

Original Research Article

***Barringtonia Asiatica* Seed Extract Induces G1 Cell Cycle Arrest in *Saccharomyces Cerevisiae* and Exhibits Cytotoxicity in A2780 Human Ovarian Cancer Cells**

ABSTRACT

Barringtonia asiatica, is traditionally used as an herbal plant in the Philippines; unfortunately, there is limited evidence supporting its anticancer capability. Thus, our study investigated the cytotoxic effect of *B. asiatica* seed extract against ovarian carcinoma A2780 (ECACC 93112519) and *Saccharomyces cerevisiae* as a cancer model organism. Seeds of the fruit of *B. asiatica* from Iloilo City, Philippines, were collected by cutting the fruit in half, followed by extraction in methanol. The seed extract was investigated for its effect on the cell cycle in *S. cerevisiae* by utilizing yeast budding experiment and imaging flow cytometry. Its cytotoxicity activity was also tested as well as a live/dead assay was conducted against ovarian cancer cells. Findings revealed the anticancer activity of *B. asiatica* and its capability to induce G1 cell cycle arrest in *S. cerevisiae*. Thus, *B. asiatica* seeds may serve as a potential source of natural compounds towards anticancer drug discovery.

Keywords:

Barringtonia asiatica, herbal plants; cancer yeast model; yeast budding experiment, antiproliferative assay; cytotoxicity

Abbreviations: PI, propidium iodide; ATCC, American Type Culture Collection; ECACC, European Collection of Authenticated Cell Cultures; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC₅₀, 50% cytotoxic concentration; PBS, phosphate buffer solution, CO₂, carbon dioxide; RPMI, Roswell Park Memorial Institute; RT, room temperature

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1. Introduction

The global incidence of ovarian cancer in 2020, according to the World Health Organization, has reached an estimation of 308,069 patients with a mortality rate of 62% [1] thus making ovarian cancer the eighth leading cause of cancer-related death globally [2]. The hallmark of ovarian cancer consists of the widespread and rapid proliferation of metastatic cells in the peritoneal cavity coupled with uncontrolled dissemination of metastatic cells and poor disease prognosis [3]. Patients receive the standard treatment for ovarian cancer including surgery followed by platinum-based chemotherapy [4]. However, the survival rate remains low (ca. 47%), predominantly due to the recurrence of chemotherapeutic resistance within several years after the initial treatment [2]. The resistance mechanism is due to the partial restoration of homology-directed DNA repair in cancer cells [3]. The high mortality rate in ovarian cancer, which is associated with recurrence brought by chemotherapeutic resistance, requires alternative treatment to prolong the overall survival of patients.

Historically, plants are known to provide effective drugs against cancers. The plant-derived drugs such as vinca alkaloids vinblastine and vincristine isolated from *Catharanthus roseus* [5,6], camptothecin, isolated from the bark of *Camptotheca acuminata* [7,8], and Paclitaxel

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(Taxol®) from Pacific yew tree (*Taxus brevifolia*) [9] have been clinically used in the cancer treatment as they affect the regulation of different molecular pathways in cancer progression. In recent years, many plant derived-natural products have played a promising vital role in cancer prevention and therapy, and these include quercetin [10], rhein [11], (endo)cannabinoids [12], resveratrol [13], curcumin[14], and green tea [15]. These phytochemicals modify normal cell proliferation and regulation, boost immune response, and involve a wide range of signaling pathways modified during carcinogenesis [10-15].

There are also medicinal and herbal plants known to have potential activities against ovarian cancer. For example, the bark, leaf, and seeds of *Azadirachta indica*, an evergreen plant that belongs to the Meliaceae family geographically found in tropical and subtropical areas, are traditionally exploited for its anticancer activity due to the polyphenols found in these plant parts [16,17]. In Chinese medicine, *Coptis chinensis* is a valuable plant that contains an isoquinoline alkaloid berberine found to arrest the tumor cycle in ovarian cancer cells [18]. The valuable benefits of medicinal plants in the treatment of cancer are also recognized in Ayurvedic medicine. For example, the locals use the leaves, roots, and stem extracts of *Withania somnifera*, commonly known as ashwagandha, to treat ovarian cancer [18]. The extract contains a family C-28 steroidal lactone triterpenoids known as withanolides found to be highly cytotoxic against cancer cells [19]. These reports demonstrate that medicinal and herbal plants are known traditionally to have anticancer activities.

The genus *Barringtonia*, a flowering plant belonging to the family Lecythidaceae, has a long history for its medicinal uses due to its numerous phytochemicals [20]. Majority of *Barringtonia*

species are native in the tropical and subtropical regions growing in Africa, southern Asia (Malaysia, Indonesia, and Philippines), Australia, and various islands of the Pacific and Indian Oceans. More than 60 species have been identified under this genus with nutritional and medicinal values [20]; however, *B. racemose* [21-24], *B. asiatica* [24-26], and *B. acutangular* [27-31] are the most well-studied species. In the Philippines, *B. asiatica* is known as a folkloric medicine [24-26] that can cure several ailments. One widespread use of *B. asiatica* based on traditional users and folkloric testimonies is scrapping the contents of the *B. asiatica* fruit and applying it as a poultice to treat cysts, goiter, boils, abscesses, and tumors [26]. With its known folkloric healing outcomes, the fresh *B. asiatica* fruit is sold in farmers' markets among the traditional medicinal plants and herbs in the Philippines. There were few studies documented on the anticancer activity of *B. asiatica*. Germanicol, a pentacyclic triterpenoid isolated from *B. asiatica* leaves, was found to be active against the HCT 116 cell line [32]. It was also studied that a mixture of betulinic acid and 22-O-tigloylcamelliagenin A showed cytotoxicity against the non-small cell lung adenocarcinoma (A549) cell line [33]. Due to limited evidence supporting the anticancer of *B. asiatica* seed extract, we investigated its effect on the cell cycle using budding yeast *S. cerevisiae* as a cancer model organism and its cytotoxicity against ovarian carcinoma.

2. Materials and Methods

2.1 Plant and Seed Collection

Mature fruits (5 kg) of *B. asiatica* were collected from the campus grounds of University of San Agustin (USA) located in Iloilo City, Philippines. The plant sample was authenticated by the National Plant Quarantine Services Division of the Department of Agriculture, Bureau of Plant

Industry, Iloilo City, Philippines. The collected fruits were cleaned, and the seeds were obtained by cutting the fruit in half and scraping off the seeds. The seeds were stored in a -80 °C freezer before extraction.

