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Original Article

Primmorph extracts and mesohyls of marine sponges inhibit proliferation and migration of hepatocellular carcinoma cells *in vitro*

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ABSTRACT

Cancer recurrence and severe side effects of currently being used chemotherapeutic agents reduce their clinical efficacy. Thus, there is a constant need to develop alternative anticancer drugs. Sustainable supply is an important challenge facing marine-based drug discovery. Primmorph, a 3D cell culture system, could provide a sustainable source to produce metabolites for anticancer drugs from marine sponges. In the present work, the anticancer activity of primmorph extracts and mesohyls of *Negombata magnifica*, *Hemimycle arabica*, *Crella spinulata*, and *Stylissa carteri* sponges was evaluated. Antiproliferative activity was studied in terms of cytotoxicity, colony formation, cell cycle, and apoptosis. Migration was assessed by migration assay and matrix metalloproteinase activity. The expression of proliferation and migration-related genes was analyzed using real time PCR. Migration and proliferation activities of HepG2 cells were inhibited by treatment with primmorph extracts and mesohyls of *N. magnifica*, *H. arabica*, and *C. spinulata*. The mesohyl of *S. carteri* did not show any anticancer activity although the primmorph extract led to cell cycle arrest. Among the selected sponge species, the primmorph extract of *C. spinulata* was the most promising anticancer agent regarding antiproliferative and antimigratory activities. In addition, primmorph extracts have the advantage of working under well-defined and controlled conditions, which allows the easy application as a bioreactor.

1. Introduction

Molecular targeting based on genetic changes must provide an ever-increasing contribution to drug development. Current clinically used anticancer agents do not effectively treat most cases of cancers. Therefore, there is an increasing need to reassess the overwhelming emphasis on molecular targeting [1]. Many strong anticancer agents isolated from marine sponges have potential clinical value [2–7]. The quantity of extractable sponge biomass available from the sea cannot meet the demand for large-scale research or clinical development of anticancer compounds. This is often referred to as a "supply problem" [8]. One of the most promising approaches to this problem is the application of *in vitro* culture of sponge primmorph for the production of bioactive compounds in bioreactors. Sponge primmorph is a special form of sponge cell aggregates formed from the mesohyl. Primmorphs provide the possibility to work under well-defined and controlled conditions and facilitate the optimization of target product formation for the purpose of pharmaceutical production [9,10].

Tumor cell migration is a critical factor in the context of the metastatic activity, and migration activity is under intense investigation as the underlying cause of cancer metastasis [11]. Matrix metalloproteinases (MMPs) are the primary proteases responsible for extracellular matrix (ECM) degradation during cancer metastasis. Cells have been proposed to employ either protease-dependent or protease-independent modes of migration [12]. Cells that lack ECM proteases or those treated with protease inhibitors use mechanical forces to physically reorganize the matrix, while simultaneously deforming their cell body to migrate in an amoeboid-like manner [13]. When metastases occur, tumor cells have already acquired resistance to inducers of apoptosis and cytotoxic drugs. Most cancer deaths are caused by cancer metastasis, not the primary tumor. Therefore, an anti-migratory strategy targeting metastatic tumor cells is highly desirable [14].

Degradation of the ECM releases basement membrane-

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sequestered growth factors, such as fibroblast growth factor (FGF4) and transforming growth factor β1 (TGFβ1) [15]. Insulin-like growth factors (IGFs) suppress apoptosis and promote cell cycle progression and metastasis in many cancers. FGF4, IGF1, and TGFβ1 are cytokines that regulate cell migration and proliferation [16,17], and thus therapeutic strategies targeting FGFs, IGF1 and TGF have been developed for the treatment of cancer.

Endoglin (CD105) is an accessory receptor for TGFβ1 and its expression is upregulated in actively proliferating endothelial cells [18]. The adhesion molecule CD44, a surface receptor for hyaluronan, is known to be involved in cancer metastasis. Increased expression of CD44 is correlated with high metastatic activity. TGFβ1 was found to induce the expression of membrane type 1 MMP (MT1-MMP), leading to CD44 cleavage which in turn enhances the migration of cancer cells [19].

To the best of our knowledge, the present work is the first to use primmorph extracts and mesohyls of four Red Sea sponges (class Demospongiae), namely, *Negombata magnifica* (family:-Podospongiidae), *Hemimycale arabica* (family: Hymedesmiidae), *Crella spinulata* (family: Crellidae), and *Stylissa carteri* (family: Dictyonellidae). We aimed to develop new anticancer agents with antiproliferative and antimigratory activities which could be more effective in cancer patients. The study also aimed to compare the anticancer activities of primmorph ethyl acetate extract and mesohyl of the same sponge species. The use of primmorphs as a source of anticancer agents has the advantage that it can easily be translated to applications in industrial drug production, where the primmorphs can act as bioreactors.

2. Materials and methods

2.1. Sample collection

Samples of Red Sea sponges *N. magnifica*, *H. arabica*, *C. spinulata,* and *S. carteri* were 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 18 m. Sponge species were identified by Dr. Mohamed Ez El-Arab, The National Institute of Oceanography and Fisheries (NIOF), Hurghada, Egypt.

