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Research Article

Phytochemical characterization and mechanistic analysis of solvent-derived extracts from Malaysian mangroves *Sonneratia alba* and *Bruguiera cylindrica* for potential anticancer properties

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Abstract
Mangroves have garnered considerable attention in anticancer research due to their abundance of phytochemicals. However, *Sonneratia alba* and *Bruguiera cylindrica*, two native mangrove species from Pengkalan Gelap, Setiu, Terengganu, Malaysia, remain underexplored. This study examined the cytotoxic effects, apoptosis-inducing potential, and antioxidant activity of twig extracts from these species. Extracts were prepared using methanol, ethyl acetate, and hexane, and tested in vitro against human breast cancer cells (MCF-7) and a non-cancerous rat muscle cell line (L6). Cytotoxicity was evaluated using the MTS assay, apoptosis by Annexin V-FITC staining, cell viability by trypan blue exclusion, and antioxidant activity against DPPH radicals. Among the six extracts, the hexane extract of *S. alba* (SAH) demonstrated the strongest cytotoxicity with an IC_{50} of 3.47 µg/mL, followed by the hexane extract of *B. cylindrica* (BCH, IC_{50} = 7.76 µg/mL) and the ethyl acetate extract of *S. alba* (SAEA, IC_{50} = 27.8 µg/mL). All extracts exhibited minimal toxicity toward L6 cells. Apoptosis induction by BCH, SAH, and SAEA was confirmed, while trypan blue staining revealed less than 50% viability of MCF-7 cells after 72 hours. Notably, only SAEA displayed significant antioxidant activity with an IC_{50} of 0.125 mg/mL. These findings underscore the potent cytotoxic and apoptosis-inducing properties of *S. alba* and *B. cylindrica* twig extracts, which selectively target breast cancer cells and hold promise as potential sources of novel anticancer agents.

Keywords: mangrove, phytochemicals, natural product, anticancer, breast cancer

Introduction

Cancer is defined as a disease of uncontrolled cell proliferation that can metastasise to other parts of the body. The top five global cancer incidences include lung cancer (12.4%), female breast cancer (11.6%), colorectal cancer (9.6%), prostate cancer (7.3%), and stomach cancer (4.9%) [1]. Notably, breast cancer accounts for the majority of cancer-related fatalities among women [1]. Statistics indicate that breast cancer predominantly affects women (99%), with only 0.5–1% of cases involving male breast cancer [2]. According to the World Health Organization (WHO), breast cancer is the most commonly diagnosed cancer in 157 out of 185 countries and was responsible for 670,000 deaths among women in 2022. This disease represents a significant challenge in women's health and is ranked as the second leading cause of mortality among women worldwide [3,4].

Among conventional cancer treatment regimens, chemotherapy remains the most widely prescribed globally due to its efficacy in managing metastatic breast cancer [5]. However, prolonged use of synthetic chemotherapeutic agents is associated with adverse side effects such as hepatotoxicity, nausea, vomiting, diarrhoea, hair loss, infertility, extreme fatigue, myelosuppression, and necrotic cell death [6,7]. Furthermore, synthetic analogue drugs often lack specificity in targeting malignant cells, leading to recurrence and deterioration of overall patient health [6]. These limitations highlight the urgent need for safer and more effective therapeutic alternatives.

Consequently, oncology research has increasingly focused on the development of natural-based chemotherapeutic agents capable of delivering targeted and apoptosis-inducing therapy [8]. Natural products are widely recognised for their bioactive compounds that treat a variety of human diseases, including cancer, immunological disorders, cardiovascular complications, and infectious diseases [9]. Approximately 60% of approved cancer drugs in clinical use are derived from natural sources or are semi-synthetic derivatives [10,11]. For centuries, mangroves have been traditionally utilised to treat various ailments. Nevertheless, mangrove forests in Malaysia remain insufficiently explored and investigated for their therapeutic potential.

Mangroves are trees and shrubs that thrive along shorelines in tropical and subtropical regions. Adapted to stressful environments such as high salinity and fluctuating tides, mangroves synthesise bioactive substances to enhance their survivability [12]. Previous studies have identified a wide range of compounds isolated from mangrove extracts, including alkaloids, flavonoids, tannins, saponins, phenolics, and triterpenes [13,14]. Rich in phytochemicals and bioactive compounds, mangroves are well known for their diverse biological activities, including antibacterial [15], antiviral [16], antifungal [17], anti-inflammatory [18], anticancer [19], antioxidant [20], and insecticidal properties [21].

Mangroves have increasingly been recognised as valuable sources of bioactive compounds with anticancer potential, owing to their diverse phytochemical profiles such as flavonoids, tannins, and triterpenoids. Recent studies have highlighted several mangrove species for their cytotoxic and apoptosis-inducing properties against cancer cell lines. In particular, *Bruguiera cylindrica* has been reported to contain metabolites with significant cytotoxic activity against breast cancer cells, while *Sonneratia alba* has demonstrated antioxidant and anticancer effects attributed to its phenolic constituents [22-25]. Extracts from mangrove species of the genera *Sonnerati*a and *Bruguiera* have documented traditional medicinal uses and emerging evidence of bioactive compounds with antioxidant, cytotoxic, and antiproliferative properties, largely attributed to their phenolic, flavonoid, and terpenoid content [24-26]. Although direct evidence for *S. alba* and *B. cylindrica* remains limited, these species are considered promising based on their ethnomedicinal applications and ecological abundance. *S. alba* is traditionally used for treating wounds, bruises, and inflammation [23], whereas *B. cylindrica* is used for haemorrhage and ulcers [24]. Both species are abundant in the Setiu Wetlands, Terengganu, Malaysia, providing ecologically justified and reproducible sampling opportunities [22]. These factors support their prioritisation for the present phytochemical and anticancer evaluation, addressing the existing gap in systematic in vitro studies.

Therefore, this study investigates the anticancer potential of mangrove extracts through apoptotic pathways. Their antioxidant properties may enhance cancer cell sensitivity while protecting healthy cells, potentially reducing chemotherapy-associated side effects. These findings may contribute to the development of mangrove-derived anticancer agents for breast cancer, while emphasising the importance of conserving Malaysian mangroves to preserve biodiversity and promote sustainable research.

Materials and Methods

Mangrove twigs crude extraction

Twigs of both *B. cylindrica* and *S. alba* were collected from Pengkalan Gelap, Setiu, Terengganu (GPS: 5°40'07"N, 102°43'49"E) and identified by botanist Encik Muhamad Razali bin Salam from the Faculty of Science and Marine Environment, Universiti Malaysia Terengganu. The twigs of both species were dried in oven at maximum temperature of 35ºC before grinded into powder. Afterward, the powdered twigs were then soaked in hexane overnight at ratio of 1:4. soaking process were repeated until 3 times or until extract appeared colourless, indicating the solvent had extracted most of the compounds. The solvents were then filtered into a round bottom flask using Whatman filter paper No.1 before concentrated using rotary evaporator at 40ºC. In this study, exhaustive extraction method was utilised, therefore the same twigs were then soaked in ethyl acetate and lastly with methanol. The extracts were then dried in 40ºC to evaporate the excessive solvent before the completely dry crude samples were stored in 4ºC chiller. Therefore, this study yielded a total of 3 distinct types of extracts for each species.

