

***Bursatella leachii* purple ink secretion extract exerts cytotoxic properties against human hepatocarcinoma cell line (HepG2): In vitro and in silico studies**

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Abstract

Liver cancer is the third leading cause of cancer death worldwide. Marine mollusc-derived extracts have gained attention as new potential natural-based anticancer agents to overcome the side effects caused by conventional chemotherapeutic drugs during cancer therapy. We evaluated the cytotoxic effects of a crude extract from the purple-ink released by the sea hare named *Bursatella leachii* (*B. leachii*) against human hepatocarcinoma cell line (HepG2) and explored the underlying mechanisms causing the programmed cell death (*i.e.*, apoptosis). Expression of cleaved-caspase-8 and cleaved-caspase-3, key cysteine-aspartic proteases involved in the initiation and completion of the apoptosis process, appeared after HepG2 cell exposure to *B. leachii* extract. Gene expression levels of pro-apoptotic *BAX*, tumour suppressor *TP53* and *Cyclin D1* were increased after treatment with *B. leachii*. Using liquid chromatography-mass spectrometry, the main biomolecules in the *B. leachii* extract were identified as hectochlorin, malyngamide X, malyngamide S, bursatellin, and lymbyatoxin A. Applying *in silico* approaches, the high scores predicted bioactivities for the five compounds were protease and kinase inhibitors. The ADME and cytochrome profiles for the compounds were also predicted. Altogether, the cytotoxic *B. leachii* extract presents high pro-apoptotic potentials, suggesting it as a promising safe natural product-based drug for the treatment of liver cancer.

1. Introduction

Liver cancer is the sixth most commonly diagnosed cancer and the third leading cause of cancer death worldwide in 2020 [1]. Liver cancer has a variable geographical distribution, which predominantly overlays with the geographic incidence of viral hepatitis (*i.e.*, hepatitis B virus and hepatitis C virus) and of human immunodeficiency virus [2]. This viral infection to the patients leads to liver cancer onset and progression from chronic hepatitis, liver cirrhosis to heterogeneous hepatocellular carcinoma (HCC) [3]. Liver cancer onset can also be due to aging, exposure to toxic compounds, autoimmunity, and metabolic diseases [4]. Conventional treatments such as surgery, radiotherapy and chemotherapy including gene and immune-based therapeutic drugs are currently used [5]. Cytotoxic chemotherapy is not the first-line treatment (*i.e.*, protein kinase inhibitor, Sorafenib) for HCC, the main type of primary liver cancer, which is considered as a chemotherapy-refractory tumor [6]. However, there is an intensive discovery of natural bioactive compounds as neo-adjuvant agents inhibiting liver cancer cell growth, which would enhance liver cancer prevention, overcome hepatotoxicity side effects from conventional therapy and liver cancer recurrence [7].

So far, an alternative treatment for liver cancer prior to transplantation is highly on demand. Natural product extracts have been widely studied for their bioactive compounds endowed with anti-proliferative activity and pro-apoptotic effects, revealed by tyrosine kinase inhibition [8] and by caspase activation, cell cycle- and apoptosis-related gene up-regulation [9]. The main advantage of the induction of apoptosis is the absence of inflammation reaction, well known to be triggered by necrotic cells [10]. Hence, some natural bioactive compounds have been discovered for their anti-inflammatory and anti-angiogenic effects, which prevent cancer progression [11].

Molluscs are the second largest animal phylum on earth and provide a rich source of medicinal natural bioactive molecules [12]. Opisthobranch molluscs are a subclass of *Gastropoda* family *Aplysiidae*, order *Anaspide* genus and species *Bursatella (B.) leachii* commonly known as sea hares [13]. Sea hare-derived bioactive compounds, including soblidotin (dolastatin-10 derivative), synthadotin/ ILX₆₅₁, cemadotin and kahalalide F, have been discovered for their anticancer activity and are currently in clinical trials [14]. The sea hares release a purple ink like that of squid to fend off predators [15]. We previously reported *B. leachii* purple ink-derived anti-HIV protein [16], 7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione, and dioxigenin acetate as potent anti-inflammatory compounds [17]. However, so far, *B. leachii* purple ink-derived extracts, including the presence of these recently identified anti-inflammatory compounds, have not been studied for their potential anticancer activity. Therefore, we evaluated the potential cytotoxic effects of a crude extract of species *B. leachii* against the growth of the human hepatocellular carcinoma (HCC) cell line HepG2. Protein and gene expression levels of apoptosis and cell cycle regulatory markers in the *B. leachii* extract-treated HepG2 cells were assessed. The chemical analysis of *B. leachii* extract and several biological target predictions were performed as well.

2. Results

2.1. Cytotoxic effect of the *B. leachii* extract

The effect of *B. leachii* extract on HepG2 cell proliferation was tested at different concentrations and time periods. The increasing concentrations (from 10 to 1,000.0 µg/mL) of the *B. leachii* extract and the elongation of exposure time periods (from 24 to 72 h) resulted in a decrease of the viability of HepG2 cells based on ATP generated by living cells, compared to the control cells, which described a dose- and time-dependent effect (Figure 1). The IC₅₀ values of *B. leachii* extracts required to inhibit 50% of HepG2 cell growth were determined at each exposure time. The treatment of HepG2 cells with *B. leachii* extract for 72 h of exposure displayed the lowest IC₅₀ value of 242.9 µg/mL while the highest IC₅₀ value was recorded after 24-h exposure with 465.4 µg/mL followed by 48-h exposure with an IC₅₀ value of 447.5 µg/mL of *B. leachii* extract.

