mixture was analysed by GC using an HP 5890 series II gas chromatograph equipped with a flame ionisation detector and a 30 m 0.32 mm i.d. Tracsil TRB-5 (95% dimethylpolysiloxane 5% diphenyl, film thickness 0.25 lm) capillary column (Teknokroma, Barcelone, Spain). The chromatographic conditions were as follows: injector 300 °C, isothermal column 275 °C, and detector 300 °C. The split ratio was 1:50 and the hydrogen flow rate of 1.0 ml/min, 130 Kpa. The chromatographic conditions for alcohol determination were the same as those mentioned above for sterols, except that the oven temperature was as follows: 215 °C (5 min); 3 °C/min increase to 290 °C and held for 2 min.

The quantification of tocopherols was based on the comparison of the peak areas with those of an external standard curve of R-tocopherol and identified by high-performance liquid chromatography (HPLC) chromatograms (AOCS Ce 8-89). The test sample was prepared as a dissolution to 10% by weight in hexane (Panreac) and analysed in a HPLC system (Hewlett–Pack-ard, Minnesota, US 1050) equipped with a fluorescence detector (Shimadzu RF-535), with the excitation wavelength set at 290 nm 170, and emission wavelength at 330 nm and HPLC analytical column silica (250 mm 4 mm i.d. 5 lm) (Merck Superspher Si60 Darmstadt, Germany), at a temperature of 30 °C. A flow rate of 1 ml/min, 400 bar was used. Results are expressed as mg/100 g GSO (**Table 1**).

Blood collection and isolation of human monocytes

This study was conducted according to Good Clinical Practice Guidelines and in line with the principles outlined in the Helsinki Declaration of the World Medical Association. Informed consent for the study was obtained from healthy male blood donors (age <35 years) at the University Hospital Virgen del Rocio, Seville. Participants declared that they were non-smokers and were not taking any medication. Peripheral blood samples were drawn from a large antecubital vein and collected into K3EDTA-containing tubes (Becton Dickinson, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples by centrifugation over a Ficoll-Histopaque (Sigma-Aldrich, Madrid, Spain) gradient. Monocytes were isolated from PBMCs using CD14 microbeads and LS columns on a midiMACS system (Miltenyi Biotec, Madrid, Spain) according to the manufacturer's instructions. The purity for CD14 monocyte isolations was routinely >90% by flow cytometry (FACScanto II flow cytometer and FACSDiva software, BD). Following isolation, monocytes were suspended in a RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin and 10% heat-inactivated foetal bovine serum.[14] For treatments, 5×10^5 of purified monocytes, after in vitro stimulation with or without LPS (100 ng/mL), were exposed to GSOUF at 10-100 mM for 24 h.

Cell viability assay (MTT)

Cells were incubated with the MTT solution (Sigma) until a purple precipitate was visible. MTT-formazan crystals were solubilized with DMSO (Sigma), and then measured with a microplate reader at 570 nm corrected to 650 nm.[15] Cell survival was expressed as the percentage of absorbance compared with that obtained in control, non-treated cells.

Immunostaining of circulating monocytes by FACS

Monocyte membrane expression of CD16 (PE anti-human CD16, Miltenyi) and CD14 (APC-Cy7 anti-human CD14, Miltenyi) was assessed by flow cytometry. According to the manufacturer's instructions, cells were incubated with antibodies at room temperature and in the dark for 15 min; erythrocytes were removed with FACS lysing solution (BD). Mean fluorescence intensity (MFI) was measured by using a FACSCanto II flow cytometer (BD) and calibrated by using a FACSDiva software (BD). MFI of 10^4 counted cells was assessed for each sample. Monocytes were gated as forward scatter^{high} (FSC^{high})-side scatter^{high} (SSC^{high})-cells.[10] Expression levels are presented as MFI corrected for nonspecific binding of isotope control antibodies.

