

In Vitro Anti-Proliferative Activity of Selected Plant Extracts Against Cervical and Prostate Cancer Cell Lines

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Abstract

Prostate and cervical cancers are among the leading cancers in men and women respectively. In 2012 the world health organization reported an estimated 266,000 deaths from cervical cancer worldwide, while 1.1 million men worldwide were diagnosed with prostate cancer. The current conventional cancer therapies, chemotherapy and radiotherapy, present severe side effects and in many developing countries are inaccessible to many cancer patients. The alternative traditional medicine offers the much-needed hope. This study investigated the anti-proliferative activity of methanolic and water extracts from four plant species namely *Aloe secundiflora*, *Maytenus obscura*, *Vernonia zanzibarensis* and *Dichrostachys cinerea* using prostate cancer cells (DU145 and 22Rv1), cervical cancer cells (HeLa) and African green monkey cells (Vero) cell lines using the MTT assay. All extracts suppressed the growth of the cancer cells in a dose-dependent manner at concentrations of 1.37 µg/ml to 1000 µg/ml. The methanol extract of *D. cinerea* stem bark had the highest anti proliferative activity among the plant extracts studied with an IC50 of 8.04 ± 2.02 µg/ml against the 22Rv1 cells and a low cytotoxic effect against the Vero cells with CC50 of 812.1 ± 12.72 µg/ml. The study indicates that the methanol extract of *D. cinerea* stem bark has potential anti-proliferative activity with low cytotoxicity to normal cells. Our results validate the ethnomedicinal use of these plants for management of cancer. The active elements in the extracts studied here need to be isolated and purified to investigate the synergy and additive pharmacological effect in killing cancer cells.

Keywords: Cancer; Anti proliferation; Extract; Cytotoxicity; DU145; Vero; 22Rv1; HELA; IC50

Introduction

Cancer is a significant disease worldwide with considerable geographical variations in incidence, mortality and survival. As of 2012, there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer worldwide [1].

Surgery is often the first treatment option. However, the simple excision with extensive resection does not prevent future recurrence and metastasis [2]. Chemotherapy and radiotherapy are alternative measures of cancer management. However, they are often limited by the toxicity to non-target tissues in the body [3]. The alternative traditional medicines offer the much-needed hope. Plants hold a vast cheap potential source for new anticancer compounds with the reduced toxicity of conventional chemotherapeutic drugs [4].

Herbalists from the Lake Victoria region of Kenya have traditionally used *A. secundiflora* to treat various ailments such as chest problems, polio, malaria and stomach ache but with little scientific knowledge on their modes of action [5]. Plants of the genus *Maytenus* have antiseptic, antiasthmatic, fertility-regulating agents and antiulcer properties [6]. The *M. buchananii* contain the compound Maytansine, a highly potent microtubule inhibitor [7]. The traditional healers from Nairobi use *M. obscura*, and *M. buchananii* extracts for the palliative care of cancer patients discharged from the hospitals [8]. In Kenya and Tanzania, the

Vernonia zanzibarensis extracts are used to treat pimples, skin rashes and stomach pains [9]. Extracts from *Dichrostachys cinerea* bark and leaves are active against pathogens causing diseases such as dysentery, elephantiasis, gonorrhoea and boils [10].

We hypothesized that the methanolic and water extracts from *Aloe secundiflora*, *Maytenus obscura*, *Vernonia zanzibarensis* and *Dichrostachys cinerea* have anti-proliferative activity. The extracts *in vitro* anti-proliferative activity against DU145 and 22Rv1 (Prostate cancer), HeLa (cervical cancer) and Vero (African green monkey), cancer cells was investigated using the MTT assay. In this study we found significant anti-proliferative activity of the organic extract of *D. cinerea* stem bark against the 22Rv1 extracts.