2.2 Extraction of *B. asiatica* seeds

The frozen seed samples were lyophilized (Martin Christ Alpha1-2 LD Plus Freeze-dryer) for several days until dry and ground into a powder using a mechanical grinder. The seed powder was then macerated in methanol for three days and filtered before concentrating. The filtrate was concentrated *in vacuo* to obtain the crude extract. The crude extract was stored in a -80 °C freezer.

2.3 Reagents, Standards, and Cell Lines

The RPMI-1640 medium, fetal bovine serum, penicillin, streptomycin, cisplatin, methylthiazolyldiphenyl-tetrazolium bromide, methylene blue, Live/Dead Cell Double Staining Kit, tetracycline, triton X, Sabouraud dextrose agar (SDA), and Sabouraud dextrose broth (SDB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The human ovarian carcinoma cells A2780 (ECACC 93112519) was purchased from Merck (Germany). *Saccharomyces cerevisiae* ATCC 20784 was purchased from ATCC (USA).

2.4 Antiproliferation Assay using *S. cerevisiae* cells

The 48 h broth culture of *S. cerevisiae* ATCC 20784 (0.05 OD cell density at 600 nm) in Sabouraud Dextrose Broth (SDB) was prepared and exposed to different treatments, namely water (negative control) and *B. asiatica* seed extract in two-fold serial dilution concentrations with a

dilution range of 3125 µg/mL – 6.1 µg/mL in a 96-well plate. The treated cells were incubated for 48 h at 35 °C and were harvested after the incubation period by centrifugation (3000 rpm for 1 min at 4 °C). The pelleted cells were resuspended in phosphate buffer solution (PBS, pH 7.4), and 20 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye was added accordingly. The mixture was incubated for 2-4 h in the dark, and absorbance of each treatment was measured at 570 nm using the microplate reader (BMG Clariostar Multimode Labtech, Germany) after incubation. Antiproliferation activity of the crude extract was determined by calculating the percent (%) growth inhibition and comparing it with the % growth inhibition of negative control. The experiment was done in triplicates for three trials.

2.5 Yeast Viability Assay with methylene blue

Here, broth culture of *S. cerevisiae* ATCC 20784 (0.3-0.4 OD cell density at 620 nm) was prepared by growing the cells in SDB for 35 °C for 48 h. After the incubation period, the cells were harvested by centrifugation (13,000 rpm for 10 min). The pelleted cells were resuspended in 190 µL of *B. asiatica* seed extract (5 mg/mL), and the mixture was incubated at 35 °C for 3 h. The treated cells were harvested by centrifugation (13,000 rpm for 10 min) and resuspended in 100 µL PBS (0.1 M). Methylene blue dye (0.1 mg/mL in 0.1 M phosphate buffer with pH 7.2) was added to the cells and incubated for 5 min at RT. Five µL of the mixture was mounted onto a glass slide and was observed under a microscope [34]. According to yeast budding patterns, the cells were classified and counted, namely unbudded cells, cells with a single small bud, cells with a single medium sized-bud, and cells with a single large bud [35]. Percent (%) accumulation of the different budding patterns was calculated, and the pattern with the highest % accumulation

was considered the target morphological stage of the crude extract. The experiment was done in triplicates for three trials.

2.6 Cell Viability of *S. cerevisiae* by Live/Dead Fluorescence Assay

Broth culture of *S. cerevisiae* ATCC 20784 (0.5 OD cell density at 620 nm) was prepared and exposed to DMSO (negative control), 5% Triton X (positive control), and *B. asiatica* extract (5 mg/mL) for 35 °C for 12 h. The treated cells were harvested by centrifugation (13,600 rpm for 3 min) and resuspended in 200 µL PBS. The cell suspension was added with 5 µL of dye mixture (equal volume of 1 mg/mL propidium iodide (PI) and 3 µM Syto-9 dyes) and incubated for 30 min in the dark. The dyed cells were harvested by centrifugation (13,600 rpm for 3 min) and resuspended in 20 µL PBS. The cells were then mounted onto a glass slide, and cell viability was observed using a fluorescence microscope (IX83 Olympus Inverted Fluorescence Microscope, USA) equipped with 60x magnification. The percent cell viability of the extract was then calculated and plotted in comparison to DMSO (negative control). The experiment was done in triplicates for three trials.

2.7 Flow Cytometry Imaging

The culture of *S. cerevisiae* ATCC 20784 in SDB (0.5 OD cell density at 620 nm) was prepared by allowing the cells to grow for 35 °C for 48 h. The inoculum (195 µL) was transferred onto 1.5 mL tubes, and DMSO (negative control) or *B. asiatica* extract (in 5, 2.5, and 1.25 mg/mL concentration) was added accordingly. The cells with treatments were incubated overnight at 35 °C. After incubation, cells were collected by centrifugation (8000 rpm for 5 min) and were washed twice with PBS (pH 7.4). The washed cells were resuspended in 200 µL of dye solution

(5 μ L of propidium iodide (1 mg/mL) and 5 μ L of calcein AM (4 mM) diluted in PBS to make 5 mL solution) and incubated in the dark for 15 min. Single-stained cells were also prepared for compensation in the acquisition of results. For single staining, another tube containing DMSO treated cells (negative control) was stained with calcein AM (0.8 μ M) only, and another tube of *B. asiatica* (5 mg/mL) treated cells were stained with propidium iodide (2 mg/mL). The cells were then collected, washed twice with PBS, and resuspended in 200 μ L PBS. Results were acquired using Amnis™ FlowSight imaging flow cytometer equipped with 488 nm laser. Ten thousand (10,000) cell events were acquired, and 93 % cell events were analyzed to eliminate cell debris. Propidium iodide fluorescence was measured at 642-745 nm band (Channel 5), and 86x calcein fluorescence was measured at 505-560 nm band (Channel 2). Analysis was done using IDEA.6.2.188 application software, considering zero rfu as the lowest fluorescence unit. Propidium iodide fluorescing cells (R1) were gated as dead cells, double-stained cells (R2) were gated as live cells with permeant cell membranes, and calcein fluorescing cells (R3) were gated as live cells ^[36]. Three technical replicates were analyzed to ensure the reproducibility of clustering patterns and images.

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2.8 Cell Culture

The human ovarian carcinoma cells A2780 (ECACC 93112519) were cultured and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 units/mL of penicillin, 100 μ g/mL of streptomycin and incubated in humidified 5% CO₂ at 37 °C for 24 h.

