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Anticancer attributes of *Illicium verum* essential oils against colon cancer

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ABSTRACT

This study investigated the antioxidant and *in vitro* cytotoxic effects of essential oils (EOs) obtained from the fruits of *Illicium verum*. The solvent extraction method was used to extract oils from the fruits. Antioxidant properties were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) assays followed by estimation of the total phenolic and total flavonoid contents and gas chromatography–mass spectrometry (GC-MS) analysis. Anti-proliferative effects were studied by MTT assay. Fluorescent dyes were used to study the apoptotic effects of EOs. Furthermore, *in vitro* cell migration, matrigel invasion, and colony formation assays were employed to study the anti-metastatic properties of EOs. Three concentrations of EOs (25, 50, and 90 μg/mL), 0.5% dimethyl sulfoxide in media, and 5-Fluorouracil (5 μg/mL) were used as the test sample, negative control, and positive control, respectively. EOs showed promising antioxidant effects in the DPPH and FRAP models; GC-MS analysis identified trans-anethole as one of the major compounds. Among the cell lines tested, EOs showed the greatest cytotoxicity towards HCT 116, with an IC_{50} value of 50.34 ± 1.19 μg/mL. Distinct nuclear morphological changes and a reduction in mitochondrial membrane potential was observed in the treated cells in a dose-dependent manner. Similarly, dose-dependent inhibition of cell migration, invasion, and colony formation was observed in the treated cells. To conclude, the cytotoxic effect of *Illicium verum* EOs may be due to multiple mechanisms such as the induction of apoptosis and inhibition of key steps of metastasis.

1. Introduction

During the past few decades, cancer has emerged as one of the most alarming diseases throughout the world. It is a multifactorial syndrome characterized by uncontrolled cellular growth, local tissue invasion, and distant metastasis of abnormal cells (Chabner, 2006; Gautam et al., 2014). One of the most rational approaches to arrest tumorigenesis is by interfering with the modulation steps (initiation, promotion, and progression) as well as those associated with signal transduction pathways (Bhall et al., 2013). Natural products have the capacity to halt these steps either alone or in combination with conventional chemotherapies. Among several benefits of using natural products for cancer prevention and cure are their relatively less toxic and ingestive nature (Amin et al., 2009). Safety and efficacy of these phytochemicals in regression and control of numerous cancers has been accessed in number of controlled clinical trials and findings of the studies show that these agents have promising effects with tolerable side effects (Dhillon et al., 2008; Shimizu et al., 2008; Khan et al., 2009; Carroll et al., 2011; Howells et al., 2011); however, this is not always true and each phytochemical should be thoroughly studied for its safety and efficacy before human use.

According to recent estimates, more than 5000 individual phytochemicals have been identified and this number is continuously increasing due to the introduction of new and efficient isolation and characterization techniques. These novel phytomedicines are broadly classified as alkaloids, carotenoids, essential oils (EOs), glycosides, nitrogen-containing compounds, phenolics, saponins, triterpenes, and vitamins and have shown promising anticancer activities in *in vitro* and *in vivo* models (Adorjan and Buchbauer, 2010).

The lipophilic nature of EOs enables them to cross the cell membrane and reach the cell interior. Therefore, extensive research is being

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conducted to explore their anticancer role. Several studies have reported the anticancer potential of EOs against breast, brain, colon, lung, liver, mouth, and prostate cancers (Cha et al., 2010; Zu et al., 2010; Akrout et al., 2011; Gomes et al., 2013; Jayaprakasha et al., 2013; Nanyonga et al., 2013). EOs such as α-bisabolol, α-humulene, α-pinene, α-thujene, β-elemene, β-caryophyllene, carvacrol, citral, eugenol, eucalyptol, farnesol, geraniol, germacrone, limonene, menthol, myrcene, nerolidol, perillyl alcohol, santalol, thymol, thymoquinone, and viridiflorene have demonstrated cytotoxic effects against a variety of cancers. Free radical scavenging, the induction of apoptosis, cell cycle arrest, and inhibition of tumor metastasis are among the few mechanisms proposed to be responsible for the antitumor activities of EOs. Furthermore, these agents have shown synergistic effects when combined with each other or with standard chemotherapy (Lesgards et al., 2014).

Illicium verum (*I. verum*) Hook (Illiciaceae), commonly known as star anise or Chinese star anise, is an aromatic evergreen tree that is distributed primarily in China, Pakistan, and other Asian countries. In 2002, *I. verum* was categorized as "both food and medicine" by the Ministry of Health, People's Republic of China, implying its low toxicity to humans (Wei et al., 2014). Its main chemical constituents include monoterpenoids, sesquiterpenoids, phenylpropanoids, lignans, flavonoids, and volatile compounds. According to previously published reports, fruits contain bitter principles, tannins, and EOs. Major EOs identified were trans-anethole, α-pinene, limone, β-phellandrene, α-terpineol, farnesol, and safrol (Chouksey et al., 2010).

Numerous studies have reported the antioxidant, antimicrobial, antifungal, anti-inflammatory, analgesic, anticonvulsive, insecticidal, and sedative activities of *I. verum* (Kim et al., 2009 and Yang et al., 2012). Significant growth inhibitory effects were observed in sarcoma-180, a mouse tumor model treated with polysaccharides isolated from the fruits of *I. verum* (Shu et al., 2010). Trans-anethole, one of the major constituents of *I. verum* EOs, has been reported to have antitumor activity against, breast cancer, cervical carcinoma, fibrosarcoma, and Ehrlich ascites tumor (Howes et al., 1990; Al-Harbi et al., 1995; Choo et al., 2011 and Carvalho et al., 2015).

