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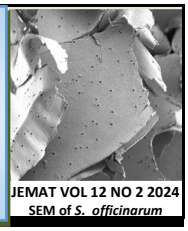

Cytotoxic Effects of *Cananga odorata* Essential Oil on p53 Protein and *TP53* Gene Dynamics in Non-Melanoma Skin Cancer

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ABSTRACT

Skin cancer remains one of the most common cancers worldwide. *Cananga odorata* essential oil (CO EO) has shown potential anticancer and antioxidant properties, suggesting its application as a topical treatment for skin cancer. The objective of this study is to assess the cytotoxic effect of CO EO on non-melanoma skin cancer, *TP53* gene expression, and p53 protein expression. The methodology involves HFF1 (human foreskin fibroblast) and A431 (human epidermoid squamous carcinoma) cell lines treatment with CO EO at 125, 250, and 500 µg/mL for 24 h. Morphological changes were observed under light microscopy. p53 protein expression and secretion were assessed using western blot. *TP53* gene expression was quantified using real-time PCR. Our results show that CO EO treatment caused dose-dependent morphological changes in A431 cells, with increased non-viable cells observed. There was a 1.3-fold increase in p53 protein secretion in A431 cells treated at 250 µg/mL of CO EO. *TP53* gene expression in A431 cells was significantly downregulated at 125 µg/mL ($p = 0.03$), while in HFF1 cells, *TP53* was upregulated. To conclude, CO EO demonstrates cytotoxic effects on A431 cells, associated with altered p53 protein secretion and *TP53* gene expression. Further studies are needed to elucidate its molecular mechanisms and therapeutic potential.

INTRODUCTION

Skin cancer, characterized by uncontrolled growth of abnormal cells, remains a significant global health concern, with over 1.5 million new cases reported in 2020 [1]. While treatments like chemotherapy and topical 5-fluorouracil (5-FU) are available, they are associated with high costs and adverse effects, such as cardiotoxicity and severe skin irritation [2,3]. Essential oils (EOs) are emerging as promising candidates for cancer therapy due to their bioactive compounds, such as phenols and aldehydes, which can disrupt cancer development pathways. They are complex mixtures of volatile compounds with variable odorous impact isolated physically via steam distillation, dry distillation, or mechanical treatment, from plants of known taxonomic origin [4]. Classically, EOs are used as perfumes or cosmetics [5]. Beyond their aromatic properties, many studies have highlighted their potential in medical applications including antimicrobial and anticancer properties [6]. These mechanisms include inducing apoptosis, arresting the cell cycle, and inhibiting metastasis and angiogenesis [7]. Their topical application, in particular, offers a potential route for targeted, non-invasive skin cancer treatment. *Cananga odorata* (CO), commonly known as ylang-ylang, is a flowering plant native to tropical Asia, including such as Malaysia and Indonesia [8]. It is well known for its fragrant flowers, widely used in the food, perfume, and aromatherapy industries [9].

Research have identified that CO EO as having antibacterial, antioxidant and anticancer properties [10,11]. However, there was no commercial drug or pharmaceutical product derived from CO EO in the market for skin cancer treatment at present. Its potential as a therapeutic agent in skin cancer remains largely unexplored, making it a promising candidate for further investigation. p53 protein is important for the process of tumor suppression in different cell types by inducing apoptosis [12]. The p53 protein has the function of ceasing cell growth which subsequently prevents mutated cell progeny from dividing [13].

Although several literatures highly suggest that CO EO has antioxidant and anticancer properties on several cell lines, its effect on the *TP53* gene for skin cancer cells is lacking [6]. This study aims to evaluate the cytotoxic effects of *Cananga odorata* essential oil on non-melanoma skin cancer by examining its influence on *TP53* gene expression and p53 protein dynamics, addressing the need for safer and more cost-effective therapeutic options.

METHODOLOGY

Material

All reagents and instruments were available in the Microbiology Pharmacology Physiology and Biochemistry Molecular and Proteomic laboratories of the Department of Basic Medical Sciences, International Islamic University Malaysia. All reagents and chemicals were purchased from Sigma-Aldrich, United States.