2.9 Cytotoxicity Assay

The cytotoxicity of *B. asiatica* was evaluated by MTT assay using the method of Dai et al. (2013)^[37], with modifications. Cells were plated in a 96-well plate in 100 µL medium. After 24 h of incubation, the medium was removed and replaced by a fresh medium containing *B. asiatica* crude extract (previously diluted in sterile distilled water) at various concentrations (0.49 to 2000 µg/mL) together with cisplatin as the positive control (4.1 µg/mL) diluted in 0.9 % sodium chloride and incubated for another 24 h in humidified 5% CO₂ at 37 °C. Subsequently, the medium was removed and replaced again with a fresh medium, and 10 µL of MTT (5 mg/mL) was added to each well, incubated for 4 h in 5% CO₂ at 37 °C. The medium with MTT was carefully removed and 100 µL of DMSO (100%) was added to dissolve the formazan crystals. The absorbance of dissolved formazan was measured at 570 nm using a microplate reader (BMG Clariostar Multimode Labtech, Germany). The IC₅₀ value was defined as the sample concentration that inhibited cell growth by 50% and was calculated using GraphPad Prism 8.4.3. Each test was conducted in triplicates. Percent cell viability was computed as follows:

$$\text{Cell viability (\%)} = ((AT - AB) / (AT_u - AB)) \times 100$$

Where in: AT is Absorbance of treated cells; AT_u is Absorbance of untreated cells; AB is Absorbance of blank

3.0 Cell Viability of Ovarian Carcinoma (A2780) by Live/Dead Fluorescence Assay

Ovarian carcinoma cells (A2780) at a cell density of 3.5 x 10⁴ cells/well were seeded in a 96-well plate and grew for 24 h at 37°C, 5% CO₂, and 85-90% relative humidity. After incubation, the media in each test well was removed and replaced with a freshly prepared RPMI medium. The wells were treated with *B. asiatica* seed extract at concentrations of 2000 µg/mL, 41.7 µg/mL, and 0.49 µg/mL. Cisplatin at a concentration of 8.2 µg/mL and sterile water were used as

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positive and negative controls, respectively. After adding the treatments, the 96-well plate was incubated for another 24 h at 37 °C, 5% CO₂, 85-90% relative humidity. After 24 h exposure to the treatments, cells were stained according to Live/Dead Cell Double Staining Kit (Sigma-Aldrich, Missouri, USA). First, the treated cells were washed twice with 1X PBS. After washing, the cells were resuspended in 100 µL of stain solution containing 1 µM of calcein-AM and 0.75 µM of propidium Iodide (PI). The 96-well plate was then incubated at 37 °C, 5% CO₂, 85-90% relative humidity for exactly 10 min. Cells were then observed, at 20x and 60x magnification, under a fluorescence microscope (IX83 Olympus Inverted Fluorescence Microscope, USA) in channels, U-FBNA for calcein-AM, and U-FGNA for propidium iodide.

3. Results

3.1 Antiproliferation Activity on *S. cerevisiae*

The yeast *S. cerevisiae* was used as the cancer model test organism to screen the antiproliferation activity of *B. asiatica* seed extract. The assay results showed that *B. asiatica* seed extract at 3125 µg/ mL to 195.3 µg/mL demonstrated 97%-99% growth inhibitory activity (**Fig 1**). Concentrations 48.8 µg/mL to 6.1 µg/mL showed 100% cell viability hence these values were considered non-toxic. *B. asiatica* seed extract is toxic against *S. cerevisiae* at concentrations of 195 µg/mL to 3125 µg/mL producing almost 100% growth inhibition.

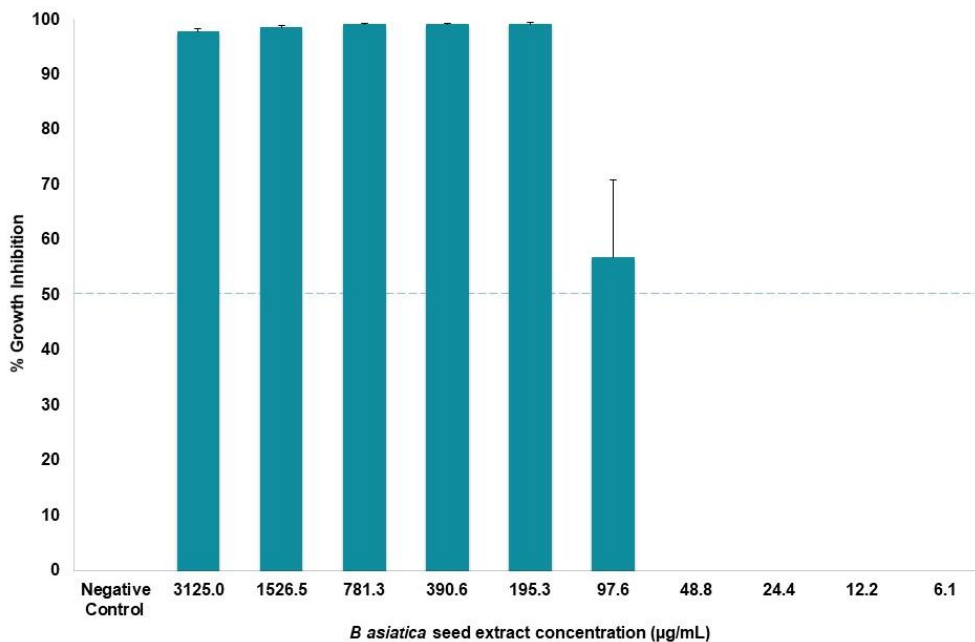


Fig 1: Antiproliferative activity of *B. asiatica* seed extract against *S. cerevisiae*. *B. asiatica* extract concentration from 98-3125 µg/mL showed growth inhibition greater than 50% with concentrations 195.3, 390.6, 781.3, 1526.5, and 3125 µg/mL exhibiting high antiproliferative activity of greater than 95% While concentrations of 6.1-48.8 µg/mL showed no activity against *S. cerevisiae*.

3.2 Cell Cycle Arrest Assay using *S. cerevisiae*

A budding yeast experiment using methylene blue dye was performed to determine which cell cycle stage is targeted by *B. asiatica* seed extract. Methylene blue is a basic dye that can only penetrate nonviable cells producing blue fluorescence, while the absence of the blue coloration indicates viable cell [34]. The results showed that *B. asiatica* seed extracts caused a high accumulation of single dead cells at 84.3% of the total population. A small fraction (10.37%) of dead cells with small bud, 4.17% of dead cells with medium bud, and 1.16% of dead cells with large bud formation were observed in the treatment of *B. asiatica* crude extract (**Fig 2**).

According to the yeast morphology (**Fig 3**), the budding pattern is dictated by the cell cycle process, which is depicted by the different phases (G1, S1, G2, M) of the yeast cell cycle.