To the best of our knowledge, no study has reported the anticancer effects of *I. verum* EOs against human colon cancer. Therefore, the present study was designed to investigate the chemical composition, antioxidant, and *in vitro* anticancer activities of these EOs. Furthermore, *in vitro* apoptotic assays were performed to explore the possible mechanism of cell death.

2. Experimental

2.1. Extraction of essential oils

Fruits of *I. verum* Hook. were procured from the local market in Malaysia and were identified by Mr. Shanmugan A/P Vellosamy, the School of Biological Sciences, Universiti Sains Malaysia. After removing dust and other foreign matter, the fruits were crushed into a coarse powder with an electric grinder (National, MJ-176NR, China). Approximately 500 g of powder was soaked in 2 L of analytical grade ethanol in an airtight container. Subsequently, the container was placed in a shaker at 40 °C for 48 h. The soaked material was then filtered using a Whatman grade 1 filter paper, and the reddish brown filtrate was evaporated using a rotary evaporator to form a thick, viscous, dark brown mass. Upon further drying in a hot air oven at 35 °C, the crude extract was separated into two portions, i.e., oily and dark brown solid mass. The oils were then separated from the residue, and the extracts were kept in a fridge at 4 °C in airtight containers until further experimentation. The stock solution of EOs, i.e., test sample (10 mg/mL), was prepared in DMSO for *in vitro* assays (Asif et al., 2014; Wong et al., 2014).

2.2. Chemical characterization of essential oils

An Agilent gas chromatography–mass spectrometer (Agilent 6890 N/5973I) coupled with electrospray ionization was used for the phytochemical analysis. Helium gas with a flow rate of 1.2 mL/min was used as a carrier, and 1 μL of sample dissolved in ethanol was injected for analysis. A single quadrupole detector was used for the separation of various phytochemicals using an HP-5 MS capillary column (0.25 mm × 30 m × 0.25 μm film thickness). The oven temperature was initially maintained at 70 °C for 2 min and then gradually increased at the rate of 20 °C/min to 305 °C for 30 min with a 1-minute hold at each point. The mass spectrometer electrospray ion source operated at 70 eV, and the acquisition range was between 35 m/z and 700 m/z, with a scan rate of 1 scan/s. Mass spectral correlations were performed using the NIST02 database.

2.3. Estimation of polyphenols

The total phenolic and flavonoid content of EOs was estimated using the following method.

2.3.1. Estimation of total phenolic content

The Folin–Ciocalteu method was used to estimate the total phenolic content of EOs. Folin–Ciocalteu reagent, sodium carbonate, standard/EOs, and distilled water were mixed in a ratio of 5:15:1:79 to make up a total volume of 200 μL in Eppendorf tubes separately for EOs and standard. After mixing, the contents were incubated at room temperature in the dark for 2 h. Absorbance was measured at 765 nm and the results were expressed as μg gallic acid equivalent mg^{-1} dry extract ($n = 3$) (Kumaran and Karunakaran, 2007).

2.3.2. Estimation of total flavonoid content

The aluminum chloride method was employed to estimate the flavonoid contents of EOs. The standard or EOs (100 μL) were mixed with 20 μL of (10%, w/v) aluminum chloride, 20 μL (1 mol L^{-1}) sodium acetate, 300 μL methanol, and 560 μL distilled water in Eppendorf tubes, followed by incubation at room temperature for 30 min in the dark. The absorbance of the reaction mixture was measured at 415 nm. Quercetin was used as a reference standard and the results were expressed as μg quercetin equivalent mg^{-1} dry extract ($n = 3$) (Orhan et al., 2011).

2.4. Antioxidant assays

The antioxidant capacity of EOs was assessed using three different models, i.e., 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays following well-established protocols (Al-Mansoub et al., 2014).

2.4.1. DPPH radical scavenging assay

A total of 100 μL of each test sample (six concentrations) and DPPH (200 μmol L^{-1} in methanol) reagent were added into each well of a 96-well plate followed by incubation at room temperature for 30 min in the dark. The absorbance of remaining DPPH was determined at 517 nm and the results were expressed as IC_{50}, i.e., concentration required to inhibit the formation of DPPH radicals by 50%. Ascorbic acid was used as a reference standard.

2.4.2. ABTS radical scavenging assay

In 180 μL of ABTS working solution, 20 μL of test samples (six concentrations) was added in each well of a 96-well plate separately. The absorbance of the samples was taken at 734 nm after 6 min of incubation at room temperature. Ascorbic acid was used as a standard and the results were expressed as IC_{50} values ($n = 3$) (Re et al., 1999).

2.4.3. Measurement of ferric reducing antioxidant power (FRAP)

A total of 150 μL of FRAP working solution and 50 μL of test sample were added to each well of a 96-well plate. The absorbance of the reaction mixture was measured at 600 nm after 8 min of incubation. A standard curve was constructed using ferrous sulfate ($FeSO_4 \cdot 7H_2O$) as a reference standard, and the results were expressed as nmol Fe^{+2} equivalent μg^{-1} dry extract (Benzie and Strain, 1996).

