Research Article

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Antimicrobial activity, induction of ROS generation in HepG2 liver cancer cells, and chemical composition of *Pterospermum heterophyllum*

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Abstract: The objective of this investigation was to assess the inhibitory effects of extracts derived from *Pterospermum heterophyllum* on bacteria and fungi, as well as their capacity to impede the proliferation and migration of HepG2 liver cancer cells. Extracts from the leaves and stems of *P. heterophyllum* revealed the ability to resist all three bacterial strains, including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Streptococcus aureus* ($p < 0.05$). Additionally, *P. heterophyllum* also inhibited the growth of three fungal strains: *Aspergillus brasiliensis*, *Candida albicans*, and *Aspergillus flavus*. Evaluation of anticancer activity showed that extracts from *P. heterophyllum* significantly reduced the proliferation of HepG2 cells, with IC_{50} values for leaf and stem extracts being 35.5 and 47.17 µg/mL, respectively. Remarkably, the extract of this herbal species inhibited the migration ability of HepG2 cells after 24 h of treatment. Fluorescence analysis of cells using 2-HDCFDA showed a considerable elevation in reactive oxygen species production within HepG2 cells treated with extracts, which is hypothesized to contribute to a reduction in cell viability. Subsequent chemical compound analysis utilizing GC-FID spectra identified some main compounds present in the extracts from *P. heterophyllum*. This study highlights the potential antimicrobial and anticancer activities of *P. heterophyllum*.

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1 Introduction

Hepatic carcinoma represents a major worldwide health issue, ranking sixth among diagnosed cancer cases and third among cancer-related deaths [1]. Hepatocellular carcinoma (HCC), a form of liver cancer, constitutes 90% of all cases [2]. Infections caused by the hepatitis B or C virus, excessive alcohol intake, and fatty liver ailment have been recognized as the main contributors to HCC [3]. Currently, there have been significant advancements in the treatment of liver cancer, including surgical resection, targeted therapy, immunotherapy, chemotherapy, and radiation therapy [4]. However, modern treatment modalities still carry significant risks of adverse effects and complications in patients, with high rates of recurrence and limited response rates [5–8]. Additionally, increasing treatment costs present significant challenges for patient populations, particularly in developing countries and economies [9]. This underscores the urgent need to promote research on new cancer treatment methods that can address and improve the shortcomings of current therapies. Traditional herbal medicine research has garnered attention for its efficacy in cancer cell destruction with fewer adverse effects, as well as its ability to improve drug resistance and recurrence post-treatment [10]. Several herbs have been analyzed for their botanical chemistry and molecular mechanisms of action in liver cancer [11,12]. Some herbs, such as *Trametes robiniophila*, *Brucea javanica,* and *Patrinia scabiosaefolia,* have been utilized in the production of cancer treatment drugs [13].

Pterospermum heterophyllum (*P. heterophyllum*) is a flowering plant species belonging to the Sterculiaceae family. It is a medium- to large-sized plant primarily distributed in China

[14]. Species within the *Pterospermum* genus are commonly used to treat ulcers, blood disorders, hemorrhages, leprosy, measles, gastrointestinal disorders, and earaches [15]. Several species of this genus exhibit antifungal and antibacterial properties. Many studies have indicated that extracts from *P. heterophyllum* possess activity against *S. aureus*, *E. coli*, *P. aeruginosa*, *A. parasiticus*, and *R. oryzae* [16]. *P. heterophyllum* can inhibit the growth of *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, and *C. albicans* [17]. Additionally, *Pterospermum diversifolium* and *Pterospermum subpeltatum* have also demonstrated significant antibacterial capabilities [18,19].

Some species within this genus exhibit anticancer effects. Betulonic acid in *Pterospermum truncatolobatum* Gagnep inhibits cancer in the cell lines KB (epidermoid carcinoma), LU (lung carcinoma), MCF-7 (breast carcinoma), and HepG2 (liver carcinoma) in humans [20]. *Pterospermum acerifolium* is cytotoxic to cancer cells A549 (lung carcinoma) and PANC-1 (pancreatic carcinoma). Phenolic-rich extracts from the leaves of *Pterospermum lanceifolium* have been tested in mice and exhibit the ability to kill HepG2 liver cancer cells. Particularly, the inhibitory effects on cancer cells of some species within the *P. terospermum* genus have been shown to be associated with increased production of free radicals [21,22]. For *P. heterophyllum*, the roots of the plant are commonly used in traditional medicine to treat rheumatoid arthritis [23]. Previous phytochemical studies have identified and isolated triterpenoids, flavonoids, and phenolic acids from this plant. Specifically, extracts and triterpenoid components from the roots of *P. heterophyllum* have been shown to be cytotoxic to several cancer cell lines such as A549, HCT-8, Bel7402, BGC-823, and A2780 [14]. However, the antibacterial and antifungal effects and mechanism of cytotoxicity on HCC cells of leaf and stem extracts from *P. heterophyllum* have not been extensively studied. This research aimed to assess the antimicrobial properties against bacteria and fungi, as well as the chemical composition of extracts from *P. heterophyllum*. Additionally, this research aimed to assess the suppressive impact on growth and migration and to identify the underlying mechanisms in HepG2 cells.

2 Methods

2.1 Extraction preparation of *P. heterophyllum*

Leaf and stem samples of *P. heterophyllum* were collected from Cu Van commune, Dong Hy district, Thai Nguyen Province, Vietnam (latitude of 21°61′38.50″N, and longitude of 105°72′77.56″E), in May 2023. The samples were stored at Thai Nguyen University under voucher specimen TNUE2023.05. Each sample consisting of 500 g of stem or leaf of *P. heterophyllum* was thoroughly rinsed and then dehydrated at 50°C for 48 h. After drying, the stems and leaves were mechanically disrupted into a homogeneous powder. Ten grams of this powder were weighed into individual samples. Each sample was then treated with 30 mL of 90% ethanol and shaken overnight. The mixture was filtered using Grade 1, Whatman® qualitative filter paper (diam. 185 mm, Merck, Germany) to remove the solids. Ethanol was then completely evaporated using a Rotavapor R100 vacuum evaporator (Buchi, Switzerland). The obtained extract was stored at 4°C for several months.

2.2 Antibacterial activity

The test panel included three standard bacterial strains: *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *S. aureus* (ATCC 25923). They were obtained from the Microbiology faculty of Thai Nguyen Central General Hospital, Vietnam, and stored at Thai Nguyen University of Education. The disc diffusion method was employed to assess the antibacterial activity of the extracts [24]. To evaluate the inhibitory efficacy of the extracts, the size of the inhibition zones was determined by measuring their diameters. The extracts were prepared in 0.2% DMSO at three concentrations: 25, 50, and 100 µg/mL. *E. coli*, *P. aeruginosa*, and *S. aureus* strains were cultivated overnight (24 h) at 30°C on nutrient agar medium. Subsequently, the cultures were diluted in fresh LB to achieve a final concentration of approximately 10^8 CFU/mL. About 0.1 mL of the bacterial suspension was distributed uniformly onto a nutrient agar plate. Five wells (6 mm in diameter) were created in the agar. Subsequently, 50 µL of the essential extracts prepared in 0.2% DMSO were introduced to each well and stored for 1 h at 4°C. A negative control containing 0.2% DMSO (the solvent used for the extracts) and a positive control containing ampicillin 50 µg/mL were placed in the agar plate. The plates were cultured at 37°C for 24 h to facilitate bacterial growth. The diameter of the clear zones (inhibition zones) surrounding the wells was measured in centimeters to quantify the antibacterial activity of the extracts. All experiments were performed in triplicate.

