

RESEARCH ARTICLE

Editorial Process: Submission:09/14/2025 Acceptance:07/08/2026 Published:07/26/2026

Exploring the Antimitotic Potential of Benincasa Hispida Seed Extract on HepG2 Cells

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Abstract

Objective: Cancer continues to pose a significant worldwide health concern, underscoring the need for the identification of innovative, safer, and more effective therapeutic agents. Phytochemicals obtained from medicinal plants are progressively acknowledged for their anticancer efficacy. This work investigates the antimitotic and antiproliferative properties of the ethanolic extract of Benincasa hispida seeds (*EeBHS*), known as winter melon, from the Cucurbitaceae family. **Methods:** Antimitotic activity was assessed using the Allium cepa root tip meristem test and the seed germination assay. The mitotic index and the distribution of cells throughout several mitotic stages were examined after treatment with *EeBHS* at varying doses. Methotrexate, a recognized antineoplastic agent, served as the standard control. The antiproliferative activity of *EeBHS* was evaluated using the MTT assay on HepG2 liver cancer cell lines to determine cytotoxicity and growth suppression. **Results:** *EeBHS* exhibited a notable dose-dependent suppression of mitotic activity in Allium cepa root tips and sprouted seeds ($p < 0.001$). The mitotic index significantly decreased in *EeBHS*-treated groups (57 ± 0.5 in root tips and 44 ± 1.2 in seeds) relative to controls (67 ± 1.2 and 96 ± 1.2 , respectively). Methotrexate also lowered the mitotic index, thereby corroborating the concept. An increased percentage of cells in prophase and metaphase in treated groups signified mitotic arrest. In HepG2 cells, doses of *EeBHS* ranging from 5 to 7 ng/ μ L markedly suppressed cell growth, indicating potent cytotoxicity. **Conclusion:** *EeBHS* has significant antimitotic and antiproliferative properties, presumably attributable to its bioactive constituents, including flavonoids, alkaloids, and phenolics, underscoring its potential as a natural anticancer agent that merits further investigation.

Keywords: Anti-mitotic activity- Benincasa hispida Seed Extract- Allium cepa root tip assay- seed germination assay

Asian Pac J Cancer Prev, 27 (7), 2535-2540

Introduction

Cancer constitutes a significant worldwide health issue and persists as a primary source of illness and mortality, resulting in millions of humanities each year. It is marked by the atypical growth of cells and the avoidance of intrinsic cell death processes like apoptosis [1]. Natural products, especially those sourced from medicinal plants, have historically been fundamental in medication discovery and development. Historical and ethnopharmacological data substantiates the utilization of several plant species in the treatment of ailments, including cancer [2]. A substantial percentage of contemporary chemotherapeutic drugs, originate from plants [3]. By 2050, almost 35 million additional cancer cases are anticipated, representing a 77% rise over the projected 20 million cases in 2022, underscoring the pressing demand for novel treatment agents [4].

Plant secondary metabolites, including alkaloids, flavonoids, terpenoids, phenolics, and glycosides,

demonstrate a wide range of pharmacological activity, such as antioxidant, anti-inflammatory, antibacterial, and antimitotic effects [5]. These phytochemicals can influence signalling pathways related to cell cycle control, apoptosis, and metastasis, rendering them intriguing candidates for anticancer drug development.

The essential principles of mitotic processes are most effectively examined in the actively proliferating parts of plants, such as the shoot or root apex. These investigations often utilize substances that alter the typical progression of mitosis [6]. A diverse array of secondary metabolites derived from plants is evaluated for their efficacy in cancer treatment. Numerous plant-derived anticancer agents are recognized for their efficacy against growing cells. They demonstrate a cytotoxic impact by disrupting cell-cycle dynamics. These pharmaceuticals are efficacious against proliferating cells and exert cytotoxic effects either by destroying DNA during the S-phase of the cell cycle or by inhibiting the development of the mitotic spindle in the M-phase [7]. However, the majority of cytotoxic agents

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demonstrate adverse consequences; hence, there is a want for medications that are effective and display fewer side effects. The root tip model of *Allium cepa* L. is frequently employed to assess pollutants from the environment, the toxicity of chemical substances, and to evaluate possible anticancer characteristics. It has been in use since 1938. It is quite convenient due to the simplicity of preparing onion roots. They consist of very homogeneous meristematic cells, with just 16 chromosomes that are notably elongated, highly observable, and readily stained. The test is a rapid and cost-effective approach, facilitating the exploration of universal processes in meristematic plant cells and their generalization to animal cells. The objective of this study was to examine the antimitotic action of *Benincasa hispida* [8].

