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Anticancer and Antioxidant Activities of Haplanthodes verticillatus: Insights from In Vitro and In Vivo Studies

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ABSTRACT

Breast cancer (BC) continues to be the foremost cause of cancer-related fatalities in women, with triple-negative breast cancer (TNBC) presenting distinct therapeutic difficulties owing to its aggressive characteristics and restricted therapy alternatives. This study assesses the anticancer efficacy of the ethyl acetate extract derived from *Haplanthodes verticillatus*, an indigenous plant recognized for its traditional therapeutic applications. The antioxidant activity of the extract was evaluated by the DPPH radical scavenging assay, demonstrating efficacy similar to ascorbic acid with an IC_{50} of 63.53 µg/mL. In vitro cytotoxicity assays on MDA-MB-231 breast cancer cells demonstrated a dose-dependent decline in cell viability, with an IC_{50} value of roughly 130 µg/mL. Microscopic investigations revealed apoptosis-like morphological alterations in treated cells. A xenograft mouse model was utilized in vivo to evaluate the extract's impact on tumor volume and weight. The ethyl acetate extract exhibited substantial, dose-dependent tumor inhibition, with the high-dose cohort attaining tumor reductions akin to those produced by cisplatin, a conventional chemotherapeutic drug. Moreover, the extract demonstrated enhanced tolerability, as indicated by better body weight preservation in treated mice compared to that administered cisplatin. Statistical analyses validated the extract's effectiveness and dose-dependent effects on multiple parameters, including tumor volume, tumor weight, and alterations in body weight. The results underscore the therapeutic promise of *Haplanthodes verticillatus* as a natural anticancer drug, demonstrating significant effectiveness and decreased systemic toxicity.

Keywords: *Haplanthodes verticillatus, Antioxidant activity, Anticancer activity.*

1. INTRODUCTION

Breast Cancer (BC), a frequent affliction in women and uncommon in males, is a heterogeneous condition comprising different subtypes, including hormone receptor-positive, HER2-positive, and triple-negative Breast Cancer (TNBC), with invasive ductal carcinoma being the most prevalent.(Katsura et al., 2022; Łukasiewicz et al., 2021) The development of BC is influenced by genetic predispositions, hormonal imbalances, lifestyle factors such as obesity, inactivity, and alcohol consumption, early menstruation, late menopause, nulliparity, delayed childbirth, and exposure to ionizing radiation, resulting in a complex risk profile for the disease. (Łukasiewicz et al., 2021) BC is the most prevalent cancer worldwide, with 2.3 million new cases each year. It is more common in industrialized nations owing to lifestyle variables and sophisticated diagnostic technology. Nevertheless, low- and middle-income nations experience a greater death burden, underscoring the necessity for fair healthcare resources and preventive strategies. (Crane and Baker, 1999; Mokhatri-Hesari and Montazeri, 2020) BC treatment involves a multidisciplinary approach, including surgical options like lumpectomy and mastectomy, systemic therapies like chemotherapy, hormone therapy, and targeted treatments like anti-HER2 drugs, radiotherapy for local disease management, immunotherapy for certain cases, and emerging targeted therapies. (Crane and Baker, 1999; Hong R., 2022; Smolarz et al., 2022) BC management faces challenges due to aggressive TNBC, limited therapeutic options, potential resistance to endocrine treatments, disparities in care access, and severe side effects of systemic therapies. Enhanced research into predictive biomarkers and personalized medicine is crucial for improving outcomes and addressing these challenges, especially in resource-poor settings. (Houghton and Hankinson, 2021; Mokhatri-Hesari and Montazeri, 2020)

Haplanthodes is a genus of angiosperms confined to peninsular India. It is one of the 49 designated genera and is predominantly located in the low-altitude areas of the Western Ghats region. (Singh et al., 2022; Surveswaran et al., 2022, 2020) Haplanthodes belongs to the Acanthaceae family, comprising 242 genera and 3,947 species categorized into four subfamilies: Acanthoideae, Avicennioideae, Nelsonioideae, and Thunburgioideae.

(Gnanasekaran et al., 2016; Surveswaran et al., 2022) The Haplanthodes species comprise: 1) *H. neilgherryensis* (Wight) R.B. Majumdar, 2) *H. plumosa* (T. Anderson) Panigrahi & G.C. Das, 3) *H. tentaculata* (L.) R.B. Majumdar, and 4) *H. verticillata* (Roxb.) R.B. Majumdar. The genus is located in the states of Goa, Gujarat, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Rajasthan, and Tamil Nadu. (Gnanasekaran et al., 2016; Singh et al., 2022; Surveswaran et al., 2022; Wood, 2014)

