RESEARCH ARTICLE

Editorial Process: Submission:07/07/2021 Acceptance:09/22/2022

Hemimycale Arabica Induced Non-Cytotoxic Anti-Migratory Activity in Hepatocellular Carcinoma *In Vitro*

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Abstract

Objective: In this work, we represented new non-cytotoxic treatments to avoid serious side effects of current used cytotoxic anticancer drugs. These treatments can compensate in finding convenient treatment for each individual case using a single agent from marine sponge *Hemimycale arabica*. **Methods:** The ethanol extract was partitioned by cold sequential liquid-liquid extraction to afford petroleum ether, diethyl ether, dichloromethane and ethyl acetate fractions. Chemical composition of H. arabica was performed by gas-liquid chromatography and gas chromatography-mass spectroscopy. Anticancer activity was evaluated by means of cytotoxicity, apoptosis induction, tumor cell migration inhibition and expression analysis of proliferation and migration-related genes. **Results:** Our results revealed that all treatments were non-cytotoxic except for dichloromethane fraction which exhibited moderate cytotoxic activity. Caspase-independent apoptosis was induced by total ethanol and dichloromethane fractions while ethyl acetate fraction induces caspase-dependent apoptosis. All treatments inhibited matrix metalloproteinase-independent migration. Petroleum ether and dichloromethane inhibited migration through the down-regulation of FGF and it could be used as anticancer therapy for VEGF-resistance patients. While ethanol inhibited tumor cell migration through down-regulation of all tested genes expression. Ether and ethyl acetate fractions exerted anti-migratory activity without affecting the tested genes. All resuls were statistically significant at $p<0.05$. **Conclusion:** Total ethanol extract is a promising non-cytotoxic anticancer agent because of its powerful apoptosis induction and capability to block tumor cell migration. Petroleum ether and ether fractions area weak non-cytotoxic anti-migratory agents. Dichloromethane could be a moderate cytotoxic anti-migratory agent induced caspase-independent apoptosis. It could be used in anticancer therapy for VEGF-resistance patients through downregulation of FGF. Ethyl acetate fraction considered a non-cytotoxic agent exerting moderate anti-migratory activity. The new sponge-derived treatments can solve different resistance problems to find a convenient treatment for each individual case using a single agent.

Keywords: *Hemimycale arabica*- migration- non-cytotoxic- growth factors- surface markers- GC-MS

Asian Pac J Cancer Prev, **23 (9)**, 2921-2928

Introduction

There is a pressing need to develop non-cytotoxic anticancer drugs that have the potential to be effective in targeted cancer therapy avoiding severe effects of current anticancer drugs. Sponges (phylum - Porifera) have emerged as a valuable source for discovering anticancer drugs owing to their diverse content of secondary metabolites. Many compounds under active research or in preclinical phases modulate one or more hallmarks of cancer and become promising anticancer drug candidates (Ruiz-Torres et al., 2017). Food and Drug Administration (FDA) approved drugs derived from marine sponges have the ability to reduce metastatic breast cancer and malignant lymphoma (Calcabrini et al., 2017; Gordon et al., 2016). Discodermolide a polyketide in phase I/II trials isolated from the marine sponge inducing cell cycle arrest at the G2/M phase (Shaw, 2008). Bryostatin 1 a polyketide isolated from the bryozoan Bugula neritina is currently undergoing phase I trials for assessment as a treatment for cancer (Mayer et al., 2010). Bastadin 6, a macrocyclic tetramer alkaloid was isolated from the marine sponge *Lanthella* sp., has an anti-angiogenic effect through the inhibition of the vascular endothelial growth factor (VEGF)- and basic fibroblast growth factor (bFGF)-dependent proliferation of human umbilical vein endothelial cells (HUVECs). Laulimalide is another macrolide (polyketide) isolated from marine sponge *Cacospongia mycofijiensis* that inhibit cell proliferation and VEGF-induced endothelial cell migration (Churchill et al., 2015). FDA approved anti-angiogenic agents inhibit vascular endothelial growth factor (VEGF) pathway as a targeted therapy alternative to chemotherapy or as adjuvant therapy (Al-Abd et al., 2017). Some patients

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acquire resistance to VEGF-inhibi¬tors treatment. To overcome this problem, several VEGF-independent were discovered. Fibroblast growth factors (FGF)-dependent inhibitors could be an effective alternative to VEGF-targeted inhibitors (Mitsuhashi et al., 2015). Also targeting TGF-β could inhibit migration which considered an important target for new therapies (Pang et al., 2016).

