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"Enhancing Skincare Efficacy: A Synergistic Formulation of N-Acetylneuraminic acid and Ozonated Oil" (Topic: Formulation)"

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1. Introduction

Ozone (O3) is an inorganic and highly oxidative gas composed of three oxygen atom[1]. It was first applied to O3 as a gaseous form in World War I for treating German soldiers affected by gaseous gangrene[2]. Ozonated oil is one form of O3 application, which is produced using an ozone generator and bubbling ozone gas into a natural oil for a specified duration in a reaction chamber followed by a controlled cooling process to stabilize the O3 within the product[3]. Ozonated oils, though still lack large randomized controlled trials, showed promising positive effects as an antimicrobial and anti-inflammatory system on skin problems such as ulcers, burns, atopic dermatitis, tinea pedis, hand sanitation, diabetic foot ulcers, and other dermatologic conditions [4-6]. The mechanism of these applications can be due to the greater oxidizing properties of the zone, which destructs bacterial cell walls and the cytoplasmic membrane[7]. In addition, ozone has shown promise in helping the innate immune system against microorganisms and improving bacteriostatic and bactericidal activity [8, 9].

In general, the ozone reacts with the double bonds of fatty acids in vegetable oils and forms ozonides and peroxides such as polymer peroxides and organic peroxides[3, 8]. The ozonated oil also improves the stability of handling with rapid degradation compared to the gaseous form. The first vegetable ozonated oil, OLEOZON®, was invented in Cuba and categorized as a medicinal product for oral and topical therapeutic purpose[9]. This product is not classified as an acute toxicity substance and has a high safety tolerance until 2000 mg/kg body weight. Unfortunately, while most research on ozonated oils has focused on their medical use for skin diseases, ozonated oils show a great possibility in the cosmetics

industry due to their skin repair application since many people now suffer from sensitive and fragile skin problems[10]. More importantly, despite the rapidly increasing ozone oil sales, many claimed efficacy and benefits are controversial without thorough investigation.

Therefore, in this study, we investigated a new form of ozonated olive oil (INCI: OLEA EUROPAEA (OLIVE) FRUIT OIL), and thoroughly investigated the composition of ozonated olive oi by UPLC-MS analysis and RNA-Seq analysis. We found that there is a special ingredient have been produced by ozonating the olive oil, which showed a strong effect in promoting cell growth and recovery.We also found that there are a lot of combination of ingredients that target the RNA of fibroblast growth factors. In addition, we found a formulation combinding ozonated olive oil and N-Acetylneuraminic Acid showed a combination effect of skin repair and antiaging.

2. Materials and Methods

Prepartion of Ozonated Olive oil.

Ozonated olive oil was prepared by bubbling ozone gas through high-quality olive oil using an ozone generator. The process was conducted in a reaction chamber at controlled temperatures to ensure the stability of the ozonides formed. The ozonation parameters were optimized based on previous studie**s**[12] to maximize the therapeutic potential while maintaining the integrity of the oil.

Deodorization process

Oxygen-enriched oil was slowly heated to 110°C under a vacuum of no more than 300 Pa. Pure steam (generated by heating pure water in a water bath at above 55°C) was introduced. The temperature was then slowly increased to 130°C and maintained for 1 hour. Subsequently, the temperature was further slowly raised to 140°C and held for 30 mins. The pure steam was turned off, and the vacuum (maintained at no more than 300 Pa) was continued for dehydration for 10 minutes, after which the temperature was reduced to 35°C to break the vacuum. The deodorized oil and its distillate were collected separately.

UPLC-MS analyses and data preprocessing.

Chromatography was performed using an ACQUITY UPLC I-Class HF system (Waters,Milford, MA, USA). ACQUITY UPLC HSS T3 (100 mm × 2.1 mm, 1.8 um; Waters, Milford, MA, USA) was used as a separation column. The elution solution consisted of deionized water contains 0.1% formic acid (A) and acetonitrile (B) and separation was achieved using the following gradient: 0.01 min, 5% B; 2 min, 5% B; 4 min, 30% B; 8 min,50% B; 10 min, 80% B; 14 min, 100% B; 15 min, 100% B; 15.1 min, 5% and 16 min, 5% B. The rate was set at 0.35 mL/min, and the injection volume was 5 mL. Mass spectrometric experiments were performed using Obritrap-QE (Thermo Fisher Scientific, Watham, MA, USA) in negative mode.

