

Antiproliferative activity of ethyl acetate fraction of *Euphorbia ingens* against prostate cancer cell line: An in silico and in vitro analysis

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ABSTRACT

Current prostate cancer (PCa) treatments often lead to severe side effects, prompting the exploration of safer alternatives. *Euphorbia ingens* is a medicinal plant used for cancer treatment in African communities; however, there is no scientific validation of its anticancer activity. This study therefore evaluated the antiproliferative activity of *E. ingens* on the DU-145 human PCa cell line. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) bio-assay was used to determine the antiproliferative activity of *E. ingens* ethyl acetate-, and water-fractions. Only the ethyl acetate fraction was considered to be active. It inhibited DU-145 cell growth

selectively without toxicity to non-cancerous Vero E6 cells. The IC₅₀ was 9.71 ± 0.4 µg/ml and had a selectivity index of 8.26, indicating promising efficacy and cellular safety. Qualitative phytochemical screening identified the presence of phenols, terpenoids, flavonoids, tannins, sterols, and saponins in the ethyl acetate fraction. An additional 18 chemical compounds with potential synergistic roles were identified through gas chromatography-mass spectrometry analysis. Network pharmacology was then employed to predict molecular targets and mechanisms of action for drug-like chemical compounds, implicating key targets such as ESR1, IL6, MMP9, CDK2, MAP2K1, AR, PRKCD, CDK1, CDC25B, and JAK2 with regulation of PI3K/Akt, MAPK, and p53 signaling pathways suggested to be the potential mechanism of action. Further, gene expression analysis of selected targets through reverse transcription-quantitative polymerase chain reaction revealed downregulation of AR and BCL2 levels, along with upregulation of p53 and caspase-3 in fraction-treated DU-145 cells compared to the 0.2 % DMSO-negative control. In conclusion, the findings of this study validate the traditional use of *E. ingens* in cancer management.

The identified drug-like compounds, their targets, and associated signaling pathways could serve as a foundation for developing novel strategies for prostate cancer management. However, we recommend additional in vitro and in vivo studies to further substantiate these findings.

Introduction

Cancer is one of the major causes of death globally and a significant contributor to decreased life expectancy. Cancer of the prostate is an adenocarcinoma that often forms in the glandular prostate, it can remain contained in the prostate for a long time or advance by metastasizing outside the prostate. According to the World Health Organization (WHO), in 2020, there were 1414,259 new cases of prostate cancer (PCa); it is the second most common cancer in men and the fifth most significant cause of cancer-related mortality globally [1]. Due to a lack of effective preventive and treatment strategies, data in 2020 show that 77,300 new cases were estimated to have occurred in sub-Saharan Africa [2].

Prostatectomy and local radiation are used to treat localized PCa, which accounts for 90% of PCa cases. However, PCa is diagnosed at an advanced stage in around 90% of men with the disease. The common treatment options for advanced PCa are androgen deprivation therapy (ADT) and chemotherapy. ADT treatment leads to recurrent androgen-independent prostate cancer within 2–3 years, with frequent metastases to regional lymph nodes or the pelvis. Although there are several prostate cancer chemotherapy treatments, there is still a knowledge gap on the evolution of treatment interventions resistance coupled with limited evidence of the

therapeutic options for advanced prostate cancer. Drugs that target rapidly proliferating cancer cells, such as docetaxel and paclitaxel, can also damage healthy cells, causing fatigue, hypertension, hot flushes, arthralgia, fractures, peripheral edema, and rash. Therefore, there is an urgent need for better therapeutic interventions against PCa, and herbal plant sources provide the greatest promise and the “lowest hanging fruits”.

The use of herbal plants in cancer has gained substantial attention, and recently, ongoing research with the US National Cancer Institute (NCI) have played a pivotal role in promoting the use of traditional medicine to treat cancer [3]. Herbal plants have various advantages over chemical products due to their lower chances of inducing adverse effects, cost-effectiveness, tolerability, and reduced chances of developing resistance. Plants exercise their anticancer properties owing to the existence of phytochemicals that act by combating lipid peroxidation through their anti-oxidative properties, repairing damaged DNA, boosting the immune system, inducing apoptosis, and suppressing the cell cycle [4].