Haplanthodes verticillata (Roxb.) R.B. Majumdar (H. verticillate) is an erect annual herb that attains a height of approximately 0.5 meters. The slender, oval leaves are placed oppositely and measure 5 to 10 centimeters in length. Each leaf possesses two spiny projections at the apex and is adorned with elongated, spreading trichomes. The sessile blooms are located in the leaf axils, densely aggregated toward the apex of the stem. Despite the appearance of five petals, the flowers possess just two petals, which are bifurcated into two and three lobes. These dark blue flowers possess a pale green throat and are typically located on rocky hillsides in the Western Ghats. (Sonawane et al., 2020) The *H. verticillate* locally known as kalu kariyatu (Gujarati); kala kiriyat, kastula (Hindi); kalem kiraytem (Konkani); jakara (Marathi) and ottu mudi kurinji (Tamil). (P R Kanthale and Biradar, 2012; Sonawane et al., 2020) Emeka and his colleagues performed a study on the ethanol extract of *H. verticillata* leaves to ascertain the presence of phytoconstituents by GCMS analysis. The researchers recorded the presence of several bioactive chemicals in the ethanol extract obtained from the leaves. The ethanolic extract of *H. verticillata* leaves comprises various compounds, with the predominant ones being 3.beta.,17.beta.-dihydroxyestr-4-ene (10.89%), hexadecanoic acid, ethyl ester (7.49%), 1-methylbicyclo[3.2.1]octane (7.28%), linoleic acid ethyl ester (6.90%), ethyl oleate (5.65%), and caryophyllene oxide (5.29%). (Emeka et al., 2022) Kanthale and Biradar (2012) performed an ethnobotanical evaluation of the plant species present in the Mahur range forest in Nanded District. The researchers recorded the conventional medicinal use of *H. verticillata* for asthma treatment. The advised dosage for asthma treatment is to orally ingest one tablespoon of root extract combined with honey twice day for seven days. (Biradar, 2014; P. R. Kanthale and Biradar, 2012; LABHANE and DONGARWAR, 2014; Mubarak, 2021) Dhole et al. conducted a survey from 2007 to 2010 to investigate the diversity of weeds in multiple maize crop fields in the Nanded district. The objective was to identify the predominant weed species present in kharif maize crops. The survey identified *H. verticillata* as a weed occurring in maize cultivation regions. (Dhole et al., 2013)

The present research is driven by the distinctive ecological and therapeutic significance of the genus Haplanthodes, particularly *Haplanthodes verticillata*. This species is indigenous to the peninsular region of India and predominantly inhabits the Western Ghats. Despite the comprehensive documentation of its traditional therapeutic applications and phytochemical ingredients, there is a deficiency of thorough research regarding its potential pharmacological effects. This study sought to examine the anticancer activity of the ethyl acetate extract of *H. verticillatus* using both in vitro and in vivo models. The study precisely assessed the extract's cytotoxicity, antioxidant properties, and capacity to diminish tumor growth and weight in xenograft models, contrasting its efficacy and safety profile with that of cisplatin, a conventional chemotherapeutic agent.

2. MATERİAL AND METHODS

Collection & Authentication of plant material

In August 2023, a whole plant of *Haplanthodes verticillatus* (*H. verticillatus*) had been collected from the Northern Western Ghats (Nashik district, Maharashtra, India). The botanist at Sandip University in Nashik, India, verified the plant, and the herbarium was archived under voucher specimen number SUN20230028.

Preparation of Plant material and Extraction of Phytoconstituents

The collected plants were dehydrated/dried in a shaded location/area. The complete plant was dried, pulverized, and ground into powder. After sufficient drying, the entire plants were pulverized into a fine powder utilizing a mechanical grinder. One thousand grams of *H. verticillatus* plant material was extracted utilizing a Soxhlet apparatus with solvents of ascending polarity (Hexane > Chloroform > Ethyl Acetate > Methanol > Water). Subsequent to the preliminary phytochemical analysis, the plant material was extracted separately utilizing all aforementioned solvents for further investigation.

Concentration of Extracts

Each solvent extract was concentrated at reduced pressure with a rotary evaporator to eliminate the solvent, resulting in crude extracts. The concentrated extracts were thereafter gathered and preserved in airtight containers at low temperatures to avert degradation and contamination.

3. IN-VİTRO STUDY

Antioxidant activity :DPPH radical scavenging activity

The different extract of *H. verticillatus* were analyzed using a 1,1-diphenyl-2-picryl hydrazyl (DPPH) method. The stock solution was produced by dissolving 24 milligrams of DPPH in 100 mL of methanol. The *H. verticillatus* extracts (100 µL) were combined with 3 mL of DPPH working solutions in a test tube. A standard/blank is typically prepared by combining 3 mL of a solution containing DPPH with 100 µL of methanol. Subsequently, the tubes were subjected to a period of 30

minutes in which they were maintained in a state of total absence of light. The measurement of absorbance was consequently conducted at a wavelength of 517 nm. The ability to scavenge the DPPH radical was expressed as percentage inhibition and calculated using the following equation: (Akgül et al., 2022; Baliyan et al., 2022; Chaves et al., 2020)

DPPH radical scavenging activity % = [(Ac−As)/Ac] × 100

Where, Ac is the absorbance of the control reaction, As is the absorbance of *H. verticillatus* extracts.

***In vitro* cytotoxicity (MTT Assay)**

Cell cultures

The MDA-MB-231 cell lines were obtained from the National Centre for Cell Sciences (NCCS), Pune, India, and cultured in Dulbecco's modified Eagles medium (DMEM).

Procedure

For the in vitro anticancer activity using the MDA-MB-231 cell line, the cells are cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. The cells are maintained in a humidified atmosphere at 37°C with 5% CO2 to ensure optimal growth conditions.

For the MTT assay to assess cell viability, MDA-MB-231 cells are seeded into 96-well plates at a density of 5×10^3 cells per well. After allowing the cells to adhere for 24 hours, the cells are treated with varying concentrations of the ethyl acetate extract of *H. verticillatus* (25 µg, 50 µg, 100 µg, 200 µg, and 400 µg). After the treatment period, 20 µL of MTT solution (5 mg/mL) is added to each well, and the plate is incubated at 37°C for an additional 2 to 4 hours to allow the MTT to be reduced by viable cells, forming formazan crystals. The medium is then removed, and 100 µL of dimethyl sulfoxide (DMSO) is added to dissolve the formazan. The absorbance is measured at 570 nm using a plate reader, and cell viability is calculated by comparing the treated wells to untreated control cells.