2.3 Antifungal activity

The antifungal activity test method was described by Jiao and colleagues [24]. The antifungal activity was evaluated against three fungal strains: *Aspergillus brasiliensis*, *Candida albicans*

ATCC 10231, and *Aspergillus flavus* ATCC 204304, which were obtained from the Microbiology faculty of Thai Nguyen Central General Hospital. Three fungal strains were cultivated on potato dextrose agar (PDA) for 72 h. Mycelium plugs from the colony periphery were transferred to 100 mL of potato dextrose broth and incubated at 30°C with shaking at 200 rpm for 3–5 days. Subsequently, 100 µL of the resulting spore suspension was spread onto PDA plates. After solidification, wells were created in agar using a sterile cork borer. Each well received 50 µL of the extract at concentrations of 50, 100, and 150 µg/mL. The plates were incubated at 30°C for 3 days, and the diameter of the resulting zones of inhibition was measured. Negative controls contained only 0.2% DMSO (solvent for the extracts), while positive controls included amphotericin B 50 µg/mL. All experiments were performed in triplicate.

2.4 Cell viability assessment and MTT assay

In each well of a 96-well plate, 10,000 cells were placed in 100 µL of RPMI 1640 medium. After 24 h of incubation, the medium in the wells was replaced with fresh medium supplemented with *P. heterophyllum* extract (PHE) at concentrations of 0, 10, 50, 100, 200, and 500 µg/mL. Cell morphology was examined using a Ts2 NIKON inverted microscope (Tokyo, Japan) during a 48-h observation period.

Cell proliferation was evaluated using the MTT method. The medium in each well containing PHE was replaced with 100 µL of medium supplemented with 10% MTT at a concentration of 5 mg/mL, followed by incubation in the dark at 37°C for 4 h. Next, the MTT-containing medium was thoroughly removed, and 100 µL of DMSO solution was introduced to each well. Optical density (OD) was measured using a Multiskan Sky Microplate Spectrophotometer (Thermo Scientific™) at a wavelength of 570 nm. The percentage of cell proliferation was determined using the equation: cell proliferation (%) = (treated sample OD value)/(control sample OD value) × 100%. GraphPad Prism 9.5.0 software was utilized to compute the IC_{50} value.

2.5 Migration analysis

A 96-well culture plate was used for cell seeding, with each well containing 10,000 cells. After 48 h, when the cell density reached 90–95%, a 200 µL tip was used to create a migration area, followed by two washes with 1× PBS buffer solution. Then, 100 µL of culture medium containing various concentrations of PHE was added. Control samples were cultured under conditions without the extract. After a 24-h period, cell migration was examined and quantified using an inverted Ts2 NIKON microscope (Tokyo, Japan) in conjunction with ImageJ software.

2.6 Analysis of the generation ability of reactive oxygen species (ROS)

Cells were plated at a density of 40,000 per well in a 24-well plate and allowed to adhere for 24 h. Following this, the cells were exposed to RPMI 1640 medium containing PHE at 50 and 100 µg/mL concentrations for a period of 48 h. After the treatment, the cells were exposed to 2′,7′-dichlorodihydrofluorescein diacetate (2-HDCF-DA) at a concentration of 2.5 µg/mL and incubated for 30 min at 37°C. After two washes with 1× PBS buffer, the cells were stained with Hoechst 33342 at a concentration of 5 µg/mL for 15 min. Cellular observation and evaluation were performed under a fluorescence inverted microscope (NIKON T2U, Tokyo, Japan) with a 20× objective lens.

2.7 GC/FID and GC/MS analysis

The leaves and stems of *P. heterophyllum* were extracted using ethanol. After concentrating the ethanol extract, *n*-hexane was added, and the mixture was centrifuged to separate the *n*-hexane phase containing the volatile compounds. This *n*-hexane phase was subsequently analyzed using GC-MS. A Hewlett Packard 5890 Series II w/HP 5971 MSD GC/MS System, coupled with a quadrupole MS system (Agilent), was employed for GC/MS analysis. The setup featured an electron impact source operating at 200°C. The analysis utilized a fused silica-capillary column with an apolar stationary phase HP5-MS, measuring 30 m × 0.25 mm, with a 0.25 µm film thickness. Helium (99.99% purity) was used as the carrier gas at a constant flow rate of 0.9 mL/min for the separation of compounds in essential oils. The oven program was 50°C (3 min), 3°C/min to 200°C, and 10°C/min to 240°C (3 min) (60 min for analysis). A flame ionization detector (FID) was employed to identify compounds by gas chromatography (GC-FID) [25]. Mass spectra were obtained at 70 eV ion voltage, with a mass range of 50–2000 uma [25]. Compound identification was achieved by comparing retention indices (Ris) on an HP-5MS column with data from the NIST Chemistry WebBook (http://webbook.nist.gov/chemistry/, accessed on 18–22 December 2022). To further verify the results,

the mass spectra of the compounds were matched against those in the Wiley NBS75K.L and NIST/EPA/NIH mass spectral databases.

2.8 Statistical analysis

Statistical assessments were carried out using the Mann–Whitney *U* test or one-way ANOVA with subsequent *post-hoc* comparisons, utilizing GraphPad Prism software (version 10.3, GraphPad Software Inc., San Diego, USA). Results are reported as mean ± standard deviation.

3 Results and discussion

3.1 PHE exhibits antibacterial activity

Ethanol extracts obtained from the leaves and stems of *P. heterophyllum* exhibited promising antibacterial activity against all three tested bacterial strains. *E. coli* displayed the highest susceptibility, followed by *P. aeruginosa*. *S. aureus* demonstrated the least susceptibility. Notably, the extract retained good activity against all strains, particularly *E. coli*, even at a concentration of 20 µg/mL (Figure 1).

