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An *Ex vivo* Apoptotic and Cytotoxic Effects of Frankincense on Oral Squamous Cell Carcinoma Cell Line

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Abstract

BACKGROUND: Oral squamous cell carcinoma is accounting for almost 90% of oropharyngeal cancer diagnoses. Natural herbal medicine can use as an alternative, or complementary, or adjunctive for cancer treatment. Frankincense and its combination with myrrh have anticancer effects on different cancer types.

METHODS: In this research, aqueous and methanolic extracts of frankincense and the combination of aqueous extract of frankincense and myrrh were applied on tongue squamous cell carcinoma cell line to study their cytotoxic and apoptotic effect by the assessment of cell viability and cytotoxicity, caspase 3 and 8 activation, reactive oxygen species activity, mitochondrial membrane potential, morphological changes, and nuclear area factor measurements.

RESULTS: The result showed that aqueous and methanolic extracts of frankincense have cytotoxic and apoptotic effects in a concentration-dependent manner with an IC_{50} value of 21.05 ± 1.27 μM for aqueous extract, 36.72 ± 2.07 μM for methanolic extract, and IC_{50} value of 1.31 ± 0.04 μM for combination of aqueous extract of frankincense and myrrh extract after 24 h.

CONCLUSION: Different extracts of frankincense and the combination of aqueous extract of frankincense and myrrh extract exhibited cytotoxic and apoptotic effects by reducing the cell viability and activating caspases 3 and 8 causing intrinsic- and extrinsic-mediated apoptosis pathways activation with the involvement of oxidative stress that was conceivable with cytonuclear morphological alterations results.

Introduction

Oropharyngeal cancer accounts for approximately 4% of all cancers [1], and most of diagnosed cases are squamous cell carcinoma (SCC) [2].

Different therapeutic modalities (e.g., surgery, radiotherapy, and chemotherapy) have been used to treat oral SCC (OSCC) with harmful side effects [1].

The most frequent side effects of chemotherapy and radiotherapy are high costs and long therapy courses, nausea, vomiting, and non-specific cellular effects, leading to diffuse cell death and tissue toxicity, oral side effects (oral mucositis, hyposalivation, loss of taste, dental caries, osteoradionecrosis, and trismus), and development of multidrug resistance [3].

Therefore, it might be helpful to use herbal medicine as an alternative, complementary, or adjunctive treatment to treat cancer as natural compounds might reduce the adverse side effects [4], [5].

Frankincense and myrrh have been used as incense in religious and cultural ceremonies since the beginning of written history. Their common medicinal properties are used in the treatment of inflammatory conditions, some cancerous diseases, and wound healing [6].

Frankincense (also known as olibanum (oleo-gum)) is an aromatic resin obtained from trees of the genus *Boswellia* in the family *Burseraceae* which has been used since ancient Egyptians [7].

Different extracts from frankincense have been exhibited several health-supporting properties such as anti-inflammatory, antibacterial, antifungal, and anticancer activities [8], [9], [10].

Frankincense was previously reported to exhibit cytostatic and apoptotic effects in multiple human cancer cell lines, including melanoma [11], hepatocellular carcinoma [12], breast cancer [13], and colon cancer [14].

The combination of frankincense and myrrh is very popular and has attracted worldwide attention not only because of their complementary scents also because of their synergy that produces even greater benefits as anti-inflammatory, analgesic, antioxidant, and antitumor effects [15], [16].

The aqueous extract of the combination of frankincense and myrrh is widely used in clinics to obtain synergistically pain relief [17] and accelerate healing of oral ulcers [18].

The biological activities of both plants have motivated us to search for their effects and their combination effects on OSCC.

Methods

We designed the study to evaluate the anticancer properties of frankincense and the combination of aqueous extract of frankincense and myrrh on the OSCC cell line.

Frankincense and myrrh extract preparation

The dried frankincense and myrrh were bought from the herbs market. The two plants were authenticated and identified by associate professor at the Pharmacognosy Department, Faculty of Pharmacy, Beni-Suef University, Egypt. The extracts were prepared at the Pharmacognosy Department, Faculty of Pharmacy, Assiut University, Egypt.

Preparing aqueous extract

The fine powder of dried frankincense and myrrh was macerated in cold filtered water (1:1 resin: water by volume) for 3 days with shaking frequently to ensure complete extraction. The solutions were filtered through filter paper, after which clear aqueous extract was obtained.

Preparing methanolic extract

The fine powder of dried frankincense was macerated in 90% methanol for 7 days, then, the methanolic extract was filtered and evaporated using a rotary evaporator and freeze dryer to give a dried methanolic extract then suspended in distilled water.