Sponges were photographed *in situ* using an underwater camera. Identification of sponge species was made on the basis of microscopic examination of skeletons and siliceous and calcareous spicules. Category and size of the spicule were examined and detected using a binocular microscope with a range of 400×magnification as described by Borges et al. [20]. Brief morphological description and sufficient documentation regarding locality, depth, and habitat were also used in identification. The categories and arrangements of the spicules, as well as fiber forms, provided important information for the identification of sponges. The specialized bibliography Systema Porifera, a basic guide for the classification of sponge specimens, was employed.

2.2. Primmorph extraction and mesohyl preparation

Sponge specimens were dissected under sterile conditions and prepared as previously described by Rady et al. [21]. The mesohyl was aspired carefully and collected with a sterile syringe taking special care not to aspirate any other fluids than the mesohyl. For primmorph production from the selected species, according to our previous work [22], fresh mesohyl was diluted 1:2 using sterile natural sea water (total cell density was approximately 3×10^8 cells/mL) and then incubated at 16 °C with discontinuous gentle agitation. Primmorphs were counted and weighed, and then extracted by ethyl acetate three times at room temperature. The solvent was completely evaporated under reduced pressure at 40 °C to afford 0.08 g solid extract. Primmorph extract and mesohyl of each sponge species were dissolved in incomplete RPMI-1640 medium (Gibco BRL, Gaithersburg, Md.) and subjected to the bioassays.

In our previous work, microscopic studies suggested that primmorphs are formed in four stages: amorphous large cell floc within 1–3 h; small irregular cell aggregations in 1 day; large primary cell aggregations and finally round-shaped primmorphs after 3 days. Primmorphs of *C. spinulata* and *S. carteri* remained alive for 3–6 months. The primmorphs of *H. arabica* remained alive for 1 month. In the case of *C. spinulata* and *S. carteri* mesohyls, a cell density range of $(3–7) \times 10^8$ cells/mL was found to be suitable for morphogenesis of cell aggregations and developed into primmorphs with diameters ranging from 400 to 500 μm, while in the case of *H. arabica* and *N. magnifica*, the suitable cell density range was $2–6 \times 10^8$ cells/mL, which resulted in primmorphs with a diameter range of 50–300 μm [22].

2.3. Cell line propagation and treatment

Human hepatocellular carcinoma cell line (HepG2) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, Md.), 1% penicillin/streptomycin (5 mg/mL each) and 2% L-glutamine (2 mM) at 37 °C in a humidified atmosphere of 5% CO_2. Cells at approximately 80% confluence were trypsinized, seeded and incubated at 37 °C and 5% CO_2 overnight. Cells were then treated with sub-lethal concentrations (1, 5, 5 or 2 μg/mL of primmorph extract or 1, 1, 5 or 5 μL/mL of mesohyl *of N. magnifica*, *H. arabica*, *C. spinulata,* and *S. carteri*, respectively) dissolved in RPMI-1640 medium.

2.4. MTT assay

Cytotoxicity against HepG2 cells was assessed by MTT [3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay according to Hwang et al. [23]. Cells were seeded in 96-well microplates (3×10^3 cells/well) in 100 μL RPMI-1640 culture medium and incubated at 37 °C and 5% CO_2 overnight. Cells were treated and re-incubated for 24 h, and 100 μL MTT (0.5 mg/mL) solution was added to each well and incubated until purple formazan crystals appeared. The medium was discarded and 100 μL of DMSO was added to dissolve the crystals. The optical density (OD) of solubilized formazan was measured at 592 nm using an automated microplate, Sunrise™ ELISA reader (TECAN, Austria, GmbH Untersbergstrasse Switzerland). The absorbance was correlated to the relative number of viable cells. Results are expressed as a percent of untreated control.

2.5. Colony formation assay

Based on the method described by Franken et al. [24], cells were seeded into culture flasks and incubated at 37 °C and 5% CO_2 overnight then treated with the tested agents for 3 days. After 14 days, colonies were counted, and the results were expressed as a percent of untreated HepG2 cells. The experiments were performed independently three times.

2.6. Caspase activity

Caspase-Glo® Assay was used to measure caspase 2 and 3/7 activity according to the manufacturer's protocol. Briefly, 50 μL of Caspase-GloR 2 reagent was added to each well of a 96 well plate

containing 50 μL of blank or control (untreated cells). The plate was gently mixed and incubated at room temperature for 2 h. Luminescence was recorded with a plateluminometer (PerkinELmer, Waltham, United States).

2.7. Apoptosis detection

HepG2 cells were treated for 72 h, then collected and re-suspended at a concentration of 1.5×10^5 cells in 500 μL of 1 × binding buffer. Five microliters of annexin V-FITC and 5 μL of propidium iodide (PI, 50 μg/mL) were added, then incubated at room temperature for 5 min in the dark. Annexin V-FITC binding and PI staining was measured on a Epics XL (Beckman Coulter) flow cytometry instrument (FITC: Ex = 488 nm, Em = 530 nm; PI: Ex = X nm, Em = X nm) according to the method described by Lu et al. [25].