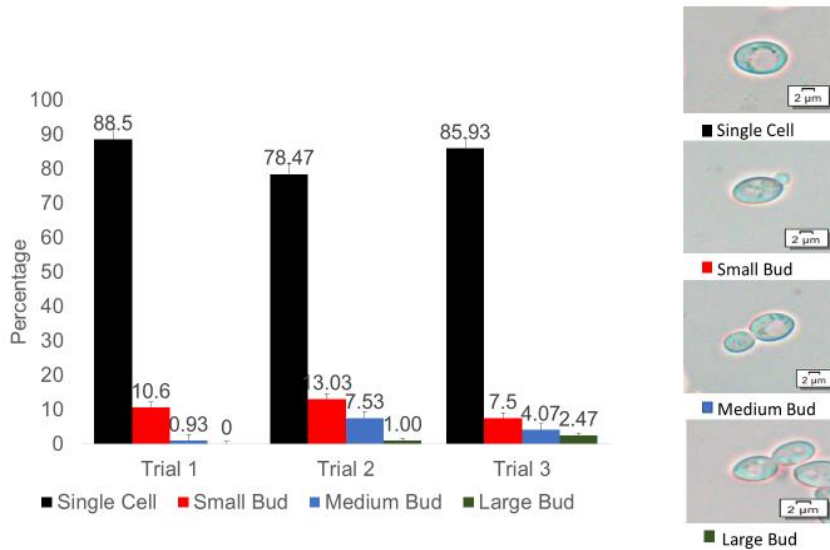


Fig 2: Percentage of Nonviable *S. cerevisiae* According to Budding Morphology. In a population of dead cells, the single cells were accumulated as dead cells as shown by the black bar compared to small bud, medium bud and large bud. The experiments were done in triplicates and three trials. Images were acquired using Olympus cellSens Dimension software version 1.18.

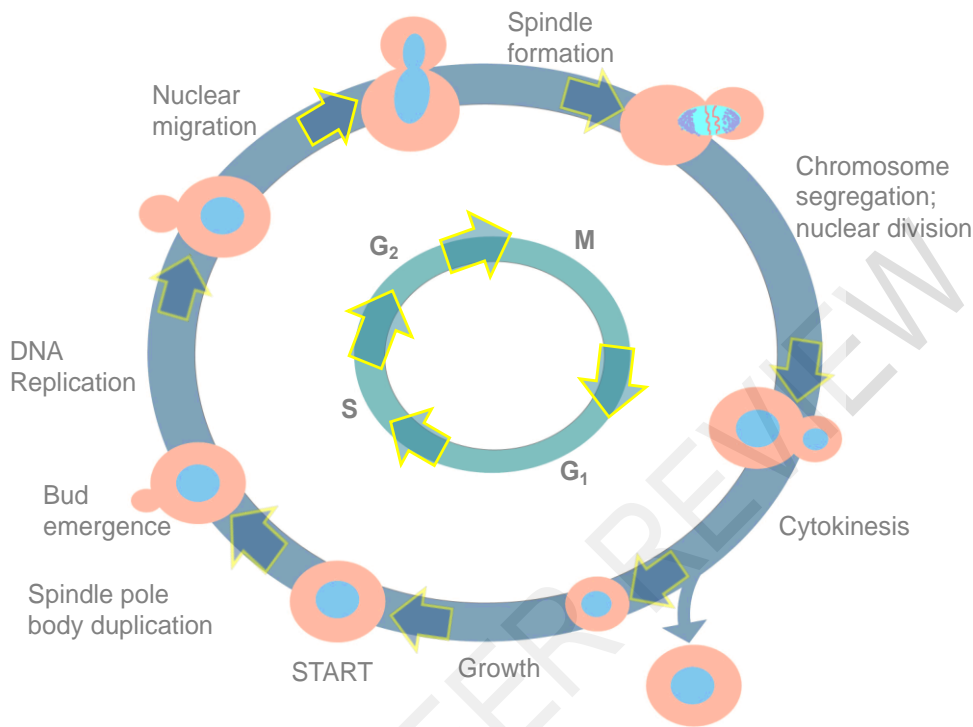


Fig 3: Yeast cell cycle. The process starts with G₁ phase followed by S phase, G₂ phase and M phase.

3.3 Cell Viability of *S. cerevisiae* (Live/Dead Fluorescence Assay)

The effect of *B. asiatica* seed extract on the integrity of the cell membrane was investigated using a live/dead staining assay utilizing a mixture of two fluorescent dyes. Syto 9, a membrane-permeable dye, stains viable/live cells with intact membranes emitting green fluorescence. On the other hand, propidium iodide is a membrane-impermeable dye that can only penetrate damaged membrane fluorescing red as it intercalates with the DNA [38-39]. The uptake of propidium iodide and Syto 9 by treated *S. cerevisiae* cells were visualized using an inverted fluorescence microscope (IX83 Olympus Inverted Fluorescence Microscope, USA) in U-FBNA and U-FGNA channels. DMSO treated *S. cerevisiae* displayed green fluorescence as shown in

Fig 4, indicating viable cells. On the other hand, the *B. asiatica* seed extract (5 mg/mL) treated *S. cerevisiae* cell showed red fluorescence indicating nonviable cells. To measure the fluorescence of each dye, viable and nonviable cells were counted and showed that 99% of the treated cells were able to uptake propidium iodide. However, only 1% of the treated cells were able to uptake Syto 9. This result suggests that *B. asiatica* seed extract damaged the cell membrane of *S. cerevisiae* that consequently led to cell death.

