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Rosmarinus Officinalis L, Eriobotrya Japonica and *Olea Europaea L* Attenuate Adipogenesis in 3T3-L1-Derived Adipocytes and Inflammatory Response in LPS-Induced THP-1-Derived Macrophages

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Abstract: This study aims to *in vitro* evaluate the impact of three traditionally used anti-obesity medicinal plant extracts on inflammatory mediators in the THP-1 cell line and adipocyte differentiation and fat accumulation in the 3T3-L1 cell line. After extract preparation, an *in vitro* evaluation of cell viability using MTT on THP-1 and 3T3-L1, NO production levels using Griess reagent and cytokines production in THP-1 cells, and adipogenicity evaluation using Oil Red O on 3T3-L1 cells were performed. *O. europaea* showed the highest cytostatic on THP-1 cells with IC_{50} of 290.6 µg/mL, while *R. officinalis* was the highest on 3T3-L1 cells with IC_{50} of 486.6 µg/mL. All extracts significantly reduced dose-dependently nitric oxide (NO) production by THP-1-derived macrophages from 80 µM to control levels after treatment with 125 µg/mL. Similarly, all extracts decreased the levels of TNF-α and IL-6 production dose-dependently, with the highest effects reached at 250 µg/mL. all extracts increased the production levels of IL-10 from 32 pg/mL to 106.8 pg/mL, 83.5 pg/mL and 87 pg/mL at 125µg/mL, respectively. After treatment with plant extracts, the adipocyte differentiation and fat accumulation in 3T3-L1 were reduced to 20% of the control values. All three medicinal plants exhibit their traditionally known anti-obesity effects through cytostatic effects, modulation of proinflammatory and anti-inflammatory cytokine production levels, and reduction of adipocyte differentiation and fat accumulation.

Keywords: *Rosmarinus officinalis L*; *Eriobotrya japonica; Olea europaea L;* anti-inflammatory; anti-obesity; THP-1; 3T3-L1.

1. Introduction

Plants have been historically known for their medicinal benefits. Ancient societies worldwide have embraced traditional herbal medicine to treat a wide range of diseases for centuries. Nowadays, herbal medicine has been seen as a potential future medicine for improving healthcare care, owing to the enhanced efficacy in pharmacological effects of medicinal plants [1]. Recently, there has been a shift in the global trend from synthetic to herbal treatment, termed the "Return to Nature". Medicinal plants have been used since ancient times and are highly valued worldwide as a rich source of medicinal compounds for disease prevention and treatment. As per the World Health Organization (WHO), herbal medicine is

used by 60% of the world's population, and it is used by over 80% of the population in developing nations for primary health care [1]. Phytocompounds and their chemical analogs have produced a variety of clinically relevant medications for treating chronic and acute disorders. And research into additional therapeutic compounds derived from medicinal plants is also an ongoing process [1].

Obesity develops as a result of adipose tissue growth caused by adipocyte hypertrophy (cell size enlargement) or adipocyte hyperplasia (cell number increase), or a combination of both of two resulting in excessive lipid accumulation [2]. Adipogenesis is the mechanism by which undifferentiated preadipocytes develop into mature adipocytes, lipid-storing adipocytes. It consists of two major steps: Terminal differentiation and preadipocyte replication [3,4]. To reach terminal differentiation, preadipocytes will go through a complex process that involves the use of adipogenic transcription factors such as sterol regulatory element-binding protein-1c (SREBP-1C), peroxisome proliferator-activated receptor-γ (PPAR-γ), and CCAAT/enhancer binding protein-α (C/EBP-α) [5-7].

Inflammation is now recognized as a critical component of obesity, type II diabetes, and insulin resistance, which is characterized by irregular cytokine production, high acute-phase reactants (APR), and other mediators, as well as activation of inflammatory signaling pathways [8]. Furthermore, increased weight gain leads to abnormalities in the normal cell composition of adipose tissue as a result of macrophage infiltration, which is induced by tissue damage caused by anoxia, apoptosis, or necrosis inside the rapidly expanding adipose tissue [9], which increases the production of several inflammatory cytokines, for example, interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis alpha (TNF-α), [10,11]. This elevation in cytokine production leads to low-grade chronic inflammation inside adipose tissue, which promotes the development of metabolic syndrome and obesity, generating a vicious endless loop [12].

Recent medication studies have concentrated on herbal remedies due to the negative side effects of many anti-obesity pharmaceuticals [13]**.** Plants have been utilized as traditional natural remedies to treat various disorders due to their potential for enhancing and sustaining human health, as well as their low negative effects and inexpensive cost. Traditional medicinal plants, particularly, are said to have biological effects [14].

R. officinalis L., *Eriobotrya japonica*, and *O. europaea L.* are well-known traditional and contemporary medicinal herbs utilized worldwide in undeveloped, developing, and even developed regions [15-17].

R. officinalis L. (Rosemary) is a perennial shrubby plant belonging to the Lamiaceae family. Its aerial parts have a long history in traditional medicine and cookery. Traditional medicine uses decoctions and infusions to treat various illnesses, including cold cure and prevention, rheumatism, and muscle and joint pain [18]. Nowadays, *R. officinalis* is considered one of the major sources of natural active compounds which utilized in many pharmacological activities such as antidiabetic, anti-obesity, anti-bacterial, anti-tumor, anti-inflammatory, and antioxidant, among others [19-28]

Eriobotrya japonica (Loquat), a subtropical evergreen fruit tree belonging to the Rosaceae family, originates in southern China. It has been cultivated in China for over 2000 years. It is commercially grown in over 30 countries worldwide, including Japan, Brazil, Pakistan, Spain, Italy, Turkey, Palestine, and others. *Eriobotrya japonica* is a plant with high therapeutic potential and several components that have been utilized in traditional medicine for thousands of years. In Chinese traditional medicine, loquat extract has been used to treat

inflammation, diabetes, cough, chronic bronchitis, and cancer [16]. The medical effectiveness of *Eriobotrya japonica* is supported by modern scientific analysis regarding active pharmacological compounds in plant extract as it proved the anti-inflammatory, antidiabetic, anticancer, antioxidant activities along with other bioactivities such as liver, lung, renal and neuronal cells function improvement, anti-obesity and hypolipidemic activity, antithrombotic, anti-nociceptive, antithrombotic, anti-allergic and anti-aging activity [16].