Some studies on the *Pterospermum* genus show the ability to inhibit bacterial growth. According to Syed Hidayathulla and colleagues in 2011, methanol extract from *Pterospermum diversifolium* has a strong inhibitory ability against bacterial strains *E. coli*, *S. aureus*, *B. subtilis*, and *P. aeruginosa* [19]. Jitendra Jena and colleagues in 2023 reported that *Pterospermum acerifolium* has good effects on human pathogenic microorganisms such as *S. flexneri*, *B. licheniformis*, *B. brevis*, *P. aeruginosa*, *S. yellow*, *S. epidermidis*, *B. subtilis*, and *E. coli* [26]. The increasing emergence of antibiotic-resistant bacterial strains is a growing concern, leading to increased treatment costs. Consequently, the exploration of plant-based sources with antibacterial properties is gaining attention as natural antibiotics and a promising alternative to traditional antibiotics, offering the potential to reduce treatment expenses and minimize undesirable side effects [27,28]. Moreover, combining plant extracts with antibiotics to enhance therapeutic efficacy and reduce the required antibiotic dosages is also considered a highly viable strategy [29]. In this study, we demonstrated that PHE effectively inhibits all three strains of *E. coli*, *P. aeruginosa*, and *S. aureus*, highlighting its potential in combating pathogenic microorganisms in humans. In our study, PHE also showed antimicrobial effects against strains of *E. coli*, *P. aeruginosa*, and *S. aureus*.

3.2 PHE exhibits antifungal activity

The findings (Figure 2) demonstrated that PHE exhibited growth-inhibiting properties against three fungal species examined: *A. brasiliensis, C. albicans ATCC 10231,* and *A. flavus ATCC 204304*. Figure 2 shows that the fungal inhibitory activity of ethanol extract from the leaves and stems of *P. heterophyllum* ranges from 100 to 150 µg/mL. The fungal growth inhibitory activity increases with the concentration of ethanol extract. *P. heterophyllum* also increased. However, the inhibitory ability between ethanol extracts from leaves and stems of *P. heterophyllum* is different. For the ethanol extract from *P. heterophyllum* leaves, the best inhibition was with the fungal strain *A. flavus,* followed by *A. brasiliensis* and *C. albicans.* For the ethanol extract from *P. heterophyllum* stems, the best inhibition was observed with *C. albicans* strain, followed by *A. flavus* and *A. brasiliensis*. According to previous studies, the genus *Pterospermum* has very good antifungal activity. In 2011, Sowmya G. Shetty and colleagues reported that *in vitro* antibacterial activity of leaf and stem extracts (ethanol and water) of *Pterospermum reticulatum* can inhibit the growth of three fungal strains (*Aspergillus niger, Candida albicans,* and *Trichoderma viridae*) [17]. Saboo et al. also reported that ethanol and water extracts of *Pterospermum acerifolium* had the ability to inhibit the growth of yeast cells with IC_{50} of 47.88 and 39.15 mg/mL [30]. In our study, the ethanol extract of leaves and stems of *P. heterophyllum* collected in Thai Nguyen also had good antifungal activity against three fungal strains: *Candida Albicans* ATCC 10231, *Aspergillus flavus* ATCC 204304, and *Aspergillus brasiliensi*.

3.3 PHE suppresses cell proliferation in HepG2 liver cancer cells

To initiate the investigation of the effect of PHE on HepG2 liver cancer cells, we assessed its potential to inhibit cell proliferation using the MTT assay. HepG2 cells were cultured in media containing extracts from either the leaves or stems of *P. heterophyllum* at doses ranging from 0 to 500 µg/mL. The results in Figure 3a and b indicate that in samples treated with the extract at a concentration of 10 µg/mL, the number of viable cells reduced slightly compared to the control after 48 h. However, at concentrations ranging from 50 to 500 µg/mL in both leaf and stem samples, cell viability significantly decreased. The IC_{50} values were determined to be 35.5 µg/mL for the leaf extract and 47.17 µg/mL for the stem extract (Figure 3c and d). Morphological observations of cells (Figure 3e and f) after 48 h of

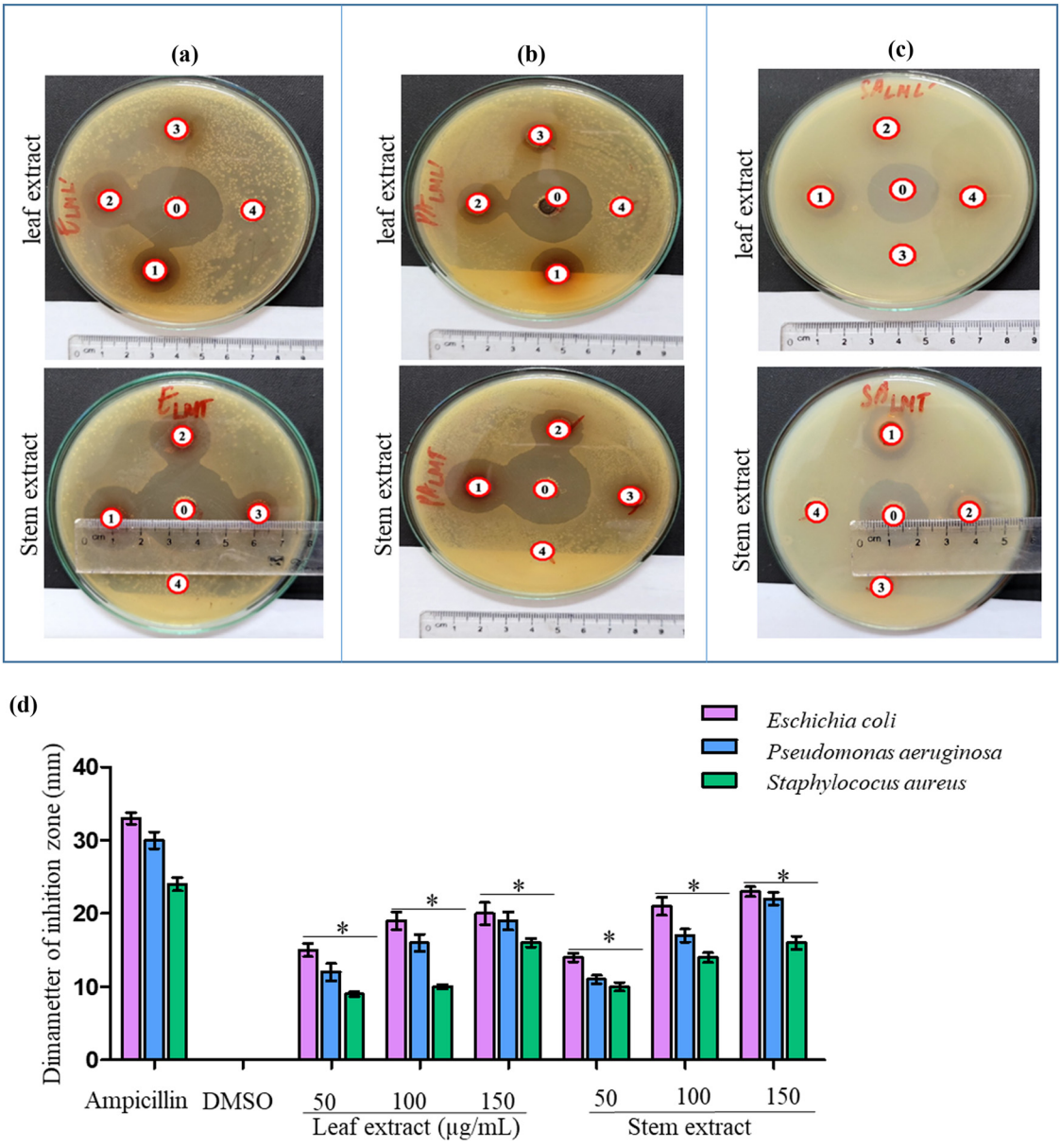

Figure 1: Antibacterial activity of PHE on *E. coli* (a), *P. aeruginosa* (b), and *S. aureus* (c), and the diameter of the inhibition zone (d). Ampicillin (50 µg/mL) (0); ethanol extract from stems or leaves at 25 µg/mL (3); 50 µg/mL (2); 100 µg/mL (1); and 0.2% DMSO (4). One-way ANOVA with subsequent *post-hoc* comparison, $*p < 0.05$.