The original LC-MS data were processed using Progenesis QI V2.3 software (Nonlinear Dynamics, Newcastle, UK) to perform baseline filtering, peak identification, integral, retention time correction, peak alignment, and normalization. Using the LuMet-TCM, Animal_DB, and Herb databases, compounds were identified using exact mass-to-charge ratios (M/z), secondary fragments, and isotopic distributions. Qualitative compounds found in the QI search database are preserved as the original ingredient if their overall score exceeds 40 points and their secondary matching score exceeds 50 points. To produce a qualitative and quantitative result data matrix, set the entire content of the relative peak area of the metabolites to 100%.

Cell culture conditions

Human fibroblast cells HFF-1[American Type Culture Collection (ATCC), CRL-3216] cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; catalog no. S711-001, LONSERA). All cells were cultured at 37°C with 5% CO2.

When not specified, human fibroblasts (HFF-1) were treated with 0.1% oxygen enriched oil (oxygen enriched oil group), 0.2% Sialic acid (SA group), and a combination of 0.05% oxygen enriched oil plus 0.1% Sialic acid (oxygen enriched oil and SA group).
Human malignant melanoma cells A-375 [American Type Culture Collection (ATCC), CL-0014] cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; catalog no. S711-001, LONSERA). All cells were cultured at 37°C with 5% CO2. When not specified, human malignant melanoma cells were treated with 0.1% oxygen enriched oil (oxygen enriched oil group), 0.2% Sialic acid (SA group), and a combination of 0.05% oxygen enriched oil plus 0.1% Sialic acid (oxygen enriched oil and SA group).

RNA-Seq and data analysis

Total RNA was isolated using a RNeasy Kit (Qiagen), and RNA quality was checked using an Agilent Bioanalyzer 2100. The mRNA was purified using KAPA mRNA Capture Kits (Roche), and cDNA libraries were prepared using KAPA RNA Hyper Prep Kits (Roche) at Metware (China). Equal amounts of cDNA library from each sample were pooled for sequencing on an Illumina HiSeq X platform (150-bp paired-end sequencing). Samples were sequenced with a median read of 112.213107 M (range: 82.544278–123.336120 M), and the raw reads were deposited to the Gene Expression Omnibus with the accession numbers GSE179134. Reads were mapped to the GRCh38 genome assemblies using Hisat2 v2.1.0 with the default settings. The aligned reads were converted to bigwig coverage files using reads per million. Genome annotations were extracted from ensemble GRCh38 Ens_96 and used to count reads with htseq-count v0.13.5. R version 3.6.0 and Deseq2 were used for differentially expressed gene analysis. Differentially spliced events were analyzed using rMATS, and significant differentially spliced events were screened using the following conditions: $|\Delta PSI| > 0.05$ and FDR < 0.05. Materials and Methods section should provide enough detail to enable others to replicate and build upon the published results. Interventionary studies involving animals are not permitted, and any work involving animal testing will be rejected from presentation at the congress (fish embryo, pig skin, Het-cam, C.elegans, immortalized cell lines are not considered as animal testing and allowed in the congress).

Cell viability assay

Cell viability was measured with CCK-8 kit（Biosharp, China）, followed the manufacturer. Briefly, Human fibroblast cells or Human malignant melanoma cells seeded in 96-well plates were either treated with oxygen enriched oil or Sialic acid at serial concentrations for 24 h. After treatment for indicated time, CCK- 8 solution was added and incubated with cancer cells for 1 h. The percentages of cell survival was measured by SpectraMax190 microplate reader (Molecular Devices) based on the absorbance.