Olea europaea L (Olive) is a small tree belonging to the family Oleaceae. It grows mainly in the eastern Mediterranean basin's coastal regions, as well as the nearby coastal areas of southeastern Europe, western Asia, the Arabian Peninsula, India and Asia, and northern Africa, as well as northern Iran at the Caspian Sea's southern edge. The olive tree is economically significant in the Mediterranean since it is a major source of olive oil. Also, in Palestine olive tree is considered a national symbol, and many parts of it are widely used in traditional medicine; for instance, fruits and leaves were used to treat liver disease, urinary and gall bladder stones, skin diseases, burns, high cholesterol level, eczema, foot pain, emollient, damaged hair, arteriosclerosis, skin rash, and anti-psoriasis.

This study assessed the effects of three commonly used anti-obesity medicinal plant extracts on the levels of proinflammatory and anti-inflammatory cytokines in human monocytic cell line THP-1, along with adipocyte differentiation and fat accumulation in 3T3-L1 mouse embryo fibroblasts cell line.

2. Materials and Methods

2.1. Plant extract.

2.1.1. *R. officinalis, Eriobotrya japonica,* and *O. europaea*.

The aerial parts of *R. officinalis* and the leaves of *Eriobotrya japonica and O. europaea* Plants were collected in the Jenin district of Palestine early in July 2019. Plant components were gathered, cleaned, and dried in the shade Plant voucher number are Pharm-PCT-2732, Pharm-PCT-2785, and Pharm-PCT-1664, respectively. Forty-five grams of air-dried powdered plants were put in a 500 mL Erlenmeyer flask with 300 mL of 1:1 ethanol/water and let to boil for 15-20 minutes in a 70°C water bath to produce a dark green extract. In an aseptic environment, the extract was filtered using medical gauze and squeezed manually by hand before being centrifuged twice at 3000 rpm for 15 minutes. The stock extracts were stored at -20°C in a falcon tube until use.

2.2. Cell culture.

2.2.1. 3T3-L1.

Cells from the 3T3-L1 Mouse embryo fibroblasts cell line (Zenbio, USA SP-L1-F) were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10%(v/v) FBS, 1%(v/v) penicillin-streptomycin, 1% (v/v) amphotericin B and 1%(v/v) non-essential amino acids and L-glutamine then sustained in the incubator in a humidified atmosphere at 37°C with 5% CO_2.

2.2.2. THP-1.

The human monocytic cell line THP-1was obtained from ATCC (ATCC 202-TIB, American Type Culture Collection, Manassas, VA, USA). For decades, these cells were employed as a model for inflammatory disorders as they express several receptors in normal monocytes. THP-1 cells were cultivated in Roswell Park Memorial Institute (RPMI 1640) medium with a high-glucose content (4.5 g/L) and 10% v/v fetal bovine serum, 1% non-essential amino acids, 1% glutamine, 100 U/mL penicillin, and 10µg/mL streptomycin. THP-1 cells were transferred into 24-well plates at a cell density of 2 X 10^5 cell/mL and stimulated with Phorbol-12-Myristate-13- Acetate (PMA) (100 ng/mL) (Sigma-Aldrich, USA) and Vitamin D3) (0.1 µM) (Sigma-Aldrich, USA) for 24 h. Then they were treated with the plant extracts (0 – 1000 µg/mL) in a new medium without serum.

2.3. Cell viability tests.

A functioning mitochondrion may convert the water-soluble yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a purple water-insoluble formazan crystal by succinate dehydrogenase enzyme that gives a quantitative determination of viable cells [29].

Cells were seeded in a 96-well plate and incubated for 24 h; cells were then treated with different concentrations of the prepared plant extracts and incubated for another 24 h at 37°C. After removing the treatment solution, 100 µl of (0.5 mg/mL) MTT was done according to Sieuwerts, A.M. *et al.* method [29].

2.3.1. Cytotoxic effects.

Cells at 75-85% confluence were trypsinized and separated from the culture flask by removing the culture medium and washing the cells with PBS or fresh serum-free culture medium, then adding 0.05% trypsin-EDTA (this step was done only with 3T3-L1 attached cells as THP-1 cells are suspended cells and don't need trypsinization). 100 µL of (2.0×10^4 cells/well) suspension were seeded in a 96-well plate and incubated for 24 h at 37°C. After media removal, cells were treated with plant extracts serially diluted to several concentrations, then incubated for 24 h at 37°C to perform an MTT assay.

2.3.2. Cytostatic effects.

The cytostatic effect was determined by using a lower density of cells seeded in each well (1.0×10^4 cells/well) and treated with the same concentrations of the plant extract that used in the cytotoxicity assessment, then incubated for 72 h at 37°C to do the MTT assay and evaluate the antiproliferative activity.

2.4. NO production.

Griess reagent is used to evaluate nitric oxide levels *in vitro* by converting nitrite into a purple-colored azo compound and can be quantified by a spectrophotometer at 550 nm [30]. This reagent is composed of two solutions, A (0.1% W/V Naphthylethylenediamine dihydrochloride in distilled water) and B (1% W/V Sulphanilamide in 3% V/V Phosphoric acid and distilled water), the two solutions are much stable separately and have a larger shelf life, mixing them induce auto-oxidation and cannot be stored for a long more than 30 min.

The THP-1 cells were differentiated by using 100 nM/mL Phorbol-12-myristate-13-acetate (PMA) and 4 µ/mL 1,25-dihydroxy vitamin D3 (VD3) overnight, then activated using 5 µg/mL of bacterial lipopolysaccharides (LPS) along with different concentrations of the test plants then seeded in a 96-well plate (1.0×10^4 cells/well) for 72 h. In an empty 96-well plate, 50 µL of the supernatant were mixed with 100 µL of solution A and 100 µL of solution B (Griess reagent) and incubated at room temperature for 20 min in the dark. Absorbance at 550 nm was measured by an ELISA plate reader.

2.5. Adipogenicity assessment.

2.5.1. 3T3-L1 differentiation.