treatment with the extract at concentrations of 50 and 100 µg/mL revealed that most cells lost their ability to adhere to the culture plate surface, with cells exhibiting a rounded shape or undergoing fragmentation into smaller fragments. When observing the nuclear morphology using DAPI (4′,6-diamidino-2-phenylindole) staining under a fluorescence microscope (Figure 3g and h), the number of cells displaying fragmented nuclei, a characteristic feature of apoptosis, was markedly increased compared to that in the control. The observed cellular morphology and nuclear patterns aligned with the MTT analysis results presented above.

The research findings indicate that extracts derived from *P. heterophyllum* leaves and stems demonstrate antiproliferative and pro-apoptotic effects on HepG2 liver cancer cells. The leaf extract showed greater potency with an IC_{50} of 35.5 µg/mL, while the stem extract exhibited an IC_{50} of 47.17 µg/mL, highlighting their potential as anticancer agents. Several herbal species commonly used in the treatment of liver cancer, such as *Pinus kesiya*, *Catimbium speciosum*, *Glochidion daltonii*, *Cladogynos orientalis*, *Acorus tatarinowii*, and *Amomum villosum*, exhibit IC_{50} values ranging from 50 to 500 µg/mL [31]. Various species belonging to the *Pterospermum*

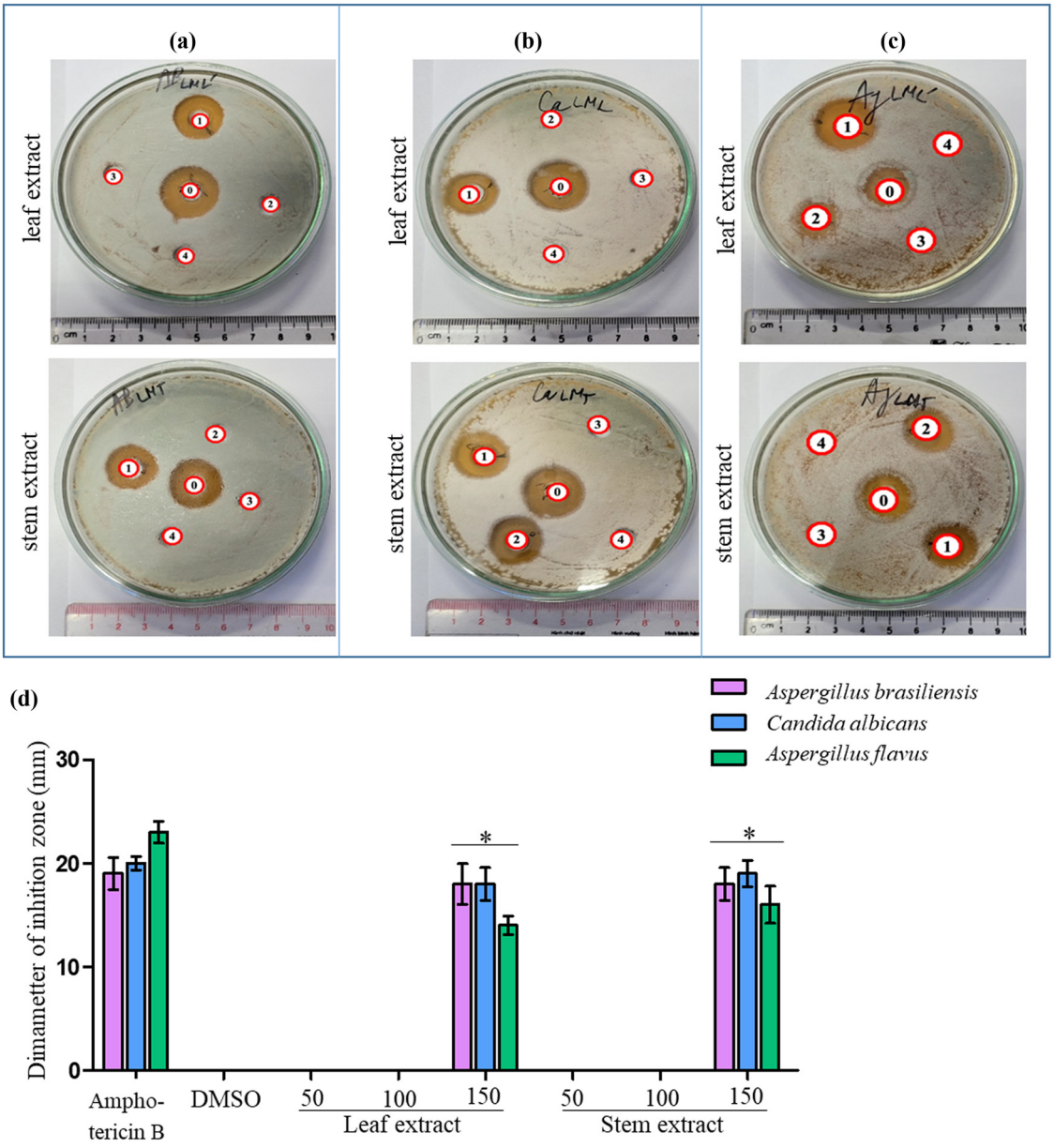

Figure 2: Antifungal activity of PHE on *Aspergillus brasiliensis* (a), *Candida albicans* ATCC 10231 (b), and *Aspergillus flavus* ATCC 204304 (c), and the diameter of the inhibition zone (d). Amphotericin (50 µg/mL); ethanol extract from stem or leaves at 50 µg/mL (3); 100 µg/mL (2); 150 µg/mL (1); DMSO (0.2%) control (4). One-way ANOVA with subsequent *post-hoc* comparison, $^{*}p < 0.05$.

genus have also shown inhibitory effects on HepG2 cell lines, such as *Pterospermum lanceifolium*, *Pterospermum acerifolium*, and *P. truncatolobatum* Gagnep [20,22,32]. Previous reports indicate that triterpenoid components isolated from the roots of *P. heterophyllum* have been demonstrated to possess cytotoxic activity against certain cancer cell lines with IC_{50} values ranging from 0.21 to 1.22 µM, where the IC_{50} value against the Bel7402 hepatoma cell line is 0.4 µM [14].