The lethal outcome of cancer is due to spreading of primary tumor cells and outgrowth of secondary tumors (Kessenbrock et al., 2010). In order to metastasize through surrounding tissue, tumor cells employ mechanisms that utilize matrix metalloproteases (MMPs) to digest extracellular matrix (ECM). Regarding the failure of MMP-inhibitors as targets for anticancer therapy in clinical trials, cells can act independently of their proteolytic activity and migrate through ECM (Fingleton, 2008). Metastatic cancer cells can switch from protease-dependent to protease-independent invasion program utilizing amoeboid migration mode (Friedl and Wolf, 2010).

Migration of hepatocellular carcinoma (HCC) cells are increased by various growth factors and cytokines such as vascular endothelial growth factor (VEGF) (Zhan et al., 2013), transforming growth factor β1 (TGF-β1) (Koudelkova et al., 2017) and basic fibroblast growth factor (bFGF) (Zheng et al., 2016). FDA approved the first anti-angiogenic agent, bevacizumab, in combination with chemotherapy, results in increased survival in patients relative to chemotherapy alone (Ferrara et al., 2004). In addition, CD44 is a widely expressed cell surface hyaluronan receptor plays a key role in mediating cell migration (Gao et al., 2015).

Presented work aimed to gain insights into the anti-proliferation and anti-migration potential of total ethanol extract and its successive fractions; petroleum ether, diethyl ether, dichloromethane, and ethyl acetate as well as the chemical composition of *Hemimycale arabica* sponge. The chemical composition of *H. arabica* was performed by gas-liquid chromatography and gas chromatography-mass spectroscopy. Anticancer activity was evaluated by means of cytotoxicity, apoptosis induction, migration inhibition and expression analysis of proliferation and migration-related genes, *CD105, CD44, VEGFA, TGFβ1,* and *FGF4.*

Materials and Methods

Sponge collection, preparation and identification

Sponge sample was collected in March 2015, from the Red Sea at the site of the Marine Biological Station, Hurghada- Egypt (geographical coordinates: Latitude 27° 15' 26 N, Longitude 33° 48' 46 E) using SCUBA at depths between 10 and 18m. Sponge species was identified by Dr. Mohamed Ez El-Arab, The National Institute of Oceanography and Fisheries (NIOF), Hurghada, Egypt. A fresh sample of *Hemimycale arabica* was cut into small pieces, blended then extracted with ethanol. The solvent was evaporated till dryness to get total ethanol extract. The ethanol extract was suspended in distilled water and subsequently extracted with petroleum ether, diethyl ether, dichloromethane and ethyl acetate.

Qualitative Chemical analysis

Preliminary phytochemical screening of the fractions was carried out to determine the qualitative estimation of alkaloids, tannins, flavonoid, steroid, terpenoids and phenolic compounds contents using standard procedures (Usman et al., 2009).

Investigation of the liposoluble matter

Petroleum ether fraction was subjected to saponification to give unsaponifiable matter and free fatty acids fraction which subjected to methylation (Helal et al., 2006). GLC analysis of unsaponifiable matter and fatty acid methyl esters was carried out using nitrogen gas as a carrier gas. Capillary column, HP-1 methyl siloxane was used for unsaponifiable matter whereas, capillary column polyethylene glycol used for fatty acid methyl ester analysis. The oven temperature was 50°C/11.7 minutes from 80-325oC and injection, detector temperature was 300°C.

Gas chromatography/mass spectroscopy analysis (GC/MS)

Dichloromethane fraction was subjected to GC/MS analysis using NIST, WILLY library data of GC/MS system by Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, TG-5MS fused silica capillary column. Helium was used as carrier gas at a flow rate of 1ml /min. the column temperature was programmed from 70°C, raised to 260 °C at a rate of 10°C/min, and held for 10 min. The injector temperature was 280°C. The sample was injected with a split ratio of 1:30 (v/v) and constituents were identified by comparison of their spectral fragmentation patterns with database libraries, Wiley (Wiley International, USA) and NIST (Nat. Inst. St. Technol., USA). Quantitative determination was carried out on the basis of peak area integration (Helal et al., 2006).