Cell culture

The human breast cancer cell line MCF-7 and the rat skeletal muscle myoblast (*Rattus norvegicus*) cell line L6 were cultured using the methods reported by Khoeliqoh [27] and Oskal [28]. Both of the cell lines were obtained from Institute of Climate Adaptation and Marine Biotechnology (ICAMB), Universiti Malaysia Terengganu, Malaysia. MCF-7 is a widely used human breast adenocarcinoma cell line as its estrogenic receptor-positive has been extensively employed in studies of breast cancer biology, hormone responsiveness, and anticancer drug screening. MCF-7 cell line was cultured in T25 flask containing 5 mL Roswell Park Memorial Institute-1640 (Gibco) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) Penicillin-Streptomycin (Pen-Strep). The flask was then left incubated in 37 ºC and 5% (v/v) CO_2. Meanwhile L6 cells was cultured in 5 mL Dulbecco's Modified Eagle Medium (D-MEM,

Gibco) and similarly supplemented with 10% (v/v) FBS and 1% Pen-strep before incubated in 37 ºC and 5% (v/v) CO_2. As cell line reach confluency, MTS assay, apoptosis assay and trypan blue assay were further conducted according to methods documented [27,28].

MTS assay

The desired cell density for MCF-7 was $2x10^4$ cells per well, while for L6 it was $2x10^5$ cells per well, seeded at 100 µL per well into a 96-well plate. The well plates were incubated for 24 hours in a 5% (v/v) CO_2 incubator at 37ºC prior to treatment. Treatment was conducted with dosage ranging from the highest at 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125µg/mL, 1.5625 µg/mL, 0.78125 µg/mL and the lowest at 0.390625 µg/mL. Vincristine sulphate used as positive control in this study. Negative control is untreated cell in complete media. Both MCF-7 and L6 cells viabilities were detected using CellTiter 96® AQueous One Solution Proliferation Assay (MTS Assay Promega, USA). After the 72h treatment incubation period, 20 µL of MTS reagent were pipetted into each well and incubated for additional 1 ½ hour. Subsequently, the viability was assessed at 490 nm absorbance for both cells using microplate reader. The inhibitions of cells were calculated using the formula:

$$Inhibition\ (\%) = \frac{[(OD\ control - background) - (OD\ test - OD\ background)]}{(OD\ control - OD\ blank)} \times 100\% \quad (1)$$

The reading of control samples was taken from untreated cells. Percentage of inhibition vs (1og10) sample concentration (µg/mL) was plotted using GraphPad Prism. From the graph, IC_{50} value was measured based on sigmoidal-dose response curve.

Apoptosis assay

Apoptotic activity in MCF-7 cells were assessed by utilising the Annexin V Apoptosis Detection Kit, FITC, from eBioscience. 2 x 10^4 MCF-7 cells were seeded into a 96-well plate. Following a 24 h incubation period, cells were then treated with the IC_{50} dosage obtained from the preceding MTS assay. Before each observation, the media were withdrawn, and the cells were rinsed with 100 µL of PBS. Afterward the cells were washed and resuspended in 1X binding buffer, 100 µL each step. 5 µL of Annexin V FITC dye staining were conducted and the cells were incubated for 15 minutes. Subsequently, the binding buffer was withdrawn again prior to washing the cells and resuspending them in 100 µL of 1x Binding Buffer. Stained MCF-7 were then additionally stained with 5 µL of Propidium Iodide. Analysis of apoptotic activity in MCF-7 cells was conducted under a fluorescence microscope in 12 h, at every 4 h interval. Quantification of apoptotic cells were made using ImageJ (Fiji, NIH, USA).

Trypan blue exclusion assay

100 µL (2 x 10^4 cells per well) of MCF-7 were seeded into 96-well plate and incubated for 24 hours prior to treatment. Samples having positive anticancer activity, acquired from the MTS assay, were diluted two-fold based on the determined IC_{50} value and administered as treatment for 24 h. Following the incubation period, the cells were washed and eventually trypsinised using 10 µL of trypsin (Gibco), then incubated until complete detachment of the cells was observed. Subsequently, trypan blue staining was performed by combining trypan blue dye with trypsin in a 1:1 ratio. Rapid observations were conducted with the automated cell counter, DeNovix.

DPPH radical scavenging assay

The antioxidant properties of the sample were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, as documented [29, 30]. To prepare a 6 x 105 M DPPH solution, 2.37 mg of DPPH powder was dissolved in 100 mL of methanol. 10 mg/mL of the selected crude sample was prepared, and 1 mg/mL of quercetin served as the positive control. Samples and quercetin were initially pipetted into the 96-well plate following the two-fold dilution procedure depicted in the figure below, with treatment dosages ranging from 0.156 mg/mL to 10 mg/mL, and 0 mg/mL designated as the blank control. Finally, 200 µL of DPPH in MeOH was added to each well and incubated in the dark at room temperature for 30 minutes. The absorbance measurements were measured at 517 nm via a multiplate reader. The percentage of DPPH radical scavenging activity was calculated using the formula below:

$$DPPH\ scavenge\ activity\ (\%) = \frac{Ao - A}{Ao} \times 100\% \quad (2)$$

Ao = Control absorbance

A = Sample absorbance

LC-MS/MS

Referring to the methods outlined by Zakaria [31], 100 mg of selected samples were diluted in 1 mL of methanol, followed by vortexing for 30 minutes and an additional sonication for 30 minutes. Then, the samples were filtered through 0.2 µM PTFE membrane into a vial before metabolomics profiling using Ultra-High Performance Liquid Chromatography- Tandem Mass Spectrometry (UHPLC-MS/MS) equipped with Orbitrap MS/MS through electrospray ionization (HESI) probe heating. Mass spectrometry data was obtained using a complete MS scan, succeeded by data-dependent tandem mass spectrometry (ddMS2) utilising Xcalibur 4.2.27 software (Thermo Scientific). A comprehensive MS scan was acquired at a resolution of 60,000 with a mass/charge (m/z) range of 100-1,000, whereas the ddMS2 scan was obtained at a resolution of 15,000 with stepwise normalised collision energies of 20, 40,

and 60 AU. The data generated by LCMS were pre-processed with MS-DIAL (v5.2.240424.3) according to Tsugawa et al., in 2015. Metabolite identification relied on the MS2 public database utilising legitimate standards from the MS-DIAL website (version17; https://systemsomicslab.github.io/compms/msdial/main.html).

Statistical analysis

All experiments were conducted in triplicates and the data were statistically analysed using one-way ANOVA and T-test before completed with Dunnett's Multiple Comparison post-hoc test. P-values <0.05 were regarded as statistically significant.