Measurement of intracellular ROS

Intracellular ROS production was measured using 2',7'-dichlorfluorescein-diacetate (DCFH-DA) and CellROX Green Reagent (ThermoFisher Scientific, Madrid, Spain).[16] DCFH-DA penetrates into the cells and is hydrolyzed by intracellular esterases to the non-fluorescent 2,7-dichlorofluorescein (DCFH), which can be rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of ROS. The fluorescence intensity was measured as described previously Cardeno *et al*.[17] In addition, intracellular ROS production was measured with CellROX Green Reagent (5 μM) for 30 min. Cells were washed with PBS, fixed with 3.7% formaldehyde, and the fluorescence signal was analyzed in a Fluoroskan Microplate Fluorometer (ThermoFisher Scientific) equipped with a 485/555 excitation/emission filter set. The auto-fluorescence of cells was measured in the same conditions but without adding CellROX Green Reagent.Results were expressed as intracellular ROS production percentage compared with LPS control cells (stimulated non-treated cells). H_2O_2 (100 μM, 30% pure) (Panreac) was used as pro-oxidant positive control (data not shown).

Measurement of nitrite production

Cells in 24-well plates were untreated or treated with different concentrations of squalene (12.5, 25 or 50 μM), and 30 min later stimulated with LPS for 18 h. The culture supernatants (100 μl) were transferred to a 96-well assay plate mixed with Griess reagent (Sigma®, St Louis, MO, USA) and incubated for 15 min at room temperature. The amount of nitrite, as an index of NO generation, was determined by a spectrophotometric method using the Griess reaction and obtained by extrapolation from a standard curve with sodium nitrite.[18] The absorbance at 540 nm was measured by an enzyme-linked immunosorbent assay reader (BioTek®, Bad Friedrichshall, Germany). Results were expressed as the nitrite production percentage compared with LPS control cells (stimulated untreated cells). 1 μM Dexamethasone (Sigma) was used as positive control (data not shown).

RNA isolation and qRT-PCR analysis

Total RNA was extracted by using Trisure Reagent (Bioline), as instructed by the manufacturer. RNA quality was assessed by A260/A280 ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Madrid, Spain). Briefly, RNA (1 μg) was subjected to reverse transcription (iScript, Bio-Rad, Madrid, Spain). An amount of 20 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (Bio-Rad). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad) containing the primer pairs for either gene or for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping genes (**Table 2**). All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method.[19] All data were normalized to endogenous reference (GAPDH) gene content and expressed as percentage of controls.

Measurement of cytokine release

The levels of IL-1β, IL-6, and TNF-α in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA), following the indications of the manufacturer (Diaclone, Besancon, France).[20] The cytokine concentrations were expressed in pg per mL, as calculated from the calibration curves from serial dilution of human recombinant standards in each assay.

Data analysis

All values are expressed as arithmetic means ± standard deviations (SD). Data were evaluated with Graph Pad Prism Version 5.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), following Tukey multiple comparisons test as post hoc test. *P* values less than 0.01 were considered statistically significant.

RESULTS AND DISCUSSION

The interest in GSO as a functional food product has increased, especially because of its high levels of hydrophilic constituents, such as phenolic compounds, and lipophilic constituents, such as vitamin E, unsaturated fatty acids, and phytosterols.[21] In the present work has been isolated the UF from GSO and for the first time has been explored the effect of this fraction, on inflammatory response and reprogramming towards functional phenotypes in primary human monocytes. After 24 h of treatment, GSOUF at concentrations up to 200 µg/mL of dry residue had no significant effects, by means of Trypan Blue exclusion test, on viability of human primary monocytes (data not shown). Several evidences indicate that blood monocytes consist on several subpopulations of cells, which differ, in size, nuclear morphology, granularity, and functionality.[22] Our study also undertook to explore whether GSOUF can affect to monocyte subset distribution. We determinate three different subsets of human monocytes: classical, defined as $CD14^{++}CD16^{-}$ cells, intermediate, defined as $CD14^{++}CD16^{+}$ cells, and non-classical $CD14^{+}CD16^{++}$. After 24 h of treatment, GSOUF induced a decrease of CD14 and an increase of CD16 surface expression in LPS-treated human primary monocytes (**Fig. 1**). These effects resulted in a decrease of the relative proportion of the classical and intermediate (**Fig. 1B**) monocyte subsets and an increase of the relative proportion of the nonclassical monocyte subset (**Fig. 1C**). The classical and intermediate monocyte subsets has a pro-inflammatory phenotype that actively produces TNF-α (in response to LPS), IL-1β, and IL-6, and leads to the progression of numerous inflammatory disorders such as atherosclerosis.[23] Therefore, a proper balance among the different monocyte subsets may be critical to prevent persistent inflammation and to achieve controlled repair. This study is the first to demonstrate that UF isolated from GSO may regulate the CD14/CD16 balance in human monocytes.