2.2. Induction of apoptosis by *B. leachii* extract

As one of the cell death mechanisms, the potential induction of apoptosis in HepG2 cells treated with 100 µg/mL and 400 µg/mL of the *B. leachii* extract was investigated using Western blot analysis. After 24 h of exposure, the expression levels of pro-apoptotic proteins, such as cleaved caspase-8 (key enzyme prompting extrinsic apoptotic pathway) and cleaved-caspase-3 (key enzyme resulting in the completion of apoptosis), were evaluated in *B. leachii*-treated cells in comparison with untreated cells. Used as a positive control, staurosporine (STS) led to a cleavage of both caspase-8 and caspase-3 in HepG2 cells while, as expected, no cleaved-caspase-8 and cleaved-caspase-3 expression was detected in untreated cells (Figure 2). A cleavage of both caspase-8 and caspase-3 was observed in *B. leachii* extract-treated HepG2 cells (Figure 2). Higher expression levels of cleaved-caspase-8 was observed in HepG2 cells treated with 400 µg/mL of *B. leachii* extract as compared with that detected in cells treated with 100

$\mu\text{g}/\text{mL}$ of *B. leachii* extract (Figure 2). A similar expression level of cleaved-caspase-3 was noticed in HepG2 cells exposed to *B. leachii* extract concentrations (Figure 2).

2.3. Modulation of gene expression levels of apoptotic and cell cycle regulatory genes by *B. leachii* extract

The pro-apoptotic effect of *B. leachii* extract on HepG2 cells was evaluated by monitoring the expression levels of apoptosis and cell cycle-related genes, including *BCL-2*, *BCL-xL*, *TP53*, *BAX*, *CDKN1A*, *Cyclin A*, *Cyclin D1* and *Survivin* using RT-qPCR. The treatment of HepG2 cells with 400 $\mu\text{g}/\text{mL}$ of *B. leachii* extract significantly enhanced the expression level of pro-apoptotic genes *BAX* (2.6-fold, $p = 0.000012$), *TP53* (2.3-fold, $p = 0.00023$), and *Cyclin D1* (2.1-fold, $p = 0.0012$), compared with the basal gene expression level detected in untreated cells (Figure 3). A significant up-regulation of the gene expression level of anti-apoptotic *BCL-xL* (2.0-fold, $p = 0.0043$) was noticed in *B. leachii* extract-treated HepG2 cells (Figure 3). Of note, up-regulation of pro-apoptotic *Cyclin A* (1.77-fold) and anti-apoptotic *Survivin* (1.64-fold) gene expression levels remained non-significant (Figure 3).

2.4. Chemical identification of *B. leachii* extract using LC-QTOF

The crude extract of the *B. leachii* was subjected to total ion current spectra (TIC) raw data (See Figure 4). The data-analysis program Mass Hunter (Agilent Technologies) qualitative and quantitative analysis software were also used. After conducting a mass screening on the below spectrum (Figure 4), the chemical features were extracted from the liquid chromatography-mass spectrometry (LC-MS) data using the Molecular Features Extraction (MFE) algorithm and the recursive analysis workflow. Features were extracted by screening the detected nodes at various retention time per minutes, with a minimum intensity of 6,000 counts and aligned with previously detected compounds considering adducts ($[\text{M}+\text{K}]^+$, and $[\text{M}-\text{H}]^-$). The tentatively identified compounds were hectochlorin (Figure 4, section A), malyngamide X (Figure 4, section B), bursatellin (Figure 4, section C), malyngamide S (Figure 4, section D), and lymbatoxin A (Figure 4, section E).

2.5. *B. leachii* extract bioactivity predictions

In addition to exploring the pro-apoptotic effects of *B. leachii* extract, we sought to determine the bioactivity score of each bioactive metabolite identified in *B. leachii* extract. These bioactivity predictions gave more information about which molecule in the extract could contribute to the observed anticancer activity. Thus, the bioactivity score of the five bioactive molecules was investigated using the PASS online webserver. Our results showed that hectochlorin and malyngamide S exhibited the highest bioactivity score (Table 1) with Pa 0,933 and Pa 0,747 respectively, suggesting a promising anti-neoplastic activity for these two molecules. Additionally, the anticancer activity of *B. leachii* extract could contribute to the presence of hectochlorin and malyngamide S metabolites. The remaining molecules exhibited a lower bioactivity score, and no predicted score was identified for bursatellin.

Table 1
The bioactivity scores of identified metabolites from *B. leachii* extract using PASS online webserver.

Antineoplastic Activity	Probability of being Active (Pa)	Probability of being Inactive (Pi)
Hectochlorin	0,933	0,002
Malyngamide X	0,295	0,231
Malyngamide S	0,747	0,019
Bursatellin	not applicable	not applicable
Lyngbyatoxin A	0,169	0,075

2.6. Molecular target predictions of *B. leachii* extract

To investigate the possible molecular targets that could mediate the observed and predicted anticancer activity, Molinspiration was utilized for this evaluation. Each bioactive molecule was evaluated as a G protein-coupled receptors (GPCR) ligand, ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease, and enzyme inhibitors. Interestingly, most of the five molecules exhibited a positive bioactivity score as protease inhibitors with malyngamide X, malyngamide S, and lyngbyatoxin A possessing the highest scores (0.46, 0.32, and 0.36 respectively). Moreover, malyngamide, malyngamide S, and lyngbyatoxin A demonstrated high bioactivity scores as Enzyme inhibitors with values of 0.32, 0.32, and 0.35, respectively. Other possible targets such as ion channels, GPCR, and kinase inhibition were seen with malyngamide X, malyngamide S, and lyngbyatoxin A, suggesting that these bioactive molecules could regulate several molecular targets. Additional target mapping was conducted using SWISS target prediction and the results were comparable with Molinspiration webserver with a high probability of targeting proteases and kinases, as summarized and shown in Table 2.