Results and Discussion

Mangrove crude extract yield

Multilevel maceration was performed using solvents of increasing polarity (hexane–ethyl acetate–methanol) to extract a wide range of secondary metabolites, as these compounds can only be dissolved in solvents of similar polarity. Table 1 presents the crude extract mass (g) obtained from the three solvents and their respective percentage yields (%). The percentage yield (%) was calculated as follows:

$$\% \text{ Yield} = (\text{Weight of Dried Extract} / \text{Weight of Dried Sample}) \times 100 \quad (3)$$

As indicated in **Table 1**, the crude mass and percentage of yield obtained for mangrove sample extraction vary in each solvent. Using methanolic extract, both *B. cylindrica* and *S. alba* achieved the highest crude yield of 5.84% and 5.86% respectively. In the meantime, the lowest percentage of yield was obtained by hexane extracts with *B. cylindrica* (1.02%) and *S. alba* (1.24%). The ethyl acetate extracts exhibited intermediate yield percentages with *B. cylindrica* (1.53%) and *S. alba* (2.14%) among the three solvents. This finding aligned with previous research [32]. The most polar ethanolic extracts used in the study dominated the yield from mangrove wooden slabs of several origin, [*B. cylindrica* (5.58%, 5.67%), *R. apiculata* (8.33%), *B. gymnorhiza* (2.81%)] in comparison with lower polarity ethyl acetate [*B. cylindrica* (1.32%,1.98%), *R. apiculata* (0.94%), *B. gymnorhiza* (0.58%)] and non-polar hexane [*B. cylindrica* (1.07%, 0.82%), *R. apiculata* (0.05%), *B. gymnorhiza* (0.73%)]. Malik [33] reported a similar pattern, with *B. sexangula*, *B. cylindrica*, *R. apiculata*, *A. alba*, and *L. racemosa* showing variation in percentage yield within each species, despite sampling from the same location, Setiu Wetlands, Terengganu. Past research has proven that extracted yield of mangroves species *B. cylindrica*, *R. apiculata and B. gymnorrhiza* have different crude extraction yield [34].

Assessment of cell viability using the MTS assay

The cytotoxicity of six twig extracts, *S. alba* hexane (SAH), *S. alba* ethyl acetate (SAEA), *S. alba* methanol (SAM), *B. cylindrica* hexane (BCH), *B. cylindrica* ethyl acetate (BCEA), and *B. cylindrica* methanol (BCM), was evaluated by the MTS assay. MCF-7 is a widely used human breast cancer cell line in anticancer research, yielding results comparable to those of previous studies. L6 rat skeletal muscle myoblasts (*Rattus norvegicus*) were included as a non-cancerous cell type to provide a non-tumorigenic reference with robust growth characteristics and well-established culture conditions, enabling assessment of differential cytotoxicity between malignant and non-malignant cells. Although cross-species differences exist, rat muscle cells are frequently used as a surrogate for normal mammalian tissue in in vitro assays due to their reproducibility and availability as a reference to assess selectivity and potential off-target effects, reflecting the baseline susceptibility of normal tissue, as cancer-related systemic impacts often lead to skeletal muscle wasting and atrophy. As shown in **Figure 1**, all six crude twig extracts from *B. cylindrica* and *S. alba* dose-dependently inhibited both MCF-7 and L6 cells. Several crude extracts from the two selected species exhibited anticancer activity against breast cancer cell lines. Vincristine sulphate cytotoxicity was assessed in both cell lines to determine the relative effects on normal versus cancerous cell proliferation.

Table 1. Mangrove crude yield (%) obtained. Each value represented mean ± S.D. (n=2). (Two independent experiments)

Species	Dry Weight (g)	Yield (%)		
		Methanol	Ethyl acetate	Hexane
B. cylindrica	50 ± 0.00	5. 84 ± 0.11	1.53 ± 0.16	1.02 ± 0.05
S. alba	50 ± 0.00	5.86 ± 0.07	2.14 ± 0.12	1.24 ± 0.07

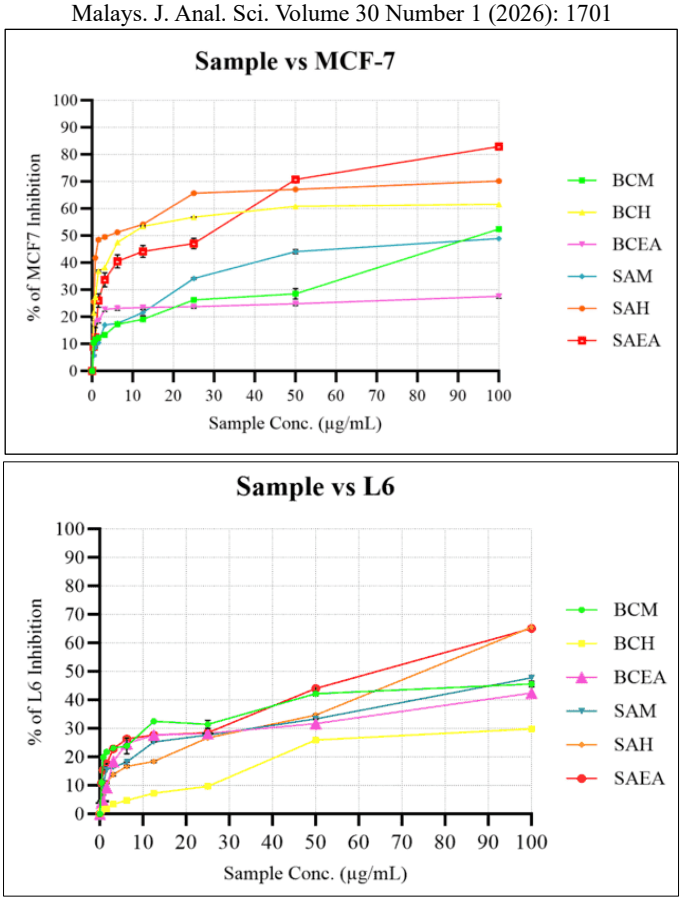

Figure 1. Dose responses curve of mangrove crude extracts against MCF-7 and L6 cell inhibition after 72h. Each value represented mean ± S.D (n=3) (three-independent experiments)

Only the BCH, BCM, SAH, and SAEA extracts successfully inhibited MCF-7 (>50%) after 72 h of treatment. BCH, at a low dosage of 12.5 µg/mL and up to the highest dosage of 100 µg/mL, inhibited more than 50% of MCF-7. BCM demonstrated successful inhibition of MCF-7 cells, reaching 52.43%. SAEA at the second-highest dosage of 50 µg/mL successfully inhibited 69.56% of MCF-7 cells. SAH inhibited MCF-7 cells by more than 50% at a concentration as low as 3.125 µg/mL. Meanwhile, for L6, only at the highest concentration (100 µg/mL) were normal cells inhibited by more than 50%. Interestingly, concentrations of mangrove crude extracts ranging from 0 to 90 µg/mL demonstrated relatively weak to non-toxic effects on normal line proliferation. Vincristine sulphate (VS) inhibited both MCF-7 and L6 cell lines by more than 50%, even at the lowest concentration of 0.391 µg/mL. At the highest treatment concentration of 100 µg/mL, VS inhibited 61.23% (L6) and 69.87% (MCF-7) in a dose-dependent manner. Surprisingly, the trend of VS inhibition against MCF-7 was only slightly higher than that against L6. The IC_{50} values were calculated and presented in Table 2.

