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Article

Antiproliferative Activity of *Euphorbia ingens* Extract against Prostate Cancer Cell Line: An *in silico* and *in vitro* Analysis

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Abstract: *Euphorbia ingens* is traditionally used to treat and manage cancer among Ambeere community of Embu County in Kenya. Whilst research has demonstrated bioactivities of *E. ingens* including antimicrobial, antitubercular and antifungal activities, scientific validation of its anticancer properties is limited. This study evaluated the antiproliferative potentials of *E. ingens* on human prostate cancer cell line (DU-145). The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay was used to assess the antiproliferative activity, chemical constituents were analysed by qualitative colour method and Gas Chromatography-Mass Spectrometry analysis while investigation of putative molecular targets and mechanisms of action of *E. ingens* was done through network pharmacological analysis. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was carried out to validate the network predictions of putative targets. Our result showed *E. ingens* ethyl acetate inhibited DU-145 growth (IC_{50} of 9.71 ± 0.4 µg/ml) with a high selectivity index of 8.26. There was presence of phenols, terpenoids, flavonoids, tannins, sterols, and saponins; additional 18 compounds identified by GC-MS approach. ESR1, IL6, MMP9, CDK2, MAP2K1, AR, PRKCD, CDK1, CDC25B, and JAK2 were indicated as key targets of *E. ingens* against prostate cancer with the PI3K-AKT, MAPK, and p53 signalling pathways identified as the most probable mechanisms of action. There was significant downregulation of AR and BCL2, and upregulation of p53 and caspase-3 in *E. ingens*-treated DU-145 cells compared to 0.2 % DMSO negative control. Our results suggest that *E. ingens* has phytochemical compounds efficacious at inhibiting the proliferation of DU-145 cells; therefore, the plant can be considered a potential source of compounds that may be used to manage and/or treat prostate cancer; however, further *in vivo* evaluations are needed.

Keywords: *Euphorbia ingens*; Anticancer; Antiproliferative; Prostate Cancer; Network Pharmacology; Phytochemicals; Mechanism of action; Molecular targets

1. Introduction

Cancer is one of the major cause of death globally, and a significant contributor to decreased life expectancy [1]. Cancer of the prostate is an adenocarcinoma that often forms in the glandular prostate and can be identified by its characteristic glandular patterns under the microscope [2]. This cancer can advance by spreading outside the prostate or to remain contained in the prostate for a very long

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time. The lymph nodes and bone are common sites where prostate cancer metastasizes; it is hypothesized that the prostatic venous plexus's connection to the spinal veins contributes to the latter [3]. According to the World Health Organisation (WHO), there are 1,414,259 new cases of prostate cancer (PCa) each year, making it the second most common cancer in men and the fifth greatest cause of cancer-related mortality globally [4]. In Africa, the rate of prostate cancer is 26.6% per 100,000 people, and in sub-Saharan Africa, there are an estimated 70,000 new cases per year [5]. Countries with low socio-demographic indexes such as Nigeria and Kenya are affected the most by prostate cancer, and this has been attributed to the lack of effective preventive and treatment strategies in the countries [6].

Prostatectomy and local radiation are used to treat localised PCa, which accounts for 90 % of PCa cases. However, PCa is diagnosed in an advanced stage in around 90 % of men with the disease, when androgen deprivation therapy (ADT) and chemotherapy are common treatment options. [5,7]. ADT treatment leads to recurrent androgen-independent prostate cancer within 2–3 years, with frequent metastases to regional lymph nodes or the pelvis, making the disease advanced [8]. Although there are several prostate cancer chemotherapy treatments, there is still a gap in 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 oedema and rash [9]. Therefore, there is a serious need to prospect 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, research is ongoing, with the US National Cancer Institute (NCI) playing a pivotal role in the research of traditional medicine to treat cancer [10]. Herbal plants have various advantages over chemical products due to their lower chances of inducing adverse effects, cost-effectiveness, their tolerability, and their reduced chances of developing resistance [11]. Findings have shown that herbal plants exercise their anticancer properties owing to the existence of phytochemicals which act by creating antioxidant effects, repairing damaged DNA, boosting the immune system, inducing apoptosis and suppressing cell cycle [12,13].

Euphorbia ingens E.Mey. ex Boiss belongs to the plant family of *Euphorbiaceae* [14], it is known to contain latex, which is applied in traditional medicine for the treatment of cancer and other abnormalities including swellings, fistula, lesions, wounds, abscesses and burns [15,16]. In East Africa, *E. ingens* is also used for the treatment of snakebites suggesting that the plant may possess an anti-venom for snakes[17]. Additionally, there are scientific data demonstrating ichthyoidal, antitubercular, antimicrobial and antifungal activities of *E. ingens* [18–20]. We have previously shown that *E. ingens* extract contain phytochemicals including phenols, tannins, terpenoids, flavonoids and saponins, which are generally associated with anticancer activity [21]. However, despite the aforementioned roles of *E. ingens* as a viable target for the management and treatment of diseases, scientific validation of its ethnobotanical use in cancer management and treatment is lacking. Therefore, we hypothesised that *E. ingens* has the ability to selectively stop the proliferation of prostate cancer cells while not harming normal cells.

To test this hypothesis, we first applied network pharmacology approaches to determine whether compounds in *E. ingens* extract have molecular targets associated with PCa and further investigated the antiproliferative activity of the extract so as to validate the putative activity and targets. We showed that *E. ingens* has selective antiproliferative effect against prostate cancer cell line, with no toxicity towards the non-cancerous Vero cell line.

2. Results

2.1. Plant extract preparation

The percentage yield of the dichloromethane:methanol root extract of *E. ingens* was 36.25%. Of the four fractions (crude dichloromethane:methanol, hexane, ethyl acetate and water) obtained after solvents partitioning, the crude and hexane fractions dissolved sparingly in dimethyl sulfoxide

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(DMSO), and so, they were not considered for further bioassays as account for their concentrations would not be possible.

2.2. Antiproliferative & cytotoxic activity

The ethyl acetate and water fractions were evaluated in a blind screening for their potential antiproliferative activity against DU-145 at a fixed concentration of 200µg/ml (Figure 1). Only the ethyl acetate fraction displayed antiproliferative activity of <50% at 200 µg/ml, and therefore, it was prioritized for concentration-dependent testing through 2-fold serial dilution.

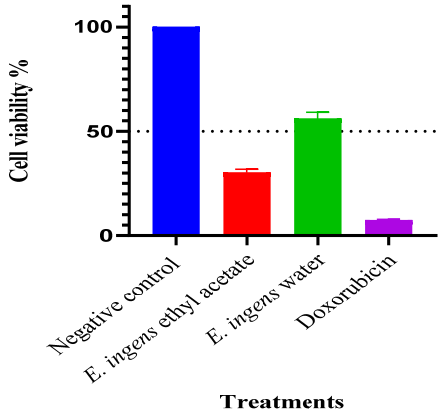

Figure 1. Screening for cellular proliferation inhibition of *E. ingens* fractions: Inhibition of cellular proliferation following 48 h treatment at 200 µg/ml concentration of the water and ethyl acetate fractions of *E. ingens* on DU-145. Doxorubicin at 200 µg/ml was used as positive control, and 0.2% DMSO as negative control. Values are expressed as Mean ± SEM. All treatments were done in triplicates (n = 3).

