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Chemo-preventive Properties of Essential Oils Obtained from *Boswellia carterii* and *B.* sacra in Combination with Insoluble β-Glucan

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Abstract

Background: *B. carterii* (Frankincense) and *B. sacra* (Sacred) essential oils (EOs) have been used for many centuries in several medicinal applications. More recently, those EOs were investigated for their anticancer properties and used in combination with other natural compounds resulting in enhanced biological activities. To that extend, yeast-derived β-glucan have shown remarkable anticancer and chemo-preventive potential in the past decades whether tested alone or in combination. In this context, the *in vitro* chemo-preventive, antiradical and antiproliferative effects of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs used alone and in combination with insoluble β-glucan from *Saccharomyces boulardii* on colorectal cancer (CRC) were investigated.

Methods and findings: Eos and insoluble β-glucan from *Saccharomyces boulardii* cell wall were assayed for their capacity to increase the specific activity of NAD(P)H: quinone reductase (QR), scavenge radicals and inhibit growth of human CRC cells (CHO-K1 and HT-29 cells). Results demonstrated that *B. carterii* (Frankincense) and *B. sacra* (Sacred) scavenged superoxide anions and similarly inhibited growth of two human CRC cell lines. This study also reported the increase of QR activity as a novel mechanism of action of these EOs in cancer prevention and demonstrated that insoluble β-glucan from *S. boulardii* cell wall enhanced the capacity of *B. carterii* (Frankincense) EO to increase QR specific activity as opposed to Sacred oil. Finally, Sacred oil efficiently scavenged superoxide anions and expressed cancerous cell-specific cytotoxicity when opposed to *B. carterii* (Frankincense) EO.

Conclusion: Results obtained in this study represent the first evidence that *Boswellia* EOs can enhance QR activity when used alone or in combination with insoluble β-glucan from *S. boulardii* cell wall, hence suggesting a novel combination to investigate in future investigations. Complete characterization of *Boswellia* EOs and further biological analyses will be required to identify component(s) that are potentially responsible for such chemoprevention activities.

Keywords: *Boswellia* essential oils; Chemoprevention; Anti-radical; Anti-proliferative; NAD(P)H: Quinone reductase; β-glucan; Apoptosis

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Abbreviations

EOs: Essential Oils; 5-FU: 5-Fluorouracil; CRC: Colorectal Cancer; MTT:3-(4,5dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; NADP: Nicotinamide Adenine Dinucleotide Phosphate; QR: NAD(P)H: Quinone Reductase; X/XO: Xanthine/Xanthine Oxidase; DPPH: A,A-Diphenyl-β-Picrylhydrazyl; PI: Propidium Iodide; CI: Combination Index; IC50: Concentration that Inhibits 50% of the Cellular Growth; SC50: Concentration that Scavenges 50% of Formed Radicals.

Introduction

The colorectal cancer (CRC) is the second most deadly cancer in males and the third in females [1] and is the third most prevalent cancer in Canada [2]. The increase of CRC in Canada and USA has generated an increased interest in the consumption of natural products to prevent the development of this disease. Thus, prevention seems to be the most efficient approach since treatments for CRC can be expensive and invasive for patients. Chemoprevention consists of using natural or synthetic materials to prevent the progression of cancer [3]. Many essential oils (EOs) and their constituents have been reported to be chemo-preventive agents due to their abilities to affect phase I and II enzymes, prevent lipid peroxidation, suppress cyclooxygenase-2 activity and exhibit anticancer properties such as *in vivo* antitumoral activities, apoptosis and cancerous cell specific cytotoxicity [4,5]. More specifically, EOs from *Boswellia* spp. are well known for their tumor cell specific cytotoxicity and their capacity to induce apoptosis in cancerous cells [6,7]. In this aspect, the use of *Boswellia* spp. EOs as chemo-preventive agents toward CRC appears to be relevant. Moreover, EOs exhibiting enhanced chemo-preventive properties obtained by combination with known chemo-preventive agents is also a relevant approach to reduce CRC development.

EOs obtained from *Boswellia* trees have been used for many centuries in religious rituals and medicinal applications such as inflammation, immune support, skin health and more recently cancer treatment. As found with others EOs, the biological properties of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs vary according to many factors such as plant species, plant organs, extraction methods, soil composition, vegetative cycle stage and climate of harvesting [8]. In this context, many scientists, botanists and governments tend to consider *B. carterii* and *B. sacra* as the same species whereas several studies proved the opposite using chemical characterization [9]. Despite these evidences, very few studies have investigated differences between *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs regarding their biological activities toward colorectal cancer (CRC), which may serve to deepen knowledge on chemo-preventive properties of these EOs.

Yeast cell walls mainly consist of mannoprotein, chitin and β-(1→3)-D-glucan with β-(1→6)-D-glucan ramifications [10]. Yeast insoluble β-glucan are known for their strong immunomodulatory properties [11] and can be easily extracted from spent yeast [12]. Furthermore, several investigations revealed the capacity of yeast-derived β-glucan to prevent and treat different types of cancer both in humans and rats [11,13]. Those properties depend on the physicochemical nature and integrity of the β-glucan structure, which vary according to growth conditions, extraction methods, and yeast species [14-16]. More recently, the chemo-preventive potential *in vitro* and *in vivo* of insoluble β-glucan from *S. boulardii* cell wall was demonstrated. Notably, this specific extract appeared to be an inducer of NAD(P)H: quinone reductase (QR), a phase II detoxification enzyme (EC 1.6.99.2) *in vitro* and *in vivo* and could significantly reduce aberrant crypt count in 1,2-dimethylhydrazine-treated rats [17,18].

To substantiate differences between *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs, this study investigated the differences and the mechanism of action of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs regarding chemo-preventive, antiradical and antiproliferative properties toward CRC. In this context, EOs were evaluated for their capacity to induce QR activity, which has never been evaluated before, and to scavenge superoxide anions ($O_2^{\cdot-}$) and DPPH radicals, which are known to be involved in CRC carcinogenesis. Moreover, EOs were tested for their antiproliferative activities against cancerous and non-cancerous cells to reveal a cancerous cell specific cytotoxicity. Then an, apoptosis assay was conducted to determine if this mechanism was involved. Finally, efforts were successfully invested to enhance biological activities of EOs through addition of insoluble β-glucan to *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs.