Cell Culture

Commercially available HFF1 cells (human foreskin fibroblasts) and A431 cells (human epidermoid squamous carcinoma) (Shanghai iCell Biotechnology Co. Ltd, Shanghai) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Biosera, France) supplemented with 10% Fetal Bovine Serum (FBS) and 100 IU/mL penicillin-streptomycin in a 75 cm^2 flask [14]. Cells were incubated in a humidified 5% CO_2 incubator at 37°C and subcultured when it was 80-90% confluent as per standard method.

Cell morphology study and cell harvesting

Cells were seeded in 6-well plates at 3.0 x 10^7 cells/well and incubated at 37°C in 5% CO_2. After 72 h, the cells were treated with CO EO dissolved in DMSO at 500, 250, and 125 µg/mL, and cisplatin at 125 µg/mL in triplicates for 24 h [15]. The final DMSO concentration was kept at 0.1% in all treatment groups. Cells in 0.1% DMSO (vehicle) were the negative control in all experiments. The images of the cells were captured at 10-20x magnification using an inverted light microscope (Olympus, Japan). Adherent cells were harvested using TRIzol (Thermoscientific, USA) and mechanical scraping (Biosera, France). The cell lysate was stored at -20°C for protein and gene expression studies. The media was kept at -20°C for protein secretion study.

Total RNA and protein expression extraction from cell lysate

RNA was extracted from the cell lysate in TRIzol (Thermoscientific, USA) using innuPREP RNA Mini Kit (Analytik Jena) as per the manufacturer's protocol [16]. The extracted RNA was eluted in 30 µl elution buffer and stored at -80°C. Protein was extracted from the second filtrate from the RNA extraction using cold acetone, as described previously [17]. The resulting protein pellet was eluted in 30 µl RIPA extraction and lysis buffer and stored at -20°C.

Protein extraction from conditioned media

The collected media was added to four volumes of 100% cold acetone and incubated at -20°C for 1 h [18]. The mixture was centrifuged at 13000 *g* using an ultracentrifuge (Beckman Coulter Life Sciences, USA) as described previously [17,19]. The resulting protein pellet was air-dried and suspended in 200 µl RIPA extraction and lysis buffer (Thermoscientific, USA) and stored at -20°C.

Western Blot

Cellular protein for protein expression and secreted protein for protein secretion assays were analyzed separately. In A431 cells, the positive control group was treated with 125 µg /mL cisplatin. The protein extract concentrations were quantified using a Protein Assay BCA Kit (Nacalai Tesque, Japan). Fifty µg protein samples denatured in 2x Laemmli (BioRad, USA) were loaded and separated in 10% acrylamide gel (BioRad, USA) with protein molecular weight marker (BioRad, USA) in the first well. Electrophoresis was run at 100V for 10 minutes, followed by 120V for one hour.

After completion of electrophoresis, the protein was transferred to a PVDF membrane using the Trans-Blot Turbo Kit (BioRad, USA) at 13V for 20 min (20]. Subsequently, the membrane was blocked with 1% bovine serum albumin (BSA) at room temperature for 1 h. The membrane was cut between 42 and 53 kDa for the protein expression assay by referring to the protein molecular weight marker. Membranes were incubated with p53 antibody (sc-263, Santa Cruz Biotechnology, USA) and ACTB antibody (C4, Santa Cruz Biotechnology, USA) at 1:1000 dilution overnight at 4°C [21]. The membrane was incubated with a secondary antibody (m-I g G $_{2^a}$ sc-542731, Santa Cruz Biotechnology, USA) at 1:25 dilution at room temperature for 1 h. Finally, the protein band was detected using the Chemi-Lumi One Series for HRP (Nacalai Tesque, Japan). The signals were captured and quantified using the ChemiDoc Imaging System Machine and ChemiDoc XRS software (BioRad, USA).

cDNA conversion

The total RNA concentration was quantified using a NanoDrop 1000 Spectrophotometer (Thermoscientific, USA). One thousand ng of RNA was converted to 20 µL cDNA using SensiFAST™ cDNA Synthesis Kit (BioLine, UK) and stored at -20°C until use [22].