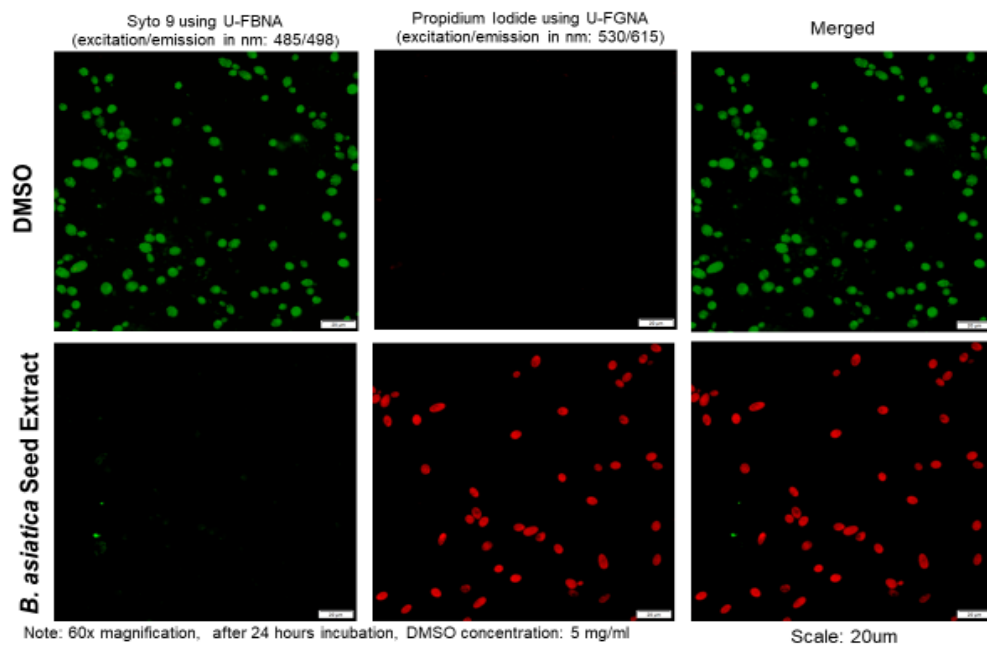


Fig 4: Live/Dead assay fluorescence microscopy of *S. cerevisiae* treatment with DMSO and *B. asiatica* seed extract. Syto 9 stains viable cells with green fluorescence which is exhibited by DMSO treatment shown on the 1st panel where the cells are alive. While propidium iodide stains nonviable cells with red fluorescence which is exhibited by *B. asiatica* seed extract treatment as shown on the 2nd panel where 99% were killed (displaying red fluorescence) and 1 % live (showing green fluorescence). Scale bar: 20 μ m. Images were acquired using Olympus cellSens Dimension software version 1.18.

3.4. Flow Cytometry

To further validate the effect of *B. asiatica* seed extract on cell membrane integrity or permeability of *S. cerevisiae* and confirm the cell cycle arrest results, flow cytometry imaging using calcein AM and propidium iodide as fluorescent probes were performed. Intracellular esterases of viable cells convert calcein AM (non-fluorescing compound but membrane permeant) to green fluorescing calcein (membrane impermeant), while propidium iodide only fluoresces (red) when it enters the cell, through damaged membrane or membrane with compromised permeability, and intercalates with the DNA of *S. cerevisiae* [40]. Cell populations were clustered into three regions where propidium iodide fluorescing cells (R1) were gated as dead cells, double-stained cells (R2) were gated as live cells with permeant cell membranes, and calcein fluorescing cells (R3) were gated as live cells. Results suggest that 90.55 % of the cell population treated with DMSO (negative control) were fluorescing with calcein (**Fig 5A - R3**) indicating that these cells were alive, while *B. asiatica* seed extract causes compromised cell membrane integrity or permeability on *S. cerevisiae* cells in a concentration-dependent manner, as shown in the scatter plot of *B. asiatica* – treated cells (**Fig 5**). *S. cerevisiae* cells treated with 1.25 mg/mL and 2.5 mg/mL concentration of the extract exhibited the same live cells with permeant membrane population (79.4 %) (**Fig 5B and 5C - R2**). This cell population has compromised cell membrane allowing the influx of propidium iodide dye, thus fluorescing with calcein and propidium iodide dyes. The highest concentration of *B. asiatica* seed extract at 5 mg/mL also exhibited the highest population (97.27 %) of dead cells with damaged membranes (**Fig 5D - R2**). Further analysis of this population of dead cells by flow cytometry suggests that 52 % belong to the G1 phase morphology (**Fig 6**).

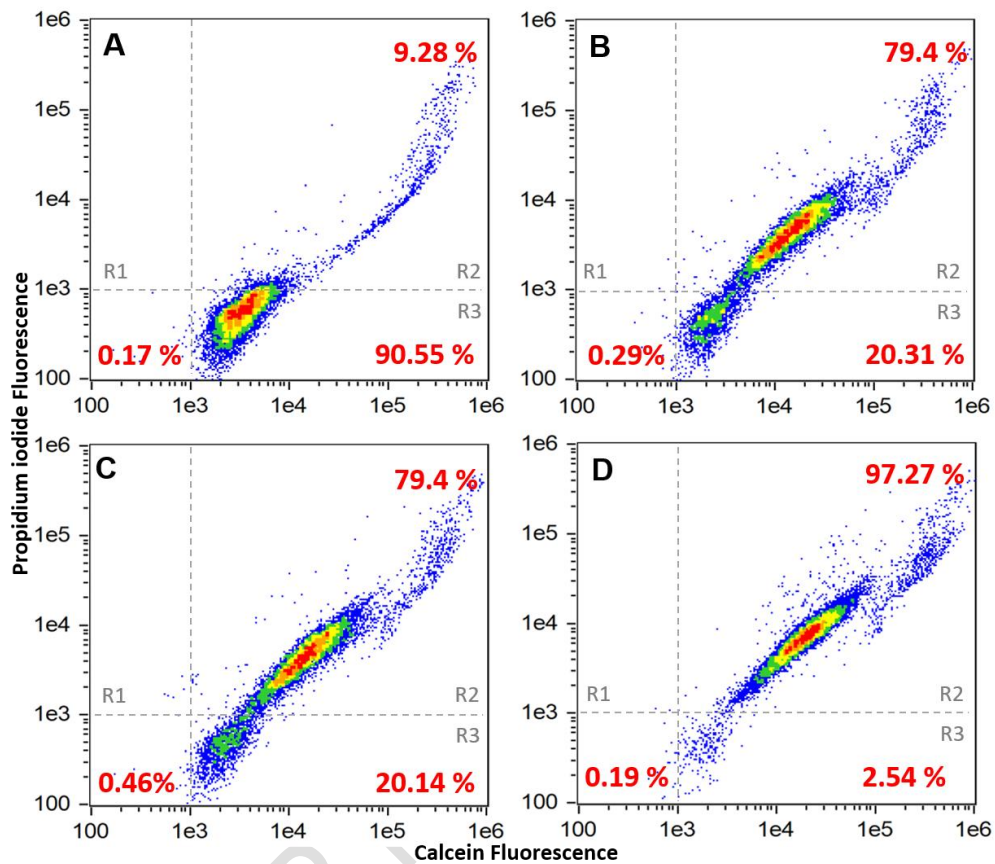


Fig 5: Concentration-dependent cell membrane permeability effect of *B. asiatica* seed extract. Data presented are the scatter plot of *S. cerevisiae* cells treated with (A) DMSO (negative control), (B) 1.25 mg/mL, (C) 2.5 mg/mL and (D) 5 mg/mL of *B. asiatica* crude extract. Cells were exposed to the treatment for 24 h incubation period and cell population were determined using calcein and propidium iodide fluorescent dyes. Results were acquired using Amnis™ FlowSight imaging flow cytometer and data was analyzed using IDEA.6.2.188. Release.86x application software considering zero rfu as the lowest fluorescence unit.

