

Gene Expression Mediated Antiproliferative Potential and Safety of Selected Medicinal Plants Against Cancerous and Normal Cells

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Abstract

Globally, approximately 13% of all deaths annually are attributed to cancer. Surgery, radiation and chemotherapy are the current treatment techniques for cancer, however these methods are expensive, have high failure rates and have been associated with detrimental side effects. Plant derived products could be good candidates in alleviating challenges being experienced with these current methods. This study aimed at evaluating the phytochemistry, antiproliferation potential, and probable mechanism of action of *Albizia gummifera*, *Rhamnus staddo* and *Senna didymobotrya* plant extracts. Phytochemical screening was done as per standard procedures. The common 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium (MTT) dye was used in the determination of the antiproliferative activity of the extracts. Extracts induction of *VEGF* (angiogenesis) and p53 (apoptosis) genes' expression was evaluated using Real Time Polymerase Chain Reaction. Phytochemical screening revealed presence of alkaloids, tannins, glycosides, flavonoids, terpenes, phenolics and saponins in the plants extracts. *A. gummifera*'s stem bark methanol: dichloromethane extract had the highest activity against the cancerous cell lines tested: HCC1395 (IC_{50} 6.07±0.04µg/ml), DU145 (IC_{50} 3.34±0.05µg/ml), CT26 (IC_{50} 5.78±0.08µg/ml) and Hep2 (IC_{50} 7.02±0.01µg/ml). *R. staddo* root bark methanol: dichloromethane extract had an IC_{50} value of 15.71±0.04µg/ml on HCC, 9.81±0.09µg/ml on Hep2 and 11.14±0.39µg/ml on CT26. *S. didymobotrya* root bark methanol: dichloromethane extract inhibited HCC with an IC_{50} of 65.06±0.07µg/ml, CT26 with an IC_{50} of 15.71±0.04µg/ml and Hep2 with an IC_{50} of 62.10±0.11µg/ml. From the results obtained, the plants exhibited selective toxicity to cancer cells while sparing the normal cells ($SI \geq 3$). *A. gummifera* and *S. didymobotrya* and *R. staddo* plant extracts upregulated p53 and down-regulated *VEGF* genes. In conclusion, this study confirms that these plant extracts could be potential candidates for development of drugs for the management of breast, prostate, colorectal and throat cancer.

INTRODUCTION

Cancer is among the leading causes of morbidity and mortality worldwide. Approximately 14 million new cases and 8.2 million cancer related deaths occurred in 2012 [1]. Cancer new cases are anticipated to rise by 70% in the next two decades [1]. In Kenya, cancer ranks third after cardiovascular and infectious diseases as cause of mortality. Approximately, 28,000 cases are recorded annually [2]. The factors that have been associated with high cancer risk include high body mass index, low fruit and vegetable intake, lack of physical activities, environmental pollution, tobacco use and alcohol [1].

Chemotherapy, radiation, surgery and hormonal therapy are the main strategies employed in the management of cancer. Despite their effectiveness, they have various side effects such as hair loss, peripheral neuropathy and cardiac damage among others [3]. Due to these challenges, people have turned to the use of medicinal plants as alternative therapies because they are thought to be cheap, effective, safe and easily accessible [3]. It has been estimated that over 30% of the plant's species contain secondary metabolites which are useful in treatment of various diseases such as cancer [3]. The use of naturally derived products from medicinal plants that selectively induce apoptosis and reduce angiogenesis could serve as an alternative to the current cancer treatment regimes. The discovery of cancer treatment drugs vincristine and vinblastine from *Catharanthus roseus* has prompted medicinal plants research for new leads in cancer treatment and management [4].