The most notable bioactive property of grape seeds is their antioxidative capacity. This property has been widely studied in grape seed extracts whose compounds are capable of scavenging ROS and inhibiting lipid peroxidation.[24] GSOUF suppressed the intracellular production of ROS induced by LPS in human primary monocytes (**Figs. 2A-B**). In line with these effects, GSOUF induced a dose-dependent decrease of nitrite release to the medium in LPS-treated human primary monocytes (**Fig. 2C**). Similar effects were observed for *Nos2* gene expression (**Fig. 2D**). Xia *et al.*[25] compared the antioxidant capacity of grape and its by-products, including leaves, skin, wine, and seeds. The highest antioxidant capacity, measured by oxygen radical absorbance capacity assay, was found in grape seeds. This high antioxidant capacity is related to the high content of gallic acid, catechin, epicatechin, procyanidins, and proanthocyanidins in grape seed and GSO[26] and may be a result of the synergistic combination of these phenolic compounds. However, we are the first to demonstrate the antioxidant capacity of the UF isolated from GSO.[27]

It has also been established that grape seeds exhibit anti-inflammatory *in vitro* and *in vivo* properties.[1] Recognition of LPS by mainly TLR4 initiates several signalling cascades leading to the activation of NF-κB and MAPK pathways that mediate the expression of inflammatory cytokines for instance, TNF-α, IL-1β, and IL-6.[28] After 24 h of treatment, GSOUF induced a dose-dependent decrease of *Tlr4* mRNA levels in LPS-treated human primary monocytes (**Fig. 3A**). Similar effects were observed for *Tnfalpha*, *Il1beta*, and *IL6* genes (**Figs. 3B-D**). This ability of GSOUF to decrease the transcriptional activity of such pro-inflammatory genes was also accompanied by a reduced release of TNF-α, IL-1β, and IL-6 to the medium (**Figs. 4A-C**). In this context, Olas *et al.*[29] observed that GSO decreased inflammation in vitro, showing more effectiveness than pure resveratrol. In addition, the polyphenols present in GSO are able to inhibit the release of arachidonic acid, responsible for the production of leukotrienes and prostaglandins, which in turn activates the inflammatory response.[30] Zhao *et al.*[31] demonstrated that GSO reduced the IL-6 and IL-8 gene expression and cytokine secretion in primary human adipose-derived stem cells (hASCs). Therefore, our findings suggest that the above *in vitro* antioxidant and anti-inflammatory activities of GSOUF could participate, at least partly, in the health benefits of GSO.

Taken together, our results suggest that UF isolated from GSO has significant potential for management of inflammatory and oxidative conditions characterized by an over-activation of monocytes and thereby for the efficient termination of the inflammatory response and offer novel benefits derived from the consumption of GSO in the prevention of atherosclerotic disease and other inflammatory-related conditions

Conflicts of interest

The authors state no conflict of interest.

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Figure legends

Figure 1. Effect of unsaponifiable fraction isolated from grape seed oil (GSOUF) on monocytes subsets. FACS analysis (MFI) of monocyte surface markers CD14 and CD16 after 24 h incubation with or without LPS (100 ng/mL) and GSOUF at 10-100 μg/mL. (**A**) Classical $CD14^{++}CD16^{-}$ monocytes, (**B**) intermediate $CD14^{++}CD16^{+}$ monocytes, and (**C**) non-classical $CD14^{+}CD16^{++}$ monocytes. (**D**) Representative CD14/CD16 dot plots of monocyte subsets. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($P < 0.01$).