Table 2. IC_{50} value of respective mangrove extracts against MCF-7 and L6. (Each assay was conducted in triplicate, n=3. The IC_{50} values are stated in Mean ± S.D)

Sample	IC_{50} ± S.D (µg/mL)	
	MCF-7	L6
BCH	7.76 ± 0.18	75.86 ± 0.21
BCEA	-	-
BCM	94.5 ± 0.14	-
SAH	3.47 ± 0.20	74.13 ± 0.17
SAEA	27.8 ± 0.11	63.09 ± 0.17
SAM	-	-
VS	0.063 ± 0.12	0.104 ± 0.12

The dash symbol (-) represented sample failed in achieving IC_{50}. Vincristine sulphate IC_{50} value serves as comparison between sample and positive control

For mangrove anticancer activity against MCF-7, the *S. alba* hexane extract showed a very strong IC_{50} of 3.47 ± 0.20 µg/mL, whereas the *S. alba* ethyl acetate extract showed a weak IC_{50} of 27.8 ± 0.11 µg/mL. *B. cylindrica* hexane extracts showed a strong IC_{50} value of 7.76 ± 0.18 µg/mL against MCF-7. *B. cylindrica* methanol extract exhibited a weak IC_{50} value of 94.5 ± 0.14 µg/mL. BCH, BCM, SAH, and SAEA cytotoxicity effects were compared with the negative control (complete media), indicating no relative toxicity against the MCF-7 cell proliferation after 72 h. BCH ($p < 0.01$), SAH ($p < 0.001$), and SAEA ($p < 0.05$) exhibited anticancer activity, with SAH being the most potent anticancer agent. Meanwhile, BCM was found to be insufficient as an anticancer agent ($p > 0.05$), as its effects were not significantly different from those of complete media. Furthermore, BCH, SAH, and SAEA were relatively compared with VS (positive control) to statistically determine the effects of natural products and semi-synthetic chemotherapy drugs against normal cell proliferation, where $p<0.05$ is statistically significant.

Overall, this cytotoxicity screening demonstrated that the *B. cylindrica* hexane extract (BCH), *S. alba* hexane extract (SAH), and *S. alb*a ethyl acetate extract (SAEA) possessed anticancer activity. Interestingly, there are no reports of cytotoxicity for these two native mangrove twig extracts, even though they are commonly distributed in Malaysia's mangrove forests. Therefore, in accordance with the American National Cancer Institute (NCI) guidelines, BCH, SAH, and SAEA have the potential to be further developed into anticancer drugs, as the achieved IC_{50} values were below 30 µg/mL [35]. Thus, these three selected extracts were further investigated in an apoptosis assay to determine the mechanism of cell death in MCF-7 cells. In line with the MTS assay analysis, the hexane extract showed higher efficacy in delivering anticancer effects against the MCF-7 cell line through the MTS assay. This finding is supported by a previous study [36], which found that nonpolar, hydrophobic solvents such as hexane can extract anticancer compounds. *S. alba* exerted a promising anticancer effect in two solvents, hexane and ethyl acetate. This finding aligned with [37], which reported that the fruit of S. alba moderately inhibited breast cancer cells (MCF-7). *B. cylindrica* hexane extracts showed a strong IC_{50} value of 7.76 ± 0.18 µg/mL against MCF-7, similar to a previous study [38], where they demonstrated that *Bruguiera gymnorrhiza* leaves exhibited IC_{50} values corresponding to the extractive solvent, where the butanol extract (3.39 µg/mL), diethyl ether extract (16.22 µg/mL), and methanol extract (37.15 µg/mL) inhibited MCF-7 proliferation through the apoptosis pathway. *B. cylindrica* methanol extract exhibited a weak IC_{50} value of 94.5 ± 0.14 µg/mL. Similarly, *B. gymnorrhiza* fruit and root extracts appear to be weak and non-toxic against the A549 cell line, with IC_{50} values of 130 µg/mL and 140 µg/mL, respectively [39]. This finding indicates that SAH, BCH, and SAEA are not cytotoxic to normal cell line proliferation. Therefore, mangrove extracts are unlikely to produce the same toxic effects as vincristine sulphate, including an unpredictable and uncertain pharmacological profile that can lead to neurotoxicity, myelosuppression, alopecia, and gastrointestinal toxicity [40].

Assessment of cell death via apoptosis assay

Interestingly, MCF-7 at IC_{50} concentrations of BCH (7.76 ± 0.18 µg/mL), SAH (3.47 ± 0.20 µg/mL), and SAEA (27.8 ± 0.11 µg/mL) underwent distinct morphological changes before death. As shown in **Figure 2**, MCF-7 cells became detached and rounded, with blebbing of the cell membrane, shrank in size, and lost volume. The mentioned morphological changes positively aligned with apoptotic cell characteristics. Thus, to further confirm that BCH, SAH, and SAEA induced cytotoxicity in the breast cancer cell line via the apoptosis pathway.

These three extracts were subjected to an Annexin V FITC binding assay (**Figure 3**). The Annexin V Apoptosis Detection Kit FITC involved staining of treated MCF-7 with two different fluorescence dyes, namely Annexin V FITC (green-fluorescent protein stain) and propidium iodide (red-fluorescent DNA stain). Annexin V FITC dye binds to phosphatidylserine of apoptotic membrane plasma [41]. Meanwhile, propidium iodide (PI) stains the DNA of dying cells but appears completely impermeable to the membranes of living cells [42]. Thus, positive staining for both Annexin V-FITC and PI indicated apoptosis.

BCH, SAH, and SAEA triggered MCF-7 cell death via apoptosis over the 4-12 h treatment period, similar to that induced by vincristine sulphate (**Figure 3**). No apoptosis occurred in the negative control. Annexin V-FITC staining for phosphatidylserine was observed in the 4 h–6 h treatment period, indicating an early apoptotic event [43]. At 8 h, the staining with both Annexin V-FITC and PI became moderately visible, indicating a mid-apoptotic event. Late apoptotic events were observed after 10 h, as evidenced by overlapping staining in both the membrane and the nucleus, and by a strongly stained red nucleus without the green-stained membrane in MCF-7 [44,45].

Furthermore, there was an observable trend in apoptosis between treatment dosage and incubation period. Therefore, the number of apoptotic cells was quantified in **Figure 3**, as shown in **Figure 4**, indicating that the treatment concentration influences the rate of apoptosis. SAH, with a very strong IC_{50} (3.47 ± 0.20 µg/mL), induced the highest level of apoptotic death, whereas fewer apoptotic cells were observed in the strong IC_{50} BCH (7.76 ± 0.18 µg/mL) and weak IC_{50} SAEA (27.8 ± 0.11 µg/mL) treatments.