Reactive oxygen species (ROS) detection

The production of ROS was detected by DCFH-DA diacetate. After treatment, the cells were cultured with complete medium containing 0.1% DCFH-DA and protected from light at 37 °C for 30 min. After incubation, cells were washed twice with PBS and re-suspended in PBS. The cells were then analysed by FACSCalibur flow cytometer (Becton Dickinson, USA). The percentage of ROS positive cells was analysed by using FlowJo software 10.4 (TreeStar, USA).

3. Results and discussion

Identification of Unique Ingredients in Ozonated Olive Oil Using HPLC-MS

To investigate the compositional changes induced by ozonation, we employed high-performance liquid chromatography-mass spectrometry (HPLC-MS) equipped with a

comprehensive fatty acid and ingredient library comprising over 10,000 reference compounds. This robust analytical platform enabled precise identification and characterization of components within the ozonated olive oil samples.

Interestingly, our analysis (Figure 1) revealed that ozonation led to the formation of a unique bioactive molecule: convallaria saponin. Convallaria saponin, a triterpenoid saponin primarily known from the Convallaria majalis (Lily of the Valley) plant, is rare across common botanical raw materials. Structurally, saponins are glycosylated compounds characterized by their amphiphilic nature, enabling interactions with both lipid membranes and aqueous environments. The presence of this saponin in ozonated olive oil suggests that the ozonation process not only modifies existing fatty acid profiles but also promotes the generation of novel bioactive molecules. Given the scarcity of convallaria saponin in most conventional botanical sources, its emergence here highlights the distinct chemical transformations that ozonation induces and may provide novel functional properties to ozonated olive oil products. Ozonation produces a unqiue ingredient in olive oil.

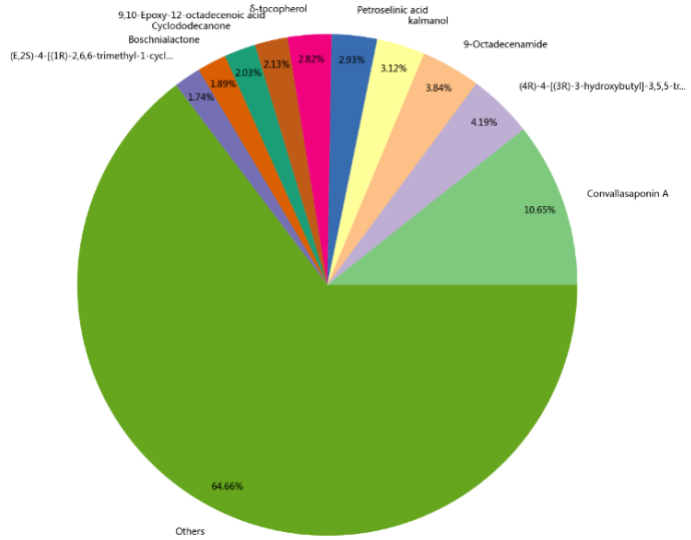

Figure 1. UPLC-MS analysis of ozonated olive oil

Molecular Docking Reveals Convallaria Saponin's Potential Interaction with Fibroblast Growth Factors

To elucidate the potential skin-related bioactivities of convallaria saponin identified in ozonated olive oil, we employed high-throughput molecular docking analyses targeting key proteins involved in skin regeneration. The docking results indicated a strong binding affinity of convallaria saponin to both acidic fibroblast growth factor (FGF1) and basic fibroblast growth factor (FGF2), suggesting a modulatory role in fibroblast-mediated skin repair processes.

FGF1 and FGF2 are critical regulators of skin homeostasis and wound healing. FGF1 has been shown to accelerate dermal wound healing by stimulating angiogenesis, granulation tissue formation, and epithelial growth . Similarly, FGF2 promotes keratinocyte proliferation, enhances skin barrier recovery, and facilitates tissue remodeling . The interaction of convallaria saponin with these growth factors may potentiate their biological effects, thereby promoting deeper skin recovery and regeneration.