The percentage of viability was calculated using the formula:

% of viability = (Mean OD Samples/ Mean OD of control group) x 100

(Arunasree, 2010; Grbović et al., 2013; Phromnoi et al., 2009; Wang et al., 2020)

4. INVİVO STUDY

Animal Model

Xenograft animal model built according to the methods described in Literature with some modification. Human breast cancer cells MDA-MB-231 (5×10^6 in 0.2 mL phosphate-buffered saline) will be injected into the left fifth mammary fat pad of each female BALB/c mouse. Tumor establishment will be confirmed by the presence of palpable tumors growing consistently to a diameter of 3–5 mm. Once the tumors are established, the xenograft mice will be randomly and equally divided into four groups (n=10/6 per group) for the study of treatment effects. A control group (Group I) consisting of female BALB/c mice without MDA-MB-231 cell inoculation will also be included (n=6).

The experimental groups will receive the following treatments for 25 days (from day 23 to day 47 post-inoculation): Group I (Control, no tumor): 100 µL sterile water/10 g body weight once daily by gavage; Group II (Vehicle control): 100 µL sterile water/10 g body weight once daily by gavage; Group III (Test extract): low, medium, and high concentrations of the test extract (e.g., 300 mg/kg, 500 mg/kg, and 700 mg/kg, respectively) will be administered in 100 µL solutions by gavage once daily; and Group IV (Standard group): cisplatin (2.0 mg/kg) will be administered intraperitoneally once every 3 days. All mice will have unrestricted access to standard food and filtered water throughout the study.

Palpable tumors will be measured thrice weekly using a digital vernier caliper, and tumor volume will be calculated using the formula: $V=(L\times W^2)/2$, where L represents the long diameter and W represents the short diameter.

5. RESULT

Collection & Authentication of plant material

The entire plant of *Haplanthodes verticillatus* (*H. verticillatus*) was gathered from the Northern Western Ghats (Nashik district, Maharashtra, India) in August 2023. The plant was verified by the botanist at Sandip University, Nashik, India, and a herbarium specimen was deposited under voucher number SUN20230028.

Extraction of Phytoconstituents

The gathered specimens of *H. verticillatus* underwent a careful drying process under shady settings to maintain the integrity of the phytoconstituents. After drying, the entire plants were carefully crushed and finely powdered to enhance extraction efficiency. A total of 1000 grams of powdered plant material underwent sequential solvent extraction utilizing a Soxhlet system, employing solvents of ascending polarity, commencing with non-polar solvents and progressing to polar solvents.

The plant material was extracted utilizing various solvents, including hexane, chloroform, ethyl acetate, methanol, and water. Each cycle was performed for 24 to 48 hours to extract phytoconstituents. The extracts were concentrated under decreased pressure, resulting in crude extracts. These were preserved at low temperatures to avert disintegration and contamination. The yeild of various extracts were as follows: Hexane (1.7 %); Chloroform (0.8 %); Ethyl Acetate (1.9 %); Ethanol (2.7 %); Water (3.4 %). The phytochemical investigation of all the extracts was performed using GC-MS analysis. The GC-MS analysis revealed the presence of diverse phytochemicals in the ethyl acetate extract compared to the other extracts, which led to the selection of the ethyl acetate extract for further study.

The systematic extraction method of *H. verticillatus* involved phytochemical screening for diverse phytoconstituents, enabling the identification of novel bioactive chemicals with therapeutic significance, thereby enhancing the plant's medicinal potential.

6. IN-VİTRO STUDY

Antioxidant activity- DPPH radical scavenging activity

The antioxidant efficacy of the ethyl acetate extract of *H. verticillatus* was assessed utilizing the DPPH radical scavenging assay, with ascorbic acid as the standard reference. The findings revealed significant differences between the ethyl acetate extract and ascorbic acid, as evidenced by the IC_{50} values and percentage inhibition (the concentration necessary to block 50% of DPPH radicals) (Figure 1). Ascorbic acid demonstrated a dose-dependent enhancement in DPPH radical scavenging activity, with inhibition levels varying from 21.73% at 20 µg/mL to 84.68% at 120 µg/mL, and an IC_{50} value of 65.53 µg/mL. The ethyl acetate extract exhibited significant antioxidant activity, with percentage inhibition values between 30.54% and 72.49% across the identical concentration range, and an IC_{50} value of 63.53 µg/mL. The results demonstrate that the ethyl acetate extract has radical scavenging capability akin to that of ascorbic acid.

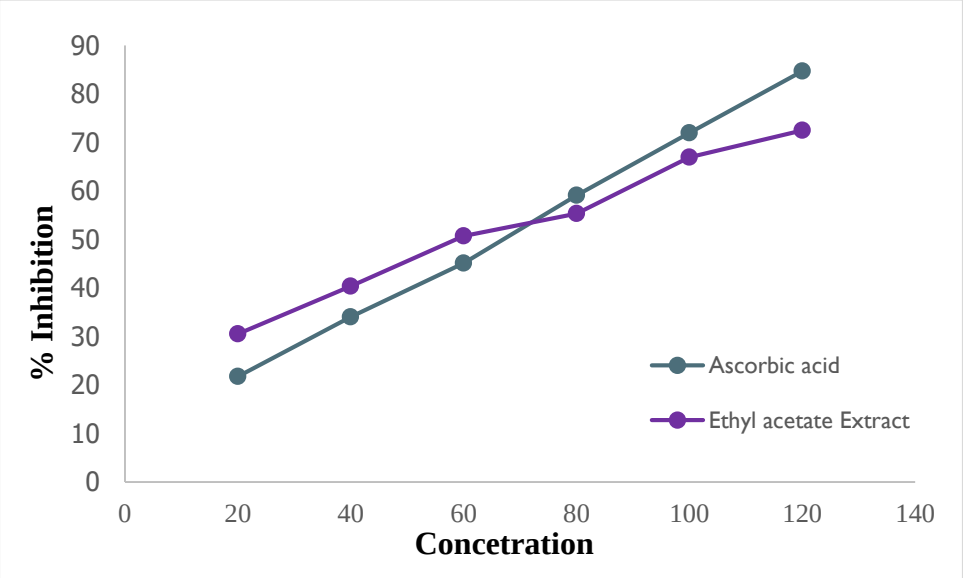

Figure 1: Percentage inhobition of ethyl acetate extract for DPPH method

In vitro cytotoxicity (MTT Assay)