Experimental drugs and reagents

Methanol (Fisher Scientific, Waltham, Massachusetts, USA), penicillin, fetal bovine serum (FBS), and streptomycin. MTT-based toxicology assay kit (SIGMA, Saint Louis, Missouri, USA). RNeasy Mini Kit (QIAGEN, Germany). BIORAD iScriptTM one-step RT-PCR Kit (USA). Reactive oxygen species (ROS). Detection Assay Kit (BIOVISION, California, USA). Mitochondrial Membrane Potential Assay Kit (Cell Signal, Danvers, Massachusetts, USA).

Cell line and cell culture protocol

Human tongue SCC cell line (SCC-25) (ATCC® CRL-1628™, American Type Culture Collection, Manassas, VA, USA) was cultured at 37°C in a humidified 5% CO_2 atmosphere for 24 h in 10 μg/ml of insulin (Sigma, Saint Louis, Missouri, USA), Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, and 1% penicillin-streptomycin. The cells were seeded in either 6-well or 96-well plates for further tests.

Grouping

- Group I includes untreated (SCC-25) cell line as a control group.
- Group II includes (SCC-25) cell line treated with different concentrations (pre-IC_{50}, IC_{50}, and post-IC_{50}) of frankincense aqueous extract for 24 h.
- Group III includes (SCC-25) cell line treated with different concentrations (pre-IC_{50}, IC_{50}, and post-IC_{50}) of the combination of aqueous extract frankincense and myrrh for 24 h.

Cell viability and cytotoxicity assay (MTT assay)

After cell culturing, 1.2–1.8 × 10^3 SCC-25 cells/well were seeded in 96-well culture plates, then incubated with a 4-fold serially diluted concentrations of the frankincense methanolic and aqueous extracts starting from 100 to 0.39 μg at 37°C. After incubation for 24 h, cell viability was assessed using MTT assay kit. Spectrophotometric absorbance was measured using an ELISA Plate Reader spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 430 nm with the reference of 630 nm. The assay was performed in triplicate for each concentration. The percentage of cell viability at different extracts concentration was obtained using this formula: Percentage cell viability $(A_t - A_b)/(A_c - A_b) \times 100\%$, where, A_t is the absorbance value of the test compound, A_b is the absorbance value of the negative control (blank), and A_c is the absorbance value of the positive control. Data were expressed relative to the mean optic density found in the untreated cells, which was arbitrarily defined as 100%.

The IC_{50} value was calculated using a linear regression equation after plotting the percentage of cell viability against drug concentration.

The more effective extract was chosen according to the cell viability% and IC_{50} value for further assays and the combination extract was prepared by

mixing the aqueous extract of frankincense and myrrh extract according to the IC_{50} values of each extract, MTT assay was performed for the combination of aqueous extract of frankincense and myrrh as mentioned previously.

Cells were then treated for 24 h with pre-IC_{50}, IC_{50}, and post-IC_{50} concentrations aqueous extract of frankincense and combination of aqueous extracts of frankincense and myrrh which were determined depending on the results of MTT assay for further assays as pre-IC_{50} is the half of the IC_{50} value, and the post-IC_{50} is 1.5 of the IC_{50} value.

Caspase 3 and 8 activity assays by real-time PCR

3×10^6 SCC-25 cells/well were seeded in 6-well culture plates then treated with the indicated groups. RNA isolation and extraction (spin technology) were performed using RNeasy Mini Kit (QIAGEN, Germany). Reverse transcription and amplification were performed using BIORAD iScriptTM One-Step RT-PCR Kit (USA). In relative quantification, all samples were normalized to a constantly expressed housekeeping mRNA (reference mRNA) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous expression standard. Only one reference gene was used in the present study because of limitation for interpretations.

The following gene-specific primers were used:

Caspase 3 F 5′-CTCGGTCTGGTACAGATGTCGA-3′
Caspase 3 R 5′-CATGGCTCAGAAGCACACAAAC-3′.
Caspase 8 F 5’-ACAATGCCCAGATTTCTCCCTAC-3’
Caspase 8 R 5’-CAGACAGTATCCCCGAGGTTTG-3’
GAPDH F 5’-GCA AGT TCA ACG GCA CGA TCA AG-3’
GAPDH R 5′-CTA CTC AGC ACC AGC ATC ACC-3’

The n-fold change in mRNAs expression was determined according to the comparative cycle threshold method ($2^{-\Delta\Delta CT}$) [19]. Data are expressed relative to the caspase-3 and 8 activities of the untreated cells, which were arbitrarily defined as 1.