The inhibition of cell proliferation by the ethyl acetate fraction in a concentration-response dependent manner against DU-145 is shown in (Figure 2); whereby the IC_{50} of the fraction was calculated at 9.71 ± 0.4 µg/ml while the standard reference drug, doxorubicin had a IC_{50} of 5.30 ± 0.11 µg/ml (Supplementary Figure S1). There was significant (p = 0.0115) difference when the two treatments were compared (Table 1).

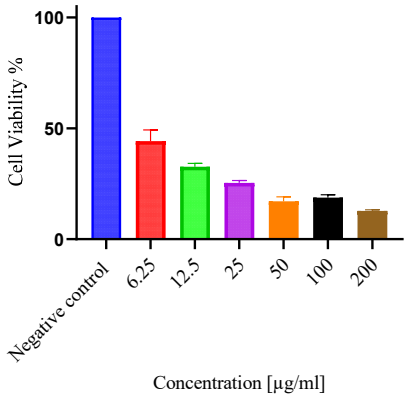

Figure 2. Inhibition of cellular proliferation by *E. ingens* ethyl acetate fraction: Inhibition of cellular proliferation following 48 hours treatment with serial concentrations of *E. ingens* ethyl acetate fraction on DU-145 so as to determine IC_{50}. 0.2% DMSO acted as negative control. Values are expressed as Mean ± SEM. All treatments were done in triplicates (n = 3).

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The fraction *in vitro* safety was determined using non-cancerous Vero E6 cells (Figure 3). The cytotoxic concetration killing 50% of treated cells (CC_{50}) of the *E. ingens* ethyl acetate was 80.19 ± 6.12, which is significantly ($p < 0.05$) different as shown in Table 1 from the doxorubicin drug (176.10 ± 8.09) (Supplementary Figure S2).

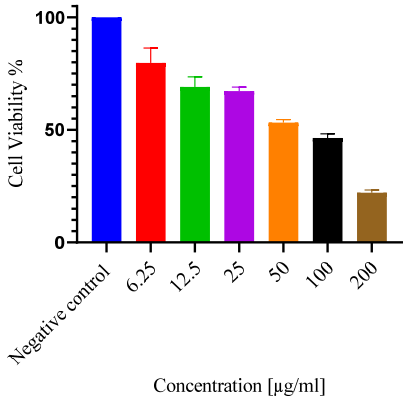

Figure 3. Cellular safety: The potential cellular safety of *E. ingens* ethyl acetate was measured using noncancerous Vero E6. Cells were treated with serial concentrations of the *E. ingens* extract fraction for 48 hours. Values were expressed as Mean ± SEM, all treatments were done in triplicates (n = 3).

2.3. Selectivity index(SI)

The IC_{50} and CC_{50} values for the tested ethyl acetate fraction were determined from the data extracted in Figures 2 and 3 respectively; while for doxorubicin from Supplementary Figures S1 and S2 respectively, and the SI calculated. Table 1 shows the summary of all the values, whereby, the IC_{50} of 9.71 µg/ml and CC_{50} 80.19 µg/ml of the ethyl acetate fraction of E. ingens gave an SI of 8.26; the SI calculated for doxorubicinfor an IC_{50} of 5.30 µg/ml and CC_{50} µg/ml of 176.10 is 33.23.

Table 1. Summary of IC_{50}, CC_{50} and selectivity index values.

Extract	IC_{50} (µg/ml)	CC_{50} (µg/ml)	SI
E. ingens ethyl acetate	9.71 ± 0.40^{a}	80.19 ± 6.12^{a}	8.26
Doxorubicin	5.30 ± 0.11^{b}	176.10 ± 8.09^{b}	33.23

Values that bear another superscript different from that of doxorubicin in a column differ significantly ($p<0.05$) from doxorubicin. Values are expressed as Mean ± SEM.

2.4. Identification and analysis of E. ingens bioactive compounds

2.4.1. Qualitative colour method

The results of the qualitative phytochemical screening to identify the class of compounds that to some extent could be responsible for the observed antiproliferative activity of the ethyl acetate fraction is shown in Table 2, whereby, tannins, terpenoids, flavonoids, saponins and sterols were found to be abundant with phenol moderately present. Alkaloid and quinones were not detected.

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Table 2. Phytochemical profile of *E. ingens* ethyl acetate fraction.

Phytoconstituent	*E. ingens* ethyl acetate
Alkaloids	-
Phenols	++
Tannins	+++
Terpenoids	+++
Flavonoids	+++
Saponins	+++
Quinones	-
Sterols	+++

+++= highly abundant, ++= moderately abundant, += less abundant, - = absent.

2.4.2. In-depth compounds characterisation by Gas chromatography–mass spectrometry (GC-MS)

To further identify the specific compounds that were present in the ethyl acetate fraction, GC-MS was used. The chromatogram of GC-MS spectra of compounds is shown in Figure 4. The individual compound's identification was established on the basis of the peak area, and retention time and the details are presented in Table 3. Twenty two peaks were observed in the chromatogram (Figure 4) comprising 18 compounds (Table 3), majorly terpenoids. The most abundant compounds include 6-pentylidene-4,5-secoandrostane-4,17.beta.-diol (55.39 %), 2-bornanol (16.75 %) and 1-octadecene appearing two times to make up 8.1 %.

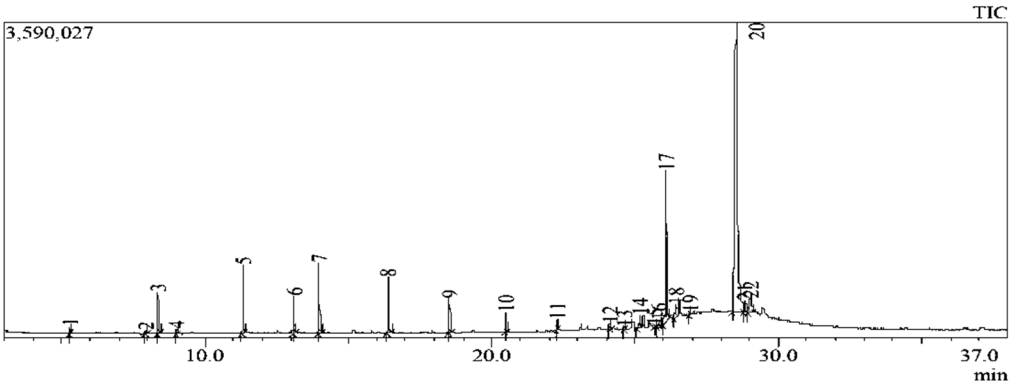

Figure 4. GC-MS chromatogram of the chemical constituents present in *E. ingens* ethyl acetate fraction.