Methods

Chemicals

Chemicals and media were obtained as follows: essential amino acids, sodium pyruvate, fetal bovine serum (FBS), minimum essential medium-Earle's balanced salt solution (MEM-EBSS), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (MEMF/12), Ham's F-12 medium, Dulbecco's Modified Eagle Medium low glucose, Hank's balanced salt solutions (HBSS), trypsin, Pierce®BCA Protein assay, glycine, 25 cm^2 flask, 96-well and 6-well microplates were purchased from Fisher Scientific (Ottawa, ON, Canada). Activated carbon, digitonin, bovine serum albumin (BSA), glucose-6-phosphate, thiazolyl blue tetrazolium bromide (MTT), menadione, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP) and flavin adenine dinucleotide (FAD), Tween 80, sodium azide, 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT), xanthine, sodium carbonate buffer (pH 10·2), xanthine oxidase, superoxide dismutase, N-methyl-2-pyrrolidone (NMP), α,α-diphenyl-β-picrylhydrazyl (DPPH) and 5-fluorouracil (5-FU) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Lecithin was purchased from ADM (Calgary, AB, Canada). *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs were graciously provided by Young Living Essential Oils (Lehi, UT, USA). Annexin V-FITC/PI Dead Cell Apoptosis kit was purchased from Invitrogen (Burlington, ON, Canada). Polymethyl methacrylate (PMMA) was obtained from Agilent Technologies (Mississauga, ON, Canada).

EOs preparation

Boswellia carterii and *B. sacra* were harvested in Kenya and Oman respectively to obtain Frankincense and Sacred EOs using the steam distillation method and were kindly provided by Young Living Essential Oils (Lehi, UT, USA). *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs were prepared under oil-in-water emulsion containing 1% (v/v) Tween-80 and 1% (w/v) lecithin as emulsifying agents. More specifically, Tween 80 and lecithin were solubilized into water using magnetic stirrer resulting in a solution termed emulsifying solution. Then, EOs were added slowly to the stirred solution until a final concentration of 68,800

ppm was reached. The O/W emulsion was covered from light and roughly stirred until complete homogenization (approximatively 45 min). Then, the resulting emulsion was filtered through a 0.2 µm filter. In order to assay various concentrations of EOs, this emulsion was diluted with sterile emulsifying solution until desired concentration under sterile condition. For combined treatments containing EOs and insoluble β-glucan, an emulsion was prepared as mentioned above but filtration was replaced by the addition of sodium azide (20 ppm) to prevent microbial contamination. The addition of pre-weighed insoluble β-glucan (40,000 ppm) from *S. boulardii* cell wall and SA under sterile condition was performed following complete homogenization of EO in emulsifying solution then stirred for another 5 min. For antiradical assays, EOs were serially diluted in anhydrous ethanol to a final concentration of 34,400 ppm.

Extraction of insoluble β-glucan from *S. boulardii* cell wall

Extraction of insoluble β-glucan was performed as described by Fortin, Aguilar-Uscanga, Vu, Salmieri and Lacroix [18]. Briefly, *S. boulardii* cells were grown in yeast peptone media containing 1% (w/v) dextrose and collected in early stationary phase. The cell suspension was centrifuged at 9,000 × g for 10 min at 4°C and the resulting biomass was washed twice with sterile phosphate buffer 50 mM, pH 7·2. Then, the wet biomass was suspended in sterile water (15% w/v) and autolyzed for 24 h at 50°C under agitation at 200 rpm. The autolyzed biomass was then centrifuged at 9,000 × g for 10 min at 4°C and 500 ml of 1 mol/l NaOH was mixed with 100 g of wet autolyzed cells for 1 h at 90°C without agitation. Finally, the resulting suspension was centrifuged as described above and the precipitate was washed twice with distilled water and then freeze-dried.

Cell lines and cells maintenance

Hepa 1c1c7 ATCC CRL-2026, HT-29 ATCC HTB-38, CHO-K1 and CaCO-2 cell lines were purchased from American type culture collection (ATCC) (Manassas, VA, USA). All cell lines were cultivated in 25 cm^2 cellular flasks (Corning, NY, USA) at 37°C in a humidified incubator with an atmosphere of 5% CO_2 and 95% air. Hepa 1c1c7 and HT-29 cells were grown in complete MEM-EBSS and complete MEMF/12 media, respectively (0.1% essential amino acids, 0.1% sodium pyruvate, 10% FBS). CaCO-2 cells were grown in Dulbecco's Modified Eagle Medium low glucose (0.1% essential amino acids, 0.1% sodium pyruvate, 20% FBS) and CHO-K1 cells were grown in Ham's F-12 media (20% FBS). At a confluence of 80 to 90%, cells were treated with 1X trypsin-EDTA for 12 min at 37°C in presence of 5% CO_2. Finally, trypsin was inactivated with 2 ml of respective media and 1 ml of the resulting suspension was used to inoculate 5 ml of fresh media.