Cell line propagation and treatment

Human hepatocellular carcinoma cell line (HepG2) was purchased from The Egyptian Company for Production of Vaccines, Sera and Drugs (VacSera). Cells were cultured in RPMI-1640 medium (Roswell Park Memorial Institute) supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, Md.), 1% penicillin/streptomycin and 2% L-glutamine at 37°C and 5% CO2. Cells at 80% confluence were trypsinized, seeded and incubated overnight then treated with sub-lethal concentrations (10-100 µg/ml) of candidate treatments.

Anti-proliferation assays

Cytotoxicity (MTT assay)

Cytotoxicity against HepG2 cells was assessed by MTT [3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay (Hwang et al., 2007). Briefly, cells were seeded in 96-well microplate in RPMI-1640 culture medium and incubated at 37ºC and 5% CO_2 overnight. Cells were treated with different fractions dissolved in dimethyl sulphoxide (DMSO) then re-incubated for 24 and 48 h. with 100 µl MTT (0.5 mg/ml) solution/well, and incubated overnight until purple formazan crystals

appeared. The medium was discarded; 100 µl of DMSO was added to dissolve crystals. Optical density (OD) of solubilized formazan was measured at 570 nm using an automatic microplate reader. Results are expressed as a percent of control.

Caspase activity

Caspase-Glo® Assay was used to measure caspase 2, 3/7 activity according to the manufacturer. HepG2 cells were cultured in a 96-well plate and incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37°C. The cells were then treated with candidate treatments (in serum free media) at treated with sub-lethal concentrations of candidate treatments. Finally, the luminescence was measured using a microplate reader (Tecan Infinite M 200 PRO, Männedorf, Switzerland).

Apoptosis detection

Treated cells were collected and re-suspend at a concentration of 1.5 x 10^5 cells in 500 µl of Binding Buffer. Five microliters of annexin V-FITC and 5 µl of propidium iodide (50µg/ml) were added, then incubated at room temperature for 5 min in dark. Analysis of annexin V-FITC binding by flow cytometry (Ex = 488 nm; Em = 530 nm) was performed using FITC signal detector and PI staining by phycoerythrin emission signal detector (Lu et al., 2011).

Reverse transcription and real-time PCR

Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA, USA). RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer. For quantitative real-time PCR, amplification mixtures were prepared using KAPA SYBR_FAST q PCR master mix (Kapa Biosystem). GAPDH was used as an internal reference gene to normalize expression of CD105, CD44, VEGFA, TGFß1 andFGF4. Results were expressed as the ratio of reference gene mRNA to target gene mRNA using $2^{-\Delta\Delta Ct}$ method. Primers are listed in Table 1.

Transwell migration assay

Migration assay was performed in 24-well transwell using polycarbonate membranes with 8-µm pores (Corning Costar, Cambridge, MA). Cells were serum-starved and kept at 37 °C and 5 % CO_2 incubator for 24 h. Cells (6 × 10^5 cells/ml) were placed in the upper chamber of the transwell assembly. The lower chamber contained 650 µl of RPMI medium. After incubation of 24 h, the upper surface of the membrane was scraped gently to remove non-migrating cells and washed. The membrane was then fixed and stained with hematoxylin/eosin. Cells were imaged and counted using image J (Lu et al., 2016).

MMP2 activity

Matrix metalloproteinase 2 (MMP2) activity was measured by the RayBio Human MMP2 ELISA Kit (RayBiotech), according to the manufacturer. Briefly, cells are pipetted into wells and MMP2 of the sample is bound to wells by the immobilized antibody. Wells are washed and biotinylated anti-human MMP2 antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was added. Wells were washed, TMB substrate solution was added to wells and the color developed in proportion to the amount of MMP2 bound. Stop solution (0.18 M sulphuric acid) changed color from blue to yellow. Color intensity was measured at 450 nm.

Statistical Analysis

Statistical analysis was done using SPSS version 16 one way ANOVA Tukey analysis, where $P<0.05$ was considered to indicate a statistically significant difference.

Results

Anticancer drugs in the early period were generally cytotoxic without specific molecular targets. Most of these cytotoxic drugs target rapidly dividing cells, resulting in severe adverse effects and high frequency of tumor recurrence. New targeted therapies are therefore an urgent unmet medical need for cancer treatment (Nakano et al., 2016).