Table 3. The GC-MS identified compounds from *E. ingens* ethyl acetate fraction.

Peak Nr.	Rt (min)	Compound identified	Peak Area %	MW (g/mol)	MF	Structure Type
1.	5.302	1-decene	0.29	140	$C_{10}H_{20}$	Alkene
2.	7.952	Bicyclo[3.1.1]heptan-3-ol	0.23	152	$C_{10}H_{16}O$	Terpenoid
3.	8.381	1-dodecene	2.11	168	$C_{12}H_{24}$	Alkene
4.	9.034	Bicyclo[3.1.1]hept-3-en-2-one	0.25	150	$C_{10}H_{14}O$	Terpenoid
5.	11.339	1-tridecene	3.64	182	$C_{13}H_{26}$	Alkene
6.	13.123	2,4-di-tert-butylphenol	2.03	206	$C_{14}H_{22}O$	Phenol
7.	14.003	1-octadecene	4.05	252	$C_{18}H_{36}$	Alkene
8.	14.003	1-octadecene	4.05	252	$C_{18}H_{36}$	Alkene
9.	18.543	1-heneicosanol	1.86	312	$C_{21}H_{44}O$	Fatty alcohol
10.	18.543	1-heneicosanol	1.86	312	$C_{21}H_{44}O$	Fatty alcohol
11.	18.543	1-heneicosanol	1.86	312	$C_{21}H_{44}O$	Fatty alcohol
12.	24.128	Octadecyl trifluoroacetate	0.37	366	$C_{20}H_{37}F_3O_2$	Fatty Acid
13.	24.639	Prasterone	0.14	288	$C_{19}H_{28}O_2$	Sterol

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14.	25.177	Andrographolide	1.22	350	$C_{20}H_{30}O_5$	Diterpenoid
15.	25.695	Ferruginol	0.13	286	$C_{20}H_{30}O$	Diterpenoid
16.	25.834	(1R,7S,E)-7-isopropyl-4,10-dimethylenecyclodec-5-enol	0.63	220	$C_{15}H_{24}O$	Sesquiterpenoid
17.	26.079	2-bornanol	16.75	348	$C_{16}H_{20}N_4O_5$	Terpenoid
18.	26.435	11-oxoandrosterone	1.53	376	$C_{22}H_{36}O_3Si$	Sterol
19.	26.933	Squalene	0.20	410	$C_{30}H_{50}$	Triterpenoid
20.	28.535	6-pentylidene-4,5-secoandrostane-4,17.beta.-diol	55.39	362	$C_{24}H_{42}O_2$	Sterol
21.	28.827	17.beta.-hydroxy-6.alpha.-pentyl-4-nor-3,5-secoandrostan-3-oic acid, methyl ester	1.78	378	$C_{24}H_{42}O_3$	Fatty acid methyl ester
22.	28.827	17.beta.-hydroxy-6.alpha.-pentyl-4-nor-3,5-secoandrostan-3-oic acid, methyl ester	1.78	378	$C_{24}H_{42}O_3$	

Key: Rt, retention time; MW, Molecular Weight; MF, Molecular Formular.

2.5. In silico work

2.5.1. Screening for drug-like compounds in E. ingens ethyl acetate fraction

Out of the 18 *E. ingens* ethyl acetate compounds that were identified through GC-MS analysis, only 7 were considered ideal drug-like candidate using the Lipinski rule of 5; which includes the compounds inability to penetrate the blood brain barrier, as a non-inhibitors of cytochrome P450 enzymes and having topological polar surface area that were less than 140 square angstrom ($Å^2$). The prioritized 7 compounds (1-dodecene, 1-heneicosanol, 1-octadecene, octadecyl trifluoroacetate, andrographolide and squalene) are presented in Table 4 while detailed results on all 18 compounds are shown in Supplementary Table S1.

Table 4. *E. ingens* ethyl acetate compounds with ideal drug candidate qualities.

S/N	Compounds	MW (g/mol)	HD	HA	Mol LogP	Lipinski's Rule (Violation)	BBB	CYP2D6	CYP3A4	TPSA
1.	1-dodecene	168.32	0	0	5.25	Yes; 1	No	No	No	0
2.	1-heneicosanol	312.6	1	1	5.62	Yes; 1	No	No	No	20.23
3.	1-octadecene	252.5	0	0	6.77	Yes; 1	No	No	No	0
4.	1-tridecene	182.35	0	0	5.52	Yes; 1	No	No	No	0
5.	Octadecyl trifluoroacetate	366.5	0	5	5.47	Yes; 1	No	No	No	26.30
6.	Andrographolide	350.4	3	5	1.98	Yes; 0	No	No	No	86.99
7.	Squalene	410.7	0	0	3.59	Yes; 0	No	No	No	0

Keys: MW, molecular weight; HD, number of hydrogen bond donors; HA, number of hydrogen bond acceptors; MolLogP lipophilicity; BBB, blood brain barrier; CYP2D6, Cytochrome P450 2D6; CYP3A4 Cytochrome P450 3A4 and TPSA, total polar surface area.

2.5.2. The targets of E. ingens prioritized drug-like compounds and therapeutic targets for prostate cancer

From the SWISS TargetPrediction (STP) and BindingDB (BDB) databases, a total of 87 potential targets were identified as targets for the 7 prioritized *E. ingens* compounds (Supplementary Table S2). 76 total targets were retained after removing duplicated targets. Additionally, we retrieved a total of 12,674, and 4,389 target genes that are closely related to PCa from GeneCards and DisGenet, respectively. A total of 12,889 genes were identified after duplicates were eliminated (Supplementary Table S3). Gene datasets obtained from the obtained 76 *E. ingens* compounds-related targets and the

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12,889 PCa-related targets were imported into an online Venn diagram, overlapped and a total of 65 intersecting targets were obtained. The analysed relationship of gene targets between *E. ingens*(drug) and disease resulted into 65 intersection genes (key targets) shown in Figure 5, and further details are presented in Supplementary Table S4.

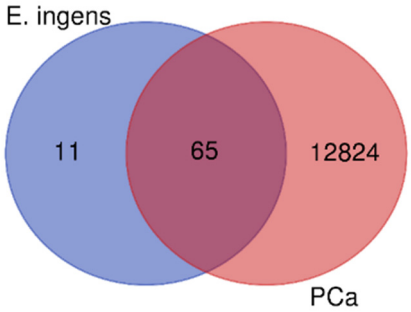

Figure 5. Compound-disease targets Venny intersection diagram, with the compound targets on the left, the disease (PCa) targets on the right, and the two intersection targets in the middle.