The apoptosis assay confirmed that BCH, SAH, and SAEA induced apoptosis, arresting MCF-7 proliferation. SAH, with a very strong IC_{50} (3.47 ± 0.20 µg/mL), triggered the highest level of apoptotic death. Quantitatively, the number of apoptotic cells was lower in the strong IC_{50} BCH (7.76 ± 0.18 µg/mL) and the weak IC_{50} SAEA (27.8 ± 0.11 µg/mL), indicating that the apoptosis was dose-dependent. Moreover, the apoptosis rate was time-dependent, with the death of MCF-7 cells increasing proportionally with the duration of treatment. The study reported in [40] observed a similar trend of apoptosis across several cancer cell lines, namely breast, cervical, and liver. This finding also aligned with research conducted [41], which reported that the methanolic extract of *B. gymnorrhiza* induced apoptosis in breast and liver cancer cell lines. In addition, *Bruguiera* sp. was found to induce apoptosis in cancerous cell lines, including MCF-7, HepG-2, and HeLa [42,43]. Another study reported a similar finding, showing that triterpenes and sterols from *S. alba* root, fruit, and leaf extracts induced apoptosis in H22 and BCG-803 cell lines [44, 45].

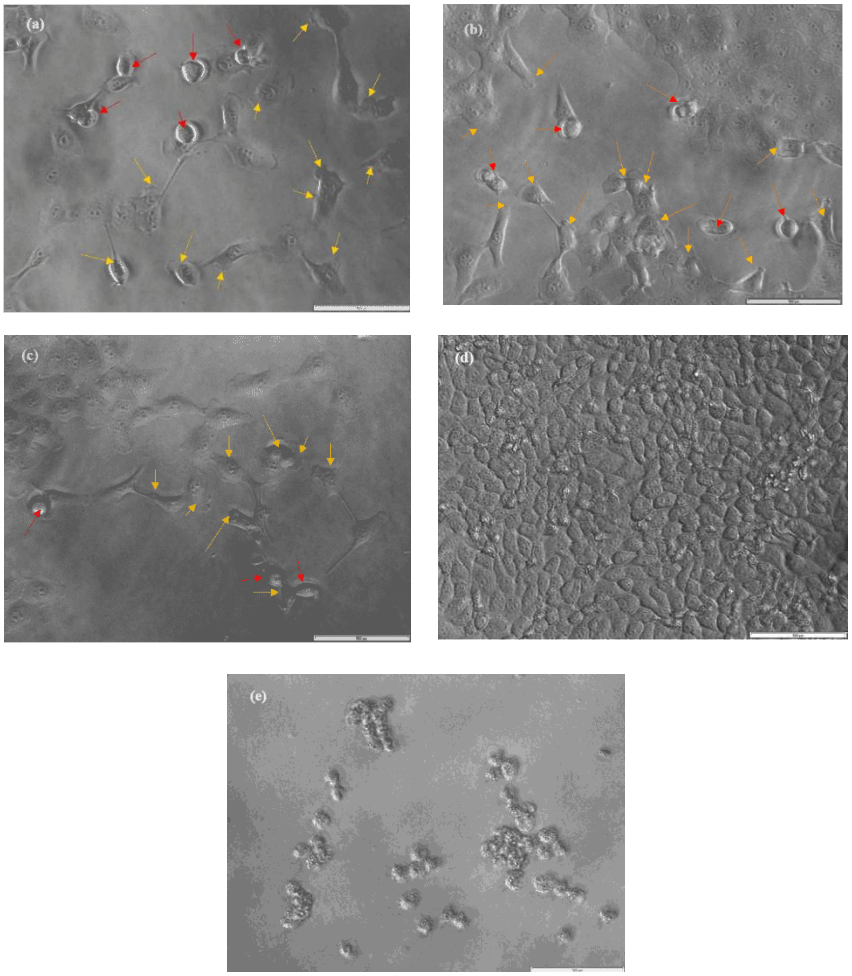

Figure 2. The presence of apoptosis sign in treated MCF-7 at IC_{50} value, observed under light microscope at 200X magnification. (a) MCF-7 treated with BCH, (b) MCF-7 treated with SAH (c) MCF-7 treated with SAEA (d) Negative control (e) Positive control. The arrow in red indicates apoptotic body while the arrow in yellow indicates membrane blebbing in MCF-7

