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Exploring the therapeutic potential of avocado oil: Insights into obesity metabolism and immune regulation through *in vitro* models

Manuela Machado *, Eduardo M. Costa, Sara Silva, Ana Maria Gomes, Manuela Pintado

Universidade Católica Portuguesa, CBQF Centro de Biotecnologia e Química Fina-Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal

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A B S T R A C T

Avocado oil boasts a compelling nutritional profile characterized by a richness of unsaturated fatty acids and key lipophilic compounds. This study aimed to explore the potential of avocado oil in obesity-related metabolic processes and immune responses modulation in *in vitro* cellular models. Results obtained unveiled avocado oil capacity to effectively regulate triglyceride accumulation in differentiated 3T3-L1 adipocytes, showcasing a remarkable five-fold increase in adipolysis rate at 15 mg/mL, and an increase in glucose uptake in insulin-stimulated cells. Furthermore, avocado oil exhibited significant immunomodulatory effects in IL-1β-stimulated Caco-2 cells, as it reduced IL-6 (by 11%) and IL-8 (by 12%) production at 10 mg/mL, while only a marginal uptick in TNF-α secretion was observed at the same concentration. These findings underscore the potential of avocado oil to be a beneficial component in functional foods and nutraceuticals targeted at obesity management.

1. Introduction

Obesity is a health condition linked with unhealthy lifestyles, marked by excessive caloric intake and insufficient physical activity (Liu et al., 2020), which leads to the accumulation of excess lipids in adipose tissues, particularly in visceral fat, and is directly associated with various diseases such as cardiovascular, hypercholesterolemia, and type II diabetes (Ravaut et al., 2021). One of the factors associated with obesity is a fatty acids' rich diet. This high intake has been shown to impair adipocytes' ability to oxidize in mitochondria, leading to elevated circulating free fatty acids. These free fatty acids will in turn accumulate in organs like the liver and muscles, causing abnormal fat deposits, a condition called steatosis (Ason et al., 2011; Bonen et al., 2000; Kojta et al., 2020; Liu et al., 2020; Ravaut et al., 2021). Additionally, the buildup of long-chain fatty acids in non-adipose cells can generate toxic lipids such as ceramides and cholesteryl esters, triggering lipotoxicity and adverse metabolic consequences like endoplasmic reticulum stress and inflammation (Kojta et al., 2020). So the question arises, how do we counter it?

The most obvious answer in the context of obesity prevention is dietary change and one way to achieve and which has gained significance in later years is the development of functional foods and nutraceuticals targeting obesity (Kumar et al., 2019; Vermaak

* Corresponding author. Escola Superior de Biotecnologia Universidade Católica Portuguesa | ; Rua de Diogo Botelho, 1327 4169-005, Porto, Portugal.

E-mail address: mmachado@ucp.pt (M. Machado).

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et al., 2011). Within this logic consumers are particularly attracted to natural anti-obesity compounds due to the perception that these are simultaneously effective and safer than conventional treatments (Vermaak et al., 2011). Among these natural compounds one family of particular interest are vegetable oils, as they are rich in essential compounds such as carotenoids, phytosterols, tocopherols, tocotrienols, bioactive fatty acids, all of which have been shown to offer promising health benefits (Vergallo, 2020). Within these oils, virgin avocado oils (AO) stand out due to their high levels of oleic acid and other bioactive compounds like alpha-tocopherol and beta-sitosterol. These components make AO a potential functional ingredient for managing health conditions such as hypercholesterolemia, diabetes, and fatty liver disease (Flores et al., 2019; Tan, 2019). However, and despite some evidence from *in vivo* studies, there is a need for a more in-depth mechanistic analyses to establish precise dose-effect relationships.

Thus, this research aimed to investigate, how avocado oil may influence obesity-related metabolism in an *in vitro* context. Our approach involved assessing its chemical composition, antioxidant capacity, cytotoxicity, and genotoxicity, followed by studying its effects on lipid accumulation, lipolysis, glucose uptake, and gut epithelial inflammation using *in vitro* cellular models.

2. Materials and methods

2.1. Chemicals and reagents

Commercial avocado oil was obtained from Sovena (Portugal). Phosphate buffered saline (PBS), Supelco standard 37, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), rosiglitazone and insulin were acquired from Sigma (USA). Tocopherols and phytosterol standards (α-tocopherol, Δ-tocopherol, γ-tocopherol, β-sitosterol, and cholesterol) were obtained from Extrasynthese (Lyon, France). Dulbecco's Modified Eagle's Medium (DMEM), and Non-essential amino acids were purchased from Gibco (ThermoScientific, USA), IL-1β and 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) from Invitrogen (USA), Fetal Bovine Serum (FBS) from Biowest (France), Penicillin-Streptomycin-Fungizone from Lonza, (Belgium) and Iron-fortified Calf Bovine Serum from ATCC (USA). Adipolysis kit, cellular antioxidant activity kit and Human IL-6 Kit High Sensitivity were obtained from Abcam (UK) while Legend Max Human Elisa Kit IL-8 and Legend Max Human Elisa Kit TNF-α were obtained from Biolegend (USA). Ames FT™ Mutagenicity Test Kit was obtained from Moltox (USA) and the BCA Pierce Assay Kit from ThermoScientific (USA).