Euphorbia ingens E. mey. ex Boiss belongs to the plant family Euphorbiaceae. It is known to contain latex; the leaves, stem, root, and whole plant are applied in traditional medicine for the treatment of cancer and other pathologies, including swellings, fistula, lesions, wounds, abscesses, burns and mental disorders [5,6]. In East Africa, *E. ingens* is also used for the treatment of snakebites, suggesting that the plant may possess an anti-venom property. Additionally, there are scientific data demonstrating the ichthyoidal, antitubercular, antimicrobial, and antifungal activities of *E. ingens* [7–9]. However, despite the aforementioned roles of *E. ingens* as a viable therapeutic option for the management and treatment of diseases in ethnomedicine, there is no scientific validation of its ethnobotanical use in cancer management, and treatment. In this context, we hypothesized that *E. ingens* could selectively abrogate the proliferation of prostate cancer cells.

Various plant parts, including stems, leaves, bark, fruits, and seeds, are employed in the formulation of cancer treatments; the roots, as highlighted by Koduru et al. [6], are mostly used. Also, previous studies have reported *E. ingens*’ stems and leaves to be inactive against human hepatoma, human breast adenocarcinoma, and human colon adenocarcinoma cell lines [10]. To test our hypothesis, we prepared crude (dichloromethane-methanol (1:1, v/v)) extract, hexane-, ethyl acetate-, and water-fractions from the roots of *E. ingens*, assessing their inhibitory effects on DU-145 prostate cancer cells. Subsequently, we evaluated the cellular safety of the ethyl acetate

fraction—identified for its robust antiproliferative activity—in non-cancerous Vero E6 cells. Employing network pharmacology, we probed the likely molecular targets and anticancer mechanisms of action associated with the chemical compounds identified within the efficacious ethyl acetate fraction. Using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), we determined the gene expression levels of selected molecular targets and genes known to play pivotal roles in prostate cancer initiation and progression. Collectively, our study scientifically validates the traditional use of *E. ingens* in treating and managing cancer, positioning the plant as a promising source for discovering bioactive compounds tailored for the treatment and/or management of prostate cancer. This is in line with Sustainable Development Goal 3, Target 3.4, which aims to reduce the mortality rate attributed to cancer by one-third by 2030. It is worthy of note that the goal coincides with goal 3 of Africa Agenda 2063, an aspiration to achieve a population that is healthy and well-nourished across the African continent.

Materials and methods

Plant collection and processing

Fresh root samples of *E. ingens* were obtained on March 26, 2022, from Embu County, Kenya (0° 46′ 27.0″ South, 37° 40′ 54.9″ East), where it grows naturally. Plant identification and authentication were carried out at Egerton University, Kenya by a plant taxonomist, and a voucher specimen number NSN9 was subsequently deposited in the same place. Approximately 2 kg of plant roots were carefully taken, washed thoroughly with water to remove sand and any other contaminants. They were cut into little pieces and shade dried at room temperature for 21 days, with the samples regularly turned upside down to avoid fungal growth. The dried samples were then milled into fine powder using an electric grinder (Christy 8 MILL, serial number 51,474). The powder of *E. ingens* (411 g) was soaked in a volume of 1 L of dichloromethane-methanol (1:1, v/v) solvent. Following percolation and intermittent agitation, the resulting liquid was filtered with Whatman No. 1 filter paper. This was done repeatedly until the initial deep color of the filtrate faded to indicate an exhaustive solvent extraction. The filtrate was successively evaporated to dryness at 57 °C using a 5-liter rotary vacuum evaporator (Rotavapor R-300; Buchi, Switzerland). The obtained extract was 149 g and the yield percentage

were calculated using the following

equation:

Percentage yield of extracts = [(Weight of the obtained extract material/Weight of original fine plant powder used)* 100]

The crude extract was further partitioned into hexane, ethyl acetate, and water fractions as previously described by Beesoo et al.

[11] with slight modification. The dichloromethane and methanol mixture was used to establish an intermediate polarity between the

non-polar and polar solvents, which allowed for the extraction of a significant quantity of chemical compounds [12]. Hexane, ethyl

acetate, and water were used to purify and concentrate non-polar compounds in hexane and polar ones in ethyl acetate and water. Also,

Euphorbia species are known to be rich in essential oils and fats, and hexane, ethyl acetate, and water are defatting solvents [13].

20 g of dried crude extract were weighed and dissolved in 50 ml of a 1:1 solution of dichloromethane and methanol. In a separating

funnel, 300 ml of hexane was vigorously mixed through shaking with the solution that was made.