2.5. In vitro anticancer activities of essential oils

2.5.1. MTT assay for measuring cell viability

In vitro cytotoxic effects of EOs were evaluated by MTT assay against two human colon cancer cell lines, viz. HCT 116 (colorectal carcinoma, ATCC® CCL-247) and HT-29 (colorectal adenocarcinoma, ATCC® HTB-38). CCD-18Co (human normal colon fibroblast, ATCC® CRL-1459) was used as a normal cell line. A total of 10×10^4 cells/well were treated for 48 h with different concentrations of EOs. Dimethyl sulfoxide (DMSO) (0.5%) in media and 5-Fluorouracil (5-FU) were used as a negative and positive control, respectively. The absorbance of dissolved formazan crystals in DMSO was read using a microplate reader (TECAN Infinite Pro® M200, Switzerland) at 570 nm, with 620 nm as the reference wavelength. The results were presented as percent viability compared to the negative control (mean ± SD, $n = 3$) (Nassar et al., 2012).

2.5.2. Apoptotic properties of essential oils

Rhodamine 123 and Hoechst 33258 probes were used to study the effects of EOs on mitochondrial membrane potential and chromatin condensation, respectively. HCT 116 cells (10×10^4 per well) were treated with 25, 50, and 90 μg/mL (IC_{25}, IC_{50}, and IC_{90}) of EOs and 5 μg/mL of 5-FU for 24 h. Afterwards, cells were washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 20 min, and stained with Rhodamine 123 (1 μg/mL) and Hoechst 33258 (10 μg/mL) for 20 min in the dark. After washing twice with PBS, cells were examined under an inverted fluorescent microscope (EVOS *fl*, Digital microscopy group, USA) using red and blue filters for Rhodamine 123 and Hoechst 33258 stains, respectively. The number of cells with apoptotic morphology was counted in five randomly selected fields per well. The apoptotic index was calculated as percentage of apoptotic cells compared to the total number of cells. The results were presented as mean ± SD ($n = 3$) (Aisha et al., 2012).

2.6. Anti-metastatic studies

The effects of EOs on three major steps of cancer metastasis, i.e., cell invasion, migration, and colony formation were studied using established protocols. The Boyden chamber method with slight modifications was used to study the inhibitory effects of EOs on the invasion of HCT 116 cells. A total of 50 μL of Matrigel (5 mg/mL, diluted with RMPI 1:1) was loaded into each well of a 96-well plate and allowed to polymerize for 45 min at 37 °C in an incubator. A total of 5×10^3 cells in 150 μL of RPMI medium containing 25, 50, and 90 μg/mL of EOs (IC_{25}, IC_{50}, and IC_{90}) were added to each well separately and incubated for 24 h. Cells with 0.5% DMSO in media and 5-FU (5 μg/mL) were used as the negative and positive control, respectively. At the end of the incubation period, non-invading cells were washed with PBS and the number of invading cells was determined under inverted light microscopy. The results were presented as the mean percentage inhibition compared to untreated cells ($n = 3$).

To further study the inhibitory effects of EOs on cell migration, HCT 116 cells were seeded in a 6-well plate and allowed to become 100% confluent. The monolayer of cells was scratched using a micropipette tip, followed by washing with PBS to remove dead and detached cells. Cells were treated with three doses of EOs (25, 50, and 90 μg/mL) and 5 μg/mL of 5-FU in fresh medium. Media containing 0.5% DMSO was used as a negative control. The resultant wounds were then photographed using an inverted microscope (EVOS *fl*, Digital microscopy group, USA) at 0 h and 24 h. The size of cell-free wounds was then measured using Leica QWin image analysis software (Leica Microsystems Inc., Buffalo Grove, Illinois), and the percentage inhibition of wound closure was calculated relative to zero time.

For clonogenic assay, 1000 cells per well were plated in single cell suspension in a 6-well plate. After 24 h, attached cells were treated with different concentrations of EOs (25, 50, and 90 μg/mL). 5-FU (5 μg/mL) and media containing 0.5% DMSO were used as the positive and negative control, respectively. After 48 h of treatment, old medium was aspirated, cells were washed twice with PBS, and fresh medium without any test sample/drugs was added. On the 10th day, the cells were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet for 30 min at room temperature. Colonies of 50 cells or more were counted, and the plating efficiency (PE) and the survival fraction (SF) were calculated using standard equations. The results were expressed as mean ± SD ($n = 3$) (Aisha et al., 2014; Nassar et al., 2012).

3. Statistical analysis

Descriptive statistics was used to present the findings. The results are presented as mean ± SD. One-way ANOVA was performed using Graph Pad Prism (Graph PAD, San Diego, USA) software to test the differences between groups. A p value of less than 0.05 was considered statistically significant.

4. Results and discussion

Carcinogenesis is a multistep process, and oxidative damage is linked to the formation of tumors through several mechanisms. Natural products derived from different plants contain a wide variety of secondary metabolites with antioxidant activities that are capable of halting various steps involved in the development of cancer (Rajput and Mandal, 2012). It is a fact that a single assay cannot represent the behavior of free radicals and antioxidants in a living system. Therefore, we performed an array of assays in the present study (i.e., DPPH, ABTS, and FRAP models) to investigate the oxidant scavenging potential of EOs. Our findings indicated good antioxidant activity of EOs in the FRAP model with a value of 359.11 ± 2.28 nmol Fe^{+2} Eq/mg extract and moderate activity in the DPPH and ABTS models with IC_{50} values of 47.01 ± 2.42 and 75.90 ± 2.29 μg/mL, respectively, indicating that the pharmacological effects of EOs might be due to multiple antioxidant mechanisms. The results of our DPPH assay are consistent with other studies in which polar extracts and the polar fractions of *I. verum* showed almost the same IC_{50} values (Yang et al., 2012).