2.2. Fatty acids profile

The avocado oil fatty acids profile was evaluated by gas chromatography after transesterification according to the method previously described (Machado et al., 2022a). In brief, 5 mg of oil were combined with 200 μL of tritridecanoin, followed by 2.26 mL of methanol, 800 μL of hexane, and 240 μL of sodium methoxide (5.4 M). The mixture was then vortexed and incubated at 80 °C for 10 min. After cooling on ice, 1.25 mL of DMF and 1.25 mL of sulfuric acid (3 M) in methanol were added. The samples were then vortexed and incubated at 60 °C for 30 min. Following cooling, the samples were vortexed and centrifuged (1250 g; 18 °C; 5 min). The upper layer containing the fatty acid methyl esters was collected and analyzed using an HP6890A gas chromatograph (Hewlett-Packard, Avondale, PA, USA), equipped with a flame ionization detector and a BPX70 capillary column (60 m × 0.25 mm x 0.25 μm; SGE Europe Ltd, Courtaboeuf, France). The analysis was conducted under the following conditions: the injector was set to a split ratio of 25:1 with an injection volume of 1 μL; injector and detector temperatures were 250 °C and 275 °C, respectively. Hydrogen served as carrier gas at a flow rate of 1 mL/min. The oven temperature was initially set at 60 °C and gradually increased to 225 °C. Fatty acids were identified using Supelco 37 reference material.

Based on the fatty acids profile the nutritional quality index (the atherogenic index (AI), the thrombogenic index (TI), and the hypo-/hypercholesterolemic ratio (HH) were calculated according to the following equations:

$$AI = \frac{[C12:0 + 4 \times (C14:0) + C16:0]}{(\sum MUFA + \sum PUFA\ n6 + \sum PUFA\ n3)} \quad \text{Equation 1}$$

$$TI = \frac{(C14:0 + C16:0 + C18:0)}{\left[0.5 \times \sum MUFA + 0.5 \times \sum PUFA\ n6 + 3 \times \sum PUFA\ n3 + \left(\frac{\sum PUFA\ n3}{\sum PUFA\ n6}\right)\right]} \quad \text{Equation 2}$$

$$HH = \frac{(C18:1n9 + C18:2n6 + C18:3n3 + C20:4n6 + C20:5n3)}{(C14:0 + C16:0)} \quad \text{Equation 3}$$

MUFA – Monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; n3 – omega 3; n6 omega 6.

2.3. Tocopherols and sterols quantification

High-performance liquid chromatography coupled with fluorescence and diode array detectors were used for tocopherols and sterols quantification as previously described (Machado et al., 2022a). Samples were diluted 1:1 (v/v) in dichloromethane and filtered using a 0.22 μL syringe filter. A 5.0 μL sample was used for chromatographic analysis. Phytosterols were quantified using an HPLC system (Agilent 1260) with a diode array detector (Model 1260 DAD WR, Agilent Technologies, Palo Alto). Tocopherols were analyzed

with a separate HPLC system (Beckham 126) paired with a fluorescence detector (Varian). Separation was achieved using an ACE equivalence 5, C18, 250 × 46 mm column (VWR, USA). The mobile phase was methanol in isocratic elution with a flow rate of 1.0 mL/min, and the column temperature was maintained at 30 °C. Tocopherols were detected by fluorescence (excitation at 294 nm, emission at 326 nm), while phytosterols were detected by absorbance at 210 nm. Calibration curves (10–0.625 mg/mL) of α-tocopherol, Δ-tocopherol, γ-tocopherol, β-sitosterol, and cholesterol were used for quantification.

2.4. Genotoxicity

The genotoxicity assessment was performed according to OECD guideline 471 (*Test No. 471: Bacterial Reverse Mutation Test, 2020*). The Ames fluctuation test was used to screen for any possible sample's genotoxic effect against two *Salmonella typhimurium* strains (TA100 and TA98) using Moltox's Ames FT™ Mutagenicity test kit, as per the manufacturer's instructions.

Briefly, the microorganisms were exposed to samples at concentrations of 20, 15, 10, and 5 mg/mL (prepared in exposure media provided with the kit), solvent controls (plain water), or genotoxicity controls (genotoxic agents supplied in the kit) for 90 min at 37 °C with constant stirring in 24-well microplates (Nunc Nunclon, ThermoScientific). Subsequently, reversion media was added to each well, and 48 aliquots (50 μL) were transferred to 384-well plates (Nunc Nunclon, ThermoScientific), followed by incubation at 37 °C in a zip-lock bag. After 48 h, the number of revertant wells (wells that changed color from purple to yellow) was counted. A sample is considered genotoxic if there is a ≥2-fold increase in the number of revertants compared to the solvent control, according to the manufacturer's instructions.

2.5. Cell lines

Human Caucasian colon carcinoma epithelial cells (Caco-2, ECACC 86010202 acquired from the European Collection of Authenticated Cell Cultures) and mouse pre-adipocytes 3T3-L1 (ATCC CL-173) acquired from the American Type Culture Collection were used in this work. Human cells were cultured using DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) of Penicillin-Streptomycin-Fungizone and 1% of non-essential amino acids. Pre-adipocytes were cultured in DMEM with 10% (v/v) of iron-fortified Calf bovine serum and 1% (v/v) of Penicillin-Streptomycin-Fungizone. All cell lines were incubated at 37 °C under a humidified atmosphere comprised of 5% CO_2 and 95% air.