2.5.3. Compound-disease target protein–protein interaction (PPI) network

A total of 65 key genes were obtained by mapping the *E. ingens* drug-like compounds targets to the PCa disease targets. The 65 targets were imported into the STRING database and imported into Cytoscape for visualization and analysis, and a network consisting of 65 nodes and 195 edges was obtained (Figure 6). The average node degree is 6 and average local clustering coefficient of 0.48; the PPI enrichment p-value was < 1.0e-16; thus, proteins have more interactions among themselves than would be expected for a random set of proteins of similar size drawn from the genome. Such a significant enrichment indicated that the proteins are at least partially biologically connected as a group. TTL, ACVRL1, PDE10A and SQLE were not analysed in the PPI network, as they do not interact with other proteins. In the network, the top ten targets with the highest the Maximal Clique Centrality (MCC) scores were estrogen receptor alpha (ESR1), interleukin-6 (IL6), matrix metalloproteinase 9 (MMP9), cyclin-dependent kinase 2 (CDK2), mitogen-activated protein kinase kinase 1 (MAP2K1), androgen receptor (AR), protein kinase C delta (PRKCD), cyclin-dependent kinase 1 (CDK1), Cell Division Cycle 25B (CDC25B) and tyrosine-protein kinase JAK2 (Figure 7).

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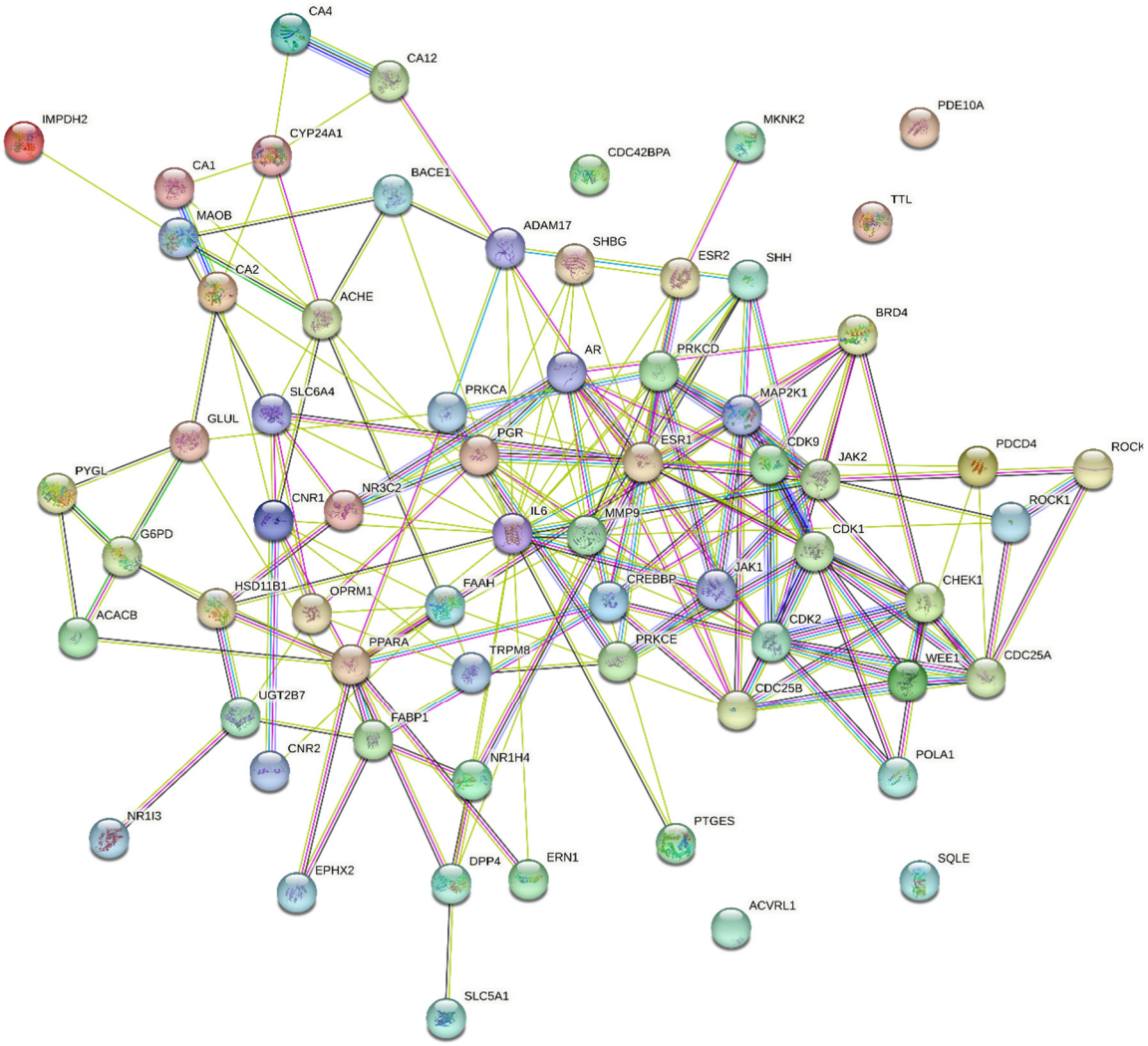

Figure 6. PPI network of the key targets in *E. ingens*' action against human prostate cancer.

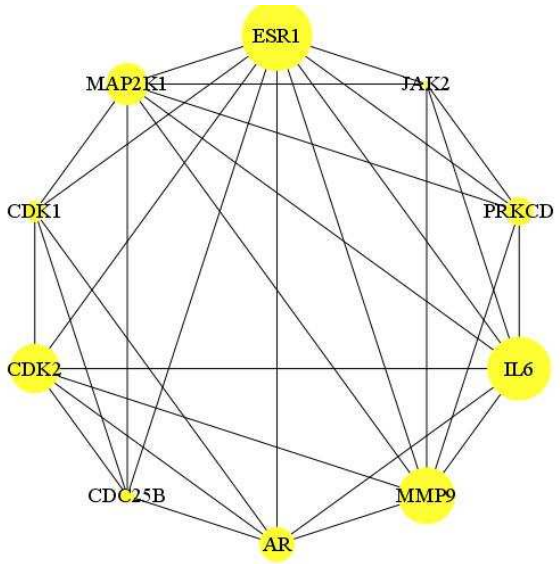

Figure 7. Obtained ten hub genes. The larger the node is, the more important the target in the network.

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2.5.4. GO and KEGG pathway enrichment

The 65 key targets of *E. ingens* against PCa were enriched in 1,418 GO (gene ontology) terms, including 1000 for biological processes (BP) terms, 107 for cellular component (CC) terms, and 311 for molecular functions (MF) terms ($p < 0.05$). The top 20 significant GO terms for each category are shown in the dot plot chart (Figure 8A-C). The y-axis represents the enriched categories and the x-axis represents the number of enrichment and the order of importance was ranked from the top to bottom by −Log10 (p value). GO analysis showed that the key targets mainly play a role in the biological processes of responding to oxygen-containing compound, lipid and organic cyclic compound. CC was mainly enriched in the membrane raft, membrane microdomain and cyclin-dependent protein kinase holoenzyme complex. MF analysis associated the key targets to protein kinase, phosphotransferase and nuclear receptor activities. KEGG (Kyoto encyclopedia of genes and genomes) signalling pathway analysis indicated that the key targets were significantly enriched in 170 pathways, the top 20 signalling pathways were screened by - LogP values (Figure 9A), and the prostate cancer pathway was selected for analysis as the relevant molecular pathways affected by *E. ingens* drug-like compounds treatment on the prostate cancer cell line (Figure 9B).

