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Dose-dependent *in vitro* cellular effects of peppermint, clove and garlic essential oils on caprine dermal fibroblasts

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Abstract

Objective

Peppermint (PO), clove (CO), and garlic (GO) essential oils are widely recognized for their therapeutic properties in traditional medicine. However, their biological effects are highly concentration-dependent—higher concentrations may exert cytotoxic effects, while lower concentrations may reduce therapeutic efficacy. This study aimed to evaluate the *in vitro* dose-dependent effects of PO, CO, and GO on caprine dermal fibroblasts (cdFibroblast cells).

Methods

Cells were exposed to serial dilutions of each oil (1:2.5K, 1:5K, 1:10K, and 1:20K), and biological responses were analysed, including cell viability, cytotoxicity, colony-forming ability (CFU), and the expression of key genes associated with fibroblast identity, apoptosis, oxidative stress, and cellular stress response (vimentin, FSP-1, CuZnSOD, BAX, BCL-6, and HSP-72).

Results

Lower dilutions (1:2.5K and 1:5K) showed significant cytotoxicity, whereas moderate dilutions (1:10K and 1:20K) enhanced cell viability and CFU formation. PO and CO at 1:10K demonstrated optimal modulatory effects, while GO was more effective at 1:20K. Gene expression analysis further supported the concentration-dependent response, with upregulation of antioxidant and anti-apoptotic markers at non-toxic concentrations.

Conclusion

These findings suggest that these essential oils exert dose-dependent effects on caprine dermal fibroblasts, with optimized dilutions promoting cell viability and protective gene expression, supporting their potential use in tissue engineering.

INTRODUCTION

Essential oils, extracted from plants, have long been valued in traditional medicine for their therapeutic properties, including their ability to treat various skin ailments, promote healing, and improve overall skin health. These natural compounds exhibit a diverse array of biological activities, including antimicrobial and antioxidant properties, making them potential candidates for various skin conditions [1]. Among the most studied essential oils are CO (*Syzygium aromaticum* (L.), PO (*Mentha piperita* L.), and GO (*Allium sativum* L.), each possessing unique bioactive properties that can significantly impact dermal cells and contribute to skin health [2]. The names of clove, peppermint and garlic has been checked and confirmed

to be consistent with the data in MPNS (http://mpns.kew.org). Their ability to protect against various skin conditions has sparked interest in their inclusion in topical treatments and formulations aimed at improving skin integrity and addressing dermatological disorders [3].

Dermal cells, particularly fibroblasts, play crucial roles in maintaining skin integrity and function, primarily through their synthesis and maintenance of the extracellular matrix, which provides structural support and mediates cellular communication [4]. Additionally, fibroblasts contribute to the skin's barrier function by contracting the wound and promoting re-epithelialization, thus protecting against external injuries [5]. Essential oil can modulate the behaviour of these cells, influencing processes like cell viability [6].

For centuries, cloves have been utilized in traditional medicine to manage conditions such as nausea, vomiting, flatulence, and various digestive and liver disorders. Additionally, they have been recognized for their stimulating effects on the nervous system. In both Indian and Chinese traditional medicine, cloves are valued for their warming and invigorating properties [7]. CO, which is rich in eugenol, has demonstrated antioxidant and antiseptic properties that are beneficial for maintaining healthy skin and addressing issues such as acne or skin infections [8, 9]. Similarly, PO has been traditionally employed to address issues like loss of appetite, sinusitis, bronchitis, common colds, fever, nausea, vomiting, and digestive ailments [10, 11]. Furthermore, GO has traditionally been employed in the management of numerous ailments, such as fever, diabetes, rheumatism, intestinal parasitic infections, colic, flatulence, dysentery, liver disorders, tuberculosis, facial paralysis, hypertension, and bronchitis [12]. In Ayurveda and Siddha medicine, garlic juice has been traditionally used to relieve sinus congestion, while Unani practitioners have employed garlic for conditions such as paralysis, memory loss, tremors, colic pain, internal ulcers, and fevers [13].

CO, rich in eugenol, has demonstrated antioxidant and antiseptic properties, which are beneficial for maintaining healthy skin and addressing issues such as acne or skin infections [11–13]. PO, with menthol as the major component, is a favourable candidate for use in skin care products with anti-inflammatory properties [14]. Furthermore, GO, known for its potent antimicrobial and anti-inflammatory properties, has been studied for its effectiveness in combating skin infections [15–16]. Together, these oils play a vital role in modern dermal therapy [17].

However, the therapeutic utility of these oils is limited by their concentration-dependent effects, which can lead to cytotoxicity at high concentrations and limited regenerative effects at low concentrations. Understanding the optimal conditions for their use is crucial for maximizing therapeutic benefits while minimizing adverse effects. Furthermore, there is a lack of studies comparing these oils at different concentrations to define their cytotoxic effects on dermal cells. Investigating how these essential oils interact with fibroblasts could reveal their potential therapeutic applications in dermatology and anti-aging treatments. Moreover, understanding the interaction of these oils with fibroblasts could result in the development of safer and more effective formulations, balancing their therapeutic benefits with potential cytotoxicity. Therefore, further research is needed to understand the implications of these essential oils on fibroblast function and to establish safe and effective usage parameters.

Therefore, this study aimed to investigate the *in vitro* cellular responses of cdFibroblast cells, in terms of cell viability, CFU, and *in vitro* cell cytotoxicity to essential oils (PO, CO, and GO). Furthermore, we also evaluated the effect of different dilutions of PO, CO, and GO on the differential expression of fibroblast marker genes (vimentin and FSP-1), antioxidant enzyme (CuZnSOD), apoptosis related genes (BAX and BCL-6), and HSP-72. To the best of our knowledge, this study provides a novel comparative insight into the dose-dependent effects of peppermint, clove, and garlic essential oils on cdFibroblast cells. The outcome of the study may provide valuable insights into the *in vitro* effects of PO, CO, and GO on fibroblasts, paving the way for their potential applications in regenerative medicine and tissue engineering.

MATERIALS AND METHODS

All chemicals were procured from Sigma-Aldrich unless otherwise stated.