The in vitro cytotoxicity of the ethyl acetate extract of *H. verticillatus* was assessed on the human breast cancer cell line MDA-MB-231 with the MTT assay. This assay quantifies cell viability by measuring the reduction of MTT to formazan, indicating the metabolic activity of living cells. The study investigated the impact of ethyl acetate extract concentrations (25 µg/mL to 400 µg/mL) in comparison to untreated control cells and cisplatin (standard chemotherapeutic agent) at 25 µM. The findings revealed a dose-dependent reduction in cell viability, suggesting the extract's potential anticancer effects.

Untreated MDA-MB-231 cells exhibited a mean optical density (OD) of 0.907, normalized to 100% cell viability, indicating substantial growth. Cisplatin, serving as the positive control, decreased viability to 64.71% (mean OD: 0.564), so confirming the validity of the experimental procedure. The ethyl acetate extract exhibited dose-dependent cytotoxicity. At a concentration of 25 µg/mL, viability was 79.93% (mean optical density: 0.697), suggesting modest effects. At a concentration of 50 µg/mL, cell viability decreased to 74.88% (mean optical density: 0.653), indicating significant cytotoxicity. At a concentration of 100 µg/mL, viability diminished to 63.64% (mean OD: 0.555), similar to that of cisplatin. Increased dosages had more pronounced effects, with 48.51% viability at 200 µg/mL (mean OD: 0.423) and 20.99% at 400 µg/mL (mean OD:

0.183). The IC50 value, calculated using a sigmoidal dose-response curve, was approximately 130 µg/mL, indicating the concentration required to inhibit 50% of cell viability. The results underscore the extract's potent antiproliferative effect, especially at elevated concentrations. (Table 1)

Microscopic examination of MDA-MB-231 cells treated with the ethyl acetate extract of *H. verticillatus* revealed dose-dependent cytotoxic effects (Figure 2). Untreated cells exhibited intact morphology, while cisplatin-treated cells showed apoptosis-associated changes like shrinkage and detachment. The extract induced mild rounding at 25–50 µg/mL, with significant effects, including membrane blebbing and detachment, at 100–400 µg/mL. At 400 µg/mL, the extract reduced cell viability to 20.99%, surpassing cisplatin's efficacy. These findings suggest apoptosis or necrosis as the underlying mechanism and highlight the extract's potential as a natural anticancer agent.

Table 1: Results of the ethyl acetate extract of *H. verticillatus* on MDA-MB-231 cell viability at various concentrations

	Blank	Untreated	Cisplatin 25 µM	Concentration of Extract (µg/mL)				
				25	50	100	200	400
Reading 1	0.03	0.912	0.594	0.729	0.684	0.591	0.464	0.215
Reading 2	0.04	0.901	0.604	0.735	0.691	0.589	0.451	0.221
Mean	0.035	0.907	0.599	0.732	0.688	0.590	0.458	0.218
Mean OD - Mean B		0.872	0.564	0.697	0.653	0.555	0.423	0.183
Standard deviation		0.008	0.007	0.004	0.005	0.001	0.009	0.004
Standard error		0.006	0.005	0.003	0.003	0.001	0.006	0.003
Viability %		100	64.71	79.93	74.88	63.64	48.51	20.99

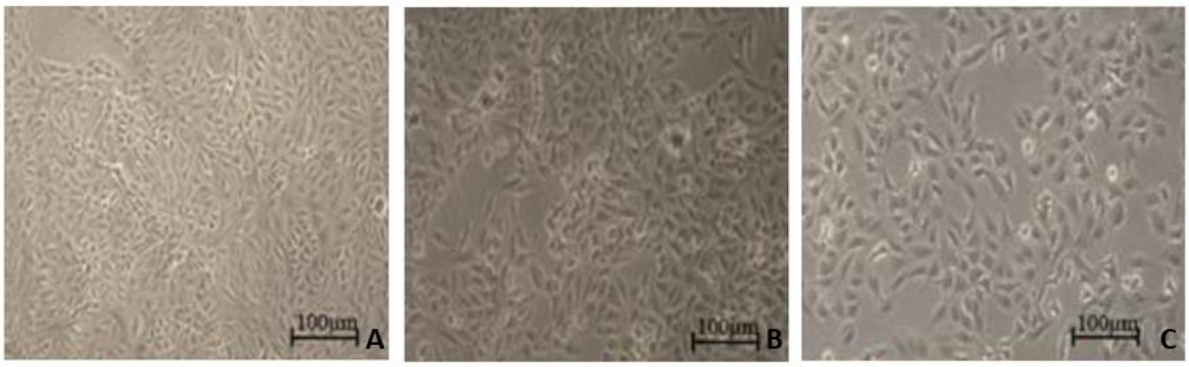

Figure 2: Morphological characteristics of MDA-MB-231cell line treated with ethyl acetate extract of *H. verticillatus* and (Std) cisplatin (25 µM) (A- Untreated B- Std, C- Extract)

Invivo Study

Subsequent to the encouraging in vitro results, the in vivo anticancer efficacy of the ethyl acetate extract of *H. verticillatus* was assessed utilizing a xenograft mouse model. The research entailed the inoculation of MDA-MB-231 human breast cancer cells into immunocompromised mice to facilitate tumor development. The extract was delivered in low, medium, and high doses to evaluate its effectiveness in decreasing tumor volume over time. Tumor proliferation was assessed at consistent intervals, and the impact of the extract was juxtaposed with untreated controls and standard chemotherapy (cisplatin). The aim of the in vivo investigation was to confirm the therapeutic efficacy of the ethyl acetate extract in a biological context and to explore its mechanisms of action further.