Materials and Methods

Reagents

The study used the following cancer cell lines: human prostate carcinoma epithelial cell line; 22Rv1 (ATCC[®]CRL-2505TM) and DU-145 (ATCC[®]HTB-81TM), human cervical carcinoma cell line; HeLa (ATCC[®]CCL-2TM) and African Green Monkey Kidney Epithelial Cell line; Vero (ATCC[®]CCL-81TM) cells. ATCC-formulated Eagle's Minimum Essential Medium (MEM), RPMI-1640 medium and fetal calf serum (Gibco Laboratories Grand Island, NY, USA). The MTT (3-(4,5-dimethyl thiazolyl)-2, 5-diphenyl-tetrazolium bromide), dimethyl

sulfoxide (DMSO) and other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Collection and preparation of plant material

Plant material of *M. obscura*, *A. secundiflora*, *D. cinerea* and *V. zanzibarensis* were collected from a natural reserve in Kibwezi, Kenya and identified by a plant taxonomist. After washing the plant parts, the selected components were dried and ground using a laboratory mill (Christy and Norris Ltd., Chelmsford, England), weighed, packaged in brown bags and stored for further use.

Aqueous extraction

The aqueous fractions were prepared using distilled water as the solvent. Briefly, 100 ml of distilled water was added into individual flasks containing 50 grams of the plant part. The flasks were capped with cotton wool and placed into a water bath at 60°C for 2 hours. After filtering the mixture into the round bottom flask, the contents were frozen in dry ice. The frozen extracts were lyophilized in a freeze dryer machine (Edwards freeze dryer Modulyo) for 48 hours. The final product weighed and stored in a -20°C freezer for the subsequent assays.

Organic solvent extraction

For each plant part, 50 grams of the dried-ground material were weighed and put in a conical flask. A volume of 200 ml of methanol was added to the plant material in a conical flask and capped with cotton wool to prevent evaporation of the methanol. The methanol containing the extract was filtered after incubating the mixture at room temperature for 24 hours. The filtrate was evaporated (concentrated) to dryness under reduced pressure using a rotatory evaporator set at 60°C. Fresh methanol (100 ml) was added to the macerate and extracted for 48 hours. The mixture was then filtered and mixed with the previous extract after solvent evaporation. The extracts obtained were labelled and stored in a -20°C freezer for further assays.

Preparation of test extract

10 mg of the test extracts were dissolved in 100 µl DMSO and topped up with 900 µl of PBS to make a stock solution with a final concentration of 10 mg/ml. Before using the stock solution, the mixture was filter sterilized using 0.22 µm Millex[®] syringe driven filter unit. The stock solution was kept at 4°C fridge in a dark-colored Eppendorf tube.

Anti-proliferative assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye reduction assay (Sigma, USA) was used to evaluate the cell viability. This colorimetric assay utilizes the enzymatic activity of mitochondrial succinate dehydrogenase enzyme found in living cells. The enzyme reduces the yellow water-soluble MTT to formazan insoluble purple colored crystals that are measured using a spectrophotometer [11,12]. The activity of the enzymes to produce formazan is directly proportional to the level of cell viability and inversely proportional to the level of cell inhibition (WST-8 patent) [13].

Cells were maintained as a monolayer culture in their respective cell culture media supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified incubator at 5% CO₂. The cells were

seeded in 96-well plates to a final volume 100 µl media containing 2.4×10^4 cells per well, enumerated using the trypan blue dye exclusion method. The plates were incubated for 24 hours, then the extracts were added in row H and serially diluted up to row B in their respective wells at concentrations of 1000 µg/ml, 333.33 µg/ml, 111.11 µg/ml, 37.04 µg/ml, 12.35 µg/ml and 4.12 µg/ml. Rows A, of the 96 well plates were left untreated to serve as a negative cell control. After 48 hours of incubation, the culture medium in the plates was discarded, followed by washing step using phosphate buffered saline (PBS). A volume of 10 µl of PBS solution containing 5 mg/ml MTT dye (5 mg of MTT, dissolved in 1 ml serum-free PBS) was added to all the wells and incubated for another 4 hours after which 100 µl of 100% DMSO was added. The plates were read using a scanning multiwell spectrophotometer (Multiskan Ex lab systems) at 562 nm and 690 nm as reference. The proliferation rate of the cells was calculated using the formula by Patel [12].

$$\text{Proliferation rate} = \frac{At - Ab}{Ac - Ab}$$

$$\text{Percentage viability} = \frac{At - Ab}{Ac - Ab} \times 100$$

$$\text{Percentage inhibition} = 100 - \frac{At - Ab}{Ac - Ab} \times 100$$

Where,

At = Absorbance value of test compound

Ab = Absorbance value of blank

Ac = Absorbance value of negative control (cells plus media)

Results and Discussion

Plant products have been used in traditional medicine to manage a variety of diseases including cancer. Despite the potential of plants to provide useful pharmaceutical agents, the medicinal potential of various herbal drugs is not yet scientifically studied. In recent times there has been a great interest in the search for novel medicinal plant products as the number of chronic diseases such as cancer develop resistance to conventional drugs. Plants represent a vast potential source for anticancer compounds that can boost the activity of traditional chemotherapeutic drugs [4].