Particularly, silver nanocomplexes synthesized from *Pterospermum acerifolium* exhibit potent cytotoxicity against HepG2 cells, as demonstrated by an IC_{50} value of 4.76 ± 1.1 µg/mL, while showing no proliferation inhibition against HEK-293 cells [33]. Thus, extracts from various parts of *P. heterophyllum*, including roots, stems, and leaves, exhibit inhibitory effects on proliferation and induce apoptosis in HepG2 cancer cells. One of the leading causes of cytotoxicity and inhibition of cell proliferation is the ability to induce the apoptosis pathway in cells [34]. Apoptosis is the process of eliminating damaged cells and preventing tumor growth. In cancer cells, the apoptosis pathway is inhibited, leading to a prolonged

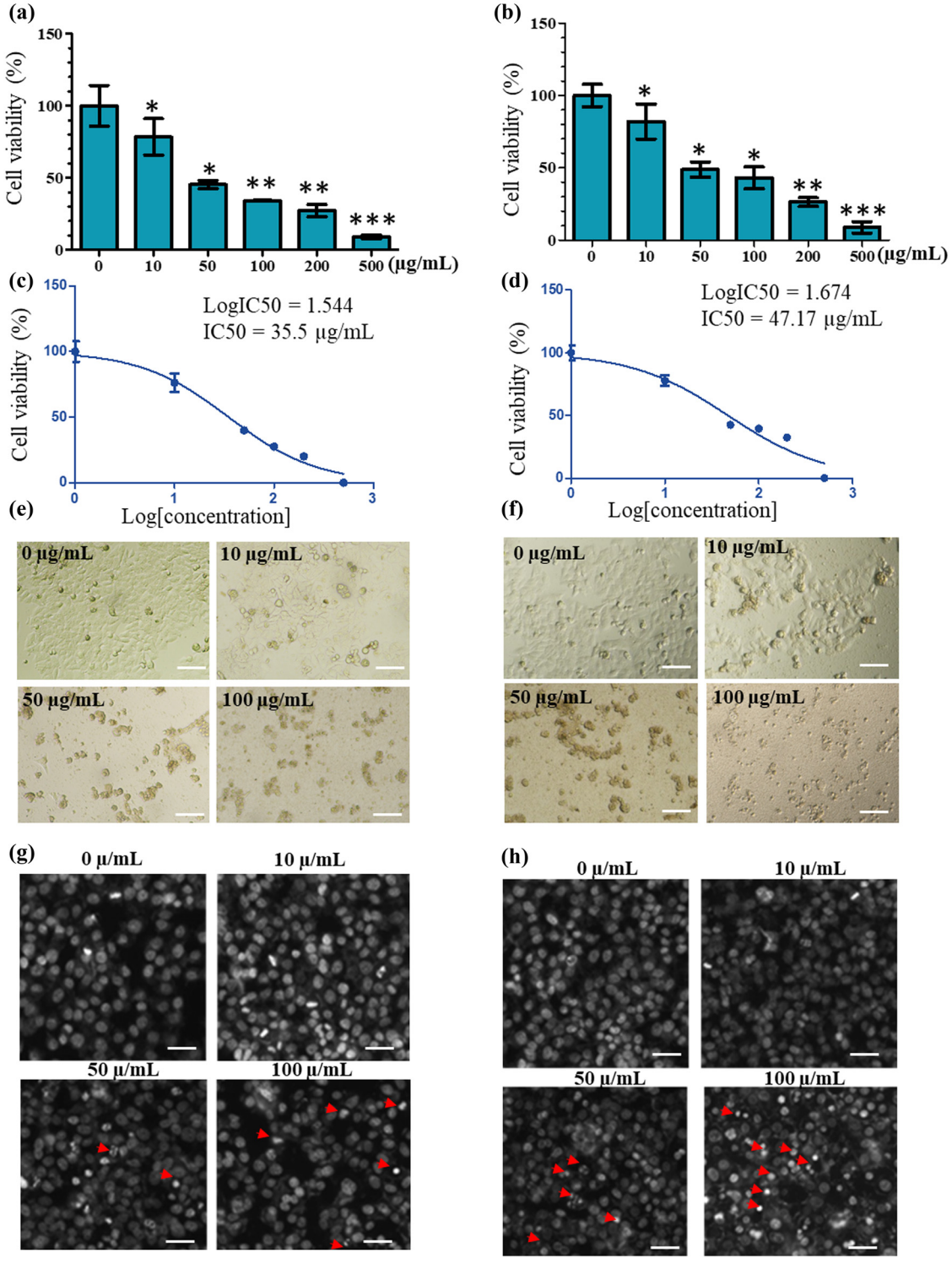

Figure 3: Influence of PHE on HepG2 cell growth and morphology. Evaluation of extracts from leaves (a) and stems (b) on HepG2 cell viability at various concentrations over a 48-h period, Mann–Whitney test, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus control. The IC_{50} values for leaf (c) and stem (d) extracts were determined after 48 h of treatment. Effects of leaf (e) and stem (f) extracts on cellular morphology, scale bar = 50 µm. Effects of leaf (g) and stem (h) extracts on nuclear morphology. Red arrows show the nuclear morphology of apoptotic cells. Scale bar = 50 µm.

accumulation of mutations that disrupt cell cycle regulation and increase cell invasion and metastasis [35]. Therefore, inducing apoptosis and causing cytotoxicity are primary targets in cancer treatment strategies. Commonly used cancer drugs such as doxorubicin, 5-fluorouracil, zerumbone, and platinum lead to apoptosis pathways in liver cancer cells [36–39]. In our study, cells undergoing apoptosis were identified using DAPI nuclear staining. The results demonstrate a notable increase in apoptotic nuclei-containing cells when exposed to *P. heterophyllum* leaf and stem extracts at concentrations of 50 and 100 µg/mL, as compared to the control group. This suggests that extracts from both leaves and stems of *P. heterophyllum* have the ability to induce the apoptosis process, thereby causing cytotoxicity and eliminating HepG2 cancer cells. Several species of the *Pterospermum* genus, such as *Pterospermum acerifolium*, *Pterospermum truncatolobatum*, and *Pterospermum Schreb*, also have the ability to induce the apoptosis pathway in certain cancer cell lines [21,32,40].