2.8. Cell cycle analysis

According to the method described by Guo et al. [26], HepG2 cells were incubated at 5×10^5 cells/well in 6 well plates with RPMI-1640 medium for 12 h and then treated for 72 h. Cells were harvested and fixed in 70% ice cold ethanol at −20 °C overnight. After fixation, cells were washed with phosphate buffer saline (PBS), re-suspended in 1 mL PBS containing 1 mg/mL RNase (Sigma) and 50 μg/mL PI (Sigma) and incubated at 37 °C for 30 min in the dark. Samples were analyzed for DNA content by Epics XL (Beckman Coulter). Cell cycle phase distributions were analyzed with the Cell Quest acquisition software (BD Biosciences).

2.9. Reverse transcription and quantitative real-time PCR

Total RNA was isolated using 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's protocol. For quantitative real-time PCR, amplification mixtures were prepared using KAPA SYBR_FAST qPCR master mix (Kapa Biosystem). *GAPDH* was used as an internal reference gene to normalize the expression of *CD105*, *CD44*, *TGFß1*, *FGF4*, and *IGF1*. Results were expressed as a ratio of reference gene mRNA to target gene mRNA using the $2^{-\Delta\Delta Ct}$ method. Primers are listed in Table 1 [27–32].

2.10. Transwell migration assay

Migration assay was performed using Thincerts™ (Greiner Bio-One) in 24 well plates. HepG2 cells were serum-starved by incubating cells in serum free media and kept in a 37 °C and 5% CO_2 incubator for 24 h. The cells were then seeded in the upper chamber of the transwell assembly at a density of 6×10^5 cells/mL in 100 μL of serum free medium. The lower chamber contained 650 μL of RPMI-1640 medium. After incubation at 37 °C and 5% CO_2 for 24 h, the upper surface of the membrane was scraped gently to remove non-migrating cells and washed with PBS. The membrane was then fixed in 4% paraformaldehyde for 15 min and stained with hematoxylin and eosin. The cells were then imaged in five fields for each membrane and counted using Image J software according to the method described by Lu et al. [33].

2.11. MMP2 activity

Matrix metalloproteinase 2 (MMP2) activity was measured by RayBio Human MMP2 ELISA Kit (RayBiotech), according to manufacturer's protocol. Standards and samples were pipetted into the wells and the MMP2 in each sample was bound to the wells by the immobilized antibody. Wells were washed and biotinylated anti-human MMP2 antibody was added. After washing away unbound antibody, HRP-conjugated streptavidin was added. The wells were washed, then a TMB substrate solution was added to the wells and the color developed in proportion to the amount of MMP2 bound. Addition of stop solution changed the color from blue to yellow, and the intensity of the color was measured at 450 nm absorbance.

2.12. Statistical analysis

Statistical analysis was made using SPSS (version 16) One-way analysis of variance (ANOVA)- Tukey analysis, where $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Cytotoxicity

Mesohyl of *H. arabica*, *C. spinulata,* and *S. carteri*, exhibited cytotoxic activities against HepG2 cells with IC_{50} values of 27, 38.3 and 39.6 μL/mL, respectively. Primmorph extracts of *C. spinulata* and *S. carteri* displayed weak cytotoxicity with IC_{50} values of 57.9 and 45.5 μg/mL, respectively. Primmorph extract of *H. arabica* had no cytotoxic activity. Our previous work revealed that IC_{50} values of primmorph extract and mesohyl of *N. magnifica* were 5 and 3 μg/mL, respectively [21]. For further bioassays, cells were treated with sub-lethal concentrations 1, 5, 5 or 2 μg/mL of primmorph extract or 1, 1, 5 or 5 μL/mL of mesohyl of *N. magnifica*, *H. arabica*, *C. spinulata,* and *S. carteri*, respectively (Fig. 1).

3.2. Inhibition of colony formation

The ability of colony formation of HepG2 cells treated with sub-lethal doses of primmorph extracts and mesohyls of all selected species was significantly inhibited. In the case of *C. spinulata* primmorph extract, colony formation was completely blocked (Fig. 2).

3.3. Cell cycle arrest

Our previous work [21] revealed that mesohyls of *N. magnifica* did not exhibit any noticeable effect on cell cycle phases, while primmorph extract decreased the cell population in S-phase and arrested cells in G0–G1. Herein, the cell number in S-phase was decreased and the cells were arrested in G0-G1 after treatment

Table 1
List of primers of genes used in RT-PCR.

Gene	Forward primer	Reverse primer	Reference
GAPDH	5′-ACCCACTCCTCCACCTTTGAC-3′	5′-TGTTGCTGTAGCCAAATTCGTT-3	[27]
CD105	5′- CTCTGCTGCTGAGCTGAATG-3	5′-GATCTGCATGTTGTGGTTGG-3	[28]
CD44	5′-AGAAGGTGTGGGCAGAAGAA-3′	5′-AAATGCACCATTTCCTGAGA-3′	[29]
TGFß1	5′-AAGGACCTCGGCTGGAAGTGC-3′	5′-CCGGGTTATGCTGGTTGTA-3′	[30]
FGF4	5′-GACTACCTGCTGGGCATCAA-3′	5′-TGCACTCATCGGTGAAGAAG-3′	[31]
IGF1	5′-GCAATGGGAAAAATCAGCAG-3′	5′-GAGGAGGACATGGTGTGCA-3′	[32]