3T3-L1 preadipocytes were cultured in the same conditions as previously mentioned. In order to differentiate preadipocyte to adipocyte cells, they were cultured before reaching full confluence (day 0); the medium was replaced by an induction medium that contained 1 µg/mL Insulin, 500 µM 3-Isobutyl-1-methylxanthan (IBMX) and 1µM dexamethasone in DMEM medium supplemented with 10% FBS for 2 days. The induction medium was replaced with a maintenance medium which contained 1.5 µg/mL insulin only, and DMEM medium with 10% FBS every 2-3 days for 10 days [31].

2.5.2. Effect of plant extracts on lipid accumulation and adipogenesis on 3T3-L1 preadipocyte.

To examine the effect of both adipogenic potential and fat accumulation by using Oil Red O staining (ORO), cultured cells were treated by using different concentrations of plants extracts along with a differentiation induction medium and maintenance medium from day 0 to day 10 for adipogenesis assessment, while for lipid accumulation extracts were added to fully differentiated adipocytes from day 10 to day 15. Cells then were stained with Oil Red O and photographed using an inverted microscope (Olympus, Inverted microscope L0390117 supported with Optika, Microscope camera F0480255) at 200x and 400x magnification.

2.5.3. Oil Red O staining.

The stock solution was prepared by dissolving 0.5 g of Oil Red O powder (O0625, Sigma-Aldrich Company, USA) in 100 mL isopropanol, then filtered using a 0.22 µm filter. To prepare 6 mL of working solution, the stock solution was diluted 2:3 with distilled water, left for 30 min at room temperature, and then filtered using a 0.22 µm filter before use.

Cells were cultured in 96-well plates, washed 3 times with phosphate-buffered saline (PBS), then fixed with 4% formaldehyde in PBS for 15 min, and washed two times with distilled water, the cells were then stained with a freshly prepared working solution, and the stain was filled to cover the bottom of the plate (0.132 mL/cm2) for 30 min and washed 3 times with distilled water, the dye maintained in cells was dissolved by adding 100% isopropanol (0.263 mL/ cm2), and optical density was determined at 510 nm [32]. The relative lipid content percentage was calculated using the following equation:

Relative lipid content (%) = (Sample OD/ Control OD) × 100%

2.6. Determination of TNF-α, IL-6, and IL-10 production.

The anti-inflammatory properties of the medicinal plant extracts were analyzed using THP-1 cells. As previously indicated, cells were initially differentiated into macrophages in a 24-well plate (105 cells/well). THP-1 cells were treated with a non-toxic concentration of plant extracts (0, 125 and 250 µg/mL). After that, the cells were treated with 5 µg/mL LPS. LPS-stimulated cells with no plant extract were used as a positive control. TNF-α, IL-6, and IL-10 releases were evaluated using an ELISA commercial kit (available from Sigma-Aldrich, USA) in the supernatants of stimulated cells after 4 h, 6 h, and 24 h of treatment, according to the manufacturer's procedure. Cytokine levels were calculated based on the standard curve and expressed in pg/mL.

2.7. Statistical analysis.

Each experiment was conducted in three replicates or duplicates, as mentioned in the results, and the values reported are expressed as mean ± SD. Data for cell viability, level of adipogenesis, and lipid accumulation were expressed as the percentage of optical density of treated cells relative to control. The IC_{50} value (concentration of extract at which the reduction of the cell viability at the half-maximal levels) was calculated using a non-linear regression curve fit of normalized optical density values (absorbance) versus log (concentration of extract) using GraphPad PRISM 8. Nitric oxide production levels and cytokine production levels were calculated by plotting the mean of optical density values (absorbance) to a standard curve generated by interpolating a standard curve using GraphPad PRISM 8. Multiple comparisons were performed by one-way ANOVA followed by Dunnett's test using GraphPad PRISM 8. The significance has been demonstrated at $P < 0.05$.

3. Results and Discussion

Obesity is an epidemic and costly disease affecting 13% of the world's adult population, related to adipose tissue growth and expansion and pathologic endocrine changes in adipose tissue, including local and chronic systemic low-grade inflammation. Furthermore, chronic inflammation is a contributing factor to both insulin resistance and obesity-related diseases. The conventional approaches to preventing or treating this syndrome and its associated diseases include a balanced diet, increased body activity, and healthy lifestyle modification. Several pharmacological and non-pharmacological therapies have been developed to reduce the difficulties associated with obesity. The utilization of functional foods and their bioactive components has been considered a new approach to preventing and managing this disease. *In vitro* and clinical studies show that medicinal plants, diet, and bioactive compounds can prevent obesity and adipose tissue inflammation [4,6].

Adipose tissue is a type of active endocrine tissue that contains macrophages, preadipocytes, mature adipocytes, and endothelial cells. Under normal physiological and pathological conditions, adipokines such as adipose hormones and cytokines are generated and controlled by cells in the adipose tissue. The chronic inflammatory response in obesity increases macrophage infiltration, decreases adiponectin release, and elevates levels of proinflammatory cytokines such as TNF-α and IL-6 [4,6]. Investigating paracrine linkages between adipocytes and macrophages is crucial for improving chronic inflammation in obesity. As a result, we chose THP-1-derived macrophages and 3T3-L1-derived adipocytes to evaluate

the potential multi-target effects of *R. officinalis*, *Eriobotrya japonica*, and *O. europaea* extracts.

3.1. Cytotoxic and cytostatic effects.

The core mechanisms of obesity include excessive development, differentiation, and hypertrophy of adipocytes, the primary cellular component in fat tissue. Adipogenesis, or preadipocytes' maturation (proliferation and differentiation) into adipocytes, is a fundamental component in developing obesity and overweight [34]. Hence, inhibiting this pathway represents a possible target in treating obesity. Several cell lines have recently been found to undergo *in vitro* lipogenic differentiation into adipocytes. Among them, the 3T3-L1 preadipocyte is a well-studied cellular model for adipogenesis. Typically, a culture media supplemented with 1 g/mL insulin, 500 M 3-Isobutyl-1-methylxanthan (IBMX), and 1M dexamethasone is used to stimulate the differentiation of these cells to mature adipocytes. The mature 3T3-L1-derived adipocytes exhibit an adipocyte phenotype with triglyceride buildup, one of adipogenesis characteristics [5].