Isolation, culture, and enrichment of cdFibroblast cells

cdFibroblast cells from Barbari goats (age 5–6 years, n = 4) were isolated, cultured and enriched according to a previously described method [18]. The skin biopsy samples were collected from animals reared at the institutional research farm of ICAR-Central Institute for Research on Goats, Makhdoom, Mathura, India. The skin samples from the tail base of adult bucks were collected in DPBS (DPBS; Gibco, Catalog#14040-117), washed with 70% ethanol and DPBS, and the epidermis was removed using a sterile scalpel. After further rinsing with DPBS containing gentamycin (10 µg/mL; Catalog#G1264) and antibiotic-antimycotic solution (10 µL/mL; Catalog#A5955), the skin was trimmed into 2–3 mm^2 fragments and cultured in cell culture media [DMEM/F-12 (Catalog#D8437) supplemented with gentamycin (10 µg/mL; Catalog#G1264), non-essential amino acids (NEAA; 0.5%, Catalog#M7145), antibiotic-antimycotic solution (10 µL/mL; Catalog#A5955), and 15% FBS (Gibco, Invitrogen, Catalog#10082-147)] at 38.5°C with 5% CO_2 for 48 hours to assess fibroblast migration. Fibroblasts (cdFibroblast cells) were enriched via magnetic-activated cell sorting following centrifugation and filtering steps. Labelled cells were separated using an MS column and eluted for subsequent experiments.

Establishing secondary cultures

On reaching confluency, the monolayer was rinsed via gentle agitation of the cell culture plate with DPBS lacking calcium and magnesium (Gibco, Invitrogen, Catalog#14190-136). The DPBS was then discarded, and the cell sheet (monolayer) was incubated with 1 mL of trypsin-EDTA solution (0.25%) (Gibco, Invitrogen, Catalog#25200-072) for 3–5 min in a CO_2 incubator at 38.5°C with 5% CO_2 and humidified conditions. Cells were observed intermittently under a microscope for partial dislodgment from the surface. cdFibroblast cells became rounded and detached from the plastic surface of the culture plate. Once approximately 70% of the cells were detached, 2 mL of wash medium (DMEM/F-12 supplemented

with 10 µg/mL gentamycin, 0.5% NEAA solution, 10 µL/mL antibiotic-antimycotic solution, and 10% FBS) was added to the plate. The cells were gently collected in a 15 mL centrifuge tube and centrifuged at 2655 × g for 10 min at 25°C to form a cell pellet. The cells were then expanded through serial passaging. After three passages in a similar manner, almost all the cells were confirmed as cdFibroblast cells through double immunofluorescence immunostaining for the expression of vimentin and FSP-1.

Immunocytochemistry (ICC) of cdFibroblast cells

Immunofluorescence staining of the cdFibroblast cells was conducted with adjustments to the protocol outlined by Singh et al. [19]. Briefly, the fibroblast monolayer was fixed with citrate-acetone-formaldehyde, permeabilized using 0.5% Triton X-100, and blocked with 2% BSA to prevent nonspecific binding. Cells were then incubated with primary antibodies [mouse anti-vimentin (Invitrogen RV203, Catalog#MAI-06908) and rabbit anti-FSP-1 (Merck S100A4, Catalog#ABF32)] and corresponding fluorescent secondary antibodies [Alexa Fluor® 488 donkey anti-mouse (Invitrogen, Catalog#R37114) and Alexa Fluor® 555 goat anti-rabbit IgG (Cell Signalling Technology, Catalog#4413S)], followed by DAPI (1 µg/mL, Catalog#D8417) nuclear staining. Immunofluorescence imaging was conducted using a Zeiss Axiovert A1 fluorescence microscope.

Experimental design

To evaluate the effect of three different essential oils [peppermint oil (Catalog#W284815), clove bud oil (Catalog#W232300) and garlic oil (Catalog#W250320)] on cdFibroblast cells, the cells were cultured in culture media (DMEM/F-12 supplemented with NEAA (0.5%), L-Glutamine (0.5%), antibiotic solution (10 µL/mL) gentamycin (10 µg/mL) and FBS (10%). The working dilutions of essential oils used in this study (1:2.5K, 1:5K, 1:10K and 1:20K) were selected based on preliminary dose-response experiments performed in our laboratory. An initial screening was conducted using a wider concentration range (1:500 to 1:30K) to evaluate cytotoxic effects on cdFibroblast cells. Dilutions higher than 1:2.5K resulted in significant cytotoxicity, whereas concentrations beyond 1:20K showed minimal cellular response. Based on these observations, different dilutions of these essential oils were chosen and categorized into the following groups:

Essential oils	Dilutions			
	1:2.5K	1:5K	1:10K	1:20K
Peppermint oil	PO1	PO2	PO3	PO4
Clove oil	CO1	CO2	CO3	CO4
Garlic oil	GO1	GO2	GO3	GO4

GC-MS analysis of essential oils. The chemical constituents of the essential oils employed in this study were characterized by gas chromatography–mass spectrometry (GC-MS) using a triple quadrupole detector (GC-MS TQ8030, Shimadzu, Kyoto, Japan). The identified compounds from PO, CO, and GO are listed in Supplementary Table 1. Analyses were performed in Q3 scan mode with a Restek Rxi-5 ms column (30 m length, 0.25 µm film thickness). The chromatographic run was carried out from 3 to 71 min with a scan speed of 2500 and a mass range of m/z 40–700. Ionization was achieved at 70 eV. Compound identification was based on comparison of retention times and fragmentation spectra with those available in the NIST mass spectral library integrated into the GC-MS/MS system.

In vitro cytotoxicity assay

The *in vitro* cytotoxicity assay was conducted using an MTT-based toxicology assay kit (Catalog#TOX-1) following the manufacturer's protocol, with appropriate controls included. Cells were seeded at a density of approximately 40,000 cells per well in 24-well culture plates and incubated for 24 hours. After this period, the culture medium was replaced with media containing PO, CO, or GO, according to the experimental design. Following a 96-hour incubation, MTT reagent (Catalog#M-5655) was added to each well at a volume equivalent to 10% of the culture medium, and the plates were incubated for an additional 3 hours. After incubation, formazan crystals were dissolved by adding MTT solubilization solution (Catalog#M-8910) in an amount equal to the initial culture medium volume. Gentle mixing was performed to ensure complete dissolution of the formazan crystals. Absorbance was then recorded at 570 nm using a multimode spectrophotometer.

Measuring cell viability and cell viability assay

To assess the impact of PO, CO, and GO on cell viability and cell viability, cells were cultured as mentioned for the expansion protocol. For measuring cell viability, approximately $7.21 \pm 0.46 \times 10^4$ cells were cultured in culture media for 24 h in 24-well plates. After 24 h, the culture medium was replaced with CO, PO and GO-containing media as per the experimental design. Cell counting was done as described by Pathak et al. [20]. Total viable cells in each well were counted on d-3 and d-5.

CFU assay

This assay aimed to determine whether a single cdFibroblast cells could form a colony in different oil-based environments. Following trypsinization, approximately 40,000 cells per well (P-4) were seeded into 6-well plates containing culture media supplemented with PO, CO, or GO. The cells were then incubated at 38.5°C with 5% CO_2 for 48 hours. To accurately assess colony formation, a low cell density was used to facilitate the identification of individual colonies. After the incubation period, the colonies were fixed and stained with 1% crystal violet solution (Catalog#V5265) for 25 minutes. The excess stain was removed by washing thoroughly with distilled water, and the colonies were then examined under an

inverted microscope. For quantitative analysis, the stain was solubilized in a 1% SDS solution for 10 minutes at RT, and absorbance was measured using a spectrophotometer.