ROS assay

This method was performed by ROS detection assay kit, according to the manufacturer’s instructions. The SCC-25 cells (1.2×10^4/well) were seeded and allowed to adhere overnight in a 96-well dark-sided culture plate, then incubated with ROS assay buffer in the dark for 45 min at 37°C. The cells were washed, the fluorescence intensity was measured immediately. Then, the cells were treated with the indicated groups for 24 h. The fluorescence was measured using Tecan Spark® multimode microplate reader (Männedorf, canton of Zürich, Switzerland) at 495 nm excitation and 529 nm emission wavelength. All treatments were done in triplicate. Data were expressed relative to relative fluorescence units (ΔRFU) of the untreated cells, which was arbitrarily defined as 100%.

Mitochondrial transmembrane potential ($\Delta\Psi_m$) assay

This method was performed by Mitochondrial Membrane Potential Assay Kit, according to the manufacturer’s instructions. SCC-25 cells (1.2×10^4/well) were plated and incubated in a 96-well plate in a warm culture medium overnight, then treated with the indicated groups. A 10 μl of 2 μM tetramethylrhodamine, ethyl ester, perchlorate labeling solution (TMRE) was added and placed in an incubator (37°C and 5% CO_2) for 20 min then washed 3 times with warm phosphate-buffered saline. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) solution used as a positive control. Intensities of the fluorescence were measured using BD FACSCalibur™ Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA) (excitation about 550 nm and emission about 580 nm). The assay was performed in triplicate. Data were expressed relative to ΔRFU of the untreated cells, which was arbitrarily defined as 1.

Microscopic examination

SCC-25 cells were treated with the indicated groups as described above then applied on heat-sterilized coverslips. The SCC-25 treated cells were stained with (H&E) to provide a qualitative analysis of the morphology of these cells according to Fischer *et al.*, 2008 [20], procedures.

The stained cells were viewed, studied, and photomicrographed at the power of X1000 (Oil immersion) using a digital video camera (C5060, Olympus, Japan) which is mounted on a light microscope (BX60, Olympus, Japan).

Nuclear morphometric analysis

The photomicrographed images were analyzed using image analysis software (Image J 1.53k, NIH, USA) in the Faculty of Dentistry, Beni-Suef University, Egypt.

The images were corrected for contrast and brightness automatically, converted to 8-bit grayscale. The threshold was applied to select the SCC-25 nuclei. From these threshold images, ImageJ measured the surface area and circularity of the nuclei. The data were tabulated in a Microsoft Excel sheet (Microsoft Office 356). Nuclear area factor (NAF) will be calculated using the formula: NAF = Circularity * Surface area.

Statistical analysis

Statistical analysis was performed using the Statistical Program Social Sciences (SPSS) for Windows version 24 (SPSS Inc., Chicago, IL, USA).

Statistical significance between groups was determined using one-way ANOVA and *post hoc* (Tukey B) tests. Graphics were done by Excel Microsoft Office 365. Statistical significance was defined as $p < 0.05$ and indicated by an asterisk (*).

Result

MTT – Cell viability assay

Both aqueous and methanolic extracts of frankincense exhibited cytotoxic effects on SCC-25 cells viability in a concentration dependent manner (Figure 1).

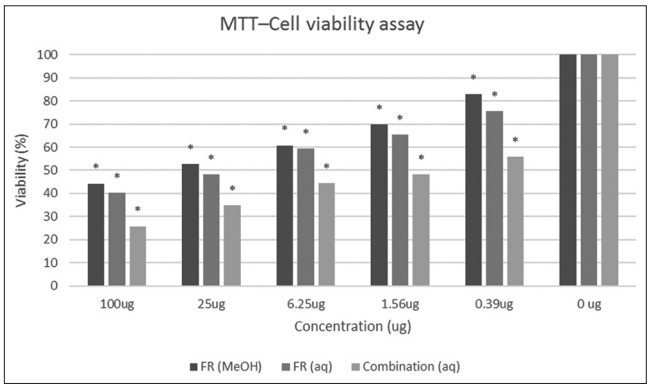

*Figure 1: Bar chart illustrating the concentration-dependent inhibitory effect of different concentrations of methanolic and aqueous extracts of frankincense and combination of aqueous extracts of frankincense and myrrh after 24 h on SCC-25 cell viability using MTT assay. *Frankincense methanolic extract = FR (MeOH), frankincense aqueous extract = FR (aq), and combination of aqueous extract of frankincense and myrrh = Combination (aq)*

We chose to continue our research work on aqueous extracts as it was more cytotoxic on SCC-25 than methanolic extracts according to MTT assay results (Figure 2).