In this study, we used the MTT test to analyze *R. officinalis*, *Eriobotrya japonica*, and *O. europaea* extracts for inhibitory effects on cells from the THP-1-derived macrophages, 3T3-L1 cell line after 24h (cytotoxic effects) and 72h (cytostatic effects) treatment with increased concentrations of leaves and fruit extracts of the three extracts. Figure 1 and Figure 2 show that *R. officinalis*, *Eriobotrya japonica*, and *O. europaea* exhibited cytotoxic effects in a dose-dependent manner in both cell lines.

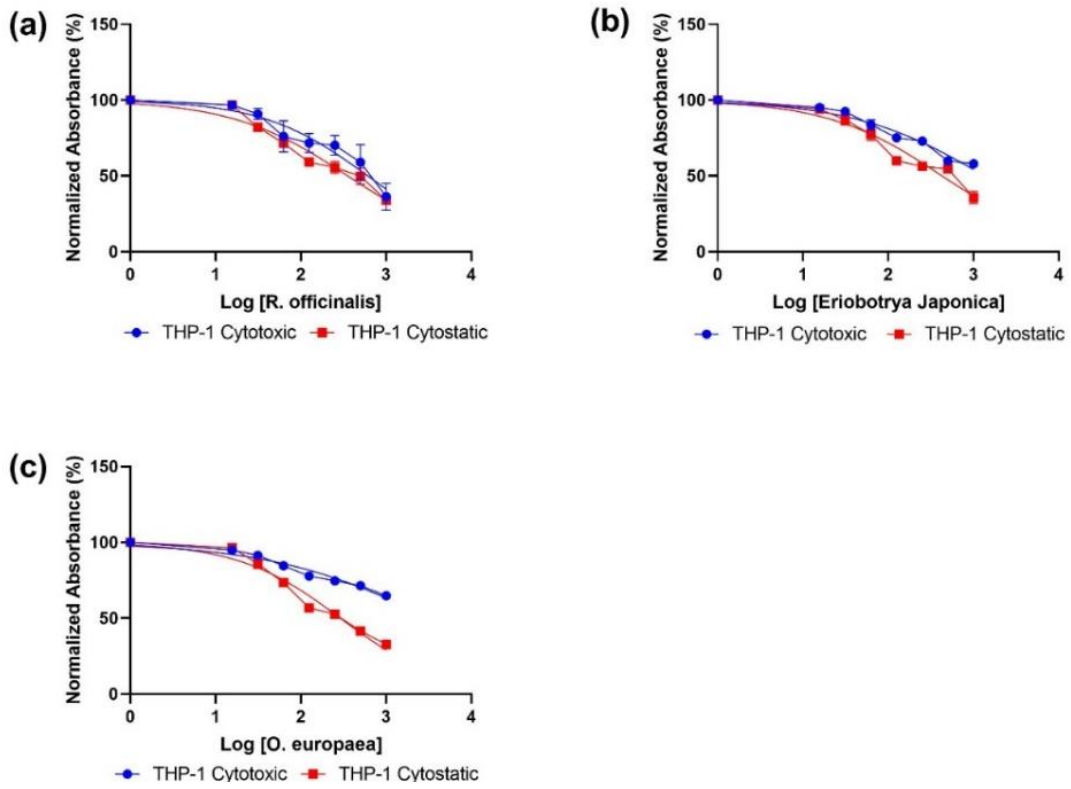

Figure 1. Cytotoxic and cytostatic effects assessed by MTT assay on THP-1 cells using different concentrations of plants extracts (1000 µg/mL – 0 µg/mL (control)): **(a)** *R. officinalis*, **(b)** *Eriobotrya japonica* and **(c)** *O. europaea*; extract concentration expressed as Log [concentration] against normalized optical density, where control value set to be 100%, IC_{50} values (µg/mL), were calculated using non-linear regression curve fit using GraphPad PRISM 8. The data shown represent the mean of three independent experiments carried out in triplicates.

Cytotoxicity was not seen in THP-1 cells at any concentration below 608.7 µg/mL, 1356 µg/mL, and 3296 µg/mL for *R. officinalis*, Eriobotrya *japonica*, and *O. europaea*, respectively. Similarly, no significant reduction in cell viability was seen when 3T3-L1 cells were exposed to concentrations below 6216 µg/mL, 2086 µg/mL, and 1979 µg/mL of *R. officinalis*, *Eriobotrya japonica*, and *O. europaea*, respectively. Table 1 summarizes the IC_{50} values measured in both cell lines. The hydroethanolic extract of *R. officinalis* exhibited the highest cytotoxic effects in THP-1 cell lines with an IC_{50} value of 608.7 µg/mL, while *O. europaea* exhibited the highest cytotoxic effects in 3T3-L1 cell line with 1979 µg/mL (Table 1).