2.6. Cytotoxicity

Avocado oil cytotoxicity was evaluated based on its impact on cellular metabolism using Thiazolyl Blue Tetrazolium Bromide (MTT) as a viability dye, following the protocol described by (Machado et al., 2022a). Briefly, cells were seeded 1.5×10^4 cells/well in 96 tissue cultures plates (Nunc Nunclon, ThermoScientific). After 24 h, cells were exposed to AO (20–10 mg/mL, prepared in culture media). Plain media was used as a growth control, and DMSO (40% v/v) served as a dead control. MTT (0.5 mg/mL) was added after 24 h, and the cells were incubated 2 h at 37 °C. Formazan crystals were dissolved in DMSO, and the absorbance was read at 570 nm using microplate reader (Synergy H1, Biotek Instruments, USA).

2.7. Cellular antioxidant assay

Cellular antioxidant activity was performed according to the kit manufacturer's instructions. Briefly Caco-2 was seeded (2.5×10^4 cells/well) in a black 96-well microplate (Nunc Nunclon, ThermoScientific) and grown until confluence. After 24 h cells were washed 3 times with PBS, and 50 μL of Dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe and 50 μL of each quercetin standard (0–2000 μM) or AO were added. The plate was incubated at 37 °C for 60 min, then cells were washed 3 times with PBS, and 100 μL of free radical initiator was added to all wells. Fluorescence (480 nm/530 nm) was immediately controlled with a microplate reader at 37 °C, with measurements being carried out in increments between 1 and 5 min for a total of 60 min.

2.8. Adipolysis

Differentiated adipocytes were used for adipolysis assay according to the method described by (Machado et al., 2022a). Briefly, differentiated adipocytes were exposed to AO (20–10 mg/mL) or 10 μM isoproterenol (used as an assay control). After 24 h of exposure, 25 μL of cell supernatant was collected. The glycerol concentration was evaluated by adding 100 μL of glycerol-free reagent to cell supernatant and glycerol standards (0–125 μg/mL). Samples were incubated for 15 min at room temperature and the absorbance at 520 nm was read using a microplate reader (Synergy H1, Biotek Instruments, USA).

2.9. Glucose uptake activity using 2-NBDG

3T3-L1 cells were seeded at 2×10^4 (cells/well) and differentiated as described above. Glucose uptake in differentiated 3T3-L1 adipocytes was measured using a fluorescent glucose analog and rosiglitazone (100 nM) was used as a positive control. Differentiated adipocytes were treated with AO (20–10 mg/mL) or rosiglitazone (RG 100 nM). After 24 h, the cells were incubated in low glucose media with AO and RG for 3 h. Subsequently, the cells were stimulated with insulin (10 μg/mL) for 10 min and then treated with 100 μM of 2-NBDG for 1 h. The fluorescence intensity of 2-NBDG in the cells was then recorded using a BD Accuri™ C6 flow cytometer (BD,

Franklin Lakes, NJ, USA).

2.10. Immunomodulation in Caco-2

The AO immunomodulation potential was assessed according to the method proposed by (Machado et al., 2022). In brief, Caco-2 cells were seeded into 24-well tissue culture plates (Nunc Nunclon, ThermoScientific) and after 24 h exposed to AO (15 and 10 mg/mL), plain culture media (basal expression control). For anti-inflammatory activity evaluation 10 ng/mL IL-1β was added to all conditions. After 24 h of exposure, the supernatants were collected, centrifuged to remove cellular debris, and stored at - 80 °C until analysis.

Table 1
Chemical composition of avocado oil and its nutritional indexes. All results are presented in mean ± standard deviation.

Fatty acids (mg/g)	
C14	0.62 ± 0.04
C16	169.92 ± 8.36
C16:1 *t*9	1.65 ± 0.15
C16:1 *c*7	1.09 ± 0.12
C16:1 *c*9	53.23 ± 2.27
C17:1 *c*10	0.91 ± 0.02
C18	33.33 ± 1.45
C18:1 *t*6 or *t*9	28.39 ± 0.17
C18:1 *c*9	590.92 ± 30.27
C18:1 *t*15	54.57 ± 2.17
C18:1 *c*11	1.61 ± 0.07
C18:1 *c*12	0.79 ± 0.07
C18:1 *c*13	1.24 ± 0.11
C18:2 *t*9*t*12	1.10 ± 0.17
C18:2 *c*9*t*12	3.13 ± 0.21
C18:2 *c*9*c*12	106.68 ± 4.63
C18:3 *c*9*c*12*c*15	5.19 ± 0.16
C20:1	2.88 ± 0.16
C20:3	2.47 ± 0.12
C24	1.74 ± 0.09
∑Fatty acids	1062.05 ± 50.68
∑SFA	205.61 ± 9.94
∑MUFAS	737.27 ± 35.44
∑PUFAS	119.17 ± 5.30
∑*n3*	5.19 ± 0.16
∑*n6*	111.51 ± 5.02
Nutritional Quality indexes	
AI	0.80 ± 0.001
TI	0.46 ± 0.001
HH	4.12 ± 0.002
Tocopherols (mg/mL)	
γ-tocopherol	0.68 ± 0.001
δ-tocopherol	0.014 ± 0.002
Sterols (mg/mL)	
β-Sitosterol	0.015 ± 0.001
Stigmasterol	0.62 ± 0.02