Gene expression study

Gene expression study was conducted using predesigned *TP53* and *ACTB* primers and probes (IDTDNA, USA) as per standard protocol [23,24]. Primer sequences were as followed:

ACTB-F, ACAGAGCCTCGCCTTTG;
ACTB-R, CCTTGCACATGCCGGAG;
ACTB probe,
/56-FAM/TCATCCATG/ZEN/GTGAGCTGGCGG/3IABkFQ/;
TP53-F, CAAGCAGTCACAGCACATGA;
TP53-R, AATACTCCACACGCAAATTTCC;
TP53 probe,
56-FAM/CCTCCTCAG/ZEN/CATCTTATCCGAGTGGA/3IABkFQ/.

PCR was performed in a 10 µl reaction of 20x primers and probes, PrimeTime™ Gene Expression Master Mix 2x (IDTDNA, USA), cDNA (1:10 dilution), and RNAse-free water. The amplification condition followed the recommended protocol: 95°C for 3 min for polymerase activation, 95°C for 15 sec for denaturation, and 58°C for 1 min for annealing using the CFX96 Touch Real-Time PCR Detection System (BioRad, USA). The cycle was repeated for 50 cycles. Gene expression analyses were conducted using the CFX Manager™ Software (BioRad, USA).

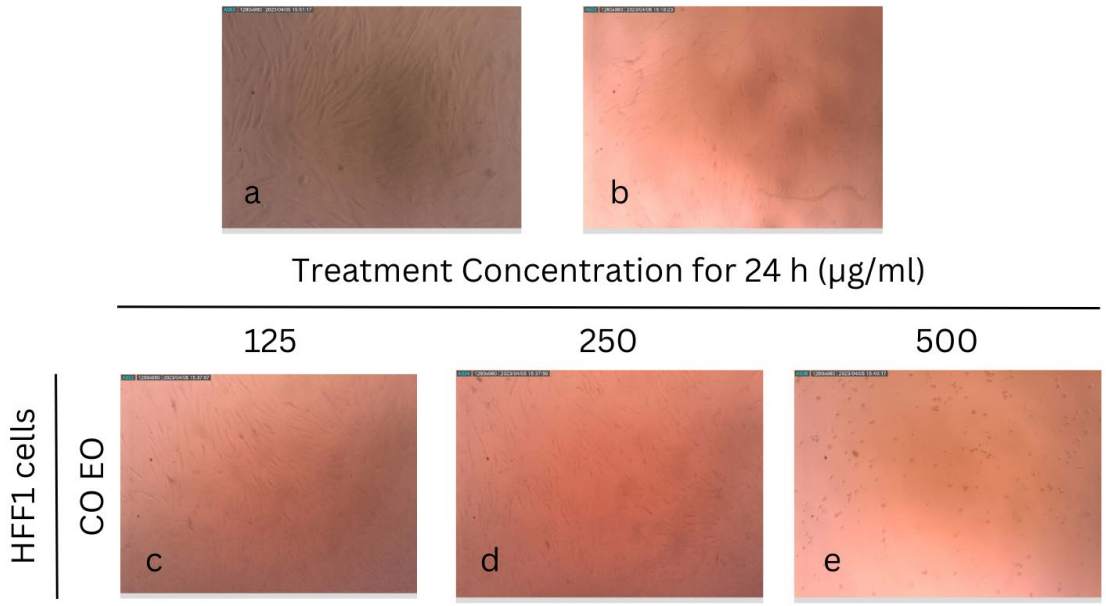

Fig. 1. HFF1 cells after 24 h of a) no intervention, b) control (0.1% DMSO), c) 125 µg/mL CO EO, d) 250 µg/mL CO EO, and e) 500 µg/mL CO EO respectively. Cells were observed under 20x magnification. CO EO = *Cananga odorata* essential oil.