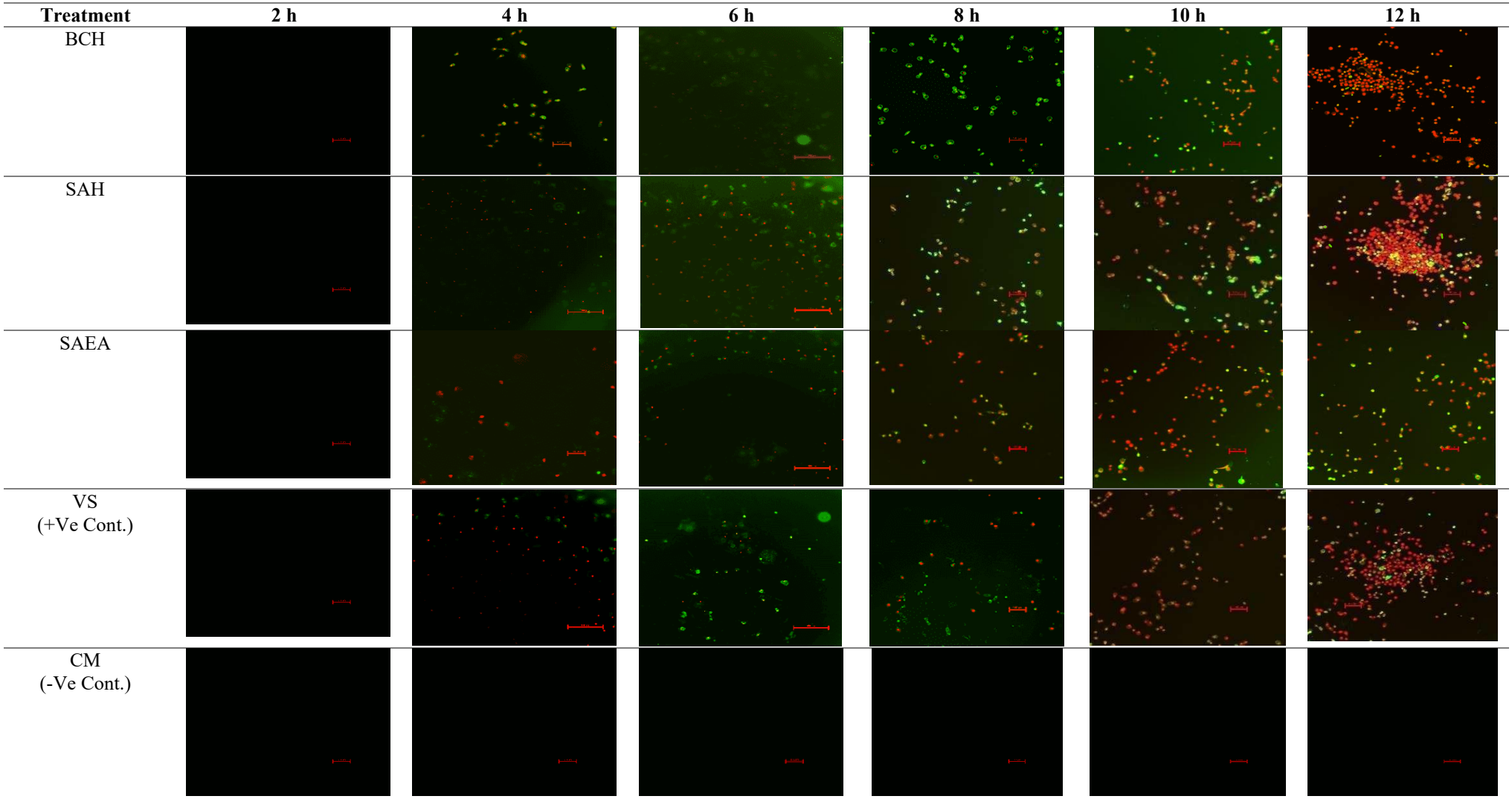

Figure 3. Apoptosis detection in MCF-7 cells treated with BCH, SAH, and SAEA at IC_{50} concentrations determined from the MTS assay. Observations were recorded every 2 h over a 12 h period using a fluorescence microscope (magnification 100×), with vincristine sulphate serving as the positive control for apoptosis and untreated cells in complete media as the negative control.

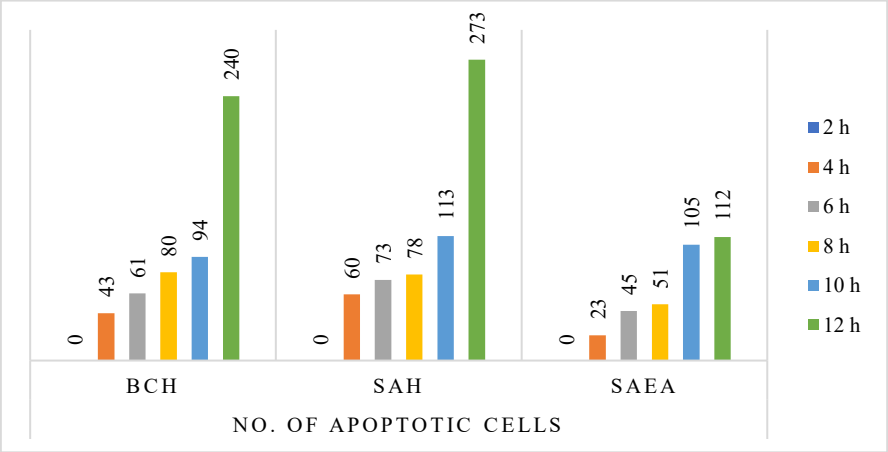

Figure 4. Quantification of MCF-7 cells undergoing apoptosis following treatment with BCH, SAH, and SAEA at their respective IC_{50} concentrations. Cell counts were obtained from **Figure 3** to assess the effects of incubation period and the apoptosis-inducing potential of the extracts

Evaluation of cell viability by trypan blue exclusion

To further demonstrate the efficacy of BCH, SAH, and SAEA as potent anticancer agents, these three extracts at their IC_{50} concentrations were tested in a cell viability assay to assess their ability to reduce the MCF-7 population over time. In this study, MCF-7 treated with IC_{50} concentrations of BCH (7.76 ± 0.18 µg/mL), SAH (3.47 ± 0.20 µg/mL), and SAEA (27.8 ± 0.11 µg/mL) were stained with trypan blue dye at a ratio of 1:1 at 24 h, 48 h and 72 h. The number of cells was automatically counted using DeNovix CellDrop™ (**Figure 5**).

The numbers of living and dead MCF-7 cells varied with the type of treatment and the treatment period. All three extracts reduced MCF-7 viability compared with the negative control, with the SAH extract being the most effective. Relative to complete media (negative control) that maintained MCF-7 viability within the range of 96% to 97% throughout the 3-day treatment, all three extracts reduced MCF-7 viability below 50% after 72 h. BCH reduced cell viability from the initial 92.15% (24 h) to 63.96% (48 h) and 48.76% (72 h). A similar trend was observed for SAH treatment, with MCF-7 being 90.70% viable at 24 h, decreasing to 73.16% at 48 h, and 47.66% after 72 h. SAEA treatment reduced cell viability from 83.81% to 73.16% and 48.79% throughout the 72-hour treatment period.

The trypan blue exclusion assay results further supported the MTS and apoptosis assay results, confirming that the twig extracts of *B. cylindrica* and *S. alba* have promising anticancer properties. This finding aligned with Chaudry [46], where a reduction in MCF-7 cells was observed to be time-dependent after treatment with *B. gymnorrhiza* leaf extracts. Huang [47] reported a comparable finding: the mangrove ethyl acetate extract from *Avicennia marina* leaves reduced the proliferation of breast cancer cell lines. Taken together, this evidence confirms the anticancer potential of BCH, SAH, and SAEA.

Antioxidant analysis

Next, the antioxidant activities of BCH, SAH, and SAEA were evaluated using a DPPH radical scavenging assay and quercetin as the positive control (**Table 3**). Among the extracts, only SAEA exhibited notable antioxidant activity, comparable to that of quercetin. It was observable that *S. alba* in mid-polar ethyl acetate possessed antioxidant activity, but neither *S. alba* nor *B. cylindrica* in hexane extracts.

This indicates that a mid-polarity solvent exerted better antioxidant effects than a nonpolar hexane solvent [47] stated the same trend with *B. gymnorrhiza* sp., where the leaf methanolic extracts confer the highest antioxidant activity, while n-hexane is the lowest. It was reported that the antioxidant capacity is 2-18 times higher in polar solvents than in nonpolar solvents [48]. Indeed, higher-polarity solvents were found to be effective for extracting strong antioxidants, which are mostly polar [49]. This study demonstrated that SAEA showed stronger antioxidant potential than quercetin, as its achieved IC_{50} (0.125 ± 0.001 mg/ml) was lower than that of the positive control, quercetin (0.157 ± 0.002 mg/ml).

Although significant antioxidant activity was observed only in SAEA, this provides additional support for the anticancer potential of the extracts, as free radical scavenging may help reduce oxidative stress, a key contributor to cancer progression, thereby enhancing their overall therapeutic value.

