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Frankincense derived heavy terpene cocktail boosting breast cancer cell (MDA-MB-231) death *in vitro*

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ABSTRACT

Objective: To investigate the anti-cancer effect of frankincense derived heavy oil obtained by Soxhlet extraction method on breast cancer cells (MDA-MB-231), and to study its chemical profile using gas chromatography mass spectrometry analysis.

Methods: Hexane was used to extract heavy oil from frankincense resin. Chemical profiling of heavy oil was done using Perkin Elmer Clarus GC system with mass spectrometer. MDA-MB-231 cells were treated with different dilutions (1:1 000, 1:1 500, 1:1 750, 1:2 000, 1:2 250, 1:2 500, 1:2 750, 1:3 000, 1:3 250) of heavy oil for 24 h. The cells were observed by using light microscopy. Cell viability was measured by MTT assay.

Results: Gas chromatography mass spectrometry chemical profiling of frankincense derived heavy oil revealed the presence of terpenes such as α-pinene (61.56%), α-amyrin (20.6%), β-amyrin (8.1%), β-phellandrene (1.47%) and camphene (1.04%). Heavy terpene cocktail induced significant MDA-MB-231 cell death at each concentration tested. Noticeably, very low concentration of Soxhlet derived heavy terpenes elicits considerable cytotoxicity on MDA-MB-231 cells compared to hydro distillated essential oil derived from frankincense resin.

Conclusions: Extracting anti-cancer active principle cocktail by simple Soxhlet method is cost effective and less time consuming. Our *in vitro* anti-cancer data forms the rationale for us to test heavy terpene complex in breast cancer xenograft model *in vivo*. Furthermore, fractionation and developing frankincense heavy terpene based breast cancer drug is the major goal of our laboratory.

1. Introduction

Cancer is a world threatening disease despite the availability of modern therapeutic strategies. Of the different cancer types, breast cancer (BC) has attracted much attention since it is the most common malignancy in women throughout the world, and it accounts for 18% of all female cancers and there are approximately 600 000 annual deaths worldwide [1]. Early screening, adjuvant, and sequential systemic treatment, including chemotherapy, radiation, and hormonal therapy, have benefits in the treatment of BC. However, recurrence remains high, and BC remains the number one killer in women population [2]. Chemotherapy is frequently preferred by clinicians to treat BC because this strategy is feasible to handle compared to other therapeutic methods. However, failure of chemotherapy is very common due to high rate of

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recurrence after an initial response. Tumor relapse, drug resistance and metastasis are well known clinical manifestations of post BC treatment [3].

Chemotherapeutic agents (synthetic or natural products) have been a major advance in the treatment of cancer but are limited by the unresponsiveness of certain cancers due to multi subtypes, recurrence, and by severe adverse effects [4]. These issues should be addressed both *in vitro* and *in vivo* studies at the molecular level before recommending the chemotherapeutic agents for clinical trials. In this context, the use of plant based natural products for these purposes has been encouraged by World Health Organization due to very low rate of side effects. Because natural product based drugs are inherently better tolerated by the body compared to synthetic chemicals, they have higher chance to be approved as new drugs by Food and Drug Administration [5]. Extensive literature search has induced us to focus on Oman's frankincense, *Boswellia sacra* (*B. sacra*) resin since its medicinal properties are well established as an anti-microbial [6], non-cytotoxic, and food preservative agent [7] *etc.*, in particular, essential oil extracted from frankincense by hydro distillation (HD) method was proven to be an effective anti-proliferative agent against pancreatic [8] and BC (MDA-MB-231 and MCF-7) cells [9].

Suhail *et al.* reported that terpene complex of frankincense HD essential oil is responsible for observed anti-cancer activity against BC cells [9]. Achieving high yield of this terpene complex from frankincense by simple and cost effective method is of much interest for medicinal chemist. In order to accomplish this, in our study we tried to apply simple Soxhlet extraction strategy to extract heavy terpenes complex from frankincense resin as it is well known that Soxhlet extraction is a very efficient technique used to achieve high yield and it does not require sophisticated set up. We analyzed and compared the chemical profiling of heavy oil and essential oil extracted by HD method. Furthermore, we tested our frankincense heavy oil for *in vitro* BC cell (MDA-MB-231) killing effect and compared the efficacy with previous reports.