C14 myristic acid; C16 palmitic acid; C16:1 t9 trans palmitoleic acid; C16:1 c7 cis-7-hexanoic acid; C16:1 c9 palmitoleic acid; C17:1 c10 cis-10-heptadecanoic acid; C18 stearic acid; C18:1 t9 elaidic acid; C18:1 c9 oleic acid, C18:1 t15 trans-15-octadecenoic acid: C18:1 c11 cis-vaccenic acid; C18:1 c12 cis-12-oleic acid; C18:1 c13 cis-13-octadecenoic acid; C18:2 t9t12 trans-9-trans12-octadecadienoic acid; C18:2 c9t12 cis-9-trans-12-octadecadienoic acid; C18:2 c9c12 linoleic acid; C18:3 c9c12c15 α-linolenic acid; C20:1 cis-gondoic acid; C20:3 dihomo-γ-linolenic acid; C24 lignoceric acid. SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids, AI atherogenic index; TI thrombogenic index; HH hypocholesterolemic/hypercholesterolemic ratio.

Cytokine quantification was performed using commercially available kits according to the manufacturer's instructions. Interleukin (IL)-6 was measured with Abcam's Human IL-6 ELISA Kit (high sensitivity), while IL-8 and Tumor Necrosis Factor (TNF)-α were measured using BioLegend's Legend Max Human ELISA Kit for IL-8 and TNF-α, respectively. Total protein in the supernatant was determined using ThermoScientific's BCA Pierce Assay Kit, and cytokine values were expressed in pg cytokine/ng protein.

2.11. Statistical analysis

Statistical analysis was performed using Minitab 17 (Minitab, LLC, USA) software. The normality of data distribution was assessed with the Shapiro-Wilk test, and comparisons were made using one-way ANOVA followed by Tukey's multiple comparisons test to identify statistically significant differences ($p < 0.05$). All data are presented as means ± standard deviation.

3. Results and discussion

3.1. Chemical composition and antioxidant capacity

As can be seen from Table 1 AO was mainly composed by monounsaturated fatty acids, of which oleic acid represented 80%. In addition to this, AO also contains high percentages of palmitic (15%) and linoleic (10%) acids. These results are in agreement with those previously reported by other authors for this kind of oil (Tan et al., 2018a, 2018b; Wang et al., 2020). Also, in Table 1 one can see the results regarding AO's fatty acids quantitative profile and nutritional indexes. This profile is drawn through the determination of the AI (a ratio between specific saturated fatty acids (SFA), and specific unsaturated fatty acids) and of the TI, which assesses the tendency for the formation of blood clots in blood vessels. With these two indexes, it is possible to evaluate the potential capacity of fatty acids to increase the risk of cardiovascular disease. The results obtained showed that AO's fatty acid profile was characterized by low AI and TI values (0.80 ± 0.001 and 0.46 ± 0.001, respectively), thus showing that AO has a low-risk profile. On the other hand, when considering the HH ratio the results obtained showed that AO had high HH values (4.12 ± 0.002), a consequence of its high content in monounsaturated fatty acids. This is of particular importance as it is related to the impact of specific fatty acids on cholesterol metabolism and unsaturated fatty acids (MUFA and PUFA) are known to decrease serum cholesterol, while some SFA, namely, lauric (C12:0), myristic (C14:0) and palmitic acids have been reported to raise serum cholesterol (Chen and Liu, 2020; de Alba et al., 2019). Compared with the literature, the values obtained for AI are higher than the values reported for AO (0.337), on the other hand the TI values are lower than the those previously reported (0.647). Concerning the HH, the values reported are 2 times higher than the values reported in other study (Krumreich et al., 2024). These differences can be related to variety and the oil extraction process (Cervantes-Paz and Yahia, 2021; Tan, 2019). Another factor accessed was the tocopherol content of AO. The results obtained (Table 1) showed that AO was also a source of tocopherols, particularly γ-tocopherol and δ-tocopherol (0.068 ± 0.001 and 0.014 ± 0.002 mg/mL respectively). These results are similar to the ones previously obtained by Tan et al. (2018a), where the total amount of tocopherols ranged from 83 to 256 mg/kg. The data reported in this study showed a total amount of 82 mg/kg, with the differences being related to the presence of α-tocopherol in the Tan et al., 2018b study (Tan et al., 2018b). In addition to tocopherols, sterols were also quantified (Table 1) with β-sitosterol (0.015 ± 0.001 mg/mL) and stigmasterol (0.621 ± 0.02 mg/mL) being quantified. The presence of these bioactive compounds in AO is in line with what has been reported by other studies. However, no clear conclusions can be reached regarding the quantified amounts as they differ greatly between the studies, because of the different extraction methods and avocados' variety employed in them (Cervantes-Paz and Yahia, 2021; Tan, 2019).