2.7. Pharmacokinetics ADME predictions and CYP P450 enzymes inhibition profiling

To assess the potential pharmaceutical properties of these *B. leachii* extract-derived bioactive molecules, SWISS ADME was utilized to evaluate several parameters important for drug discovery. Among the five bioactive metabolites, malyngamide S, bursatellin, and lyngbyatoxin A demonstrated a molecular weight of fewer than 500 Daltons. Moreover, all the compounds that demonstrated high lipophilicity except for bursatellin exhibited a low Log P value. The solubility of the compounds was poor with exception of bursatellin that demonstrated excellent solubility (Log S -2.39). Most of the compounds were predicted to

have a peripheral effect (no blood-brain barrier (BBB) penetration except for lyngbyatoxin A) and have high gastrointestinal (GI) absorption as summarized in Table 3.

Table 3
The predicted ADME properties for the bioactive molecules identified in *B. leachii* extract.

Compound Name	Molecular Weight	Log Po/w (WLOGP)	Log S (SILICOS-IT)	BBB Permeant	GI Absorption	Rule of Five (ROF)
Hectochlorin	665.60 g/mol	5.09	-6.86 Poorly soluble	No	Low	No; 2 violations: MW>500, NorO>10
Malyngamide X	607.82 g/mol	3.98	-5.07 Moderately soluble	No	High	Yes; 1 violation: MW>500
Malyngamide S	484.07 g/mol	4.73	-6.25 Poorly soluble	No	High	Yes; 0 violation
Bursatellin	264.28 g/mol	-0.21	-2.39 Soluble	No	High	Yes; 0 violation
Lyngbyatoxin A	437.62 g/mol	4.09	-6.69 Poorly soluble	Yes	High	Yes; 0 violation

Additionally, we sought to determine and qualitatively predict the possibility of cytochrome P450 (CYP) enzymes inhibition that could be associated with these bioactive molecules, thus SWISS webserver was utilized, and five compounds were subjected for this prediction. Our results showed that hectochlorine and bursatellin did not exhibit any CYP enzymes inhibition for all the five enzymes. malyngamide X demonstrated only two inhibitions for CYP2C19 and CYP3A4. Moreover, malyngamide S was predicted to inhibit CYP2C19, CYP2D6, and CYP3A4 enzymes and lyngbyatoxin A inhibited three CYP enzymes including CYP2C19, CYP2C9, and CYP3A4 as shown in Table 4.

Table 4

The CYP enzymes inhibition profile for the bioactive molecules identified in *B. leachii* extract.

Compound Name	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
Hectochlorin	No	No	No	No	No
Malyngamide X	No	Yes	No	No	Yes
Malyngamide S	No	Yes	No	Yes	Yes
Bursatellin	No	No	No	No	No
Lyngbyatoxin A	No	Yes	Yes	No	Yes

3. Discussion

Intensive exploration of the marine ecosystem has provided an immense source of diverse bioactive compounds. Recently, a purple ink extract released by the sea hare *Bursatella (B.) leachii* has been studied and found to be endowed with anti-HIV and anti-inflammatory activities [16, 17], two prominent properties for a potential liver cancer treatment. To widen the biological activities of this *B. leachii* purple ink-derived extract as a promising natural neo-adjuvant for the treatment of liver cancer, we have investigated its potential cytotoxic effects against HCC HepG2 cells and established molecular target and pharmacokinetic predictions of identified metabolites-derived *B. leachii* purple ink-derived extract.

In this present study, human HCC cell line HepG2 exposed to the *B. leachii* purple ink-derived extract led to an inhibition of the cell proliferation in a dose- and time-dependent manner. An induction of apoptosis in *B. leachii* extract-treated HepG2 cells was observed at intermediate concentrations (100 and 400 µg/mL) of *B. leachii* extract after 24-h exposure. Among the studied time periods, *B. leachii* extract added to the HepG2 cells for 72-h treatment resulted in the lowest IC₅₀ value of 242.9 µg/mL compared with IC₅₀ values determined after 24- and 48-h exposure. A previous study conducted by Suntornchashwej and colleagues [18], reported the cytotoxicity exhibited by the ethyl acetate-derived extract of *B. leachii* against the human small cell lung cancer (NCI-H187), oral human epidermoid carcinoma (KB), and breast cancer (BC) cell lines with an effective dose (ED₅₀) value of 16.2, 7.2, and 6.6 µg/mL, respectively. A protective effect of *B. leachii* extracts against neuroblastoma cell line SH-SY5Y pre-treated with hydrogen peroxide and against microglia cells stimulated by bacterial lipopolysaccharide was studied [19]. *B. leachii* extracts were stated to be effective only against microglia cells by decreasing intracellular nitric oxide production and exhibiting an IC₅₀ value of 5.74 µg/mL while the SH-SY5Y cells have no cell response to the studied concentration of BL extracts [20]. Thus, the determination of different IC₅₀ values reflecting

the anti-proliferative activity of *B. leachii* extracts indicates the specificity of the extract to exert cytotoxicity against various cancer cell lines.

Similarly, the anticancer potential of *B. leachii* extract at 100 and 400 µg/mL through apoptosis induction in HepG2 cells treated for 24 h was confirmed using Western blot technology by qualitatively detecting the expression of the most important pro-apoptotic proteins: the initiator caspase-8 and the effector/executioner caspase 3. Apoptosis is initiated and achieved by the cleavage of initiator and executioner pro-caspases into cleaved-caspases, the activated form of the enzymes. In this present study, the cleavage of caspase-8 and caspase-3 was observed in HepG2 cells after 24 h of cell exposure to *B. leachii* extract. Equal level of cleaved-caspase-3 was observed at both *B. leachii* extract concentrations (100 and 400 µg/mL), confirming the slight decrease of HepG2 cell growth noticed at similar conditions. Tested at the same intermediate concentrations, a concomitant cleavage of pro-caspase-3 induced by *B. leachii* extract would be expected after 72-h exposure, due to the important decrease of the *B. leachii* extract-treated HepG2 cell growth. However, the degree of pro-caspase-8 cleavage increased with *B. leachii* extract concentrations, confirming the role of caspase-8 as initiator of apoptosis. In addition, the expression of cleaved-caspase-8 and cleaved-caspase-3 detected in *B. leachii* extract-treated HepG2 cells may reveal that the induction of apoptosis occurs via the activation of the extrinsic death receptor pathway by transmitting the death signal from the cell surface to the intracellular signaling pathways through tumor necrosis factor (TNF) receptor gene family, for instance [21]. Active caspase-8 either initiates apoptosis directly by cleaving pro-caspase-3 into activated cleaved-caspase-3 or through mitochondria by the cleavage of BID to induce cell death [22]. Thus, an investigation of the involvement of the mitochondrial-dependent intrinsic apoptosis pathway in *B. leachii* extract-treated HepG2 cells would be of interest.