Benincasa hispida (Thunb.), generally referred to as winter melon or ash gourd, is a member of the Cucurbitaceae family and is extensively utilized in Asia for both culinary and medicinal purposes. Historically, many components of *B. hispida*, particularly its seeds, have been utilized in Ayurvedic and traditional medicine to address ailments such as epilepsy, peptic ulcers, diabetes, and inflammation [9]. Extracts from the fruit, peel, and seeds have pharmacological activity, including hepatoprotective, diuretic, anxiolytic, and antibacterial properties. Nonetheless, its putative antimitotic and antiproliferative effects essential for impeding tumor progression yet investigated.

This work seeks to assess the antimitotic activity of the ethanolic extract of *Benincasa hispida* seeds (*EeBHS*) using *Allium cepa* root meristem and seed germination models, as well as its cytotoxic efficacy on HepG2 cells by MTT test. The results may enhance the existing evidence for the anticancer efficacy of plant-based formulations, facilitating the creation of natural and economical treatment options [10, 11].

Materials and Methods

Plant material collection and extraction

The fruit was purchased at the local market in Turkayamjal, Telangana, and authenticated by Dr. A. Vijaya Bhasker Reddy, Department of Botany, University College of Science, Osmania University, Hyderabad. The seeds of *Benincasa hispida* were dried in the shade and roughly ground. Then, using the Soxhlation method (Srinivasa merchants, Swavan instruments), the powdered material was extracted with 80% ethanol (Qualikem Vadodara). The resultant extracts were filtered and concentrated under reduced pressure utilizing a rotary flash evaporator (Adithya Scientific Pvt. Ltd, Re2A) to get the crude ethanolic extract (*EeBHS*), percentage yield was calculated and stored for further usage.

Phytochemical analysis

Preliminary phytochemical screening of *EeBHS* was performed for phenol, saponins, tannins, steroids, flavonoids, glycosides, alkaloids and carbohydrates by using standard reported methods [12].

Cell culture

HepG2 is a widely used human hepatoma (liver cancer) cell line derived from a well-differentiated hepatocellular carcinoma cell line, was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in DMEM containing 10% FBS, 1% antibiotics (Penicillin 100 IU/mL and streptomycin 100 µg/mL) in 5% CO₂. Cells were cultured to 70-80% confluence [11].

Determination of Mitotic Index [8, 9]

The red variety of *Allium cepa* (onions) was sourced from the local market and stored for the duration of the study. The bulbs were sprouted in tap water at room temperature for 48 hours. The resulting roots were then treated by immersing them in aqueous solutions of *EeBHS* at a concentration of 10 mg/ml for 2 hours [13]. Control treatments included roots dipped in distilled water as a negative control and roots treated with methotrexate (GSK, India) at 2.5 mg/ml as a positive control.

Following treatment, the root tips were carefully excised and transferred to a fixing solution composed of acetic acid (45% v/v) and ethanol (95% v/v) in a 1:3 ratio, and kept for 10-15 hours. After fixation, the root tips were softened by treating them with 1 N hydrochloric acid and incubated in an oven at 50°C for 15 minutes [14]. The softened root tips were then washed with distilled water and stained with 2-3 drops of Carmine stain (LR Central Drug House Pvt. Ltd). The stained root tips were squashed onto microscope slides and examined under a microscope.

For each treatment group, the number of cells in various stages of mitosis was counted across four different fields to assess the effects of the treatments on cell division [15].

Seed germination assay for in-vitro antimitotic and anti-proliferative activity

Experimental plant model *Vignaradiata* L. (Green-gram; Family: Fabaceae) was employed. They are of excellent quality and devoid of any external damage. It was bought from the neighborhood store. The cell cycle delay and metaphase arresting activity were measured using green-gram seedling root apical meristem cells in order to validate the antiproliferative properties.