This study reports for the first time the anti-proliferative potential of four plant species namely *A. secundiflora*, *M. obscura*, *V. zanzibarensis* and *D. cinerea*. Methanolic and aqueous extracts obtained from the four plants were tested for their anti-proliferative potential against two prostate cancer cell lines; DU145 and 22Rv1, and one human cervical cancer cell lines; HeLa. The cytotoxicity of extracts is evaluated using the Vero cells as a representative of the normal cells [14,15]. Thus, we evaluated the effect of our extracts on Vero cells with 5-Fluorouracil as a reference drug.

Total of 12 extracts from 4 plant species: *Aloe secundiflora* (leaves), *Maytenus obscura* (stem and leaves), *Vernonia zanzibarensis* (stem and leaves), *Dichrostachys cinerea* (stem and leaves) were extracted using aqueous (water) and organic (methanol) solvents. Extracts at different concentrations in a triplicate experiment per selected extract were inoculated into the three cell lines (DU145, 22Rv1 and HeLa) (Table 1). We interpreted the results as described according to Wang [16] plant extracts with IC₅₀ < 1 µg/ml described as highly active, those with IC₅₀; 1-10 µg/ml as active and the extracts with IC₅₀; 10-100

µg/ml as moderately active. Extracts with IC₅₀>100 µg/ml were considered inactive.

The methanol extract of *D. cinerea* stem bark was found to have the highest anti-proliferative activity among the 12 extracts with an IC₅₀ of 8.04 ± 2.02 µg/ml on 22Rv1 cells. This activity was higher than the reference drug (9.22 ± 1.42 µg/ml) against the 22Rv1 cells (Table 2). Other extracts with moderate activity were *D. cinerea* aqueous stem bark extract on DU145 cells (22.75 ± 4.12 µg/ml) with high anti-proliferative activity than the reference drug (25.03 ± 1.7 µg/ml). Traditionally *D. cinerea* stem bark is used for the treatment of dysentery, headaches, toothaches and elephantiasis. The leaves of *D. cinerea* are used in the treatment of epilepsy, as a diuretic and laxative [10].

Plants of the genus *Maytenus* are used in South America to prepare infusions or decoctions as anti-inflammatory and analgesic remedies for topical and oral administration. Compounds isolated from the *Maytenus* genus include mayteine and maytansine alkaloids that are documented as having anti-proliferative activity [17]. In the current study the aqueous extract of *M. obscura* stem bark had moderate activity on DU145, 22Rv1 and HeLa with IC₅₀ of 25.03 ± 3.14 , 30.88 ± 2.55 , and 23.11 ± 3.08 µg/ml respectively. The aqueous extract of *M. obscura* leaves had less activity as compared to the stem bark extract, with an IC₅₀ of 159.62 ± 13.73 , 240.18 ± 17.91 and 89.21 ± 9.14 µg/ml on DU145, 22Rv1 and HeLa respectively.

Extract	DU145	22Rv1	HeLa
<i>D. cinerea</i> Aq Leaves	132.37 ± 6.81	225.39 ± 21.79	98.86 ± 6.31
<i>D. cinerea</i> Aq stem bark	22.75 ± 4.12	113.26 ± 12.83	83.75 ± 13.87
<i>D. cinerea</i> Og leaves	147.24 ± 22.08	302.64 ± 19.43	96.74 ± 11.66
<i>D. cinerea</i> Og stem bark	104.17 ± 10.31	8.04 ± 2.02	32.53 ± 5.71
<i>V. zan</i> Aq Leaves	258.26 ± 14.16	259.27 ± 20.73	94.85 ± 18.73
<i>V. zan</i> Aq stem bark	153.74 ± 13.84	292.11 ± 22.37	287.83 ± 28.42
<i>V. zan</i> Og Leaves	92.53 ± 6.29	73.37 ± 14.52	81.31 ± 10.56
<i>V. zan</i> Og stem bark	193.22 ± 12.50	242.71 ± 19.07	281.4 ± 25.23
<i>A. sec</i> Aq	246.28 ± 12.25	317.14 ± 2.15	67.05 ± 8.65
<i>A. sec</i> Og	139.27 ± 22.85	90.42 ± 5.24	23.19 ± 3.22
<i>M. obscura</i> Aq Leaves	159.62 ± 13.73	240.18 ± 17.91	89.21 ± 9.14
<i>M. obscura</i> Aq stem bark	25.03 ± 3.14	30.88 ± 2.55	23.11 ± 3.08