3.4 PHE inhibits cell migration in HepG2 liver cancer cells

The impact of PHE on the migratory capability of HepG2 cells was assessed using the wound healing assay. The results depicted in Figure 4 revealed a significant difference in the migratory potential of cells in the samples treated

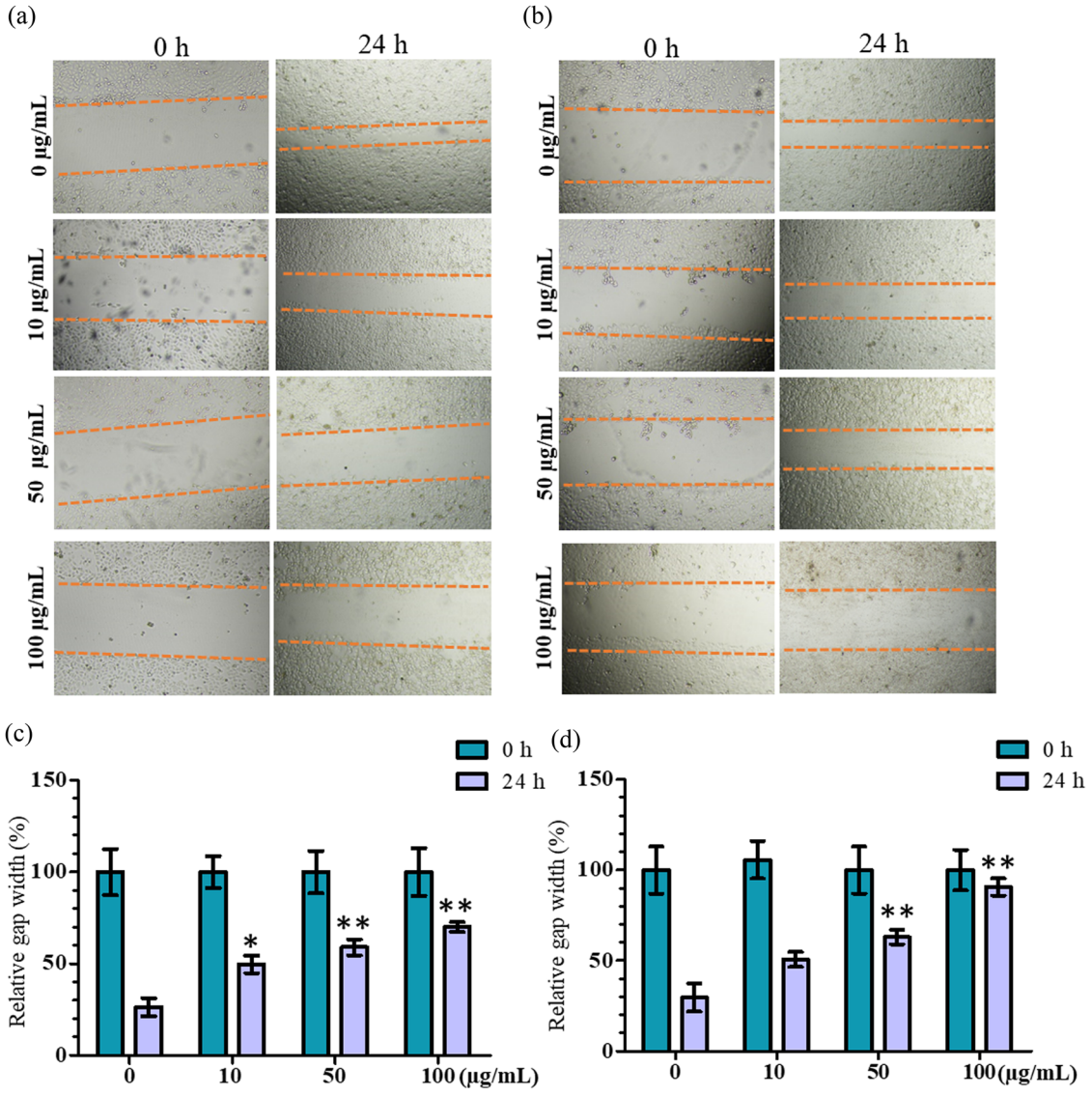

Figure 4: Impact of PHE on cell migration in HepG2 cells. Impact of leaf (a and c) and stem (b and d) extract on cell migration of HepG2 at different concentrations for 24 h. Mann–Whitney test, *$p < 0.05$; **$p < 0.01$.

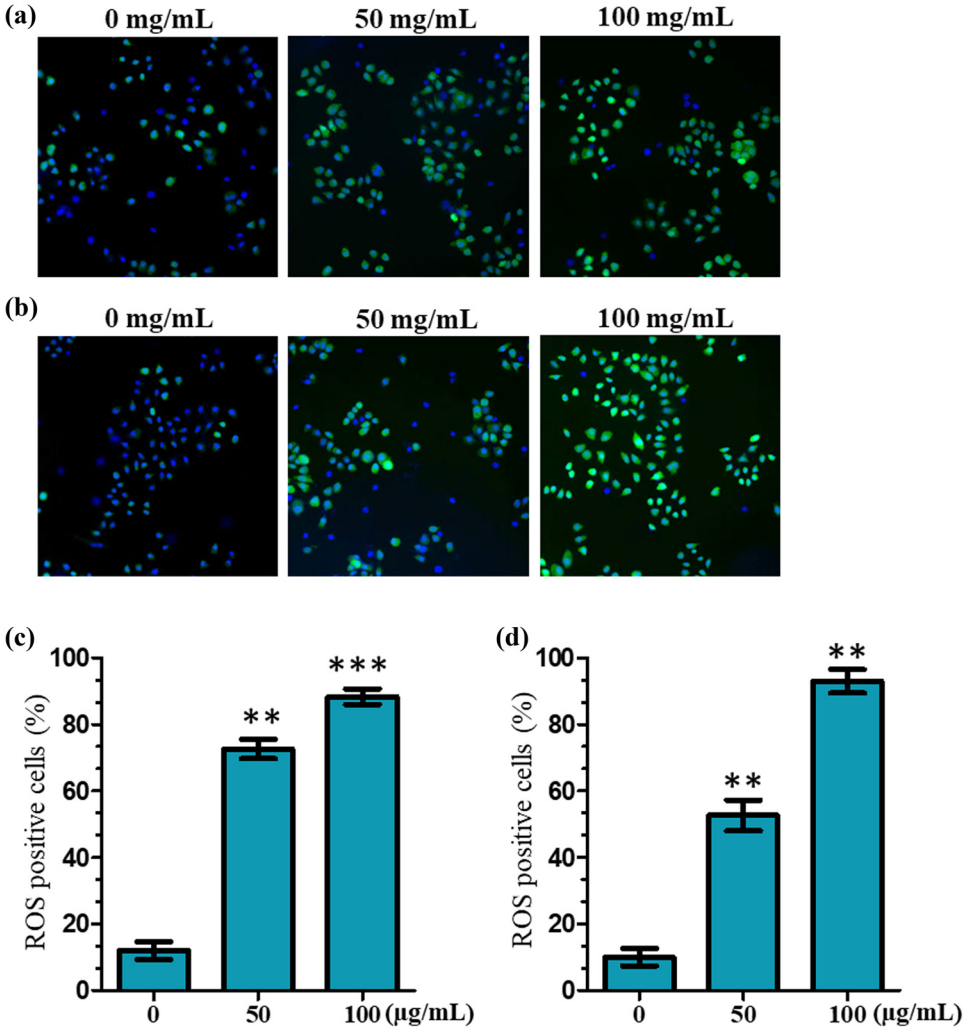

Figure 5: Impact of PHE on ROS generation in HepG2 cells. Cells treated with leaf (a and c) or stem extract (b and d) at concentrations of 50 and 100 µg/mL and for 24 h. Cell morphology was then captured using a fluorescence microscope (NIKON, Japan). Cellular nuclei were labeled with Hoechst 33342 (blue), and ROS-positive cells exhibited green fluorescence. Mann–Whitney test, $^{**}p < 0.01$.

with the extract at concentrations of 10, 50, and 100 µg/mL compared to the control group. Analysis of the migration (Figure 4c) demonstrated that after 24 h, in the control group, the reduction in the empty area was only 27.7 ± 3.1% compared to the initial time (0 h). Conversely, in leaf extract-treated samples, a more pronounced reduction in the empty area width was observed compared to the control, ranging from 50.2 ± 4.7 to 74.7 ± 3.4%, based on the concentration ($p < 0.05$ and $p < 0.01$). A similar outcome was observed when cells were treated with the stem extract (Figure 4d). Thus, extracts from the leaves and stems of *P. heterophyllum* distinctly decreased the proliferative and migratory capacities of HepG2 cells.