S. didymobotrya belongs to the family Fabaceae (Leguminosae) [5]. An ethnobotanical study conducted in Kakamega County in Kenya, showed that the leaves of *S. didymobotrya* are boiled and taken together with the leaves of *Galinsoga parviflora*, *Triumfetta rhomboidea* and *Ocimum gratissimum* for the treatment of colorectal cancer [6]. Elsewhere in Kenya, the plant has been used traditionally by the Kipsigis community in the control of malaria as well as diarrhea [7]. In

West Pokot, the pastoralists peel the bark, dry the stem and burn it into charcoal which they use in milk preservation [5]. The root decoction has been used in several countries such as Congo, Rwanda, Burundi, Kenya, Tanzania, and Uganda to treat malaria, ringworms, jaundice and intestinal worms [8].

A. gummifera belongs to the Leguminosae (Fabaceae) family and subfamily Mimosoideae [9]. The *Albizia* species have numerous ethnomedical uses. *A. gummifera* has been widely used traditionally in African countries [10]. Traditionally, the leaf and stem bark are boiled together with the leaf of *Salvia coccinea* and *Conyza sumatrensis* and taken orally for the treatment of colorectal cancer, throat cancer, breast cancer and squamous cell carcinoma of the gums [6]. The bark infusion and pounded bark is taken to treat malaria and snuffed to treat headache, respectively, in Kenya. An aqueous acetone extraction on *A. gummifera* showed *in vitro* anthelmintic activities of condensed tannins on egg hatchability and larval development of sheep *Haemonchus contortus* [11]. Compounds isolated from *A. gummifera* exhibited cytotoxicity against the A2780 human ovarian cancer cell lines [12].

R. staddo belongs to family Rhamnaceae. It is commonly known as staddo or buckthorn. It has been shown that plants from the family Rhamnaceae possess anticancer activity [13]. *Ziziphus spina christi* is an example of a plant belonging to this family. It demonstrated anti-proliferative potential on MCF-7 breast cancer cells by inducing apoptosis [14]. Traditionally, its roots are used to treat typhoid, back pain, joint pain and headache. The leaf is burned and smelt to increase male libido [15]. The root bark is also used by the Kenyan Maasai community to extend the shelf life of milk [16]. *R. staddo* has been reported to have both antifungal and antimalarial activity when used synergistically with other plants [17].

Several important bioactive compounds that produce desirable physiological activities have been derived from plants. These plants could serve as new leads and clues for modern drug design [18]. These important bioactive constituents of plants include but are not limited to alkaloids, tannins, flavonoids, terpenoids and phenolic compounds [19]. During the synthesis of compounds with specific activities to treat various diseases such as cancer, it is important to know the correlation between the phytoconstituents and the bioactivity of plants [20]. Therefore, preliminary screening of plants is the need of the hour in the discovery and development of safe novel therapeutic agents with improved efficacy.

The determination of the molecular mechanisms underlying neoplastic transformation and progression have resulted in the understanding of cancer as a genetic disease, which evolved from the accumulation of a series of acquired genetic lesions [21]. Protein 53 (p53) is a tumor suppressor that eliminates and inhibits multiplication of abnormal cells through induction of apoptosis [22]. It is one of the key orchestrators of cell signaling pathways related to apoptosis and cell cycle, which have an essential role in the development and progression of complex diseases such as cancer [23]. Studies have shown that medicinal plants can activate apoptotic genes [24].

Angiogenesis is a key process in cancer promotion. It is an important pathological event associated with tumor growth and metastasis. Vascular Endothelial Growth Factor (*VEGF*) plays an important role in this event [25]. It is a physiological process of formation of new blood vessels on already existing ones. The newly formed blood vessels facilitate the metastatic dissemination of cancer cells. In most cancers, angiogenesis correlates with disease stage and metastasis [26].

