Biomedicine & Pharmacotherapy 109 (2019) 1620–1629

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Establishment of *in vitro* and *in vivo* anti-colon cancer efficacy of essential oils containing oleo-gum resin extract of *Mesua ferrea*

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ARTICLE INFO

Keywords:
Mesua ferrea
Oleo-gum resin
Survivin, xIAP
HSPs
Isoledene
Elemene

ABSTRACT

Proven the great potential of essential oils as anticancer agents, the current study intended to explore molecular mechanisms responsible for *in vitro* and *in vivo* anti-colon cancer efficacy of essential oil containing oleo-gum resin extract (RH) of *Mesua ferrea*. MTT cell viability studies showed that RH had broad spectrum cytotoxic activities. However, it induced more profound growth inhibitory effects towards two human colon cancer cell lines *i.e.*, HCT 116 and LIM1215 with an IC_{50} values of 17.38 ± 0.92 and 18.86 ± 0.80 µg/mL respectively. RH induced relatively less toxicity in normal human colon fibroblasts *i.e.*, CCD-18co. Cell death studies conducted, revealed that RH induced characteristic morphological and biochemical changes in HCT 116. At protein level it down-regulated expression of multiple pro-survival proteins *i.e.*, survivin, xIAP, HSP27, HSP60 and HSP70 and up-regulated expression of ROS, caspase-3/7 and TRAIL-R2 in HCT 116. Furthermore, significant reduction in invasion, migration and colony formation potential was observed in HCT 116 treated with RH. Chemical characterization by GC–MS and HPLC methods revealed isoledene and elemene as one the major compounds. RH showed potent antitumor activity in xenograft model. Overall, these findings suggest that RH holds a promise to be further studied for cheap anti-colon cancer naturaceutical development.

1. Introduction

In the current scenario of cancer therapeutics, radiations, surgery, and chemotherapy are the main options available to treat different stages of carcinogenesis. Though, synthetic drugs have shown promising results in the management of a wide range of neoplastic disorders, still these agents are known to reduce only 5% of cancer-related mortalities and are associated with advent of side effects over the course of treatment [1]. In addition, majority of these therapeutic options were designed to hit a single intracellular target which makes them merely ineffective in later phases of cancers where multiple cellular components interact with each other to support the uncontrolled proliferation. Therefore, multitargeted approach in which a single compound or group of compounds can halt several abnormal cellular events simultaneously is highly desirable [2]. Plant extracts have capacity to halt multiple tumorigenic steps either alone or in combination with conventional chemotherapies and are associated with relatively tolerable side effects [3]. Induction of apoptosis, modulation of activity of cell signalling pathways, inhibition of key enzymes involved in carcinogenesis, retardation of tumor metastasis and angiogenesis are among the few mechanisms proposed to be responsible for anticancer activities of natural products [4]. Colorectal carcinoma (CRC) is the third most common cancer worldwide. In Malaysia, according to the National Cancer Registry (NCR) report 2007, CRC accounts for 12.3%

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https://doi.org/10.1016/j.biopha.2018.10.127
Received 16 July 2018; Received in revised form 21 October 2018; Accepted 21 October 2018

of all the cancer cases reported. Worldwide high prevalence of CRC demands urgent need to find new treatments to combat this multi-factorial syndrome [5]. In our previous *in vitro* study we proposed that isoledene (82% of total IR-SF by GC-MS) has potent apoptosis-inducing activity towards HCT 116 [6], however due to limited amount of compound rich sub-fraction further *in vivo* studies were not possible. Therefore, current study was designed to obtain isoledene rich oleo-gum resin extract and to study the detailed molecular interactions responsible for *in vitro* and *in vivo* anticancer activity. Moreover, to best of our knowledge this is first study that reports the detailed *in vitro* and *in vivo* anticancer efficacy of *M. ferrea* oleo-gum resin extract toward human colon cancer.

2. Materials and methods

2.1. Collection of plant material

Oleo-gum resin was collected from the incisions created on bole of well-identified *Mesua ferrea* tree (voucher number, 11,535) located in the premises of Universiti Sains Malaysia (USM), Penang, Malaysia. The crude oleo-gum resin (yellowish in colour having pleasant fragrance) was stored at 4 °C in an airtight container until further detailed studies.

2.2. Preparation of oleo-gum resin extract and fractionation

Sonication method (35 °C for 30 min) was used for preparation of crude ethanol (99.8%) extract (RCr) and subsequent fractionation of RCr into n-hexane (RH) and chloroform (RCF) fractions. Stock solutions of RCr, RH and RCF (20 mg/mL) were prepared in a dimethyl sulfoxide (DMSO) for *in vitro* anticancer assays. RCr and its resultant fractions (RH and RCF) were subject to preliminary cytotoxic screening towards human colorectal carcinoma, HCT 116 cell line using MTT assay. Based on MTT cell viability assay results, RH was selected for further experimentations. See supplementary file for details.

2.3. Chemical characterization

2.3.1. Gas chromatography mass spectrometer (GC–MS) analysis

An Agilent GC–MS (Agilent 6890 N/5973I) coupled with electrospray ionization was used for the detailed chemical characterization of active oleo-gum resin crude fraction (RH) following a reported method. Compounds having similarity 90 and above with National Institute of Standards and Technology library (NIST version 02) were considered for reporting in the present study [3]. See supplementary file for details.

2.3.2. High performance liquid chromatography (HPLC) quantification

Reported method was used for HPLC method development and quantification of marker compound i.e., Isoledene (Sigma-Aldrich, CAS No: 95910-36-4, ≥ 95.0%) [6,7]. See supplementary file for details.

2.4. In vitro anticancer activities

2.4.1. MTT cell viability assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed to estimate the extent of cytotoxicity induced by RH against a panel of human cancer cell lines, *viz.* colorectal carcinoma (HCT 116, ATCC® CCL-247; HT-29, ATCC® HTB-38; LIM1215, CBA-0161), gastric carcinoma (KATO-III, ATCC® HTB10; MKN-74, JCRB-1473), pancreatic carcinoma (PANC-1, ATCC® CRL-1469; Capan-1, ATCC® HTB-79), prostate cancer (PC-3, ATCC® CRL-1439), mammary gland carcinoma (HCC38, ATCC® CRL231; BT-549, ATCC® HTB122) and glioblastoma (U-87 MG, ATCC® HTB-14) respectively. CCD-18co (human normal colon fibroblasts, ATCC® CRL-1459) was used to estimate toxicity induced by RH against normal human cells. On the basis of IC_{50} and selectivity index (SI) values, the most susceptible cell line *i.e.*, HCT 116 was selected for further detailed studies [8].

2.4.2. Apoptosis studies

2.4.2.1. Morphological observations. Inverted light microscopic studies were conducted to study the typical changes induced by RH in the overall morphology of HCT 116 following well-established methods [1,8]. See supplementary file for details.

2.4.2.2. Quantification of apoptosis by Rhodamine 123 and Hoechst 33258 staining. Pro-apoptotic effects of RH on mitochondria and nucleus of HCT 116 cells were studied using Rhodamine 123 and Hoechst 33258 stains respectively, following reported method [9]. Cell having apoptotic features (having low mitochondrial outer membrane potential (MOMP) and condensed nuclei) were counted in five randomly selected fields per well. Results are presented as mean ± SD of % apoptotic indexes (n = 3). See supplementary file for details.

2.4.2.3. Lactate dehydrogenase (LDH) release assay. Effect of RH on release of LDH enzyme in HCT 116 was measured using Cayman® LDH assay kit following manufacturer's protocol. Results are presented as μU of LDH present in each mL of cell culture supernatant (n = 3). See supplementary file for details.

