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Original Article

GC-MS Profiling of *Caulerpa lentillifera* Hot Water Extract and it's *in vitro* Effects on Hepatocellular Carcinoma Cell Progression

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ABSTRACT

Background: Hepatocellular Carcinoma (HCC) is a highly resilient cancer associated with poor survival outcomes. Current treatment with sorafenib inhibits Raf kinases but still encounter resistance mediated by alternative survival pathways. *Caulerpa lentillifera* (*C. lentillifera*) has demonstrated anti-cancer properties. Therefore, this study aims to assess its anticancer profile in hepatocellular carcinoma HepG2 cells and characterize the metabolomic profile of C. *lentillifera* hot water extract. **Materials and Methods:** The experimental methodology was hot water extraction of C. *lentifillera* and anticancer profiling by cell proliferation, cell migration, and colony formation assays in HEPG2 cells and normal fibroblast L929 cells. Followed by identification of metabolites in hot water extract using Gas Chromatography-Mass Spectrometry (GC-MS). **Results:** Comparison of two hot water extraction temperatures indicated that higher yield was obtained at 50ºC than at 100ºC. Cell proliferation assay showed a dose-dependent inhibitory activity with stronger inhibition observed in the extract derived at 100ºC than that at 50ºC. The half-maximal inhibitory concentration at 72 hr was 5 mg/mL for the 100ºC extract and was 8 mg/mL for the 50ºC extract. Subsequent analysis utilized the 100ºC extract to determine changes in cell migration. HepG2 cell migration was reduced after 72 hr of treatment compared with L929 cells. Similar findings were observed in colony formation assay, where the number of colonies was reduced in HepG2 cells relative to L929 cells. GC-MS analysis demonstrated presence of putative constituents cyclohexasilone, cycloheptasiloxane, pentasiloxane and benzoic acid. **Conclusion:** Taken together, these effects could be attributed to the presence of putative constituents cyclohexasilone, cycloheptasiloxane, and benzoic acid detected in the 100ºC extract. Further validation studies are needed to corroborate these findings for HCC treatment.

Keywords: *Caulerpa lentillifera*, Colony, Cytotoxicity, GC-MS, Hepatocellular Carcinoma, Metabolites, Migration.

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INTRODUCTION

Liver cancer is among the top ten cancers and ranked as the third leading cause of cancer-related mortality. Hepatocellular Carcinoma (HCC) represents as major form of liver cancer (Hwang *et al*., 2024). According to GLOBOCAN 2022, there were approximately 866,136 new liver cancer cases and 758,725 related deaths (Li *et al*., 2024). Over 70% of all global HCC cases occur in Asia (predominantly East and Southeast Asia), followed by Africa at roughly 7.8% (Hwang *et al*., 2024). In Malaysia, liver cancer ranks as the eighth most common cancer. Since 1990, the mortality from liver cancer has increased by 31.5% and HCC is the most common type (Raihan *et al*., 2018). Approximately 70% of HCC patients experience recurrence within 5 years (Wen *et al*., 2018). Current therapies include liver transplantation, local ablation, surgical resection or systemic therapy. Surgical resection is classified as curative-intent treatments for Hepatocellular Carcinoma (HCC) but still encounter recurrence within 2 years after surgery, while ablation is restricted to tumor size (Hwang *et al*., 2024). Systemic therapies with multikinase inhibitor have been widely used with sorafenib as first-line targeted therapy for advanced HCC (Guo *et al*., 2023). Despite its therapeutic efficacy, only approximately 30% of patients derive clinical benefit, and nearly all responders develop acquired drug resistance within six months (Guo *et al*., 2023; Tang *et al*., 2020). Therefore, more effective therapeutic strategies are urgently needed. Currently, drug discovery is directed toward natural sources due to safety reasons (Gouthamchandra *et al*., 2024; Zhou *et al*., 2016). Marine algae are a sustainable bioresource known for its valuable bioactive compounds which can be cultivated in controlled