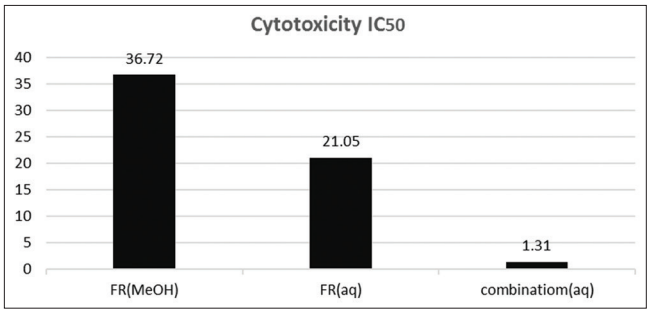

Figure 2: Bar chart showing the IC_{50} values arranged from the highest to the least (left to right)

The combination of aqueous extract of frankincense and myrrh had the most cytotoxic effect on cell viability.

There was a statistical significance difference between the control and different concentrations of each extract (Figure 1).

Caspase 3 and caspase 8 activity assay

Caspase 3 and 8 activations were detected in different concentrations. The combination of aqueous of frankincense and myrrh extract had the highest effect at all concentrations on caspase 3 and 8 activations (Table 1).

ROS activity assay

ROS activity increased in all concentrations in a concentration-dependent manner (Figure 3).

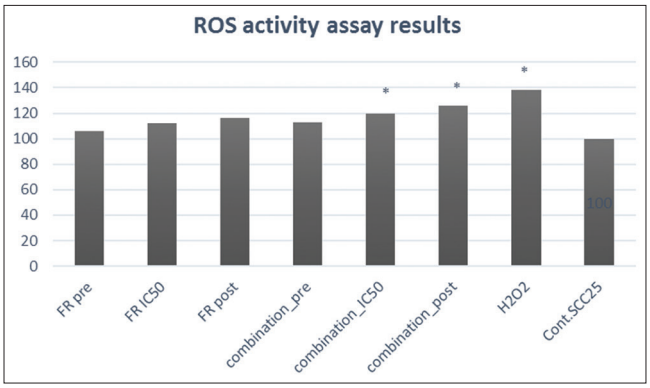

*Figure 3: Bar chart illustrating the effect of different concentrations of aqueous extracts of frankincense and the combination of aqueous extracts of frankincense and myrrh after 24 h on ROS activity. *Indicate the statistical significance with $p < 0.05$*

The combination extract had the highest effect on ROS activity at all concentrations while frankincense had the least effect on ROS activity at all concentrations.

Mitochondrial membrane potential ($\Delta\Psi_m$) assay

A concentration-dependent decrease of mitochondrial membrane potential was detected in all concentrations.

The combination of aqueous extracts of frankincense and myrrh had the highest effect at all concentrations (Figure 4).

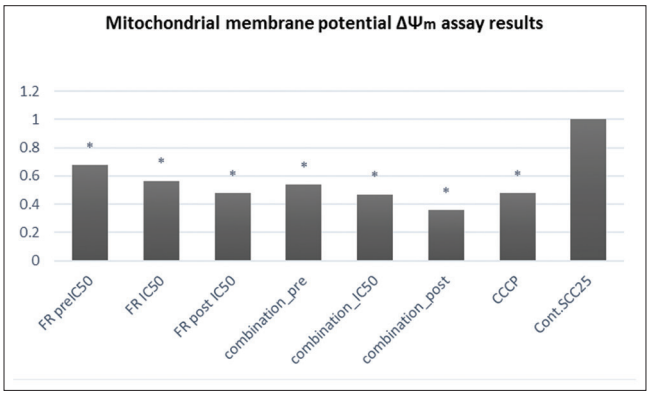

Figure 4: Bar chart illustrating the effect of different concentrations of aqueous extracts of frankincense and the combination of aqueous extracts of frankincense and myrrh after 24 h on ($\Delta\Psi_m$) activity (*) indicate the statistical significance with $p < 0.05$

Photomicrography and cytonuclear morphological changes evaluation results

Our study showed that SCC-25 cells treated with different concentrations of aqueous extracts of frankincense and the combination of aqueous extracts of frankincense and myrrh after 24 h induced morphological alterations of the cell membrane and the nucleus in comparison to control cells.