Figure 2. Effect of unsaponifiable fraction isolated from grape seed oil (GSOUF) on ROS and NO generation and iNOS expression in LPS-treated monocytes. Monocytes were treated with or without LPS (100 ng/mL) and then incubated with GSOUF at 10-100 μg/mL for 24 h. The production of intracellular ROS (**A,B**) and nitrites (**C**) was expressed as percentage of fluorescence/absorbance relative to cells treated with LPS. (**D**) Relative *Nos2* mRNA expression levels detected by qRT-PCR. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($P < 0.01$).

Figure 3. Effect of unsaponifiable fraction isolated from grape seed oil (GSOUF) on inflammatory gene expression in LPS-treated monocytes. Monocytes were treated with or without LPS (100 ng/mL) and then incubated with GSOUF at 10-100 μg/mL for 24 h. Relative expression of (**A**) *Trl4*, (**B**) *Tnfalpha*, (**C**) *Il1beta*, and (**D**) *Il6* mRNA expression levels detected by qRT-

PCR. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different (*P* < 0.01).

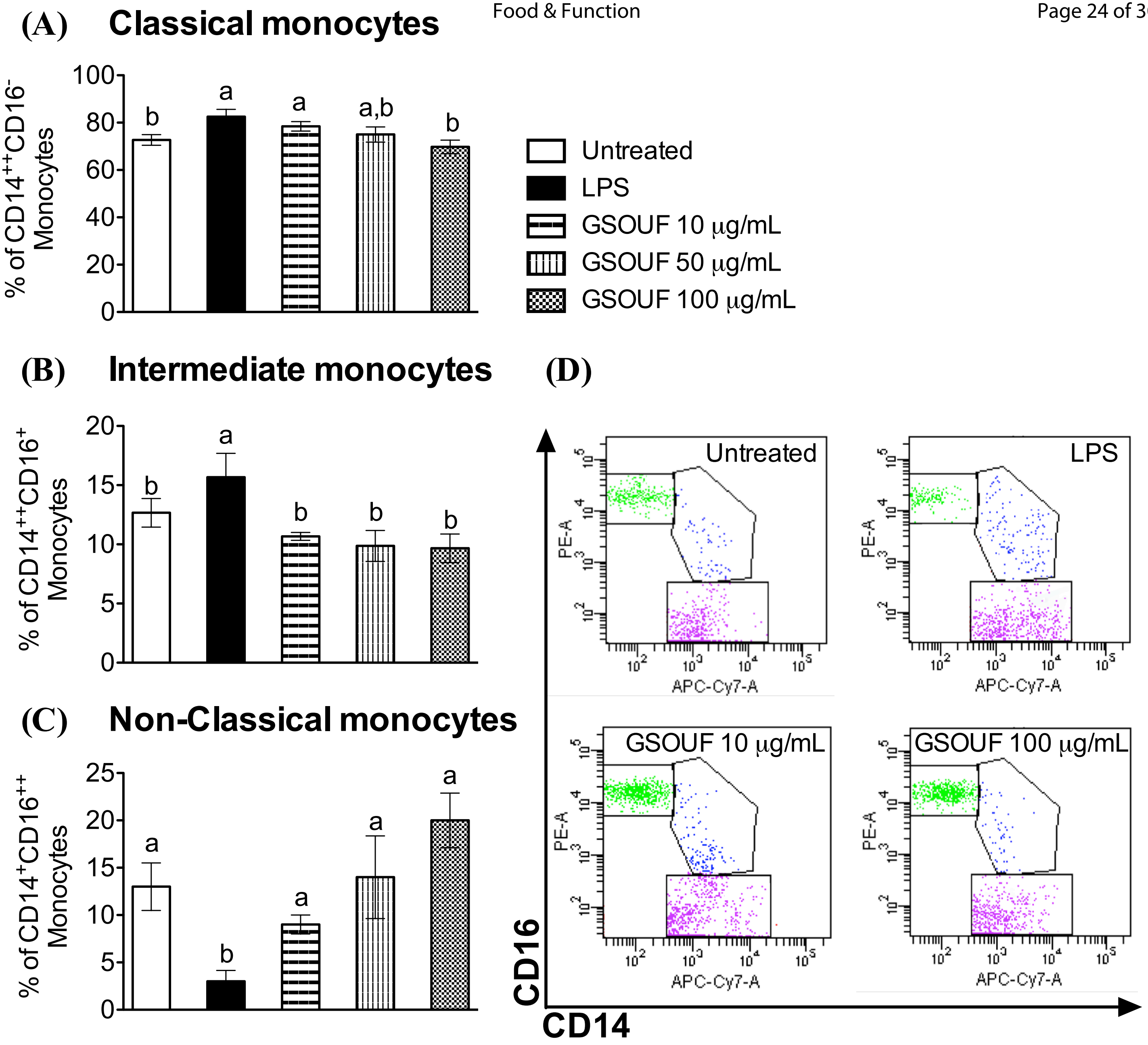
Figure 4. Effect of unsaponifiable fraction isolated from grape seed oil (GSOUF) on inflammatory cytokine secretion in LPS-treated monocytes. Monocytes were treated with or without LPS (100 ng/mL) and then incubated with GSOUF at 10-100 μg/mL for 24 h. Concentration of **(A)** TNF-α, (**B**) IL-1β, and **(C)** IL-6 was measured by ELISA in culture supernatants of LPS-treated monocytes. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different (*P* < 0.01).