Anti-radical assays (O_2^- and DPPH radicals scavenging activity)

The capacity of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs to scavenge O_2^- anions was measured using the xanthine/xanthine oxidase (X/XO) system (XTT color assay) based on Gerhäuser, Klimo, Heiss, Neumann, Gamal-Eldeen, Knauft, Liu, Sitthimonchai and Frank [19] with modification. A 20 µl sample previously diluted in ethanol was loaded in a 96-well microplate and completed to 200 µl with reactional mix (1 mmol/l XTT, 1 mmol/l EDTA, 1 mmol/l xanthine, 50 mmol/l sodium carbonate buffer (pH 10·2) and 3 mU/ml xanthine oxidase). The optical density (OD) was read at 490 nm after 20 min. Negative and positive controls consisted of ethanol and 30 U/ml of superoxide dismutase respectively. Scavenging activity was calculated as follows:

% Scavenging Activity = [(sample OD - Negative control OD)/(Positive control OD - Negative control OD)] × 100 (1)

The capacity of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs to scavenge DPPH was based on the method of Blois and Kedare and Singh with some modifications [20,21]. Briefly, 1 ml of 40 µM DPPH previously dissolved in anhydrous ethanol was added to 250 µl of serially diluted EOs (also diluted in anhydrous ethanol). The solution was mixed and kept at room temperature for 1 hour protected from lights then, optical density was read at 517 nm. The blank consisted of 1.25 ml anhydrous ethanol whereas control consisted of 250 µl of anhydrous ethanol and 1 ml of DPPH solution. The inhibition percentage (IP) of free radicals was measured by the equation proposed by Megdiche-Ksouri, Trabelsi, Mkadmini, Bourgou, Noumi, Snoussi, Barbria, Tebourbi and Ksouri [22]:

$$IP\,(\%) = ([\text{Control OD} - \text{Sample OD}]/\text{Control OD}) \times 100 \quad (2)$$

For both assays, concentrations that exhibited a scavenging activity of 50% (SC50 values) were determined.

NAD(P)H: QR assay

QR assay was based on methods from Prochaska and Santamaria and Talalay with some modifications [23,24]. Hepa 1c1c7 cells were seeded at a density of 2 ×10^3 cells/well in a 96-well plate using complete MEM-EBSS media and were incubated at 37°C in a humidified incubator with 5% CO_2. Afterward, the media was removed using a multichannel micropipette and serially diluted samples were added, then the microplate was incubated for 48 h as mentioned above. Cells were washed with 200 µl HBSS solution and 50 µl of 1.6% (w/v) digitonin were added to each well followed by a 20-min incubation. Then, 20 µl of samples were removed using a multichannel micropipette and used for total protein quantification whereas 200 µl of complete reaction mixture (0.25 mol/l Tris-HCl pH 7, 4.67% (w/v) BSA, 0.01% Tween-20, 5 mol/l FAD, 1 mmol/l glucose-6-phosphate, 30 mol/l NADP, 34.8 µmol/l MTT, 50 µmol/l menadione and 2 mU/µl glucose-6-phosphate dehydrogenase) were added to each well then incubated at room temperature for 5 min. The microplate was read at 595 nm. A protein assay was conducted using Pierce®BCA reagents using the manufacturer's instructions. Negative controls consisted of emulsifying solution whereas media was used as blank. Specific activity of QR was defined as nmol of blue formazan formed per mg protein per minute. Fold induction of QR was calculated as follows:

Fold induction = Specific Activity of Treated Group/Specific Activity of Negative Control Group (3)

Molecular weight determination by gel permeation chromatography (GPC)

Molecular weights (Mw) of insoluble β-glucan treated with *B. sacra* (Sacred) EO was analyzed by gel permeation chromatography (GPC) (Agilent Technologies 1260 infinity series, Waldbronn, BW, Germany), equipped with a quaternary pump (Model G1311B), a manual injector with a sample loop of 20 µl and a refractive index detector (Model G1362A). Two identical PL gel 5 µm Mixed-D 300 × 7.5 mm columns were used in series and mobile phase consisted of 100% N-methyl-2-pyrrolidone (NMP) containing 5% (w/v) LiCl at a flow rate of 0.5 ml/min. Both columns and detector were set at 60°C. Insoluble β-glucan (5 mg) and EOs were suspended in 5 ml of emulsion as described in section 2.2 in a proportion of 5:1 for 48 h. Then, 2 ml of 100% NMP was added to obtain a relative concentration in insoluble β-glucan of 2.5 mg/ml and the suspension was stirred for 48 h at 60°C, filtered through a nylon 0.2-µm filter and injected in the column. Polymethyl methacrylate (PMMA) was used as a standard and was prepared as indicated by the manufacturer. The equation obtained by plotting Mw with retention times of standards was used to calculate Mw of insoluble β-glucan. All extracts were injected in triplicate (n=3).

Anti-proliferative assay

Antiproliferative properties were determined by the ability of the metabolic active cells to cleave the tetrazolium salt to purple formazan crystals based on Vistica, Skehan, Scudiero, Monks, Pittman and Boyd [25]. Different cell lines were seeded at 2×10^4 cells/well of media in a 96-well plate (200 µl/well) and were incubated for 24 h at 37°C in 5% CO_2. Then, spent media were removed using a multichannel micropipette. A quantity of 100 µl of fresh media containing 10 µl of sample previously serially diluted (ranging from 3,000 to 5.85 ppm regarding both EOs and insoluble β-glucan) was immediately added in well and the microplate was then incubated for 48 h as mentioned above. Afterward, samples were removed using a multichannel micropipette and replaced with 225 µl of fresh media containing 25 µl 0.5% (w/v) MTT followed by incubation for 4 h at 37°C in 5% CO_2. Finally, the media was carefully removed using a multichannel micropipette and replaced by 225 µl of DMSO containing 25 µl of Sorensen buffer composed of 0.1 mol/lglycine and 0.1 mol/l NaCl at a pH of 10.5. The microplate was then read at 562 nm. The negative control and blank consisted of emulsifying solution and media, respectively. Growth inhibition was calculated as follows:

% Growth inhibition = 100 – (([Sample OD]/Negative control OD) × 100) (4)

Equations obtained by plotting the linear portion of growth inhibition versus increasing concentrations of samples were used to calculate concentrations that inhibit 50% of cellular growth (IC50 values). For combined treatments, concentrations corresponding to IC50 values when tested separately were serially diluted and assayed.