Metastasis is the primary cause of 90% of cancer-related deaths [41]. It is a multistep process involving the migration and invasion of cancer cells [42]. Although the survival rate of metastatic cancer patients has significantly improved in recent years due to cytotoxic therapies, researching new treatment modalities targeting metastasis remains urgent [43]. In our study, extracts from the leaves and stems of *P. heterophyllum* inhibited the migration of cancer cells. Particularly, the stem extract at a dose of 100 µg/mL markedly decreased the migration ability of cells compared to the control sample. This result indicates that extracts from both the stem and leaves of *P. heterophyllum* have the ability to suppress metastasis in HepG2 cells. Apart from *P. heterophyllum*, only *Pterospermum acerifolium* within the same genus has been extensively studied and has shown similar inhibitory effects on migration in A549 and PANC-1 cell lines [21]. The growing interest in the research and use of plants in cancer treatment highlights the importance of herbal medicine in modern healthcare [44]. Evidence shows that an increasing number of

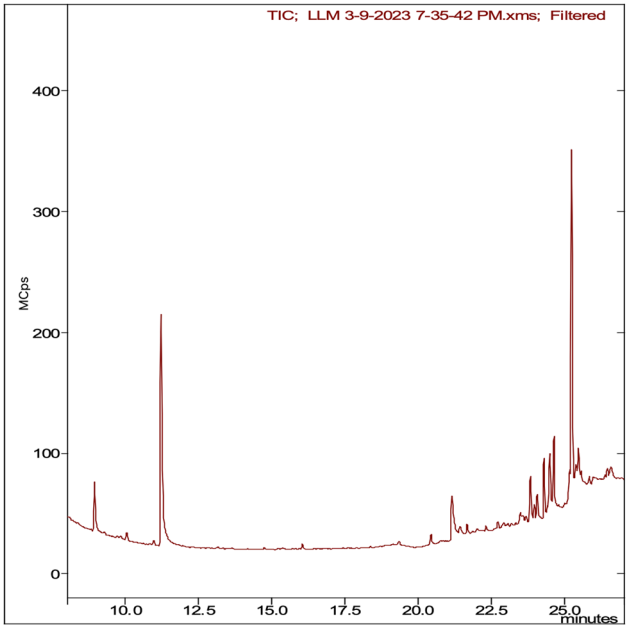

Figure 6: GC-FID spectra of the leaf extract of *P. heterophyllum*.

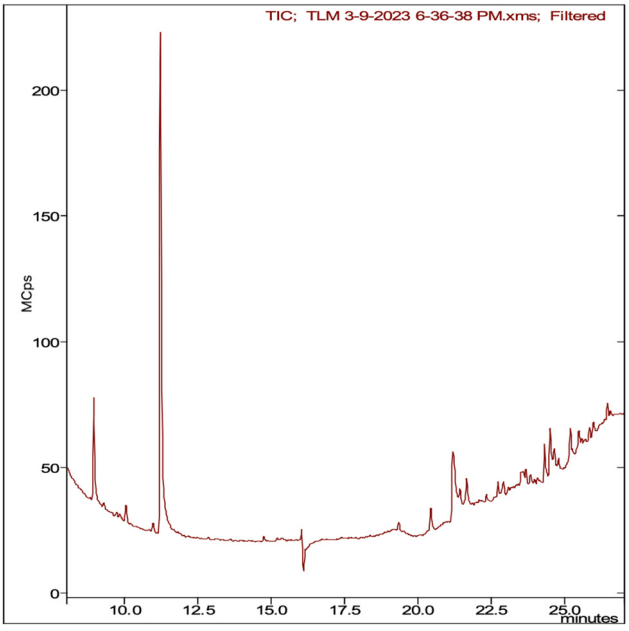

Figure 7: GC-FID spectra of the stem extract of *P. heterophyllum*.

anticancer drugs are being developed from plants due to advantages such as lower cost and fewer side effects compared to the current synthetic chemotherapeutic drugs [45]. Furthermore, the combination of plant extracts with anticancer drugs may lead to a synergistic effect, significantly enhancing the therapeutic efficacy against cancer cells [46]. In our study, extracts from *P. heterophyllum* demonstrated the ability to inhibit cell proliferation and prevent cell migration, showing its potential to block the development of breast cancer cells. Further studies in animal models and clinical trials are necessary to more accurately assess their value in medical practice.

3.5 PHE increases ROS generation in HepG2 cells

To assess the impact of PHE on the generation of ROS in the HepG2 cell line, the fluorescent dye 2-HDCFDA was employed to stain cells after 24 h of treatment with the extract. The results depicted in Figure 5 reveal that all PHE-treated samples exhibited enhanced ROS production compared to the control group ($p < 0.01$). Specifically, at a concentration of 50 µg/mL, 73.7 ± 4.8% of HepG2 cells exhibited ROS expression in leaf-treated samples, and 45.5 ± 4.7% of cells showed ROS

Table 1: Chemical composition of the leaf extract of *P. heterophyllum*

No.	Compound	Chemical formula	RT	Area	Signal/ noise	Composition (%)	Similarity index
1	Hexadecane	$C_{16}H_{34}$	20.435	7.49×10^7	105.00	2.90	900–950
2	Phenol, 2-methoxy-4-(1-propenyl)-, (*Z*)-	$C_{10}H_{12}O_2$	21.185	1.91×10^8	154.2	7.40	900–920
3	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	21.660	1.10×10^8	76.41	4.26	850–900
4	Benzenemethanamine, *N*-(phenylmethylene)-	$C_{14}H_{13}N$	23.472	1.29×10^8	2.947	5.00	800–880
5	Benzyl(phenylmethylene)amine	$C_{14}H_{13}N$	23.556	1.28×10^8	4.32	4.96	820–870
6	Neophytadiene	$C_{20}H_{38}$	23.824	1.23×10^8	44.31	4.76	880–930
7	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	24.057	7.00×10^7	25.50	2.71	850–900
8	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	24.289	1.57×10^8	62.00	6.08	910–940
9	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	24.481	2.23×10^8	62.46	8.64	920–950
10	Phytol	$C_{20}H_{40}O$	25.230	9.79×10^8	425.20	37.92	930–970
11	1-Benzoxepin-2(3*H*)-one, octahydro-	$C_{10}H_{16}O_2$	25.386	1.19×10^8	2.95	4.61	780–850
12	Ethyl iso-allocholate	$C_{20}H_{38}O_2$	25.463	6.59×10^7	55.37	2.55	850–900
13	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	24.625	2.12×10^8	77.32	8,21	910–940