In the presented study, ethanol extract of *Hemimycale arabica* sponge and four of its successive fractions were used for this aim. Our qualitative chemical composition analysis results revealed that petroleum ether, diethyl ether, dichloromethane and ethyl acetate fractions contained alkaloids, triterpenes, sterols, and cardiac glycosides. Diethyl ether and dichloromethane fractions contained flavonoids while leucoanthocyanins were presented in diethyl ether and ethyl acetate fractions. GLC analysis of unsaponifiable fraction revealed the presence of 16 compounds (Table 2) mainly hydrocarbon compounds (75.83%) and sterols 24.17 %. The total saponifiable matter contained thirteen fatty acids, five saturated fatty acids (18.53%) and eight unsaturated fatty acids (81.47%) (Table 3). Dichloromethane fraction was subjected to GC/MS analysis which detected 15 compounds comprising 6 hydrocarbons (28.80%), 3 alcohols (5.34%),

Table 1. List of Primers of Genes Used in RT-PCR.

Gene	Forward (5′—3′)	Reverse (5′—3′)	Reference
GAPDH	ACCCACTCCTCCACCTTTGAC	TGTTGCTGTAGCCAAATTCGTT	(Shao et al., 2014)
CD105	CTCTGCTGCTGAGCTGAATG	GATCTGCATGTTGTGGTTGG	(Zemel et al., 2009)
CD44	AGAAGGTGTGGGCAGAAGAA	AAATGCACCATTTCCTGAGA	(Biddle et al., 2013)
VEGFA	ATGAGGACACCGGCTCTGACCA	AGGCTCCTGAATCTTCCAGGCA	(Vizio et al., 2013)
TGFβ1	AAGGACCTCGGCTGGAAGTGC	CCGGGTTATGCTGGTTGTA	(Zaravinos et al., 2011)
FGF4	GACTACCTGCTGGGCATCAA	TGCACTCATCGGTGAAGAAG	(Johannesson et al., 2009)