These findings suggest that convallaria saponin contributes to the skin-healing properties of ozonated olive oil by targeting fibroblast growth factors, offering potential applications in dermatological therapies aimed at enhancing skin repair and resilience. Identification of

Unique Ingredients in Ozonated Olive Oil。

Target	Common name	Uniprot ID	ChEMBL ID	Target Class	Probability*	Known actives (3D/2D)
Gamma-secretase	PSEN2 PSENEN NCSTN APH1A PSEN1 APH1B	P49810 Q9NZ42 Q92542 Q96BI3 P49768 Q8WW43	CHEMBL2094135	Protease		9 / 12
Heat shock protein HSP 90-alpha	HSP90AA1	P07900	CHEMBL3880	Other cytosolic protein		13 / 3
Vascular endothelial growth factor A	VEGFA	P15692	CHEMBL1783	Secreted protein		0 / 2
Acidic fibroblast growth factor	FGF1	P05230	CHEMBL2120	Secreted protein		0 / 7
Basic fibroblast growth factor	FGF2	P09038	CHEMBL3107	Secreted protein		0 / 4
Heparanase	HPSE	Q9Y251	CHEMBL3921	Enzyme		0 / 5
Cyclin-dependent kinase 1	CDK1	P06493	CHEMBL308	Kinase		7 / 1

Figure 2.High-throughput molecular docking of Convallaria Saponin

Functional Analysis of Petroselinic Acid in Ozonated Olive Oil via High-Throughput Molecular Docking

In this study, high-throughput molecular docking was employed to investigate the potential skin-related functions of Petroselinic Acid derived from ozonated olive oil. Our analysis revealed a significant binding affinity of Petroselinic Acid with several members of the fatty acid-binding protein (FABP) family, particularly FABP3 (muscle and epidermal fatty acid-binding protein), FABP4 (adipocyte fatty acid-binding protein), and FABP5 (epidermal fatty acid-binding protein).

Among these interactions, the high-affinity binding of Petroselinic Acid to FABP3 was especially notable. FABP3 plays a crucial role in intracellular lipid transport and homeostasis, particularly within epidermal cells. The molecular docking results indicated that Petroselinic Acid possesses a structure characterized by hydrophilic terminals oriented outward, which is highly compatible with the lipid-organization domains of FABP3. This structural complementarity suggests that Petroselinic Acid can act as an effective moisturizing agent by reinforcing lipid layer organization, enhancing skin hydration, and restoring the integrity of the skin barrier.

Furthermore, by interacting with FABP3, FABP4, and FABP5, Petroselinic Acid may contribute to the regulation of epidermal lipid metabolism and the maintenance of barrier function, which are essential for preventing transepidermal water loss and alleviating skin dryness. These findings underscore the therapeutic potential of Petroselinic Acid as a bioactive lipid for deep moisturization and barrier repair in skincare formulations