Tumor Volume

The anticancer efficacy of the ethyl acetate extract of *H. verticillatus* was assessed by measuring tumor volume variations among various treatment groups (Low Dose, Medium Dose, High Dose, Standard Drug, Tumor Control, and Normal Control) over a period of 21 days. Two-way repeated measures ANOVA demonstrated significant effects of time, tumor volume, their

interactions, and variability specific to individual animals. Time represented the predominant proportion of the change (45.13%, $F(2.026, 48.63) = 21124$, $p < 0.0001$), indicating substantial tumor reduction in all therapy groups over time. Tumor volume explained 33.07% of the variance ($F(5, 24) = 3497$, $p < 0.0001$), signifying significant disparities among treatment groups. The interaction between time and tumor volume (21.71%, $F(15, 72) = 2032$, $p < 0.0001$) indicates that the tumor-reducing impact fluctuated significantly over time based on the treatment provided. Animal-specific variability was statistically significant ($F(24, 72) = 2.65$, $p = 0.0008$), however it constituted merely 0.0454% of the variation, thereby affirming the consistency of responses within groups.

Tukey's multiple comparisons test indicated clear patterns within each group. The Normal Control group exhibited no tumor growth, but the Tumor Control group demonstrated increasing tumor enlargement, with significant differences observed at all time periods, resulting in a tumor volume increase of 1740 mm^3 from Day 0 to Day 21 ($p < 0.0001$). The Standard Drug group significantly diminished tumor volume, achieving a total reduction of 602 mm^3 by Day 21, predominantly within the initial 14 days (502 mm^3). The extract demonstrated a dose-dependent anticancer effect. The Low Dose group had substantial tumor reductions, with a decrease of 1216 mm^3 by Day 21 ($p < 0.0001$), but the Medium Dose group indicated superior performance, lowering tumor volume by 795 mm^3. The High Dose group exhibited the greatest efficiency, attaining a tumor decrease of 496 mm^3 by Day 21 ($p < 0.0001$). The extract-treated groups exhibited gradual tumor suppression over time, with consistent decreases at each interval. (Figure 3 and 4)

No tumor growth was seen at any time point in the Normal Control (NCG) group, establishing a baseline for healthy physiological circumstances. Conversely, the Tumor Control (TCG) group exhibited significant tumor growth, recording the largest tumor volume of all groups by Day 21. Substantial augmentations in tumor volume were noted at each interval (e.g., Day 0 to Day 21, +1740 mm^3, $p < 0.0001$). This pronounced disparity highlights the unregulated tumor advancement without therapy and acts as a benchmark for assessing the effectiveness of the other groups.

The Standard Drug (STG) group had a significant decrease in tumor volume, with an overall reduction of 602 mm^3 by Day 21 relative to Day 0 ($p < 0.0001$). Tumor suppression in this cohort progressed consistently over time, with the most significant decrease (502 mm^3) seen between Day 0 and Day 14. The Standard Drug group, although efficient, was surpassed by the higher doses of the extract, indicating the possible superiority of the natural component compared to conventional treatment in this model. The Low Dose (LDG) group exhibited a significant and dose-dependent reduction in tumor volume. By Day 21, the tumor volume diminished by 1216 mm^3 ($p < 0.0001$) relative to Day 0, demonstrating superior efficacy compared to the Standard Drug. The decrease was gradual throughout all periods, with the most significant suppression recorded between Day 7 and Day 21 (-912 mm^3). Nonetheless, the Low Dose group exhibited reduced efficacy relative to the Medium and High Dose groups, demonstrating a dose-dependent therapeutic impact. The Medium Dose (MDG) group demonstrated superior tumor suppression relative to the Low Dose, with a total decrease of 795 mm^3 by Day 21 ($p < 0.0001$). Tumor suppression was consistently observed throughout all periods, with significant reductions during the initial therapy phase (Day 0 to Day 14). In comparison to the Standard Drug group, the Medium Dose group exhibited enhanced efficacy, indicating the extract's potential to yield improved results in tumor growth treatment.

The High Dose (HDG) group had the most significant tumor suppression compared to all other experimental groups. By Day 21, tumor volume diminished by 496 mm^3 relative to Day 0 ($p < 0.0001$), exhibiting consistent reductions at all intervals. Initial and prolonged tumor suppression was apparent, with diminished differences in tumor reduction observed between subsequent intervals (e.g., Day 14 to Day 21: -96 mm^3), indicating that the peak therapeutic impact may have been attained earlier. The High Dose group significantly surpassed the Standard Drug, Medium Dose, and Low Dose groups, highlighting the extract's efficacy at elevated doses. In the comparison of all groups, the Normal Control group exhibited stability, whereas the Tumor Control group demonstrated exponential tumor growth, establishing a baseline for untreated cancer progression. The Standard Drug group exhibited modest tumor suppression, whereas all extract-treated groups (Low, Medium, and High Doses) displayed enhanced efficacy, with the High Dose group achieving the most significant tumor decrease. A distinct dose-response relationship was seen among the extract-treated groups, with the High Dose group surpassing both the Medium and Low Dose groups. Pairwise comparisons demonstrated substantial differences between the Tumor Control group and all other groups ($p < 0.0001$), confirming the effectiveness of both the extract and the conventional medicine in diminishing tumor growth. Moreover, substantial differences were observed between the Low and Medium Dose groups, as well as between the Medium and High Dose groups ($p < 0.0001$), corroborating the extract's dose-dependent efficacy. The comparative research demonstrated that the High Dose group exceeded the Standard Drug in tumor shrinkage, highlighting the extract's significant anticancer efficacy. The time-dependent and dose-dependent effects identified in this investigation underscore the potential of the ethyl acetate extract of *H. verticillatus* as a viable candidate for anticancer therapy. These results necessitate additional investigation into the fundamental mechanisms of action and clinical validation of this extract as a therapeutic agent.

