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IN VITRO CYTOTOXIC EFFECT OF *BOSWELLIA SP.* ESSENTIAL OIL

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ABSTRACT

Boswellia species oleo-gum-resins are known for their anti-inflamatory, anti-arthritic, anti-microbial and anti-neoplastic effects. In the current preliminary study, we wanted to assess the anti-proliferative potential of Boswellia sp. essential oil on MCF7 2D cell line, Caco2 2D cell line and HDFa 2D cell line. Also, the essential oil was analyzed for its capability in inhibiting celular multiplication and adhesion in Caco2 3D cell line. The essential oil was diluted with culture media and 6 dilutions were obtained. Cell viability in 2D cultures was determined using the MTT assay. For the 3D colon adenocarcinoma culture, alamar Blue assay was used. Cell viability assessment was analyzed at 24h, 48h, 72h for MCF-7 and HDFa, respective at 48h, 72h for Caco-2, following essential oil exposure. Although the two different cancer cell lines varied in their sensitivities to the volatile oil treatment, there was a significant supressed cell viability in both of them. High cytotoxic effects were seen at the 1:200 dilution in MCF7, but potent cytotoxicity was observed at 1:400, 1:800, 1:1600 dilutions also. In Caco2 cell line, the essential oils suppressed cell viability in a more potent way following 72h exposure, at 1:200 and 1:400 dilutions. In HDFa cell line, cell proliferation was inhibited after 24h exposure especially at 1:200 and 1:400 dilutions, but the proliferation was highly visible after 72h exposure at all the dilutions tested. The cell proliferation in 3D culture was inhibited in a potent way at 1:200 dilution while the other tests had the opposite effect. All the dilutions blocked the cellular aggregation in

3D Caco2 culture. The essential oil from various Boswellia species is shown to induce tumor cell cytotoxicity in different cancer cell types and blocks the cellular aggregation in colon adenocarcinoma 3D cultures.
KEY WORDS: *anxiety as a state, anxiety as a trait, fullterm newborn, postpartum depression, premature newborn.*

INTRODUCTION

Frankincense, the oleo-gum-resin obtained from Boswellia species trees is internationally known as Indian olibanum, salai guggal, loban or kundur (Hamidpour *et al.*, 2013). Due to its economical and medical importance since ancient times, it is today one of the most important resins in the world. The family of *Burseraceae* consists of 700 species of trees and shrubs, while the number of *Boswellia* species known today is 21 (Al-Harrasi *et al.*, 2019). These species are located in Oman, Yemen, Southern Saudi Arabia, Somalia, Ethiopia and India (Iram *et al*., 2017). Although there are numerous species in the *Boswellia* genus, only a few of them are the source of economically, medically and socially important frankincense: *Boswellia carterii* (East Africa), *Boswellia freana* (Somalia), *Boswellia sacra* (Arabian Peninsula) and *Boswellia serrata* (India) (Hosain *et al.*, 2019).

The oleo-gum-resin is obtained through incisions made in the trunk of *Boswellia* trees which allow the milk like substance to ooze out. By being exposed to air, it solidifies and then it is collected in bamboo baskets, left to harden and eventually chopped in smaller tear like pieces. Frankincense varies in its physical and chemical properties, hence the numerous grades under which it can be found on the market (Al-Harrasi *et al*., 2019, Siddiqui *et al*., 2011). Factors like location, climate, harvesting conditions impact the physico-chemical properties of frankincense, but there can be noted three main components: resin (majority 60-85%), gum (polysaccharides 6-30%) and essential oil (5-9%) (Hamidpour *et al*., 2013).

The resinous part chiefly contains boswellic acids which are acid pentacyclic triterpenes with the chemical formula $C_{32}H_{52}O_4$. It is thought today that these BA are genus-specific, having been isolated solely from the resin of the frankincense tree. The major BAs are α and β-boswellic acids (BA), α and β acetyl-boswellic acids (ABA), 11-keto-β-boswellic (β-KBA) and 3-O-acetyl-11-keto-β-boswelic (β-AKBA) (Iram *et al.*, 2017, Al-Harrasi *et al.*, 2021). These BA are considered to be the main bioactive principles of frankincense.