Quantitative real-time PCR (qRT-PCR) for gene expression analyses

Total RNA was extracted from enriched P-4 cells cultured in different essential oils (PO, CO, and GO) using RNAiso Plus following the manufacturer's instructions (DSS Takara, Catalog#9108). RNA concentration and purity were measured using a spectrophotometer (Nanodrop Lite Plus, ThermoFisher Scientific). cDNA was synthesized using a cDNA synthesis kit (DSS Takara, Catalog#6110A) and a C1000™ Thermal Cycler (Biorad), and gene expression of fibroblast markers (vimentin, FSP-1), apoptosis-related gene (BAX, BCL-6), stress (HSP-72), and antioxidant enzymes (Cu-Zn SOD) was analyzed by qRT-PCR with GAPDH as the endogenous control. Reactions were performed in duplicate using TB Green® Premix Ex Taq™ (Takara, Catalog#RR42LR) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific), and relative gene expression was calculated using the ΔΔCt method.

Statistical analysis

The data are expressed as mean ± SEM from six independent experiments. Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Culture and ICC of cdFibroblast cells

Figure 1 displays representative images of explant culture, cell expansion, and double immunofluorescence staining for FSP-1 and vimentin, in cdFibroblast cells. The effect of essential oils (PO, CO, and GO) on fibroblast cell morphology showed that these essential oils had no significant detrimental effects on the morphology of cdFibroblast cells and did not alter the cell shape. Immunocytochemical analysis demonstrated elevated expression of these fibroblast-specific markers in cultured cdFibroblast cells. These findings indicate that the culture conditions and media utilized in the study successfully preserved the characteristic features of cdFibroblast cells, making them suitable for cultivation throughout the experiment.

Effect of essential oils on cdFibroblast cells morphology

The effect of used essential oils (PO, CO, and GO) on fibroblast cell morphology has shown that at used dilutions, these essential oils have no significant detrimental effects on the morphology of cdFibroblast cells and did not alter cell shape.

In vitro cytotoxicity assay

For the estimation of the cytotoxicity of these essential oils on cdFibroblast cells, the MTT-cellular viability assay was performed. This test was based on the fact that only viable cells change the relatively colourless MTT compound into dark purple formazan crystals (Fig. 2). The results are presented as absorbance values, where higher values correspond to increased cell viability (Fig. 5C). The quantitative data demonstrate that PO and CO at 1:2.5K dilution (0.16 ± 0.003 and 0.16 ± 0.001, respectively) showed cell cytotoxicity when compared to the control (0.18 ± 0.002), suggesting inhibitory effect at high concentration. Furthermore, the 1:5K dilution (0.19 ± 0.006 and 0.18 ± 0.001, respectively) also resulted in a slight increase in cell viability; however, this difference was not statistically significant. Moreover, the 1:10K dilution (0.24 ± 0.012 and 0.24 ± 0.008, respectively) resulted in significant ($p < 0.05$) enhancement in cell viability, with the highest absorbance recorded, indicating optimal conditions for cell viability. However, the 1:20K dilution (0.20 ± 0.004 and 0.22 ± 0.002, respectively) exhibited a reduction in cell viability, though still higher than the control, indicating a decrease in the effectiveness of the PO and CO at this concentration.

Furthermore, for GO, 1:2.5K and 1:5K dilutions resulted in invitro cell cytotoxicity (0.15 ± 0.004 and 0.17 ± 0.002, respectively) suggesting a strong inhibitory effect at this concentration. At the 1:10K dilution (0.21 ± 0.004), there was an increase in cell viability, indicating a favourable effect but significantly ($p < 0.05$) higher results were observed at the 1:20K dilution (0.23 ± 0.002), suggesting that higher concentration of GO results in cell cytotoxicity.

Effects of essential oils on viability of cdFibroblast cells

Figure 3 shows the representative images of cell viability evaluation of cdFibroblast cells in PO, CO, and GO by cell counting. Each culture displayed similar patterns of cell viability patterns up to day 2. This may be attributed to the cell adaptation process, where cells gradually adjust to the new culture environment. Such adaptation is essential for enhancing cell growth, productivity, and overall viability.

On comparing the data (cell number $\times 10^4$) at d-3 and d-5, at 1:2.5K dilution, a significantly ($p < 0.05$) lower number of cells were observed in PO, CO and GO (d-3: 8.53 ± 0.46, 7.65 ± 0.14, and 7.53 ± 0.21, respectively; d-5: 11.74 ± 0.51, 11.04 ± 0.36, and 10.30 ± 0.21, respectively) indicating potential cytotoxicity at this concentration when compared to control on d-3 (12.53 ± 0.14) and d-5 (15.23 ± 0.66). Interestingly, at 1:5K dilution, the number of cells in PO, CO and GO on d-3 (14.80 ± 0.14, 13.92 ± 0.52, and 9.24 ± 0.36, respectively) and d-5 (15.58 ± 0.36, 14.79 ± 0.46, and 12.39 ± 0.36, respectively) increased, suggesting a possible stimulatory effect or adaptive response.

Furthermore, at 1:10K dilution, a significantly ($p < 0.05$) higher number of cells were observed with PO and CO (d-3: 21.04 ± 0.36 and 18.99 ± 0.14, respectively; d-5: 23.64 ± 0.14, and 21.69 ± 0.36, respectively) while GO showed almost same results with control at this concentration (d-3: 12.56 ± 0.22 and d-5: 14.93 ± 0.51). However, at 1:20K dilution, the number of cells declined in PO and CO (d-3: 16.46 ± 0.22 and 16.26 ± 0.22, respectively; d-5: 18.91 ± 0.46, and 18.64 ± 0.14, respectively) while in case with GO a

significantly ($p < 0.05$) higher number of cells were observed on d-3 (17.09 ± 0.36) and d-5 (18.45 ± 0.14). These results suggest that these oils exhibit dose-dependent cytotoxicity, with lower concentrations potentially promoting cell viability while higher concentrations exert inhibitory effects (Fig. 5A).

Effect of essential oils on CFU of cdFibroblast cells

In this assay, isolated colonies of cdFibroblast cells representing a single precursor cell were observed. Images of the six-well culture plates, which contained cdFibroblast cells colonies cultured in different essential oils (PO, CO, and GO) (Fig. 4), clearly demonstrate the varying effects of peppermint, clove, and garlic oils on colony formation at different dilutions.