Plant-derived phenolic compounds such as phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, and tannins have shown promising results in different antioxidant and anticancer models (Cai et al., 2004). In an attempt to establish a link between the antioxidant effects of EOs and their polyphenolic contents, we further estimated the phenolic and flavonoid contents of EOs by adopting methods reported in the literature. The total phenolic and total flavonoid contents in EOs were 63.51 ± 3.23 μg gallic acid Eq/mg extract and 24.30 ± 0.28 μg quercetin Eq/mg extract, respectively. These findings suggest that the antioxidant activity of EOs might be mainly due to their polyphenol contents. Table 1 presents the antioxidant properties and polyphenol contents of EOs.

A detailed chemical characterization of EOs by GC-MS revealed that a total of 35 compounds were present in the sample. Compounds with a similarity index of 90 and above were considered for reporting. Trans-anethole was one of the major components (Table 2). The GC-MS spectrum of the EOs is shown in Fig. S1.

A positive relationship between polyphenol intake and reduced risk of some cancers has been reported in some epidemiological studies (Zhao et al., 2007; Prasada et al., 2009). We therefore screened the

Table 1
Polyphenolic contents and antioxidant values of *I. verum* essential oils.

Extract	Total phenolic (μg gallic acid Eq/mg extract)	Total flavonoids (μg quercetin Eq/mg extract)	DPPH IC_{50} (μg/mL)	ABTS IC_{50} (μg/mL)	FRAP (nmol Fe^{+2} Eq/mg extract)
Essential oil	63.51 ± 3.235	24.30 ± 0.28	47.01 ± 2.42	75.90 ± 2.29	359.11 ± 2.28
Standard	–	–	5.40 ± 0.07	3.90 ± 0.05	–

Table 2
Chemical composition of essential oils of *I. verum*.

S. No.	% Area	Compound	Quality
1	7.07	Tans-anethole	98
2	0.90	p-Anisaldehyde	90
3	2.23	Cyclohexanol, 2-[2-pyridyl]-	95
4	3.72	Palmitic acid,	98
5	1.05	Ethyl palmitate	97
6	5.00	Elaidic acid,	99
7	1.25	Stearic acid	99
8	1.37	Oleic acid	96
9	0.53	Cyclohexanol, 2-[2-pyridyl]-	92
10	1.38	Tricyclo[7.2.0.0(2,6)]undecan-5-ol, 2,6,10,10-tetramethyl- (isomer 2)	90
11	1.39	*N′*-(2,4,6(1H,3H,5H)-Trioxopyrimidin-5 ylidenemethyl)-2-nitrobenzhydr azide	90

in vitro anticancer effects of EOs against human colon cancer cell lines (HCT 116 and HT-29) using the MTT assay and found that the EOs of *I. verum* have moderate anti-proliferative activity against the p53 positive colon cancer cell line (HCT 116), with an IC_{50} value of 50.34 ± 1.19 μg/mL (Sylvestre et al., 2006). Similarly, the EOs were screened for toxicity against normal human colon fibroblasts (CCD-18co), and the IC_{50} was 200 ± 2.29 μg/mL (Table 3). The selectivity index of EOs against each cell line was calculated, revealing that the EOs were three times more toxic towards the HCT 116 cell line compared to normal cells. A similar trend was reported by another research group, who found that EOs showed more toxicity in cancer cells compared to a normal human cell line (de-Oliveira et al., 2015). Given the selective cytotoxic nature of EOs among the cancer cell lines tested (HCT 116 and HT-29), further detailed mechanistic studies were carried out using the HCT 116 cell line.

The induction of apoptosis in cancer cells is one of the most common approaches used to treat carcinogenesis and results in the arrest of various steps involved in tumor metastasis and the development of resistance to chemotherapy (Lowe and Lin, 2000; Zhang et al., 2000; Wong, 2011). Scientific reports have shown that the mixture of EOs and trans-anethole showed promising apoptosis-inducing and anti-metastatic activity against a variety of cancer cell lines (Lu et al., 2004; Chen et al., 2011; Choudharya et al., 2015). In an attempt to study the apoptosis-mediating effects of EOs, two fluorescent probes, i.e., Hoechst 33258 and Rodhamine 123, which have a selective affinity for nuclei and mitochondria, were used. Hoechst 33258 is a DNA-specific fluorescent dye that enters the cell nucleus and binds to DNA only when the plasma membrane is damaged, resulting in the formation of a complex with DNA due to its specific binding with A-T base pairs in ds-DNA. Apoptotic cells appear brighter when observed under the blue filter of a fluorescent microscope compared to normal cells. Moreover, the uptake of Rhodamine 123, a lipophilic cationic dye, by mitochondria was observed under the red filter of a fluorescent microscope. Loss of red fluorescent signal, which is proportional to the reduction in mitochondrial membrane potential, is used as an indicator of mitochondrial damage and involvement of the intrinsic pathway of apoptosis (Nassar et al., 2012). Our study showed that the EOs induced chromatin condensation and typical crescent-shaped nuclei in treated cells in a dose-dependent fashion. The apoptotic indices after 24 h of treatment with EOs (25, 50, and 90 μg/mL) and 5-FU were 12.55 ± 2.77%, 39.26 ± 11.69%, 54.06 ± 10.69% and 77.31 ± 12.01%, respectively, and were significantly greater ($p < 0.001$) compared to untreated cells (3.07 ± 5.69%) especially at higher doses. In addition, a reduction in mitochondrial membrane potential was observed in treated cells, indicating the involvement of the mitochondrial pathway in the induction of apoptosis. The apoptotic indices after 24 h of treatment with EOs and 5-FU were 16.70 ± 2.23%, 33.63 ± 2.16%, 50.33 ± 2.16% and 76.31 ± 2.42%, respectively (Fig. 1). A similar kind of apoptotic behavior of EOs was reported by other research groups, reflecting the medicinal importance of EOs in the treatment of cancer (Cha et al., 2009; Chen et al., 2010; Gomuttapong et al., 2012). The arrows in Fig. 1 highlight the apoptotic cells with typical morphological changes. After establishing the antioxidant and apoptotic role of EOs in colon cancer, we further studied the anti-metastatic effects of EOs against the best responding cell line.