In accordance with the 2018 International Rules for Seed Testing (ISTA) guidelines, *Vignaradiata* L. (Greengram) seedlings were treated and their germination test was conducted. For the germination of seeds, three different concentrations of 10mg/ml, 20mg/ml and 30mg/ml concentrations of *EeBHS* were placed in a 24 well microplate and *Vignaradiata* L. seed (n=30) was dropped into each well [16, 17]. The microplate was then covered with a lid and allowed to germinate at room temperature. Tap water alone in a microplate was considered as negative control. Both the test and control groups' root length and seed germination were assessed at 24, 48, and 72-hour intervals [13]. No further criteria could be measured on the seeds that failed to germinate; therefore they were just thrown away.

The following formula was used to get the mitotic index
Mitotic Index was calculated using the formula

$$\text{Mitotic Index} = \frac{\text{Number of dividing cells (P+M+A+T)}}{\text{Total number of cells}} \times 100$$

Where, P = Prophase, M = Metaphase, A = Anaphase, T = Telophase

MTT assay

HepG2 cells (1×10^6) were seeded in 96 well plates and treated with different concentrations of *EeBHS* (1, 2, 3, 4, 5, 6, and 7 $\mu\text{g}/\mu\text{L}$) and incubated for 24h at 37 °C, 5% CO₂. To determine any anti-proliferative activity of *EeBHS*, MTT reagent was added and further incubated for 3 h. Media was decanted and formazan crystals were dissolved in 1% DMSO. Absorbance was recorded at 570 nm using a Biotek multi-mode micro plate reader (Biotek instruments, Inc. USA). Cell death was calculated against the absorbance of control run well where *EeBHS* was replaced by Ethanol [18].

Results

Phytochemical profiling of *EeBHS*

Percentage yield was calculated and reported as 54.3%. *EeBHS* showed the presence of phytoconstituents like Phenols, Tannins, steroids, flavonoids, glycosides, alkaloids etc, shown in Table 1.

Assessment of anti-mitotic activity of *EeBHS* using seed germination assay

Seed germination anti-mitotic assays involve using seed germination tests to evaluate the anti-mitotic activity of various substances, serving as a useful tool for screening potential anticancer agents. These assays typically utilize easily accessible seeds such as green gram or Bengal gram, where the reduction in seed growth or germination rate indicates the presence of anti-mitotic effects shown in Figure 1.

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was applied to compare differences. *** $p < 0.001$; when compared with control. Values are represented as mean $\pm$ SD, $n = 3$. The antimitotic activity of the *EeBHS* was evaluated using green gram seeds, with different extract concentrations (10 mg/mL, 20 mg/mL, and 30 mg/mL). The control

group had the significant mitotic index (96.52%) in Figure 2, showing normal cell division. The standard drug (methotrexate) significantly reduced the mitotic index (35.31%) ($p < 0.001$), confirming its antimitotic effect. The *EeBHS* exhibited a dose-dependent decrease in the mitotic index, with 30 mg/mL showing the strongest inhibition (36.32%) ($p < 0.001$), and suggesting potential antimitotic properties of *EeBHS*.

Effect of *EeBHS* on Cell cycle phases in seed germination assay

Anti-mitotic extracts interfere with the cell cycle by disrupting specific stages of mitosis, resulting in alterations in the distribution of cells across different phases. A characteristic sign of their activity is a reduction in the percentage of cells undergoing mitosis (prophase, metaphase, anaphase, and telophase) and an increase in the proportion of cells in the G0/G1, S, or G2/M phases. This shift indicates inhibition of cell division.

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was applied to compare differences. *** $p < 0.001$; when compared with control. Values are represented as mean $\pm$ SD, $n = 3$. The treatment with different concentrations of *EeBHS* (10 mg/mL, 20 mg/mL, and 30 mg/mL) resulted in a significant effect ($P < 0.001$), Cell cycle analysis further revealed that increased the number of cells in the A and M phases in Figure 3. These findings imply *EeBHS* exhibits notable anticancer activity and holds potential for therapeutic application.