Consecutively, the quantitative expression of cell cycle and apoptotic regulatory genes in HepG2 cells treated with 400 µg/mL of *B. leachii* after 48-h exposure was analyzed using RT-qPCR. The expression of pro-apoptotic *BAX*, *TP53*, and *Cyclin D1* genes were significantly up-regulated while the anti-apoptotic *BCL-xL* gene was up-regulated at a lesser extent. The gene expression of *BCL-2*, the main regulator of apoptosis that is endowed with pro- and anti-apoptotic activities, was not modulated even after HepG2 cell exposure to *B. leachii* extract. The tumor suppressor p53 is a transcriptional protein activated by a variety of oncogenic/hyperproliferative stimuli including DNA damage or chemotherapeutic drugs [23]. The protein p53 located in the cytosol induces the activation of pro-apoptotic Bax by protein-protein interactions and with Bcl-xL and Bcl-2 by p300/CBP binding [24]. Therefore, the elevated expression levels of TP53 and BAX in *B. leachii* extract-treated HepG2 cells endorses the cell death might be due to p53-dependent apoptosis.

The chemical analysis in this study was performed using high-resolution Q-TOF analysis, which enabled to tentatively identify the chemicals with more accuracy and compare them with previous structure identification studies related to the *B. leachii* ink extract biomolecules. For example, the appeared m/z value at retention time (0.164 - 0.661) were correlated with the parent compound hectochlorin, [18] with m/z $[M+K]^+$ 703.5708 daltons and molecular formula of $[C_{27}H_{34}Cl_2N_2O_9S_2]^+$, in positive ion mode $[M+H]^+$

m/z 666.236, and $[M-H]^-$ with m/z 664.163 daltons in negative mode, indicating that the compound has a molecular weight of 665.603 g mol⁻¹. The appeared m/z value at retention time (0.164 - 0.661) were correlated with the parent compound malyngamide X, [19] with m/z $[M+K]^+$ 646.5302 daltons and molecular formula of $[C_{33}H_{57}N_3O_7]^+$, in positive ion mode $[M+H]^+$ m/z 607.420, and $[M-H]^-$ with m/z 606.822 daltons in negative mode, indicating that the compound has a molecular weight of 607.420 g mol⁻¹. The appeared m/z value at retention time (3.398 - 4.559) were correlated with the parent compound bursatellin [25] with m/z $[M+2H]^+$ 266.9169 daltons and molecular formula of $[C_{13}H_{16}N_2O_4]^+$, in positive ion mode $[M+H]^+$ m/z 264.111, and $[M-H]^-$ with m/z 263.210 daltons in negative mode, indicating that the compound has a molecular weight of 264.277 g mol⁻¹. The appeared m/z value at retention time (1.176 - 2.071) were correlated with the parent compound malyngamide S [26] with m/z $[M+K]^+$ 522.4386 daltons and molecular formula of $[C_{26}H_{42}ClNO_5]^+$, in positive ion mode $[M+H]^+$ m/z 483.279, and $[M-H]^-$ with m/z 482.109 daltons in negative mode, indicating that the compound has a molecular weight of 484.069 g mol⁻¹. The appeared m/z value at retention time (9.634 - 13.382) were correlated with the parent compound lyngbyatoxin A [27] with m/z $[M-CH_3]^+$ 426.4968 daltons and molecular formula of $[C_{27}H_{39}N_3O_2]^+$, in positive ion mode 437.304, and $[M-H]^-$ with m/z 437.304 daltons in negative mode, indicating that the compound has a molecular weight of 437.617 g mol⁻¹.

The analysis of the bioactivity predictions revealed that among the five identified compounds only hectochlorin and malyngamide S demonstrated the highest scores as anti-neoplastic agents. This is of great importance for lead optimization and further lead development. Additionally, these predictions support the observed anticancer activity of the *B. leachii* extract that could contribute to the presence of hectochlorin and malyngamide S. Additionally, the molecular target predictions established from *B. leachii* extract suggested the involvement of proteases and kinase inhibitors as potential targets that could explain the observed up-regulation of several apoptotic markers. Several studies have reported the modulation of proteases and kinases by marine bioactive molecules, which induced apoptosis in cancer cells [27–30].

The in silico ADME predictions for the identified molecules are useful for the potential use of these compounds as a lead for the discovery of a novel anticancer therapy. The ADME properties data showed that most of the bioactive molecules have acceptable pharmaceutical properties and follow Lipinski's rule of five of drugability related to absorption/permeation, molecular weight, and solubility [31] except for hectochlorine that demonstrated two violations for this rule. Moreover, the CYP enzymes inhibition profile suggests that some of the compounds could inhibit CYP2C19 and CYP3A4 activities. However, this inhibition profile could be overcome for future optimization of the lead compound.