Table 2: Chemical composition of the stem extract of *P. heterophyllum*

No.	Compound	Chemical formula	RT	Area	Signal/noise	Composition (%)	Similarity index
1	Hexadecane	$C_{16}H_{34}$	20.437	3.70×10^7	105.00	5.55	900–950
2	Phenol, 2-methoxy-4-(1-propenyl)-, (*Z*)-	$C_{10}H_{12}O_2$	21.148	1.69×10^8	148.20	25.35	900–920
3	*Trans*-Isoeugenol	$C_{10}H_{12}O_2$	21.192	1.49×10^7		2.24	890–920
4	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	21.662	5.53×10^7	76.41	8.30	850–900
5	Benzenemethanamine,*N*-(phenylmethylene)-	$C_{14}H_{13}N$	23.575	1.57×10^7		2.36	800–880
6	Neophytadiene	$C_{20}H_{38}$	23.827	1.69×10^8	44.31	25.35	880–930
7	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	24.314	5.95×10^7	49.13	8.93	910–940
8	*n*-hexadecanoic acid	$C_{16}H_{32}O_2$	24.481	5.48×10^7	62.46	8.22	920–950
9	l-(+)-Ascorbic acid 2 6-dihexadecanoate	$C_{38}H_{68}O_8$	24.498	1.74×10^7	54.27	2.61	800–870
10	7-hydroxycadallene	$C_{38}H_{68}O_8$	24.784	1.17×10^7		1.76	780–850
11	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	$C_{27}H_{42}O_4$	25.185	3.70×10^7	33.18	5.55	850–890
12	Ethyl iso-allocholate	$C_{20}H_{38}O_2$	25.463	2.53×10^7	19.65	3.80	850–900

expression in stem-treated samples (all $p < 0.01$). The proportion of ROS-expressing cells significantly increased at a dose of 100 µg/mL for both leaf and stem extracts ($p < 0.01$ and $p < 0.05$). Thus, PHE induced cellular stress by augmenting ROS production.

ROS are oxygen-derived reactive species produced in the mitochondria. Excessive cellular ROS production causes oxidative damage to biological molecules [47]. Several studies have shown that ROS acts as an antitumor agent by promoting aging, halting the cell cycle, and stimulating apoptosis [21,48,49]. Some species within the Pterospermum genus, such as *Pterospermum acerifolium* and *Pterospermum lanceifolium*, have been shown to enhance ROS production in cancer cells [21,22]. For *P. heterophyllum*, our analysis results indicate that stem and leaf extracts of the plant enhance ROS production in HepG2 cells. This increased ROS production may be the cause of the results observed in our study, initiating the apoptosis process, thereby inducing cytotoxicity and cell elimination, ultimately leading to the inhibition of cancer cell migration. Further molecular-level analyses will provide a clearer explanation of the mechanism of cytotoxicity against HepG2 cells by *P. heterophyllum*. Excessive ROS production has been identified as a crucial mechanism underlying the anticancer effects of several chemotherapy agents, such as doxorubicin, daunorubicin, epirubicin, and 5-fluorouracil [50,51]. At high levels, ROS can cause damage to mitochondrial membranes, triggering the release of cytochrome *c* and subsequent activation of caspases. This cascade of events ultimately leads to programmed cell death or apoptosis. Additionally, the accumulation of ROS alters the Bcl-2/Bax ratio, promoting apoptosis through the caspase-dependent pathway [52]. Elevated levels of ROS are utilized in cancer therapy; however, this approach may also induce inflammation and cause damage to normal cells and tissues in the body [53]. Furthermore, excessive ROS can potentially initiate the development of new cancer cells in healthy tissues [54]. Therefore, therapies utilizing ROS require careful balancing of ROS levels to optimize the damage to cancer cells while minimizing adverse effects on normal cells, particularly in critical organs such as the heart, liver, eyes, and lungs [54,55]. The application of high levels of ROS to selectively target cancer cells while avoiding side effects on healthy tissues will be a focus of research aimed at improving the efficacy and safety of treatment.

3.6 Phytochemical compounds of ethanol extract of *P. heterophyllum*

The GC/MS spectral analysis of leaf extracts obtained using alcohol extraction techniques (Figure 6 and Table 1)

revealed that the chemical composition comprised 13 distinct compounds, of which the most abundant compound was phytol ($C_{10}H_{40}O$), accounting for 37.92%, while the compound with the lowest yield was ethyl iso-allocholate ($C_{26}H_{44}O_5$), at 2.55%. Similar to the extract from *P. heterophyllum* leaves, the one extracted from the stems is liquid with a reddish-brown color. Twelve compounds were identified by GC/MS spectral analysis of extracts from the overground stems of *P. heterophyllum*, as shown in Figure 7 and Table 2. Phenol, 2-methoxy-4-(1-propenyl)-, (*Z*)-($C_{10}H_{12}O_2$), and neophytadiene ($C_{20}H_{38}$) gave the highest yield (25.35% for each compound), while 7-hydroxycadallene ($C_{38}H_{66}O_8$) gave the lowest yield (2.5678%). Overall, extracted by alcohol extraction methods from the leaves and upper stem portions of *P. heterophyllum* were phenol, 2-methoxy-4-(1-propenyl)-, (*Z*)-, and neophytadiene.

These are the major components present in the volatile fraction of the ethanol extract of *P. heterophyllum*. Previously published data have indicated that *trans*-isoeugenol [56], hexadecanoic acid [57], ethyl iso-allocholate [58], and hexadecane [59] have been identified to exhibit proliferation inhibitory activity and induce apoptosis in various cancer cell lines.

4 Conclusion

This study reveals that the extracts of leaves and stems from *P. heterophyllum* exhibited inhibitory effects against both bacterial strains, including *E. coli*, *P. aeruginosa*, and *S. aureus*, as well as fungal strains such as *A. brasiliensis*, *C. albicans*, and *A. flavus*. Additionally, this study is the first to demonstrate that the extract of *P. heterophyllum* exhibits potent inhibitory effects on cell proliferation, induces apoptosis, and inhibits cell migration. The increased generation of ROS within the cells induced by chemical components present in the ethanol extract of *P. heterophyllum* may contribute to the mechanism of inhibiting HepG2 liver cancer cells, a mechanism not previously identified in earlier studies. This study indicates the potential of *P. heterophyllum* as a promising candidate for combating liver cancer cells.

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Conflict of interest: The authors declare that there is no conflict of interest.

Ethical approval: The conducted research is not related to either human or animal use.

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