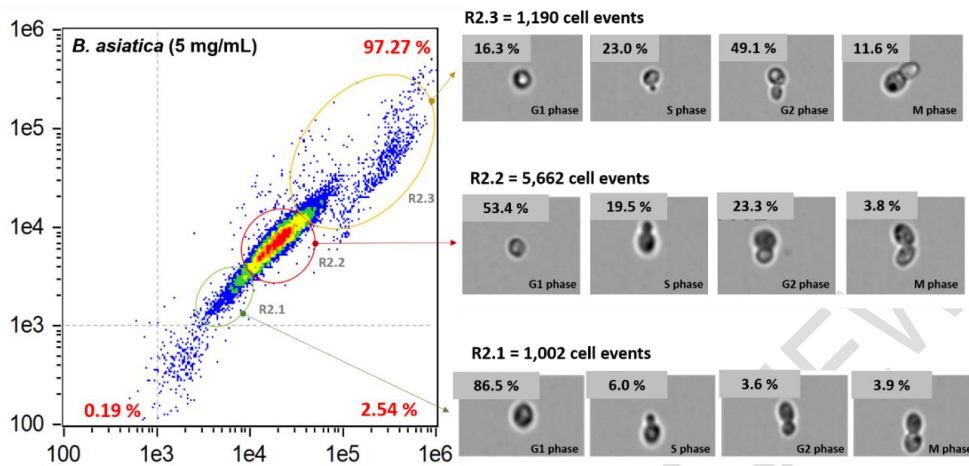


Fig 6: Morphology profile of membrane permeant *S. cerevisiae* treated with 5 mg/mL of *B. asiatica* seed extract. Data presented are the morphology profile of the different subregions of live cells with compromised cell membrane permeability treated with 5 mg/mL of *B. asiatica* seed extract. Results were acquired using Amnis™ FlowSight imaging flow cytometer and data was analyzed using IDEA.6.2.188.

3.5 Cytotoxicity Assay

Cytotoxicity assay was done to determine the anticancer activity of *B. asiatica* against human ovarian carcinoma A2780 (ECACC 93112519). The ovarian cancer cells were exposed to concentrations 0.49-2000 µg/mL (log concentration= 0-2.39 µg/mL) in two-fold serial dilution for 24 h and cell viability was evaluated using MTT assay. The results revealed that *B. asiatica* seed extract exhibited a dose-dependent decrease in human ovarian carcinoma cell viability as the concentration was increased (**Fig 7**), relative to the negative control (water). While Cisplatin at 4.1 µg/mL (log concentration= 0.61 µg/mL) concentration showed a 76% decrease in the viability of cells. The IC₅₀ value of *B. asiatica* was calculated at 35.08 µg/mL (log concentration= 1.55 µg/mL) using GraphPad Prism. 8.4.3.

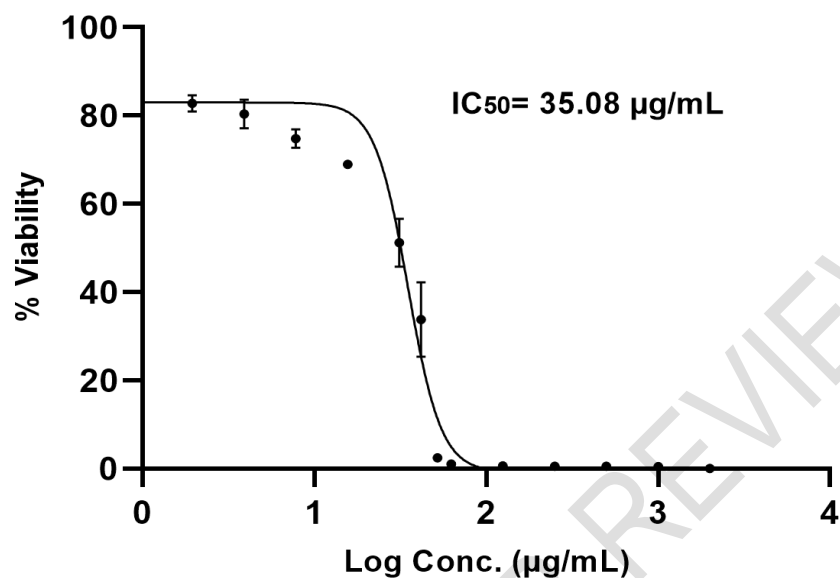
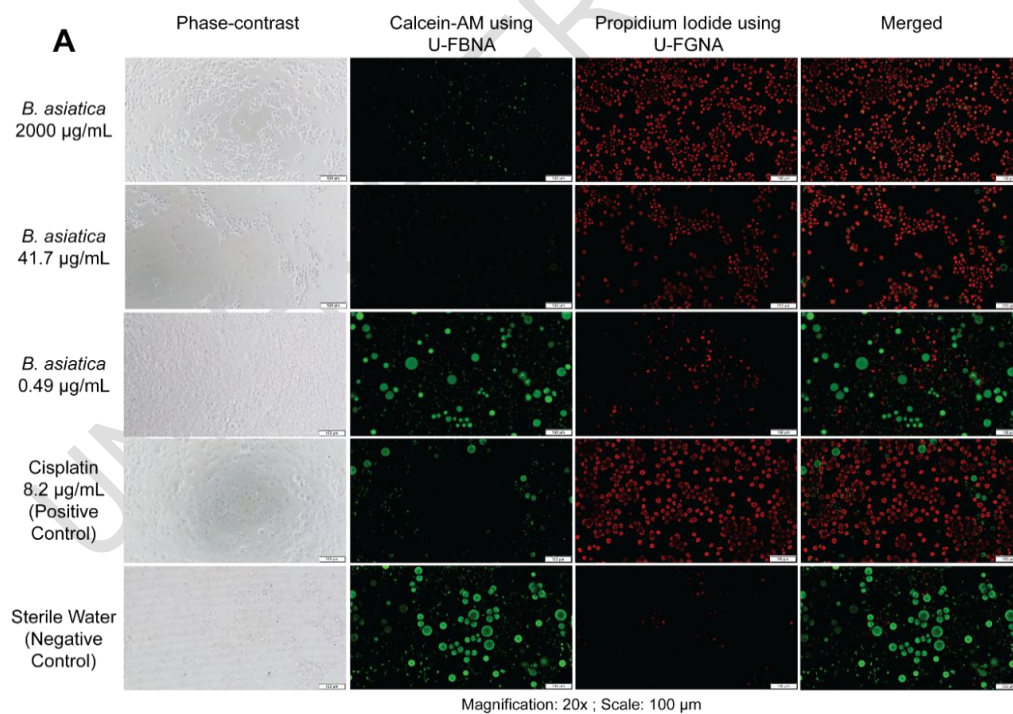


Fig 7: Percent viability of human ovarian carcinoma A2780 (ECACC 93112519) treated with *B. asiatica* seed extract. Result showed concentration-dependent decrease in percent cell viability as the concentration increases from 0.49-2000 µg/mL (log concentration= 0-3.3 µg/mL). IC₅₀ value was determined at 35.08 ug/mL (log concentration= 1.55 µg/mL) by GraphPad Prism 8.4.3 with values as ± Standard Error Mean (SEM), n=3 in triplicate.