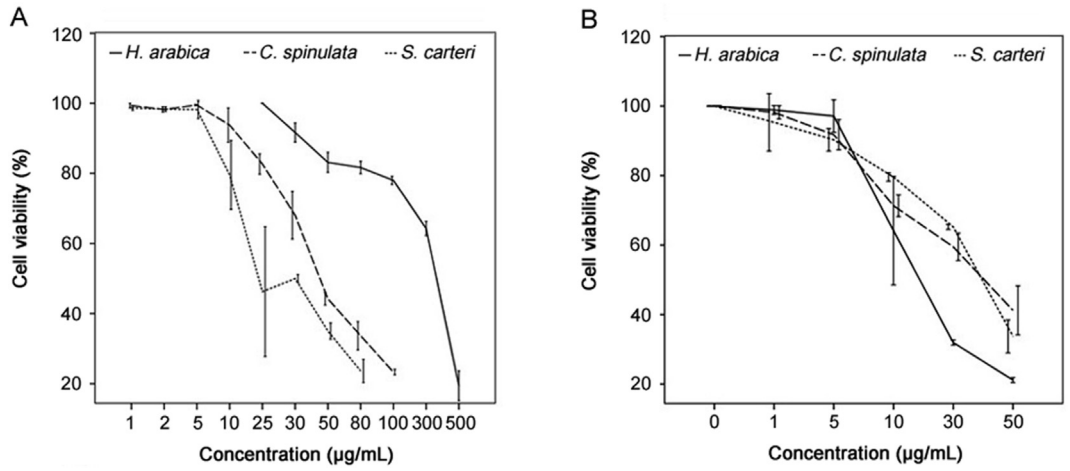

Fig. 1. Cytotoxic activity of *Hemimycle arabica*, *Crella spinulata,* and *Stylissa carteri* against HepG2 cells treated for 24 h at various concentrations of (A) primmorph ethyl acetate extracts and (B) mesohyls. Data are expressed as a percent of untreated control ± SE ($n = 3$).

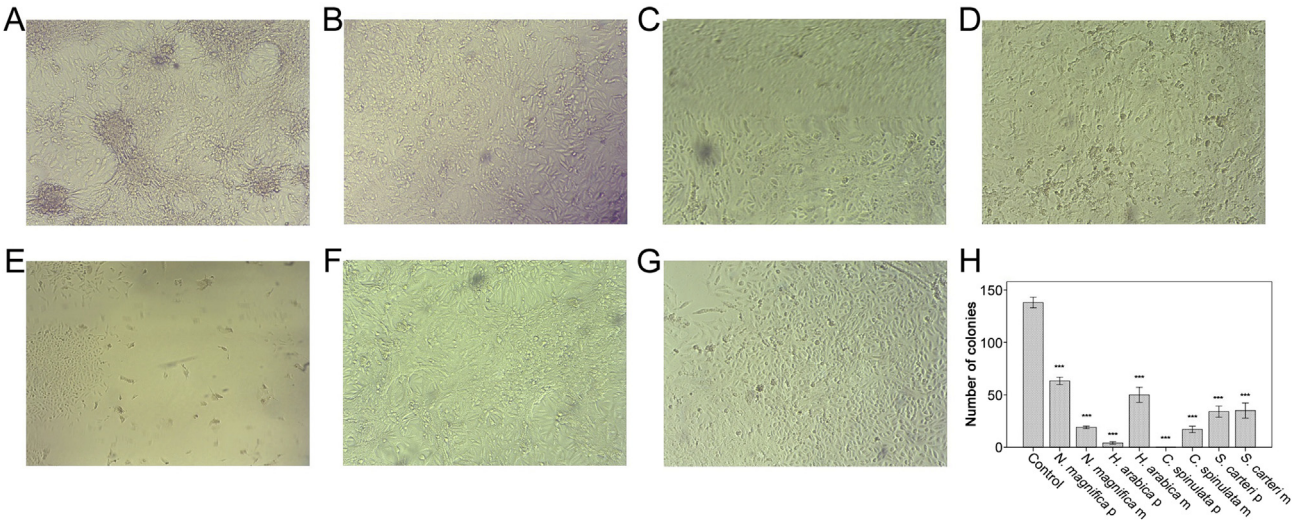

Fig. 2. Inhibition of colony formation of HepG2 cells. (A–G) Light microscope images of HepG2 cells in control (A) and after treatment with the primmorph ethyl acetate extracts (p) and mesohyls (m) of *Hemimycle arabica*, *Crella spinulata,* and *Stylissa carteri* sponges (B–G), respectively. (H) Number of colonies in control and treated HepG2 cells. Statistical analysis was conducted using One-way analysis of variance (ANOVA). Values are presented as means ± SE ($n = 3$). Asterisks above bars denote significance in change between different treatments and the control $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

with the primmorph extracts of the three sponge species. On the other hand, mesohyls did not affect the cell cycle except for that of *H. arabica* which arrested cells in G0-G1 (Fig. 3).

3.4. Apoptosis induction

On the basis of flow cytometric analysis, the primmorph extract and mesohyl of *N. magnifica* as well as the primmorph extract of *C. spinulata* increased cell number in the early and late apoptotic phases. The primmorph extracts of *H. arabica* and *S. carteri* as well as mesohyl of *C. spinulata* decreased cell population undergoing early and late apoptosis, while mesohyl of *H. arabica* decreased cell number in early apoptosis without changing the number of cells that were subjected to late apoptosis. Mesohyl of *S. carteri* affected neither early nor late percent of apoptotic cells (Fig. 4A–J).