2.4.2.4. Caspase-3/7 activity. Effects of RH on activation of caspase-3/7 were studied using Caspase-Glo® assay kit following manufacturer's (Promega, USA) protocol. Results are presented as mean ± SD of relative fold increase in the levels of caspase-3/7 in RH-treated HCT 116. See supplementary file for details.

2.4.2.5. Human apoptosis proteome profiler. Human apoptosis proteome profiler (Raybiotech®, USA) was used to study the effects of RH on expression pattern of wide range of interrelated pro-apoptotic and antiapoptotic proteins in HCT 116. Assay was performed using a 8-well antibody pre-coated glass slide array following manufacturer's protocol.

2.4.2.6. Measurement of intracellular reactive oxygen species (ROS). A cell-permeable fluorescent dye *i.e.*, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was used to study the effects of RH on generation of intracellular ROS in HCT 116 following reported methods [8,10]. Results are expressed as mean ± SD of fluorescent intensity measured in each group (n = 3).

2.5. Antimetastatic studies

2.5.1. Cell invasion assay

Modified Boyden chamber method with slight modifications was employed to study the anti-invasive potential of RH towards HCT 116. Results are presented as mean ± SD of percent inhibition of cell invasion in RH treatment groups (n = 3) [11]. See supplementary file for details.

2.5.2. Cell migration assay

In vitro scratch assay was employed to study the effects of oleo-gum resin extract on migratory potential of HCT 116 following well-established methods. Results are presented as mean ± SD of percent inhibition of wound closure (n = 3) [12]. See supplementary file for details.

2.5.3. Colony formation assay

Seeding before treatment protocol was adopted to study anti-clonogenic potential of RH towards human metastatic cell line *i.e.*, HCT 116 [12]. Results are presented as mean ± SD of percent inhibition of colony formation in the treatment groups. See supplementary file for details.

2.6. In vivo antitumor activity

In vivo antitumor efficacy of active oleo-gum resin fraction was evaluated in ectopic tumor model using athymic NCR nu/nu nude mice. Six animals of same sex were kept together in sterile cages fitted with HEPA (High efficiency particulate air) filters and were given sterile food and water. The bedding of cages was changed twice a week. All animal handling procedures were approved by animal ethics committee of USM (Approval number: USM/Animal Ethics Approval/2015/(660). In brief, 1×10^6 HCT 116 in 200 µL of RPMI-1640 media were injected subcutaneously into 6–8 weeks old mice under aseptic conditions. When average tumor size reached approximately 100 mm^3, animals were divided into four groups of six animals each. Group one (control group) was orally administrated 100 µL of distilled water containing 5% Tween 80. Group two and three were treated with 100 and 200 mg/kg of RH (100 µL/ animal). Group four was treated with 10 mg/kg of capecitabine reconstituted in distilled water. Changes in body weight, tumor volume and antitumor efficacy were calculated following reported formulas [13,14]. Keeping in view oral route of administration for RH, oral analogue of 5-FU *i.e.*, capecitabine was used as positive control.

3. Statistical analysis

Results are presented as mean ± SD. Paired *t*-test and *One-way ANOVA* followed by *post hoc-tukey* were performed using GraphPad Prism (San Diego, CA, USA) software to test the differences between groups. $p < 0.05$ was considered statistically significant.

4. Results and discussion

The current study describes the *in vitro* and *in vivo* anti-colon cancer efficacy of oleo-gum resin extract obtained from *M. ferrea*. Crude ethanol extract (RCr) prepared by sonication method was subjected to cytotoxic screening towards HCT 116 using MTT assay and was found to have an IC_{50} value of 20.29 µg/mL. Subsequent fractionation of RCr resulted in collection of two crude fractions *i.e.*, RH and RCF. Both fractions were again subjected to cytotoxic screening towards HCT 116 cell line and IC_{50} values were calculated. Data obtained showed that non-polar fraction (RH) was more active (RH IC_{50} = 17.38 µg/mL) as compared with polar fraction (RCF IC_{50} = 48.03 µg/mL). This preliminary cytotoxic screening data showed that RH has cytotoxic activity comparable to semi-pure compound (IC_{50} = 16.62 ± 0.38 µg/mL) as reported in our previous *in vitro* study [6] and thus worthy to be further studied as an alternative option of semi pure isoledene (which was difficult to isolate owing to its volatile nature and poor yield) for treatment of colon cancer. Outcome of cell viability assay corresponds well with another preliminary anticancer study in which root bark extract (non-polar; n-hexane) of *M. ferrea* was shown to be more cytotoxic towards a panel of human cancer cell lines as compared with other solvent extracts [15]. Similarly, anticancer study conducted on different species of *Mesua* genus *i.e.*, *M. daphnifolia* showed that compound isolated from n-hexane extract had potent cytotoxic activities [16]. Having established the *in vitro* cytotoxic efficacy, RH was further subjected to broad spectrum cytotoxic screening towards a panel of human cancer and normal cell lines using MTT assay. Data obtained revealed that RH had potent cytotoxic activities and induced typical morphological changes in almost all the cancer cell lines tested (Figs. 1 and 2). Order of sensitivity (higher to lower) of cancer cell lines towards RH was HCT 116 > LIM1215 > U-87 MG > KATO III > HT-29 > HCC38 > PC-3 > BT-549 > PANC-1 > MKN-74 > Capan-1 respectively (Table 1). In addition, RH induced relatively weak cytotoxicity (IC_{50} = 60.83 µg/mL) in normal human colon fibroblasts (CCD-18-co). Preliminary *in vitro* anticancer activities of different parts of *M. ferrea* towards an array of human cancer cell lines *i.e.*, Raji, CL-6, HeLa, LS-174 T, K562, SNU-1, Hep-G2, NCI-H187, NCI-H23, MCF-7, SK-MEL-28, IMR-32 and KB have been reported by number of studies [15,17–20]. However, these investigations were limited to explore cytotoxic potential (IC_{50}) only and no further mechanistic studies were conducted. The present study reports for the first time the *in vitro* anticancer activities of *M. ferrea* oleo-gum resin extract towards human colon (HCT 116, HT-29, and LIM1215), prostate (PC-3), pancreatic (PANC-1 and Capan-1), breast (HCC38 and BT-549), glioblastoma (U-87 MG) and gastric (MKN-74 and KATO III) cancer cell lines respectively. All the cell lines tested in the current study vary in their genotype and phenotype from the cell lines tested in above cited studies. Studies have shown that SI values ≥ 3 indicate good anticancer activity of plant extracts towards cancer cells while SI values < 2 indicate general toxicity of extracts [21]. SI values of RH showed that it was 3 times more toxic towards colon cancer (HCT 116) cells as compared with their normal counterparts *i.e.*, CCD-18co. After HCT 116, RH showed relatively selective cytotoxic activity towards LIM1215 and U-87 MG cell lines. This data showed that RH can induce toxicity in multiple colon cancer cell lines (HCT 116 > LIM1215 > HT-29) with favourable margin of safety (SI = 3.50 > 3.22 > 2.55). On the basis of IC_{50} (lowest) and SI (highest) values, HCT 116 cell line was chosen for further studies.