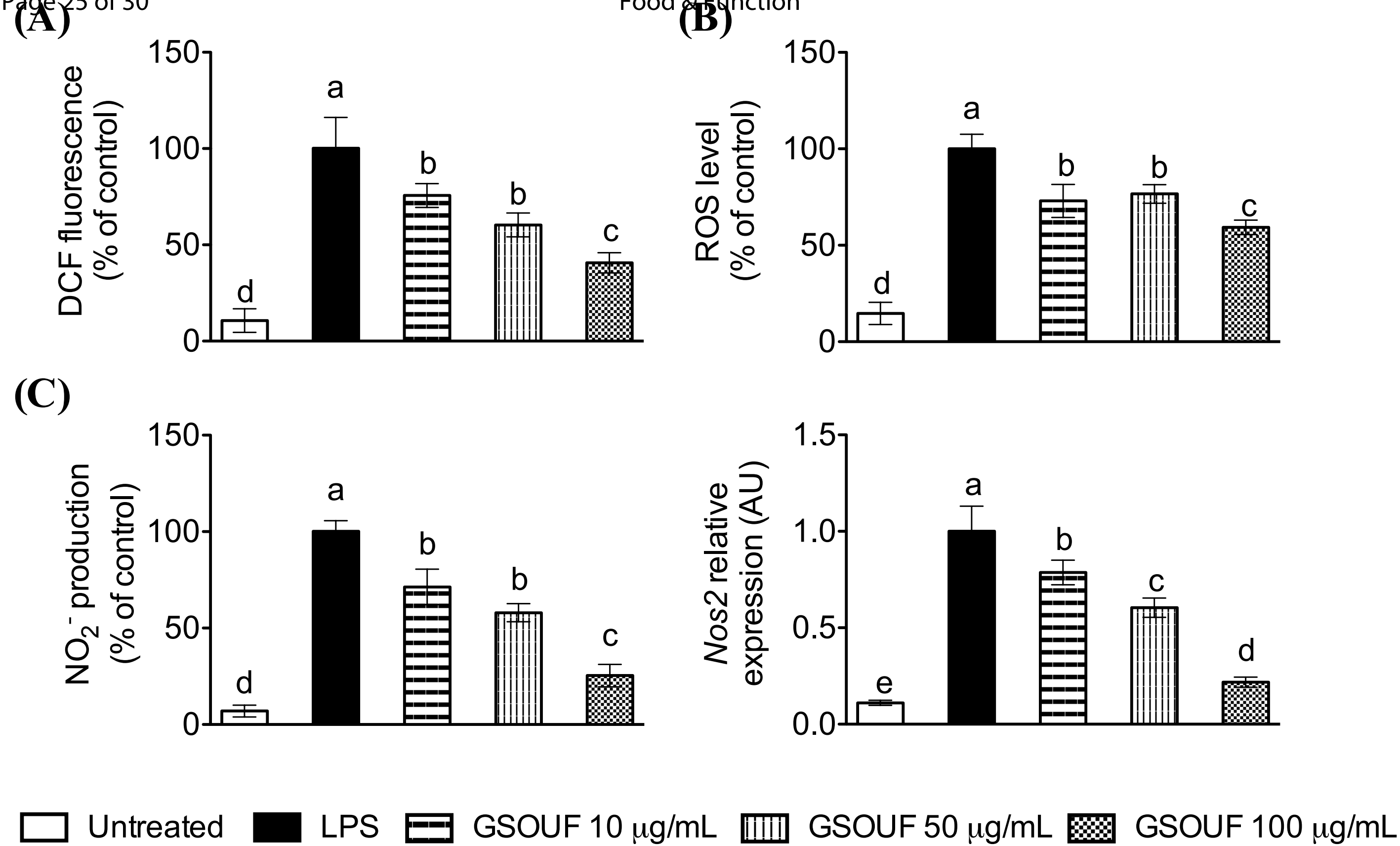
Table 1. Unsaponifiable fraction of grape seed oil composition