2. Materials and methods

2.1. Extraction of heavy oil from frankincense resin

Fresh resins of frankincense were collected from Salalah, Sultanate of Oman. Collected resins were powdered by mechanical grinding and heavy oil extracted by Soxhlet extraction using hexane for 4 h (Figure 1). After extraction, the residual solvent was removed completely from the oil by evaporation.

2.2. Chemical profiling of heavy oil by gas chromatography coupled with mass spectrometry (GC–MS)

GC–MS analysis was performed on a Perkin Elmer Clarus 600 GC System, fitted with a Rtx®-5MScapillary column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness; maximum temperature, 350 °C), coupled to a Perkin Elmer Clarus 600C MS. Ultra-high purity helium (99.999 9%) was used as carrier gas at a constant flow of 1.0 mL/min. The injection, transfer line and ion source temperatures were 270, 240 and 240 °C, respectively. The ionizing energy was 70 eV. Electron multiplier voltage was obtained from autotune. All data were obtained by collecting the full-scan mass spectra within the scan range 40–550 amu. The injected sample volume was 1 μL with a split ratio of 50:1. The oven temperature program was 60 °C and accelerated at a rate of 3 °C/min to a final temperature of 240 °C. The unknown compounds were identified by comparing the spectra obtained with mass spectrum libraries (NIST 2011 v.2.3 and Wiley, 9th edition).

2.3. Cell lines and culture method

MDA-MB-231 cells were purchased from American Type Culture Collection, USA. Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin) and maintained in humidified cell incubator at 37 °C and 5% CO_2.

2.4. Drug preparation

Stock solution of heavy oil was prepared in dimethylsulfoxide. Different dilution (1:1 000, 1:1 500, 1:1 750, 1:2 000, 1:2 250, 1:2 500, 1:2 750, 1:3 000, 1:3 250) of heavy oil prepared in cell culture medium.

2.5. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay

MDA-MB-231 cells (1×10^5/well) were seeded in 96 well plates (100 μL/well) and allowed to adhere firmly overnight in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Then cells were treated with different dilution of freshly prepared heavy oil for 24 h. Then medium was removed and cells were incubated with MTT reagent (5 mg/mL) for 4 h and violet formazan crystals were dissolved in dimethylsulfoxide and absorbance was read at 540/690 nm. Absorbance of control (without treatment) was considered as 100% cell survival. Doxorubicin was used as positive control.

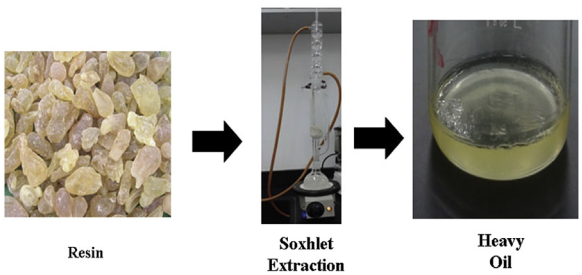

Figure 1. Soxhlet extraction of heavy oil from frankincense resin.

2.6. Statistical analysis

Three independent experiments were performed in order to ensure the reproducibility. Each experiment consisted of 4 duplicates. Experimental data were evaluated by students *t*-test and One or Two-way ANOVA. Significant difference between each set of data were considered at the confidence level of $P < 0.05$ and $P < 0.001$.

3. Results

3.1. Chemical profiling of frankincense derived heavy oil

In total, 36 constituents were identified from frankincense heavy oil. The results of GC–MS analyses showed that a major portion of heavy oil were composed of terpenes (Table 1). The main constituents were α-pinene (61.56%), α-amyrin (20.60%), β-amyrin (8.10%), β-phellandrene (1.47%) and camphene (1.04%). This observation was clearly evidenced from GC chromatogram (Figure 2). α-amyrin and β-amyrin are pentacyclic terpenes, whereas, α-pinene, β-phellandrene and camphene are monoterpenes (Figure 3).

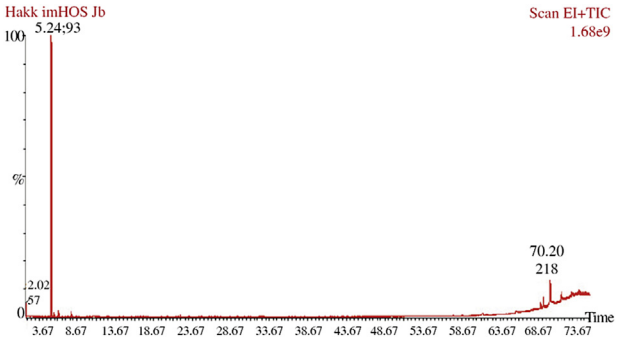

Figure 2. GC chromatogram of frankincense resin derived heavy oil.