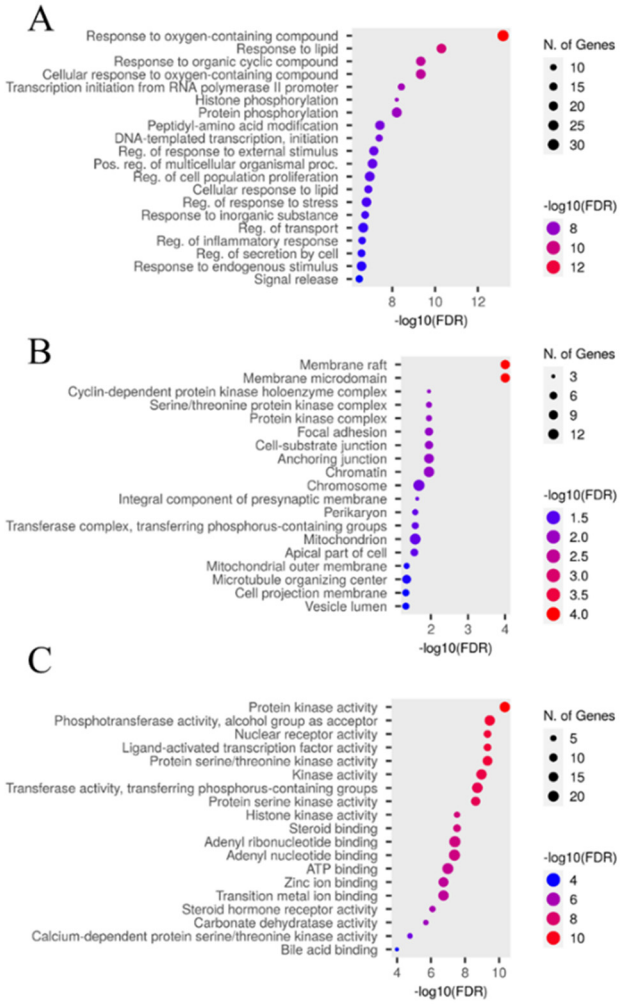

Figure 8. *Euphorbia ingens* GO enrichment analysis (**A**) BP terms (**B**) CC terms (**C**) MF terms.

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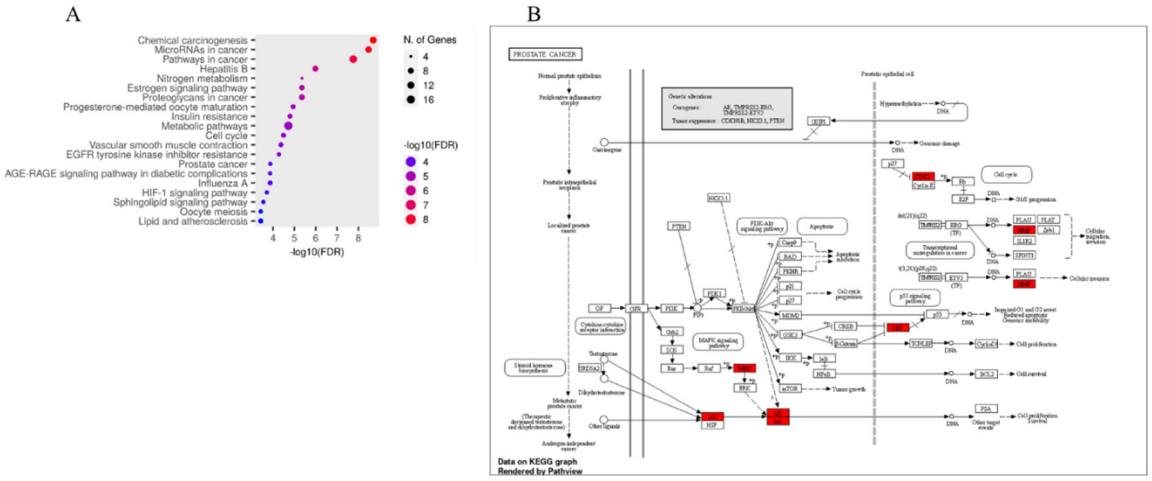

Figure 9. KEGG signaling pathway analysis and prostate cancer pathway of *E. ingens*-PCa. (**A**) KEGG signaling pathway analysis. Each bubble represents a KEGG pathway on the vertical axis. The - logP values are shown on the horizontal axis. The size of each bubble indicates the number of genes enriched in each KEGG pathway. Larger bubbles indicate more genes involved in the pathway. The colour of each bubble represents the adjusted *P*-value of each KEGG pathway, with redder color indicating smaller adjusted *P*-value (**B**) Prostate cancer pathway (red marks represent potential targets for *E. ingens* intervention).

2.6. RT-qPCR results

The mRNA expression of AR, CDK1, p53, BCL-2 and Caspase-3 was measured by RT-qPCR to validate the genes associated with the prostate cancer pathway (Figure 9B) as demonstrated by network pharmacology. AR and CDK1 were among the ten hub genes. Further, we picked p53, BCL-2 and caspase-3 genes due to their involment in tumorigenesis and cancer chemotherapy through deregulation of cell survival and apoptosis. The quantification cycle, also known as the threshold cycle (Ct), was calculated, and relative mRNA expression levels of target genes were normalized to β -Actin using the $2^{-\Delta\Delta Ct}$ method [22]. The results are presented in Figure 10.

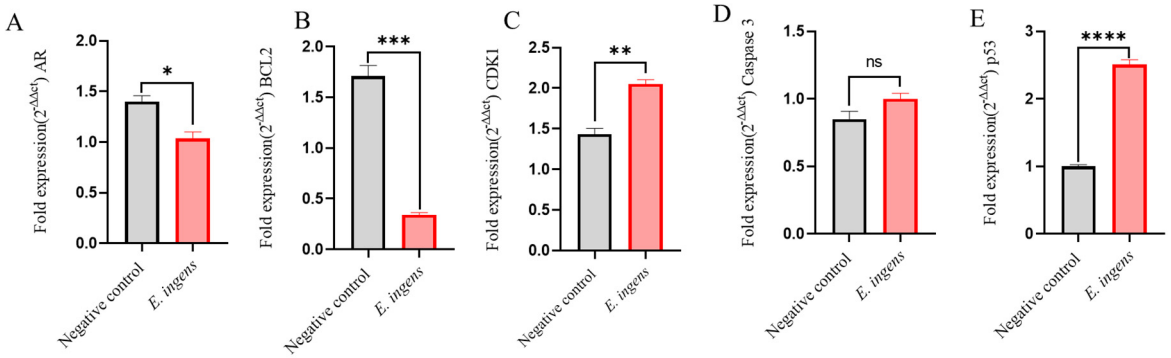

Figure 10. Relative gene expression analysis of *E. ingens* treated and untreated DU-145 cells (A) AR, (B) BCL-2, (C) CDK1, (D) caspase-3 and (E) p53. *ns* $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ as compared to untreated control.