The gum fraction of frankincense is not as intense studied as the fraction which consists of pentacyclic triterpenes, but it is known that among the polysaccharides D-

galactose, L-arabinose, 4-O-methyl-glucuronic acid and rhamonse are found (Hosain *et al.*, 2019).

The essential oil of frankincense is one of the most commonly used oil in aromatherapy. It can be obtained through hydrodistillation or steam distillation and its composition varies with the species. The oil from *Boswellia serrata* consists mainly of monoterpenes α-pinene, cis-verbenol, trans-pinocarveol, borneol, myrcene, phallendrene, while the oil from *Boswellia sacra* contains chiefly of 1-octanol and 1-octyl acetate and of small amount of cembrene, incensol, isocembrene, isoincensol and α-pinene (Iram *et al*., 2017, Al-Harrasi *et al.*, 2021).

Frankincense is known for its anti-inflammatory activity and since ancient times it has been used in India, China, Africa and Middle East as a treatment for inflammatory diseases (Hamidpour *et al*., 2013). Today, it is known that the boswellic acids found in the oleo-gum-resin are potent inhibitors of 5-lipoxygenase, a key enzyme in the biosynthesis of leukotrienes from arachidonic acid in the celullar inflammatory cascade. Also, the inhibition of nitric oxide synthetase, cyclooxygenase-2 and nuclear factor κB activities plays an important role (Al-Yasiry *et al*., 2016). The anti-inflammatory effect is being supported by clinical trials for the treatment of osteoarthritis of the knee (Majeed *et al*., 2019) and it has showed beneficial effects on the irritable bowel syndrome symptoms (Giacosa *et al*., 2022).

Along with the anti-inflammatory activity, frankincense has shown high anti-bacterial activity due to its acid fractions, anti-fungal properties, anti-depressant-like activity (Al-Harrasi *et al*., 2019, Al-Yasiry *et al*., 2016, Gupta *et al*., 2022)

The oleo-gum-resin from the *Boswellia sp*. has also shown anticancer activities. The essential oil successfully induced tumor cell-specific apoptosis in cultured human cancer breast cells (Suhail *et al.*, 2011) and in cultured human bladder cancer cells (Frank *et al*., 2009). Also, extractive solutions showed antiproliferative effect on colorectal carcinoma cells and cervical cancer cells (Zhang *et al*., 2016).

MATERIALS AND METHODS

Reagents and chemicals. Cell culture medium DMEM, Human Dermal Fibroblast Expansion Medium, fetal bovine serum (FBS), penicillin-streptomycin, phosphate buffered saline (PBS), Trypan Blue Solution 0,4%, Trypsin/EDTA 0,25%, MTT Solution, alamar Blue Cell Viability Reagent, dimethyl sulfoxide (DMSO) were purchased from Invitrogen.

Frankincense essential oil was purchased from doTERRA. The product sheet states that the oil was extracted through steam distillation from resins from *Boswellia*

carterii, sacra, papyrifera and *freana*. The main chemical components listed are α-pinene, limonene and α-thujene. 6 serial dilutions containing essential oil:growth media were made: 1:200 (D1), 1:400 (D2), 1:800 (D3), 1:1600 (D4), 1:3200 (D5) and 1:6400 (D6). The growth media used for preparing the dilutions was specific to each cell line.

Cell cultures. Human breast adenocarcinoma cell line MCF-7, colorectal adenocarcinoma cell line Caco2, human dermal fibroblasts HDFa were purchased from Invitrogen.

MCF-7, Caco2 and HDFa cell lines

MCF-7 and Caco2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. HDFa cells were cultured in Human Dermal Fibroblast Expansion Medium supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were maintained in a cell incubator at 37ºC and 5% CO2. The medium was changed every 2-3 days. When they reached 80% confluence, they were passaged. For the present study, passage 8 was used for MCF-7 cells, passage 4 for Caco2 cells and passage 3 for HDFa cells.