Increased cellular proliferation, evasion of apoptosis and metastasis are the three well-known hallmarks of cancer and are known to support various processes involved in the transformation of normal cells into malignant form [22]. Interestingly, all these steps are interlinked with each other and aberration in one cellular event leads to the concomitant abnormalities in other events. Apoptosis lies at the central axis of these processes. Therefore, in order to arrest these abnormal cellular events it is highly desirable to encourage apoptosis in cancer cells [23]. To study apoptotic changes incurred by RH at cellular and sub-cellular levels in HCT 116, an array of assays representing both morphological and biochemical characteristics of apoptosis were employed. Cell shrinkage and deformation, plasma membrane blebbing, loss of cell-cell interaction, cell detachment from substratum, chromatin condensation and margination at nuclear membrane, and formation of apoptotic bodies are among the most commonly observed morphological changes in the apoptotic cells [24,25]. Among these features; cell shrinkage, cell rounding and loss of contact between cells can be studied employing inverted light microscope [24]. Treatment with RH resulted in appearance of morphological changes in majority of cancer cell lines tested in the current study. However, more pronounced phenotypic changes (cell shrinkage and detachment) were observed in HCT 116, HT-29, KATO III, LIM1215, PANC-1 and PC-3 cell lines respectively (Figs. 1 and 2). Noteworthy, treatment with RH induced no apparent morphological changes in normal colon fibroblasts *i.e.*, CCD-18co. This finding of morphological study is in agreement with another scientific study mentioning induction of similar type of structural changes in HCT 116, MCF-7 and Ca Ski cancer cells upon exposure to *Curcuma zedoaria* rhizomes extracts when observed under inverted phase contrast microscope [1]. Collectively, findings of MTT cell viability and morphological studies highlight the relatively selective cytotoxic nature of phytochemicals present in RH.

Data of flourescent staining assays showed that RH induced biochemical and morphological changes in two important cellular organelles *i.e.*, mitochondria and nucleus (Fig. 3). Images of Rhodamine 123 staining assay showed that treatment with RH resulted in significant reduction in mitochondrial outer membrane potential (MOMP) in HCT 116. Apoptotic index (%) in HCT 116 treated with 9, 18 and 32 µg/mL of RH and 5-FU (5 µg/mL) were 60.67 ± 9.99% ($p < 0.01$), 64.50 ± 5.43% ($p < 0.001$), 68.38 ± 7.63% ($p < 0.001$) and

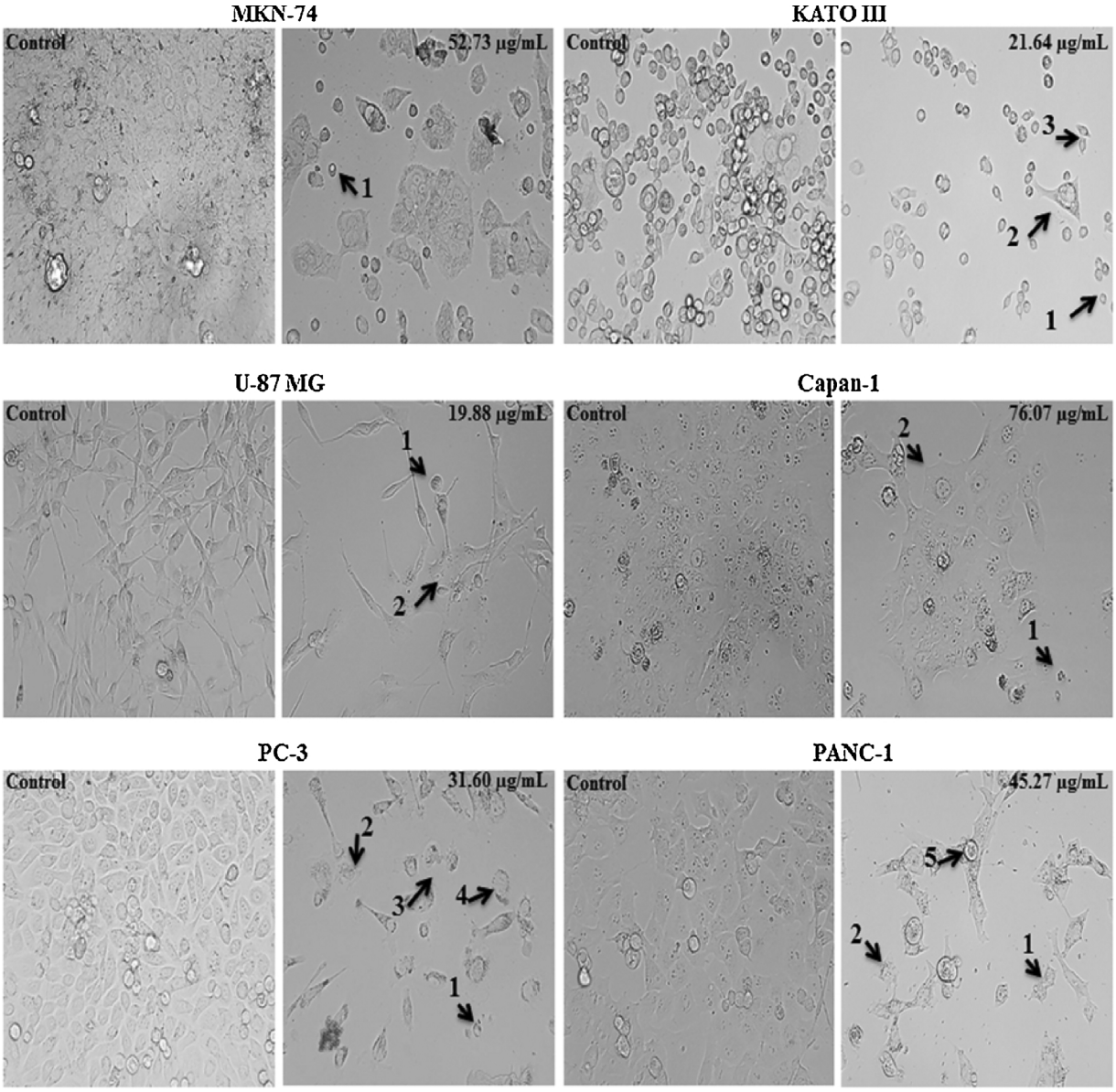

Fig. 1. Cytotoxic effects of RH towards an array of human cancer cell lines. Numbered arrows represent different apoptotic features observed in the morphology of cells. Where 1 = cell shrinkage, 2 = membrane blebbing, 3 = echinoid spikes, 4 = apoptotic bodies 5 = floating cells respectively.

71.63 ± 14.51% ($p < 0.001$) respectively, and were statistically significant when compared with apoptotic index (%) in 0.5% DMSO treated cells (9.33 ± 3.30%). 32 µg/mL of RH showed similar apoptotic effects as observed in 5-FU treated cells with no statistical differences ($p > 0.05$) **(**Fig. 3 **and S1)**. Mitochondria are regarded as powerhouse of cell. Cancer cells are shown to have high energy demand owing to their rapid proliferation rates [26]. Similarly, studies have shown that mitochondria communicate with nucleus through retrograde signalling. This communication regulates variety of processes including signal transduction, transcription factors and structure of chromatin to meet mitochondrial and nuclear requirements of cancer cells [27]. Therefore, targeting powerhouse of cancer cells by anticancer agents is expected to halt variety of oncogenic signalling pathways. Loss of mitochondrial outer membrane potential is considered as one of the early hallmarks of activation of intrinsic pathway of apoptosis [3]. This indicates that RH has potential to activate mitochondrial mediated apoptotic pathways. Treatment with RH also induced nuclear apoptosis *i.e.*, chromatin condensation and formation of typical kidney shaped nuclei in HCT 116 cells. Apoptotic index (%) in HCT 116 treated with 9, 18 and 32 µg/mL of RH and 5- FU (5 µg/mL) were 12.66 ± 2.76% ($p < 0.05$), 20.62 ± 4.99% ($p < 0.01$), 24.35 ± 3.55% ($p < 0.01$) and 81.95 ± 6.03% ($p < 0.001$) respectively, and were significantly greater when compared to untreated cells (8.53 ± 4.56%). 32 µg/mL of RH showed significantly less ($p < 0.001$) apoptotic effects as compared with those observed in 5-FU treated cells (Fig. 3 and S1). Taken together, data of fluorescent staining assays showed that RH has potential to modulate activity of multiple cell signalling pathways and their downstream proteins in HCT 116.