Assessing interaction between EOs and insoluble β-glucan regarding antiproliferative and NAD(P)H: QR assays

The assessment of interactions in combined treatments regarding QR and antiproliferative assays differed due to the nature of measured effects. The determination of combined effects concerning antiproliferative assay was based on combination index (CI) as used by Hossain, Follett, Dang Vu, Harich, Salmieri and Lacroix [26] with different upper and lower bounds suggested by Berenbaum [27] following the equation:

$$CI = [Dx/IC50x] + [Dy/IC50y] \quad (5)$$

where Dx and Dy represent concentrations of components used in combination that reached IC50 values whereas IC50x and IC50y represents concentrations of components x and y that reached IC50 values when tested separately. Based on CI values, different combined effects can be classified: CI value<1 was interpreted as a synergistic effect, a CI value equal to 1 was interpreted as an additive effect and a CI>1 was interpreted as an antagonistic effect. Concerning the QR assay, concentrations that exhibited an induction of 1.5 when tested separately were used for combined treatment assays. Determination of the combined effect was based on fold induction and assessed as follows: Fold induction from 1.5 to 3.0 was interpreted as an additive effect, fold induction ≈1.5 as no interactive effect and fold induction <1.5 as an antagonistic effect. Fold induction calculated in combined treatments was obtained as described in equation 3.

Apoptosis assay

HT-29 cells were seeded in a 6-well plate at 3×10^5 cells/well (3 ml/well) and incubated as described above for 24 h. Then, cells were incubated for 48 h at 37°C in 5% CO_2 in the presence of 450, 900 and 1,800 ppm of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs in a final volume of 3 ml to surround IC50 values obtained for this cell line. Cells present in the supernatant were harvested by centrifugation at 500 × g for 10 min at 4°C. Adhered cells were treated with 1 ml of 1x trypsin-EDTA for 12 min at 37°C. Then, 2 ml of complete MEM/F12 medium was added and cells were harvested by centrifugation as described previously. The cell-containing pellets (from the supernatant and the adhered cells) were washed twice with PBS containing 0.25% EDTA to avoid clumping and the apoptosis evaluation was performed by using Annexin V-FITC and PI double staining assays. Harvested cells were diluted in 1X binding buffer at 10^6 cells/ml and Annexin V-FITC/PI staining was performed according to the manufacturer's instructions with a total of 10,000 events by flow cytometry (Coulter Epics XL-MCL, Beckman Coulter Canada, Inc., Mississauga, ON, Canada). The 5-fluouracil (5-FU) was used as positive control and emulsifying solution was used as negative control.

Statistical analysis

All measurements were done in triplicate (n=3) and results are presented as average ± standard deviation. QR fold induction, IC50 values and percentage of apoptotic and necrotic cells were

analyzed by one-way analysis of variance (ANOVA) using PASW statistics 18 software (IBM Corporation, Somers, NY, USA) and differences among treatments were analyzed with a post hoc Duncan's multiple-range test.

Significance was considered at $P \leq 0.05$.

Results

Evaluation of the anti-radical properties of EOs

Antiradical properties of EOs from *Boswellia* spp. were investigated via their capacity to scavenge O_2^- and DPPH radicals and the results are presented in **Figure 1** *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs demonstrated a dose-dependent response in antiradical activities and were found to scavenge 50% of O_2^- anion at 4,300 and 34,400 ppm respectively in addition to demonstrate a dose-dependent response **(Figure 1A)**. These results are suggesting that *B. sacra* (Sacred) EO possess an enhanced capacity to scavenge O_2^- anion as compared to *B. carterii* (Frankincense) EO. In contrast, both EOs were not able to scavenge 50% of DPPH radical despite their high concentration ranging from 33.59 to 34,400 ppm. However, a dose-dependent response was also observed suggesting a weak capacity of *B. sacra* (Sacred) EO (18% at 34,400 ppm) to scavenge DPPH radical **(Figure 1B)**.

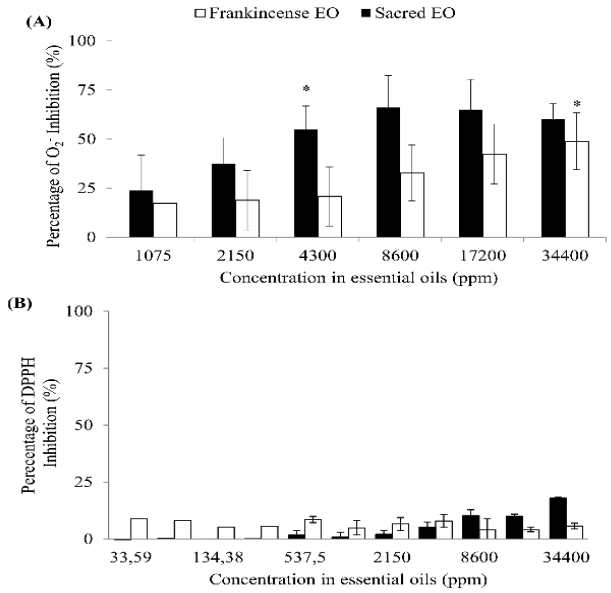

Figure 1 Effect of increasing concentration of EOs on the percentage of:
(A) O_2^-
(B) DPPH radical inhibition. EO: Essential Oil.
*Concentration that scavenged 50% of radicals formed. Error bars represent the standard deviation of the mean of at least 3 independent experiments.

Effect of EOs in combination with insoluble β-glucan on the induction of NAD(P)H: QR and molecular weight