Various studies have reported the anti-metastatic effects of EOs, including trans-anethole, against a variety of cancers (Lu et al., 2004; Choo et al., 2011). The HCT 116 cell line is classified as a model cell line for studying the molecular mechanisms involved in tumor metastasis and was used to study the anti-metastatic effects of EOs against three key steps of metastasis, i.e., migration, invasion, and colony formation (Rajput et al., 2008). The present study showed a dose-dependent decrease in cell migration. At 25, 50, and 90 μg/mL, significant inhibition of wound closure was observed (10.01 ± 3.44% ($p < 0.05$), 23.12 ± 3.05% ($p < 0.001$), 46.09 ± 3.32% ($p < 0.001$)), while in the 5-FU-treated group, 81.40 ± 9.09% inhibition of wound closure was observed at the end of 24 h, respectively. The experiment was stopped when the gap in the negative control (media with 0.5% DMSO) was closed, i.e., 24 h (Fig. 2).

Tissue invasion and metastasis are vital components in the escape and dissemination of tumor cells from the primary site into distant organs (Hanahan and Weinberg, 2011). A modified Boyden chamber method was employed to study the effects of EOs on tumor cell invasion across the Matrigel basement membrane. At the concentration of 25, 50, and 90 μg/mL of EOs, the percent inhibition of cell invasion was 33.33 ± 7.43%, 55.01 ± 3.67%, and 60.30 ± 6.73%, respectively, which was significantly higher ($p < 0.001$) from that in the control group (Fig. 3). In 5-FU-treated cells, almost 90% inhibition of cell invasion was observed. Finally, colonization of micro-metastatic lesions at distant sites was studied using a colony formation assay. The results of the study showed that the EOs significantly arrested the colony formation process in a dose-dependent manner. The percent inhibition of colonization at 25, 50, and 90 μg/mL was 17.66% ± 2.63% ($p < 0.05$), 37.87% ± 1.66% ($p < 0.001$), and 80.7% ± 19.2% ($p < 0.0001$), respectively. In 5-FU-treated wells, a 5% survival rate and 98.70% ± 2.80% ($p < 0.0001$) inhibition of colony formation were observed compared with the control group. Fig. 3 depicts the effects of EOs against HCT 116 cell invasion and colony formation. Altogether, the results of our study demonstrated that the multiple components present in EOs arrested various

Table 3
IC_{50} values of essential oils against panel of cell lines.

Cell lines	IC_{50} (μg/mL)		Selectivity index
	EOs	5-FU	
HCT 116 (colon cancer)	50.34 ± 1.19	5	3.87
HT-29 (colon cancer)	100 ± 2.19	8	2
CCD-18co (human colon fibroblast)	200 ± 2.29	–	–

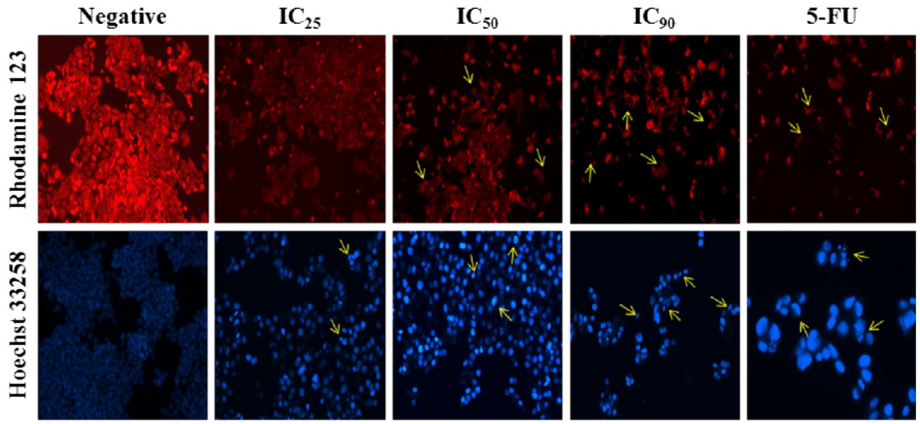

Fig. 1. Apoptotic effects of EOs after 24 h of treatment in HCT 116 cells. Yellow arrows show the apoptotic bodies (where IC_{25} = 25 μg/mL, IC_{50} = 50 μg/mL, and IC_{90} = 90 μg/mL).