Recently, the role of Vascular Endothelial Growth Factor (VEGF) in angiogenesis has attracted the attention of researchers. This gene plays an important role in regulation of physiological as

well as pathological angiogenesis [27]. Various reports have shown that plant extracts and plant derived compounds have the potential to down regulate VEGF. Octacosanol, a long-chain aliphatic alcohol derived from *Tinospora cordifolia* suppressed the expression of VEGF an activity which was attributed to the compounds ability to regulate IL-1 β , IL-6 and TNF- α cytokines levels [28, 29]. *Withania somnifera* inhibits VEGF mediated neovascularization [30] and Z-guggulsteron isolated from *Commimpora mukul* plant down regulated angiogenesis through VEGF and granulocyte colony-stimulating factor, pro-angiogenic growth factors inhibition [31]. In most cancers, angiogenesis correlates with disease stage and metastasis [32]. Metastasis is the major cause of death from cancer. The development of rich vascular networks of new lymphatic and blood vessels in the tumors results in resistance to conventional therapies such as androgen ablation and cytotoxic chemotherapy [33].

Materials and Methods

Study site

The study was carried out at the Center for Traditional Medicine and Drug Research (CTMDR), Kenya Medical Research Institute (KEMRI) Nairobi, Kenya.

Study design

In vitro laboratory based (pre-clinical) and *in vivo* experimental study design method was used.

Sample collection and preparation

The leaf, stem bark and root bark of *Senna didymobotrya* and *Rhamnus staddo* were collected from Laikipia County. *Albizia gummifera* plant parts were collected from Ngong Forest, Kajiado County. Harvesting was done sustainably. Identification of the botanical samples was conducted by a qualified botanist and voucher specimens (RAM 2017/01, RAM 2017/03 and RAM 2017/2 respectively) stored at the University of Nairobi Herbarium. The leaf, stem bark and root bark

collected were dried at room temperature and then ground into fine powder using Gibbons electric mill (Wood Rolfe Road Tolles Bury Essex, UK). The ground samples were then stored in air tight bags at room temperature until use.

Extraction

Aqueous Extraction

About 200g of each sample was weighed and submerged in 1litre of double distilled water. Extraction was done in an aqueous bath at 80°C for 2hrs. After cooling, the extract was decanted in a clean 1000ml conical flask and filtered using a Whatman No. 1 filter paper. The filtrate was then freeze dried using a freeze dryer (Modulyo Edwards high vacuum, Crawley England, Britain, Serial No. 2261). The extract was weighed and stored at 4°C in air tight vials until use [34].

Organic Extraction

Briefly, 200g of each sample was weighed, put in a flat-bottomed conical flask and solvent added to cover the sample completely and left to stand for 24hrs. A Whatman No. 1 filter paper was used to filter and the sample re-soaked again for 24hrs. Extraction was done using methanol: dichloromethane (1:1). The solvents were removed using a rotary evaporator (Büchi, Switzerland) and the concentrated extracts packed in air tight vials and stored at 4°C until use [27].

Qualitative Phytochemical Screening

Qualitative phytochemical screening of *S. didymobotrya*, *A. gummifera* and *R. staddo* was done using standard procedures [35]. Secondary metabolites tested included alkaloids, tannins, steroids, glycosides, flavonoids, phenols, saponins, and terpenoids.

Cell culturing

DU 145 (prostate cancer), HCC 1395 (breast cancer) Hep 2(throat cancer), CT26 (colorectal cancer) and Vero E6 (normal) cells obtained from ATCC (Manassas, VA, USA) were used. The cells initially stored in liquid nitrogen were removed from the tank and quickly thawed in a water bath at 37°C. The vial contents were centrifuged, supernatant removed and then transferred into growth MEM medium supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine and 1% antibiotic (Penicillin/Streptomycin) in a T75 culture flask and incubated at 37°C and 5% CO₂ to attain confluence.