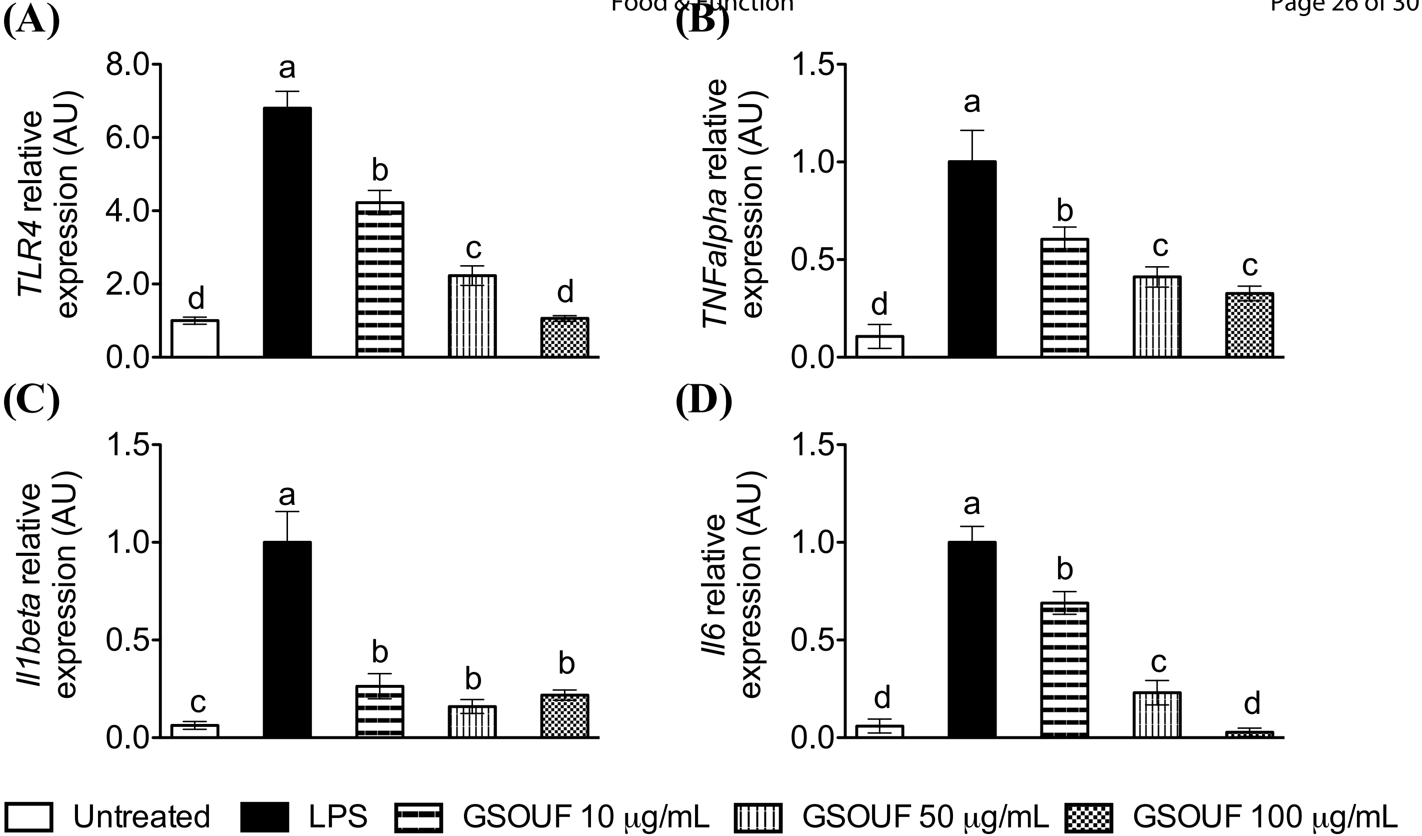
Component	mg/100 g GSO
Total sterols	418,63 ± 9.37
β-Sitosterol	292.85 ± 7.79
Total aliphatic alcohols	63.33 ± 3.18
C:26	18.15 ± 1.63
Total methylsterols	20.17 ± 2.86
Dammaradienol	3.92 ± 0.92
Total triterpenic alcohols	28.76 ± 3.17
Cicloartenol	6.98 ± 1.26
Tocopherols and tocotrienols	328.3 ± 12.21
α-Tocopherol	154.1 ± 4.94