Intrinsic and extrinsic apoptotic pathways converge at the common point *i.e.*, caspases 3/7 activation. Activated caspases 3/7 then induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins and inhibitory subunits of endonucleases family. They also have an effect on cytoskeleton, cell cycle and signalling pathways [25]. HCT 116 treatment with RH activated caspase-3/7 in a significant manner ($p < 0.05$). Relative fold increase in activity of caspase-3/7 in HCT 116 treated with 9, 18 and 32 µg/mL of RH and 5 µg/mL of 5-FU was 1.08 ± 0.028 (ns), 1.50 ± 0.12 ($p < 0.001$), 1.54 ± 0.15 ($p < 0.001$), and 1.57 ± 0.69 ($p < 0.001$) respectively. When caspase-3/7

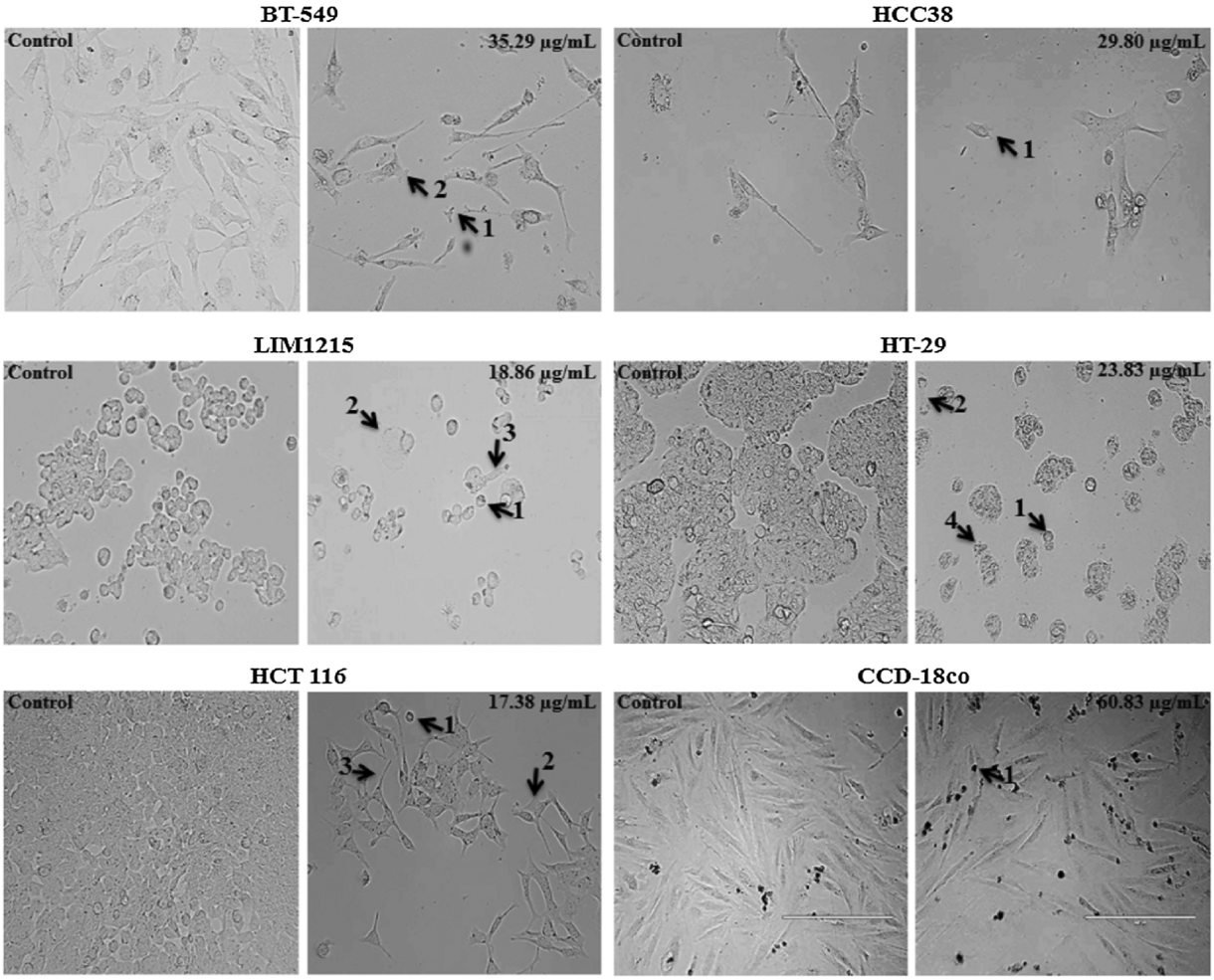

Fig. 2. Cytotoxic effects of RH towards an array of human cancer cell lines. Numbered arrows represent different apoptotic features observed in the morphology of cells. Where 1 = cell shrinkage, 2 = membrane blebbing, 3 = echinoid spikes and 4 = apoptotic bodies respectively.

Table 1
Cytotoxic activities of oleo-gum resin fraction towards a panel of human cancer and normal cell lines.

Cell lines	IC_{50} values (µg/mL)	Selectivity index
PC-3 (prostate cancer)	31.60 ± 2.09	1.92
PANC-1 (pancreatic carcinoma)	45.27 ± 0.47	1.34
Capan-1 (pancreatic ductal carcinoma)	76.07 ± 6.00	0.01
MKN-74 (gastric cancer)	52.73 ± 1.04	1.15
KATO III (gastric carcinoma)	21.64 ± 5.21	2.81
U-87 MG (glioblastoma)	19.88 ± 0.84	3.05
HCC38 (mammary ductal carcinoma)	29.80 ± 4.05	2.04
BT-549 (mammary ductal carcinoma)	35.29 ± 1.55	1.72
LIM1215 (colorectal carcinoma)	18.86 ± 0.80	3.22
HCT 116 (colorectal carcinoma)	17.38 ± 0.92	3.50
HT-29 (colorectal adenocarcinoma)	23.83 ± 0.86	2.55
CCD-18co (colon fibroblasts)	60.83 ± 0.90	———

Values shown are mean ± SD after 48 h of treatment with RH. SI index was calculated by dividing IC_{50} value of RH towards each cell line by IC_{50} value of RH towards CCD-18co cells respectively. SI value > 3 shows the promising activity.

levels in cells treated with 9 µg/mL of RH was compared with other treatment groups, a significantly higher caspase-3/7 activity was observed in cells treated with 18 µg/mL ($p < 0.01$) and 32 µg/mL ($p < 0.001$) of RH and 5 µg/mL of 5-FU ($p < 0.001$). RH at the concentration of 32 µg/mL showed comparable activity in terms of induction of caspase-3/7 with that of 5-FU (5 µg/mL) with no statistical difference ($p > 0.05$) (**Figure S2**). These activated caspases are suggested to be responsible for appearance of typical morphological changes in HCT 116 as observed in light and fluorescent staining assays. Studies have shown that apoptotic and necrotic cells share some similar characteristics. Therefore, to confirm mechanism of cell death additional studies apart from cytomorphological observations should be performed [24]. Characteristics features of necrosis are cell swelling and damage of plasma membranes which result in release of cytoplasmic contents including LDH into extracellular space. Hence, measuring levels of LDH in cell culture supernatant can help to distinguish between two types of cell death [28]. Treatment with 9, 18 and 32 µg/mL of RH and 1% triton X100 resulted in significant ($p < 0.001$) increase in release of LDH enzyme from HCT 116 when compared with LDH levels in 0.5% DMSO treated cells. However, when LDH levels in 1% triton X100 (a known necrotic agent) and RH treatment groups (9, 18 and 32 µg/mL) were compared, cells treated with 9 µg/mL ($p < 0.001$), 18 µg/mL ($p < 0.01$) and 32 µg/mL ($p < 0.05$) of RH released significantly lower LDH than triton X100 treated cells. This indicated maintenance of cell membrane integrity, especially at lower concentrations of RH (Table 2).