Antiproliferative assay

Upon attainment of confluence, cells were washed with saline phosphate buffer and harvested by trypsinization. The number of viable cells was determined using Trypan blue exclusion method (cell density counting) using a hemocytometer. An aliquot of 100µl containing 2.0×10^4 cells/ml suspension was seeded in to a 96-well plate and incubated at 37°C for 24hrs at 5% CO₂ for 24hrs. After 24hrs, 15µl of sample extracts at seven different concentrations each serially diluted were added on Row H-B. Row A, containing media and cells alone served as the negative control. The standard drug Doxorubicin was used as the positive control. The experiment was done in triplicate. The cells were incubated for 48hrs, then 10 µl of MTT dye (5mg/ml) was added and the plates incubated for 2hrs at 37°C and 5% CO₂. Mitochondrial dehydrogenase which is a biomarker of live cells interacts with MTT dye reducing it to insoluble formazan. The formazan formed is directly proportional to the number of live cells. Formazan formation was confirmed using inverted light microscope and then solubilized with 50µl of 100% DMSO and optical density (OD) read using a calorimetric reader at 540nm and a reference wavelength of 720nm. The effect of the test samples on the cancer and normal cells was expressed as IC₅₀ values (the extracts concentration

which kills 50% of the cancer cells) and CC₅₀ values (concentration of extracts that exerted cytotoxic effects to 50% of the normal cells) respectively [36].

Selectivity index (SI) which indicates the ability of the extracts to exert selective toxicity to cancer cells while sparing the normal ones was calculated using the formula:

$$SI = \frac{CC_{50}}{IC_{50}}$$

Where;

CC₅₀ – Concentration of extract that exerted cytotoxic effect to 50% of the normal cells

IC₅₀ – Concentration of extract that inhibited the growth of cancer cells by 50%

The data obtained was analysed using linear regression model to get IC₅₀ of each drug. The IC₅₀ values of the extracts were compared using Minitab Version 18 to obtain the Mean±SEM.

RNA Extraction and Gene Expression Assay

Cell Culture and RNA Extraction

The prostate cancer cell line (DU 145) was used in the evaluation of the gene expression profiles of the most active plant extracts from each of the three plants. The cell line used was selected based on the antiproliferative effect of the extracts. The cells obtained from ATCC (Manassas, VA, USA) were cultured in a T-25 culture flask. Minimum Essential media (MEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine and 1% antibiotic (Penicillin/Streptomycin) was then added and the flask incubated at 37°C and 5% CO₂ to attain confluence. After 24hrs, *S. didymobotrya*, *A. gummifera* and *R. staddo* extracts were added to the T-25 flask at appropriate concentrations. The concentrations of the extracts used in this assay were informed by the IC₅₀ values earlier obtained. To get the concentration of the extract to expose to the cultured cells and

get enough viable cells, the IC₅₀ values were reduced by 30%. This was then incubated for 48 hours. After 48hrs, the media was decanted and cells washed in PBS to remove any debris. Trypsinization of the cells was done. RNA extraction was carried out using the procedure described by Pure Link RNA mini kit (Thermo Scientific, USA). The extracted RNA was quantified and its quality examined using a Nanodrop ND-2000 spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE, USA) and the concentrations (ng/μl) obtained.

Gene Expression Assay

Reverse transcription and cDNA amplification were done in single step reaction using SuperScript IV Reverse Transcriptase and Thermo scientific Real time SYBR green Kits according to the manufacturer's instructions. A single narrow peak from each PCR product was obtained by melting curve analysis at specific temperatures. The quantitative RT-PCR data was analyzed by a comparative threshold (Ct) method, and the fold inductions of the samples compared with the untreated samples. Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control to normalize the expression of the target genes. The Ct cycle was used to determine the expression level in control and cells treated with different extracts for 48hours. The gene-expression levels were calculated as described by Yuan *et al* [30]. The data obtained was expressed as the ratio of the reference gene to the target gene using the standard formula: $\Delta Ct = Ct(\text{test gene}) - Ct(\text{GAPDH})$. To determine the relative expression levels, the following formula was used: $\Delta\Delta Ct = \Delta Ct(\text{treated}) - \Delta Ct(\text{calibrator})$. Therefore, the expression levels were presented as *n*-fold differences relative to the calibrator. The value was used to plot the expression of apoptotic and angiogenic genes using the relative quantification ($2^{-\Delta\Delta Ct}$) [37].