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environment. Studies show that algae contain numerous health benefits. Green seaweed, notably *C. lentillifera*, is consumed as salad in Southeast Asia and East Asia. It is rich in phytochemicals, including flavonoids, phenols, and fatty acids (Ismail MF *et al.*, 2020; Nurkolis *et al.*, 2023a). Its nutritional profile comprises carbohydrates, fiber, proteins, amino acids, lipids, minerals, vitamins, pigments, and antioxidants (Syakilla *et al.*, 2022). Previous studies have identified propanoic acid as the most abundant compound in ethanolic macerated extract (Nurkolis *et al.*, 2023), and polysaccharides as the predominant component in water extract (Tesvichian *et al.*, 2024). While, treatment with hexane extract of *C. lentillifera* in glioblastoma inhibited the proliferation (Tanawoot *et al.*, 2021). Solvent-based extracts of *C. lentillifera* induced apoptotic cell death in oral cancer KON cells (Manmuan *et al.*, 2025), prostate cancer cells (Wu *et al.*, 2024) and liver cancer cells (Rajasegaran *et al.*, 2024; Sangpairoj *et al.*, 2024). Furthermore, it suppressed the colony formation and migration of these cells. Polysaccharides from *C. lentillifera* played a similar inhibitory role in both monolayer and spheroid colon cancer models. While *C. lentillifera* holds therapeutic potential, its hot water extract has not yet been studied in the context of HCC progression. Here, we characterize the metabolomic profile of this extract and assess its anti-cancer potential by measuring HCC cell proliferation, migration, and colony formation

MATERIALS AND METHODS

Plant sample

Fresh *C. lentillifera* (Fisheries Research Institute, Malaysia) was washed, oven-dried at 60°C to a constant weight (Hung *et al.*, 2022), ground into a powder, and stored at -20°C until extraction and analysis.

Hot water extraction of *C. lentillifera* sample

Following a modified method, *C. lentillifera* powder (5 g) was extracted in 150 mL of Milli-Q water for 30 min at 50°C or 100°C (Azmin *et al.*, 2015). After cooling to 25°C, the mixtures were filtered through Whatman No. 1 paper. The filtrate was concentrated via rotary evaporation (60°C, 72 mbar, 30 rpm) and stored at -80°C, and subsequently lyophilized. The final extract was stored at -20°C. The percentage yield was calculated using the following formula:

$$\text{Percentage yield of } C.\ lentillifera\ (\%) = \frac{\text{Weight of dry } C.\ lentillifera \text{ extract}}{\text{Weight of dry } C.\ lentillifera \text{ sample}} \times 100\%$$

Complete media and cell culture

HepG2 (hepatocellular carcinoma) and L929 (normal fibroblast) cells were cultured in EMEM and RPMI, respectively. Both media were supplemented with 10% FBS and 1% penicillin-streptomycin, and cultures were maintained at 37°C with 5% CO_2.

Cytotoxicity analysis using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

HepG2 cells were seeded in 96-well plates (5 x 103 cells/well) and cultured overnight. Cells were treated with hot water extract (7.06-113 mg/mL, two-fold dilutions) for 24 or 48 hr, alongside medium-only blanks and untreated negative controls. Cell viability was assessed according to the manufacturer's protocol by measuring absorbance at 570 nm (Koç *et al.*, 2026). The experiments were conducted independently a minimum of three times. The percentage of cell viability was calculated based on the following formula:

$$\text{Percentage of cell viability (\%)} = \frac{\text{Average OD}_{\text{treated}} - \text{Average OD}_{\text{blank}}}{\text{Average OD}_{\text{control}} - \text{Average OD}_{\text{blank}}} \times 100\%$$

Colony Formation Assay

HepG2 and L929 cells were seeded in triplicate in 6-well plates (1 x 103 cells/well) (Abruscato *et al.*, 2023). After initial incubation, cells were treated with *C. lentillifera* extract (5 mg/mL; IC_{50}) and same concentration was used for L929 cells for screening (Tseng *et al.*, 2026), while medium-only wells served as controls. Following a 13-day incubation, cells were washed with PBS, fixed in 70% ethanol, and stained with 0.5% (w/v) crystal violet. Colonies exceeding 50 cells were quantified using a phase-contrast microscope (Olympus, Japan).