4. Materials And Methods

4.1. Collection of *B. leachii* Extract

The adult *B. leachii* sea hares were collected from intertidal waters of the Pulicat lake in the position Lat. 13.452523° N Long. 80.319133°E+/- 0.03°N/E and brought to the laboratory in live condition. The accession number was M-1697, obtained from Zoological Survey of India, Marine Biology Regional Centre (MBRC), Chennai, India and dated on 23 July 2015. Obtained by disturbing the *B. leachii*, the purple fluid ink (extract) was filtered through Whatman® filter paper. All aqueous ink-derived samples were centrifuged at 15,000×*g* for 15 min as described by Vennila and colleagues [33] and the supernatant was kept and lyophilized to extract a purple residue using a freeze dryer then stored at 4°C for further use.

4.2. Chemicals and Reagents

Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine were obtained from Gibco™ (Waltham, MA, USA). Staurosporine (STS) (>99%) was obtained from Santa Cruz biotechnology (Dallas, TX, USA). High-purity methanol (99.9%) was procured from Honeywell (Charlotte, NC, USA). Formic acid (>95.0%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was produced using a Millipore (Billerica, MA, USA) system with a resistivity reading of 18.2 MΩ·cm at 25°C.

4.3. Cell Line and Culture Medium

Human HCC cell line HepG2 (#HB-8065, American Type Culture Collections, Manassas, VA, USA) was cultured in DMEM, supplemented with 10% FBS, 100 IU/mL of penicillin and 100 µg/mL of streptomycin, and 2 mM of L-glutamine. The cells were maintained at 37°C in a 5% humidified CO₂ incubator.

4.4. Cell Proliferation Assay

HepG2 cells (5,000/0.1 mL) were seeded in white flat-bottom 96-well plates (Costar®), Thermo Fisher Scientific). After 24 h of incubation, the cells were independently treated 3 times in triplicates in the presence of various concentrations of *B. leachii* extract (10, 100, 200, 400, 500, and 1,000 µg/mL). The wells containing the culture media and the cells without treatment served as blank and control, respectively. The cell proliferation was measured after 24, 48 and 72 h using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation Inc., Fitchburg, WI, USA) and determined half-maximal inhibitory concentration (IC₅₀) value as described in [34].

4.5. Western Blot Analysis

HepG2 cells (5×10⁵/mL) were seeded in Nunc™ 12-well plates (Thermo Fisher Scientific, Inc.). After 24 h of incubation, the cells were treated in triplicates with *B. leachii* extract at 100 and 400 µg/mL along with 1 µM STS, used as a positive control. Western blot technology and image analysis were employed as previously described in [35]. Polyvinylidene difluoride membranes (Millipore, Thermo Fisher Scientific) were probed with (1:1,000 dilution) mouse anti-pro/cleaved-caspase-3, rabbit anti-pro-caspase-8, mouse anti-cleaved-caspase-8 antibodies (Cell Signaling Technology, Danvers, MA, USA) and mouse anti-GAPDH antibody (Abcam, Cambridge, UK).

4.6. Gene Expression Analysis

HepG2 cells (1.5×10^6) were seeded in Nunc™ 6-well plates. After 24 h of incubation, the cells were treated in triplicate with or without 400 µg/mL of *B. leachii* extract and incubated for a further 48-h incubation. From total RNA extraction to reverse-transcribed cDNA, RT-qPCR was carried out as previously described in [36]. Relative quantification of the mRNA expression level for the target genes are listed in the Table 1.

4.7. Chemical Analysis

The fingerprinting of *B. leachii* secretion aqueous extract was performed using the Agilent (Santa Clara, CA, USA) 1260 Infinity high performance liquid chromatography (HPLC) system coupled to Agilent 6530 Quadrupole Time of Flight (Q-TOF). The analysis was performed using Agilent SB-C18 column (4.6 mm × 150 mm, 1.8 µm) with the following elution gradient; 0–2 min, 5% B; 2–17 min, 5–100% B; 17–21 min, 95% B; 21–25 min, 5% B, using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in methanol). The injection volume was 10 µL and flow rate was set at 250 µL/min. The scanning range was set at 50-800 (m/z) and the remaining parameters were set as follows: gas temperature at 300 °C, gas flow of 8 L/min, nebulizer pressure of 35 psi, sheath gas temperature at 350 °C, and sheath gas flow rate of 11 L/min. The data was generated by Agilent MassHunter qualitative analysis software (version B.06.00).

4.8. Activity Prediction using PASS online Webserver

The antineoplastic activity of the bioactive metabolites identified from *B. leachii* extract was assessed using Pass online webserver [37]. For each compound, the SMILES (Simplified Molecular Input Line Entry System) was generated and entered in the webserver to perform the assessment. The generated results are classified based on the compound probability of being active (Pa) and inactive (Pi) at the specified activity.

4.9. Target Predictions using Molinspiration and SWISS Target Prediction Tools

To investigate the possible molecular targets for these metabolites identified from *B. leachii* extract, Molinspiration [38] and SWISS target prediction were utilized [39]. For both webserver, SMILES were used to generate data. The Molinspiration web server gives a score that reflects the bioactivity of the specified compound. The positive values represented the highest probability that the compound was active at the molecular target. For the SWISS target predictions, a general mapping of the possible molecular targets was provided for any given compound that greatly facilitate the identification of the biological targets of uncharacterized molecules [38–39].

4.10. Pharmacokinetic ADME Predictions and Cytochrome P450 Profiling using SWISS Tool

The pharmacokinetics concerning the absorption, distribution, metabolism, excretion (ADME) of the identified bioactive metabolites from *B. leachii* extract were explored using the SWISS ADME web server

that provided a detailed fast in silico predictions of the pharmaceutical profiles of bioactive compounds [40]. The selected ADME parameters for the analysis were molecular weight, lipid solubility (Log P), water solubility (Log S), blood-brain barrier (BBB) penetration, and gastrointestinal (GI) absorption. After data generation, results were compared to the established drug-likeness properties (rule-of-five, ROF) considered important for drug discovery [41].