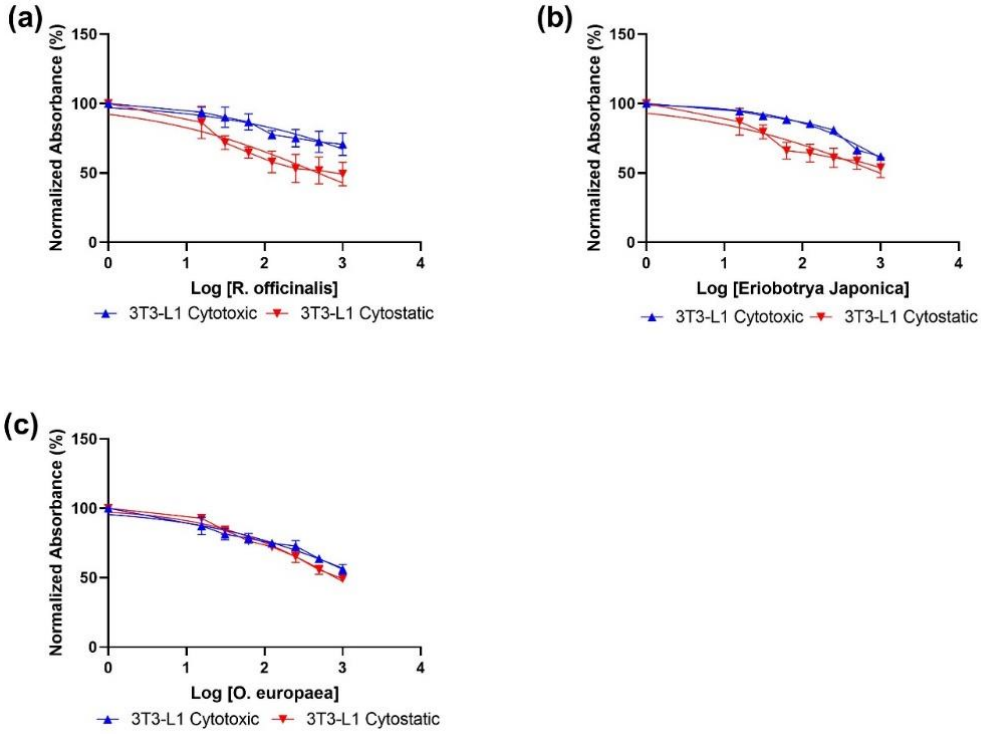

Figure 2. Cytotoxic and cytostatic effects assessed by MTT assay on 3T3L-1 cells using different concentrations of plants extracts (1000 µg/mL – 0 µg/mL (control)): **(a)** *R. officinalis*, **(b)** *Eriobotrya japonica* and **(c)** *O. europaea*; extract concentration expressed as Log [concentration] against normalized optical density, where control value set to be 100%, IC_{50} values (µg/mL), was calculated using non-linear regression curve fit using GraphPad PRISM 8. Data shown represent the mean of three independent experiments carried out in triplicates.

Table 1. IC_{50} values of test plant extract in THP-1 and 3T3-L1 cell lines. The IC_{50} values determined by fitting the dose-response curves to a non-linear regression curve fit using GraphPad PRISM 8.

Extract	Cytotoxic µg/mL		Cytostatic µg/mL	
	THP-1	3T3-L1	THP-1	3T3-L1
R. officinalis	608.7	6216	354.6	486.6
Eriobotrya japonica	1356	2086	427.5	978.7
O. europaea	3296	1979	290.6	819.2

In terms of cytostatic, the antiproliferative properties of the three extracts were assessed using the MTT test. Figures 1 and 2 reveal that all three extracts have dosage-dependent cytostatic effects. The impacts on THP-1 cell lines were much greater than the effects on 3T3-L1 cell lines. The IC_{50} values for THP-1 were 354.6 µg/mL, 427.5 µg/mL, and 290.6 µg/mL for *R. officinalis*, *Eriobotrya japonica*, and *O. europaea*, respectively (Table 1). Among the

three extracts tested, *O. europaea* was found to induce significant antiproliferative activity at the nontoxic concentration on THP-1 cells, while *R. officinalis* induced significant antiproliferative activity on 3T3-L1 cells. More research and investigation are required to comprehend the underlying processes that create this effect. Plant extracts such as *Alchemilla vulgaris*, *Olea europea*, Mentha longifolia L, and Cuminum cyminum seeds have previously been shown to decrease adipogenesis in adipocytes [33-36]. Moreover, a number of phytochemicals that increase cytostatic activity and apoptosis have been shown to inhibit preadipocyte and adipocyte proliferation. Flavonoids with cytostatic and antioxidant activities, such as resveratrol, naringin, genistein, naringenin, rutin, hesperidin, capsaicin, green tea polyphenol EGCG, and quercetin, have been demonstrated to induce apoptosis in preadipocytes. Quercetin is one of the most often detected flavonoids in fruits and vegetables. Caspase 3, Bax, Bak activation, Bcl-2 downregulation, and PARP cleavage are thought to promote apoptosis in preadipocytes. Similarly, phenolic acids such as o-coumaric acid, m-coumaric acid, and chlorogenic acid were utilized to stop the preadipocyte cell cycle during the G1 phase, demonstrating that the effect is time and dosage-dependent. More research is needed to understand the biochemical and molecular underlying mechanisms fully, and the increase in apoptosis in post-confluent differentiating cells is projected to reduce the amount of adipocytes in the long run. [36-38].