There was significant downregulation of AR (p = 0.0129) and BCL2 (p = 0.0002, relative fold change recorded was 5.0). Furthermore, while significant upregulation was also observed in the expression level of p53 ($p < 0.0001$, relative fold change of 2.5), there was no significant difference in the upregulation of caspase 3 (p = 0.1004). Though, CDK1 was observed to be upregulated, the RT-

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qPCR results still allude that drug-like compounds in *E. ingens* ethyl acetate fraction interfere with prostate cancer cells proliferation by inducing apoptosis.

3. Discussion

Active surveillance, surgery, radiotherapy, hormonal therapy, immunotherapy and chemotherapy are treatment options currently available for prostate cancer. These options have major limitations, particularly the accompanying adverse side effects; hence, prostate cancer remains to be incurable. Nevertheless, natural products provide an outlet for identification of promising new anticancer agents that are highly efficient with low toxicity [23]. Cancer cells exhibit sustained proliferation and resistance to cell death; and thus, compounds that can stop or slow down cell proliferation in rapidly dividing cells hold great promise as anticancer therapeutics [24]. Based on the cell-based MTT assay, our findings demonstrated a significant selective antiproliferative effects of extracts from *E. ingens* roots towards the DU-145 prostate cancer cell lines without affecting the non-cancerous ones (Vero E6). An $IC_{50} < 30$ µg/ml is one of the established criteria by the US National Cancer Institute (NCI) to consider crude extracts as being cytotoxic. Interestingly, we observed that the ethyl acetate fractions of *E. ingens* caused cytotoxicity in DU-145 with IC_{50} value that was less than 10 µg/mL (Table 1). Therefore, *E. ingens* has significant potential for further studies as a potential chemotherapeutic agent. The inhibitory effects of *E. ingens* ethyl acetate on DU-145 is better when compared to that reported for longifolene isolated from *Chrysopogon zizanioides* (IC_{50} of 78.64 µg/mL) on the same DU-145 cells [25]. Meanwhile, El-Hawary et al.[26] found *E. ingens* to be inactive when tested against human colon adenocarcinoma (CACO2), human hepatoma (HepG2) and human breast adenocarcinoma (MCF-7) cell lines. This might be related to the difference in the solvents extraction, hence a likely different set of chemical compounds; while they used the methanolic extract of the plant, we test the ethyl acetate fraction of crude (dichloromethane:methanol) extract. A future study to investigate the cytotoxic effect of ethyl acetate fraction of *E. ingens* on the CACO2 and HepG2 cell lines is encouraged. Meanwhile, a drug with a measure of the safety margin (selectivity index) of ≥2 is considered highly selective [27]. The ethyl acetate fraction of *E. ingens* had an SI greater than 2, an indicator of high selectivity for the cancer cells. Previous studies have also shown that *E. ingens* bioactivities are not a result of general toxicity [19].

The findings of this study depict a high abundance of terpenoids which act on different stages of tumour development and exhibit their anticancer properties by inducing autophagy in cancer cells *via* a complex signalling pathway [28,29]. In recent study, sesquiterpenoids have been shown to restrict cell cycle in prostate cancer through induction of apoptosis [30]. Flavonoids are known antioxidants under normal conditions and potent pro-oxidants in cancer cells, triggering the apoptotic pathways and downregulating pro-inflammatory signalling pathways [31,32]. Other present phytoconstituents, phenols, tannins, saponins, and sterols, were previously shown to possess anticancer properties. Phenols and tannins have been demonstrated to participate in cell cycle arrest, induce apoptosis, and suppress cancer cell proliferation and invasiveness [32,33]. Similar mechanisms as well as regulation of angiogenesis have been attributed to saponins and sterols [13,34]. Some of the GC-MS-identified compounds in this study have been isolated and reported to elicit anticancer activity while others have been identified to synergistically act with other compounds in many medicinal plants. For example, andrographolide, diterpenoid, that was isolated from *Andrographis paniculata* Nees induced cell cycle arrest and apoptosis in HT-29 human colon cancer cells [35]. By increasing intracellular reactive oxygen species, squalene induced anti-proliferative activity against ovarian, breast, lung, and colon cancers [36]. Similarly, diterpene phenol, ferruginol has pharmacological properties such as inhibition of the growth rate of cancer cells [37]. Other compounds which have also been reported to have anticancer activity include 1-octadecene, 1-heneicosanol, and 2,4-di-tert-butylphenol [38–40]. Given that these compounds are found in the ethyl acetate fraction of *E. ingens*, we suggest that they may be responsible for the demonstrated antiproliferative effects of the plant on DU-145 cells. However, further research is required to determine the antiproliferative activity of these isolated compounds, particularly on prostate cell lines.

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Network pharmacology is currently used in cancer therapy to develop new drugs [41]. The multi-target pathways application of network pharmacology is widely adopted to study the mechanism of action of traditional medicine; it identifies the active ingredients of plants, predict their targets, and subsequently combine them with disease targets to generate a presentable drug-target-disease relationship [42]. We selected 7 GC-MS-identified compounds with good absorption, distribution, metabolism, and excretion (ADME) activity using the RO5, as well as blood–brain barrier, total polar surface area, CYP2D6 and CYP3A4. Drug screening and development relies heavily on pharmacokinetic characteristics. Without appropriate pharmacokinetic qualities, drugs will fail to attain requisite concentration in the target organs where they are needed to produce therapeutic effects [43]. The candidate targets of the selected *E. ingens* bioactive compounds for prostate cancer treatment were obtained; of which ESR1, IL6, MMP9, CDK2, MAP2K1, AR, PRKCD, CDK1, CDC25B and JAK2 were at the core position in the PPI network. These targets are considered the possible molecular targets of the drug-like compounds in *E. ingens* ethyl acetate fraction against prostate cancer cells.

The MMPs family is known to have proteolytic effect on the cell membrane; member proteins such as MMP9 release proangiogenic factors which acts on endothelial cells to induce cell migration and proliferation [44,45]. An increased level of MMP9 was reported to cause metastasis in androgen-independent prostate cancer [46]. There have been reports on the role of ESR1, PRKCD, and IL6 in the proliferation and migration of PCa cells, and inhibitors of JAK2 have be suggested to be important in the treatment of advanced PCa [47–50]. Targeted inhibition of MAP2K1 expression has been shown to elicit cell apoptosis and weakening of cell proliferation in DU-145 and PC-3 prostate cancer cells [51]. The cell cycle protein-dependent kinase (CDK) and cell cycle proteins, as well as CDK inhibitors are essential in the regulation of cell cycle, hence, impairment in the activities of these cell cycle mediators is observed in many types of cancer. CDK2 is a core regulator of cell cycle through late G1-phase and S-phase. CDK2 is thought to be strongly linked to development of cancer, and accumulating evidence shows that inhibition of CDK2 induces cancer cell apoptosis without cellular damage [52]. CDC25B is a cell cycle transitions regulatory enzyme; it is an important target of the checkpoint machinery in maintaining genome stability during DNA damage [53]. Overexpression of CDC25B has been reported in many types of human cancers, and targeted cellular depletion of the enzyme in DU-145 facilitated rapamycin anticancer effects [54]. Given the clear evidence that these genes are involved in the development and progression of prostate cancer, the ability to modulate them would have contributed to the observed antiproliferative property of the ethyl acetate fraction of *E. ingens*.