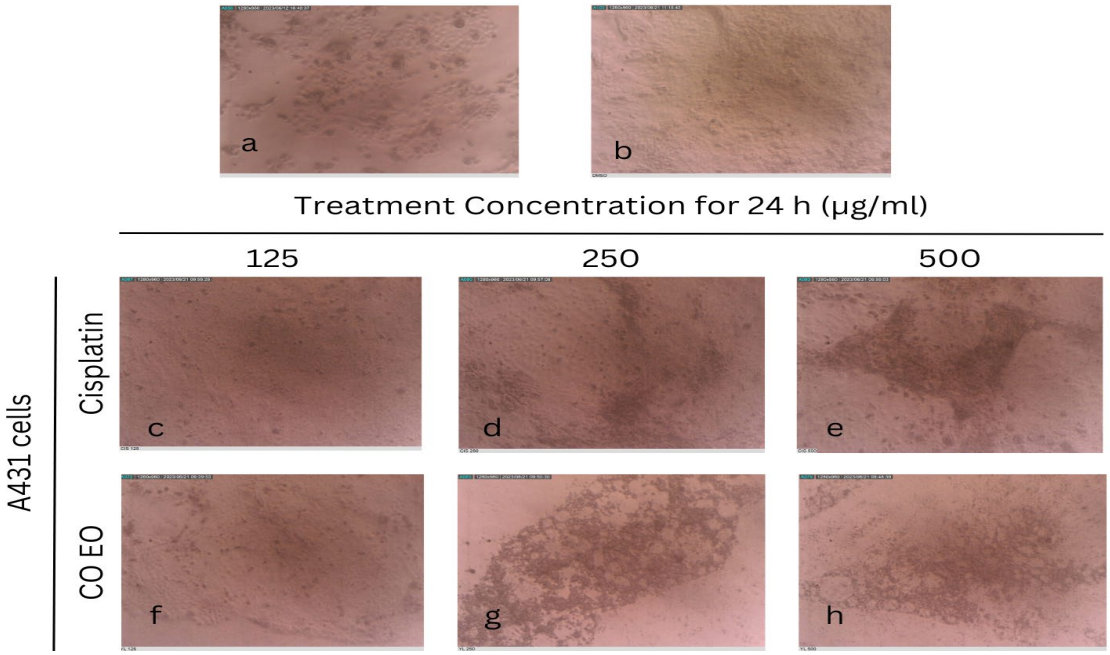

Fig. 2. A431 cells after 24 h of a) no treatment, b) control (0.1% DMSO), c) 125 µg/mL Cisplatin, d) 250 µg/mL Cisplatin, e) 500 µg/mL Cisplatin f) 125 µg/mL CO EO, g) 250 µg/mL CO EO, and h) 500 µg/mL CO EO. Cells were observed under 20x magnification. CO EO = *Cananga odorata* essential oils.

RESULTS

Effect of *Cananga odorata* Essential Oil on Cell Morphology

Untreated and control HFF1 cells displayed a typical homogenous spindle shape (**Fig. 1a-b**). Following treatment with 250 and 500 µg/mL CO EO for 24 h, there was reduced viable spindle-shaped cells and altered morphology (**Fig. 1d-e**). However, there was no significant change in the morphology of cells treated with 125 µg/mL CO EO (**Fig. 1c**). On the other hand, untreated and control A431 cells displayed an epithelial-like, flat, polygonal shape (**Fig. 2a-b**). Following 24 h treatment with cisplatin (**Fig. 2c-e**) and CO EO, there was loss of defined cell margin (**Fig. 2f-h**). A higher number of non-viable (floating) cells was observed in the treated group compared to the untreated group.