3.6 Cell Viability of Ovarian Carcinoma (A2789) (Live/Dead Fluorescence Assay)

The anticancer efficacy of *B. asiatica* seed extract was further studied through live/dead-based fluorescence assay by staining the ovarian cancer cells A2780 (ECACC 93112519) exposed to the crude extract with calcein-AM and propidium iodide, dyes which was also used and described in the flow cytometry analysis of this study. In **Fig 8**, the negative control cells treated with sterile water showed bright green fluorescence and a very low or few PI positive cells, indicating that majority of the cells are viable or live cells. Live and healthy cells are easily recognized with their large size and spherical shape. While cells treated with the positive control, cisplatin at 8.2 µg/mL, are ruptured and are strained red, indicating that cells are

nonviable. Nonviable cells with the stained nucleus are smaller in size and have an irregular shape. Cells treated with *B. asiatica* seed extract at the lowest concentration, 0.49 $\mu\text{g/mL}$, showed the presence of calcein-AM positive cells. Still, as the concentration increased to 41.7 $\mu\text{g/mL}$ the calcein-AM positive cells decreased, and the PI positive cells increased, indicating that viable cells have declined. Simultaneously, at the highest concentration tested, 2000 $\mu\text{g/mL}$, all cells are PI positive emitting red fluorescence, indicating no more viable cells are present. Closely looking into the ovarian cancer cells in **Fig 8B**, the difference between the cells treated with 2000 $\mu\text{g/mL}$ and 41.7 $\mu\text{g/mL}$ crude extract can be seen. At 41.7 $\mu\text{g/mL}$, cells clustered together and shrunk compared to cells treated with 2000 $\mu\text{g/mL}$ where no clustering and shrinking was observed with the nonviable cells.



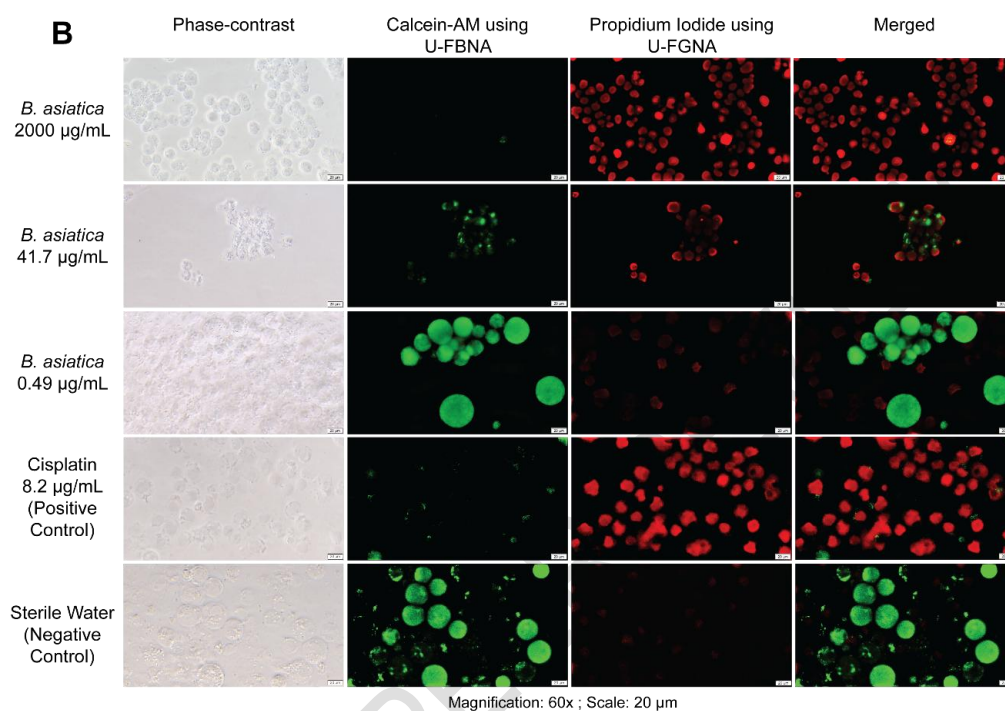


Fig 8: Live/Dead assay fluorescence microscopy of ovarian carcinoma A2780 (ECACC 93112519) treated with *B. asiatica* seed extract. (A) Cells exposed to *B. asiatica* seed extract at 2000 µg/mL and 41.7 µg/mL have shown membrane damage, with cells exhibiting red fluorescence on both concentrations tested. No viable cells can be observed at 2000 µg/mL treatment. Closer magnification (B) shows clustering and shrinking of cells that at 41.7 µg/mL and presence of live and healthy spherical shaped cells on the lowest concentration tested, 0.49 µg/mL. The seed extract of *B. asiatica* have exhibited permanent membrane damage on ovarian carcinoma A2780 (ECACC 93112519) on concentrations 2000 µg/mL and 41.7 µg/mL. Images were acquired using Olympus cellSens Dimension software version 1.18.

4. Discussion

The study on the antiproliferative activity of *B. asiatica* seeds is not well described. Thus, this study provides an insight into the ability of *B. asiatica* seeds extracts to inhibit the growth against model organism *S. cerevisiae*. The budding yeast *S. cerevisiae* was the first eukaryote to have its genome fully sequenced. It has been used successfully as an efficient tool and model

organism to study cell cycle control, DNA repair, aging, gene expression, autophagy, the molecular and cellular pathway of human diseases including cancer, as well as identification of drug targets, and mechanism of action studies [40-43]. Studies using *S. cerevisiae* as a model organism for cancer studies could represent a faster and cheaper solution to screen anticancer drugs and cytotoxicity.