3.2. Safety profile: genotoxicity and cytotoxicity

Evaluating the safety profile of AO is crucial for its potential use in functional foods or nutraceuticals. When regarding the genotoxic profile of the sample (Table 2) no deleterious effects were found for the range of concentration tested, as none of the tested concentrations of AO exerted any genotoxic effects, with and without metabolic activation, against the tested strains. No other studies were found using AMES test; however, other studies demonstrate the safety of AO using micronucleus assay (Nicolella et al., 2017; Padilla-Camberos et al., 2013), demonstrating that it was safe to use for concentrations up to 250 μg/mL, which is much lower than the

Table 2
Results obtained for avocado oil samples AMES genotoxicity assay against *S. typhymurium* TA98 and TA100. All values represent the average number of positive (revertant) wells ± standard deviation.

	TA98		TA100	
	Without S9	With S9	Without S9	With S9
Solvent control (Baseline)	1.33 ± 0.58	1.67 ± 0.58	8.33 ± 0.58	8.67 ± 2.08
Positive Control	46.67 ± 0.58	47.33 ± 0.58	19.67 ± 2.31	47.67 ± 0.58
20 mg/mL	2.23 ± 0.58	2.33 ± 0.58	6.00 ± 2.65	7.00 ± 4.58
15 mg/mL	0.67 ± 1.15	1.00 ± 1.00	7.67 ± 2.52	7.33 ± 2.31
10 mg/mL	1.00 ± 1.00	2.67 ± 1.15	6.33 ± 3.06	6.00 ± 1.73
5 mg/mL	1.00 ± 1.73	1.00 ± 0.00	8.00 ± 4.36	4.67 ± 0.58

S9 - Metabolic Activation (Phenobarbital/β-Naphthoflavone induced Sprague Dawley rat liver S9 and Cofactors).

concentrations validated in this study. Additionally, no metabolism inhibitory effects were observed in Caco-2 and 3T3-L1 cell's metabolic activity (Fig. 1). For Caco-2 cells no significant differences ($p > 0.05$) were verified between the tested concentrations. On the contrary, for 3T3-L1, significant differences ($p < 0.05$) were observed between the tested concentrations, with a slight metabolic promotion at 10 mg/mL being observed for 3T3-L1 cells.

3.3. Cellular antioxidant activity (CAA)

As verified in the chemical assays, AO showed high antioxidant capacity (Fig. 2) which was dependent on the concentration of the oil (higher AO concentration, higher CAA). These results follow those reported for Vero cells where there was a high reduction in ROS in the presence of AO at concentrations ranging from 1 to 1000 μg/mL (Queiroz Junior et al., 2021). Similar results were observed in animal studies, where AO was associated with an attenuation in ROS production in diabetic and obese mice treated with diets containing AO (de Oliveira Marques et al., 2022; Ortiz-Avila et al., 2015).

3.4. Effect on adipolysis

Adipolysis is a tightly regulated process that ensures the proper delivery of free fatty acids to the bloodstream to meet energy demands. However, an imbalance in free fatty acid levels is linked to the development of insulin resistance and elevated triglyceride levels. As can be seen from the results obtained (Fig. 3) significant ($p < 0.05$) reductions in triglycerides accumulation on 3T3-L1 treated cells were observed. The presence of AO (10 mg/mL) led to the release of 2 times more glycerol in comparison with the control (isoproterenol 10 μM). Moreover, the increase in AO concentration (10 mg/mL to 15 mg/mL) led to an increase (5 times relatively to the control) in glycerol release. Although no studies on this sample were found, it is known that oleic acid (the main constituent of AO) has a beneficial effect on the regulation of lipid metabolism in adipose tissue (Malodobra-Mazur et al., 2019). Animal diets rich in oleic acid (30–70%) positively impacted the lipolysis rate and improved the regulation of lipid metabolism in a dose-dependent manner (Malodobra-Mazur et al., 2019). Other *in vitro* studies showed that oleic acid in different concentrations 0.1–1 mM) can modulate the animals' lipidic metabolism either by stimulating lipolysis or through the increase of the oxidation of the fatty acid and lipogenesis inhibition (García-Escobar et al., 2008; Siriwardhana et al., 2013). In terms of concentration, the literature reports a wide range, but in most cases, it is lower than what was tested in this study. However, in animal studies, oleic-rich diets can reach concentrations of over 70%, as seen in diets based on olive oil. The possible mechanism of action of oleic acid in adipolysis is associated with the activation of PPAR-α, which promotes triglyceride degradation and the release of fatty acids from adipocytes for uptake in oxidative tissues.

In summary, dietary oleic acid is absorbed in the enterocyte through the FTA/CD36 transporter (Bowen et al., 2017). Triglycerides containing oleic acid are packaged into chylomicrons to enter circulation, while phospholipids containing oleic acid are metabolized to oleoyl ethanolamide (OEA). OEA can bind to nuclear PPAR-α within the enterocyte to modulate gene transcription (Bowen et al., 2017; Sihag and Jones, 2018). Additionally, it must be taken into consideration that consumption of foodstuffs with elevated oleic acid content (such as the AO used in this work) leads to a high dietary oleic acid. This in turn increases the amount of oleic acid transported for incorporation at the sn-1 position of phospholipids which, consequently, increases the synthesis of OEA. The result of this will be the activation of PPAR-α by OEA in adipocytes, which will promote triglycerides degradation, the release of free fatty acids, and increased adipolysis (Bowen et al., 2017; Sihag and Jones, 2018). The enhanced lipolysis rate facilitated by AO could be beneficial for obesity prevention, as it stimulates the breakdown of triglycerides stored in fat cells, leading to a reduction in adipose tissue fat content (Marcelin and Chua Jr, 2010; Torres-Villarreal et al., 2019).