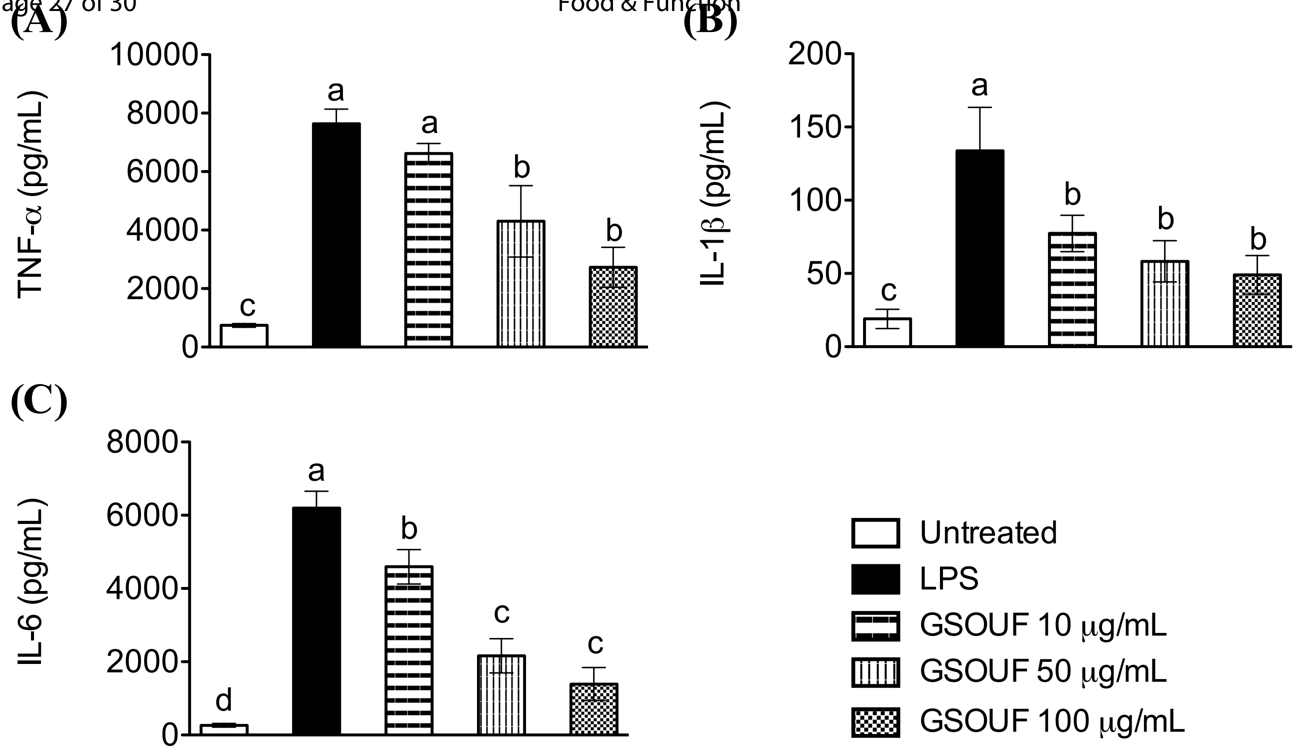
Table 2. Sequences of RT-PCR primers for gene expression analysis