3D cellular aggregates formation

The 3D cellular aggregates were generated from Caco2 cells, passage 6. The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were maintained in a cell incubator at 37ºC and 5% CO2. The medium was changed every 2-3 days.

Cell viability assay for 2D cell cultures. To evaluate the antiproliferative effect of *Boswellia sp*. essential oil, the MTT colorimetric assay was used. MCF-7, Caco2 cell lines (3x$\mathbf{10^5}$ cells/ml) and HDFa cell line (3x$\mathbf{10^4}$ cells/ml) were seeded in 96-well tissue culture plates in 100 µl growth media and were incubated. After 24 hours adherence, the medium was discharged and 100 µl varying dilutions of frankincense oil and growth media were added. The dilutions used were: 1:200, 1:400, 1:800, 1:1600, 1:3200 and 1:6400. A parallel untreated positive control lot was used. All tests and controls were done in triplicate. After 24, 48 and 72hour incubation for MCF-7, HDFa cells, respectively 48, 72 hours incubation for Caco2 cells, the test solutions and the growth media was discharged and changed with 100 µl fresh growth media. 10 µl of MTT was added to each well and incubated for 3 hours at 37ºC. After incubation time, 100 µl DMSO was added to dissolve the blue formazan crystals. The plates were mixed 15 minutes protected from light. The absorbance was measured at 590 nm with Biotek Synergy H1 microplate reader.

The antiproliferative effect was calculated using the formula: Inhibition (expressed in percentage) = [1-(A_{CT}/A_{CC})] x 100, where A_{CT} is the corrected absorbance

for the test solutions and A_{CC} is the corrected absorbance for the untreated positive control.

Cell viability assay and 3D cellular aggregate formation

To evaluate the antiproliferative effect of *Boswellia sp*. essential oil on 3D colorectal adenocarcinoma cells, the alamar Blue colorimetric assay was used. Also, the frankincense oil`s capacity to block cell aggregation was assessed, images of the 3D aggregates being captured with Biotek Lionheart FX. The Caco2 cells were seeded ($1x10^4$ cells/ml) in 96-well Nunclon™ Sphera™ plates in 100 µl growth media for 3D cell aggregates formation. After 24 hours, 10 µl of varying dilutions of frankincense essential oil and growth media were added to the wells that in the end the same six dilutions as previous mentioned were obtained: 1:200, 1:400, 1:800, 1:1600, 1:3200 and 1:6400. A parallel untreated positive control lot was used. All tests and controls were done in triplicate. After 5 days, the tests and the media was changed. The alamar Blue assay was applied after 8 days from the beginning of the experiment as follows: the test solutions and media were discharged and 90 µl growth media was added. 10 µl alamarBlue solution were added to each well and the plate was incubated for 3 hours at 37ºC. After 3 hours, the absorbance was read at 570 nm and 600 nm with Biotek Synergy H1 microplate reader.

The alamar Blue assay incorporates an oxidation-reduction indicator that changes color from blue to red when a chemical reduction of growth media resulting from cell growth is produced. The percent difference in reduction between treated and control cells was calculated using the formula: Percent difference in reduction = [$(\varepsilon OX)\lambda_2$ $A\lambda_1$ - $(\varepsilon OX)\lambda_1$ $A\lambda_2$ of test solution] / [$(\varepsilon OX)\lambda_2$ $A°\lambda_1$ - $(\varepsilon OX)\lambda_1$ $A°\lambda_2$ of untreated positive control] x 100, where λ_1=570nm, $\lambda_2 = 600nm$; $(\varepsilon OX)\lambda_1,\lambda_2$ – molar extinction coefficient of alamarBlue oxidized form; $A\lambda_1,\lambda_2$ – corrected absorbance of test wells; $A°\lambda_1,\lambda_2$ – corrected absorbance of positive growth control well.