Effect of *Cananga odorata* Essential Oil on p53 Protein Secretion

In HFF1 cells, p53 protein secretion was highest in cells treated with 500 µg/mL CO EO, followed by 125 µg/mL CO EO, 250 µg/mL CO EO (**Fig. 3a**). Compared to control, p53 protein secretion was highest (47-folds) in HFF1 cells treated with 500 µg/mL CO EO. Whereas cells treated with 250 µg/mL CO EO and 125 µg/mL CO EO secreted 24- and 30-fold p53 protein compared to control (**Fig. 3c**).

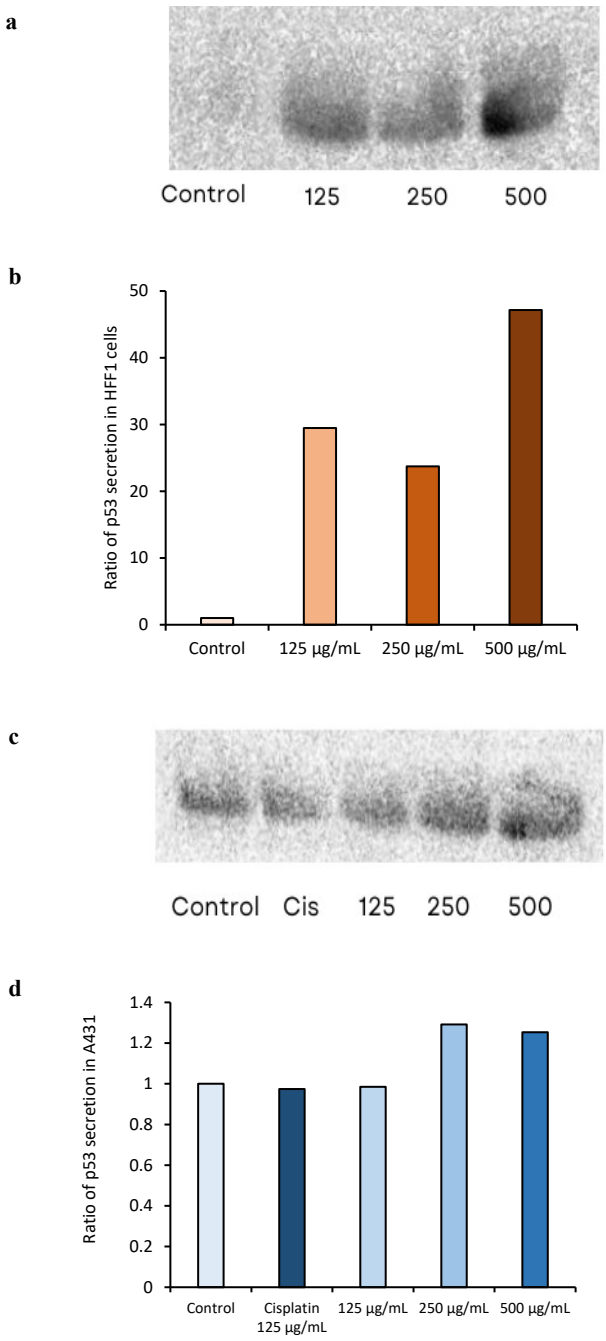

Fig. 3. Western blot analysis of p53 protein secretion and corresponding graph ratios in (a, b) HFF1 cells control (0.1% DMSO) and treated with varying concentrations of CO EO (125, 250, 500 µg/mL) and (c, d) A431 cells control (0.1% DMSO), and treated with Cisplatin (125 µg/mL), and CO EO (125, 250, 500 µg/mL).

The ratios of p53 in each treated group were compared against the control without normalization to β-actin, as β-actin is an intracellular protein and is not secreted into the extracellular media. Since secreted proteins such as p53 are measured directly in the media, normalization to an intracellular loading control like β-actin is not applicable in this context. Instead, comparisons were made relative to the untreated control to account for variations in secretion levels. Western blot analysis showed dose-dependent increases in p53 protein secretion in A431 cells, peaking at 250 µg/mL CO EO with a 1.3-fold increase compared to control (**Fig. 3b**). However, cisplatin-treated cells showed a lower secretion than untreated controls.