To determine the chemo-preventive potential of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs, their effects on QR activity was evaluated and results are shown in **Table 1**. Cells treated with emulsifying solution (control) showed a 0.8-fold induction which corresponded to a basal expression of QR in Hepa 1c1c7 cells. Moreover, *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs reached maximum fold inductions of 1.5 and 1.4 at a concentration of 27 and 54 ppm respectively. In addition, both EOs tested separately demonstrated a maximum fold induction significantly higher than control (0.8-fold induction) ($P \leq 0.05$). Those results demonstrate that *B. carterii* (Frankincense) EO (27 ppm) is twice as efficient as *B. sacra* (Sacred) EO (54 ppm) to induce QR. In order to increase the chemo-preventive potential of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs, they were combined with insoluble β-glucan of *S. boulardii* which has demonstrated an excellent chemo-preventive potential against colorectal cancer evaluated *in vitro* and *in vivo* [17,18]. Concentrations in EOs and insoluble β-glucan that exhibited similar fold inductions were mixed to determine the combined effect of EO + insoluble β-glucan on QR activity and these results are also presented in **Table 1**. The combination of *B. sacra* (Sacred) EO+ *B. carterii* (Frankincense) EO showed a similar QR activity (1.4-fold induction) as compared to each EO tested separately, hence suggesting there was no interactive effect between the two EOs. In contrast, the combination of *B. sacra* (Sacred) EO + insoluble β-glucan revealed a QR activity (0.4-fold induction) which was significantly ($P \leq 0.05$) lower than that of *B. sacra* (Sacred) EO (1.4-fold induction) and insoluble β-glucan (1.5-fold induction at 250 ppm) alone, hence suggesting an antagonistic effect. The combination of *B. carterii* (Frankincense) EO + insoluble β-glucan revealed a QR activity (2.0-fold induction) which was significantly ($P \leq 0.05$) higher than that of *B. carterii* (Frankincense) EO (1.5-fold induction) or insoluble β-glucan (1.5-fold induction) alone, hence suggesting an additive effect. These results demonstrate that insoluble β-glucan improves the capacity of *B. carterii* (Frankincense) EO in inducing QR activity. The antagonistic effect of *B. sacra* (Sacred) EO when combined with insoluble β-glucan on QR activity led to a hypothesis that *B. sacra* (Sacred) EO might degrade insoluble β-glucan. The hypothesis was verified by comparing the molecular weight (Mw) of insoluble β-glucan before and after addition of *B. sacra* (Sacred) EO. Results demonstrated that insoluble β-glucan possessed a Mw (1921 ± 13 kDa) similar as if it was combined with *B. sacra* (Sacred) EO (1904 ± 297 kDa), hence suggesting that EOs did not affect the insoluble β-glucan.

Effect of EOs in combination with insoluble β-glucan on the cellular proliferation of different cell lines

The effect of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs on the growth inhibition of human CRC HT-29 and CaCO-2 cells as well as on non-cancerous CHO-K1cells were evaluated and the results are presented in **Table 2**. Regarding HT-29 cells, IC50 values of 1,447 ppm and 1,348 ppm were found for *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs respectively.

Table 1 Effect of EOs used separately and in combination with insoluble β-glucan on the induction of QR.

Samples	IC_{50} (ppm)											
	HT-29				Caco-2				CHO-K1			
	Insoluble glucan	Total EOs	CI	CE	Insoluble glucan	Total EOs	CI	CE	Insoluble ‡ glucan	‡ Total EOs	CI	CE
Sacred EO	NA	1348 ± 107^{B}	NA		NA	1138 ± 57^{B}	NA		NA	NR	NA	NA
Frankincense EO	NA	1447 ± 86^{B}	NA		NA	$1424 \pm 206^{A,B}$	NA		NA	1689 ± 22^{B}	NA	NA
Insoluble β-glucan	108 ± 33^{a}	NA	NA		$634 \pm 242^{a,b,c}$	NA	NA		NR	NA	NA	NA
Frankincense + Sacred EOs	NA	$1465 \pm 276^{A,B}$	1.05	AD	NA	$1032 \pm 8^{A,B}$	0.82	AD	NA	704 ± 108^{A}	CI<1.0	S
Insoluble β-glucan + Sacred EO	93 ± 6^{a}	1279 ± 87^{B}	2.61	A	307 ± 36^{b}	551 ± 65^{A}	0.97	AD	611 ± 49^{c}	561 ± 45^{A}	CI<1.0	S
Insoluble β-glucan + Frankincense EO	93 ± 6^{a}	1282 ± 76^{B}	1.75	A	$337 \pm 54^{a,b}$	756 ± 122^{A}	1.06	AD	$796 \pm 279^{a,b,c}$	448 ± 157^{A}	CI<1.0	S

‡The highest concentration of insoluble glucan and Sacred EO were selected for combination treatments against CHO-K1 cells, as no IC_{50} values were observed when tested separately. EO: Essential oil. IC_{50}: Concentration that inhibits 50% of the cellular growth. CI: Combination Index. CE: Combinatory effect. NR: Not reached. NA: Not applicable. Additive effect (AD): CI ≈ 1.0. Antagonistic effect (A): CI>1.0. Synergistic effect (S): CI<1.0. Results are presented as average ± standard deviation of at least 3 independent experiments. IC_{50} values of insoluble glucan bearing different lowercase letters are significantly different ($p \leq 0.05$).
IC_{50} values of total EOs bearing different uppercase letters are significantly different ($p \leq 0.05$).

Table 2 Effect of EOs used separately and in combination with insoluble β-glucan on the cellular proliferation of different cell lines.

Samples*	Concentration (ppm)		Fold Induction	Effect
	Insoluble glucan	Total EOs		
Control	NA	NA	0.8 ± 0.4^{b}	NA
Sacred EO	NA	54	1.4 ± 0.1^{c}	NA
Frankincense EO	NA	27	1.5 ± 0.1^{c}	NA
Insoluble glucan	250	NA	1.5 ± 0.2^{c}	NA
Frankincense EO + Sacred EO †	NA	81	1.4 ± 0.2^{c}	I
Insoluble glucan + Sacred EO	250	54	0.4 ± 0.2^{a}	A
Insoluble glucan + Frankincense EO	250	27	2.0 ± 0.5^{d}	AD

*Concentrations of essential oil (EO) tested separately ranged from 3 to 1720 ppm. †: 27 ppm of Frankincense and 54 ppm of Sacred EOs were combined. Additive effect (AD): 1.5 <Fold induction >3.0. No interactive effect (I): Fold induction ≈ 1.5. Antagonistic effect (A): 1.5 >Fold induction. NA: Not applicable. Concentrations for combined treatments were chosen based on the highest induction of each compounds tested separately. Means followed by different letters are significantly different ($p \leq 0.05$). Results are presented as average ± standard deviation of at least 3 independent experiments.