Treatment with the mesohyl of *H. arabica*, primmorph extract of *C. spinulata,* and mesohyl of *S. carteri* increased caspase activity (RLU = 64.5, 66.6 and 55.6, respectively), while the mesohyl and primmorph extract of *N. magnifica* as well as the mesohyl of *C. spinulata* decreased caspase activity. No significant change was observed after treatment with the primmorph extract of *H. arabica* and *S. carteri* (Fig. 4K).

3.5. Inhibition of migration ability and MMP2 activity

Migration ability after treatment with *N. magnifica*, *H. arabica* and *C. spinulata* primmorph extracts and mesohyls was nearly blocked. Slight non-significant inhibition of migration was observed after treatment with *S. carteri* where the number of migrated cells was decreased to 72% and 75% for primmorph extract and mesohyl, respectively (Fig. 5A–J).

C. spinulata (primmorph extract and mesohyl) and primmorph extract of *H. arabica* significantly inhibited MMP2 activity, while *N. magnifica* (primmorph extract and mesohyl) had no effect on MMP2 activity. On the other hand, treatment with *S. carteri* (primmorph extract and mesohyl) increased MMP2 activity (Fig. 5K).

3.6. Downregulation of surface marker genes expression

Using 2-fold change (increase or decrease) as a cut-off value for

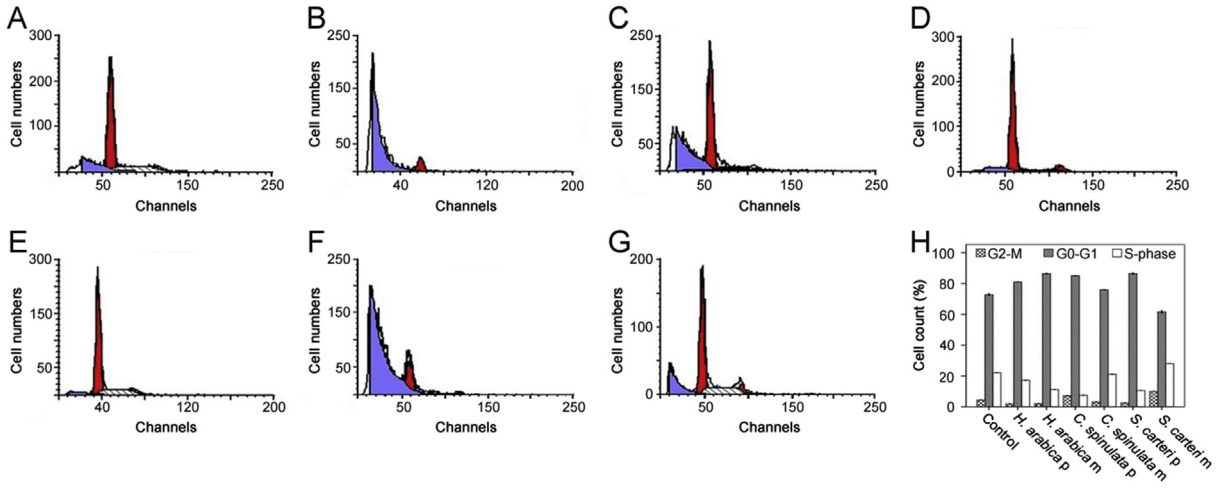

Fig. 3. Cell cycle analysis of HepG2 cells treated with sponge species for 24 h. (A) Control and (B–G) the primmorph ethyl acetate extracts (p) and the mesohyls (m) of *Hemimycle arabica*, *Crella spinulata*, and *Stylissa carteri*; (H) percentage of phases of the cell cycle. Statistical analysis was conducted using One-way analysis of variance (ANOVA). Values are presented as means ± SE ($n = 3$). Asterisks above bars denote significance in change between different treatments and the control * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

significant inhibition of gene expression, the results revealed that *N. magnifica* primmorph extract inhibited expression of *CD44* and *CD105* two folds relative to the control, while its mesohyl inhibited the expression of *CD105* only. *H. arabica* and *C. spinulata* (primmorph extract and mesohyl) significantly inhibited the expressions of *CD44* and *CD105*. *S. carteri* mesohyl decreased *CD105* two fold without affecting *CD44*, while its primmorph extract did not affect *CD105* gene expression but exceptionally increased *CD44* expression 10-fold relative to the control (Fig. 6).

3.7. Downregulation of growth factor gene expression

The expression of growth factor genes *TGFβ1*, *FGF4* and *IGF1* was investigated in treated HepG2 cells. *TGFβ1* expression was significantly inhibited 25-fold relative to control after treatment with the primmorph extract of *H. arabica*. Expressions of *FGF4* and *IGF1* were significantly (>2-fold) inhibited after treatment of HepG2 cells by *N. magnifica*, *H. arabica,* and *C. spinulata* primmorph extracts and mesohyls. On the other hand, the primmorph extract of *S. carteri* had no effect on the expression of *TGFβ1, FGF4,* and *IGF1*, while the mesohyl exhibited 4-fold downregulation of *IGF1* expression (Fig. 6).