Effect of *Cananga odorata* Essential Oil on p53 Protein Expression

We found that the protein extracted from cell lysate was very low in amount (less than 0.3 µg/mL) in the treated groups. Therefore, we were not able to proceed with Western Blot to quantify p53 protein expression. However, we predicted that the level of p53 protein expression was consistent with its secretion. If the level of p53 protein secretion increased, the level of p53 protein excretion was increased, and vice versa.

Effect of *Cananga odorata* Essential Oil on *TP53* Gene Expression

We observed increased *TP53* gene expression in CO EO-treated HFF1 cells compared to control at 125 µg/mL (2.5-fold increase, p= 0.014) and 250 µg/mL CO EO group (3.4-fold increase, $p <$ 0.001) (**Fig. 4**).

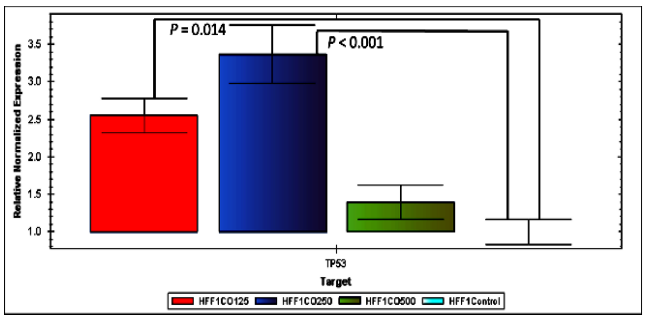

Fig. 4. Relative normalized gene expression of *TP53* in HFF1 cells after treatment with CO EO at 500, 250, and 125 µg/mL for 24 h compared to control (0.1% DMSO). HFF1CO125 = HFF1 cells treated with 125 µg/mL; HFF1CO250 = HFF1 cells treated with 250 µg/mL; HFF1CO500 = HFF1 cells treated with 500 µg/mL CO EO; HFF1Control = HFF1 cells treated with 0.1% DMSO.

In contrast, the relative normalized *TP53* gene expression in A431 cells showed significant *TP53* gene downregulation (p = 0.030) at 125 µg/mL CO EO but no significant changes at higher concentrations (**Fig. 5**).

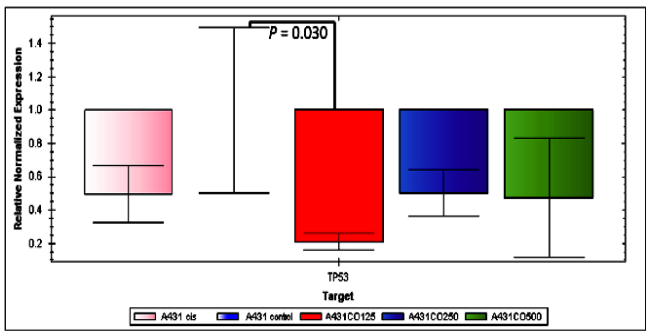

Fig. 5. Relative normalized expression of *TP53* gene in A431 cells after treatment with CO EO at 125, 250, and 500 µg/mL, and 125 µg/mL Cisplatin for 24 h compared to control (0.1% DMSO). A431 cis = A431 cells treated with 125 µg/mL cisplatin; A431CO125 = A431 cells treated with 125 µg/mL; A431CO250 = A431 cells treated with 250 µg/mL; A431CO500 = A431 cells treated with 500 µg/mL CO EO; A431 Control = A431 cells treated with 0.1% DMSO.

DISCUSSION

In this study, we assessed the effect of CO EO in normal skin cell lines (HFF1) and non-melanoma skin cancer cell lines (A431). This study highlights the cytotoxic effects of CO EO on non-melanoma skin cancer cells (A431), demonstrating its potential as a topical anticancer agent. Morphological changes and increased non-viable cells in A431 are consistent with apoptotic processes, aligning with previous findings on essential oil-induced apoptosis [25]. The study of *TP53* gene expression in A431 cells treated with 125 µg/mL CO EO showed significant downregulation but no significant changes in 250 and 500 µg/mL groups. This finding contradicts the finding in a similar study which reported significant upregulation of *TP53* gene expression in triple-negative breast cancer cells treated with CO EO [26]. The lower CO EO concentration (29.01 µg/mL) used in this study albeit at a longer period (48 h) of duration suggests that the effect of CO EO on *TP53* gene could be dose- and time-dependent [26]. This suggests that CO EO mechanisms may vary across cell types and conditions.