Target	GenBank accession number	Direction	Sequence (5'→3')
Tlr4	NM_138554	Forward Reverse	CTGCCACATGTCAGGCCTTAT AATGCCCACCTGGAAGACTCT
Nos2	NM_000625	Forward Reverse	ACCCAGACTTACCCCTTTGG GCCTGGGGTCTAGGAGAGAC
Tnfalpha	NM_000594	Forward Reverse	TCCTTCAGACACCCTCAACC AGGCCCCAGTTTGAATTCTT
Il1beta	NM_000576	Forward Reverse	GGGCCTCAAGGAAAAGAATC TTCTGCTTGAGAGGTGCTGA
Il6	NM_000600	Forward Reverse	TACCCCCAGGAGAAGATTCC TTTTCTGCCAGTGCCTCTTT
Gapdh	NM_001289746	Forward Reverse	CACATGGCCTCCAAGGAGTAAG CCAGCAGTGAGGGTCTCTCT


(A) Classical monocytes
% of CD14++CD16- Monocytes
100
80
60
40
20
0
b
a
a
a,b
b
Untreated
LPS
GSOUF 10 μg/mL
GSOUF 50 μg/mL
GSOUF 100 μg/mL
(B) Intermediate monocytes
% of CD14++CD16+ Monocytes
20
15
10
5
0
b
a
b
b
b
(C) Non-Classical monocytes
% of CD14+CD16++ Monocytes
25
20
15
10
5
0
a
b
a
a
a
(D)
Untreated
LPS
GSOUF 10 μg/mL
GSOUF 100 μg/mL
PE-A
APC-Cy7-A
CD16
CD14


(A)
DCF fluorescence (% of control)
150
100
50
0
a
b
b
c
d
(B)
ROS level (% of control)
150
100
50
0
a
b
b
c
d
(C)
NO2- production (% of control)
150
100
50
0
a
b
b
c
d
Nos2 relative expression (AU)
1.5
1.0
0.5
0.0
a
b
c
d
e
Untreated
LPS
GSOUF 10 μg/mL
GSOUF 50 μg/mL
GSOUF 100 μg/mL


(A)
TLR4 relative expression (AU)
8.0
6.0
4.0
2.0
0.0
a
b
c
d
d
(B)
TNFalpha relative expression (AU)
1.5
1.0
0.5
0.0
a
b
c
c
d
(C)
Il1beta relative expression (AU)
1.5
1.0
0.5
0.0
a
b
b
b
c
(D)
Il6 relative expression (AU)
1.5
1.0
0.5
0.0
a
b
c
d
d
Untreated
LPS
GSOUF 10 μg/mL
GSOUF 50 μg/mL
GSOUF 100 μg/mL


(A)
TNF-α (pg/mL)
10000
8000
6000
4000
2000
0
a
a
b
b
c
(B)
IL-1β (pg/mL)
200
150
100
50
0
a
b
b
b
c
(C)
IL-6 (pg/mL)
8000
6000
4000
2000
0
a
b
c
c
d
Untreated
LPS
GSOUF 10 μg/mL
GSOUF 50 μg/mL
GSOUF 100 μg/mL