RESULTS AND DISCUSSIONS

Suppresion of tumor cell viability by Boswellia sp. essential oil in MCF-7 and Caco2 cell lines

The frankincense essential oil was tested for its capability to inhibit cellular viability in MCF-7 and Caco2 cancer cell lines. 6 serial dilutions were used and using the MTT assay, the cell viability was assesed at 24, 48 and 72 hours post treatment for the MCF-7 cell line, respective at 48 and 72 hours for Caco2 cell line. Although the Boswellia sp. essential oil has not suppressed cell viability in a concentration-dependent manner in MCF-7 cell line, in all three days the most concentrated test solutions (D1,

D2 and D3) showed potent antiproliferative activity. For the Caco2 cells, the highest inhibition rates were seen after 72 hours post treatment. Again, the first three dilutions showed the most potent inhibitory activity.

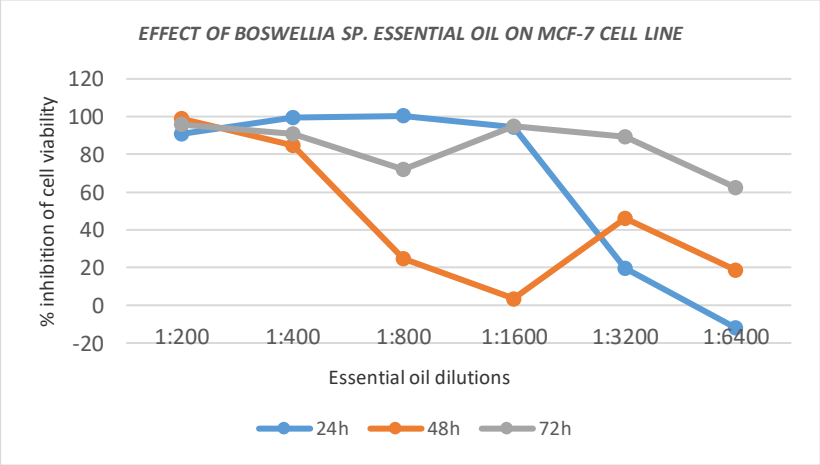


FIG 1. **Inhibition of breast cancer cell viability after Boswellia sp. essential oil treatment.**

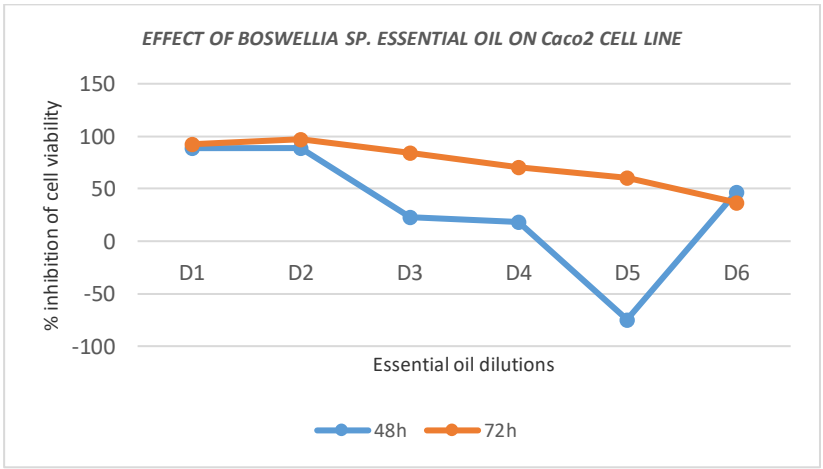


FIG 2. **Inhibition of colorectal adenocarcinoma cell viability after Boswellia sp. essential oil treatment.**

The effect of Boswellia sp. essential oil on HDFa cell line

The toxicity of *Boswellia sp*. essential oil was evaluated using normal human dermal fibrolasts. After 24h adhesion, the same six test solutions were added to the wells and using the MTT assay, the cell viability was assesed at 24, 48 and 72 hours post exposure. There was noticed a decrease in cell proliferation of fibrolasts in the first two days following exposure, but after 72 hours, all tests stimulated the cell viability in a percent >60%, confirming that the essential oil was not toxic.