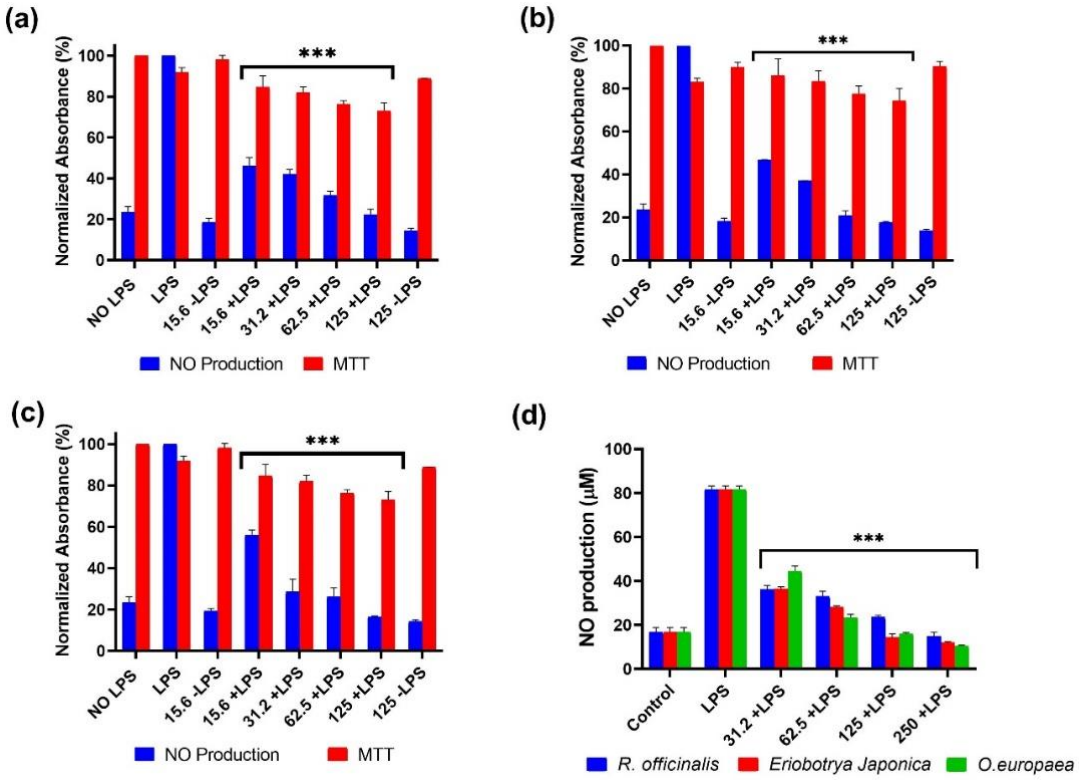

Figure 3. LPS-induced THP-1 macrophages nitric oxide (NO)-production vs. cell viability (%) after 72-h incubation with different plant extracts concentrations (125 µg/mL – 15.6 µg/mL): **(a)** *R. officinalis*, **(b)** *Eriobotrya japonica* and **(c)** *O. europaea*, the normalized optical density of (LPS treated cells) along with (No LPS MTT) values were set to be 100%. **(d)** represents LPS-induced THP-1 macrophages (NO)- production (µM) after 72-h incubation with different plant extracts concentrations (250 µg/mL – 31.2 µg/mL). Values were calculated by plotting the mean of optical density values (absorbance) to a standard curve generated by interpolating the standard curve using GraphPad PRISM 8. Each bar represents the mean ± SD of three different experiments carried out in triplicates. Asterisk indicates a statistical difference from LPS treated cells, calculated using one-way ANOVA followed by Dunnett's multiple comparisons test. (* $p<0.05$ vs LPS, ** $p<0.01$ vs LPS, ***$p<0.001$ vs LPS).

3.2. Effects of the plant extracts on the production levels of IL-6, TNF-α, IL-10, and NO production.

According to recent scientific findings, increasing levels of adipocyte-derived adipokines in obese individuals drive macrophage infiltration into adipose tissue, inducing inflammation and increased insulin resistance. TNF-α and IL-6 are proinflammatory cytokines produced in white adipose cells, and their concentrations rise as lipid accumulation increases [39,40]. Infiltration of macrophages into adipose tissue is linked to obesity-induced inflammation. Immune cell penetration and macrophage infiltration into adipose tissues are increased by inflammatory intermediates produced by adipocytes. Hence, natural products attenuating obesity-related inflammation are promising candidates for obesity management.

The anti-inflammatory effects of the three extracts were analyzed in macrophages derived by LPS-activation. TNF-α, IL-6, IL-10, and NO levels were evaluated using an enzyme-linked immunosorbent assay and Griess assay. We found increasing levels of TNF-α, IL-6, IL-10, and NO post LPS treatment (Figures 3-5).

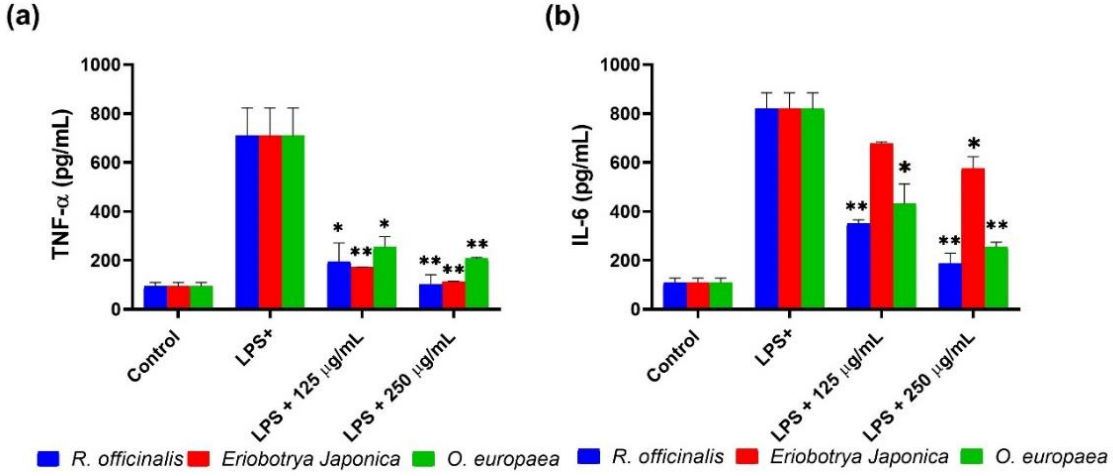

Figure 4. Effect R. *officinalis*, *Eriobotrya japonica*, and *O. europaea* extracts on LPS-induced THP-1 macrophages on proinflammatory cytokines **(a)** TNF-α and **(b)** IL-6 production (pg/mL) after a 4 h and 6 h incubation, respectively with two particular doses (250 µg/mL and 125 µg/mL). Each bar represents mean ± SD of three different experiments carried out in duplicates. Asterisk indicates a statistical difference from LPS treated cells which were calculated using one-way ANOVA followed by Dunnett's multiple comparisons test. (* $p<0.05$ vs LPS, ** $p<0.01$ vs LPS, ***$p<0.001$ vs LPS).

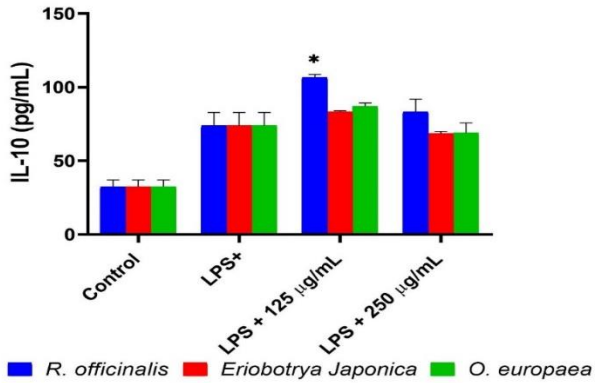

Figure 5. Effect R. *officinalis*, *Eriobotrya japonica* and *O. europaea* extracts on LPS-induced THP-1 macrophages on anti-inflammatory cytokines IL-10 production (pg/mL) after a 24h incubation with two particular doses (250 µg/mL and 125 µg/mL). Each bar represents mean ± SD of three different experiments carried out in duplicates. Asterisk indicates a statistical difference from LPS treated cells which were calculated using one-way ANOVA followed by Dunnett's multiple comparisons test. (* $p<0.05$ vs LPS, ** $p<0.01$ vs LPS, ***$p<0.001$ vs LPS).