Noteworthy, treatment of HCT 116 with 32 µg/mL of RH resulted in significant increase ($p < 0.001$) in LDH release. This data indicated possibility of activation of necrotic cell death pathway in HCT 116 as a result of exposure to RH. One possible explanation for this activity could be RH-mediated ROS generation in HCT 116 causing damage to DNA and mitochondrial membranes (**Figure S3**). These changes especially damage to mitochondrial membranes lead to reduced supply of

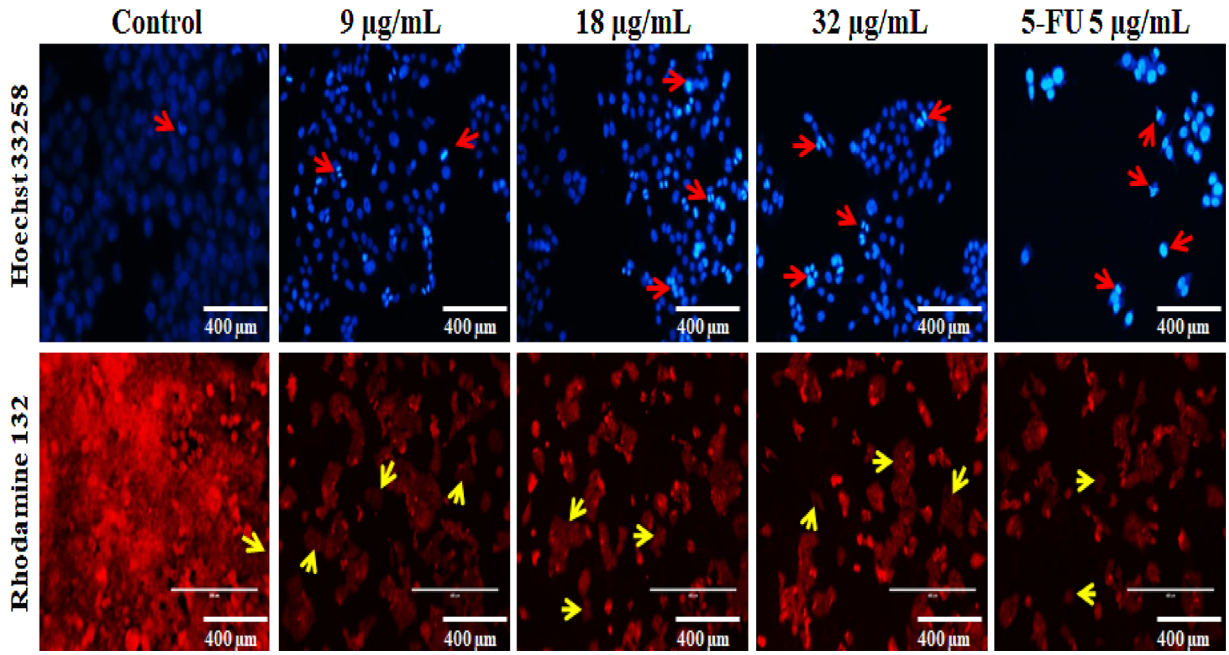

Fig. 3. Pro-apoptotic effects of the RH in HCT 116 after 24 h of treatment with three different concentrations. In Hoechst 33258 staining, apoptotic cells appear brighter in colour as compared with normal non-apoptotic cells and are highlighted by red arrows in each photo. While in Rhodamine 123 staining, apoptotic cells appear less bright in colour as compared with non-apoptotic cells and are highlighted by yellow arrows in each photo. Photos were taken at 10× magnification (scale bar 400 µm).

Table 2
Effect of oleo-gum resin extract on LDH release.

S. NO	Treatment	LDH (µU/mL)
1	0.5% DMSO	60.56 ± 4.56 [b]
2	RH (9 µg/mL	98.45 ± 1.45 [a,b]
3	RH (18 µg/mL)	99.77 ± 1.44 [a,b]
4	RH (32 µg/mL)	106.92 ± 5.79 [a,b]
5	1% Triton X100	118.19 ± 4.99

Values shown are mean ± SD of three independent experiment (n = 3). a = $p < 0.05$ when compared with the LDH values in 0.5% DMSO treated group and b = $p < 0.05$ when compared with the LDH values in 1% Triton X100 treated group.

ATP for caspases activation, causing cells to enter necrotic pathway following transient activation of apoptotic cascade. Therefore, on the basis of outcome of the LDH release assay together with the cytomorphological observations (cell shrinkage and detachment) it is suggested that phytoconstituents present in RH activate multiple cell death programmes (mainly necrosis at higher concentrations) in HCT 116.

Having established basic mechanisms responsible for cytotoxic attributes of RH in preliminary experiments, study was further extended to explore possible molecular interactions responsible for observed effects using human apoptosis proteome profiler. Relative fold change in expression of Bim, TRAIL-R2, Survivin, HSP27, HSP60, HSP70 and xIAP proteins in HCT 116 treated with RH was 1.50 ± 0.18 ($p < 0.05$), 2.77 ± 0.26 ($p < 0.05$), 0.002 ± 0.004 ($p < 0.01$), 0.51 ± 0.05 ($p < 0.05$), 0.17 ± 0.07 ($p < 0.001$), 0.46 ± 0.07 ($p < 0.001$) and 0.02 ± 0.02 ($p < 0.001$) respectively. Fold change values higher than 1 show up-regulation while values lower than 1 indicate down-regulation of protein targets (Fig. 4). Inhibitors of apoptosis proteins (IAPs) including survivin and xIAP belong to a family of proteins which act as negative regulators of apoptosis. These proteins inhibit apoptosis through two mechanisms, 1) inhibit activation of caspases and 2) degrade active caspases [25]. Studies have shown that survivin and xIAP are overexpressed in majority of CRC cases and are responsible for generation of resistance to TRAIL-induced apoptosis [29]. Apart from anti-apoptotic properties, these proteins are known to control other tumorigenic events including cell division, metastasis, and angiogenesis [30]. In addition to IAPs, heat shock proteins (HSPs) represent another class of negative regulators of apoptosis and are known to inhibit release of cytochrome c and activation of caspase-9. Furthermore, HSPs also up-regulate expression of IAPs *i.e.*, survivin in CRC [29]. Data of the present study is supported by findings of another study where similar type of multi-targeted pro-apoptotic nature of natural product was mentioned [8].

After identification of protein targets, study was further extended to identify nature of cytotoxic stimuli generated by RH which was regulating expression of these proteins. Role of reactive oxygen species (ROS) as an apoptosis inducer has already been established. Elevated levels of ROS have been shown to activate death receptor as well as mitochondrial-mediated apoptosis in tumor cells [31]. Furthermore, an inverse relationship between intracellular ROS levels and expression of negative regulators of apoptosis including HSP27, HSP60 and survivin has been highlighted by numerous studies [32,33]. In the current study significant ($p < 0.001$) intensification of intracellular ROS signal in HCT 116 treated with RH was observed as compared with ROS level in 0.5% DMSO treated cells (**Figure S3**). This observation together with findings of Rhodamine staining and human apoptosis proteome profiler array gives a hint about possible mechanism action of RH. In short, RH induced ROS generation in HCT 116. This elevated ROS resulted in mitochondrial membrane depolarization and resulted in opening of mitochondrial transition pores. From these pores, apoptogenic proteins were released into cytosol with subsequent activation of caspases-3/7. Activated caspases executed last phases of cell death *i.e.*, destruction of various cellular organelles. Findings of the current study are supported by outcome of research report mentioning selective increment in intracellular ROS in tumor cells as one main cell death mechanisms [34].