Assessment of anti-mitotic activity of *EeBHS* using The *Allium cepa* (onion) root tip assay

The *Allium cepa* (onion) root tip assay is a technique employed to evaluate antimitotic activity, which refers to a substance's capacity to inhibit cell division. Additionally,

Table 1. Preliminary Phytochemical Screening of *EeBHS*

S.no	Phytochemicals	Result
1	Phenol	+
2	Saponins	+
3	Tannins	+
4	Steroids	+
5	Flavonoids	+
6	Glycosides	+
7	Alkaloids	+
8	Carbohydrates	+

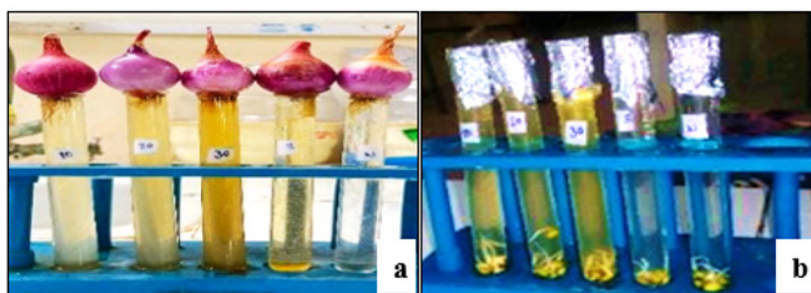


Figure 1. Anti-Proliferative Activity of *EeBHS* a. *Allium cepa*; b. green gram seed germination

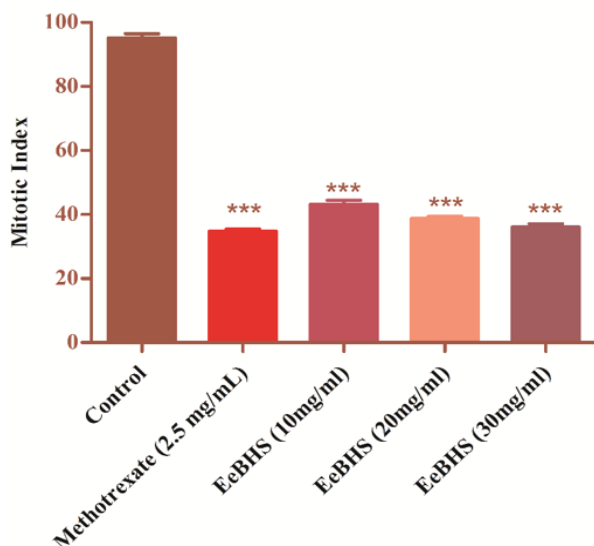


Figure 2. Mitotic Index: anti-mitotic activity observed after treating germinated seeds with different concentrations of EeBHS (10 mg/mL, 20 mg/mL, and 30 mg/mL), and standard drug methotrexate (2.5 mg/mL) [17]. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was applied to compare differences. *** $p < 0.001$; when compared with control. Values are represented as mean $\pm$ SD, $n = 3$

it can be used to assess the cytotoxic, genotoxic, and mutagenic effects of different compounds [17]. By examining changes in the root tip cells, can determine whether a substance interferes with normal cell division processes, potentially affecting growth and development.

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was applied to compare differences. *** $p < 0.001$; when compared with control. Values are represented as mean $\pm$ SD, $n = 3$. The antimitotic activity of EeBHS, assessed using *Allium cepa* root tip assays and varying extract concentrations (10 mg/mL, 20 mg/mL, and 30 mg/mL), demonstrated a significant increase in the percentage of non-dividing cells compared to the control [18]. The control group showed in Figure 4, 33% non-dividing cells, while methotrexate treatment significantly increased this to 55% ($p < 0.001$). EeBHS treatment also significantly increased the percentage of non-dividing cells in a dose-dependent