For PO and CO, the CFU assay revealed a dose-dependent response in colony formation (Fig. 5B). Compared to the control (0.30 ± 0.00), the 1:2.5K dilution (0.26 ± 0.001 and 0.25 ± 0.004, respectively) exhibited a reduction in CFU values. In contrast, the 1:5K dilution demonstrated a slight increase (0.34 ± 0.001 and 0.30 ± 0.00, respectively). Notably, the highest CFU value was recorded at the 1:10K dilution (0.46 ± 0.002 and 0.44 ± 0.006, respectively), which significantly ($p < 0.05$) higher than the control. At the 1:20K dilution (0.40 ± 0.007 and 0.38 ± 0.001, respectively), CFU values remained elevated but were lower than those observed at 1:10K. These results indicate that a moderate dilution (1:10K) optimally promotes colony formation, whereas higher concentrations may exert an inhibitory effect.

Furthermore, for GO, when compared with control, a marked decrease in CFU was observed at the 1:2.5K dilution (0.19 ± 0.003) indicating a significant suppression of colony formation. Similarly, the 1:5K dilution (0.22 ± 0.017) also resulted in a substantial reduction compared to the control. However, at the 1:10K dilution (0.34 ± 0.013), CFU values surpassed the control, suggesting a reversal of the inhibitory effect. A significantly ($p < 0.05$) higher CFU value was recorded at the 1:20K dilution (0.42 ± 0.006).

These findings suggest that garlic oil at lower concentrations (1:2.5K and 1:5K) exerts an inhibitory effect on CFU, potentially due to cytotoxic effects or interference with cellular attachment and survival. In contrast, higher dilutions (1:10K and 1:20K) appear to mitigate this inhibitory effect, facilitating a more favourable environment for colony establishment. Overall, all three oils exhibited a dilution-dependent effect, with the highest colony formation observed at 1:10K PO and CO, and at 1:20K for GO.

Quantitative real-time PCR (qRT-PCR) for gene expression analyses

The relative expression of fibroblast markers (vimentin and FSP-1), apoptosis-related genes (BAX and BCL-6), antioxidant enzyme (CuZnSOD), and stress-related gene (HSP-72) was analyzed using GAPDH as an endogenous control in cdFibroblast cells cultured with or without essential oils. Expression analysis showed significant ($p < 0.05$) upregulation of fibroblast markers (vimentin and FSP-1), anti-apoptotic gene (BCL-6), antioxidant enzyme gene (CuZnSOD) and stress related gene (HSP-72) in cdFibroblast cells cultured with lower dilutions of essential oils (Fig. 6). While, the expression of pro-apoptotic gene (BAX) was downregulated in cdFibroblast cells cultured with lower dilutions of essential oils when compared with control indicating the role of dilution in case of PO, CO and GO.

DISCUSSION

Essential oils have emerged as promising bioactive agents in dermatological applications due to their antimicrobial, anti-inflammatory, and regenerative properties [21]. Investigating the cytotoxic and regenerative effects of PO, CO, and GO on cdFibroblast cells will provide insights into their impact on cell viability and potential cellular damage, as well as their capacity to modulate wound healing processes. The results presented in this study demonstrate distinct cytotoxic effects of these essential oils on fibroblast behaviour.

Immunocytochemical analysis of cdFibroblast cells revealed enhanced expression of fibroblast-specific markers, vimentin and FSP-1, across all experimental conditions. This indicates that the culture conditions and media employed in the study were effective in maintaining the normal phenotype and functionality of cdFibroblast cells throughout the experimental period. The presence of these markers confirms the integrity of the cdFibroblast cells and their suitability for evaluating the effects of essential oils.

The MTT assay, commonly used to assess cell viability, relies on the metabolic conversion of MTT into formazan crystals by viable cells. In the present study, the MTT results were considered in conjunction with cell number data to better interpret the effects of different concentrations of essential oils on cdFibroblast cells. At the 1:10K dilution of PO and CO, a significant increase in absorbance was observed, suggesting enhanced cell viability. In the present study, PO and CO exhibited cytotoxic effects at higher concentrations (1:2.5K dilution), as evidenced by reduced MTT absorbance values when compared to control. This suggests a decline in viable cell number or metabolic activity at this dilution, potentially due to the toxic effects of certain bioactive components present at higher concentrations. At 1:5K dilution, PO and CO showed a slight increase in cell viability, although the changes were not statistically significant, indicating partial recovery or a threshold concentration below which toxicity begins to diminish. The most pronounced effect was observed at 1:10K dilution, where both PO and CO significantly enhanced MTT absorbance values compared to the control. This increase may correspond to a higher viable cell number and/or enhanced metabolic activity, implying that this dilution creates a favourable microenvironment for cdFibroblast cells viability.

This can be further clarified through the following explanation. CO, known for its high eugenol content, exhibits significant cytotoxic effects even at relatively low concentrations. Eugenol, a phenolic compound, is well-documented for its potent biological activity, including antimicrobial, anti-inflammatory, and antioxidant properties. However, its high reactivity with cellular components, such as lipids and proteins, can disrupt cellular membranes and metabolic processes, leading to reduced cell viability and potential cytotoxicity at lower concentrations [22]. This makes CO a highly effective agent in specific therapeutic contexts but requires careful dilution to minimize adverse effects on non-target cells like fibroblasts.

Furthermore, in the case of GO, cytotoxicity was observed at both 1:2.5K and 1:5K dilutions, as indicated by lower absorbance values than the control. This points toward a stronger inhibitory effect of GO at higher concentrations, potentially due to the presence of more potent cytotoxic constituents. At 1:10K, a moderate increase in viability was noted, while the highest viability was observed at the 1:20K dilution

A cell viability assay demonstrated significant differences in viability of cells when cultivated with essential oils compared with the control. CO and PO at a 1:10K dilution exhibited the most favourable effects on fibroblast viability, while GO produced optimal results at a higher dilution (1:20K). The observed differences in cytotoxicity at varying concentrations may be due to the specific bioactive compounds present in each oil and their respective concentrations.

Furthermore, the CFU assay demonstrated distinct patterns of colony formation across the oils. Like viability, PO and CO showed the highest number of colonies at a 1:10K dilution, while GO yielded maximum colony formation at a higher dilution of 1:20K. This differential response highlights the unique regenerative potential of each oil and its dependence on dilutions as the CFU ability of fibroblasts is a critical indicator of their regenerative capacity. The observed results suggest that the essential oils used in this study may have potential applications in wound healing at appropriate dilutions.

PO, primarily composed of menthol and menthone, has a comparatively milder cytotoxic profile. These compounds are known to modulate membrane fluidity and ion channel activity without causing severe damage to cell structures at similar concentrations as CO. This relatively low cytotoxicity might be attributed to the chemical stability and targeted biological action of menthol and menthone, which favour therapeutic applications such as soothing inflammation and promoting cell migration. Unlike CO, PO is better tolerated by cdFibroblast cells at higher concentrations, making it a promising candidate for applications requiring cell proliferation and repair [22].