Studies have shown that apoptosis and tumor metastasis are interlinked processes [3], and apoptosis can arrest metastatic dissemination of tumor cells by inducing death in misplaced cells [35]. Findings of modified Boyden chamber assay revealed that HCT 116 treatment with RH resulted in significant reduction in number of HCT 116 invading Matrigel matrix. At the concentration of 9, 18 and 32 µg/mL of RH and

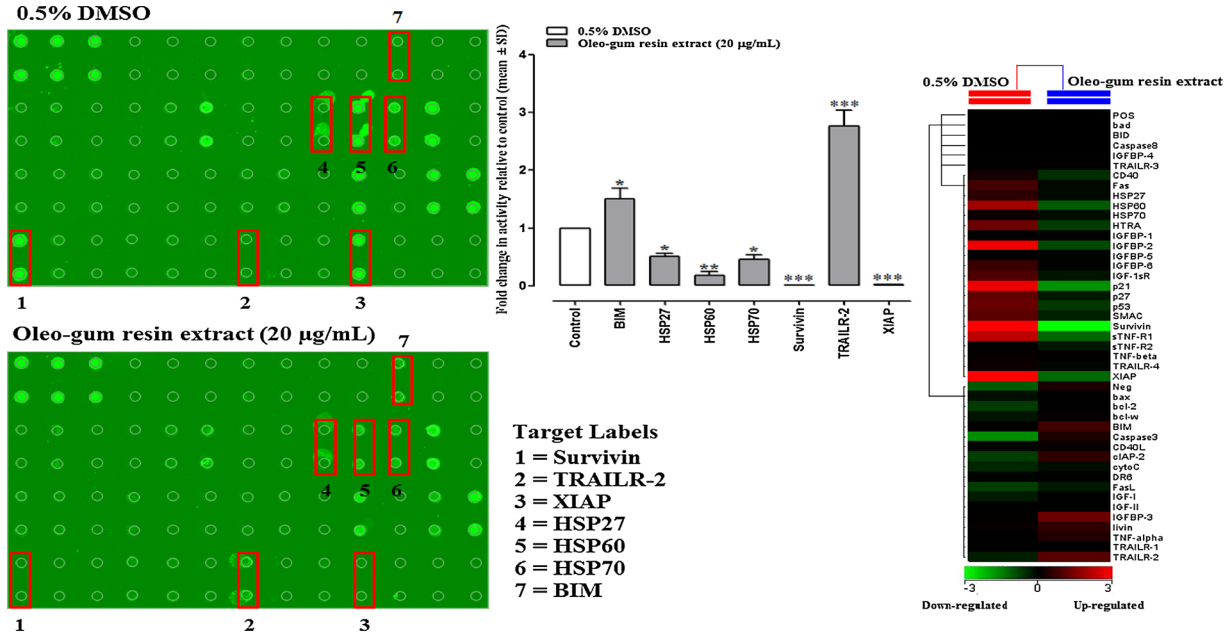

Fig. 4. Effects of oleo-gum resin extract (RH) on the relative expression pattern of multiple proteins involved in apoptotic cascade. RH modulated the activity of multiple proteins. Cluster diagram (Heatmap) shows signal intensities from each protein. Red band indicates up-regulation while green band indicates down-regulation of proteins. Labelled spots show the expression of proteins in 0.5% DMSO and RH treatment groups respectively.

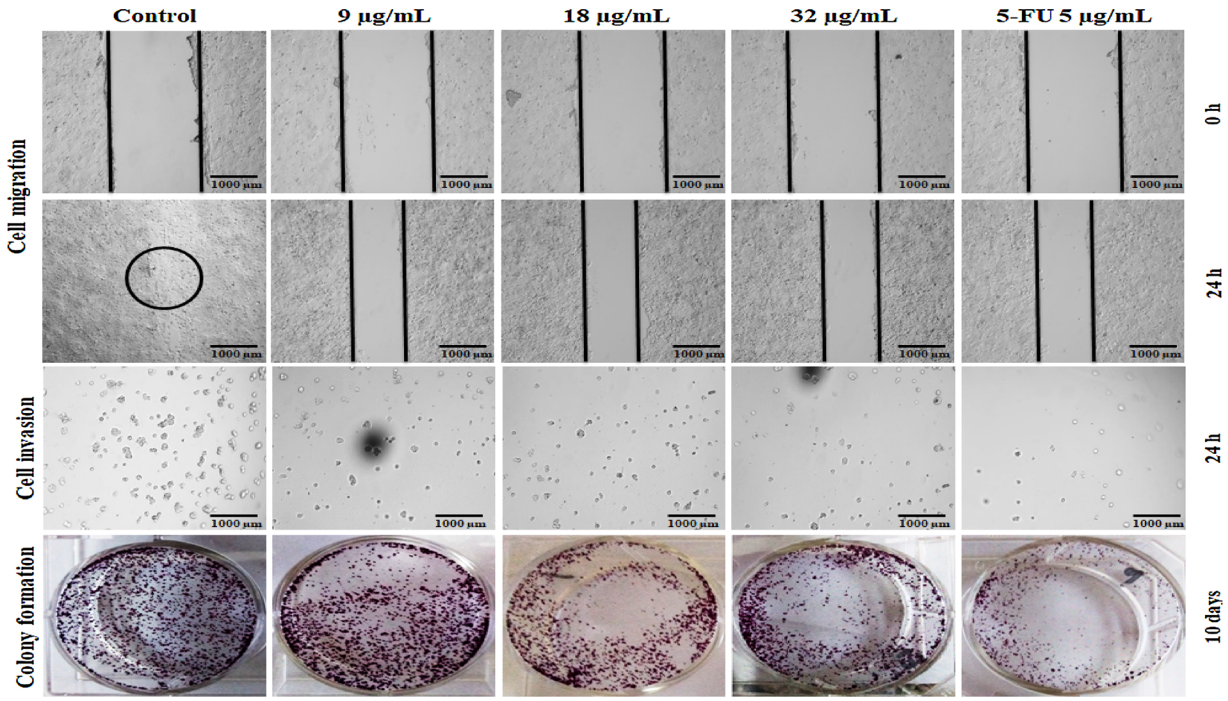

Fig. 5. Antimetastatic activity of RH towards HCT 116. Treatment with RH induced significant inhibition of different stages of colon cancer metastasis in *in vitro* assays.

5 µg/mL of 5-FU, percent inhibition of cell invasion was 21.50 ± 6.90%, 32.10 ± 6.20%, 77.87 ± 3.57% and 90 ± 4.77% respectively. A significant reduction in number of cells invading the Matrigel matrix was observed in cells treated with 9 ($p < 0.05$), 18 ($p < 0.05$) and 32 ($p < 0.001$) µg/mL of RH and 5 µg/mL of 5-FU ($p < 0.001$) when compared with number of cells invaded in 0.5% DMSO treatment group. When compared with 5-FU, RH at 32 µg/mL showed significantly lower ($p < 0.05$) activity in terms of % inhibition of HCT 116 invasion. Similarly, significant ($p < 0.05$) inhibition of cell migration across artificially created wound was observed in HCT 116 treated with different concentrations of RH. Percent inhibition of cell motility in RH (9, 18 and 32 µg/mL) and 5- FU (5 µg/mL) treated cells was 42.88 ± 6.23%, 44.73 ± 0.94%, 51.94 ± 5.21%, and 49.60 ± 7.50% respectively, which was statistically significant ($p < 0.001$) when compared with percent inhibition of cell migration in 0.5% DMSO treated cells (2.28 ± 2.17%) (Fig. 5). When compared between treatment groups, HCT 116 treated with 32 µg/mL of RH and 5 µg/mL of 5-FU showed significantly higher ($p < 0.05$) inhibition of cell migration as compared with cells treated with 9 µg/mL of RH. RH (32 µg/mL) showed same level of inhibition as observed in 5-FU treated

cells with no statistical difference ($p > 0.05$).