In our study, the action of different concentrations of aqueous extract of frankincense was mainly of apoptotic and secondary necrotic cell death mode, and as the dose increased, the secondary necrosis becomes the more prevalent mode of cell death (Figure 5a-c).

The inhibitory mechanism of the combination of aqueous extract of frankincense and myrrh seemed to be through activating of apoptosis, secondary necrosis, and necrosis by increasing the concentration (Figure 6a-c).

Results of nuclear morphometric analysis

The average surface area and circularity of the nuclei of the SCC-25 treated cells by different concentrations of frankincense aqueous extracts and the combination of aqueous extracts of frankincense and myrrh after 24 h decreased compared to control cells, resulting in dramatic reduction of NAF. These results supported the appearance of apoptotic and secondary necrotic changes during cytological examination (Figure 7).

Post hoc multiple comparison test (Tukey B) revealed a statistically insignificant difference among mean values of NAF of SCC-25 cells treated with different concentrations of frankincense aqueous extracts and combination aqueous extracts of frankincense and myrrh after 24 h to each other. However, there was a statistically significant difference among mean values of NAF of SCC-25 cells treated with different concentrations of aqueous extracts of frankincense and combination aqueous extracts of frankincense and myrrh after 24 h when compared to the mean value of control cells.

Discussion

The search for new drugs that display activity against different types of cancer has become the

Table 1: The effect of different concentrations of aqueous extracts of frankincense and the combination of aqueous extracts of frankincense and myrrh after 24 h on caspase 3 and caspase 8 activity using RT-PCR

Sample	Pre-IC_{50} concentration		IC_{50} concentration		Post-IC_{50} concentration	
	Caspase 3	Caspase 8	Caspase 3	Caspase 8	Caspase 3	Caspase 8
Frankincense (aq)	3.38	1.78	6.5	2.19	10.9	3.87
Combination (aq)	7.68	2.77	12.6	3.73	18.5	5.96