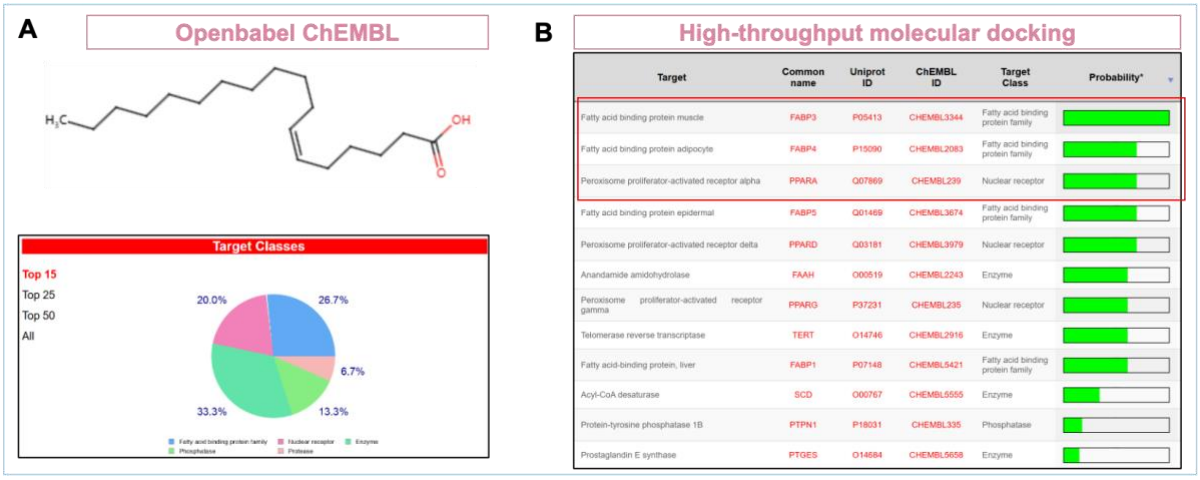

***Figure 3.(A)chemical structure of* Petroselinic Acid and (B) *High-throughput molecular docking of* Petroselinic Acid.**

Transcriptomic and Gene Enrichment Analysis of Ozonated Olive Oil Effects on Fibroblasts

To further elucidate the biological effects of ozonated olive oil on skin health, we conducted a comprehensive gene enrichment analysis and RNA-sequencing (RNA-seq) study using human fibroblast models. Comparative transcriptomic profiling between the ozonated oil-treated group and the untreated control group revealed significant modulation of pathways associated with cell proliferation and extracellular matrix organization.

Specifically, gene enrichment analysis highlighted a strong upregulation of signaling pathways related to fibroblast proliferation and skin tissue regeneration. Notably, the treatment led to enhanced expression of collagen-related genes, suggesting a promotive effect on the biosynthesis and deposition of extracellular matrix components essential for skin structure and resilience.

Mechanistic analysis further indicated that the biological activity of ozonated olive oil was mediated through the targeted regulation of SMAD proteins, critical downstream effectors of the transforming growth factor-beta (TGF-β) signaling pathway. Activation of SMAD-dependent transcription is known to be pivotal for collagen production and fibroblast differentiation, thereby implicating SMAD modulation as a key mechanism underlying the regenerative benefits observed.

Collectively, these transcriptomic findings provide strong evidence that ozonated olive oil promotes skin repair and rejuvenation by stimulating fibroblast activity, enhancing collagen synthesis, and modulating critical signaling networks involved in dermal homeostasis.

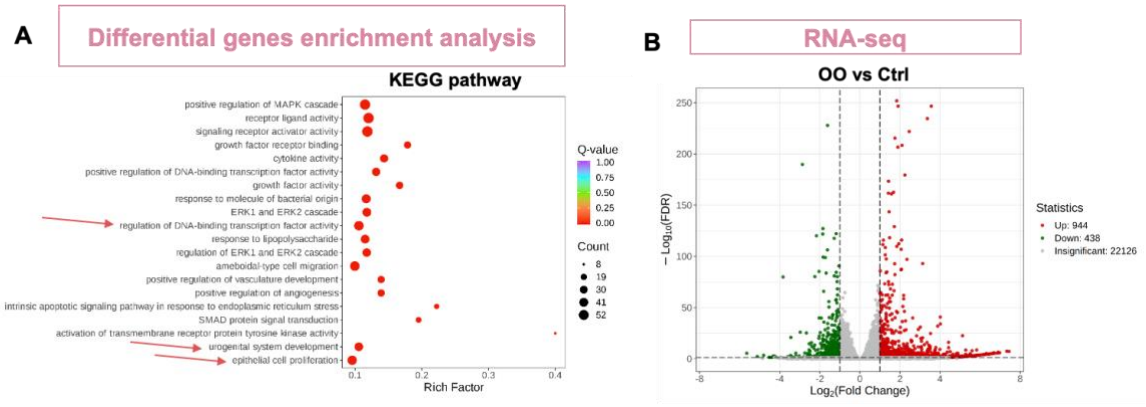

Figure 4.(A) Genes entrichment analysis and (B)RNA sequencing analysis of oznated oilve in fibroblast cell.

Multi-Omics Analysis and Functional Enrichment Clustering of Ozonated Olive Oil Effects

To achieve a comprehensive understanding of the biological impact of ozonated olive oil, we integrated multi-omics data and conducted functional enrichment clustering analysis of all differentially expressed genes (DEGs) identified in fibroblasts treated with the oxygen-enriched oil. The results demonstrated that ozonated olive oil exerts robust efficacy in promoting cellular proliferation and regeneration, underscoring its potential to enhance skin renewal processes.