Wound healing assay

HepG2 and L929 cells were seeded in 24-well plates (6×10^5 / well) and incubated overnight (Gangwar *et al.*, 2025). After a 2-hr treatment with colcemide (0.1 µg/mL) to inhibit cell division (Zhang *et al.*, 2025), a linear scratch wound was created. Cells were then treated with the 5 mg/mL of *C. lentillifera* hot water extract, using untreated wells as a control. Wound closure was imaged via an Axiocam Erc 5s rev 2.0 camera (Carl Zeiss) and quantified using ImageJ software according to equation:

$$\text{Wound closure (\%)} = \frac{T\theta - T\Delta h}{T\theta} \times 100$$

Tθ=area of the wound measured at 0 hour.

TΔh=area of the wound measured at h hours.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis of *C. lentillifera* methanol extract (0.01 g/mL) was performed using an Agilent 7890A GC coupled to a 5975C MS, equipped with a capillary column (30 m x 250 µm x 0.25 µm). Helium carrier gas (99.99%) was maintained at 1 mL/min, with an injector temperature of 250°C. The oven program started at 50°C (3 min), ramped at 10°C/min to 280°C, and held at 300°C for 10 min. MS detection used 70 eV electron ionization, a 0.2 s scan time, and a mass range of 40-600 m/z. Compounds were identified via retention times, peak characteristics, and

mass spectra by comparison with the NIST library and GCMSD software (Konappa *et al.*, 2020).

RESULTS

Preparation of hot water extract from *C. lentillifera* powder

Maceration produced a higher extraction yield at 50°C than at 100°C (Table 1). This decrease at the higher temperature is due to rapid water evaporation, which reduces the solvent volume and causes premature saturation. As a result, less water is available to solubilize and extract the target compounds. Consequently, lowering the final yield.

Cytotoxicity of *C. lentillifera* hot water extract in HepG2

Across all time points (24, 48, and 72 hr), increasing the concentration of the extract led to a progressive decrease in cell viability compared to untreated control cells (Table 2). The cytotoxic effects became significantly more pronounced with longer exposure times. As time progressed from 24 to 48 hr, the concentration required to inhibit 50% of cell growth (IC_{50}) dropped sharply, showcasing heightened toxicity over time. While both extracts showed time-dependent toxicity, the 100°C extract was notably more effective at longer intervals (48 and 72 hr). Because the 100°C extract at 72 hr demonstrated the strongest overall inhibitory activity and the lowest cell viability, it was selected as the optimal condition for subsequent analyses.

Effect of *C. lentillifera* hot water extract on colony formation

Treated HepG2 cells displayed minimal colony development compared to their untreated counterparts, which formed progressively larger and more numerous colonies (Figure 1). In contrast, L929 cells exhibited a substantially lower reduction in colony formation when exposed to the 100°C extract (Figure 2). This suggests that *C. lentillifera* demonstrated selective inhibition on HepG2 cells.

Inhibitory effect of *C. lentillifera* hot water on cell migration

In this assay, treated HepG2 cells demonstrated a marked delay in closure, while untreated control HepG2 cells exhibited progressive wound closure over time (Figure 3). It indicated robust inhibition of cell migration (Figure 3). Nevertheless, wound closure was observed in both the control and treated L929 groups (Figure 4). The extract's inhibitory effect on L929 migration was substantially less pronounced than that observed in HepG2 cells.

Identification of bioactive compounds in *C. lentillifera* sample

The GC-MS chromatograms revealed three major peaks in both extraction temperatures at retention times of 10.551, 12.750, and 14.713 min. These findings indicated the presence of three major metabolites in hot water extracts. For both the 50 and 100°C extracts, cyclohexasiloxane and cycloheptasiloxane corresponded to the major peaks with qualities of 91 and 47, respectively (Table 3). In contrast, major constituent of the third peak in the 50°C extract was pentasiloxane and for the 100°C extract was benzoic acid derivative.