B. asiatica seed extract is highly toxic in the antiproliferation assay against *S. cerevisiae* at concentrations between 3125 to 2 µg/mL, causing 96 % growth inhibition. Subsequently, *S. cerevisiae* treated with *B. asiatica* seed extract in the cell cycle arrest assay revealed that 86 % of single cells were accumulated in the population of dead cells. The cell cycle in *S. cerevisiae* is composed of 4 different phases: G1, S, G2, and M. In the G1 phase, cells prepare for duplication by reaching a threshold of “structures”, size, or organelles needed to support partition. During the S phase, the genetic information is duplicated. In the G2 phase, the cells get ready for partition. Finally, during the M phase, the initial cell is divided into two cells. The budding yeast morphology reflects the four different cell cycle phases, i.e., unbudded cells are in the G1 phase, small budded cells are in the S phase, and large budded cells are in the G2/M phase cell cycle. This study showed that *B. asiatica* seed extract targets the G1 phase, thus consequently causing permanent cell cycle arrest. The anti-proliferative activity of *B. asiatica* seed extract as indicated by the cessation of the progression of cell growth of *S. cerevisiae* at the G1 phase demonstrates its potential to target the cell cycle of mammalian cancer cells for anticancer activity.

We then determined whether *B. asiatica* seed extract affects the cell permeability of *S. cerevisiae*. In the Live/Dead Fluorescence Assay, propidium Iodide is a fluorescent dye that

cannot penetrate live cells and binds to DNA [40]. Therefore, an increase in the fluorescence of propidium iodide corresponds to an increase in the number of dead cells [44]. The result of the study conducted showed that *B. asiatica* promotes membrane damage, as demonstrated by propidium Iodide uptake. To confirm this finding, flow cytometry was performed. Flow cytometry analysis is a powerful tool utilized for the past decades to investigate cell cycle kinetics and the mechanism of action of bioactive compounds [45-49]. This present study focused on investigating the effect of *B. asiatica* seed extract on *S. cerevisiae* cell membrane by staining the cells with calcein AM to distinguish intact cell membrane and propidium iodide identify cells with damaged (raptured) cell membrane.

The cell membrane is one of the essential cell parts that serve as a barrier to the passage of ions and other compounds in and out of the cells [50]. In addition, it serves as protection to intracellular organelles and allows the maintenance of many cellular functions such as solute transport and regulation of metabolism [51]. Furthermore, the occurrence of damage membrane may be due to the formation of pores in the lipid membrane or thinning of the lipid bilayer or lipid-peptide domain formation. Cell membrane damage resulting in compromised cell membrane integrity and permeability allows intracellular leakage or abnormal cellular functions to eventually lead to cell death [52]. On an additional note, chemotherapeutic agents (anticancer agents) have two targets for action, 1) intracellular targets such as DNA, proteins and enzymes to name a few, or 2) extracellular targets such as the cell membrane, targets on membrane surfaces such as membrane proteins, or the relative abundance of lipids in the membrane [53-55]. The latter, having the cell membrane as the target, has been the less prioritized approach in searching for anticancer agents. Examples of these cell membrane targeting compounds are the less toxic peptides and have fewer side effects [55-57] and the strong antineoplastic compounds alkyl

phospholipids and alkyl phosphocholines [57]. These two compounds induce apoptosis by modulating cell membrane permeability and fluidity [58], amount of lipids in the membrane, phospholipid metabolism, and signal transduction proliferation [57] as their mechanism of action. In this context, plants belonging to the genus *Barringtonia* were already reported to have anticancer activity. A few examples to name are *B. maunwongyathiae* from Khuan Thon Forest in Thailand which was found to contain cancer chemopreventive compounds namely taraxerol, 3-(*E*)-coumaroyltaraxerol, and α -tocopherylquinone [59]; and *B. racemosa* which was found to contain gallic acid (antioxidant), lupeol (anticancer and cancer chemopreventive compound), and betulinic acid (antioxidant, antiproliferative, apoptotic, and antiangiogenic compound) [60].

The potential anticancer activity of *B. asiatica* seed extract against human ovarian carcinoma was demonstrated by the investigation conducted on *S. cerevisiae* as a cancer model organism. The MTT assay results showed dose-dependent manner cytotoxicity. It was exhibited as the percent (%) viability of cells decreased as the concentration was increased, with inhibitory concentration (IC_{50}) determined at 35.08 $\mu\text{g/mL}$. The presence of phytochemical constituents such alkaloid and flavonoid in *B. asiatica* are potential compounds eliciting the effect. The same compounds present in *B. racemosa* were previously reported to have cytotoxic activity against breast carcinoma (MCF-7) with an IC_{50} of 57.61 $\mu\text{g/mL}$ [61]. To note, *B. asiatica* also contains anticancer compounds such as polyisoprenoids which was reported to be present in the plant's leaves [62], betulinic acid (induction of apoptosis by triggering the mitochondrial pathway), α -amyrin (antitumor and cytotoxic activity), squalene (antitumor, antioxidant, and cancer chemopreventive agent), spinasterol (antiproliferative and antimutagenic), and β -sitosterol

(chemopreventive through free radical quenching) which were reported to be present in the fruits, leaves, stems, barks, and flowers [63].

The live/dead assay on the ovarian carcinoma A2780 (ECACC 93112519) demonstrated the ability of the *B. asiatica* seed extract to damage the cell membrane of the cells reducing the viable cells as the concentration of *B. asiatica* seed extract increases. The presence of numerous secondary metabolites found in *B. asiatica*, such as alkaloids, flavonoids, and glycosides, among others, have been previously reported in seed extracts. The efficacy of saponins, also present in the seed, against cancer cells has been attributed to their ability to inhibit cell proliferation, stimulate apoptosis, and reduce angiogenesis [64]. Therefore, these compounds plausibly account for the cytotoxic activity of *B. asiatica* seed extract against ovarian carcinoma (A2780) cell line in this study. Thus, purification and isolation of *B. asiatica* seed extract is imperative to determine the compound eliciting the cytotoxic effect.

5. Conclusions

We reported the anticancer potential of *Barringtonia asiatica*, a medicinal mangrove tree growing ubiquitously in the Philippines. This study demonstrates the anticancer activity of *B. asiatica* seeds against ovarian carcinoma A2780 (ECACC 93112519) and *S. cerevisiae* as cancer model organisms. Its anticancer mechanism of action is displayed by loss of cell viability, cell cycle arrest at the G1 phase, and loss of cell membrane potential. *B. asiatica* seed may, thus, serve as a potential source of lead compounds for anticancer drugs. Furthermore, the plants showing anti-proliferative activity can be used for the isolation of active phytochemicals. Further, these compounds can be tested against other human cancer cell lines for anticancer therapy. In this

study, we have also validated the use of *S. cerevisiae* as an effective and economical tool for screening anti-proliferative activities from plants towards anticancer drug discovery.

NOTE:

The study highlights the efficacy of "herbal" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Comment [A6]: Chk all references used in this study. There are total 64 references used in manuscript and list shows 63 references. Write all references in same and standard formate.

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