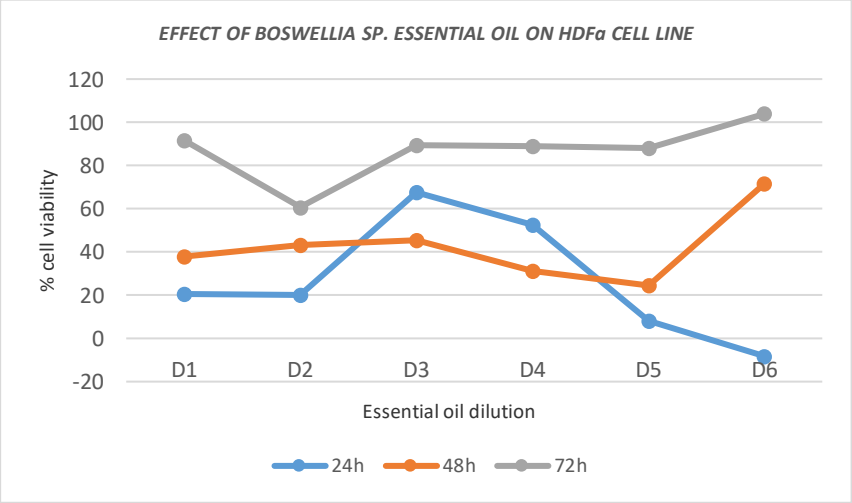


FIG. 3. **Proliferation of normal human dermal fibroblasts afterBoswellia sp. essential oil treatment**.

Suppresion of multicellular tumor aggregates growth by Boswellia sp. essential oil

Caco2 cells are known for the capacity of generating multicellular aggregates. The *Boswellia sp.* essential oil was assessed for its potential of inhibiting cellular proliferation in multicellular tumor aggregates and for its capacity to block the formation of this aggregates. For the former, the alamar Blue assay was used. The most concentrated test solution, D1, showed the most potent antiproliferative effect, while the rest of the dilutions stimulated cell proliferation. However, the frankincense essential oil blocked the aggregation of Caco2 cells, especially dilutions D1, D2 and D3.

Boswellia species extracts and essential oil were and still are widely used in traditional medicine for effects such as anti-inflammatory, anti-arthritic or antimicrobian. Although there are several *Boswellia* species, just a number are of a

medicinally and economically importance: *B. carterii, B. freana, B. sacra, B. serrata* and *B. papyrifera*. The essential oil can be obtained through different extraction methods such as hydrodistillation, steam dystillation or solvent extraction.

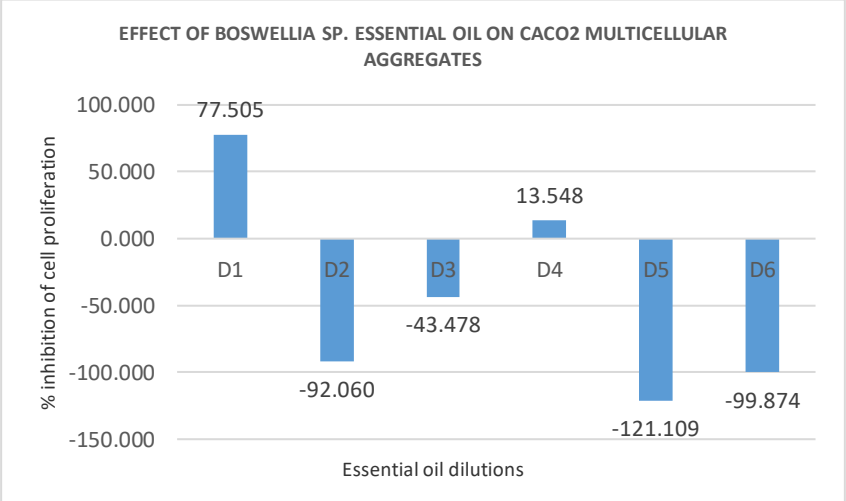
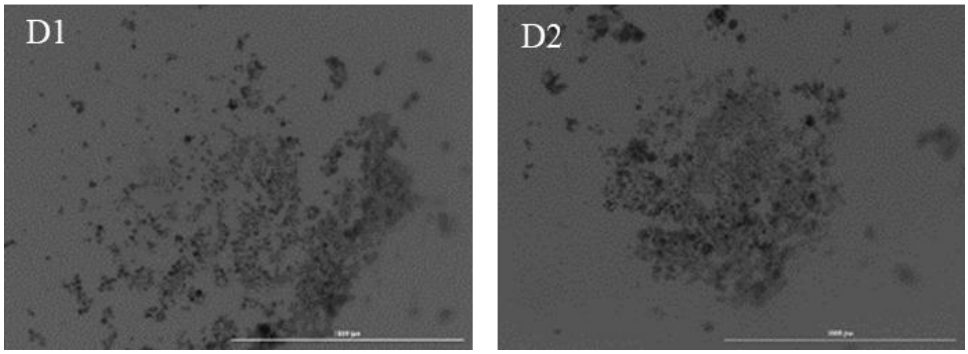