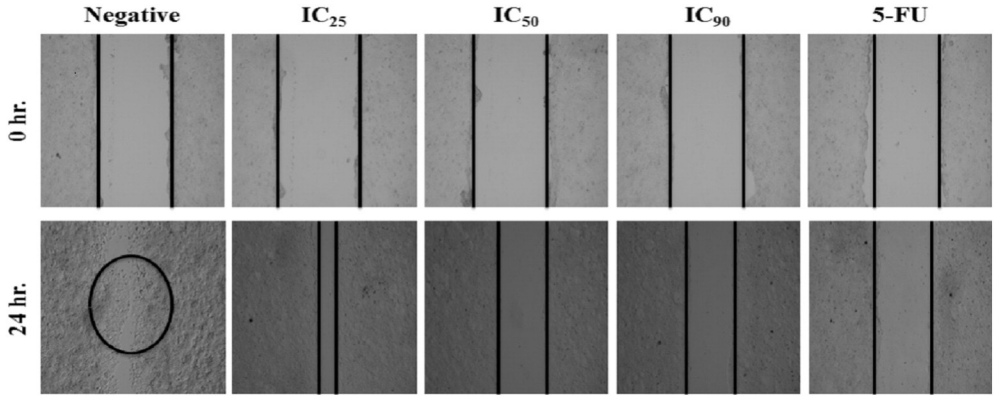

Fig. 2. Effects of EOs against HCT116 cell migration. The straight lines indicate the cell-free zone while the circle indicates the sites where cell migration has filled the free zone (where IC_{25} = 25 μg/mL, IC_{50} = 50 μg/mL, and IC_{90} = 90 μg/mL).

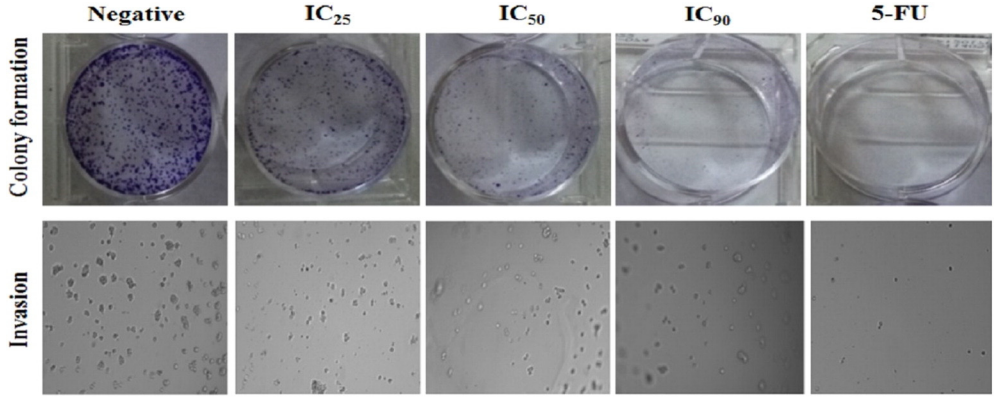

Fig. 3. Anti-metastatic effects of EOs against HCT 116 cells. Dose-dependent inhibition of colony formation and cell invasion was observed in treated groups (where IC_{25} = 25 μg/mL, IC_{50} = 50 μg/mL, and IC_{90} = 90 μg/mL).

steps of tumor metastasis, which might be due to their antioxidant and apoptosis-inducing nature. Further studies to isolate the pure compounds and study their effects against key steps in tumor apoptosis and metastasis are encouraged.

5. Conclusion

The cytotoxic effect of EOs obtained from the ethanolic extract of *Illicium verum* fruits in colon cancer cells may be attributed to multiple mechanisms such as free radical scavenging, the induction of apoptosis, and inhibition of tumor metastasis, ultimately leading to cell death. Further studies to isolate pure compounds and explore exact molecular targets are encouraged.

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References

Adorjan, B., Buchbauer, G., 2010. Biological properties of essential oils: an updated review. Flavour Fragance J. 25, 407–426.

Aisha, A.F.A., Abu-Salah, K.M., Ismail, Z., Majid, A.M.S.A., 2012. In vitro and in vivo anti-colon cancer effects of *Garcinia mangostana* xanthones extract. BMC Complement. Altern. Med. 12, 104.

Akrout, A., Gonzalez, L.A., Jani, H.E., Madrid, P.C., 2011. Antioxidant and antitumor activities of *Artemisia campestris* and *Thmelaea hirsuta* from southern Tunisia. Food Chem. Toxicol. 49, 342–347.

Al-Harbi, M.M., Qureshi, S., Raza, M., Ahmed, M.M., Giangreco, A.B., Shah, A.H., 1995. Influence of anethole treatment on the tumour induced by Ehrlich ascites carcinoma cells in paw of Swiss albino mice. Eur. J. Cancer Prev. 4, 307–318.

Al-Mansoub, M.A., Asmawi, M.Z., Murugaiyah, V., 2014. Effect of extraction solvents and plant parts used on the antihyperlipidemic and antioxidant effects of *Garcinia atroviridis*: a comparative study. J. Sci. Food Agric. 94, 1552–1558.

Amin, A., Gali-Muhtasib, H., Ocker, M., Schneider-Stock, R., 2009. Overview of major classes of plant-derived anticancer drugs. Int. J. Biol. Sci. 11, 1–11.

Asif, M., Jabeen, Q., Abdul Majid, A.M.S., Atif, M., 2014. Diuretic activity of *Boswellia serrata* Roxb. oleo gum extract in albino rats. Pak. J. Pharm. Sci. 27, 1811–1817.

Benzie, I.F., Strain, J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay. Anal. Biochem. 239, 70–76.

Bhall, Y., Gupta, V.K., Jaitak, V., 2013. Anticancer activity of essential oils: a review. J. Sci. Food Agric. 93, 3643–3653.

Cai, Y., Luo, Q., Sun, M., Corke, H., 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 74, 2157–2184.

Carroll, R.E., Benya, R.V., Turgeon, D.K., Vareed, S., Neuman, M., Rodriguez, L., Kakarala, M., Carpenter, P.M., McLaren, C., Meyskens Jr., F.L., Brenner, D.E., 2011. Phase IIa clinical trial of curcumin for the prevention of colorectal neoplasia. Cancer Prev. Res. (Phila). 4, 354–364.