The KEGG enrichment results showed that many disease pathways that were not relevant to this study were enriched, probably because the same molecular targets exist in the development of different diseases. The prostate cancer pathway was selected for analysis, the pathway showed a sets of multiple pathways, through which the therapeutic effect of *E. ingens* on prostate cancer may be produced; these include the PI3K/Akt, MAPK and p53 signalling pathways. The phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) signalling pathway is an important tumour cell pathway which participates in the occurrence, invasion, and distant metastasis of prostate cancer [55]. Long-term ADT can abnormally activate the PI3K/Akt pathway thereby enhancing the antiapoptotic ability of tumour [56]. Therefore, the PI3K/Akt pathway is an important potential target of non-AR pathway in the treatment of PCa. Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that could link extracellular signals to fundamental cellular processes such as cell growth, proliferation, differentiation, migration, and apoptosis. The p53 pathway is one of the crucial signalling pathways for cancer cell apoptosis [57], as a cancer suppressor gene, p53 regulate the downstream genes and take a part in DNA repair and regulation of the cell cycle and apoptosis. Hence, reactivation and restoration of p53 function holds great potential for the treatment of PCa.

Multiple lines of evidence in the field of cancer research have demonstrated the role of secondary metabolites in natural products is to affect gene expression by influencing significant transcription factors involved in carcinogenesis [58]. We used RT-qPCR to validate the predicted molecular targets

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of *E. ingens* in prostate cancer. Overexpression of CDK1 promotes progression of PCa, and when this kinase is hyperactivated, it mediates the phosphorylation that activates AR when there are no ligands [59,60]. AR signalling plays a crucial role in the growth of normal prostate tissue and PCa pathogenesis and progression. In advanced PCa stages, AR mutations and overexpression contribute to sustained proliferation [61]. The treatment of DU-145 prostate cancer cells with *E. ingens* resulted into a significant downregulation in AR when compared with the untreated control. Similar results were reported on the modulation of AR expression by the flavonoid genistein. [62] However, *E. ingens* showed upregulation in the expression of CDK1. This unusual finding was also observed by Mustafa et al.[63]. CDK1 has been said to be capable of avoiding an accumulation of oncogenic mutations during cell division, that might explain the result [64]. In cancer cells, the evasion of cell death is often achieved by either upregulating anti-apoptotic proteins like BCL-2 or by impairing the function of pro-apoptotic proteins such as caspase-3. BCL-2 controls the mitochondrial apoptotic pathway by binding to pro-apoptotic proteins and preventing pore formation and cytochrome c release. Apoptosis is executed by caspases and various upstream regulatory factors, including p53. We observed an upregulation in the expression of caspase-3 and p53 with concomitant downregulation of BCL-2 when *E. ingens*-treated prostate cancer cells were compared with the untreated prostate cancer cells; this pattern was also reported by Eltamany et al.[65]. Moreover, recent computational study revealed that flavonoids, found to be abundant in our study, have strong interaction with caspase-3, BCL-2 and p53 [66]. The difference in the expression level of AR, p53, BCL-2 and caspase-3 between the *E. ingens*-treated human prostate cancer cells and the untreated cells justifies the network pharmacology predictions; as these genes are distributed in the PI3K/Akt, MAPK and p53 signalling pathways, the results further suggest that *E. ingens*' antiprofelirative activity is likely by modulating the pathways to cause induction of apoptosis as well as suppression of cell cycle.

4. Materials and Methods

4.1. Plant collection and extract preparation

Fresh root samples of *E. ingens* were obtained on 26th March, 2022 from Embu County, Kenya (0° 46' 27.0" South, 37° 40' 54.9" East) where it grows naturally. Plant identification and authentication was carried out at Egerton University, Kenya by a plant taxonomist, and a voucher specimen number NSN9 was subsequently deposited in the same place. The dried roots of the plant were extracted using dichloromethane:methanol as previously described [21]. The obtained extract of 149 g and the yield percentage was calculated using the following equation [67]:

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

We further partitioned the crude (dichloromethane:methanol) extract into hexane, ethyl acetate and water fractions. 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 mixed with the solution that was made. The mixture was then left undisturbed for 30 minutes to ensure proper separation. The top hexane part was then put in a beaker. The lower part was washed over and over with hexane, and the different fractions of hexane were collected and concentrated. This process was repeated using 300 ml of ethyl acetate and distilled water (1:1). At the end, the ethyl acetate fraction was collected and concentrated, while the bottom distilled water part was taken for freeze drying. Following partitioning, the crude, and obtained hexane, ethyl acetate, and aqueous fractions were stored at -20 ˚C until further analysis.

4.2. 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 Centre for Traditional Medicine and Drug Research. The cells were grown in Modified Eagle's Medium (MEM) supplemented with 1% L-glutamine (200 mM), 10 % fetal bovine serum (FBS), 1.5 % sodium bicarbonate, 1 % HEPES (1 M), 1%

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penicillin/streptomycin, and 0.4 % phenol red at 37 °C and 5 % CO_2. The logarithmic growth phase of the cells was used in all studies.

4.3. Cellular proliferation assay

The antiproliferative activity of tested fractions was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [68]. The DU-145 (1×10^4 cells/well) were seeded in 96-well plates and grown for 24 h. The seeding medium was aspirated from the plates, and 100 µl of a 200 µg/ml working concentration of plant fractions were added for 48 h as screening treatments. The same concentration of doxorubicin was used as a positive control, while 0.2 % dimethyl sulfoxide (DMSO) was used as a negative control. 10 µl of freshly prepared MTT (5 mg/ml) was added to each well. After 4 h incubation, MTT was aspirated out and 100 µl of 100% DMSO was added to solubilize the formazan crystals. Plates were then read on an Infinite M1000 by Tecan (a plate reader) using the absorbance at 570 nm. Experiments were carried out in triplicates, and the percentage cell survival was calculated using the formula below [24]:

Percentage cell viability = [(Absorbance of treated cells –Absorbance of culture medium/Absorbance of untreated cells –Absorbance of culture medium) * 100]

The extracts with equal or less than 50% cell viability after 48 hours of treatments were considered active [69]. After the initial screening at a single concentration (200 µg/ml), only the active fraction was selected for further anti-proliferative studies using a range of concentrations (6.25-200 µg/ml) and the 50% inhibition concentration (IC_{50}) was determined. The cytotoxicity of *E. ingens* fraction was further assessed on Vero E6 cells using the MTT cell proliferation assay previously described, and the 50% cytotoxicity concentration (CC_{50}) was calculated.