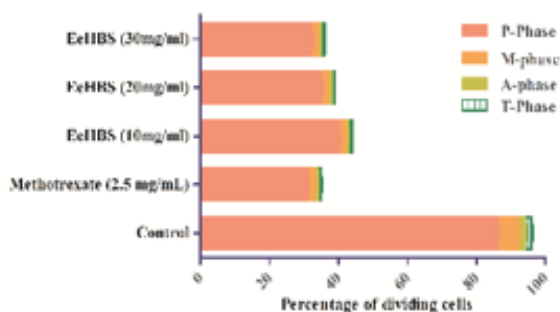


Figure 3. Percentage of Dividing Cells in Different Cell Cycle Phases

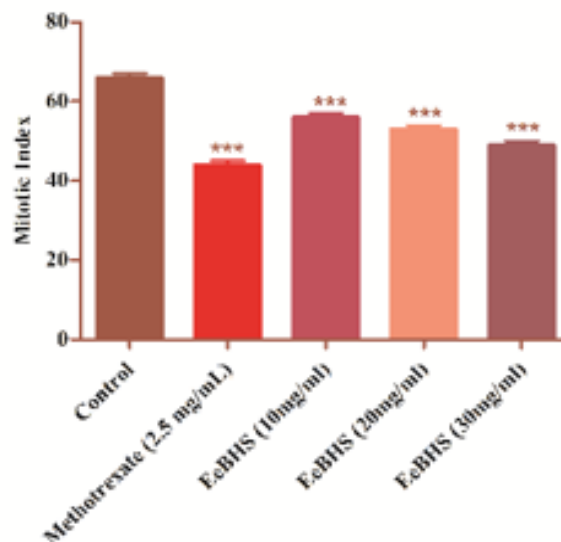


Figure 4. Mitotic Index: anti-mitotic activity observed after treating *Allium cepa* roots with different concentrations of EeBHS (10 mg/mL, 20 mg/mL, and 30 mg/mL), and standard drug methotrexate (2.5 mg/mL).

manner (43% at 10 mg/mL, 45% at 20 mg/mL, and 52% at 30 mg/mL, all $p < 0.001$). These results suggest that EeBHS possesses antimitotic potential, comparable to methotrexate, which is a known anti-mitotic agent.

Effect of EeBHS on Cell cycle phases in *allium cepa* root tip assay

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was applied to compare differences. *** $p < 0.001$; when compared with control. Values are represented as mean $\pm$ SD, $n = 3$.

The treatment with different concentrations of EeBHS (10 mg/mL, 20 mg/mL, and 30 mg/mL) resulted in a significant effect ($P < 0.001$). Cell cycle analysis further revealed that increased the number of cells in the A and M phases, suggesting induction of apoptosis. These findings shown in Figure 5 imply EeBHS exhibits notable anticancer activity and holds potential for therapeutic application.

Effect of EeBHS on HepG2 Cells

One-way analysis of variance (ANOVA) followed by

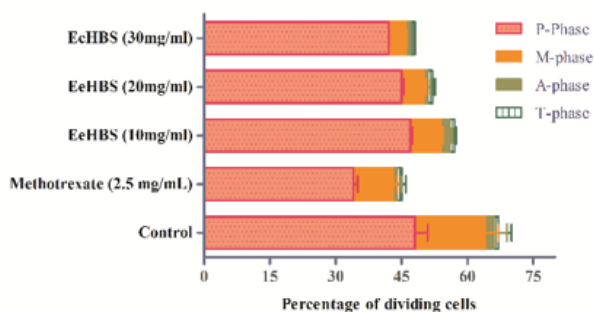


Figure 5. Percentage of Dividing Cells in Different Cell Cycle Phases.

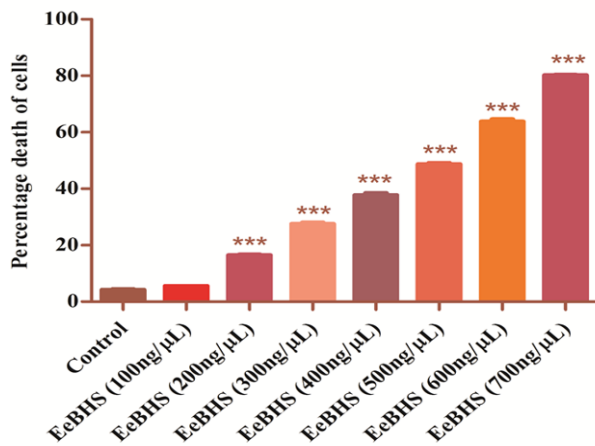


Figure 6. Anti-Proliferative Activity of EeBHS (MTT Assay)

Tukey's multiple comparison test was applied to compare differences. *** $p < 0.001$; when compared with control. Values are represented as mean $\pm$ SD, $n = 3$. The effect of *EeBHS* on HepG2 cell viability was assessed using a cell death assay. Treatment with different concentrations of *EeBHS* (1, 2, 3, 4, 5, 6, and 7 ng/ μ L) for 24 hours revealed a dose-dependent response. At lower concentrations (1 and 4 ng), cell viability remained largely unaffected, with minimal signs of cell death [19, 20]. However, higher concentrations (5, 6, and 7 ng/ μ L) induced a significant increase in cell death markers, indicating cytotoxic effects at these doses. These findings shown in Figure 6, suggest that *EeBHS* exhibits selective cytotoxic activity against HepG2 cells at higher concentrations, potentially contributing to its antimitotic or anticancer properties.