Against CaCO-2 cells, IC50 values of 1,424 ppm and 1,138 ppm were found for *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs respectively. Concerning the non-cancerous CHO-K1 cells, *B. carterii* (Frankincense) EO exhibited an IC50 value of 1,689 ppm whereas *B. sacra* (Sacred) EO showed no effect on this non-cancerous cell line at tested concentrations ranging from 21.5 to 2,752 ppm. The results hence suggest that *B. sacra* (Sacred) EO exhibited cancerous cell-specific cytotoxicity. In addition, no significant difference between IC50 values of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs tested separately were observed regarding HT-29 and CaCO-2 cell lines (P>0.05).

In order to increase the chemo-preventive potential of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs, these EOs and insoluble β-glucan were used in combination to evaluate their effect on the growth of different cell lines. **Table 2** indicates that the combination of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs against HT-29 cells exhibited a combination index (CI) of 1.05 and an IC50 value in total EOs of 1,465 ppm, thus suggesting an additive effect. In contrast, *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs in combination with insoluble β-glucan (which showed an IC50 value of 108 ppm when tested separately) presented a CI values of 2.61 and 1.75 respectively. However, combined treatments revealed that IC50 values of insoluble β-glucan (93 ppm for both EOs) and EOs (1,279 and 1,282 ppm respectively) were not significantly different from IC50 values obtained when tested separately (P>0.05). Those results suggest that EOs combined with insoluble β-glucan generated antagonistic effects against HT-29 cells.

In the case of CaCO-2 cells, combination of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs exhibited a CI value of 0.82 and an IC50 value in total EOs of 1,032 ppm, hence suggesting an additive effect which is congruent with results observed with HT-29 cells. Moreover, *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs in combination with insoluble β-glucan (which showed an IC50 value of 634 ppm when tested separately) presented CI values of 0.97 and 1.06 respectively. However, *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs in combination with insoluble β-glucan showed that IC50 values of insoluble β-glucan (307 and 337 ppm respectively) and EOs (551 and 756 ppm respectively) were lower than IC50 values obtained when tested separately. Those results

suggest that EOs combined with insoluble β-glucan demonstrated additive effects against CaCO-2 cells.

Table 2 also indicates that the combination of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs against CHO-K1 cells exhibited an IC50 value in total EOs of 704 ppm, hence suggesting a synergistic effect. Moreover, *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs in combination with insoluble β-glucan revealed IC50 values of insoluble β-glucan (611 and 796 ppm respectively) and EOs (561 and 448 ppm respectively), hence suggesting a synergistic effect since insoluble β-glucan and *B. sacra* (Sacred) EOs assayed separately showed no IC50 values against CHO-K1 at tested concentrations ranging from 21.5 to 2,752 ppm. These results reveal that CHO-K1 cells were highly sensitive to a combination of insoluble β-glucan and EOs, possibly due to an important cytotoxic effect on noncancerous cells and the loss of cancerous cell-specific cytotoxicity.

Effect of EOs on the level of apoptosis in human colorectal cancer cells

Since a cancerous cell-specific cytotoxicity was observed with *B. sacra* (Sacred) EO tested separately, Annexin V-FITC/PI double staining was performed to determine if apoptosis was involved in the growth inhibition of cancerous cells by EOs. HT-29 cells were chosen for this test since IC50 values of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs were not significantly (P>0·05) different against HT29 and CaCO-2 cell lines **(Table 2)**. Further, the effect of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs on apoptosis induction in HT-29 cells has never been investigated previously. Data in **Figure 2A** demonstrated that at the tested concentrations (450, 900 and 1800 ppm), neither *B. sacra* (Sacred) EO (percentage of apoptotic cells of 2.38, 2.96 and 3.90, respectively) nor *B. carterii* (Frankincense) EO (percentage of apoptotic cells of 4.70, 4.06 and 4.34, respectively) were able to significantly (P>0·05) induce apoptosis (Annexin V + PI - cells) in HT-29 cells at concentrations surrounding IC50 values compared to the negative control (percentage of apoptotic cells of 2.61%). In contrast, data shown in **Figure 2B** demonstrated that at the tested concentrations (450, 900 and 1,800 ppm), *B. sacra* (Sacred) EO (percentage of apoptotic cells of 18.43, 25.02 and 24.76, respectively) and *B. carterii* (Frankincense) EO (percentage of apoptotic cells of 24.11, 22.62 and 26.17respectively) were able to significantly (P ≤ 0·05) increase the percentage of necrotic cells as compared to negative control (percentage of necrosis cells of 7.57%) which corresponds to annexin V + PI + cells. Those results suggest that *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs induced cytotoxicity in HT-29 cells via necrosis rather than apoptosis, based on the analysis of externalization of phosphatidylserine on the surface of the cell membrane using Annexin V-FITC and PI double staining.

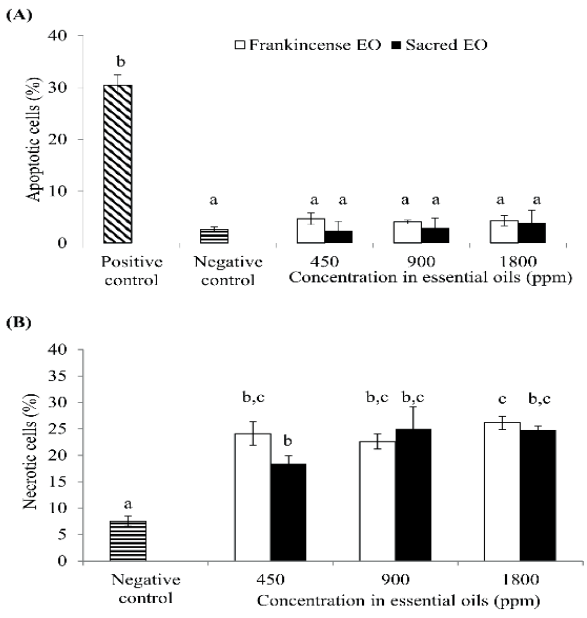

Figure 2 Effect of increasing concentration of EOs surrounding IC50 values on the percentage of:
(A) apoptotic cells (Annexin V+ PI- cells)
(B) necrotic cells (Annexin V+ PI+ cells). EO: Essential Oil. Positive control (PC): 500 µg/ml of 5-fluorouracil, Negative control (NC): emulsifying solution.
Error bars represent the standard deviation of the mean of at least 3 independent experiments. Means followed by different letters are significantly different (p ≤ 0.05)