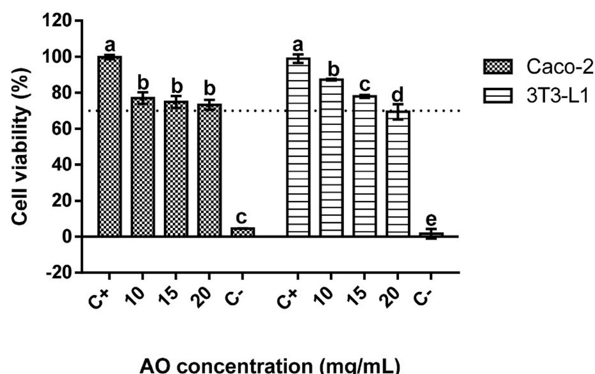

Fig. 1. Avocado oil cytotoxicity towards the target cells lines at 20, 15, and 10 mg/mL. The dotted line represents the 70 % cell viability minimal limit as defined by the ISO, 20093-5 (ISO, 20093-5 Biological evaluation of medical devices — Part 5: Tests for *in vitro* cytotoxicity, 2009). Different letters mean significant differences as determined by the one-way ANOVA test ($p < 0.05$). C+ is the growth control and C- is the dead control.

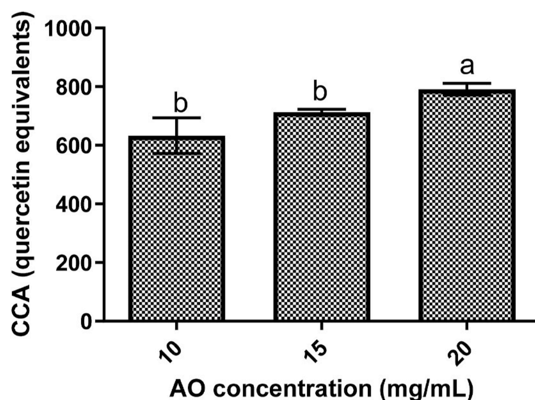

Fig. 2. – Avocado oil cellular antioxidant activity (CCA). Different letters mean significant differences as determined by the one-way ANOVA test ($p < 0.05$).

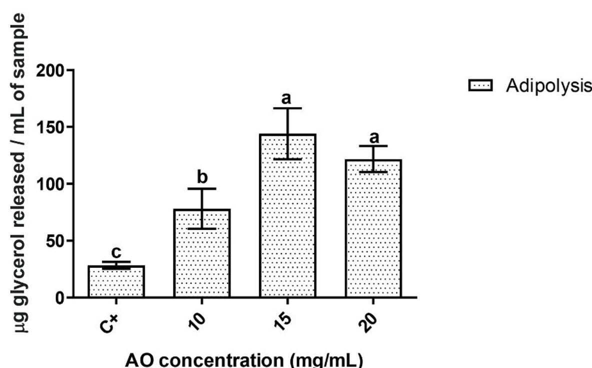

Fig. 3. Adipolysis results for the different avocado oil concentrations tested. C+ represents the positive control - Isoproterenol at 10 μM. Different letters represent significant differences as determined by the one-way ANOVA test ($p < 0.05$).

3.5. *Effect on glucose uptake*

The results indicate that AO significantly increased insulin-stimulated glucose uptake in adipocytes, however, a dose-dependent response was not verified (Fig. 4). The presence of RG increased the glucose uptake up to 190% and, in the presence of the AO this value triplicated. This data is in accordance with what has been already reported in the literature. A study published by Grigorova et al. (2022) showed that oleic acid (the main component of AO) duplicates the glucose uptake in 3T3-L1 cells (Grigorova et al., 2022). The same trend was verified in 3T3-L1 adipocytes treated with 1 μM of oleic acid (Tsuchiya et al., 2014). Moreover, animal studies corroborate this data, as for example obese mice diets enriched with AO has been shown to lead to increased glucose uptake (de Oliveira Marques et al., 2022; Del Toro-Equihua et al., 2016). The effect of AO on glucose uptake can be linked to the inhibition of protein tyrosine phosphatase 1B which results in enhanced insulin signaling along insulin receptors to facilitate the insulin-stimulated glucose uptake into adipocytes (Tsuchiya et al., 2014).

3.6. *Modulation of inflammatory response*

The results regarding the effect of AO on pro-inflammatory cytokines IL-6, IL-8, and TNF-α production in Caco-2 cells can be seen in Fig. 5. In the basal conditions (non-stimulated with IL1-β), no significant differences ($p > 0.05$) were observed in IL-6 and IL-8 concentrations in all tested conditions. On the contrary, for TNF-α a significant ($p < 0.05$) increase was observed, with levels being 24 times higher in cells treated with 10 mg/mL of AO and 30 times higher in cells treated with 15 mg/mL than the ones registered for the non-stimulated control.