Figure 3 represents the dose-dependent inhibition of the LPS-activated production of NO by *R. officinalis*, *Eriobotrya japonica*, and *O. europaea* extracts in cultured THP-1 at nontoxic concentrations. The NO reached control levels of untreated cells at a concentration of 16.60±2 µM. Similar results were recorded with many medicinal plants, crude extracts, and phytochemicals [41-45]. Using LPS/cytokine-treated macrophages or macrophage-like cell lines, varieties of flavonoids, including apigenin, luteolin, and quercetin, were found to inhibit NO production. However, the mechanisms studied have shown that flavonoids had not significantly inhibited iNOS. They were revealed to down-regulate iNOS induction and reduce NO production [41]. The action mechanism by which *R. officinalis*, *Eriobotrya japonica*, and *O. europaea* extracts inhibit the NO secretion needs to be evaluated in future experiments. These plants were reported to contain various potential antioxidant and anti-inflammatory compounds. Extract from *R. officinalis* leaves contain diterpenes (carnosic acid, carnosol, royleanonic acid, 7-methoxyrosmanol) in addition to triterpenes (oleanolic acid), rutin, quercetin and rosmarinic acid which make *R. officinalis* exhibit anti-hyperglycemic, anti-hyperlipidemic, anti-inflammatory, antioxidant, pancreatic lipase and lipid absorption inhibition, lipid peroxidation inhibition, PPARγ agonistic activity and metformin-like effects [46-52]. *Eriobotrya japonica* leaf and flower are rich in phenolics and triterpenes; fruit is rich in sugars, organic acids, carotenoids, flavonoids, phenolic acids (ursolic acids, corosolic acid, oleanolic acid, and maslinic acid), and vitamins that give *Eriobotrya japonica* anti-hyperglycemic, anti-hyperlipidemic, anti-inflammatory and antioxidant activity [16,53-59]. Oleuropein, a natural product of the secoiridoid group, is the major active component in *O. europaea* leaf and its extract. Oleuropein has a wide range of pharmacologic and health-promoting effects, including antiarrhythmic, spasmolytic, immune-stimulant, cardioprotective, hypotensive, anti-inflammatory, antioxidant, and antithrombotic activities [17,60-65]

Production levels of proinflammatory (IL-6 and TNF- α) and anti-inflammatory (IL-10) cytokines by LPS-activated THP-1-derived macrophages were measured in the culture media via commercial enzyme-linked immunosorbent assay. TNF-α and IL-6 production reached maximal levels after 4h and 6h post LPS stimulation. As shown in Figures 4 a & b, TNF-α, and IL-6 levels were significantly inhibited by test plant extracts in a dose-dependent manner. Among the three extracts tested, *R. officinalis* inhibited the production of TNF- α and IL-6 at a concentration of 250 µg/mL by 86% and 77% compared to control LPS treated cells.

In contrast to the inhibitory effects on the TNF- α, and IL-6, the anti-inflammatory cytokine IL-10 was increased after treatment of the LPS-activated THP-1-derived macrophage. Although untreated cells produced low levels of IL-10, TNF- α, and IL-6, *R. officinalis* extract performed significant inhibition. However, at a lower concentration of 125 µg/mL. *R. officinalis* extract showed a significant elevation in IL-10 production level (106.82±1.3 pg/mL) compared with untreated cells produced by LPS-treated cells (73.90±8.9 pg/mL) Figure 5. Similar results were published for several medicinal plant crude extracts [41-45]; in another study, we found that *Peganum harmala* increased levels of IL-10 protein by stimulation of mRNA expression. As for *R. officinalis*, the effects of *Peganum harmala* were evident at significantly lower crude extract concentrations (125µg/mL) compared to inhibitory effects on IL-6 and TNF- α (250 µg/mL). IL-10 is the potent immunosuppressor cytokine that inhibits the production of proinflammatory cytokines. [44,45,66]. The inhibitory effects of *R. officinalis* on the production of LPS-induced secretion of IL-6 and TNF- α may pass through the induction of IL-10 secretion. Obesity, like other inflammatory illnesses, is characterized by a very low blood level of IL-10 and a high blood level of TNF- α. Also, injections of the exogenous

recombinant version of IL-10 resulted in a decline in TNF-α levels in the blood, which has been shown to be advantageous in such disorders [67-69].

3.3. Effects of the plant extracts on adipogenicity.

To evaluate the impact of test plant extracts on 3T3-L1 cell adipogenesis, differentiation of 3T3-L1 preadipocytes to mature adipocytes was generated in the presence of different concentrations of the test plants (0-2 days), and the treatment was continued for a total of 10 days. Fat accumulation was measured in completely developed adipocytes that were treated with varying doses of the test plants for 5 days from day 10 to day 15 of development. Oil Red O staining was used to assess adipogenesis and lipid buildup using an optimized procedure [70]. At first, cells were microscopically observed and photographed, then the stained cells were eluted in 2-propanol, and the optical density values were measured using an ELISA reader. Data obtained were normalized and calculated as relative values to control (%) to quantify intracellular triglyceride accumulation. Also, ID_{50} values (Table 2) measurement for the test plants extracts were calculated at which the reduction of the cell adipogenesis and lipid accumulation at the half-maximal levels (50%) which was calculated by graphic interpolation of the dose-response curves to non-linear regression curve fit using GraphPad PRISM 8 as shown in Figure 6.

Table 2. ID_{50} values of test plant extract concentration at which the reduction of the cell adipogenesis and lipid accumulation at the half-maximal levels of 50% was determined by graphic interpolation of the dose-response curves to non-linear regression curve fit using GraphPad PRISM 8.