FIG.4. **Inhibition of cell viability in acolon adenorcarcinoma multicellular aggregates after Boswellia sp. essential oil treatment.**



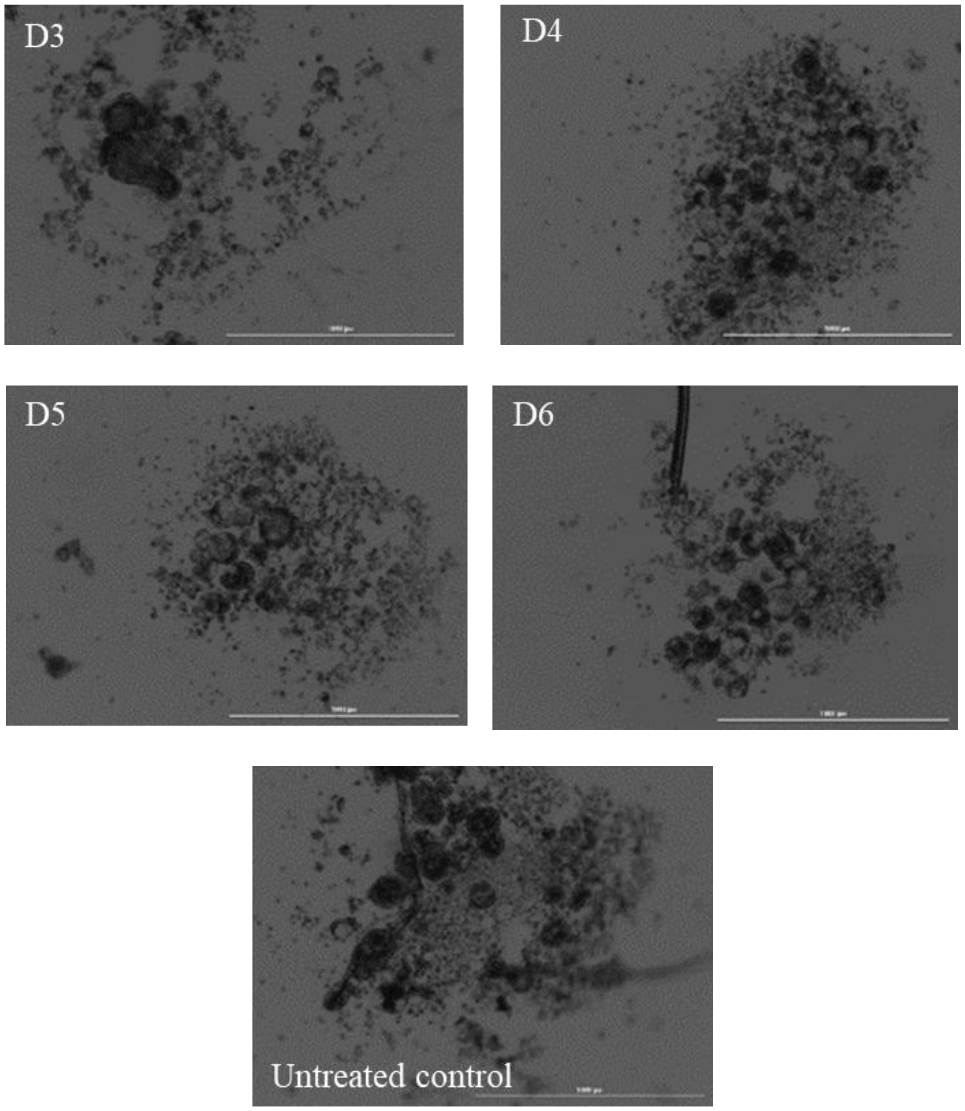


FIG. 5. The effect of Boswellia sp. essential oil on multicellular tumor aggregates. Representative images taken with Biotek Lionheart FX Microscope are presented.