3.2. Cytotoxicity of heavy terpenes on MDA-MB-231 cells

Heavy terpenes exhibited considerable cell death of each concentration tested after 24 h treatment (Figure 4). Heavy terpene treated MDA-MB-231 cells lost adherence and attained

Table 1
Chemical composition of heavy oil derived from Oman's frankincense.

Name	RT	Area	KI	%
α-Pinene	5.240	52 421 768.000	933.7	61.56
Camphene	5.615	891 046.500	948.7	1.04
β-Phellandrene	6.240	1 253 243.500	973.8	1.47
β-Pinene	6.345	824 030.250	978.1	0.96
β-Myrcene	6.685	189 273.984	991.7	0.22
α-Phellandrene	7.111	125 742.477	1 006.5	0.14
O-Cymene	7.296	37 991.113	1 012.0	0.04
(+)-4-Carene	7.491	22 333.596	1 017.8	0.02
O-Cymene	7.736	142 446.609	1 025.1	0.16
D-Limonene	7.886	1 351 695.375	1 029.5	1.58
Eucalyptol	7.986	99 737.570	1 032.5	0.11
Trans-beta-ocimene	8.146	105 841.359	1 037.2	0.12
β-Ocimene	8.501	46 717.734	1 047.8	0.05
γ-Terpinene	8.891	31 123.676	1 059.3	0.03
Myrtenyl acetate	10.712	605 367.313	1 111.5	0.71
α-Campholenal	11.347	172 759.047	1 127.6	0.20
Pinocarvone	12.789	50 390.559	1 164.5	0.05
Myrtenal	14.138	52 837.758	1 198.6	0.06
1-Verbenone	14.679	189 862.406	1 211.8	0.22
Bornyl acetate	17.810	242 122.313	1 287.8	0.28
α-Terpineol acetate	20.406	130 338.734	1 351.3	0.15
(−)-β-Bourbonene	21.822	108 914.273	1 386.0	0.12
β-Elemene	22.127	401 735.656	1 393.5	0.47
Caryophyllene	23.202	242 665.406	1 420.5	0.28
Humulene	24.548	59 947.055	1 454.6	0.07
Alloaromadendrene	24.843	26 928.473	1 462.1	0.03
γ-Muurolene	25.483	22 515.510	1 478.3	0.02
β-Eudesmene	25.838	268 771.656	1 487.2	0.31
α-Selinene	26.199	114 555.250	1 496.4	0.13
γ-Cadinene	26.939	27 214.281	1 515.8	0.03
Delta-cadinene	27.309	51 775.660	1 525.5	0.06
Trans-caryophyllene	29.530	77 984.422	1 584.1	0.09
1-Phellandrene	36.978	58 851.047	1 747.9	0.06
β-Amyrin	69.267	6 971 952.000	3 016.6	8.10
α-Amyrin	70.202	17 608 366.000	3 061.6	20.60
α-Cubebene	21.462	56 306.059	1 377.2	0.06

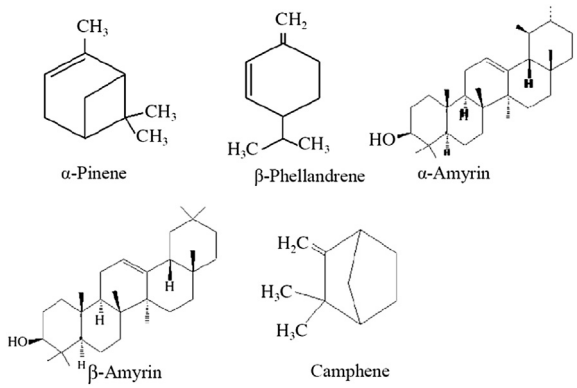

Figure 3. Structure of identified compounds.