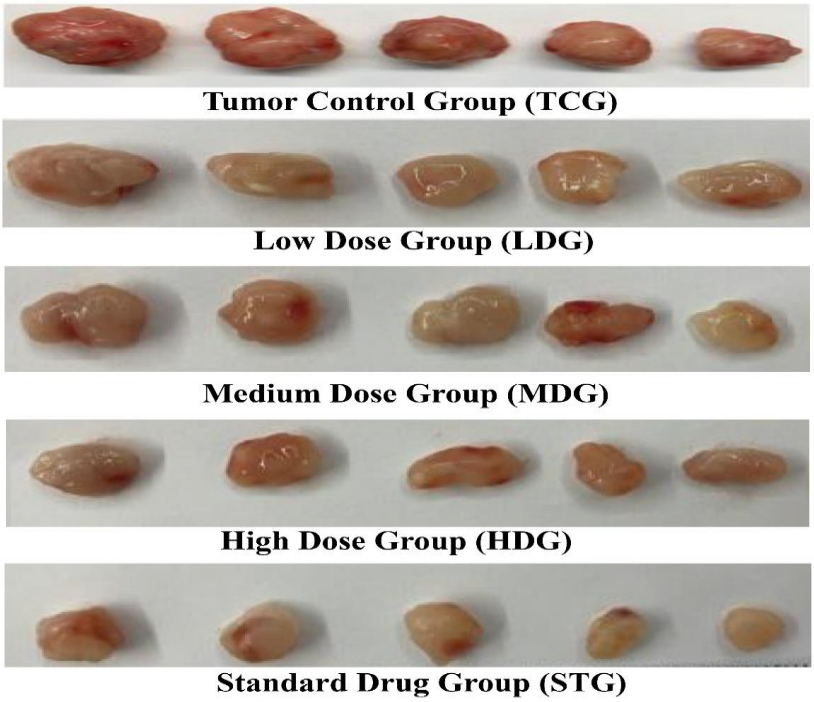

Figure 3: Tumor Changes in mice after treatment of ethyl acetate extract of *H. verticillatus*

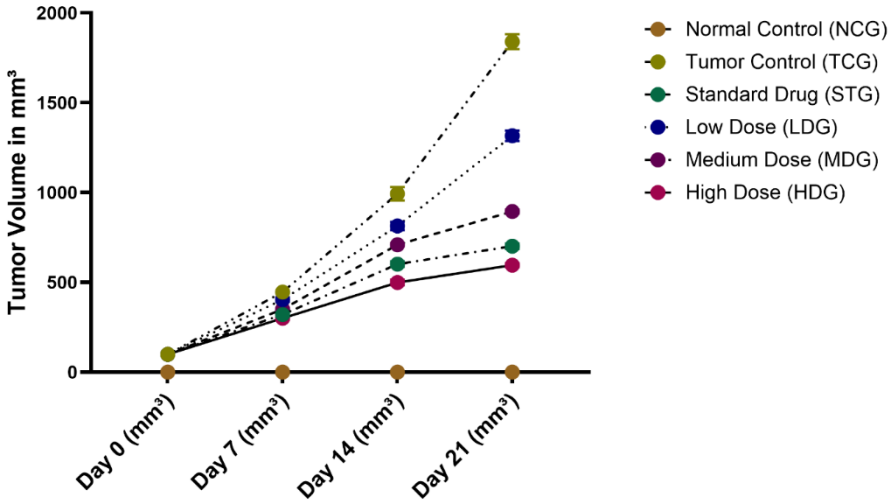

Figure 4: Tumor volume changes over time in different treatment groups.

(Tumor volumes (mm^3) were measured at Days 0, 7, 14, and 21 for all groups: Normal Control (NCG), Tumor Control (TCG), Standard Drug (STG), and ethyl acetate extract-treated groups (Low Dose, Medium Dose, High Dose). Significant differences in tumor volume were observed among groups, with extract-treated and standard drug groups showing dose- and time-dependent tumor growth suppression. Data are represented as mean ± standard deviation ($P < 0.0001$).)

Tumor Weight

The examination of tumor weight underscores the anticancer efficacy of the ethyl acetate extract of *H. verticillatus* in comparison to a conventional drug, normal control, and untreated tumor control groups. One-way ANOVA indicated significant differences among groups ($F(5, 24) = 317.7$, $p < 0.0001$), with a high R^2 value of 0.9851, signifying that 98.51% of the variability in tumor weight is ascribed to the therapies. This strong statistical outcome highlights the dependability of the therapy effects. Tukey's multiple comparison test elucidated the distinctions among groups, demonstrating both dose-dependent and treatment-specific effects.

The Normal Control (NCG) group had no tumor growth (0 g) and functioned as a baseline for comparison. Marked disparities

were noted between the Normal Control and all other groups, including the Tumor Control (TCG) group, which demonstrated the greatest mean tumor weight of 1.86 g ($p < 0.0001$). This indicates that untreated animals undergo aggressive tumor development, providing a benchmark for assessing therapy efficacy. The Standard Drug (STG) group had significant tumor suppression, decreasing tumor weight to 0.68 g. Despite being markedly elevated compared to the Normal Control (difference = -0.68 g, $p < 0.0001$), the Standard Drug substantially inhibited tumor growth relative to the Tumor Control (difference = -1.18 g, $p < 0.0001$), indicating its therapeutic efficacy. In comparison to the extract-treated groups, the High Dose (HDG) group (0.6 g) demonstrated tumor suppression akin to that of the Standard Drug (difference = +0.08 g, $p = 0.671$), indicating comparable efficacy.