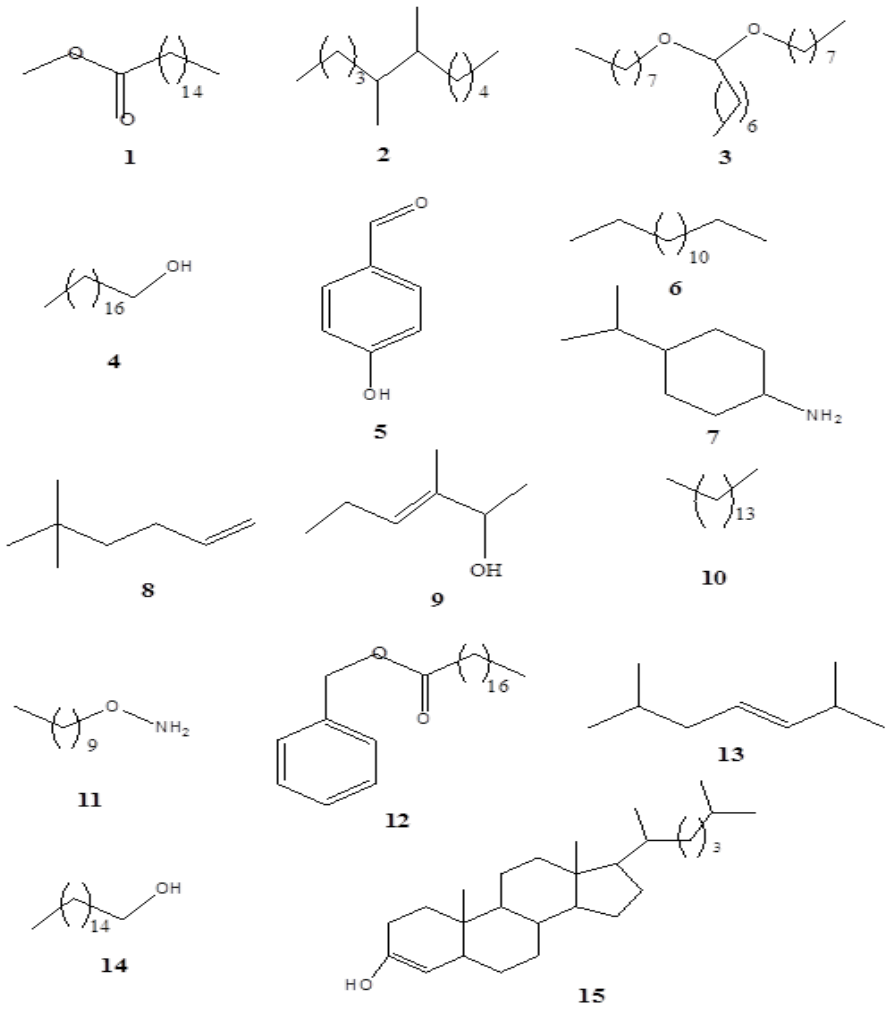

Figure 1. The Identified Compounds by GC/MS in the Dichloromethane Fraction

1 aldehyde (12.56%), 2 esters (14.01%), 2 amines (3.83%) and 1 sterol (3.10%) (Table 4, Figure 1). GLC analysis of liposoluble matter obtained from petroleum ether fraction revealed the presence of polyunsaturated,

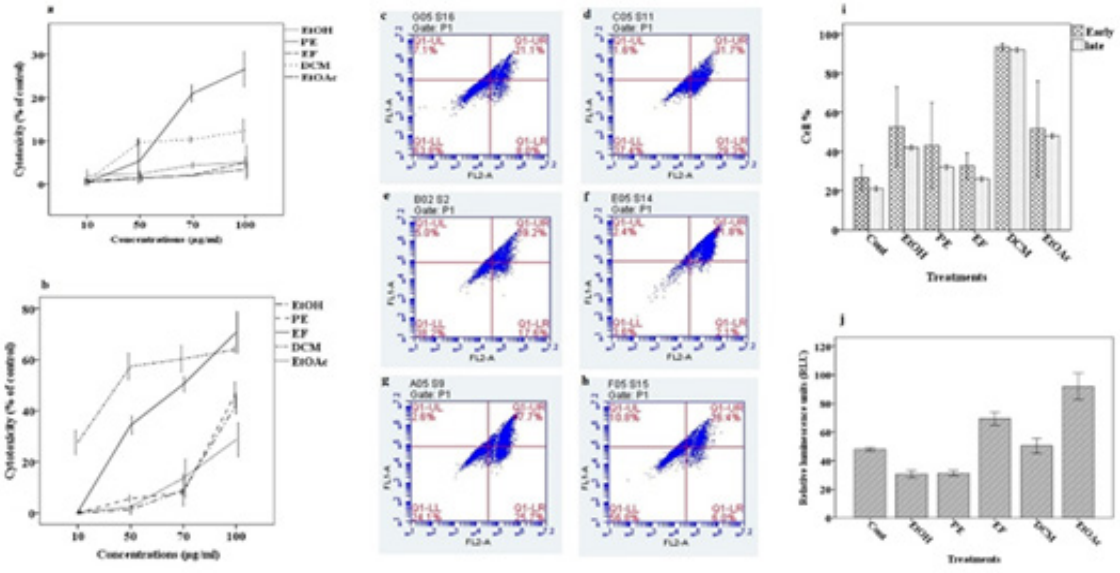

Figure 2. Anti-Proliferation Activity of Hemimycale Arabica Fractions. Cytotoxic activity against HepG2 treated for a) 24h b) 48h at various concentrations. Apoptotic activity, Annexin V-FITC/PI double staining analysis of apoptosis. c) control d) total ethanol extract (EtOH), e) petroleum ether (PE), f)diethyl ether (EF), g) dichloromethane (DCM) and h) ethyl acetate (EtOAc) fractions treated for 24 hours. i: Annexin (Percentages of apoptotic cells). j: Caspase activity of HepG2 cells control and treated. Data were expressed as a percent of control ± SE (n=3).

Table 2. GLC Analysis of the Unsaponifiable Matter

[a]RR_t	Compounds	% Peak area
0.65	n-Pentadecane	1.34
0.89	n-Octadecane	2.20
0.96	n-Nonadecane	1.57
1.00	n-Eicosane	20.06
1.12	n-Heneicosane	2.36
1.15	n-Docosane	8.84
1.21	n-Tricosane	9.53
1.27	n-Tetracosane	8.37
1.38	n-Hexacosane	3.26
1.44	n-Heptacosane	2.32
1.49	n-Octacosane	1.99
1.54	n-Nonacosane	3.44
1.60	n-Triacontane	6.28
1.69	Cholesterol	5.05
1.76	Stigmasterol	8.77
1.82	β-Sitosterol	9.02
1.49	n-Octacosane	1.99
1.54	n-Nonacosane	3.44
1.60	n-Triacontane	6.28
1.69	Cholesterol	5.05
1.76	Stigmasterol	8.77
1.82	β-Sitosterol	9.02

[a]RR_t, Retention time relative to n-eicosane (Rt = 17.08 min).