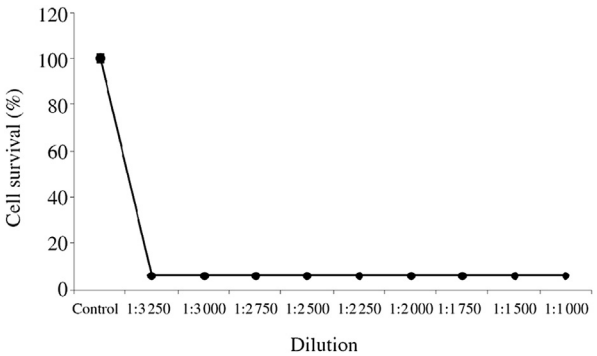

Figure 4. Anti-proliferative effect of Soxhlet extracted heavy oil from frankincense on MDA-MB-231 cells.
Values are presented as mean ± SD of 4 duplicates of 3 independent experiments.

spherical morphology compare to control cells (Figure 5). This indicated complete arrest of cell growth.

3.3. Anti-cancer effect comparison

We compared our anti-cancer data with HD essential oil (HDEO) induced cytotoxicity of MDA-MB-231 cells reported by Suhail *et al.* [9]. Interestingly, two fold dilution of our heavy oil can induce significant cell death ($P < 0.001$) than HDEO ($P < 0.05$) and doxorubicin (100 μg/mL) ($P < 0.05$) (Figure 6).

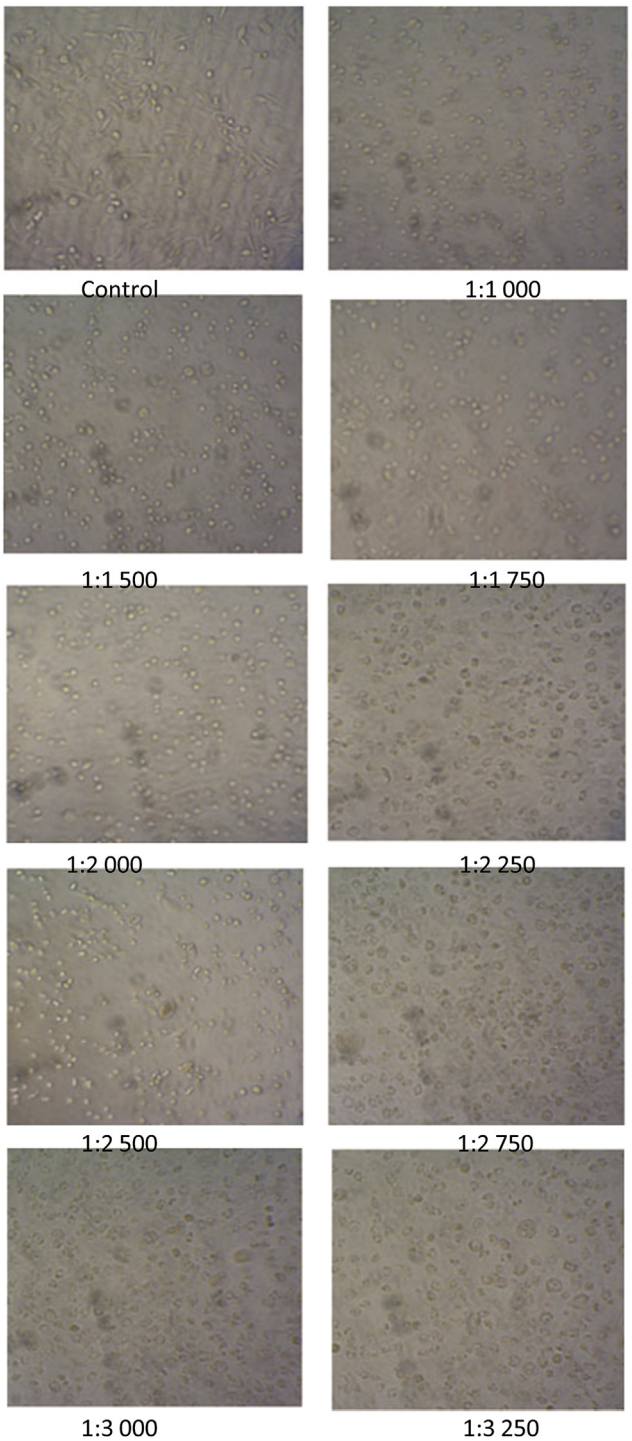

Figure 5. Morphology of MDA-MB-231 cells after 24 h treatment of frankincense resin derived heavy oil.