Additional investigations were conducted to assess the cytochrome P450 (CYP) inhibition profile of the identified bioactive molecules using the SWISS web server. Each compound was evaluated against the following CYP enzymes including CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4. The CYP enzymes inhibition profile was important for the early identification of possible significant drug interactions.

4.11. Statistical analysis

All the data are expressed as mean \pm SD of three independent experiments. The IC₅₀ values were calculated by nonlinear dose/response regression model using GraphPad Prism software version 6 for Windows (CA, USA, <http://www.graphpad.com/>). The relative changes in the expression of the gene were analyzed by $2^{-\Delta\Delta Ct}$ method [36]. Student paired *t*-test was used to calculate *p* value, and the significance was considered if *p* < 0.05.

5. Conclusion

Brusatella leachii ink extract exerts anti-proliferative and pro-apoptotic activities in human liver cancer HepG2 cell line, suggesting *B. leachii* extract as a promising safe natural-based neo-adjuvant drug for liver cancer treatment. Our computational predictions for the *B. leachii* extract-derived identified bioactive molecules suggest that these compounds have promising anticancer properties with acceptable drug-likeness profile and minimal CYP enzymes inhibition that warrants further lead optimization and development to discover novel drug entities from marine-derived natural resources. Further chemical isolation and *in vivo* studies are still needed.

Declarations

Ethical approval statement

This study was approved by the institutional review board of King Abdullah International Medical Research Center and conducted according to the Helsinki Declaration of 1975.

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Author Contributions

Conceptualization, Zeyad Alehaideb, Anuradha Venkatraman, Rasha Suliman and Sahar Alghamdi; Data curation, Mahadev Kokane, Saranya Rameshbabu and Maha-Hamadien Abdulla; Formal analysis, Maha-Hamadien Abdulla; Funding acquisition, Zeyad Alehaideb; Investigation, Zeyad Alehaideb, Anuradha Venkatraman and Sabine Matou-Nasri; Methodology, Zeyad Alehaideb, Mahadev Kokane, Syed Mohamed, Saranya Rameshbabu, Rasha Suliman, Sahar Alghamdi, Hamad Al-Eidi, Bandar Alghanem and Maha-Hamadien Abdulla; Resources, Saranya Rameshbabu, Hamad Al-Eidi and Bandar Alghanem; Software, Rasha Suliman, Sahar Alghamdi and Bandar Alghanem; Supervision, Zeyad Alehaideb, Anuradha Venkatraman and Sabine Matou-Nasri; Validation, Anuradha Venkatraman, Mahadev Kokane, Syed Mohamed, Saranya Rameshbabu, Rasha Suliman, Sahar Alghamdi, Hamad Al-Eidi, Bandar Alghanem, Maha-Hamadien Abdulla and Sabine Matou-Nasri; Visualization, Mahadev Kokane, Syed Mohamed, Saranya Rameshbabu, Rasha Suliman, Sahar Alghamdi, Hamad Al-Eidi, Bandar Alghanem, Maha-Hamadien Abdulla and Sabine Matou-Nasri; Writing – original draft, Anuradha Venkatraman and Sabine Matou-Nasri; Writing – review & editing, Zeyad Alehaideb, Mahadev Kokane, Syed Mohamed, Saranya Rameshbabu, Rasha Suliman, Sahar Alghamdi, Hamad Al-Eidi, Bandar Alghanem, Maha-Hamadien Abdulla and Sabine Matou-Nasri.

Conflict of interest

The authors have declared that there are no conflicts of interest.

Abbreviations

ADME, absorption distribution metabolism excretion; ATP, adenosine triphosphate; cDNA, complementary deoxyribonucleic acid; CYP, cytochrome P450; DMEM; Dulbecco's Modified Eagles Medium; ED₅₀, half-maximal effective dose; HCC, hepatocellular carcinoma; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IC₅₀, half-maximal inhibitory concentration; HCC, hepatocellular carcinoma; HPLC, high-performance liquid chromatography, LC-MS, liquid chromatography-mass spectrometry; MFE, molecular features extraction; mRNA, messenger ribonucleic acid; QTOF, quadrupole time of flight; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; STS, staurosporine; TNF, tumor necrosis factor; TIC, total ion current spectra

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Table 2

Table 2 is available in the Supplementary Files section.

Figures

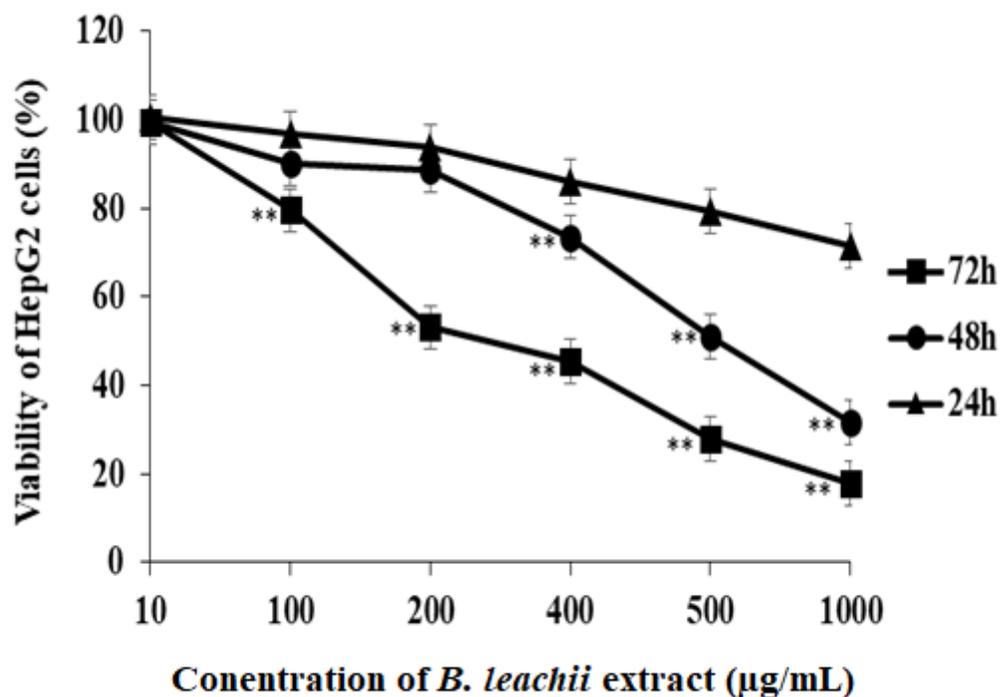


Figure 1

Effects of *B. leachii* extract on HepG2 cell viability. The cell viability was determined using CellTiter-glo® kit and expressed as percentage of the control, the untreated cell viability, corresponding to 100%.