Noteworthy, RH also inhibited last step of metastatic cascade *i.e.*, formation of colon cancer cells colonies from single cells. Percent inhibition of colonization in HCT 116 treated with 9, 18 and 32 µg/mL of RH and 5 µg/mL of 5-FU was 44.38 ± 5.16%, 55.72 ± 10.72%, 82.01 ± 5.94%, and 82.25 ± 8.03% respectively. Plating efficiency of HCT 116 in 0.5% DMSO, RH (9, 18 and 32 µg/mL) and 5-FU (5 µg/mL) treatment groups was 70.73 ± 18.38%, 24.72 ± 2.57%, 16.70 ± 2.08%, 13.03 ± 1.04% and 6.26 ± 2.83% respectively. Survival fraction (SF) of cells treated with RH (9, 18 and 32 µg/mL) and 5-FU (5 µg/mL) was 1.41 ± 0.14%, 0.95 ± 0.11%, 0.74 ± 0.05%, and 0.35 ± 0.16% respectively. When comparision made between different RH treatment groups, 9 µg/mL showed significantly lower ($p < 0.001$) anti-clonogenic activity as compared with 32 µg/mL of RH and 5 µg/mL of 5-FU, while no statistical difference ($p > 0.05$) was observed between 9 and 18 µg/mL of RH. RH at 32 µg/mL and 5-FU 5 µg/mL showed similar inhibitory activity with no statistical difference ($p > 0.05$) between two groups (Fig. 5).

Studies have also shown that metastasizing cells usually have abnormalities in multiple proteins of apoptotic cascade [35]. In this regard, up-regulation of IAPs including survivin and xIAP has been shown to promote tumor cell migration, invasion, and metastasis by providing shelter against a variety of apoptotic stimuli [36]. Therefore, agents which can regulate expression of these proteins in cancer cells can help in management of metastatic dissemination. In this prospective, RH mediated down-regulation of IAPs with simultaneous up-regulation of caspases-3/7 is proposed to be partially responsible for observed anti-metastatic effects.

A thorough phytochemical investigation of RH was carried out using GC–MS and HPLC methods. GC–MS study identified a total 15 compounds along with a multiple unidentified compounds. Isoledene and α-elemene (sesquiterpenes) were identified as one of the two marker compounds (Fig. 6). MS comparison of isoledene peak in RH with NIST02 library database is shown in Figure S4. HPLC quantification data revealed that 170.57 ± 1.37 mg of isoledene was present per gram of RH. HPLC method validation data is explained in supplementary file. *In vitro* anticancer activities of elemene (especially β-elemene in pure form and α-elemene in a mixture of essential oils) towards variety of cancer cell lines have already been reported. Induction of apoptosis, cell cycle arrest, down modulation of α-tubulin, inhibition of microtubules polymerization and increase in sensitivity of cancer cells towards radiotherapy are among the few mechanisms reported to be responsible for anticancer effects of elemene [37,38]. Similarly, recently we have reported that crude isoledene has pro-apoptotic activity towards human colon cancer cells [6]. Overall on the basis of these findings it is suggested that isoledene and elemene present in RH act synergistically to induce apoptosis in HCT 116. Thus, essential oil rich oleo-gum resin fraction can be used as an alternative to isoledene.

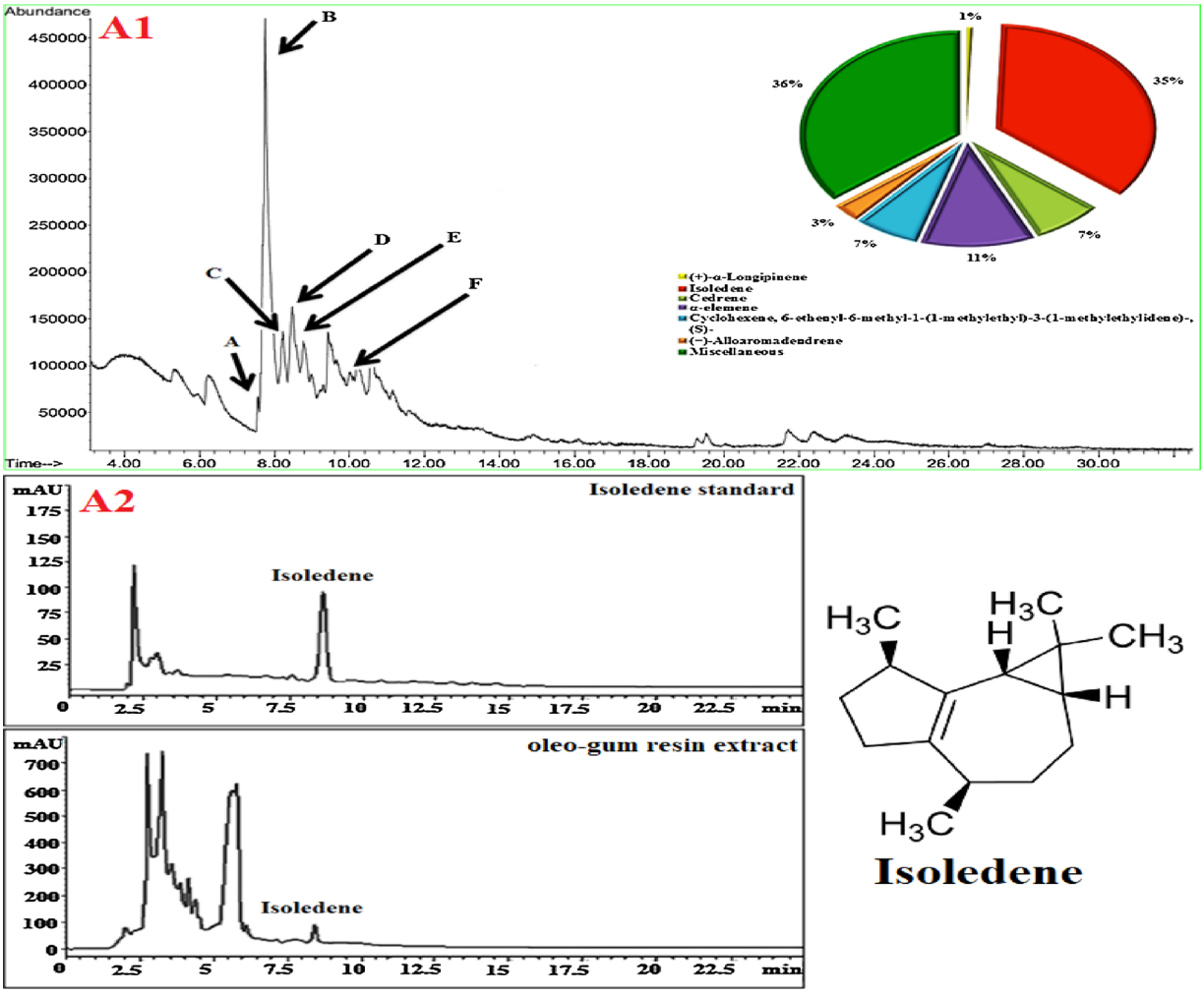

Fig. 6. GC–MS chromatogram of oleo-gum resin extract collected from the trunk of *M. ferrea* (April, 2015) (A1). Where peak A = Longipinene, B = Isoledene, C = Cedrene, D = α-elemene, E = Cyclohexene, 6-ethenyl-6-methyl-1-(1-methylethyl)-3-(1-methylethylidene)-, (S)-, F = (-)-Alloaromadendrene respectively (Note: Structure of isoledene shown in the figure is tentative, further studies are required to confirm its identity). A2: HPLC chromatogram of oleo-gum resin extract and standard Isoledene. The peak corresponding to Isoledene in RH was identified by comparing the retention time with that of the standard reference compound.