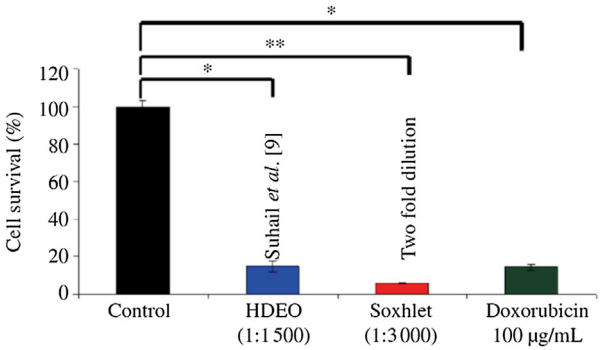

Figure 6. Anti-proliferative effect of HDEO and Soxhlet extracted heavy oil from Omani frankincense on MDA-MB-231 cells. HD data adopted from Suhail *et al.*, 2011 [9].
Values are presented as mean ± SD of four duplicates of three independent experiments. Asterisks indicates the significant difference compare to control (*: $P < 0.05$, **: $P < 0.001$).

4. Discussion

Resins of frankincense have long been used in Ayurvedic and the traditional Chinese medicine to treat different health problems. We acquired general knowledge from native people of Oman that resins mainly used for fragrance purpose and some edible variety of frankincense are reducing tumor burden. However, this kind of traditional practice needs scientific evaluation and strong evidence emerging from experiments that would allow us to translate this formulation from lab bench to clinic. Frankincense has many pharmaceutical uses such as anti-inflammatory [10] and it belongs to genus *Boswellia*. This genus consists of 18 genera and 540 species that grow mostly in tropical regions of America, China, India, North Africa, and Arabia [11]. Different strategies have been employed to extract essential oil from frankincense such as HD and microwave assisted hydro distillation. In particular, essential oil extracted by HD method at different temperatures has showed to be an effective anticancer agent against BC and pancreatic cancer cells and, furthermore this oil can modulate apoptotic and cell survival signaling cascades at intracellular level to elicit significant cell death [8,9]. GC–MS chemical profiling of frankincense HDEO revealed that α-pinene, a terpene, is a major active principle and it could be responsible for observed anti-cancer activity along with other constituents. In our laboratory, we followed simple Soxhlet extraction procedure to extract bio-active molecules from natural sources. It is well known that Soxhlet extraction is a widely used method to extract the components with high yield. Applying appropriate temperature in Soxhlet apparatus might break down complex plant cellular structure efficiently and this would facilitate the maximum extraction of components. Based on this principle and, because frankincense is a rich source of terpenes, we hypothesized that Soxhlet procedure can extract significant amount of terpenes.

To address this issue, in this study we extracted heavy terpenes from frankincense by Soxhlet method and the chemical constituents were analyzed by GC–MS. Interestingly we found that α-pinene was a major component followed by α-amyrin, β-amyrin, β-phellandrene and camphene (Table 1 and Figure 3). Ni X *et al.* reported that fractions of HD essential oil contain α-pinene in the range of 60%–80% [8]. Interestingly we found a considerable amount of α-amyrin and β-amyrin in our heavy

oil as these compounds have shown to be effective anticancer agents [12], and it is not present in essential oil extracted by HD method. Furthermore, our cytotoxicity results revealed that a very low concentration of frankincense derived heavy oil can kill MDA-MB-231 cells more effectively than HDEO reported by Suhail *et al.* [9]. Mechanisms of cell death induced by heavy oil still remain unclear. However, the major component of our heavy oil such as α-pinene, α-amyrin, β-amyrin could facilitate significant cell death by synergistic mode.

α-Pinene is reported to be an efficient antibacterial agent and it elicits cytotoxicity against different array of tumor cell lines such as human ovarian cancer (SK-OV-3 and HO-8910), human hepatocellular liver carcinoma (Bel-7402) [13] and neuroblastoma cells [14]. Chen WQ *et al.* reported that α-pinene inhibited Bel-7402 cell proliferation by arresting cell cycle at G2/M phase and down regulating cyclin dependent protein kinase activity and Cdc25C expression [15]. Also it is reported that, fragrance enriched with α-pinene reduce tumor growth in mice [16]. In addition, α-amyrin is reported to be an abundant component of roots of *Ichnocarpus frutescens* and it has exhibited cytotoxicity on different cancer cell lines (MCF-7, BEL-7402, SPC-A-1 and SGC-7901) [12]. These reports strongly suggest that our heavy oil induced MDA-MB-231 cell death is predominantly due to the presence of α-pinene and amyrin's complex. However, fractionation of heavy oil and its efficacy in BC xenograft model along with preclinical toxicity studies is highly warranted to translate this drug formulation into clinical use.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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