Key: Aq: Aqueous extract, Og: Methanol extract, Zan: Zanzibarensis, Sec: *Secundiflora*

Table 1: Inhibition concentration 50% (IC₅₀) of extracts of *Aloe secundiflora*, *Maytenus obscura*, *Vernonia zanzibarensis* and *Dichrostachys cinerea* extracts inoculated in DU145, HeLa and 22Rv1 cell lines. The results presented as Inhibition concentration at 50% mean \pm SD (n=3).

While most of the extracts from *V. zanzibarensis* showed no activity with IC₅₀ greater than 100 µg/ml on all cell lines, the methanol extract of the stem bark had moderate activity on DU145, 22Rv1 and HeLa with IC₅₀ values of 92.53 ± 6.29 , 73.37 ± 14.52 and 81.31 ± 10.56 µg/ml respectively. Traditionally, a decoction of the *V. zanzibarensis*

roots are used as a remedy for pain in the spleen or kidneys, relieve strangulated hernia and for chest diseases while the leaves are powdered, mixed with hot water and drunk twice a day for coughs [18,19].

For the *A. secundiflora* only the leaves were used for this study. Traditionally the diluted leaf sap is drunk as a cure for malaria, typhoid fever, diarrhea, oedema, swollen diaphragm, nose bleeding, headache, pneumonia, chest pain and as a disinfectant. The significant components of the leaf exudate of *Aloe secundiflora* are the anthrones aloenin, aloenin B, aloin A (barbaloin) and other aloin-derivatives [20]. Barbaloin possesses anti-inflammatory, antibacterial, antiviral and anticancer activities mediated by the anti-oxidative capacity [21-25]. In the current study both the methanolic and aqueous extracts of the *A. Secundiflora* showed moderate anti-proliferative activity against the three cancer cell lines; DU145, 22Rv1 and HeLa.

We evaluated the 50% cytotoxic concentration (CC₅₀) of extracts of *D. cinerea* stem bark Og, *D. cinerea* stem bark Aq and *M. obscura* stem bark Aq, against the Vero cell line. The relative activity of the extracts and the reference drug 5-Fluorouracil against DU145, 22Rv1, HeLa and Vero cell lines indicate a dose-dependent cytotoxic effect (Figure 1).