We observed an increase in p53 protein secretion in treated A431 cells at 250 and 500 µg/mL CO EO concentration. This could indicate that there is an increased rate of apoptosis in these cancerous cells induced by p53 by stimulating growth arrest and cell death in response to cellular stress [27]. Contradictions between *TP53* gene downregulation and increased p53 protein secretion in A431 cells could stem from post-transcriptional regulation or stabilization mechanisms. One possible explanation for this conflicting finding is that there could be the role of other apoptotic regulator genes, such as the *BCL2, BAX,* and *MdmX*- [28,29]. Further investigations into gene and protein expression levels are essential to elucidate the incomplete apoptotic pathway observed. Moreover, assessing downstream caspase activation would offer a more comprehensive understanding of apoptotic progression. Future studies could validate apoptosis in the cells through methods such as Annexin V/PI staining, TUNEL assay, or analysis of caspase-3 activation.

The observed morphological changes and reduced viable HFF1 cells at 250 and 500 µg/mL CO EO suggests that at higher concentration, CO EO imposed some degree of toxicity on these cells. However, these changes were not seen in cells treated at 125 µg/mL CO EO. Based on our observation, we confer that at this concentration, CO EO is safe to be used in non-cancerous skin cells. This is in line with previous study which reported toxicity of other Eos such as rosemary, citrus, eucalyptus, and lavender by upregulation of xenobiotic and oxidative stress genes [30,31]. The upregulation of *TP53* gene expression and increased p53 secretion in normal HFF1 cells treated with CO EO suggests a protective mechanism, consistent with its role in maintaining genomic stability under stress [32,33]. In normal cells, the secretion of p53 protein should be present but in low concentration [34]. Since we postulate that CO EO at higher concentrations could be harmful to normal skin cells, p53 protein secretion could increase in response to the treatment as a protective mechanism against genetic damage that could lead to cancerous changes.

The observed increase in p53 protein secretion in HFF1 cells treated with 500 ug/mL of CO EO could be due to accumulation of p53 protein which resulted from alteration of other p53 regulatory proteins, for example MdmX protein. The MdmX protein, in conjunction with Mdm2 and the MdmX ring finger domain, can stabilize p53, enhancing its transactivation activity and potentially leading to an accumulation of p53 protein within cells [28]. These results should be interpreted with caution as no technical replicate was performed in the protein secretion assay due to limitations in time and resources. Further data collection is required to determine CO EO mechanism of action on p53 protein expression and secretion in non-cancerous cells. This is because each EO exhibited unique biological mechanisms, targeting protein molecules involved in inflammation, immune responses, tissue remodeling, and wound healing [35].

Clinical relevance lies in CO EO's potential to act as a cost-effective, plant-derived anticancer agent. However, challenges remain in translating these findings into therapeutics, particularly regarding optimal dosing to balance efficacy and safety. In conclusion, this study demonstrates that CO EO induces cytotoxic effects in A431 cells, with associated alterations in *TP53* gene expression and p53 protein secretion. These findings imply its potential as a topical agent for non-melanoma skin cancer. Further investigation into its effect on other apoptotic regulatory genes are recommended to establish its underlying molecular mechanism.

LIST OF ABBREVIATIONS

CO EO: *Cananga odorata* essential oil
DMEM: Dulbecco's Modified Eagle's Medium
EO: Essential oil
FBS: Fetal Bovine Serum
HFF1: human foreskin fibroblasts
A431 cells: human epidermoid squamous carcinoma
5-FU: 5-fluorouracil

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