Discussion

The present study evaluated the antimitotic potential of the ethanolic extract of *Benincasa hispida* seeds (*EeBHS*) using the *Allium cepa* root tip meristem model an established biological assay for preliminary screening of antimitotic and cytotoxic compounds. The meristematic region of the onion root, due to its rapid rate of cell division, serves as an excellent model system for assessing the effects of various substances on the mitotic cycle. Its cellular processes closely mimic those of mammalian mitotic division, making it a valuable tool for early-stage anticancer screening.

In this investigation, the application of *EeBHS* resulted in a significant, dose-dependent reduction in root length and number, clearly indicating growth inhibition. This suppression of root development is a primary macroscopic indicator of cytotoxicity. Furthermore, microscopic analysis revealed a marked reduction in the mitotic index in treated root tips compared to controls, highlighting the extract's ability to interfere with cellular proliferation. This effect was comparable to that of the standard anticancer agent methotrexate, a well-known antimitotic drug.

The underlying mechanism of *EeBHS* action may involve disruption of microtubule dynamics during cell

division. Microtubules are essential for chromosomal segregation, and interference with their polymerization or depolymerisation can lead to mitotic arrest and subsequent apoptosis. Similar mechanisms are observed with colchicine, a potent antimitotic agent. Although plant cells are known to be more resistant to colchicine than animal cells, the conserved nature of mitotic pathways supports the hypothesis that substances affecting plant chromosomes may also impact mammalian systems.

In addition to mitotic arrest, the extract appeared to increase the percentage of cells in prophase and metaphase, suggesting a cell cycle arrest at these stages. This could result in failed progression through mitosis, leading ultimately to program cell death (apoptosis). Apoptosis plays a vital role in cancer control, as the unregulated proliferation of cells in tumours is often a consequence of impaired apoptotic signalling.

Phytochemical constituents such as flavonoids, phytosterols, triterpenoids, and phenolic acids present in *B. hispida* seeds may be responsible for the observed antimitotic effects. Compounds like kaempferol, quercetin, and ellagic acid, which have been reported in related plant extracts, are known to exhibit antiproliferative and pro-apoptotic effects in various cancer cell lines. Previous studies, including those by Awad et al. (2000), have demonstrated that phytosterols modulate cell cycle and apoptosis, thereby contributing to tumour growth suppression.

The similarity of our findings with those reported for *Aplotaxis auriculata* and other medicinal plants further validates the cytotoxic and antimitotic potential of *B. hispida*. Given that the extract showed comparable efficacy to methotrexate in terms of mitotic inhibition, it underscores the potential of *B. hispida* as a source of plant-derived anticancer agents. These results align with traditional medicinal claims and reinforce the plant's ethno pharmacological relevance in cancer therapy.

In conclusion, based on these findings, *EeBHS* exhibits significant, dose-dependent antimitotic and cytotoxic activities, demonstrated by its inhibitory effects on cell division in the *Allium* assay, seed germination, and reduced HepG2 cell proliferation. The extract caused a notable decrease in mitotic index and increased the proportion of cells in the prophase and metaphase stages, indicating disruption of normal mitotic processes. The cytotoxic effects observed in HepG2 cells further support its potential as an anticancer agent. These bioactivities are likely attributable to the presence of bioactive phytochemicals such as flavonoids, alkaloids, and phenolic compounds identified within the extract. Overall, *EeBHS* demonstrates promising antimitotic and cytotoxic properties, suggesting its potential utility in anticancer strategies and warranting further detailed studies. Further research involving *in vivo* models, cell cycle analysis, and molecular target identification is necessary to confirm these findings and advance toward clinical applications.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

None.

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