Carvalho, A.A., Andrade, L.N., Sousa, É.B.Vd., Sousa, D.Pd., 2015. Antitumor phenylpropanoids found in essential oils. BioMed. Res. Int. 2015, 1–21.

Cha, J.D., Moon, S.E., Kim, H.Y., Cha, I.H., Lee, K.Y., 2009. Essential oil of *Artemisia capillaris* induces apoptosis in KB cells via mitochondrial stress and caspase activation mediated by MAPK-stimulated signaling pathway. J. Food Sci. 74, 75–81.

Cha, J.D., Kim, Y.H., Kim, J.Y., 2010. Essential oil and 1,8-cineole from *Artemisia lavandulaefolia* induces apoptosis in KB cells via mitochondrial stress and caspase activation. Food Sci. Biotechnol. 19, 185–191.

Chabner, B., 2006. Cancer chemotherapy and biotherapy principles and practice clinical strategies for cancer treatment. In: Chabner, B.A., Longo, D.L. (Eds.), The Role of Drugs, 4th ed. Lippincott Williams and Wilkins, Philadelphia.

Chen, W., Hou, J., Yin, Y., Jang, J., Zheng, Z., Fan, H., Zou, G., 2010. α-Bisabolol induces dose- and time-dependent apoptosis in HepG2 cells via a Fas- and mitochondrial-related pathway, involves p53 and NFκB. Biochem. Pharmacol. 80, 247–254.

Chen, W., Lu, Y., Wu, J., Gao, M., Wang, A., Xu, B., 2011. Beta-elemene inhibits melanoma growth and metastasis via suppressing vascular endothelial growth factor-mediated angiogenesis. Cancer Chemother. Pharmacol. 67, 799–808.

Choo, E.J., Rhee, Y.H., Jeong, S.J., Lee, H.J., Kim, H.S., Ko, H.S., Kim, J.H., Kwon, T.R., Jung, J.H., Kim, J.H., Lee, H.J., Lee, E.O., Kim, D.K., Chen, C.Y., Kim, S.H., 2011. Anethole exerts antimetatstaic activity via inhibition of matrix metalloproteinase 2/9 and AKT/mitogen-activated kinase/nuclear factor kappa B signaling pathways. Biol. Pharm. Bull. 43, 41–46.

Choudharya, A., Kumarb, R., Srivastavab, R.B., Surapanenic, S.K., Tikooc, K., Singha, I.P., 2015. Isolation and characterization of phenolic compounds from *Rhodiola imbricata*, a trans-Himalayan food crop having antioxidant and anticancer potential. J. Funct. Foods 16, 183–193.

Chouksey, D., Sharma, P., Pawar, R.S., 2010. Biological activities and chemical constituents of *Illicium verum* hook fruits (Chinese star anise). Der Pharm. Sin. 1, 1–10.

de-Oliveira, F., Alves, J.M., Damasceno, J.L., Oliveira, R.A.M., Dias, H.J., Crotti, A.E.M., Tavares, D.C., 2015. Cytotoxicity screening of essential oils in cancer cell lines. Rev. Bras. Farm. 25, 183–188.

Dhillon, N., Aggarwal, B.B., Newman, R.A., Wolff, R.A., Kunnumakkara, A.B., Abbruzzese, J.L., Ng, C.S., Badmaev, V., Kurzrock, R., 2008. Phase II trial of curcumin in patients with advanced pancreatic cancer. Clin. Cancer Res. 14, 4491–4499.

Gautam, N., Mantha, A.K., Mittal, S., 2014. Essential oils and their constituents as anticancer agents: a mechanistic view. BioMed Res. Int. 2014, 23.

Gomes, M.R.F., Schuh, R.S., Jacques, A.L.B., Augustin, O.A., Bordignon, S.A.L., Dias, D.O., 2013. Citotoxic activity evaluation of essential oils and nanoemulsions of *Drimys angustifolia* and *D. brasiliensis* on human glioblastoma (U-138 MG) and human bladder carcinoma (T24) cell lines in vitro. Rev. Bras. Farm. 23, 259–267.

Gomuttapong, S., Pewphong, R., Choeisiri, S., Jaroenporn, S., Malaivijitnond, S., 2012. Testing of the estrogenic activity and toxicity of *Stephania venosa* herb in ovariectomized rats. Toxicol. Mech. Methods 22 (2012), 445–457.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.

Howells, L.M., Berry, D.P., Elliott, P.J., Jacobson, E.W., Hoffmann, E., Hegarty, B., Brown, K., Steward, W.P., Gescher, A.J., 2011. Phase I randomised double-blind pilot study of micronized resveratrol (SRT501) in patients with hepatic metastases-safety, pharmacokinetics and pharmacodynamics. Cancer Prev. Res. 4, 1419–1425.

Howes, A.J., Chan, V.S., Caldwell, J., 1990. Structure-specificity of the genotoxicity of some naturally occurring alkenylbenzenes determined by the unscheduled DNA synthesis assay in rat hepatocytes. Food Chem. Toxicol. 28, 537–542.

Jayaprakasha, G.K., Murthy, K.N.C., Uckoo, R.M., Patil, B.S., 2013. Chemical composition of volatile oil from *Citrus limettioides* and their inhibition of colon cancer cell proliferation. Ind. Crop. Prod. 45, 200–207.

Khan, N., Adhami, V.M., Mukhtar, H., 2009. Review: Green tea polyphenols in chemoprevention of prostate cancer: preclinical and clinical studies. Nutr. Cancer 61, 836–841.