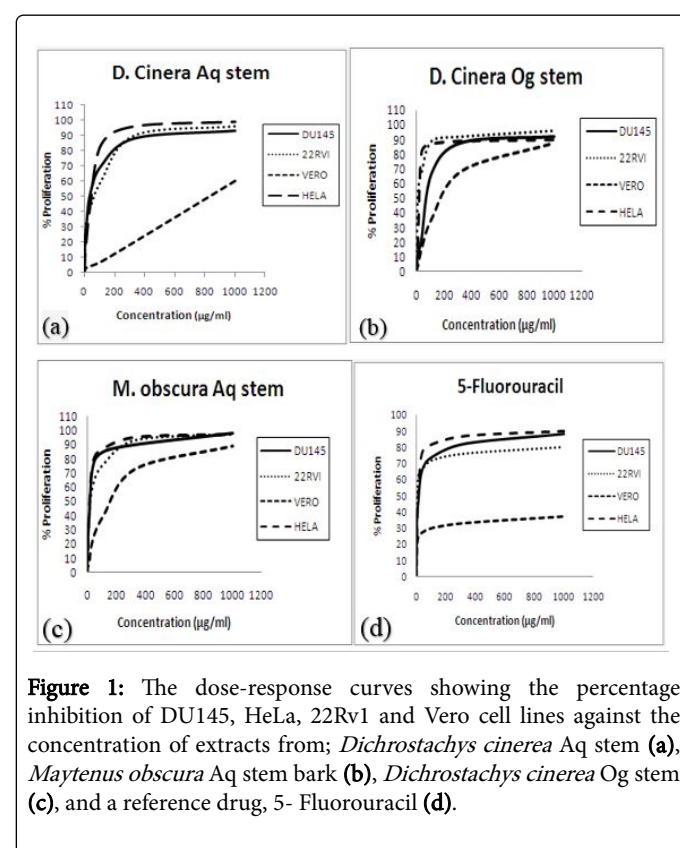


Figure 1: The dose-response curves showing the percentage inhibition of DU145, HeLa, 22Rv1 and Vero cell lines against the concentration of extracts from; *Dichrostachys cinerea* Aq stem (a), *Maytenus obscura* Aq stem bark (b), *Dichrostachys cinerea* Og stem (c), and a reference drug, 5- Fluorouracil (d).

Extracts with the least activity against the Vero cells were described to have the least toxicity to normal cells. The aqueous extract of *D. cinerea* stem bark had low toxicity to Vero cell; with CC₅₀ of 812.1 ± 12.72 µg/ml, followed by the methanol extract of *D. cinerea* stem bark (200.4 ± 7.54 µg/ml). This study indicates that the *D. cinerea* stem bark has potential anti-proliferative activity with low cytotoxicity to normal cells. Phytochemical analysis conducted on *D. Cinerea* leaves, and stem bark extracts reveal the presence of tannins, Alkaloid, flavonoids, steroids and saponins (Johnstone et al., 2012). Several alkaloids have

been shown to have *in vivo* and *in vitro* anti-proliferative activity, while others such as Camptothecin and Vinblastine, have already been successfully developed into anticancer drugs [26]. Flavonoids have been proposed to prevent the initiation stages of carcinogenesis by inhibiting ornithine decarboxylase induced by tumor promoters causing a subsequent decrease in polyamine and inhibition of DNA and protein synthesis [27]. *In vivo* study on the methanolic extract of *D. cinerea* leaves indicate significant hepatoprotective activity with a high LD50 [28].

The selectivity index is a comparison of the amount of a test compound that causes 50% inhibitory effect to the standard cells

(CC50) to the amount that causes death to the cancer cells (IC50). The selective index was calculated using the formulae: Selective Index (SI)=CC50/IC50. Samples with SI value greater than three were considered to be highly selective to cancer cells against normal cells [29]. All the test samples apart from the methanol extract of *D. cinerea* stem bark (1.92) had a selective index greater than 3 (Table 2). Among the plant extracts, the aqueous extract of *D. cinerea* stem bark had the most significant SI (35.7) on the DU145, followed by the methanol extract of *D. cinerea* stem bark (24.93) on the 22Rv1. The reference drug had SI>30 in all the three cell lines [30-37].

Extract	DU145		22Rv1		HeLa		Vero
	IC50	SI	IC50	SI	IC50	SI	CC50
<i>D. cinerea</i> stem bark Aq	22.75 ± 4.12	35.7	113.26 ± 12.83	7.17	83.75 ± 13.87	9.7	812.10 ± 12.72
<i>D. cinerea</i> stem bark Og	104.17 ± 10.31	1.92	8.04 ± 2.02	24.93	32.53 ± 5.71	6.16	200.40 ± 7.54
<i>M. obscura</i> stem bark Aq	25.03 ± 3.14	7.87	30.88 ± 2.55	6.38	23.11 ± 3.08	8.52	196.93 ± 5.31
5-Fluorouracil	25.03 ± 1.7	39.95	9.22 ± 1.42	108.45	14.23 ± 1.72	70.27	>1000
Key: Aq: Aqueous extract, Og: Methanol extract							

Table 2: Anti-proliferative activity and selectivity index of *Dichrostachys cinerea* Aq stem, *Dichrostachys cinerea* Og stem, *Maytenus obscura* Aq stem bark extracts and a reference drug against DU145, 22Rv1, HeLa and Vero cell line. The results are presented as anti-proliferative activity at 50% mean ± SD (n=3).

Conclusion

The present study supports the ethno-medicinal use of medicinal plants: *D. cinerea*, *A. secundiflora* and *M. obscura* in prostate cancer cell lines (Du145, 22Rv1) and cervical cancer cell line (HeLa) used in this study [38-43]. This study suggests wisdom in the use of these plants for further investigation in developing efficient, specific and non-toxic anticancer drugs that are affordable for developing countries.

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