Table 3. GLC Analysis of Fatty Acid Methyl Esters

[a]RR_t	Compounds	Peak area (%)
0.42	Lauric acid	2.14
0.43	Tridecanoic acid	1.72
0.49	Myristoleic acid	2.76
0.63	Palmitic acid	4.96
0.65	Palmitoleic acid	9.72
0.77	Oleic acid	14.39
0.79	Linoleic acid	7.39
0.82	Linolenic acid	3.83
0.92	11,14,17,-Eicosatrienoic acid	4.89
1.00	5,8,11,14,17-Eicosapentaenoic acid	26.08
1.09	Tricosanoic acid	3.94
1.23	Lignoceric acid	3.59
1.15	Nervonic acid	4.23

Table 4. GC/MS Analysis of DCM Fraction

[a]RR_t	Compound No.	Compound name	Peak area (%)
0.61	1	Methyl-palmitate	16.7
0.47	2	5,6- Dimethylundecane	2.27
0.62	3	1,1- Dioctyloxyoctane	7.89
0.88	4	Stearyl alcohol	4.95
0.98	5	p- Hydroxybenzaldehyde	16.25
1	6	n- Tetradecane	16.94
1.05	7	4-Isopropylcyclohexanamine	0.72
1.06	8	5,5-Dimethylhexene-1	0.97
1.07	9	3- Methyl-3-hexen-2-ol	0.97
1.11	10	Pentadecane	8.23
1.22	11	O- Decylhydroxylamine	4.23
1.25	12	Benzyl stearate	1.16
1.29	13	2,6-Dimethyl-3-heptene	1.4
1.41	14	1-Hexadecanol	0.95
1.51	15	Cholest-5-en-3-ol-(3α)	4.01

RR_t, Retention time relative to n- tetradecane (Rt = 27.58 min).

omega-3 polyunsaturated, fatty acids and linolenic acid. These long-chain polyunsaturated fatty acids are known for their anticancer activity (Biegelmeyer et al., 2015).

Discussion

Our results revealed that all treatments were non cytotoxic after 24 h diethyl ether fraction and ethyl acetate had weak cytotoxicity with IC_{50} equal to 70 µg/ml and

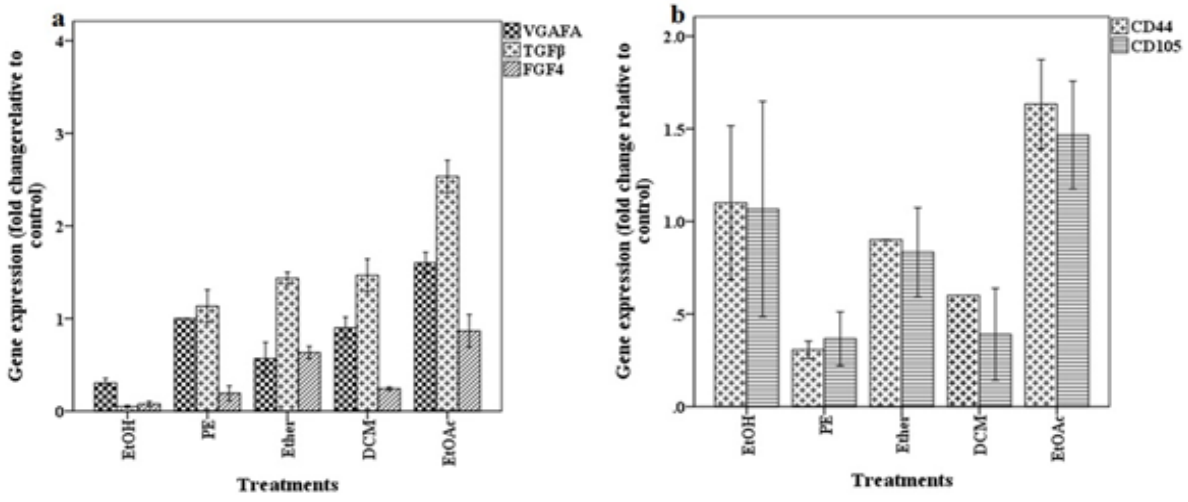

Figure 3. Real Time PCR Analysis and Gene Expression of HepG2 Cells Treated with Hemimycale Arabica Fractions. a. anti-proliferative and cytokines anti-migratory growth factor-related genes VEGFA, TGFβ1, FGF4, and IGF. b. Surface marker (CD44 and CD105. Data represented as percent of control.