At the present moment, there are several brands worldwide that market frankincense essential oil. For the current study, the doTERRA frankincense essential oil was used. The oil was extracted through steam distillation from resins from *Boswellia carterii, sacra, papyrifera and freana*, α-pinene, limonene and α-thujene being the major constituents. The *Boswellia* oil was tested on three 2D cell lines: MCF-7, Caco2 and HDFa, and on Caco2 multicellular tumor model. Six serial dilutions were made, the proportions of frankincense oil:growth media varying from 1:200 to 1:6400. No other modifications to the test solutions were made. In this preliminary study, we have not determined the exact chemical composition of the oil. In the breast cancer cell line, the inhibitory effect that was noticed was most potent in the wells treated with D1, D2 and D3, in all three days of exposure. For the Caco2 cell line, after 72 hours the highest inhibition rates were noticed. The human dermal fibrolasts were exposed to the essential oil dilution for 24, 48 and 72 hours. At first, a decrease in cell proliferation appeared, but after 72 hour, the oil stimulated cell viability by more than 60%. Although 2D cell cultures are easily to use and manipulate, they lack the complexity of malignant tumors, therefore the multicellular tumor spheroid models that mimic the three dimensionality of tumors are widely used (Nagelkerke *et al.*, 2013). For the curent study, Caco2 cells were used to create multicellular aggregates, which were blocked from forming by D1, D2 and D3 frankincense oil dilutions. D1 also inhibited cell proliferation by 77%.

There are several studies conducted on the cytotoxic effect of Boswellia resin. For example, Mahmoud M Suhail and his collaborators obtained the essential oil through steam distillation from *Boswellia sacra* resins. The oil was tested in varying concentrations on three breast cancer cells and one immortalized normal human breast cell line. It was observed that the frankincense essential oil induced specific cytotoxicity in the human breast cancer lines, while it increased the viability of the normal breast cells (Suhail *et al*., 2011).

Mark B Frank and his collaborators evaluated the effect the Young Living`s frankincense essential oil has on human bladder cancer cells and on immortalized normal bladder urothelial cells. In a similar way as the effect noticed on breast cancer cells, the oil suppressed cell viability in the cancerous bladder cells, activating genes responsible for apoptosis, cycle arrest and cell growth suppresion. The noticed effect affected just the carcinoma cells (Frank *et al*., 2009).

Consequently, the frankincense essential oil has shown on various tumor cells specific cytotoxicity. But it is not only the essential oil that possesses such effects, but also various solvent extracts. One study realized liquid extractions of frankincense resin with methanol, water, ethyl acetate, chlorofrom and hexane, these extracts being tested

on Caco2 and HeLa cell lines. An antiproliferative effect was observed, the colorectal adenocarcinoma cell line being more sensible to the extracts than the cervical carcinoma line (Zhang *et al*., 2016).

This preliminary study showed the effects that doTERRA`s frankincense essential oil has on three 2D cell lines and on one multicellular tumor model. In the right dilutions, it has successfully inhibited cell proliferation in MCF-7 and Caco2 cells and blocked the aggregation of Caco2 cells in 3D cultures. These results support the studies published on this wide theme.

CONCLUSIONS

The essential oil extracted from *Boswellia carterii, B. sacra, B. payrifera* și *B. freana* resins purchased from doTERRA, with a composition that consists of α-pinene, limonene and α-thujene, showed during this preliminary study antiproliferative activity on breast and colon adenocarcinomas cells. It also inhibited multicellular aggregation. The potential of frankincense essential oil should be further studied, studies on the cellular toxicity, chemical standardization and for elucidating the mechanism of action should be conducted.

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