Table 1: Effect of temperature on percentage of yield from *C. lentillifera* hot water extracts.

Temperature	Yield
50°C	67.8%
100°C	55.9%

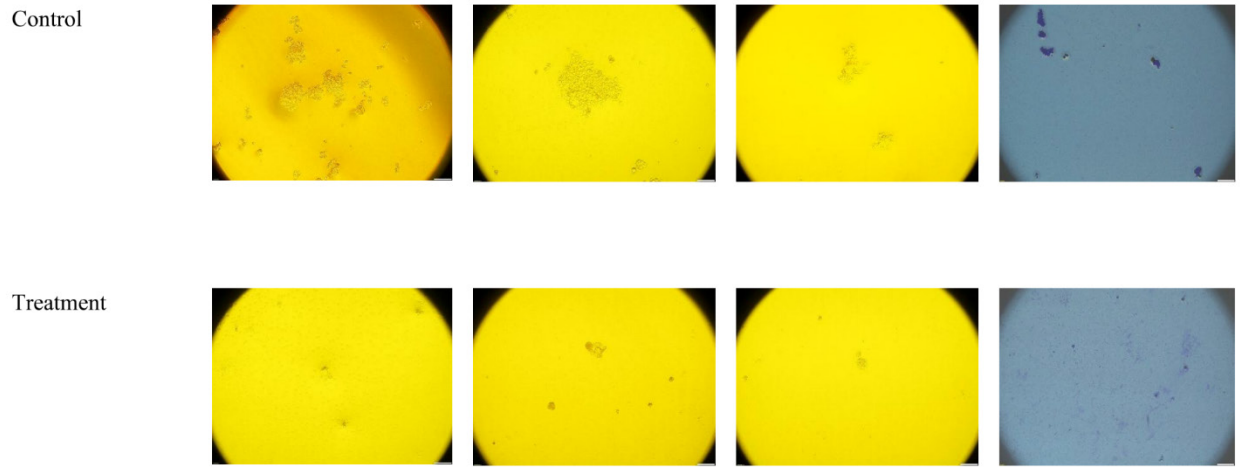

Figure 1: The colony of HepG2 cells without treatment (control) and treated with *C. lentillifera* hot water extract (5 mg/mL) after 13 days under a phase-contrast microscope (8x magnification) and crystal violet staining (4x magnification).

DISCUSSION

The present study demonstrated that the extraction temperature significantly influenced the yield of metabolites obtained from *C. lentillifera* by hot water maceration. Specifically, extraction at 50°C resulted in a high yield. In the specific context of *C. lentillifera*, polysaccharides represent a major class of bioactive water-soluble compounds. Prior study indicated that optimized conditions can yield substantial polysaccharide content up to ~25% of dry weight, further illustrating that *C. lentillifera* constituents respond differently to maceration temperature (Chaiklahan *et al*., 2020).

The *C. lentillifera* hot water extracts prepared at 50 and 100°C exhibited time- and concentration-dependent cytotoxic effects against HepG2 liver cancer cells. HepG2 cells exposed to increasing concentrations of the extracts for 24, 48, and 72 hr showed a progressive decrease in viability. These findings indicate that the cytotoxic effect was influenced by treatment duration and extraction temperature, with the 100°C extract demonstrating greater potency at later time points. Previously, aqueous extracts of C. racemosa and *Ulva lactuca* were able to reduce viability in HepG2 cells in a dose-dependent manner (Choudhary *et al*., 2021). The apparent difference in cytotoxicity between the two maceration temperatures suggests that it influences the profile of metabolites. Higher extraction temperatures may enhance the release and solubility of certain phytochemicals or secondary metabolites, resulting in more effective inhibition of HepG2 proliferation.