GO, on the other hand, is rich in organosulfur compounds such as diallyl disulphide and diallyl trisulfide. These compounds are renowned for their broad-spectrum biological activities, including anti-cancer, antimicrobial, and antioxidant effects. At lower concentrations, they can promote cell health and viability by modulating oxidative stress and inflammatory pathways. However, at higher concentrations, these sulphur-based compounds are highly reactive and can induce cytotoxicity through mechanisms such as the generation of reactive oxygen species (ROS), mitochondrial dysfunction, and apoptosis induction. These effects make GO a double-edged sword, beneficial at optimal dilutions but potentially harmful at higher doses [23].

The differential cytotoxic effects of CO, PO, and GO highlight the importance of their bioactive components and the need for precise concentration optimization in therapeutic applications. Understanding these variations is critical for harnessing their benefits in promoting fibroblast viability and wound healing; while mitigating adverse cytotoxic effects.

Furthermore, the results of the expression analysis suggest that lower dilutions of essential oils (PO, CO, GO) have a protective effect on cdFibroblast cells, as evidenced by the significant upregulation of

vimentin, FSP-1, BCL-6, CuZnSOD, and HSP-72. These genes are associated with characterization, anti-apoptotic, antioxidant, and stress response, respectively, indicating that the essential oils might enhance cell survival and resistance to oxidative stress. The upregulation of CuZnSOD supports this by suggesting that the essential oils contribute to the antioxidant defence of the fibroblasts, helping neutralize reactive oxygen species (ROS) and reduce cellular damage. The increase in HSP-72, a heat shock protein, further implies that the cells are undergoing a stress response, potentially offering protection against environmental or physiological stressors.

The downregulation of the pro-apoptotic gene BAX in the presence of essential oils suggests that these oils might play a role in reducing cell death, possibly contributing to fibroblast survival in stress conditions. This finding aligns with the upregulation of anti-apoptotic and stress-response genes. Furthermore, the observed expression patterns of vimentin and FSP-1, markers of fibroblast activation, across the experimental groups suggest a concentration-dependent response. Specifically, higher concentrations of the essential oils resulted in lower expression levels of these markers, while lower concentrations led to increased expression. This inverse relationship between oil concentration and fibroblast marker expression indicates protective effects of these essential oils are more pronounced at lower concentrations, potentially enhancing cdFibroblast cells activation and survival.

The results suggest a concentration-dependent bioactivity, which may be attributed to the antioxidant properties of essential oils by modulating oxidative stress pathways by scavenging reactive oxygen species (ROS), upregulating endogenous antioxidant enzymes (e.g., SOD, catalase), or modulating redox-sensitive signalling cascades like Nrf2 and MAPK [24, 25]. While such mechanisms can support cell survival and stress tolerance, it is important to note that increased metabolic activity in assays like MTT reflects mitochondrial function and does not necessarily correlate with functional regeneration.

Therefore, the distinct cytotoxic and regenerative effects of CO, PO, and GO on cdFibroblast cells can be attributed to their unique bioactive compounds and concentration-dependent activities. CO, with its high eugenol content, exhibits significant cytotoxicity at lower concentrations, necessitating careful dilution to avoid adverse effects on cell viability. In contrast, PO, dominated by menthol and menthone, demonstrates a more favourable cytotoxic profile, supporting fibroblast viability and repair at relatively higher concentrations. GO, enriched with organosulfur compounds such as diallyl disulphide and diallyl trisulfide, requires higher dilution to balance its potent biological activities with its potential for cytotoxicity. These findings emphasize the importance of optimizing the dilution and application of these essential oils to maximize their therapeutic benefits, particularly in wound healing contexts, while minimizing cytotoxic risks.

Conclusion

The differential cytotoxic and regenerative effects observed in this study highlight the importance of selecting appropriate dilutions of essential oils to maximize their therapeutic potential while minimizing adverse effects. Lower dilutions of the oils (1:2.5K and 1:5K) generally exhibited inhibitory effects on cell

viability and CFU ability likely due to cytotoxicity. In contrast, moderate dilutions (1:10K and 1:20K) promoted higher cell viability and enhanced colony formation, with 1:10K showing the most significant positive effect for PO and CO. GO exhibited a consistent increase in cell viability at higher dilutions (1:10K and 1:20K), with 1:20K being the most effective for promoting cell survival. Furthermore, lower dilutions resulted in the upregulation of significant upregulation of genes associated with characterization, anti-apoptotic, antioxidant, and stress response suggesting an optimal range for therapeutic use. These findings suggest that essential oils may serve as potential modulators of cellular growth, with optimal dilutions required to maximize their beneficial effects. Overall, this study provides valuable insights into the *in vitro* effects of GO, CO, and PO on cdFibroblast cells, paving the way for their potential applications in regenerative medicine and tissue engineering.

Abbreviations

CO
Clove essential oil
PO
Peppermint essential oil
GO
Garlic essential oil
cdFibroblast cells
Caprine adult fibroblast cells
CFU
Colony formation unit
ICC
Immunocytochemistry
MTT
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
GC-MS
Gas Chromatography-Mass Spectrometry
RT
Room temperature

Declarations

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

S.P.S. and R.K. conceived the study, designed the experimental protocol, and proofread the manuscript. J.P. and M.P. carried out the experiments and drafted the manuscript. Y.K.S. and S.P.S. statistically

analyzed the data. All authors read, revised, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

One supplementary file (Supplementary File 1) is provided with this manuscript, containing additional supporting data relevant to the study.

DATA AVAILABILITY

The authors declare that the data supporting the findings of this study are available within the manuscript.

ETHICS APPROVAL

The Animal Ethics Committee (IAEC) of the Institute approved the present investigation (IAEC/CIRG/22–23), and all the protocols followed were as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animal(CPCSEA) and Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

DECLARATION OF GENERATIVE AI

During the preparation of this manuscript, the authors used ChatGPT (developed by OpenAI) to assist in refining grammar and improving language clarity. The authors reviewed and edited all content to ensure accuracy and integrity, and are fully responsible for the final version of the manuscript.