Moreover, functional clustering revealed additional biological activities beyond regeneration. Notably, ozonated olive oil significantly modulated gene expression profiles associated with anti-inflammatory responses, suggesting a potential role in mitigating skin inflammation and supporting overall skin health. In parallel, pathways related to melanogenesis regulation were downregulated, indicating a skin-brightening and whitening effect, which may contribute to more uniform skin tone and reduced hyperpigmentation.

Importantly, ozonated olive oil also exhibited a strong ability to promote collagen biosynthesis, further validating its role in enhancing extracellular matrix production and reinforcing skin structural integrity. Collectively, these findings highlight the multifunctional capabilities of ozonated olive oil in skin barrier restoration, inflammation regulation, pigmentation modulation, and maintenance of dermal homeostasis.

These multi-omics insights provide compelling molecular evidence for the broad-spectrum efficacy of ozonated olive oil as a promising active ingredient in advanced dermatological and cosmetic formulations.

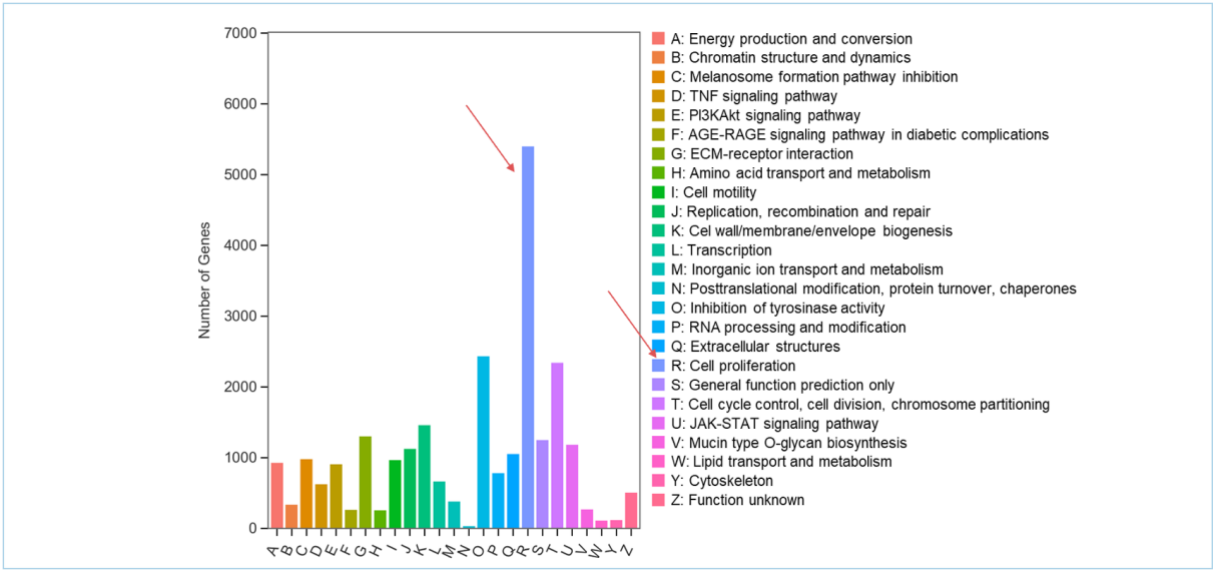

Figure 5.Multi-omics data and conducted functional enrichment clustering analysis of all differentially expressed genes (DEGs) identified in fibroblasts treated with the oxygen-enriched oil

Evaluation of Cellular Proliferation and Oxidative Stress Modulation by Ozonated Olive Oil in HFF-1 Cells

To further validate the biological efficacy of ozonated olive oil, we conducted CCK-8 assays and reactive oxygen species (ROS) detection experiments using HFF-1 human skin fibroblast cells. The CCK-8 assay results revealed that treatment with oxygen-enriched oil

significantly enhanced the proliferative capacity of fibroblasts compared to the untreated control group, suggesting a positive effect on cellular renewal and skin regeneration processes.