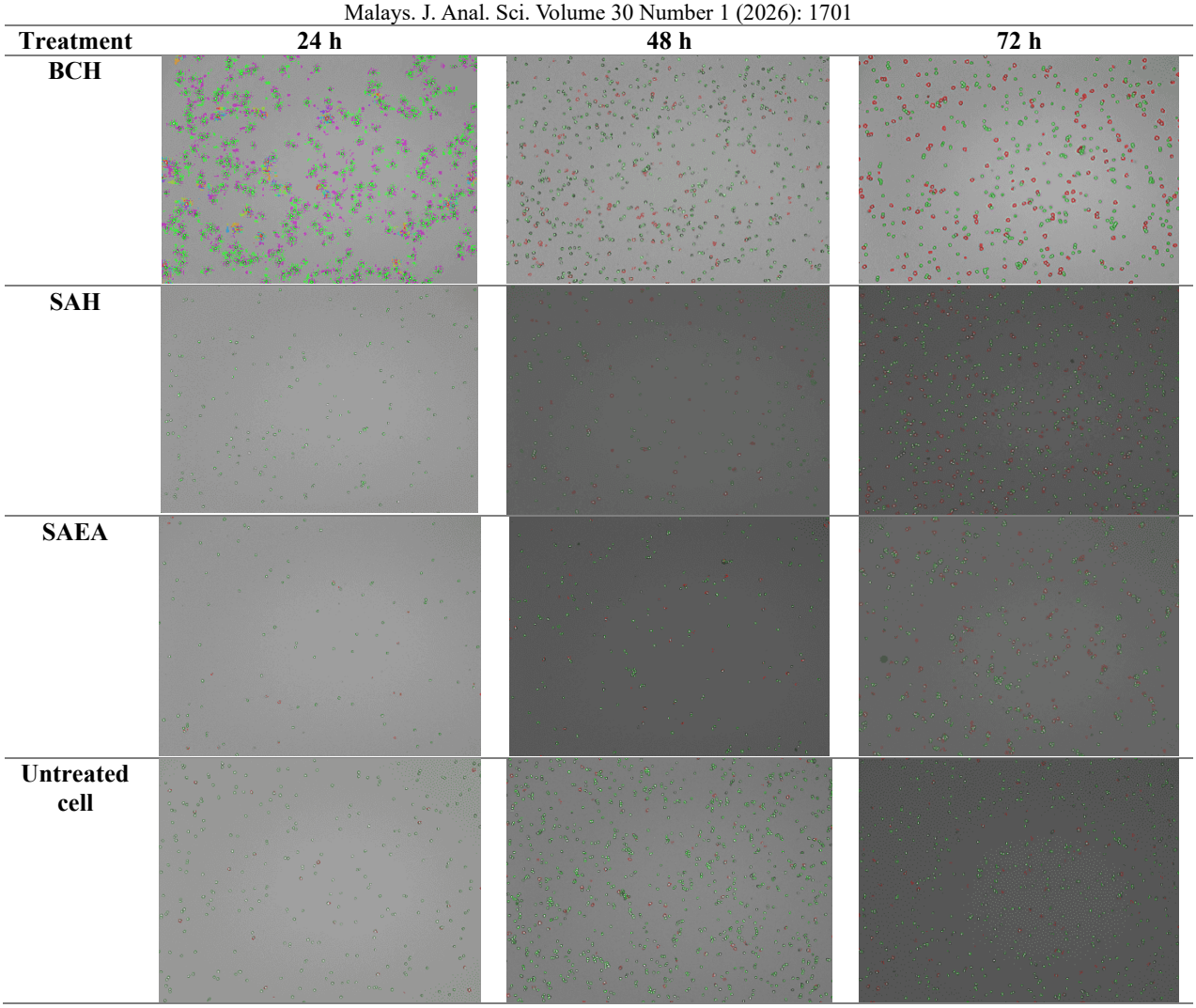

Figure 5. Enumeration of MCF-7 cells after 24 h, 48 h, and 72 h of incubation. Green markers indicate viable cells, while red markers denote non-viable cells. Untreated MCF-7 cells served as the negative control, demonstrating normal proliferation in the absence of treatment.

Table 3. IC_{50} value of DPPH Radical Scavenging Activity. (Each assay was conducted in triplicate, n=3.The IC_{50} values are stated in Mean ± S.D

Sample	IC_{50} ± S.D (mg/mL)
BCH	-
SAH	-
SAEA	0.125 ± 0.001
Quercetin (-Ve Cont.)	0.157 ± 0.002

The dash symbol (-) represented sample failed in achieving IC_{50}

Analytical evaluation of phytochemical constituents via LC–MS/MS

Liquid chromatography–mass spectrometry (LC–MS) was performed on BCH, SAH, and SAEA to identify the phytochemicals responsible for their anticancer and antioxidant effects. Each extract contained hundreds of bioactive compounds, consistent with the well-established abundance of phytochemicals in mangroves. In line with current oncology approaches that emphasize the identification of anticancer compounds with synergistic effects, the raw data were analyzed to pinpoint phytochemicals exhibiting both antioxidant and anticancer properties. These compounds are presented in **Table 4** (BCH), **Table 5** (SAH), and **Table 6** (SAEA), respectively.

Table 4. Phytochemicals responsible for both anticancer and antioxidant properties in BCH

No.	Rt (Min)	m/z	Identified Compound	Class of Compound	Formula	Mode
1	21.581	318.30032	Phytosphingosine	1,3-aminoalcohols	$C_{18}H_{39}NO_3$	+
2	17.344	338.34247	Erucamide	Fatty amides	$C_{22}H_{43}NO$	+
3	14.836	161.0596	4-Methylcoumarin	Coumarins and derivatives	$C_{10}H_8O_2$	+
4	14.697	169.01318	Gallic acid	Gallic acids	$C_7H_6O_5$	-
5	14.641	179.07013	4-Methoxycinnamic acid	Cinnamic acids	$C_{10}H_{10}O_3$	+
6	14.394	487.34378	Asiatic acid	Triterpenoids	$C_{30}H_{48}O_5$	-
7	11.41	301.21564	Grandifloric acid	Kaurane diterpenoids	$C_{20}H_{28}O_2$	+
8	6.31	137.02356	4-Hydrobenzoate	Hydroxybenzoic acid derivatives	$C_7H_6O_3$	-
9	6.177	135.11694	CAMPHOR (1R)	Bicyclic monoterpenoids	$C_{10}H_{16}O$	+
10	5.973	197.11717	Loliolide	Benzofurans	$C_{11}H_{16}O_3$	+
11	5.459	173.08118	Suberic Acid	Medium-chain fatty acids	$C_8H_{14}O_4$	-
12	5.196	153.05501	Vanillin	Methoxyphenols	$C_8H_8O_3$	+

Table 5. Phytochemicals responsible for both anticancer and antioxidant properties in SAH

No.	Rt (Min)	m/z	Identified Compound	Class of Compound	Formula	Mode
1	17.335	338.34232	Erucamide	Fatty amides	$C_{22}H_{43}NO$	+
2	16.644	149.02339	Phthalic anhydride	Phthalic anhydrides	$C_8H_4O_3$	+
3	14.67	161.06	4-Methylcoumarin	Coumarins and derivatives	$C_{10}H_8O_2$	+
4	14.67	179.07071	4-Methoxycinnamic acid	Cinnamic acids	$C_{10}H_{10}O_3$	+
5	14.53	177.05444	Ferulate	Hydroxycinnamic acids	$C_{10}H_{10}O_4$	+
6	12.756	297.24347	Ricinoleic acid	Lineolic acids and derivatives	$C_{18}H_{34}O_3$	-
7	12.257	279.23218	Linolenic acid	Lineolic acids and derivatives	$C_{18}H_{30}O_2$	+
8	11.019	170.09679	Diphenylamine	Aniline and substituted anilines	$C_{12}H_{11}N$	+
9	6.416	177.05496	Coniferyl aldehyde	Methoxyphenols	$C_{10}H_{10}O_3$	-
10	6.379	179.07079	Coniferaldehyde	Methoxyphenols	$C_{10}H_{10}O_3$	+
11	6.185	187.0966	Azelaic acid	Medium-chain fatty acids	$C_9H_{16}O_4$	-
12	5.961	197.11781	Loliolide	Benzofurans	$C_{11}H_{16}O_3$	+
13	5.317	173.08072	Suberic acid	Medium-chain fatty acids	$C_8H_{14}O_4$	-
14	5.185	153.05498	Vanillin	Methoxyphenols	$C_8H_8O_3$	+
15	3.227	122.09679	Phenylethylamine	Phenylethylamine	$C8H_{11}N$	+