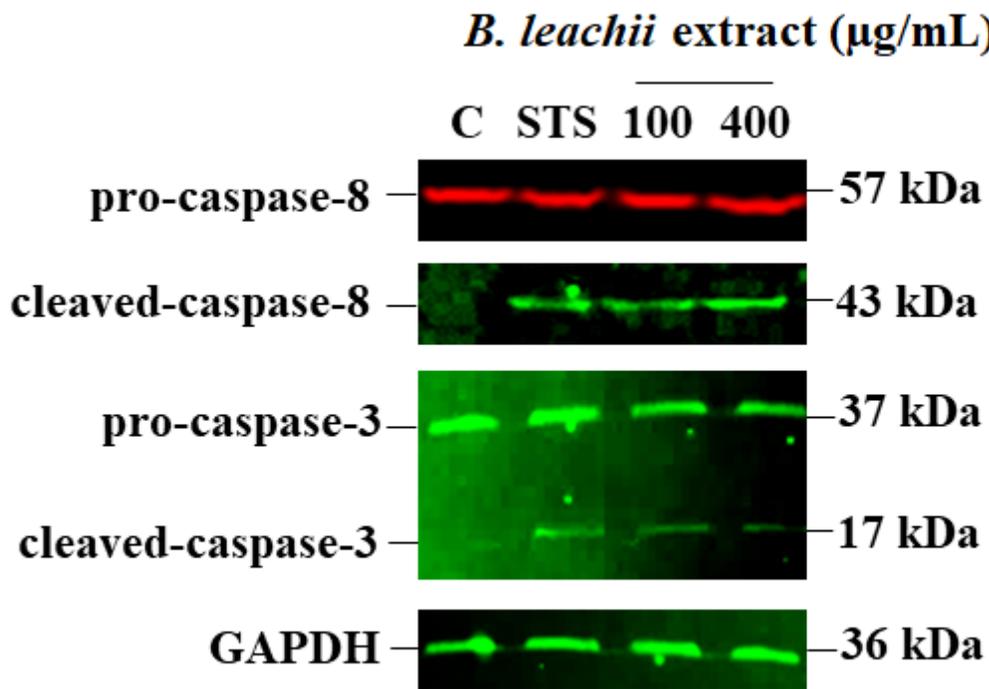


Figure 2

B. leachii extract triggers caspase-8 and caspase-3 cleavage in HepG2 cells. Representative Western blot gels showing the detection of cleaved-caspase-8 and of cleaved-caspase-3 in HepG2 cells after 24 h of treatment with 100 and 400 mg/ml of *B. leachii* extract along with 1 mM STS, used as a positive control. No cleavage of caspase was observed in untreated HepG2 cells, the control (C).