Regarding the IL-1β stimulated Caco-2 cells, the presence of 10 mg/mL AO led to a significant ($p < 0.05$) decrease in IL-6 secretion (around 11%). A similar behavior was observed for IL-8 production with reductions of 12 % and 5 % for 10 mg/mL and 15 mg/mL of AO, respectively. When regarding TNF-α levels a slight increase (1.6 and 1.3 times more than the control for 10 mg/mL and 15 mg/mL, respectively) was observed in the presence of AO, although no significant differences ($p > 0.05$) were observed relatively to the stimulated control. According to these results, a dose of 10 mg/mL seems to be the most adequate to obtain a favorable modulation of the targeted cells inflammatory response. Despite a lack of studies about AO's *in vitro* immune response modulation, human and animal

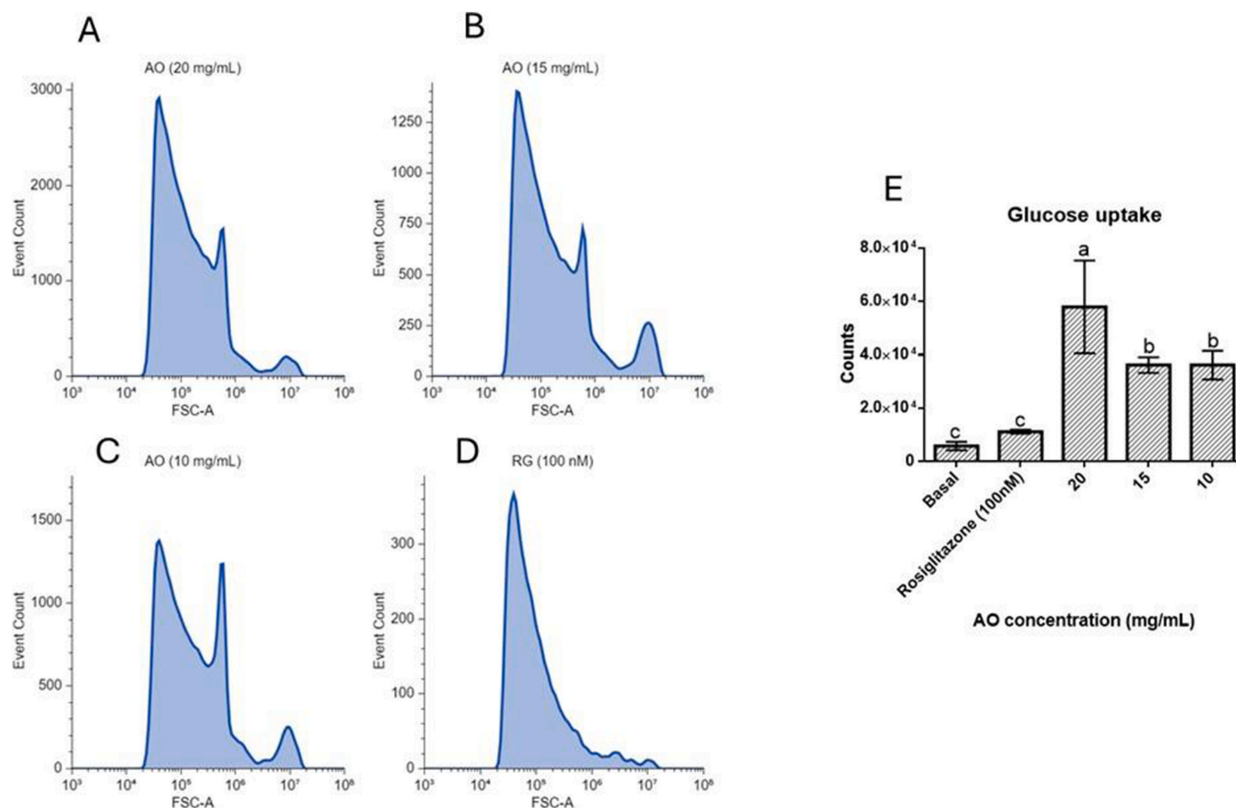

Fig. 4. – Glucose uptake in adipocytes results for the different avocado oil concentrations tested. A; B; C; D - 2-NBDG uptake activity in various groups (20 mg/mL; 15 mg/mL; 10 mg/mL and RG (rosiglitazone) 100 nM. E – glucose uptake based on fluorescence counts. The representative histogram shows cells with fluorescent intensity, which indicates the glucose uptake activity. Different letters represent significant differences as determined by the one-way ANOVA test ($p < 0.05$).

studies have previously shown that a diet rich in monounsaturated fatty acids (such as the ones found in the tested AO composition) generally contributes to the reduction of pro-inflammatory cytokine levels (such as IL-6 and TNF-α) (Konstantinidou et al., 2010; Schwingshackl et al., 2015). The potential mechanism of action related to these activity may be associated with the high content of this sample in monounsaturated fatty acids and their ability to inhibit various inflammatory responses pathwways, namely: the activation of nuclear factor-kappaB (NF-kB), the NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) through direct binding to G-protein coupled receptor 120 (GRP120), the peroxisome proliferator-activated receptors (PPARs), particularly PPARγ, as well as through AMP-activated protein kinase (AMPK) phosphorylation (Ravaut et al., 2021).