Table 6. Phytochemicals responsible for both anticancer and antioxidant properties in SAEA

No.	Rt (min)	m/z	Identified Compound	Class of Compound	Formula	Mode
1	21.564	318.30017	Phytosphingosine	1,3-aminoalcohols	$C_{18}H_{39}NO_3$	+
2	10.601	269.04565	Aloe-emodin	Anthraquinones	$C_{15}H_{10}O_5$	-
3	9.322	371.03943	Norstictic acid	Diaryl ethers	$C_{18}H_{12}O_9$	+
4	7.536	271.06049	Naringenin	Flavanones	$C_{15}H_{12}O_5$	-
5	7.099	285.03958	3',4',5,7-tetrahydroxyflavone	Flavones	$C_{15}H_{10}O_6$	-
6	6.425	301.03467	Quercetin	Flavonols	$C_{15}H_{10}O_7$	-
7	6.191	275.0914	Phloretin	2'-Hydroxy-dihydrochalcones	$C_{15}H_{14}O_5$	+
8	6.148	177.05476	Coniferyl aldehyde	Methoxyphenols	$C_{10}H_{10}O_3$	-
9	6.062	137.02354	4-Hydroxybenzoate	Hydroxybenzoic acid derivatives	$C_7H_6O_3$	-
10	5.947	197.11711	Loliolide	Benzofurans	$C_{11}H_{16}O_3$	+
11	5.883	193.04951	Ferulic acid	Hydroxycinnamic acids	$C_{10}H_{10}O_4$	-
12	4.601	167.03429	Vanillic acid	M-methoxybenzoic acids and derivatives	$C_8H_8O_4$	-
13	4.485	179.03415	Esculetin	6,7-dihydroxycoumarins	$C_9H_6O_4$	+
14	4.467	177.01871	6,7-Dihydroxycoumarin	6,7-dihydroxycoumarins	$C_9H_6O_4$	-
15	4.217	135.04417	4-Hydroxyacetophenone	Alkyl-phenylketones	$C_8H_8O_2$	-
16	4.217	165.05452	3-hydroxy-1-(4-hydroxyphenyl) propan-1-one	Alkyl-phenylketones	$C_9H_{10}O_3$	-
17	4.191	183.02901	Methyl gallate	Galloyl esters	C8H8O5	-
18	3.903	137.02312	Salicylic Acid	Salicylic acids	$C_7H_6O_3$	-
19	3.19	122.09677	Phenylethylamine	Phenethylamines	$C_8H_{11}N$	+

From BCH, 12 phytochemicals with both anticancer and antioxidant activities were identified. These included major phytochemical classes such as coumarins (e.g., 4-methylcoumarin), triterpenoids (asiatic acid), kaurene diterpenoids (grandifloric acid), and methoxyphenols (vanillin). In SAH, 15 compounds exhibiting dual anticancer and antioxidant properties were detected. SAH was found to contain key phytochemical classes such as coumarins, terpenoids, and phenols, along with an alkaloid, phenylethylamine. LC–MS/MS analysis of SAEA revealed 19 bioactive compounds with synergistic anticancer and antioxidant effects. Notably, SAEA contained three potent antioxidant compounds: aloe emodin, quercetin, and phloretin.

Interestingly, *B. cylindrica* and *S. alba* may possess therapeutic potential due to their abundance of phytochemicals that act synergistically as both anticancer and antioxidant agents in suppressing breast cancer proliferation. Studies have demonstrated that coumarins are particularly significant anticancer compounds, as they inhibit key cell proliferation pathways such as PI3K, AKT, and mTOR, while simultaneously activating apoptotic proteins in lung cancer and regulating ROS levels [50,51]. Terpenoids and their derivatives have been shown to suppress tumors at early stages by inducing cell cycle arrest and apoptosis, thereby preventing further angiogenesis and metastasis. Plant phenolics are reported to alter the genomic content of cancer cells, disrupting signal transduction and ultimately leading to apoptosis [52-54]. Phytosphingosine has been found to induce cell cycle arrest at the G_2/M phase and promote oxidative stress in cancer cells, triggering the intrinsic apoptosis pathway in both lung and breast cancers [55]. Erucamide mediates mitochondrial-dependent apoptosis through downregulation of UNC5B receptors [56]. Ferulate delivers anticancer properties through cell cycle arrest in the G_0/G_1 phase, further inhibiting cancer from proliferating and becoming invasive [57]. Reported in a study by [58], norstictic acid was found to inhibit the MDA-MB-231 cancer cell line while showing low cytotoxicity towards the normal mammary cell line (MCF-10). It was demonstrated that naringenin, a type of flavanone, causes cell cycle arrest at G_0/G_1 in MDA-MB-231, causing the cancer cell to die in the apoptosis pathway [59,60].

From this study, *B. cylindrica* in hexane extract, and *S. alba* in hexane and ethyl acetate extract possessed potential for further study in the development of supplemental, natural based chemotherapeutic agent in the treatment of breast cancer. Overall, the major phytochemical classes identified in the extracts are consistent with those previously reported for *B. cylindrica* and *S. alba*. Minor variations in individual compounds are expected due to differences in extraction methods, plant origin and analytical conditions. These observations collectively support the reliability of the present phytochemical profiles.

Conclusion

The anticancer and antioxidant potential of *Bruguiera cylindrica* and *Sonneratia alba* extracts against MCF-7 cells was elucidated. Hexane and ethyl acetate twig extracts of both species successfully inhibited more than 50% of MCF-7 cell growth. These extracts were confirmed to induce cell death through the apoptosis pathway. Further testing demonstrated that the extracts significantly reduced MCF-7 viability to below 50% after a 72-hour treatment period. Notably, the ethyl acetate extract of *S. alba* exhibited strong antioxidant activity, which may synergistically contribute to reducing cancer progression. The presence of major phytochemical compounds in *B. cylindrica* and *S. alba*, including phenols, alkaloids, flavonoids, terpenoids, and their derivatives, highlights mangroves as promising candidates for the development of supplementary chemotherapeutic agents in breast cancer treatment. To further validate the efficacy of mangroves in oncological applications, additional studies using animal models are required before advancing to standard clinical procedures, thereby enabling a deeper understanding of their anticancer mechanisms of action.

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