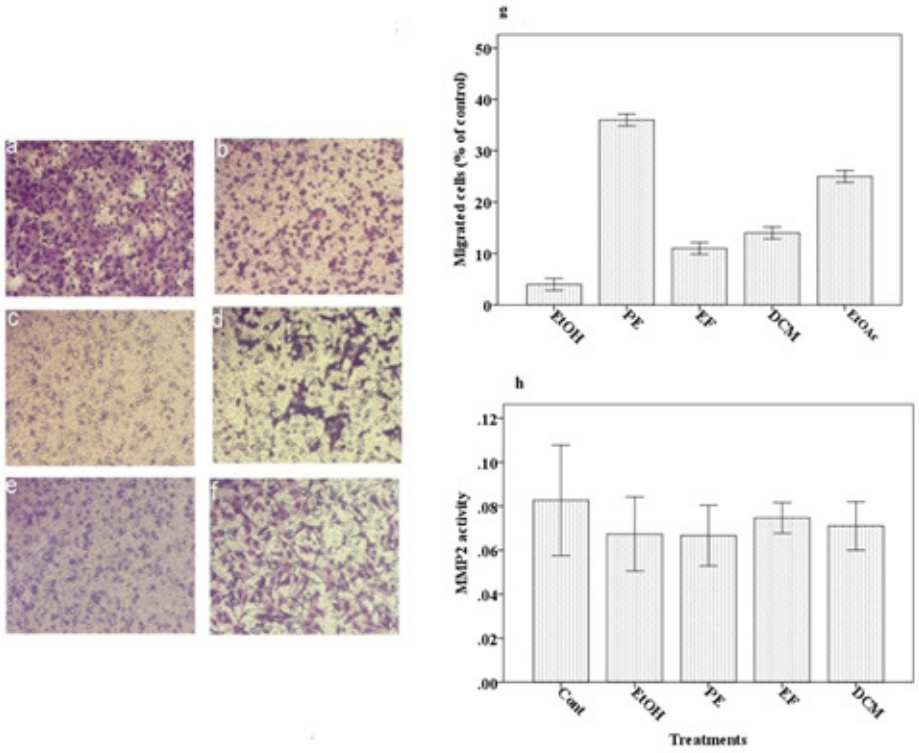

Figure 4. Migration capability of HepG2 Cells in Response to Treatment with *Hemimycale Arabica* Fractions. The appearance of HepG2 cells (haematoxylin- eosine stained) on the underside of the membrane in the migration assay. a) control. b) total ethanol extract (EtOH), c) petroleum ether (PE), d) diethyl ether (EF), e)dichloromethane (DCM) and f) ethyl acetate (EtOAc) fractions. g) Migrated cells percent in response to different treatments. h) MMP-2 activity percent of control.

dichloromethane fraction exhibited moderate cytotoxicity with IC_{50} of 50 µg/ml. Other fractions (total ethanol and petroleum ether fractions) were non-cytotoxic. For further bioassays cells were treated with 100 µg/ml in case of ethanol and petroleum ether fractions, 50 µg/ml of diethyl ether and ethyl acetate fractions and 25 µg/ml of dichloromethane fraction (Figure 2). Our results were concomitant with that found by Youssef et al., (2015) who reported the anti-proliferative activity of isolated alkaloid from *H. arabica* against human cervical carcinoma cell line (Youssef et al., 2015).