The extract-treated groups demonstrated dose-dependent antitumor efficacy. The Low Dose (LDG) group decreased tumor weight to 1.54 g, which is considerably lower than that of the Tumor Control group (difference = -0.32 g, $p < 0.0001$), although greater than the Medium Dose (MDG) and High Dose (HDG) groups. The Medium Dose (MDG) group decreased tumor weight to 1.00 g, demonstrating considerably better suppression relative to the Low Dose (difference = -0.54 g, $p < 0.0001$). The High Dose (HDG) group had the most significant tumor suppression, decreasing tumor weight to 0.60 g. This was markedly lower than both the Low Dose (difference = -0.94 g, $p < 0.0001$) and Medium Dose (difference = -0.40 g, $p < 0.0001$), while demonstrating comparable efficacy to the Standard Drug ($p = 0.671$). The dose-dependent response noted among the Low, Medium, and High Doses highlights the therapeutic efficacy of the extract at elevated concentrations. (Figure 5)

A comparative investigation among all groups reveals distinct differences. The Tumor Control group demonstrated the most accelerated and unrestrained tumor progression, with a tumor weight markedly greater than that of the other groups ($p < 0.0001$). The Low Dose group, while beneficial in diminishing tumor weight, had inferior potency compared to the Medium and High Doses, indicating that greater amounts of the extract are necessary for maximal efficiency. The Medium Dose group exhibited moderate tumor suppression, exceeding the Low Dose but failing to achieve the effectiveness of the High Dose or the Standard Drug. The High Dose group had the most significant tumor suppression among the extract-treated cohorts, equaling the efficacy of the Standard Drug. The Normal Control group exhibited no tumor growth, establishing a baseline, whereas the Tumor Control group highlighted the aggressive advancement of untreated tumors.

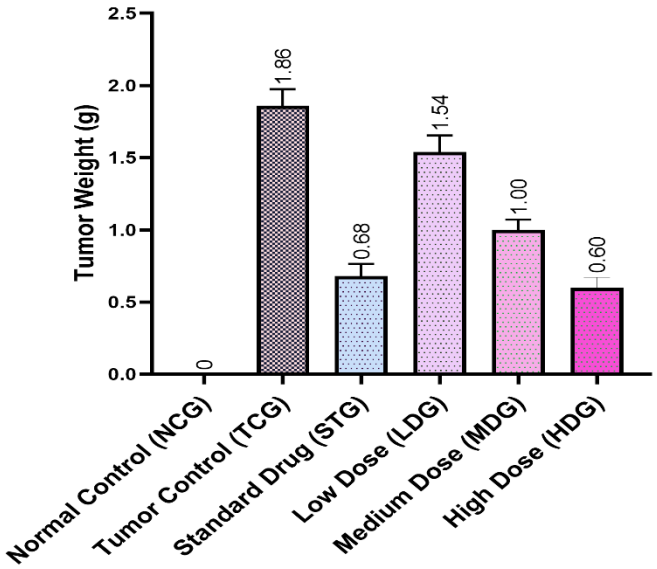

Figure 5: Tumor weight comparison among treatment groups at the end of the study.

(Tumor weights (g) were recorded for all groups: NCG, TCG, STG, and extract-treated groups (Low Dose, Medium Dose, High Dose). The Tumor Control group showed the highest tumor weight, while the High Dose group demonstrated tumor weight reduction comparable to the Standard Drug group. Statistical significance was determined using ANOVA and Tukey's post hoc test (**P** < 0.0001). Data are expressed as mean ± standard deviation.)

Body Weight Changes

The body weight study offers essential insights into the tolerance and systemic effects of the ethyl acetate extract of *H. verticillatus* in comparison to the Standard Drug, Tumor Control, and Normal Control groups. Significant effects of time, treatment (column factor), and their interaction on body weight changes during the study were discovered using a two-way repeated measures ANOVA. The interaction between time and treatment explained 46.06% of the overall variation (F(5, 24)

= 1071, $p < 0.0001$), indicating that the therapies' effects on body weight significantly fluctuated with time. The treatment (column factor) independently explained 42.43% of the overall variation ($F(5, 24) = 86.7$, $p < 0.0001$), whereas the effect of time alone accounted for 8.95% ($F(1, 24) = 1040$, $p < 0.0001$). Animal-specific variability was statistically significant, however it constituted a minor fraction of the variation (2.35%, $F(24, 24) = 11.37$, $p < 0.0001$), suggesting consistency among groups.

Tukey's multiple comparison test indicated no significant variations in beginning body weights among groups ($p > 0.05$), confirming uniform distribution of animals at the study's commencement. Notable disparities were noted in final body weights, indicating the effects of therapies and tumor advancement. The Normal Control (NCG) group demonstrated the greatest body weight, establishing a benchmark for healthy specimens. In comparison to the Normal Control, the Tumor Control (TCG) group exhibited a substantial decrease in body weight (mean difference = 2.02 g, $p < 0.0001$), underscoring the physiological impact of untreated tumor advancement. The usual Drug (STG) group demonstrated significant weight loss relative to the Normal Control (mean difference = 3.76 g, $p < 0.0001$), indicating systemic toxicity linked to the usual anticancer therapy. The extract-treated groups, including the Low Dose (LDG), Medium Dose (MDG), and High Dose (HDG), exhibited reductions in body weight compared to the Normal Control (mean differences = 0.96 g, 1.00 g, and 1.30 g, respectively; $p < 0.0001$ for all comparisons). The results indicate that the extract-treated groups underwent minor weight loss, which was considerably less pronounced than that of the Standard Drug group. (Figure 6)

Significant weight discrepancies were noted when contrasting the Tumor Control group with the treated groups across all regimens. The Tumor Control group exhibited reduced body weights relative to the Low Dose (mean difference = -1.06 g, $p < 0.0001$), Medium Dose (mean difference = -1.02 g, $p < 0.0001$), and High Dose (mean difference = -0.72 g, $p < 0.0001$) groups. This suggests that the extract-treated groups were more effective in preserving body weight despite tumor advancement. The Tumor Control group saw less significant weight loss compared to the Standard Drug group (mean difference = 1.74 g, $p < 0.0001$), hence reinforcing the concept of systemic toxicity linked to the standard treatment.