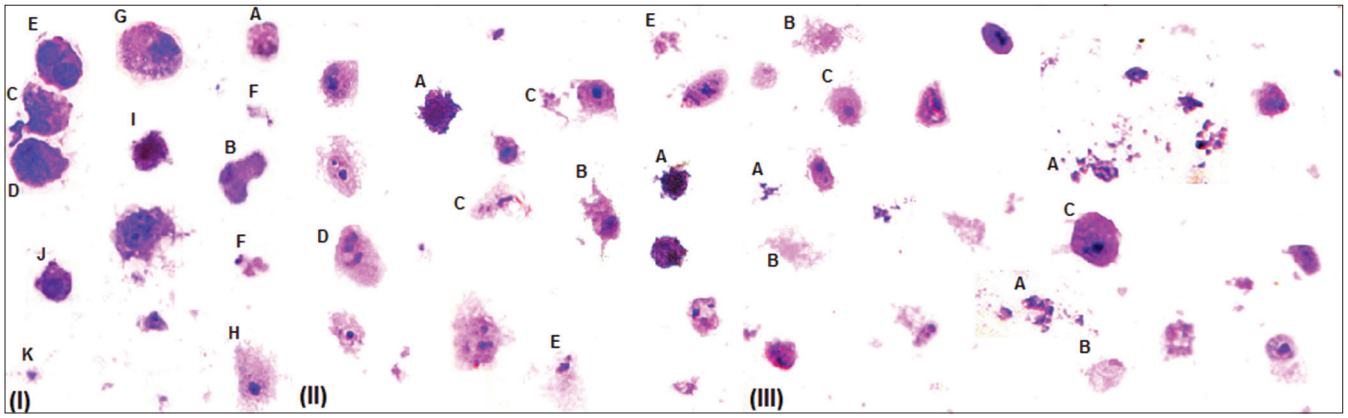

Figure 5: (I) A photomicrograph of SCC-25 cells 24 h after treatment with pre-IC_{50} of frankincense aqueous extract showing (a) cell rounding and cup shape chromatin condensation, (b) marked membrane irregularity, blebbing, peripheral chromatin condensation, and nuclear pyknosis, (c) membrane blebbing contains organelles and fragmented nucleus and nuclear fragmentation (karyorrhexis) of apoptotic cell, (d) cell rounding and nuclear fragmentation (Karyorrhexis), (e) secondary necrotic cell (fragmentation and intense chromatin condensation), (f) apoptotic body, (g) secondary necrosis with intense chromatin condensation and cell swelling, (h) advanced secondary necrosis with chromatolysis and cell swelling, (i) apoptotic cell with marked membrane blebbing and nuclear fragmentation (Karyorrhexis), (j) cellular shrinkage, membrane irregularity, blebbing, and nuclear pyknosis (k) cell debris (H and E ×1000 oil). (II) A photomicrograph of SCC-25 cells 24 h after treatment with IC_{50} of frankincense aqueous extract showing (a) cellular shrinkage, membrane irregularity, plasma membrane blebbing contain organelles and fragmented nucleus, and nuclear fragmentation (Karyorrhexis), (b) echinoid spikes, (c) apoptotic body, (d) apoptotic secondary necrosis, showing nuclear apoptotic changes (fragmentation and intense chromatin condensation) and a lysed cytoplasm. (e) Cell debris (H and E ×1000 oil). (III) A photomicrograph of SCC-25 cells 24 h after treatment with post-IC_{50} of frankincense aqueous extract showing (a) apoptotic body, (b) cell debris, and (c) apoptotic secondary necrosis, showing nuclear apoptotic changes (fragmentation and intense chromatin condensation) and a swelled lysed cytoplasm (H and E ×1000 oil)

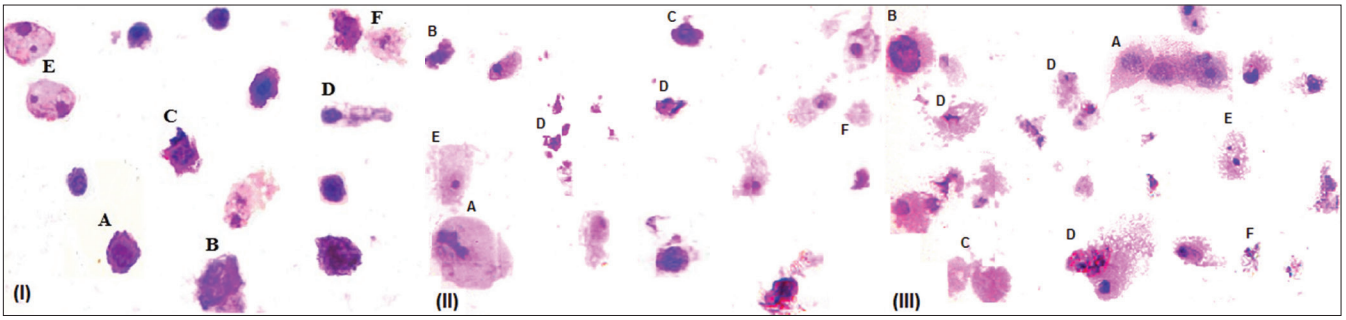

Figure 6: (I) A photomicrograph of SCC-25 cells 24 h after treatment with pre-IC_{50} of combination aqueous extract showing (a) membrane irregularity and nuclear fragmentation, (b) more membrane irregularity and nuclear fragmentation (karyorrhexis), (c) cellular shrinkage, blebbing formation, and nuclear fragmentation, (d) cell shrinkage, vacuolation of cytoplasm, and nuclear pyknosis, (e) apoptotic secondary necrosis, showing nuclear apoptotic changes (intense chromatin condensation and a swelled lysed cytoplasm, (f) A more advanced secondary necrosis with chromatolysis and cell swelling (H and E ×1000 oil). (II) A photomicrograph of SCC-25 cells 24 h after treatment with IC_{50} of combination aqueous extract showing (a) apoptotic secondary necrosis, showing nuclear apoptotic changes (fragmentation and intense chromatin condensation), and cell swelling, (b) membrane irregularity, cellular shrinkage, and nuclear fragmentation (karyorrhexis), (c) membrane irregularity plasma membrane blebbing and nuclear fragmentation karyorrhexis (d) apoptotic body, (e) apoptotic secondary necrosis with a lysed cytoplasm with nuclear apoptotic changes, (f) cell debris (H and E ×1000 oil). (III) A photomicrograph of SCC-25 cells 24 h after treatment with post-IC_{50} of combination aqueous extract showing (a) cell lysis and nuclear fading (karyolysis), (b) marked nuclear swelling with cell burst, (c) cell debris, (d) advanced secondary necrosis with chromatolysis, cell swelling, and lysed cytoplasm, (e) a more advanced secondary necrosis with chromatolysis, cell swelling, and lysed cytoplasm, (f) apoptotic body (H and E ×1000 oil)