4. Discussion

Dysregulation of cellular proliferation and apoptosis is frequently associated with hepatocellular carcinoma progression [34]. Cancer cells harbor alterations that result in impaired apoptosis signaling, which facilitates tumor development and metastasis [35]. Control of tumor metastasis is important to identify anticancer agents with antimigratory potency [36]. Some anticancer drugs are not intrinsically toxic but directly induce apoptosis of cancer cells. Since cancer therapy that depends on cytotoxicity is inherently more likely to cause the death of more normal cells, the development of non-cytotoxic anticancer agents with high apoptosis induction potential is an important goal for targeted cancer therapy [37].

In this work, we illustrated the anticancer potential (antiproliferative and antimigratory) of primmorph ethyl acetate extracts and mesohyls of *N. magnifica*, *H. arabica*, *C. spinulata,* and *S. carteri* against a hepatocellular carcinoma cell line (HepG2) *in vitro*. Antiproliferative activity was studied in terms of cytotoxicity, colony formation, cell cycle analysis, and apoptosis induction, whereas antimigration activity was assessed by migration assay and MMP2 activity. Gene expression of growth factors/cytokines and surface markers involved in proliferation and migration was analyzed.

In our previous work, antiproliferative activity of *N. magnifica* against HepG2 cells was observed through its cytotoxic activity. Primmorph extract arrested the cell cycle in G0-G1 while the mesohyl induced apoptosis [21]. In the present study, mesohyl of *N. magnifica* induced caspase-independent apoptosis while primmorph extract induced non-apoptotic antiproliferation. The anticancer activities of primmorph extract and mesohyl of *N. magnifica* may be due to the presence of latrunculin A and B, which are known cytotoxic growth inhibitors and apoptosis inducers [38].

Primmorph extract of *H. arabica* has a non-cytotoxic antiproliferative activity, which was concomitant with the findings by Hishita et al. [39], who found that colony formation ability could be inhibited by non-cytotoxic agents. On the other hand, we found that mesohyl of *H. arabica* had a powerful cytotoxic antiproliferative activity that caused cell cycle arrest. The primmorph extract and the mesohyl of *H. arabica* did not induce apoptosis; however, the mesohyl increased caspase activity, which can regulate cell growth through non-apoptotic functions [40,41].

Although the primmorph extract of *C. spinulata* possessed moderate cytotoxic activity, it was considered a powerful anticancer agent because it exerted a promising antiproliferative activity through caspase-dependent apoptosis induction and cell cycle arrest. On the other hand, the mesohyl exhibited its anticancer activity through moderate cytotoxicity, migration inhibition, MMP2 inhibition, and colony formation inhibition. Meanwhile, it did not induce apoptosis and had no effect on cell cycle.

Quantitative measurement of *in vitro* cell migratory behavior in three-dimensional biomimetic matrices is a direct approach to determining the efficacy of antimetastatic drugs on cancer cell migration. Recently, tumor therapeutic targets have expanded into the non-cytotoxic domain with the objective of curbing cancer metastasis [2]. Development of anti-metastatic drugs has the potential to greatly improve cancer prognosis. In this study, all treatments inhibited tumor cell migration (except for *S. carteri*) via either MMP-dependent or MMP-independent mechanisms. Migration inhibition was mediated through downregulation of gene expression of growth factors and cytokines and suppression of surface markers gene expression. These results were in agreement with the trend that many natural products have been reported to improve the efficacy of cancer chemotherapeutics through targeting of growth factors and surface markers, where some drugs enter