In parallel, ROS detection assays demonstrated a marked reduction in intracellular ROS levels in cells treated with ozonated olive oil and a formulation of ozonated olive oil and sialic acid. Since excessive ROS accumulation is a major contributor to photoaging and oxidative skin damage, this finding indicates that ozonated olive oil possesses strong antioxidative properties capable of mitigating ROS-mediated cellular stress. By decreasing ROS levels, ozonated olive oil may help protect skin cells against environmental aggressors, thus enhancing skin resilience and promoting healthier, more youthful skin.

Together, these results provide direct experimental evidence that ozonated olive oil not only stimulates fibroblast proliferation but also confers antioxidative benefits, positioning it as a promising multifunctional ingredient for skincare formulations aimed at skin repair and anti-aging..

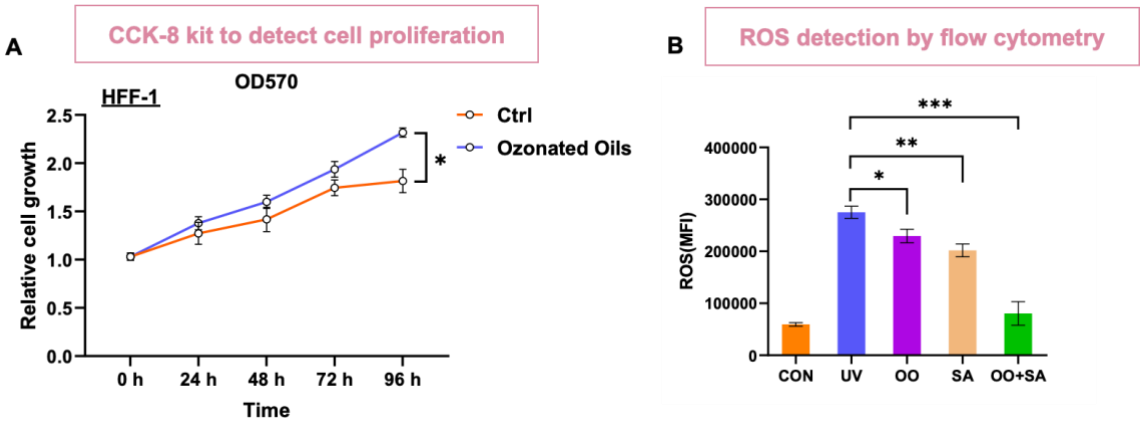

Figure 6.(A)Cellular proliferation of ozonated olive oil in HFF-1 human skin fibroblast cells. (B) Ros detection of flow cytometry formulation of ozonated olive and salic acid in treating the HFF-1 human skin fibroblast cells.

4. Conclusion

In this study, we explored the biological effects of ozonated olive oil, identifying unique bioactive compounds such as convallaria saponin and petroselinic acid, which are formed through the ozonation process. Our findings demonstrate that ozonated olive oil possesses multifaceted skin benefits, including enhanced fibroblast proliferation, collagen synthesis, and skin regeneration. The interaction of convallaria saponin with fibroblast growth factors (FGF1 and FGF2) and the binding affinity of petroselinic acid with fatty acid-binding proteins (FABPs) further supports its potential for improving skin barrier function, hydration, and resilience.

Transcriptomic analysis revealed that ozonated olive oil modulates key signaling pathways related to cell proliferation, collagen production, and extracellular matrix organization, while also exhibiting anti-inflammatory and skin-brightening effects. Moreover, the oil demonstrated potent antioxidative properties, reducing reactive oxygen species (ROS) levels and providing protection against oxidative skin damage, which is crucial in the context of photoaging.

These findings collectively underscore the promising potential of ozonated olive oil as a multifunctional bioactive ingredient in dermatological and cosmetic formulations aimed at skin regeneration, anti-aging, and barrier repair. The study lays the foundation for future research

to optimize ozonated olive oil formulations and assess their clinical efficacy in diverse skincare applications.

5. References

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