This capacity of AO to regulate the immune response is crucial for managing obesity, as IL-6 production is a critical step in obesity related inflammation. The production of this interleukin by various cell types, including immune cells and adipose tissue, and the presence of IL-6 receptors in several brain regions, such as the hypothalamus, will influence appetite and energy intake by suppressing lipoprotein lipase activity and thereby playing a role in energy homeostasis regulation (Ellulu et al., 2017). Furthermore, elevated TNF-α levels decrease adiponectin secretion and induce insulin resistance by inhibiting the insulin receptor substrate 1 signaling pathway (Rodríguez-Hernández et al., 2013).

4. Conclusion

In conclusion, the study provides a comprehensive assessment of the chemical composition, bioactivity, and safety profile of avocado oil (AO), highlighting its potential as a functional food ingredient. AO's high monounsaturated fatty acid content, particularly oleic acid, combined with low AI and TI values, indicated a low cardiovascular risk profile, making its potential application in functional foods nutritionally advantageous. Its significant tocopherol and sterol content further contributes to its antioxidant properties and aligns with previous research findings, suggesting that AO can help mitigate oxidative stress.

The safety evaluation, which included genotoxic and cytotoxic analyses, confirmed that AO does not exhibit genotoxicity and shows minimal cytotoxic effects, supporting its potential for safe consumption at the tested concentrations. Furthermore, AO demonstrated concentration-dependent antioxidant capacity, improved glucose uptake in adipocytes, and promoted triglyceride degradation, which are beneficial for metabolic health, particularly in contexts of obesity and insulin resistance. Its capacity to modulate inflammatory responses, especially the reduction of IL-6 and IL-8 secretion in Caco-2 cells, indicates potential anti-

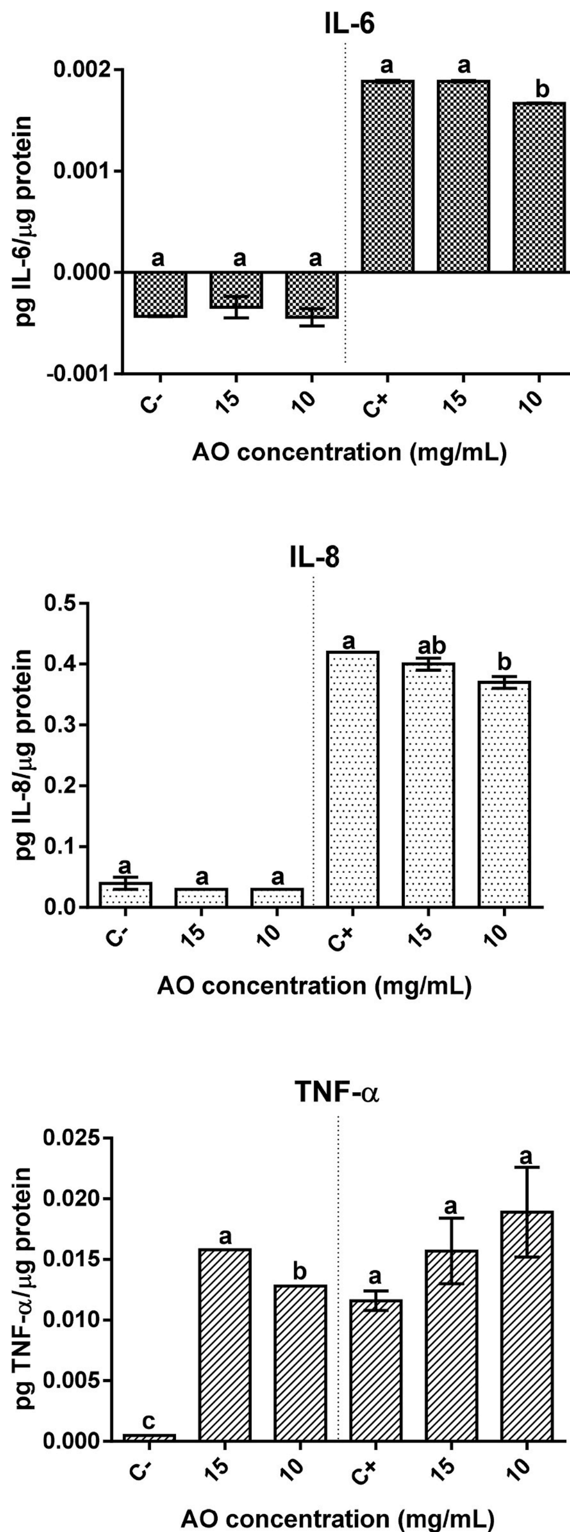

Fig. 5. Modulation of inflammatory response in Caco-2 cells by avocado oil. The left part of all graphs corresponds to the non-stimulated cell's response, and the right is related to the anti-inflammatory effect (IL1-β stimulated cells). C- is the basal activity of non-stimulated cells, C+ is the basal activity of stimulated cells. Different letters represent significant differences as determined by the one-way ANOVA test ($p < 0.05$).

inflammatory effects.

The findings suggest that AO has potential uses in nutraceuticals and functional foods, especially for managing obesity and improving heart health. Further research should explore how AO's metabolic and anti-inflammatory effects work.

CRediT authorship contribution statement

Manuela Machado: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Eduardo M. Costa:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Sara Silva:** Writing – review & editing, Conceptualization. **Ana Maria Gomes:** Writing – review & editing, Supervision, Conceptualization. **Manuela Pintado:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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