The mixture was then left undisturbed

for 30 min to ensure proper separation. The upper hexane part was then decanted in a beaker. This was done two more times to

ensure complete partitioning of the hexane fraction. The process continued by introducing 300 ml of ethyl acetate and distilled water

(1:1) into the remaining lower part, from which the hexane fraction was washed off. The ethyl acetate fraction settled atop the distilled

water part, and the two layers were collected separately in beakers. The collected hexane and ethyl acetate fractions were concentrated

by the rotary vacuum evaporator, while the water part was taken for freeze drying. Following partitioning, the crude and obtained

hexane, ethyl acetate, and water fractions were stored at $\pm 20^{\circ}\text{C}$ until further analysis.

Cell culture

A human prostate carcinoma cell line (DU-145) and a kidney epithelial cell line derived from African green monkeys (Vero E6) were

purchased from the American Type Culture Collection (ATCC) and cultured at the Kenya Medical Research Institute's center for

Traditional Medicine and Drug Research. The cells were grown in Modified Eagle's Medium (MEM, Sigma-Aldrich, USA) supplemented

with 1 % L-glutamine (200 mM) (Sigma-Aldrich, USA), 10 % fetal bovine serum (FBS, Gibco, USA), 1.5 % sodium bicarbonate (Loba

chemie, India), 1 % HEPES (1 M) (GoldBio, USA), and 1 % penicillin-streptomycin (Sigma-Aldrich, USA), at 37°C and 5 % CO_2 . The

DU-145 cell line was selected as it has moderate metastatic potential, while Vero E6 is the non-cancerous cell (employed to assess the cellular toxicity of the plant's fraction) [14,15]. The passage numbers of the DU-145 and Vero E6 cell lines used in this study were 30 (DU145HTB-81/P-30/06/22) and 14 (VeroE6/P-14/08/22), respectively; and were sub-cultured twice a week. The logarithmic growth phase of the cells was used in all studies.

Cellular proliferation assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to test how well the plant fractions stopped cell growth. The DU-145 cells at 80 % confluence were washed with phosphate buffered saline (PBS, Sigma-Aldrich, USA) and detached by a 0.25 % trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) solution (Solarbio, China). 100 µl of cell suspensions was taken into a sterile Eppendorf tube, and 50 µl of trypan blue (Loba chemie, India) was added and mixed. The hemocytometer was then used to count the number of viable cells. Cell suspensions with a cell density of 1×10^4 cells/well were seeded onto flat-bottomed 96-well plates and grown for 24 hours. A stock solution of 100,000 µg/ml was prepared by dissolving 30 µg of plant fractions in 300 µl of 100 % dimethyl sulfoxide (DMSO, Finar Chemicals, India) and diluted appropriately with growth medium so that the final concentration of DMSO in the test sample is 0.2 %. The seeding medium was aspirated from the plates, and 100 µl of a 200 µg/ml (in culture medium) working concentration of plant fractions were added for 48 h as screening treatments. The same concentration of doxorubicin (Solarbio, China) was used as a positive control, while 0.2 % DMSO was used as a negative control. After 48 h of exposing the cells to the treatments, 10 µl of freshly prepared MTT (5 mg/ml) was added to each well and incubated for 4 hours. Following that, MTT was aspirated out, and 100 µl of 100 % DMSO was added to solubilize the formazan crystals. Then, using the absorbance at 570 nm, a plate reader (an Infinite M1000 by Tecan) read the 96-well plates. Experiments were carried out in triplicate, and the percentage cell survival was calculated using the formula [14]:

$$\text{Percentage cell viability} = \left[\frac{\text{Absorbance of treated cells} - \text{Absorbance of culture medium}}{\text{Absorbance of untreated cells} - \text{Absorbance of culture medium}} \right] \times 100$$

After the initial screening at a single concentration (200 µg/ml), the fractions with equal or less than 50 % cell viability after 48 h of treatment were considered active and selected for further antiproliferative studies using a range of concentrations (6.25–200 µg/ml)

[16]. The 50 % inhibition concentration (IC₅₀) was then determined. The cytotoxicity of the E. ingens fraction was further assessed on Vero E6 cells using the MTT cell proliferation assay previously described, and the 50 % cytotoxicity concentration (CC₅₀) was calculated.

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