Kim, J.Y., Kim, S.S., Oh, T.H., Baik, J.S., Song, G., Lee, N.H., Hyun, C.G., 2009. Chemical composition, antioxidant, anti-elastase, and anti-inflammatory activities of *Illicium anisatum* essential oil. Acta Pharma. 59 (3), 289–300.

Kumaran, A., Karunakaran, R.J., 2007. In vitro antioxidant activities of methanol extracts of five *Phyllanthus* species from India. LWT- Food Sci. Technol. 40, 344–352.

Lesgards, J.F., Baldovini, N., Vidal, N., Pietri, S., 2014. Anticancer activities of essential oils constituents and synergy with conventional therapies: a review. Phytother. Res. 28, 1423–1446.

Lowe, S.W., Lin, A.W., 2000. Apoptosis in cancer. Carcinogenesis 21, 485–495.

Lu, X.G., Zhan, L.B., Feng, B.A., Qu, M.Y., Yu, L.H., Xie, J.H., 2004. Inhibition of growth and metastasis of human gastric cancer implanted in nude mice by d-limonene. World J. Gastroenterol. 10, 2140–2144.

Nanyonga, S.K., Opoku, A., Lewu, F.B., Oyedeji, A.O., Singh, M., 2013. Chemical composition, antioxidant activity and cytotoxicity of the essential oils of the leaves and stem of *Tarchonanthus camphoratus*. Afr. J. Pharm. Pharmacol. 7, 360–367.

Nassar, Z.D., Aisha, A.F.A., Idris, N., Ahamed, M.B.K., Ismail, Z., Abu-Salah, K.M., Alrokayan, S.A., Shah Abdul Majid, A.M., 2012. Koetjapic acid, a natural triterpenoid, induces apoptosis in colon cancer cells. Oncol. Rep. 27, 727–733.

Orhan, N., Orhan, I.E., Ergun, F., 2011. Insights into cholinesterase inhibitory and antioxidant activities of five *Juniperus* species. Food Chem. Toxicol. 49, 2305–2312.

Prasada, K.N., Haoa, J., Shib, J., Liua, T., Lia, J., Weia, X., Qiua, S., Xueb, S., Jiang, Y., 2009. Antioxidant and anticancer activities of high pressure-assisted extract of longan (*Dimocarpus longan* Lour.) fruit pericarp. Innov. Food Sci. Emerg. Technol. 10 (4), 413–419.

Rajput, S., Mandal, M., 2012. Antitumor promoting potential of selected phytochemicals derived from spices: a review. Eur. J. Cancer Prev. 205–215.

Rajput, A., Martin, I.D.S., Rose, R., Beko, A., LeVea, C., Sharratt, E., Mazurchuk, R., Hoffman, R.M., Brattain, M.G., Wang, J., 2008. Characterization of HCT116 human colon cancer cells in an orthotopic model. J. Surg. Res. 14, 276–281.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26, 1231–1237.

Shimizu, M., Fukutomi, Y., Ninomiya, M., Nagura, K., Kato, T., Araki, H., Suganuma, M., Fujiki, H., Moriwaki, H., 2008. Green Tea extracts for the prevention of metachronous colorectal adenomas: a pilot study. Cancer Epidemiol. Biomarkers Prev. 17, 3020–3025.

Shu, X., Liu, X., Fu, C., Liang, Q., 2010. Extraction, characterization and antitumor effect of the polysaccharides from star anise (*Illicium verum* Hook.). J. Med. Plant Res. 4, 2666–2673.

Sylvestre, M., Pichette, A., Longtin, A., Nagau, F., Legault, J., 2006. Essential oil analysis and anticancer activity of leaf essential oil of *Croton flavens* L. from Guadeloupe. J. Ethnopharmacol. 103, 99–102.

Wei, L., Hua, R., Li, M., Huang, Y., Li, S., He, Y., Shen, Z., 2014. Chemical composition and biological activity of star anise *Illicium verum* extracts against maize weevil, *Sitophilus zeamais* adults. J. Insect Sci. 14, 80.

Wong, R.S., 2011. Apoptosis in cancer: from pathogenesis to treatment. J. Exp. Clin. Cancer Res. 30, 87.

Wong, Y.C., Lee, P.P., Nurdiyana, W.A.W., 2014. Extraction and antioxidative activity of essential oil from star anise (*Illicium verum*). Orient. J. Chem. 30, 1159–1171.

Yang, C.H., Chang, F.R., Chang, H.W., Wang, S.M., Hsieh, M.C., Chuang, L.Y., 2012. Investigation of the antioxidant activity of *llicium verum* extracts. J. Med. Plant Res. 6, 314–324.

Zhang, Y.A., Nemunaitis, J., Scanlon, K.J., Tong, A.W., 2000. Anti-tumorigenic effect of a K ras ribozyme against human lung cancer cell line heterotransplants in nude mice. Gene Ther. 7, 2041–2050.

Zhao, M., Yang, B., Wang, J., Liu, Y., Yu, L., Jiang, Y., 2007. Immunomodulatory and anticancer activities of flavonoids extracted from litchi (*Litchi chinensis* Sonn) pericarp. Int. Immunopharmacol. 7, 162–166.

Zu, Y., Yu, H., Liang, L., Fu, Y., Efferth, T., Liu, X., Wu, N., 2010. Activities of ten essential oils towards *Propionibacterium acnes* and PC-3, A-549 and MCF-7 cancer cells. Molecules 15, 3200–3210.