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Figures

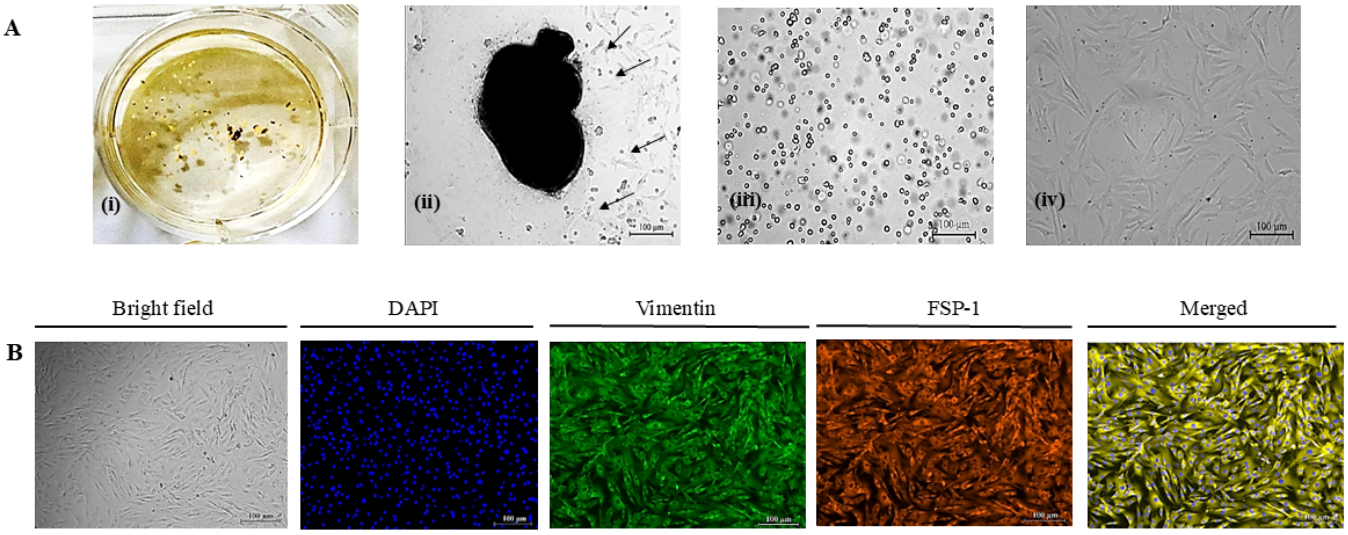


Figure 1 (A-B). The representative images for caprine dermal fibroblast (cdFibroblast cells) culture from explants (P-0) and characterization by immunostaining. [A(i-iv)] Outgrowth of cdFibroblast cells from explant (P-0) and culture. A(i). Caprine skin explants after mincing were transferred in 6 well cell culture plates in culture media and incubated at 38.5 °C, 5% CO2 in humidified conditions for adherence and attachment of tissue A(ii). Caprine skin explant with the migration of spindle-shaped cdFibroblast cells (black arrows) at d 8 of culture. Dark-shaded area in the image is the skin explant. A(iii). Image of cdFibroblast cells in inverted light microscopy after enrichment. For enrichment, after trypsinization with 0.25% trypsin-EDTA solution, fibroblast cells were enriched with magnetic microbeads labeled and Fibroblast Specific Protein (FSP-1) antibodies through magnetic-activated cell sorting (MACS). A(iv). Image of cdFibroblast monolayer formed by culturing magnetically labeled enriched FSP-1+ cells at 38.5 °C, 5% CO2 in humidified conditions. B. The representative images for characterization of cdFibroblast cells by immunostaining. Relative fluorescence positivity with vimentin and FSP-1 was observed. Scale bar = 100 µm.

Figure 1

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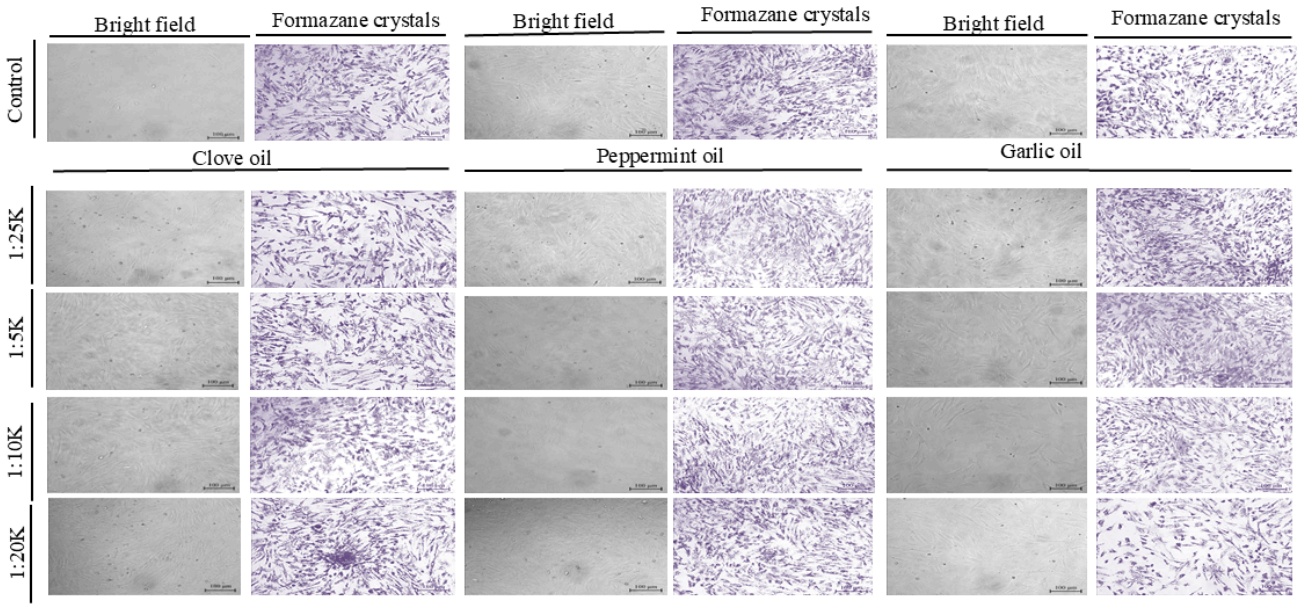


Figure 2. *In vitro* cytotoxicity assay with MTT in response to peppermint oil (PO), clove oil (CO), and garlic oil (GO). *In vitro* cytotoxicity assay was performed with MTT based *in vitro* toxicology assay kit with appropriate controls. Cells were seeded in 24 well culture plates (~ 40,000 cells/well) for 24 h. After 24 h, culture medium was replaced with PO, CO, and GO containing media at 1:2.5K, 1:5K, 1:10K and 1:20K dilutions as per experimental design. After 96 h, MTT in an amount equal to 10% of the culture medium volume was added in each well followed by incubation for 3 h. Formazan crystals formed were dissolved by adding MTT Solubilization Solution equal to the original culture medium volume. Multimode spectrophotometer was used to measure absorbance at the wavelength of 570 nm.