The colony formation assay determines whether cells retain the ability to undergo unlimited division and form colonies, which is critical in assessing the potential of an anticancer agent to prevent metastasis (Brix *et al*., 2021). In this study, treatment of HepG2 liver cancer cells with 5 mg/mL *C. lentillifera* hot water

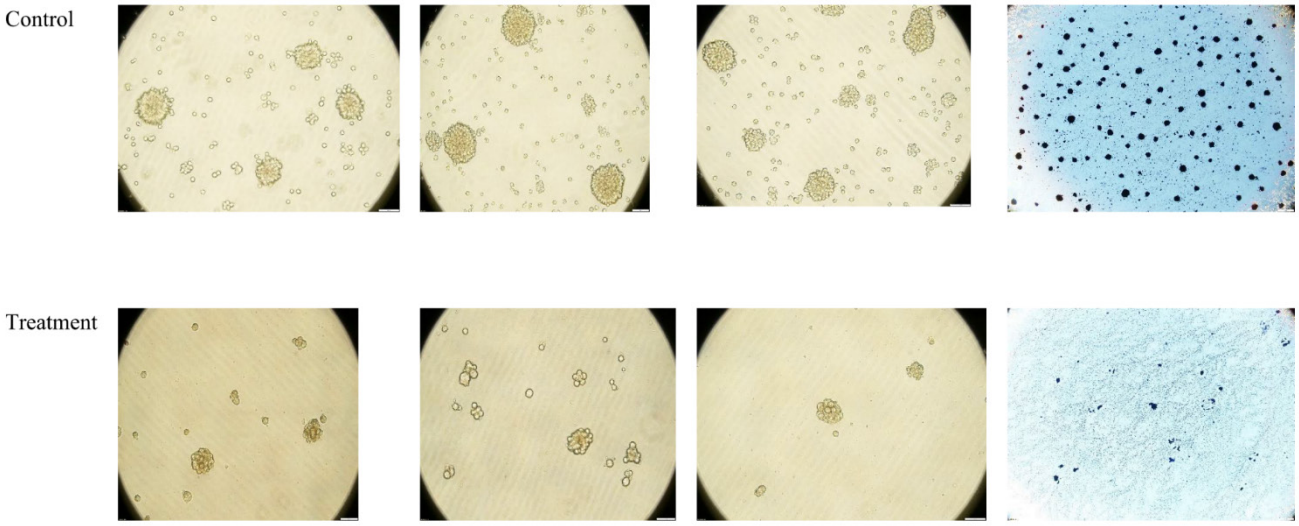

Figure 2: The colony of L929 cells without treatment (control) and treated with *C. lentillifera* hot water extract (5 mg/mL) for 13 days under a phase-contrast microscope (8x magnification) and crystal violet staining (4x magnification).