Extracts	ID_{50} Adipogenesis µg/mL	ID_{50} Lipid Accumulation µg/mL
R. officinalis	32.12	35.87
Eriobotrya japonica	183.7	406.9
O. europaea	488.2	164.9

Microscopic observation of 3T3-L1 cells was exposed to different concentrations of tested plant extract (31, 125, 500 µg/mL) and stained with Oil Red O displayed the differentiation process along with intracellular triglyceride accumulation produced a dose-dependent effect in the number of fully differentiated cells which had accumulated lipid droplets Figure 6 a-c, no cytotoxicity was indicated by comparing the morphology of control and treated 3T3-L1. A significant decrease in cytoplasmic lipid droplet accumulation was observed as the doses increased with different intracellular morphology with smaller lipid droplets or even lacking it. These observations were confirmed by eluting the stain and reading the optical density after microscopic observation Figure 6.

The average absorbance of the undifferentiated preadipocyte medium was 0.202 ± 0.09 when compared with the control group of differentiated cells medium was 0.523 ± 0.02, indicating that lipid accumulation increased by 258.91% and that differentiation developed efficiently. In the test plants group, lipid accumulation was significantly decreased in all samples in a dose-dependent manner, as shown in Figure 6. Table 2 showed ID50 for test plants, indicating that *R. officinalis* has the most effect on differentiation inhibition and lipid accumulation suppression with nontoxic concentrations of 32.12 µg/mL and 35.87 µg/mL, respectively.

Additional research should be conducted to see whether these extracts may be combined to form a synergistic composition that inhibits adipogenesis more effectively than the separate extracts. Furthermore, adipogenic signals regulate adipocyte differentiation and

intracellular fat deposition during adipogenesis by activating transcriptional activators, mostly from the PPAR and C/EBP families. These two nuclear factors sync up the complex operation of adipogenic gene expression throughout terminal preadipocyte differentiation by activating the expression of multiple adipogenic gene products such as ADRP, aP2, CD36, and perilipin, which all work together to achieve the adipocyte phenotype [2-4]. Future research will look into the role of these transcription factors in our findings.

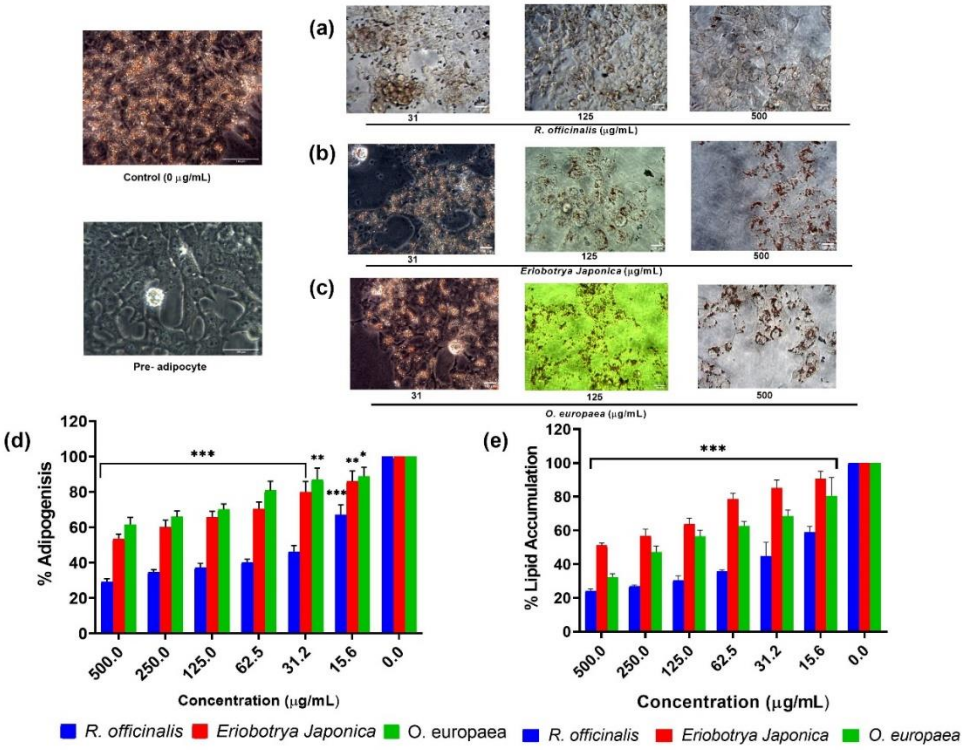

Figure 6. Effect of **(a)** *R. officinalis*, **(b)** *Eriobotrya japonica*, and **(c)** *O. europaea* extracts on fat accumulation in 3T3-L1 cells using Oil red O staining of the intracellular triglycerides. 3T3-L1 cells were treated with (31, 125, 500 µg/mL) during differentiation, and the assays were performed on fully differentiated adipocytes (day 12). **(d)** The relative density of the lipid contents in 3T3-L1 cells were treated with different extracts concentrations (500 µg/mL - 0 µg/mL) during differentiation. (e) Relative density of lipid contents in fully differentiated adipocytes treated for 5 days (day 10 to day 15) with different extracts concentrations (500 µg/mL - 0 µg/mL). Each bar represents the mean ± SD of three experiments carried out in triplicates. Asterisk indicates a statistical difference from control (untreated cells), calculated using one-way ANOVA followed by Dunnett's multiple comparisons test. (* $p<0.05$ vs ctrl, ** $p<0.01$ vs ctrl, *** $p<0.001$ vs ctrl).

4. Conclusions

This *in vitro* study aimed to evaluate the impact of *Rosmarinus officinalis L*, *Eriobotrya japonica*, and *Olea europaea L* extracts' inflammatory mediators of human monocytic cell line THP-1 and adipocytes differentiation and fat accumulation in 3T3-L1 mouse embryo fibroblasts cell line. All three extracts presented a significantly elevated cytostatic effect on both cell lines compared to cytotoxic effects. All three extracts significantly reduced dose-dependently the production levels of proinflammatory mediators (TNF-α, IL-6, and NO) and reduced the adipocytes' differentiation and fat accumulation in 3T3-L1. Plant extracts increased the production levels of IL-10. These results suggest that *Rosmarinus officinalis L*, *Eriobotrya japonica*, and *Olea europaea L* exhibit their traditionally known anti-obesity effects through cytostatic modulation, modulation of proinflammatory and anti-inflammatory cytokine production levels, and reduction of adipocyte differentiation and fat accumulation.

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Conflicts of Interest

The authors declare that they have no competing interests.

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