4.4. Selectivity index

The selectivity index (SI) for each of the tested plant fraction was determined by dividing the IC_{50} by the corresponding CC_{50}.

4.5. Identification of plant compounds

The plant fraction that showed antiproliferative activity was subjected to the colour change qualitative phytochemical screening and GC-MS analysis as previously described [21]. This was to identify the compounds responsible for the activity as well as the class of the bioactive compounds.

4.6. In silico work

4.6.1. Drug candidate screening test

The Canonical SMILES (Simplified Molecular Input Line Entry System) of the GC-MS identified compounds were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The SMILES were then submitted to the Swiss ADME (absorption, distribution, metabolism, and excretion) tool (http://www.swissadme.ch/index.php) to predict the drug likeness and physicochemical properties of the compounds [70]. The prediction was based on the parameters of the blood–brain barrier, total polar surface area, cytochrome P450s (CYP2D6 and CYP3A4) and Lipinski's rule of five (RO5) (a drug candidate should have a molecular weight of fewer than 500 daltons, a number of rotatable bonds of less than 10, a number of hydrogen bond donors of less than 5, a number of hydrogen bond acceptors of less than 10, and a lipophilicity (log P) value of less than 5). It was predicted that a molecule would be a non-orally accessible medicine if two or more of the RO5 were not met [71].

The polar atoms of a molecule add up to the topological polar surface area (TPSA), which can be used to predict how the drug will be transported. In most cases, the amount of TPSA in an approved medicine was below 140 Å2 [70]. Substances that are able to breach the blood-brain barrier (BBB) can be hazardous to the nervous system because they can pass from the more hydrophilic blood to the more lipophilic brain. A drug candidate should not block cytochrome P450 enzymes (such as CYP2D6 and CYP3A4) because they are essential for drug metabolism [70].

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4.6.2. Identification of candidate targets in Euphorbia ingens against Pca

Compounds targets were predicted using BindingDB (https://bindingdb.org/rwd/bind/chemsearch/marvin/FMCT.jsp) correspondence to the known ligand molecules having minimum similarity of >0.7 and their Gene IDs were retrieved from UniProtKB (https://www.uniprot.org) database [72]. Similarly, the SMILES of the compounds were uploaded to the Swiss TargetPrediction (http://www.swisstargetprediction.ch/)database, with "humans" (Homo sapiens) as the study species, and the probability of each potential target was determined to be >0 [73]. The retrieved targets were converted into standardized abbreviations by UniProt. The resulting predicted compounds targets from the two databases were pooled together and duplicates were removed [74].

Disease targets of PCa were collected from the GeneCards (https://www.genecards.org/) and the DisGeNET (https://www.disgenet.org/) databases [75]. The targets from the databases were searched using the keyword "prostate cancer"; retrieved results were merged, and ensured there was no repeats. Finally, the targets of active compounds of *E. ingens* and disease targets of PCa were intersected using the bioinformatics and evolutionary genomics platform (https://bioinformatics.psb.ugent.be/webtools/Venn/).

4.6.3. Construction of the protein-protein interaction (PPI) network

The intersection obtained, which are considered the common potential targets were up loaded to the STRING 11.5 database (https://string-db.org/), the species was set as "*Homo sapiens*", and the minimum interaction threshold was set to 0.4. The cytohubba plug-in in Cytoscape software (version 3.9.1) was used to to analyse the topology of the network and the Maximal Clique Centrality (MCC) algorithm was used to filter out the top 10 key targets [76].

4.6.4. Gene Ontology (GO) & Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis

The GO and KEGG enrichment analyses for the targets of *E. ingens* for PCa treatment were conducted in the enrichment tool ShinyGO version 0.76 (http://ge-lab.org/go/). The following parameters were considered for the analyses: species = Human, false discovery rate (FDR) cut-off = 0.05, and number of pathways to show = 20. The biological process (BP), the cellular component (CC), and the molecular function (MF) categories were considered for the gene ontology analysis.

4.7. Quantitative real-time polymerase chain reaction (RT-qPCR)

Eighty (80) % confluent DU-145 in T-25 flask were treated with *E. ingen* fraction at concentration equivalent to the calculated IC_{50}. Negative control cells were exposed to fresh growth media with 0.2 percent DMSO. The cells were incubated for 48 h. Cells were collected for the extraction of total RNA and the reverse transcription was conducted using FIRE Script RT cDNA synthesis kit (Solis BioDyne, Estonia). Luna Universal qPCR Master Mix (New England Biolabs) was used for RT-qPCR) detection. The primer sequences (Macrogen Europe BV, Netherlands) are listed as follows:

Table 5. Primers sequences.

Genes	Primers
AR	Forward- GCTTTATCAGGGAGAACAGCCT Reverse- TGCAGCTCTCTCGCAATCTG
BCL2	Forward- GGCCTCAGGGAACAGAATGAT Reverse- TCCTGTTGCTTTCGTTTCTTTC
CDK1	Forward- GAACACCACTTGTCCCTCTAAGAT Reverse- CTGCTTAGTTCAGAGAAAAGTGC
Caspase-3	Forward- CAAAGAGGAAGCACCAGAACCC Reverse- GGACTTGGGAAGCATAAGCGA

P53	Forward- CTTCGAGATGTTCCGAGAGC Reverse- GACCATGAAGGCAGGATGAG
β –Actin	Forward- GCCAACTTGTCCTTACCCAGA Reverse- AGGAACAGAGACCTGACCCC

4.8. Statistical analysis

GraphPad Prism version 8.4.0 software (San Diego, CA, USA) was used for statistical analysis. Data was presented as the mean ± SEM. An independent-sample T test was used for the comparison between two groups and one-way ANOVA was used for the comparison between multiple groups, and $p < 0.05$ was considered as statistically significant.

5. Conclusions

Our findings are the first to demonstrate that *E. ingens* has a selective cytotoxicity effect against prostate cancer cell line. Network pharmacology pointed to ESR1, IL6, MMP9, CDK2, MAP2K1, AR, PRKCD, CDK1, CDC25B and JAK2 as likely molecular targets of *E. ingens* against prostate cancer, with the regulation of the PI3K/Akt, MAPK and p53 signalling pathways as the plant's molecular mechanism of action. Interestingly, the expression level of AR, p53, caspase-3 and BCL-2 was reversed in *E. ingens*-treated prostate cancer cells when they were compared with the untreated cancer cells. Additional use of other prostate cancer cell lines (PC3, and LNCaP) might have further added to the robustness of our data; this we considered the limitation of this study. Nonetheless, ethyl acetate fraction of *E. ingens* could be a source of new effective and safe alternative to current prostate cancer treatments. However, additional *in vivo* research is necessary to better enhance the potential clinical use of the plant.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: I.O.: Conceptualization; Methodology; Data curation; Writing—Original draft preparation. F.N.: Conceptualization; Supervision; Reviewing and Editing. M.K.: Conceptualization, Supervision; Reviewing and Editing. J.O.: Conceptualization, Reviewing and Editing. S.N.: Conceptualization; Methodology; Supervision; Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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