Discussion

Evaluation of the antiradical properties of EOs

Antiradical assays revealed that *B. sacra* (Sacred) EO were more efficient than *B. carterii* (Frankincense) EO to scavenge O_2^- anion. This fact underlies the differences between biological activities between them even if both EOs were ineffective to scavenge DPPH radical. Many studies reported the weak capacity of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs to scavenge DPPH radical, which is in congruence with our results [28,29]. For instance, it was found that *B. sacra* EO reached 8% scavenging activity at 1,000 ppm [28]. Moreover, it was reported that an EO from *B. carterii* scavenged 50% of DPPH radical at 15,210 ppm (15.21 mg/ml), which is at very high concentration [29]. In addition, it was reported that an EO from *B. sacra* exhibited a greater capacity to scavenge O_2^- anion (56.40%) as compared to DPPH radical (16.30%) at tested concentrations, similarly to results obtained in the present study [30]. This difference in capacity to scavenge O_2^- anion more efficiently than DPPH radical can be explained by the lower reactivity of DPPH radical compared to reactive oxygen species (ROS) such as O_2^- anion [31-33]. Likewise, it is generally accepted that ROS, especially the O_2^- anion, are the most important free radicals in many diseases including cancer [34,35]. Such O_2^- anion scavenging activity of *B. sacra* (Sacred) EO may be due to its high content of terpene compared to *B. carterii* (Frankincense) EO, such as α-pinene, as reported by other authors [9,36]. It will be necessary to quantify the exact content and nature of terpenes found in both EO to verify this hypothesis.

Effect of EOs in combination with insoluble β-glucan on the induction of NAD(P)H: QR and Mw determination

The results of QR assays results show that *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs were able to induce QR activity at low concentrations (54 and 26 ppm respectively). An increase of the QR specific activity by *Boswellia* EOs has never been reported until now, hence revealing a novel chemo-preventive property of these EOs. Thus, mechanisms by which EOs from *Boswellia* spp. induce QR are worthy of further discussion. The increase of gene transcription coding for phase II enzymes depends on the destabilization of Keap1/Nrf cytoplasmic complex which triggers antioxidant response element (ARE) release. This destabilization is related to an α,β-unsaturated ketone moiety of an inducer reacting with the cysteine thiol of Keap1 [37-39]. Unsaturated ketones (enones) are known to be present in *Boswellia* EOs. Indeed, traces of rotundone and mustakone, two sesquiterpene ketones (aromatic enones), in *B. sacra* (Sacred) EO were detected [40].

Moreover, *Boswellia* EO contains keto-β-boswellic acid (K-BA) and acetyl-keto-β-boswellic acid (AK-BA) which also possess aromatic enone functional groups [41]. Thus, such molecules in *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs might explain the increase of QR activity observed in the present study especially since other organic acids and triterpenoid compounds were proven to be QR inducers such as fumaric acid derivatives, coussaric acid A and bardoxolone methyl [42-45].

In order to increase the chemo-preventive potential of *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs, they were combined with insoluble β-glucan of *S. boulardii*'s cell wall. In previous studies, this insoluble β-glucan showed the most relevant chemo-preventive properties *in vitro* and *in vivo* [17,18]. The combination of insoluble β-glucan and *Boswellia* EO as potential chemo-preventive agents has never been tested before. Our results show that insoluble β-glucan enhanced QR activity induced by *B. carterii* (Frankincense) EO whereas such an enhancement was not observed on *B. sacra* (Sacred) EO. The degradation of β-glucan by *B. sacra* (Sacred) EO was not found to be responsible for the observed differences between EOs since the Mw of insoluble β-glucan was similar despite mixing with *B. sacra* (Sacred) EO. Moreover, many studies demonstrated that EOs may be encapsulated in polysaccharide-basedgels without negatively affecting their properties [46,47], which rejects the hypothesis of insoluble β-glucan degradation. However, divergence of combinatory effect between EOs regarding the QR assay may be due to an increased sensitivity of Hepa 1c1c7 cells toward *B. sacra* (Sacred) EO leading to a weak induction. Thus, future studies on this aspect are necessary.

Effect of EOs in combination with insoluble β-glucan on the cellular proliferation of different cell lines

Many studies demonstrated that *Boswellia* EOs exhibit cytotoxic effects (antiproliferative activity) toward different cancerous cell lines. It was demonstrated that an EO from *B. sacra* exhibited IC50 values varying from 1:1680 (1264 ppm) to 1:1800 (477 ppm) toward human breast cancer cells [41]. Moreover, 4 EO fractions from *B. sacra* exhibiting IC50 values varying from 1:270 (3,185 ppm) to 1:1560 (551 ppm) toward human pancreatic cancer cells was obtained [48].

Finally, it was demonstrated that an EO from *B. carterii* exhibited an IC50 value of 1:1250 (688 ppm) toward human bladder cancer cells whereas in another research an IC50 value of 1:600 (1,433 ppm) against the same cell line was obtained [6,7]. These investigations confirm the congruence of IC50 values obtained in the present study with the scientific literature.