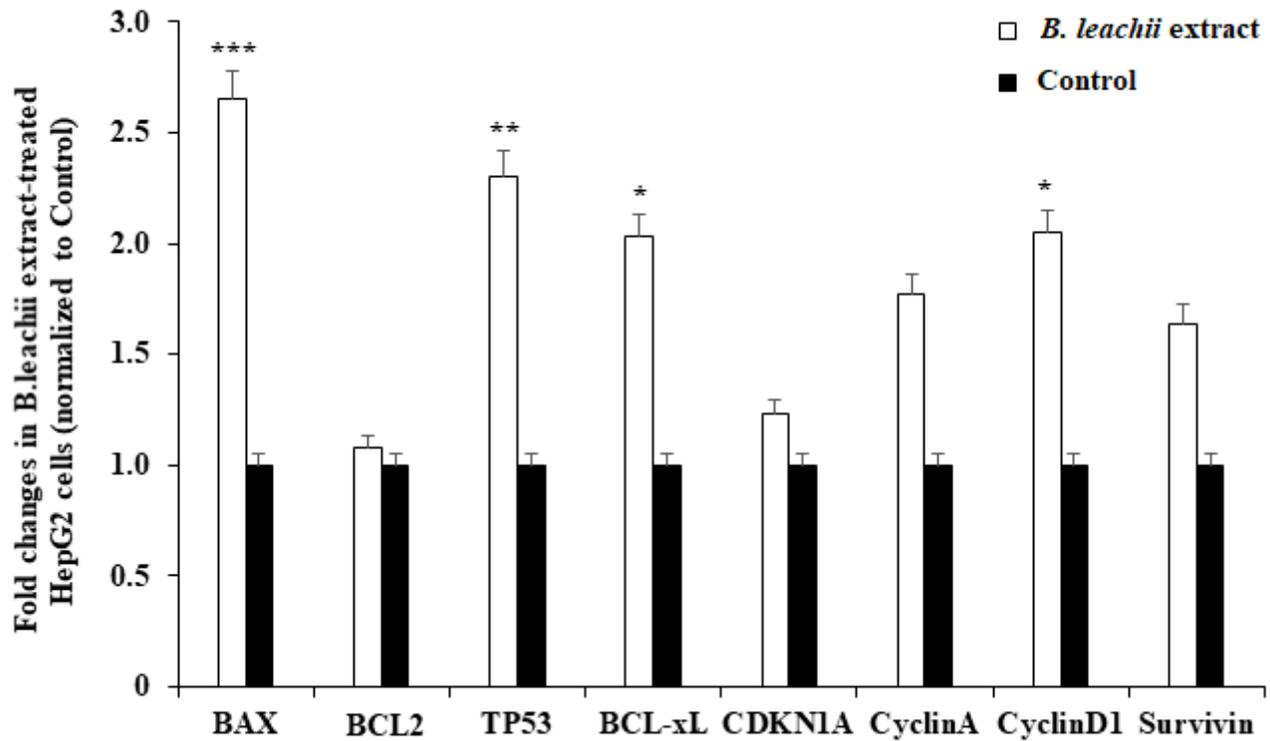


Figure 3

Effects of *B. leachii* extract on apoptosis and cell cycle regulatory gene expression detected in HepG2 cells. The bar graph shows the relative expression of apoptotic (Bax, Bcl2, Bcl-xL, Survivin), tumour suppressor TP53, and cell cycle (cyclin A, cyclin D1, cyclin-dependent kinase inhibitor CDKN1A) regulatory transcripts determined by RT-qPCR analysis in HepG2 cells treated with 400 mg/ml of *B. leachii* extract, as compared with the basal level of gene expression monitored in untreated HepG2 cells, the control. (*), (**), and (***) signify a statistically significant difference ($p < 0.01$, $p < 0.001$, and $p < 0.0001$) compared with the control.

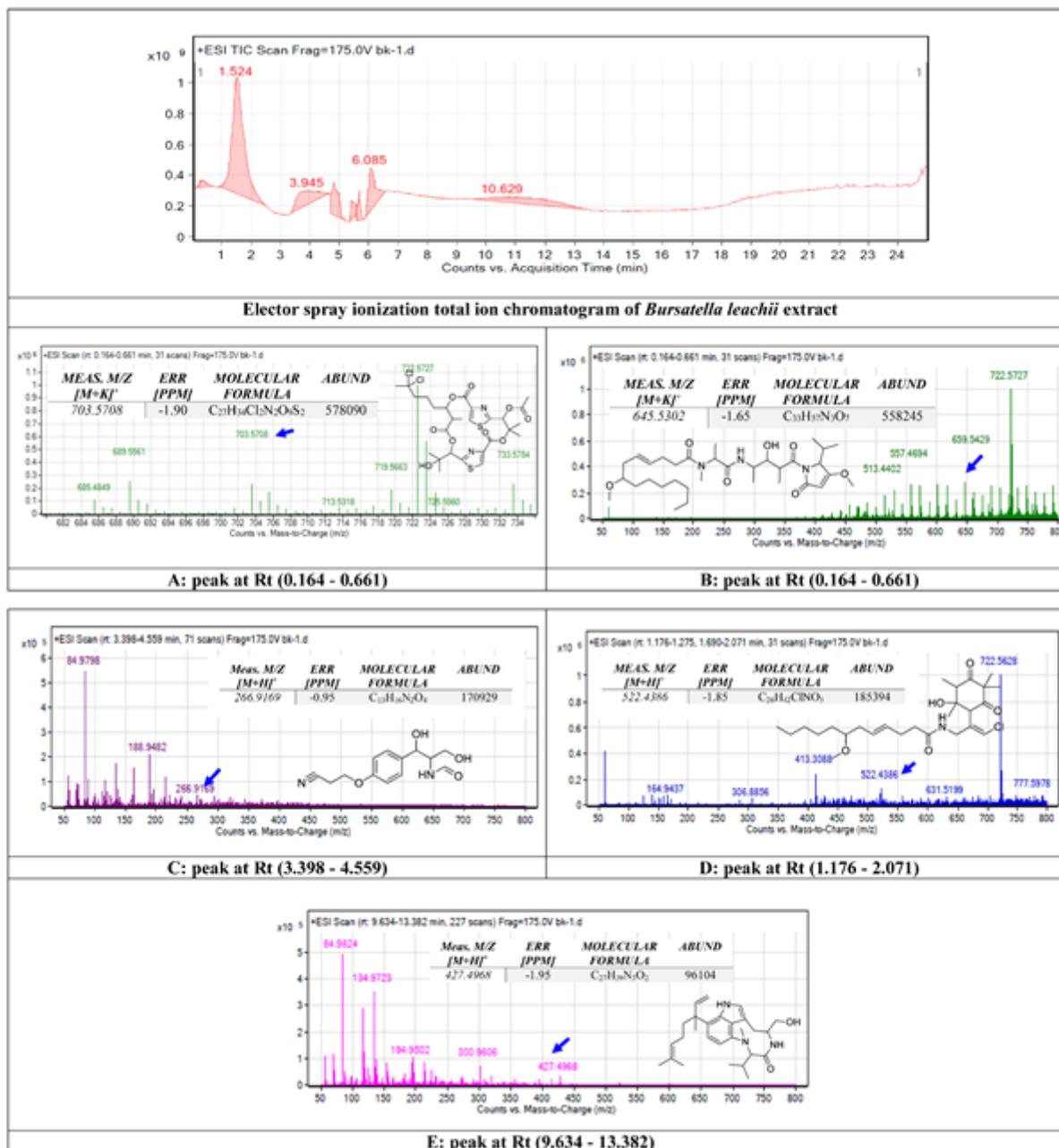


Figure 4

Chemical analysis of *B. leachii* purple ink secretion aqueous extract. Base peak chromatogram of *B. leachii* extract were extracted and tentatively identified secondary metabolites are hectochlorin (A), malyngamide X, (B) malyngamide S (C), bursatellin (D) and lyngbyatoxin A. Means m/z implies measured m/z.

Supplementary Files

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- [Table2.docx](#)