Using flow cytometric technique, ethanol, dichloromethane and ethyl acetate fractions induced apoptosis as observed by an increasing number of cells undergoing early (positive for annexin V) and late apoptosis (positive for annexin V and PI). Ethyl acetate fraction raised caspase activity while diethyl ether fraction increased caspase activity without a change in cell number in early or late apoptosis. Petroleum ether fhad no effect on apoptosis whereas no change in cell number positive for annexin V or PI (Figure 2). Caspase-independent apoptosis was induced by total ethanol extract and dichloromethane fraction while ethyl acetate fraction induces caspase-dependent apoptosis. Ether fraction induced non-apoptotic caspase activity which known as a nonlethal role of caspase where its activation leads to a subset of apoptotic morphological changes without causing cell death (Nakajima and Kuranaga, 2017).

Total ethanol extract blocked migration activity and this persisted in its sub-fractions in different proportions where dichloromethane and ether fractions equally inhibited migration ability more than ethyl acetate and petroleum ether fractions. All treatments inhibited migration through down-regulation of one or more of tested gene expression. Except for ethanol fraction, which decreased *VEGFA* expression (3.3 fold), other fractions did not affect *VEGFA* expression. Ethanol fraction down-regulated *FGF4* and *TGF-β* expression. Petroleum ether fraction down-regulated expression of surface marker genes *CD44* and *CD105* as well as *FGF4*, whereas dichloromethane down-regulated expression of *FGF4* and *CD44* only. Diethyl ether and ethyl acetate had no effect on growth factors and surface markers genes (Figure 3). Total ethanol extract exhibited a powerful inhibition of cell migration through tested growth factors resulted in almost complete blockage of migration ability which was concomitant with the hypothesis that hitting multiple aspects of tumor angiogenesis, with a cocktail of compounds create more effective treatment (Huang et al., 2018; Wang et al., 2015). *FGF4* was found to be targeted by petroleum ether and dichloromethane fractions with VEGF- independent inhibition of migration, which concomitant with the fact that FGFs may promote VEGF-independent angiogenesis (Cao et al., 2012).

Surface markers were not affected by either ethanol extract, ether nor ethyl acetate fractions. In contrast, petroleum ether fraction suppressed gene expression of surface markers while dichloromethane fraction suppressed *CD44* without affecting *CD105* (Figure 3).

All fractions exerted anti-migratory activity on

hepatocellular carcinoma cells. They decreased migrated cells percent to 4, 35, 11, 14 and 25% when treated with ethanol, petroleum ether, diethyl ether, dichloromethane and ethyl acetate, respectively. The induction of the anti-migratory effect was performed via different molecular mediators. The common feature in their mode of action is the absence of matrix metalloproteinase 2 (MMP2) role in mediating the anti-migratory activity (Figure 4). MMP-independent migration induction, called chemotactic induction of migration, could represent events induced by treatments resulting in amoeboid-like migration via cell squeezing through matrix pores without degradation of ECM (Friedl and Wolf, 2003).

In conclusion, Total ethanol extract is a promising non-cytotoxic anticancer agent because of its powerful apoptosis induction and capability to block tumor cell migration. Petroleum ether and ether fractions area weak non-cytotoxic anti-migratory agents without apoptosis induction. Dichloromethane could be a moderate cytotoxic anti-migratory agent induced caspase-independent apoptosis. It could be used in anticancer therapy for VEGF-resistance patients through downregulation of FGF. Ethyl acetate fraction considered a non-cytotoxic caspase-dependent apoptotic inducer exerting moderate anti-migratory activity. In this work, we represent new sponge-derived treatments which can solve different resistance problems to find a convenient treatment for each individual case using a single agent without serious side effects of the current used cytotoxic drugs.

Author Contribution Statement

H. Rady the, corresponding author, participated in the design of the study, the coordination of the study, participated in the practical work, collecting data, performed the statistical analysis and reviewed the manuscript. S. Salem participated in the design of the study, the practical work, participated in collecting the data and writing and reviewed the manuscript. A. Hassan design of the study, the practical work participated in the coordination of the study and reviewed the manuscript. H Abd-Alla reviewed the manuscript. H. Raouf participated in the practical work of the study. All authors made a significant contribution to the work reported, read, revised, and agreed the final manuscript before submission.

Acknowledgments

Funding

This work was supported by the National Research Center-Egypt [grant number 10160003].

Ethical Approval

Ethical approval not applicable.

Data Availability

All relevant data are within the paper. The study was approved by any scientific Body and it is not a part of an approved student thesis.

Conflict of intrest

The authors declare that there is no conflict of interest in this work including any financial, personal or other relationships with other people or organizations.

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