It is largely accepted that *B. carterii* is merely a synonym for *B. sacra*. However, significant differences were observed in the composition of EOs obtained from these both plants [9]. The authors reported that *B. sacra* (Sacred) EO differed from *B. carterii* (Frankincense) EO on higher optical rotation values (+30.1 and -13.3° respectively), enantiomeric ratios values and α-pinene content (79.0 and 48.2% respectively), which confirmed that both species are distinct. Terpenes contained in *Boswellia* EOs are known to influence cancerous cell-specific cytotoxicity. Indeed, 2 EOs from *B. sacra* were extracted and it was observed that EOs with higher boswellic acid (BA) content exhibited higher cancerous cell-specific cytotoxicity in breast cancer cells. EO from *B. sacra* was extracted containing a high concentration of α-pinene (62%) and α-amyrin (21%) which exhibited a more efficient antiproliferative effect on a human breast cancer cell line as compared to a similar EO containing less α-amyrin, hence suggesting a combined effect of terpenes [41,49]. Moreover, enantiomeric ratios can influence biological activities of EOs. Indeed, it was demonstrated that α- and β-(+)-pinenes, which are the most abundant terpenes in Boswellia EOs [50], exhibited minimal inhibitory concentration (MIC) values against *Candida albicans*, *Cryptococcus neoformans, Rhizopus oryzae* and Methicillin-resistant *Staphylococcus aureus* whereas α- and β-(-) pinenes showed no effect at tested concentrations. Those investigations might explain the divergence in biological activities between EOs observed in the present study notably regarding the cancerous cells specific cytotoxicity of *B. sacra* (Sacred) EO.

Combined treatments demonstrated that the effect of EOs and insoluble β-glucan had different effects on the viability of CaCO-2 and HT-29 cell lines whereas no difference ($P>0.05$) was observed when tested separately. Since no study has investigated the combined effect of EOs and insoluble β-glucan on cell viability, it may be hypothesized that combining these compounds might create different chemical species that acted differently on cell lines. Since it was confirmed that EOs did not affect the Mw of insoluble β-glucan, further investigation will be necessary to understand such an effect. Finally, results showed that all combinations exhibited IC50 values toward CHO-K1 cells, hence suggesting that combinations affected the growth of non-cancerous cells since insoluble β-glucan and *B. sacra* (Sacred).

EO exhibited cancerous cell-specific cytotoxicity when tested separately. Such synergistic effects in CHO-K1 cells might be due to multiple mechanisms triggered by combined treatments

in non-cancerous cells, which suggests important side effects of these combinations.

Effect of EOs on the level of apoptosis in human colorectal cancer cells

Cytotoxic activities of *Boswellia* EOs are known to be mainly due to pro-apoptotic properties [6,7,41,48]. However, it is interesting that no apoptotic activity was detected in the present study. Such divergence with the scientific literature may be explained by the fact that no study investigated the apoptosis activities of whole *Boswellia* EOs (which contain many bioactive compounds) on HT-29 cells. However, it was demonstrated that boswellic acid (BA), keto-β-boswellic acid (K-BA) and acetyl-keto-β-boswellic acid (AK-BA) induced apoptosis in a dose-dependent manner in HT-29 cells [51]. Also, it has been reported that 5 µg/ml (5 ppm) of boswellic acid were able to induce phosphatidylserine exposure at the outer membrane of erythrocytes, hence indicating undergoing suicidal erythrocyte death [52]. Those previous studies suggest that *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs used in the present study did not contain sufficient amounts of those specific triterpenes to induce apoptosis in HT-29 cells. Indeed, BA and its derivatives have been frequently reported to correlate with apoptotic activity depending on cell lines and concentrations used. Also, researchers have measured the BA content of two EOs from *B. sacra* obtained at different temperatures and investigated their apoptotic activity in human breast cancer cells [41]. Extracts obtained at 100°C exhibited the highest content in BA (30.1 mg/ml) and showed the most relevant results regarding DNA fragmentation, caspase activation and cell cycle arrest as compared to EOs obtained at 78°C (19.6 mg/ml boswellic acid). Moreover, researchers have obtained 4 EO fractions from *B. sacra* gum resins and reported that fractions (III and IV) containing high content of BA exhibited apoptosis activity in four different human pancreatic cancer cells [48]. The authors also noticed that expression patterns in time function of pAkt, cdk4 and cyclin D1 proteins differ among those human pancreatic cancer cell lines using cell cycle arrest assay upon treatment with fractions III and IV. More recently, research studies have shown to have treated several cancer cell lines with acetyl-lupeolic acid obtained from the resin of *B. carterii*, and have assessed the capacity of this compound to inhibit cellular growth and induce apoptosis [53]. The authors noticed an important variability among IC50 values obtained. More importantly, they have observed that apoptosis was not induced in the only cell line resistant to the acetyl-lupeolic acid treatment (i.e., no IC50 value obtained). Those studies revealed that induction of apoptosis by Boswellia EOs may vary upon terpene composition and cancerous cell lines.

Conclusion

This study confirmed that *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs exhibited different chemo-preventive, antiradical and anti-proliferative properties toward CRC. This study also reported for the first time that *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs could induce QR activity which is one of the important mechanisms in cancer chemoprevention. *B. carterii* (Frankincense) EO was more effective than *B. sacra* (Sacred) EO in increasing QR specific activity and combining *B. carterii* (Frankincense) EO with insoluble β-glucan obtained from cell wall of *S. boulardii* enhanced the capacity of this EO to increase QR specific activity. Moreover, *B. sacra* (Sacred) EO efficiently scavenged $O_2^{\cdot}$ anion and demonstrated cancerous cell-specific cytotoxicity as opposed to *B. carterii* (Frankincense) EO. Also, additive antiproliferative effects were observed by combining *B. carterii* (Frankincense) and *B. sacra* (Sacred) EOs toward human colorectal cancer cells and by combining EOs with insoluble β-glucans toward CaCO-2 cells. It is worth noting that even *B. sacra* (Sacred) EO or insoluble β-glucan alone expressed cancerous cell-specific cytotoxicity effects, their combinations were found to be more efficient in affecting the viability of CHO-K1. Finally, based on the Annexin V-FITC/PI double staining assay, the induction of apoptosis in colorectal cancer cells might not be responsible for the cell growth inhibitory effect observed by both EOs.

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Conflict of Interest

The authors have no conflict of interest to declare.

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