Table 3
Volumes of subcutaneous tumors in different treatment groups.

Treatments	Tumor volume (mm^3)			
	Day 0	Day 7	Day 14	Day 21
Vehicle (Tween 80)	99.5 ± 53.5 ^{ns}	191 ± 20.5	415 ± 101	1057 ± 53.70
RH 100 mg/kg	99.2 ± 33.3 ^{ns}	190 ± 54.8^{ns}	282 ± 109 ^{ns}	277 ± 102 ***
RH 200 mg/kg	83 ± 14.2 ^{ns}	158 ± 99.7^{ns}	158 ± 56.2 **	113 ± 76.5 ***
Capecitabine 10 mg/kg	151.4 ± 56.5 ^{ns}	225 ± 39.2^{ns}	253 ± 63.2 *	240 ± 81.8 ***

Values shown are mean ± SD (n = 6). The study was terminated when the average volume of tumor in any of the treatment group reached the limit of 1000 mm^3. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

However, it is noteworthy to mention here that this the first study that reports the *in vitro* and *in vivo* anticancer activities of oleo-gum resin extract of *M. ferrea* towards human colon cancer.

Data from ectopic tumor model revealed that treatment with RH resulted in reduced growth of subcutaneous tumors as compared to vehicle treated animals. ΔT/ΔC values at 21st post–cell inoculation day in RH-treated (100 and 200 mg/kg) and capecitabine-treated (10 mg/kg) groups were 41.57, 3.16 and 9.25 respectively, mentioning a potent antitumor efficacy of RH (at 200 mg/kg) and capecitabine (Table 3, Fig. 7). Histopathological examination of tumor sections showed reduction in viable tumor cells with subsequent increase in percentage of necrotic area in treatment groups especially at higher dose. Moreover, H&E staining also gave a clue about reduction in number of intratumor blood vessels in RH and capecitabine treatment groups. However, further studies in this regard are required to confirm effects of RH on blood vessesls outgrowth in tumors. Tumor sections of vehicle treated group on the other hand, showed tightly packed healthy tumor cells with only small necrotic areas and plenty of intratumor blood vessels (Fig. 7). Body weight changes are an indicator of adverse side effects, as the animals that survive cannot lose more than 10% of the

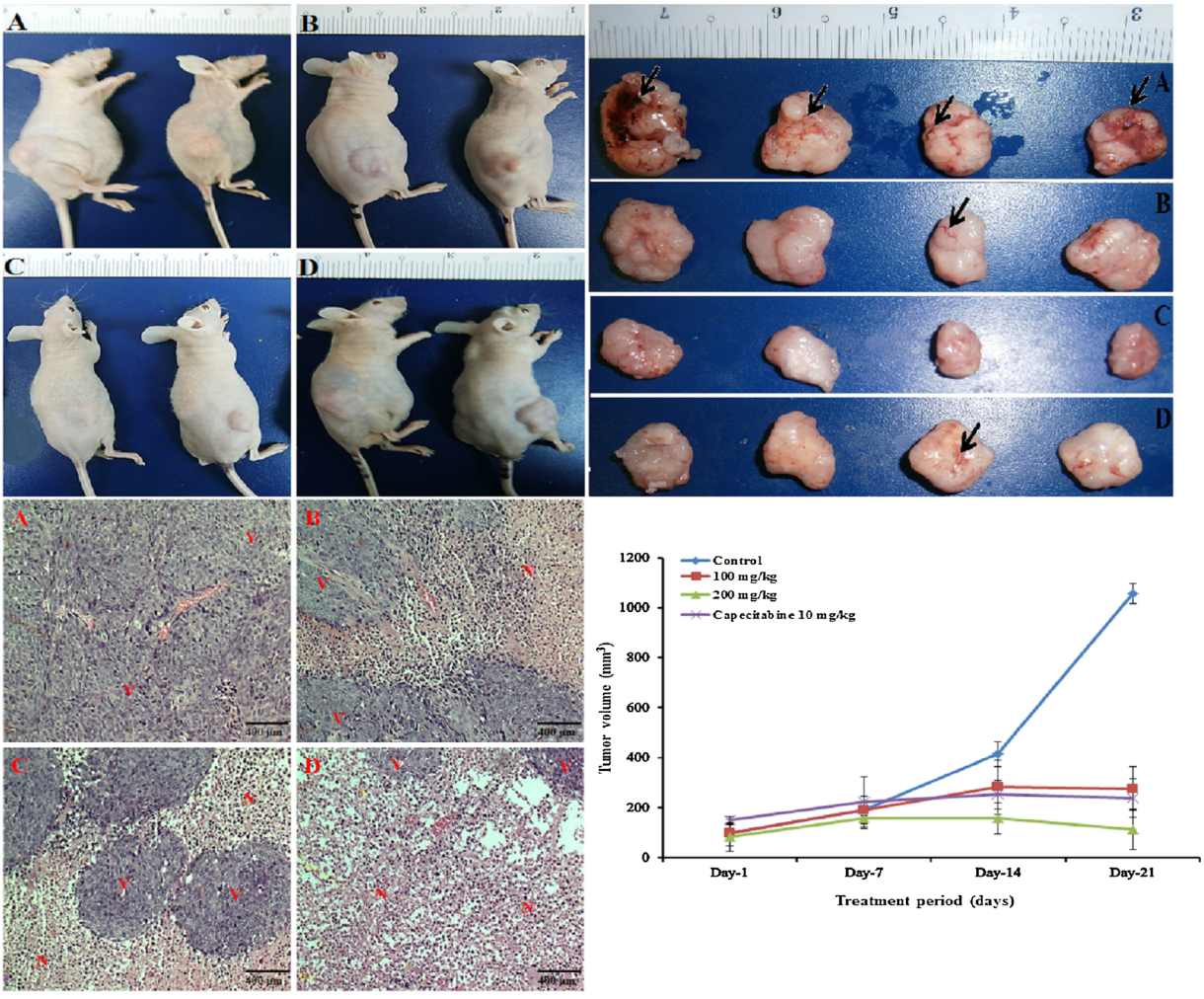

Fig. 7. *In vivo* anti-colon cancer efficacy of oleo-gum resin extract in ectopic nude mice model. Photos of tumors harvested from test animals showed that RH treatment reduced size of tumors. Pointed arrows in harvested tumors show marked reduction in blood vessels in RH-treated animals as compared with vehicle-treated animals. Histological examination show marked reduction in number of viable cells in tumor sections of treated animals. Where A = vehicle-treated, B = 100 mg/kg RH, C = 200 mg/kg RH and D = capecitabine 10 mg/kg. N = necrotic areas and V = viable cells.

initial body weight [39]. Loss of body weight in all the RH and capecitabine treatment groups was less than 10% representing relatively safe nature of RH (Figure S5).

5. Conclusion

Overall, outcome of the present study demonstrates that RH has cytotoxic and antimetastatic activities towards human colon cancer cells. These activities are suggested to be result of treatment-related changes in the expression pattern of multiple proteins including survivin and xIAP.

Acknowledgement and funding

The authors would like to acknowledge the Institute of Postgraduate Studies at Universiti Sains Malaysia for providing a USM Fellowship (P-FD0009/12(R)). We would also like to acknowledge USM for providing funding through a University Grant (RUT 1001/PFARMASI/851001).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.10.127.

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