Figure 2

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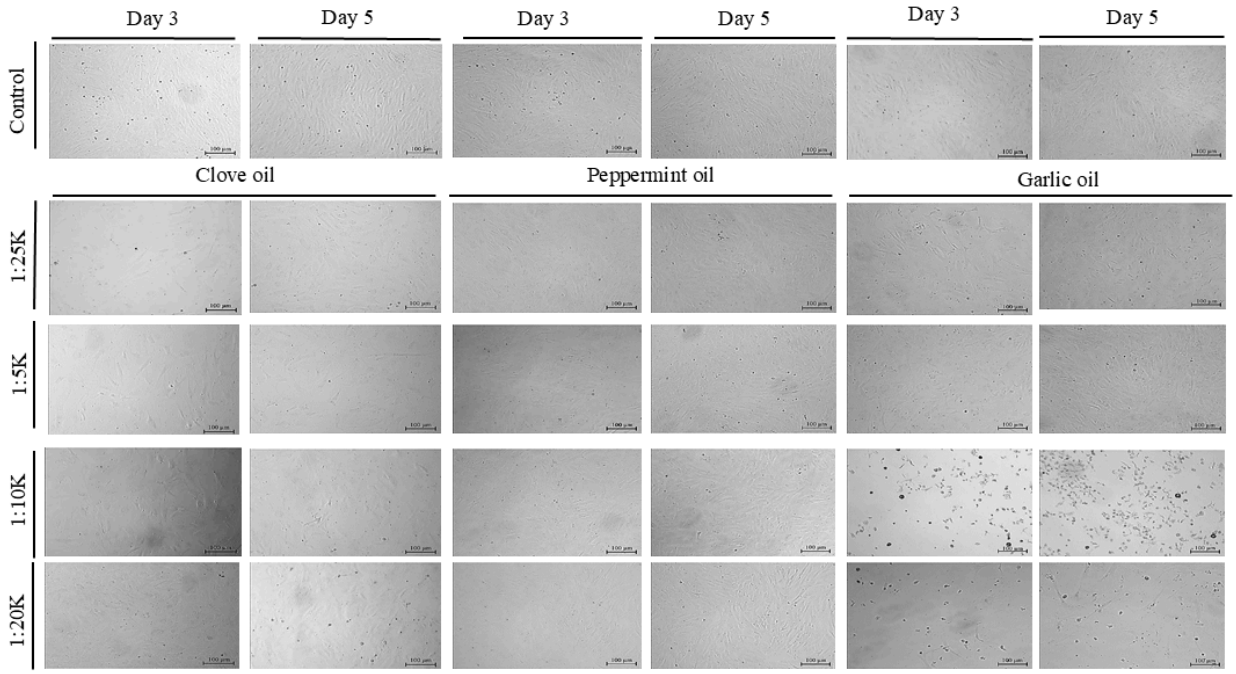


Figure 3. Representative images of cell viability evaluation of caprine dermal fibroblast (cdFibroblast) cells in different essential oils [Peppermint oil (PO), clove oil (CO), and garlic oil (GO)] by cell counting. For measuring the effect of essential oils on cell viability, after trypsinization with 0.25% trypsin-EDTA solution, approximately 7.21±0.46×104 cells were seeded at P-4 in culture medium (DMEM/F-12 supplemented with 10% FBS, 0.5% L-Glutamine, 0.5% NEAA, 10 µg/mL Gentamycin and 10 µL/mL antibiotic solution) for 24 h. After 24 h, culture medium was replaced with PO, CO, and GO at 1:2.5K, 1:5K, 1:10K and 1:20K dilutions in culture media as per experimental design. Total viable cells in each well were counted on d 3 and d 5 (through trypsinization) by automatic cell counter.

Figure 3

See image above for figure legend.

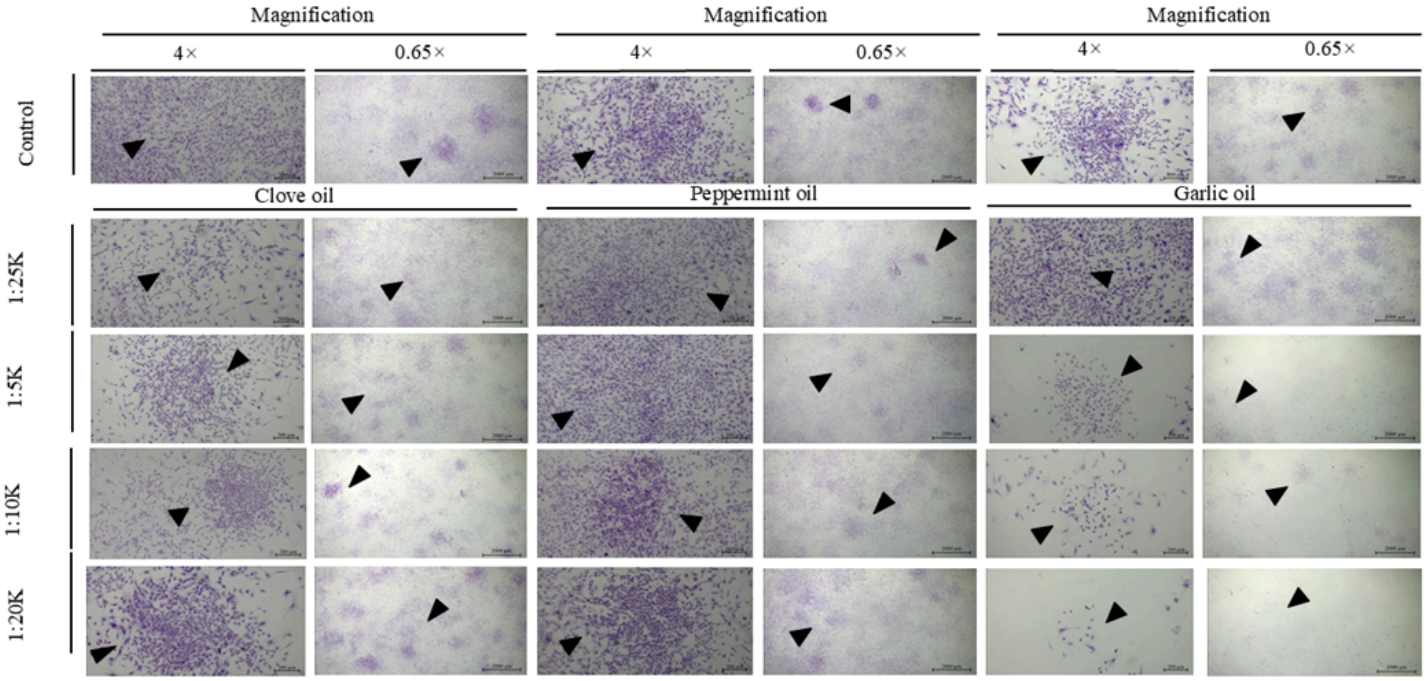


Figure 4. Representative images of the effect of peppermint oil (PO), clove oil (CO), and garlic oil (GO) on colony forming unit (CFU) efficiency of caprine adult dermal fibroblast (cdFibroblast) cells. Images showing the CFU ability of cdFibroblast cells by crystal violet staining. After trypsinization, approximately 40,000 cells per well (P-4) in culture media were cultivated in 6 well plates with 5% CO2 at 38.5 °C in a CO2 incubator for 24 h. After 24 h, culture medium was replaced with PO, CO, and GO at 1:2.5K, 1:5K, 1:10K and 1:20K dilutions in culture media as per experimental design. Colonies were washed and stained with 1% crystal violet solution for 25 min. The colonies were thoroughly washed with distilled water before being examined under an inverted microscope. Arrowheads indicate individual colonies.