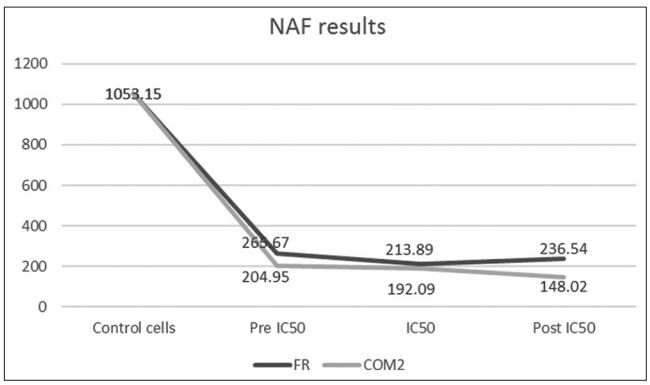

Figure 7: Line chart illustrating the decrease of mean values of NAF of SCC-25 treated cells after 24 h

most interesting subjects. In this area, plants and phytochemicals have played a dominant role in the development of therapeutic drugs, especially those with a long history in the treatment of cancer through controlling several molecular pathways which are associated with cancer growth [4], [21], [22], [23].

In this study, cell viability and cytotoxicity, caspase 3 and 8 activation, ROS activity, ($\Delta\Psi_m$), morphological changes, and NAF were assessed to find out the likely mechanisms of cytotoxicity, growth inhibition, and apoptosis of the studied extracts.

In our study, the whole extract was used. According to results of Xia *et al.*, 2017 [24]; Ni *et al.*, 2012 [25]; and Suhail *et al.*, 2011 [26], the bioactivity of frankincense may not depend on only one compound but also depend on several compounds or synergistic effects of different compounds or potential metabolite resulting in a complex action mechanisms.

The polar extracts of plants (such as aqueous and methanolic) have more anticancer effects than nonpolar ones [27]. Hence, we used water and methanol as a solvent.

Studies evaluating frankincense effect on OSCC are so deficient.

Similar to our result, Jaafari-Ashkavandi *et al.*, 2017 [28] showed anticancer effects of frankincense aqueous extract on OSCC as it inhibited cell growth and viability in a concentration-dependent manner.

Zhang *et al.*, 2011 [29], demonstrated the chemopreventive effect of boswellic acid (a resin acid of frankincense) on 7, 12-dimethylbenzanthracene (DMBA)-induced OSCC in the hamster cheek pouch model.

Similar to our result, Jasim *et al.*, 2019 [30], stated that frankincense (aqueous and methanolic extracts) suppressed viability and induced cytotoxicity and apoptosis in pancreatic and breast cancer cell lines. Likewise, Goa *et al.*, 2020 [31], revealed that frankincense ethanolic extract had an anti-proliferation effect on multiple myeloma cells. Alipanah and Zareian, 2018 [32], declared that frankincense alcoholic extract had anticancer properties on the breast cancer mouse model as it can reduce tumor growth. Huang *et al.*, 2000 [33], stated that frankincense methanolic extract inhibited human leukemia cells through the inhibition of DNA synthesis.

According to Al-Harrasi *et al.*, 2018 (A and B) [34], [35]; Xu *et al.*, 2018 [36], the aqueous extract of frankincense offers a novel and practical strategy for hepatocellular carcinoma therapy considering the cancer-related inflammation.

In our study, the aqueous extract was more effective than methanolic extract, in accordance with a by Jasim *et al.*, 2019 [30], and Zhang *et al.*, 2016 [37], study results.

Unrelated to our results, Namdarian *et al.*, 2018 [38], revealed that frankincense alcoholic extract

had better results than the aqueous extract in cytotoxicity induction on fibroblast cells.

The pharmacological properties of the combination are mostly synergistic, including synergistic anticancer, synergistic anti-inflammatory, synergistic analgesic, synergistic antibacterial, and synergistic blood-activating effects [6], [15], [16], [17].

To the best of our knowledge, no research was conducted to investigate the effect of the frankincense and myrrh combination in relation to inhibition of cancer cell growth in OSCC as this was the 1st time that the combination has been used. Hence, the current *in vitro* results are supported and correlate with researches using different cancer cells.

Analogous to our results, Cao *et al.*, 2019 [16]; Xu *et al.*, 2018 [36]; Cheng *et al.*, 2016 [39]; Guo *et al.*, 2015 [40]; and Qin *et al.*, 2015 [41], showed that the combination of frankincense and myrrh exerts a synergistic anticancer effect on the same cell line.

In contrast to our results, Chen *et al.*, 2013 [42], reported no synergistic effect between the frankincense and myrrh combination. Moreover, Goa *et al.*, 2020 [31], showed that the combination of aqueous extracts was less efficacious than the combination of ethanolic extract.