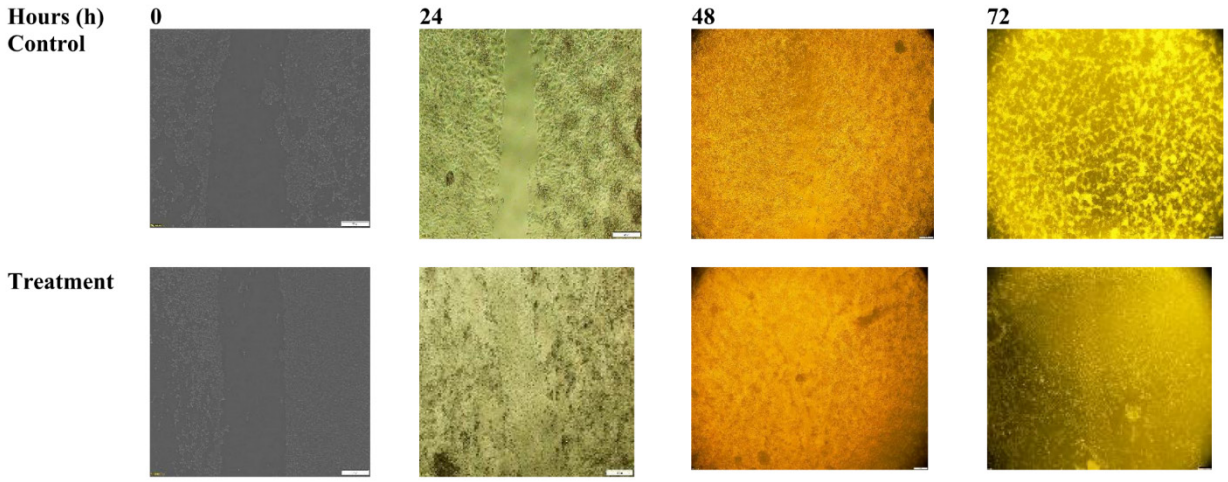

Figure 3: The wound healing effect of HepG2 cells without treatment (control) and treated with *C. lentillifera* hot water extract (5 mg/mL) under Olympus phase-contrast microscope (4x magnification).

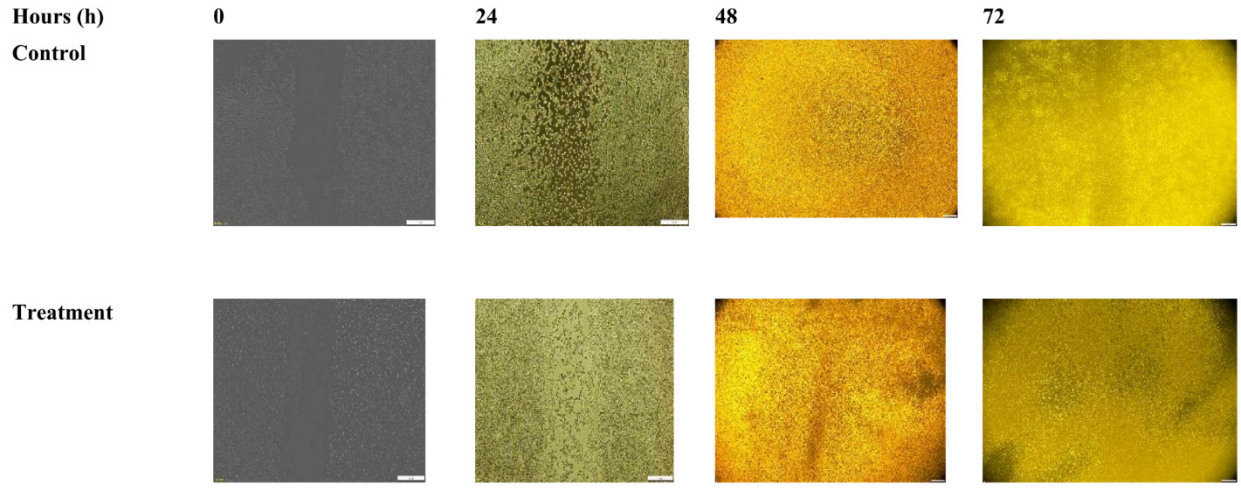

Figure 4: The wound healing effect of L929 cells without treatment (control) and treated with *C. lentillifera* hot water extract (5 mg/mL) under Olympus phase-contrast microscope (4x magnification).

Table 2: Dose and time-dependent responses on HepG2 cells.

Extraction temperature	24 Hr	48 Hr	72 Hr
50°C extract	12 mg/mL	6 mg/mL	8 mg/mL
100°Cextract	13 mg/mL	5 mg/mL	5 mg/mL

Table 3: Metabolites detected by gas chromatography MS analysis.

Temperature	Peak 1		Peak 2		Peak 3	
	Name	Quality	Name	Quality	Name	Quality
50°C	Cyclohexasiloxane, dodecamethyl-	91	Cycloheptasiloxane, tetradecamethyl	49	1,3,5,7,9-Pentaethylbicyclo [5.3.1] pentasiloxane	47
100°C	Cyclohexasiloxane, dodecamethyl-	91	Cycloheptasiloxane, tetradecamethyl-	91	Benzoic acid, 2,4-bis(trimethy|silyl) oxy]-, trimethylsilyl ester	47