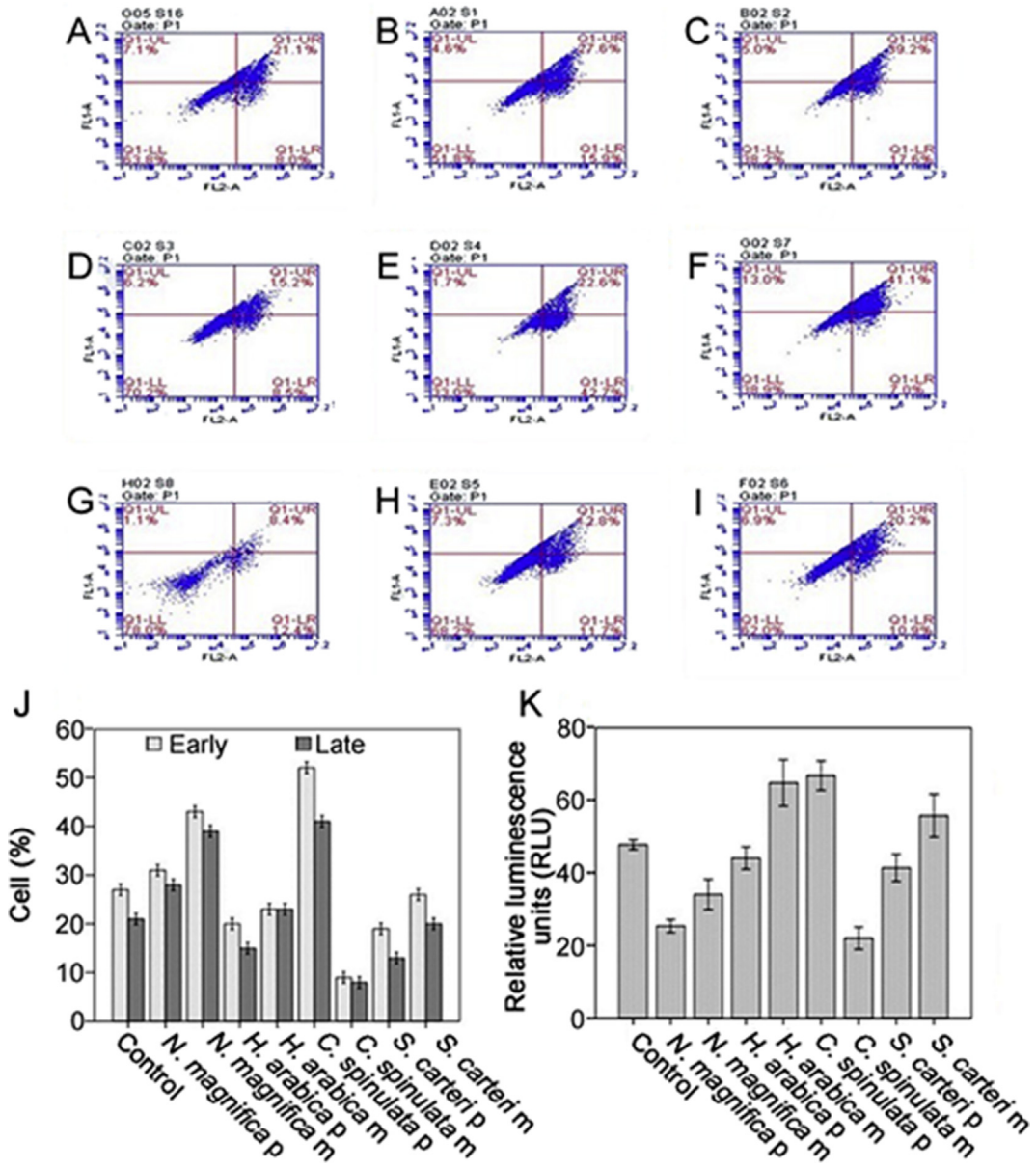

Fig. 4. Apoptotic activity of HepG2 cells in response to treatment with the primmorph ethyl acetate extracts (p) and mesohyls (m) of the selected sponges. A–I: Annexin V-FITC/PI double staining analysis of apoptosis in control (A) and treated cells with *Negombata magnifica*, *Hemimycle arabica*, *Crella spinulata*, and *Stylissa carteri* (B–I) for 24 h. J: Annexin percentages of apoptosis in HepG2 cells. K: Caspase activity of HepG2 treated and control cells. Statistical analysis was conducted using one-way analysis of variance (ANOVA). Values are presented as means ± standard error (SE) ($n = 3$). Asterisks above bars denote significance in change between different treatments and the control * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

clinical trials targeting these growth factors/cytokines [42,43].

The primmorph extract and the mesohyl of *S. carteri* could not be considered as an anticancer agent, as it exerts weak cytotoxic activity and does not promote apoptosis induction. Moreover, they do not inhibit migration of the tumor cells but do arrest the cell cycle.

Our results also revealed that there is no relationship between the anticancer activity of the primmorph extract and that of the mesohyl of the same sponge species. Notably, the primmorph extract of *C. spinulata* has the potential as a powerful anticancer agent (antiproliferation and antimigration) while the mesohyl is considered to be a weak growth inhibitor and a powerful anti-migratory agent. In addition, the mesohyl of *H. arabica* possesses cytotoxic activity, while the primmorph extract is non-cytotoxic and the primmorph extract of *N. magnifica* arrests the cell cycle and decreases cell number in the S-phase, but the mesohyl does not affect the cell cycle.

5. Conclusion

This study presented new candidates for cancer treatment from the selected marine sponge species that act on different targets in tumor cells requiring more effective treatments. Primmorph extracts and mesohyls of *N. magnifica, H. arabica*, and *C. spinulata* could be considered as promising anticancer agents. The primmorph extract of *C. spinulata* exhibited the most promising anticancer potential among the investigated treatments and is considered to be a powerful antiproliferative, apoptosis inducer and antimigratory agent. On the other hand, *S. carteri* could not be used as an anticancer agent because it did not exert any anticancer activity except for cell cycle arrest. Primmorph extracts have the advantage of well-defined and controlled conditions, which allows optimization of target product formation and can be applied as bioreactors.