A comparative investigation of the extract-treated groups indicated a dose-dependent trend in alterations of body weight. The Low Dose group demonstrated the minimal weight loss compared to the other two doses, whereas the High Dose group displayed marginally greater weight loss, with significant differences observed between the Low and High Doses (mean difference = 0.34 g, $p = 0.0155$) and the Medium and High Doses (mean difference = 0.30 g, $p = 0.0445$). Nonetheless, no substantial change was observed between the Low and Medium Doses (mean difference = 0.04 g, $p = 0.9986$), indicating comparable tolerability at both dosage levels. In comparison to the Standard Drug, all extract-treated groups had considerably reduced weight loss ($p < 0.0001$), underscoring the extract's improved safety profile.

In the comparison of all groups, the Normal Control group exhibited the highest body weight, highlighting the lack of physiological stress. The Tumor Control group saw mild weight loss attributable to tumor growth, whereas the Standard Drug group displayed the most pronounced weight loss across all groups, presumably due to its systemic toxicity. The extract-treated groups, especially the Low and Medium Doses, demonstrated enhanced body weight maintenance, indicating higher tolerability relative to the Tumor Control and Standard Drug groups. The High Dose group, although beneficial in diminishing tumor size, exhibited somewhat more weight loss compared to the lower doses, suggesting a dose-dependent impact on tolerance.

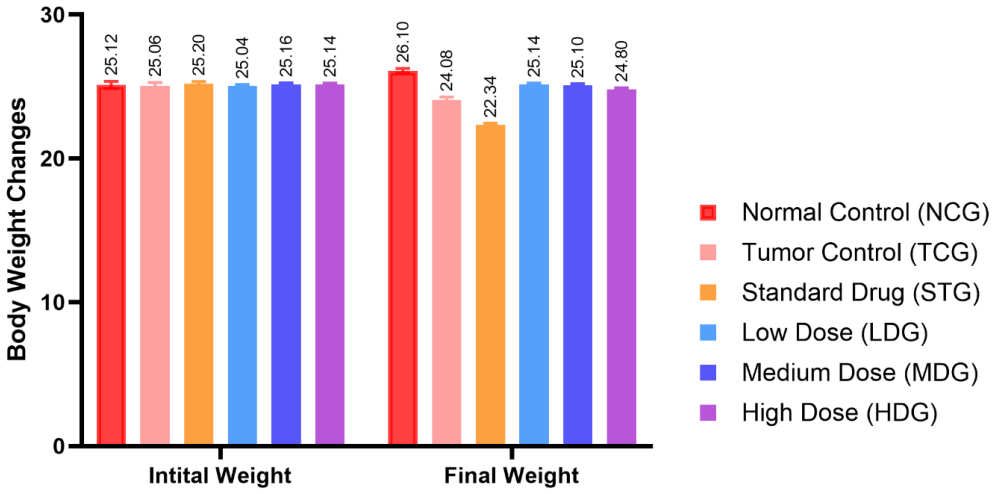

Figure 6: Body weight changes over time across different treatment groups.

(Body weights (g) were recorded at baseline (Day 0) and at subsequent time points (Days 7, 14, and 21) for all groups. The Normal Control group maintained stable body weight, whereas tumor-bearing animals exhibited weight loss. Extract-treated groups, particularly the High Dose group, mitigated weight loss in a dose-dependent manner compared to the Standard Drug group. Statistical significance was determined using two-way ANOVA ($P < 0.0001$). Data are presented as mean ± standard deviation.)

7. CONCLUSION

The research underscores the anticancer efficacy of the ethyl acetate extract of *H. verticillatus* by thorough in vitro and in vivo studies. The extract exhibited strong antioxidant activity in DPPH experiments, with an IC_{50} value akin to ascorbic acid, and notable cytotoxicity against the MDA-MB-231 breast cancer cell line in a dose-dependent manner, attaining an IC_{50} value of roughly 130 µg/mL. Microscopic analysis demonstrated apoptosis-like morphological alterations, highlighting the extract's possible mechanism of action.

In vivo tests utilizing a xenograft model validated the extract's dose- and time-dependent effectiveness in diminishing tumor volume and mass. The high-dose group (HDG) demonstrated the most significant tumor reduction, with results akin to the conventional chemotherapeutic drug, cisplatin. Analysis of tumor volume demonstrated substantial interaction effects between time and treatment, with the extract exhibiting enhanced efficacy at elevated doses. Analysis of tumor weight confirmed these findings, revealing significant reductions in tumor weight among extract-treated groups, especially at medium and high doses, underscoring its therapeutic potential.

The research additionally assessed alterations in body weight as an indicator of systemic toxicity and the effects of treatment. The extract-treated animals, particularly the HDG, exhibited superior weight preservation relative to the standard medication group, suggesting less systemic toxicity. The data indicate that the ethyl acetate extract of *H. verticillatus* is a promising natural anticancer drug, exhibiting significant anticancer efficacy, dose-dependent effects, and little systemic toxicity, thereby necessitating further investigation for therapeutic applications.

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