extract prepared at 100°C resulted in a marked reduction in colony formation than untreated HepG2 cells over the incubation period. Untreated HepG2 cells exhibited progressive colony growth with a higher number of colonies per well, indicating preserved clonogenic survival. In contrast, treated HepG2 cells formed markedly fewer or no visible colonies, demonstrating a strong inhibitory effect. This suppression of the colony-forming ability suggests that the extract effectively impaired the reproductive integrity of HepG2 cells rather than merely inducing transient cytotoxic effects. Another study demonstrated that seaweed-derived polysaccharides significantly reduced colony formation in HepG2 cells, indicating long-term growth suppression (Kumarasinghe *et al.*, 2025). Likewise, extract from C. racemosa significantly inhibited colony formation in cancer cell lines (Permatasari *et al.*, 2022b). L929 cells showed only minimal reduction, indicating that normal cells retained greater proliferative capacity under the same treatment conditions. Such selectivity is a desirable characteristic in anticancer agents, as it indicates reduced toxicity toward normal tissues.

In the present study, the wound healing assay demonstrated that *C. lentillifera* hot water extracts inhibited migration of HepG2, while L929 normal fibroblast cells were able to migrate. The untreated HepG2 control cells exhibited progressive wound closure over time, reflecting active migratory capacity. In contrast, HepG2 cells treated with *C. lentillifera* hot water extract had a pronounced delay in wound closure. The reduced migration is unlikely to be solely due to cytotoxicity, as the assay was conducted under conditions that prevented proliferation. However, further analysis is required to confirm its anti-metastatic features. A study demonstrated that extracts of *C. racemosa* significantly inhibited migration as evidenced by delayed wound closure in treated cells (Permatasari *et al.*, 2022a). Similarly, seaweed-derived bioactive compounds reduced migration of HepG2 cells in wound healing assays through modulation of matrix metalloproteinases and epithelial-mesenchymal transition -related proteins (Cho *et al.*, 2022). The selective anti-migratory effect observed in this study, where HepG2 cells were more strongly inhibited than L929 normal fibroblast cells, is particularly noteworthy.

GC-MS analysis was employed to profile the volatile and semi-volatile compounds present in *C. lentillifera* hot water extracts prepared at 50°C and 100°C. Chromatographic profiles of both extracts revealed three major peaks at retention times

of 10.551, 12.750, and 14.713 min, indicating the presence of three dominant metabolites. For both extraction temperatures, cyclohexasiloxane and cycloheptasiloxane were identified as the primary putative constituents. Similar siloxane derivatives have been detected in the GC-MS profiles of Caulerpa species and other green algae, suggesting these compounds may represent common volatile constituents extracted through aqueous and thermal processes (Li *et al*., 2021).

Interestingly, the identity of the third major peak diverged between the two extraction temperatures. In the 50ºC extract, the dominant compound was putatively identified as pentasiloxane, whereas the 100ºC extract yielded a benzoic acid derivative. This variation suggests that extraction temperature influences the relative abundance and type of extractable compounds, particularly those sensitive to thermal conditions. Because both extracts shared identical retention times but differed in peak intensities, it indicates that the hot water extraction temperature did not alter the qualitative composition of the extracts but rather influenced extraction efficiency.

The enhanced anticancer effects observed for the 100ºC extract in cytotoxicity, clonogenic, and migration assays may be ascribed to higher concentrations or improved availability of specific bioactive compounds, such as the detected benzoic acid derivative, rather than the formation of entirely new metabolites. However, because these profiles are based on preliminary GC-MS library matching, cyclohexasiloxane, cycloheptasiloxane, and the benzoic acid derivative remain putative constituents. Hence their specific contributions to the observed anticancer effects require further rigorous validation and compounding isolation.

CONCLUSION

This study demonstrated that hot water extract of *C. lentillifera* exhibited HCC cell progression which needs further investigation to confirm the significance of these metabolites.

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ABBREVIATIONS

C. lentillifera: *Caulerpa lentillifera*; **HCC:** Hepatocellular Carcinoma; **GC-MS:** Gas Chromatography-Mass Spectrometry; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; **IC_{50}:** Half-maximal inhibitory concentration.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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