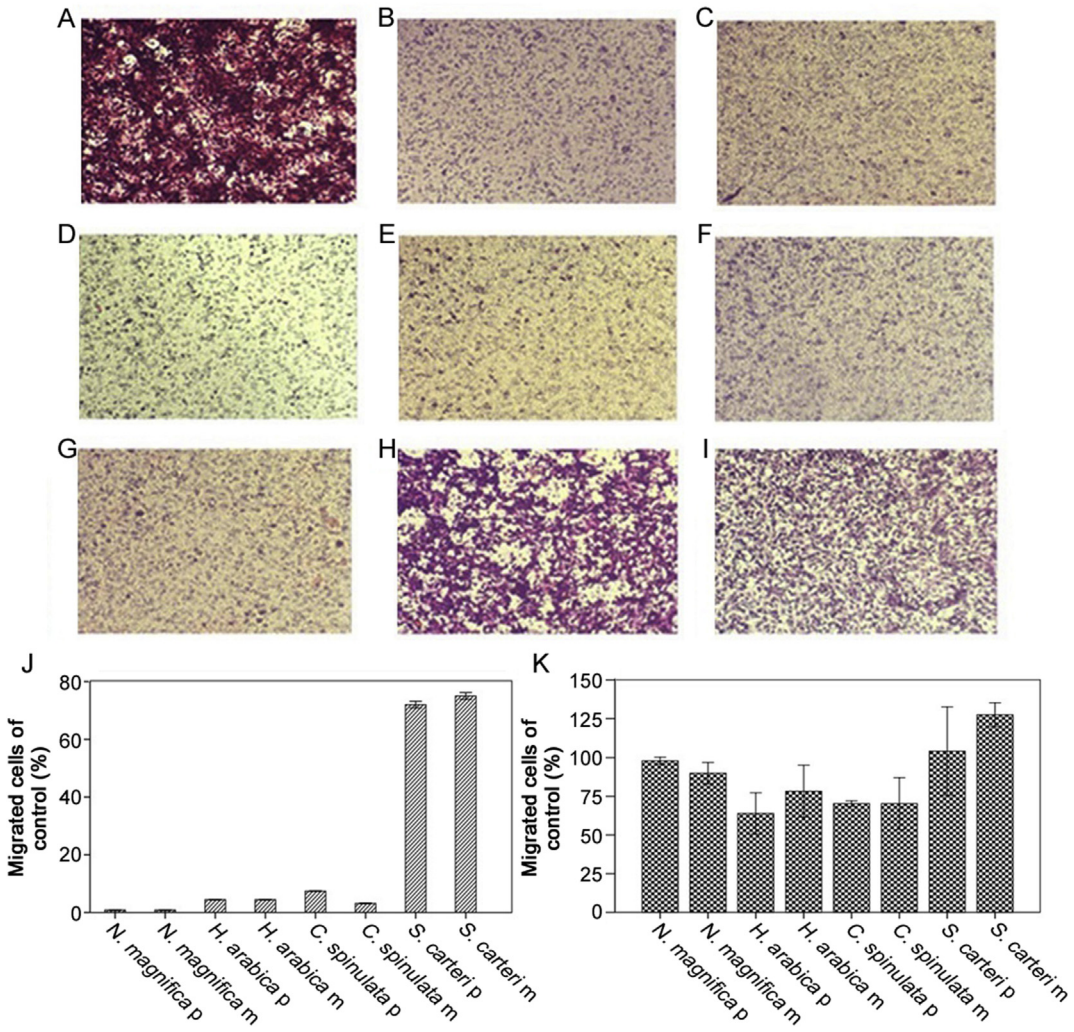

Fig. 5. Migration capability of HepG2 cells in response to the treatment with ethyl acetate extract of primmorph extracts (p) and mesohyls (m) of the selected sponges. (A–I) The appearance of HepG2 cells (hematoxylin & eosin stained) on the underside of the membrane in the migration assay in control (A) and after treatment with *Negombata magnifica*, *Hemimycle arabica*, *Crella spinulata*, and *Stylissa carteri* (B–I). (J) Migrated cells percent in response to different treatments relative to untreated control cells (%). (K) MMP2 activity relative to control (%). Statistical analysis was conducted using one-way analysis of variance (ANOVA). Values are presented as means ± standard error (SE) ($n = 3$). Asterisks above bars denote significance in change between different treatments and the control $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

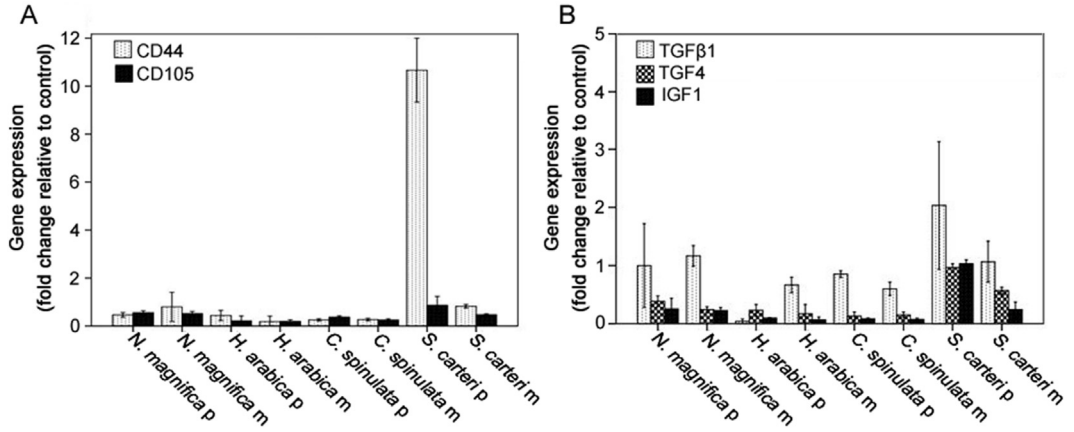

Fig. 6. Real-time PCR analysis and gene expression of HepG2 cells treated with primmorph ethyl acetate extract (p) and mesohyls (m) of the selected sponges. (A) Surface marker (*CD44* and *CD105*). (B) Antiproliferative and antimigratory growth factor related genes *TGFβ1*, *FGF4*, and *IGF*. Data are represented as percent of untreated control.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2019.03.008.

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