Figure 4

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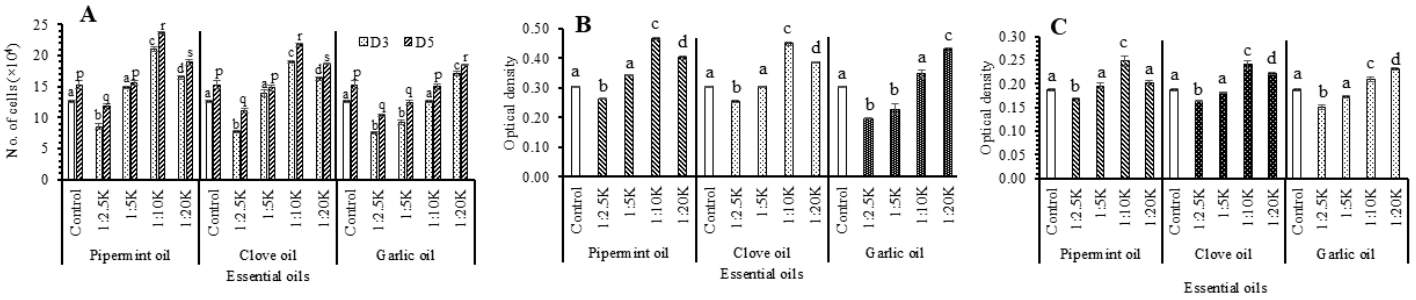


Figure 5 (A-C). Comparative analyses of the effect of essential oils (Peppermint oil, Clove oil and Garlic oil) on viability, colony forming unit (CFU) and MTT assay of caprine adult dermal fibroblast (cdFibroblast) cells. A. Effect of essential oils on viability of cdFibroblast cells. Cell counting was done at d 3 and d 5 by automatic cell counter. B. Comparison of optical densities showing the effect of essential oils on CFU efficiency of cdFibroblast cells. Optical density was measured after solubilization of stain (crystal violet) with 1% SDS solution. C. Quantitative assessment of cell cytotoxicity (MTT assay) by essential oils on cdFibroblast cells. It was measured by the ability of the cells to convert the yellow MTT to its purple formazan metabolite, which appears as dark granules and crystals. The optical densities of the cdFibroblast cells growing in different essential oils were compared after solubilization of stain. Different lowercase letters (a–d and p–s) indicate statistically significant differences ($p < 0.05$) among various dilutions of each essential oil when compared with the respective control group at the same time point. Bars sharing the same letter are not significantly different, while those with different letters represent significant variation, highlighting the dose-dependent effects of peppermint, clove, and garlic oils.

Figure 5

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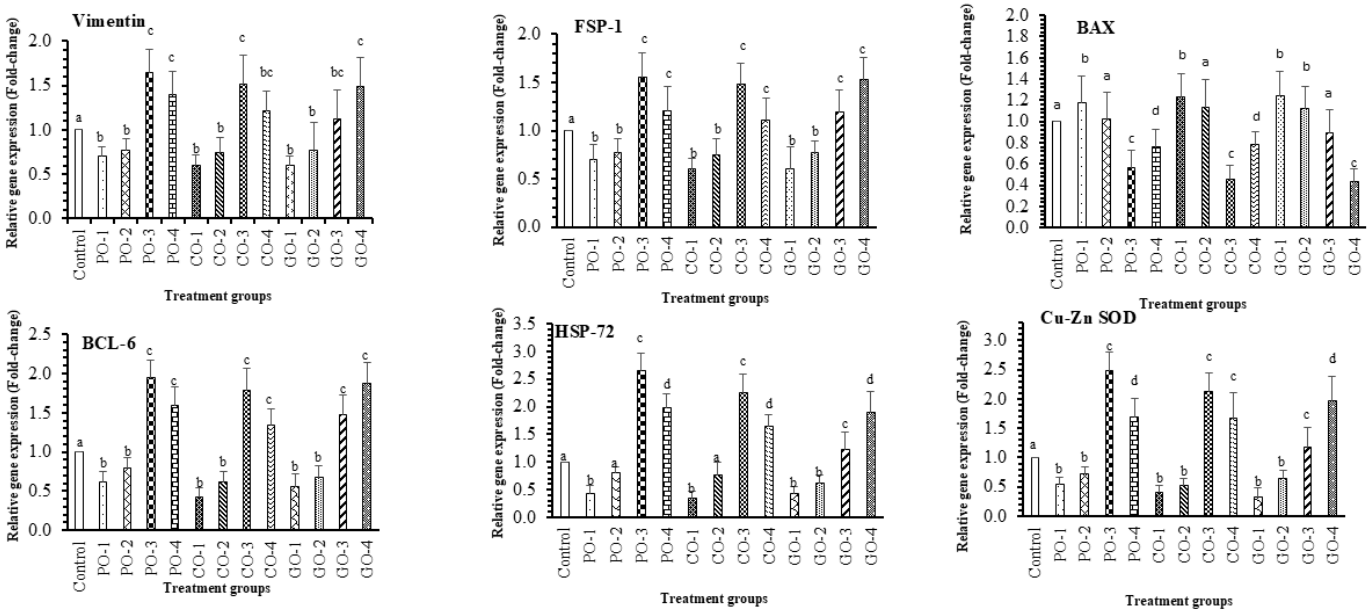


Figure 6. Comparative evaluation of the effect of essential oils (Peppermint oil, Clove oil and Garlic oil) on the differential expression of marker genes associated with typical fibroblasts [vimentin and Fibroblast specific protein-1 (FSP-1)], apoptosis (BAX and BCL-6), stress (HSP-72), and antioxidant enzyme (CuZnSOD) in enriched cdFibroblast cells at P-4 culture. The differential expression levels were assessed using real-time qPCR with GAPDH as an internal calibrator and cells in culture media without essential oil as a control. Different lowercase letters (a–d) indicate statistically significant differences ($p < 0.05$) among various dilutions of each essential oil when compared with the respective control group at the same time point. Bars sharing the same letter are not significantly different, while those with different letters represent significant variation, highlighting the dose-dependent effects of peppermint, clove, and garlic oils. Peppermint oil = PO, Clove oil = CO, and Garlic oil = GO.

Figure 6

See image above for figure legend.