The discrepancy among the results may be attributed to the presence of various frankincense trees that produce different resin types with variable biochemical properties, due to geographical sources of resins (different soil and climatic conditions), harvest time, and storage conditions [26], [43], [44]. Other factors including methods of extract preparations as temperature and concentration of the solvent, solvent-to-resin ratio, extraction time, and phytochemicals in plant extraction which play a role in that divergence of the results. Moreover, sensitivity to specific treatment may vary between different cancer cell lines and even between cancer cell lines' different types.

In our research, it was confirmed that the mitochondrial-mediated pathway was responsible for apoptosis induction by the observed loss of the ($\Delta\Psi_m$) and the concentration-dependent increased expression of caspase 3 and also mediated by death receptor-mediated pathway through caspase-8 increased expression.

Similar to our result, Jaafari-Ashkavandi *et al.*, 2017 [28], showed that the anticancer effects of frankincense aqueous extract seem to be more related to the induction of apoptosis in OSCC.

Frankincense and its isolated components induce apoptosis (intrinsic and extrinsic) in a dose-dependent way in different cancer cell lines [14], [45], [46], [47] through activation of caspases 3 and 8 [25], [26], [48], [49].

Comparable to our result, Ni *et al.*, 2012 [25], and Frank *et al.*, 2009 [44], stated that frankincense suppressed cancer cell viability through a combination of induction of cell membrane damages, reduced cell growth, activation of apoptotic death, enhancement of tumor cell death, and cytotoxicity.

Like our result, Hakkim *et al.*, 2020 [11], explained that frankincense had caspase-dependent effect and appeared to be mediated by the mitochondrial pathway.

The roles of ROS are complicated and act as a diversified biochemical entity in cancer progression [50]. Hence, reducing (antioxidation) or increasing intracellular ROS levels would be a potential strategy to prevent or treat cancer [51].

Similar to our result, several studies have indicated that frankincense and its components enhanced apoptosis through ROS-dependent production [26], [47].

Aside from changes in energy-dependent molecular pathways (restricted to one apoptotic stage), apoptosis induction results in distinct and stage-dependent morphological alterations such as nuclear morphology changes, cellular morphology changes, and modification of cytoplasmic organelles.

Most of changes observed in frankincense extracts treated cells were of apoptotic and secondary necrotic nature. On the other hand, combination aqueous extract treated cells demonstrated necrotic changes in addition to apoptotic and secondary necrotic changes.

Morphological features of apoptosis were found to be very similar in different treated groups in our study similar to results observed by Sayed Abdul Rahman *et al.*, 2013 [52].

Similar to our study results, Hakkim *et al.*, 2020 [11], Jasim 2019 [30], Suhail *et al.*, 2011 [26], and Frank *et al.*, 2009 [44] revealed that frankincense treatment-induced dose-dependent cell shrinkage and an apoptotic cells nuclear damage and DNA fragmentation (a hallmark of apoptosis).

There are numerous biochemical and image-based essays for apoptosis that differs greatly in complexity, specificity, and cost [53].

Calculation of NAF is relatively simple and could easily be used with nuclear dye such as hematoxylin which we used here and can be used as a marker for apoptosis [53], [54], [55].

The mean values of NAF of SCC-25 treated cells decreased significantly compared to control untreated cells, especially at post-IC_{50} concentration of frankincense and combination of aqueous extract of frankincense and myrrh which were supported by the appearance of apoptotic and secondary necrotic changes during cytological examination demonstrating that the apoptosis is the a leading cause of cell death rather than the necrosis.

Conclusion

Frankincense different extracts have cytotoxic effects, and the aqueous extract was more effective. The combination of aqueous extracts has the most cytotoxic and apoptotic effect than other extracts.

This study suggested that frankincense and combination extract causes oxidative stress through ROS-regulated activation, which induces intrinsic and extrinsic apoptotic signaling cascades in OSCC cells with apoptotic and secondary necrotic cytonuclear morphological alterations.

Declarations

Ethics approval and consent to participate

Not applicable: This article does not include any human or animal studies with human or animal subjects, simply human tongue SCC cell lines (SCC-25) obtained from the American Type Culture Collection VACSERA-Egypt.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions

Resources, data analysis, and writing original draft: Sarah Mostafa said; review and editing: Sarah Mostafa Said and Enas Alaa Eldin Abd El-Aziz; and supervision: Amr Helmy Moustafa El Bolok, Ahmed Nabil Fahmi, and Enas Alaa Eldin Abd El-Aziz. All authors read and approved the final manuscript**.**

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