Results

IC₅₀ and CC₅₀ values for the plants extracts

On the prostate cancer cell line (DU 145), the stem bark extract of *A. gummifera* MeOH: DCM exhibited the highest cell inhibitory effects with an IC₅₀ value of 3.34±0.05µg/ml followed by *R. staddo* root bark methanol dichloromethane and *A. gummifera* aqueous stem bark extracts at IC₅₀ values of 9.36±0.10µg/ml and 18.29±0.02µg/ml, respectively (Table 1). *A. gummifera* MeOH: DCM root bark had the lowest activity exhibiting the lowest IC₅₀ value of 79.71±0.10µg/ml. Amongst all the *S. didymobotrya* extracts tested on the prostate cancer cell line, only the leaf MeOH: DCM extract portrayed activity with an IC₅₀ value of 65.72±0.01µg/ml (Table 1). There was a significant difference among the IC₅₀ values of all the extracts and the positive control (p≤0.05) against DU 145 cell line.

On the breast cancer cell line (HCC 1396), *A. gummifera* root bark MeOH: DCM extract had the highest cell inhibition with an IC₅₀ value of 2.38±0.01µg/ml. This was then followed by the *A. gummifera* stem bark MeOH: DCM extract *R. staddo* root bark MeOH: DCM extract with IC₅₀ values of 6.07±0.04µg/ml and 15.71±0.04µg/ml respectively. Among the *S. didymobotrya* extracts, the leaf MeOH: DCM extracts had the greatest activity with an IC₅₀ value of 32.32±0.03µg/ml. *A. gummifera* stem bark and root bark aqueous extracts exhibited cell growth inhibition with IC₅₀ values of 21.38±0.03µg/ml and 35.58±0.25µg/ml, respectively (Table 1). Amongst all the extracts tested on the HCC 1396 cell line, *S. didymobotrya* root bark MeOH: DCM extract had the lowest activity with an IC₅₀ value of 65.06±0.07µg/ml (Table 1). A significant difference was observed between all the extracts and the positive control (p≤0.05).

On the throat (larynx) cancer cell line (Hep 2), the *A. gummifera* extracts, the MeOH: DCM stem bark and root bark extracts were shown to have IC₅₀ values of 7.02±0.01µg/ml and

12.15±0.07µg/ml, respectively. *A. gummifera* stem bark aqueous extract had an IC₅₀ value of 23.41±0.21µg/ml. The root bark MeOH: DCM extract of *R. staddo* also inhibited the growth of the throat cancer cells with an IC₅₀ value of 9.81±0.09µg/ml. *S. didymobotrya* leaf MeOH: DCM extract exhibited an IC₅₀ value of 39.17±0.29µg/ml. The stem bark MeOH: DCM extract of *S. didymobotrya* showed the lowest activity with an IC₅₀ value of 66.66±0.35µg/ml (Table 1). There was significant difference among the IC₅₀ values of all the extracts and the positive control (Table 1; p≤0.05).

A. gummifera stem bark and root bark MeOH: DCM extracts had the highest inhibition on colorectal (CT26) cancer cell line with IC₅₀ values of 5.78±0.0µg/ml and 6.04±0.17µg/ml respectively. *A. gummifera* stem bark aqueous extract had an IC₅₀ value of 27.00±0.12µg/ml. Root bark MeOH: DCM extract of *R. staddo* also displayed an IC₅₀ value of 11.14±0.39µg/ml (Table 1). The root bark MeOH: DCM extract of *S. didymobotrya* showed the highest an IC₅₀ value of 15.13±0.03µg/ml against colorectal (CT26) cancer cell line. The lowest cell inhibition was shown by *A. gummifera* leaf MeOH: DCM extract at an IC₅₀ value of 79.86±0.14µg/ml. A significant difference was observed among the IC₅₀ values of all the extracts and the positive control (p≤0.05). The CC₅₀ values of the plant extracts varied depending on the plant extract and the solvent of extraction used. It was observed that most of the aqueous extracts were not cytotoxic (CC₅₀≥100) and compared to the MeOH: DCM plant extracts. All the *S. didymobotrya* extracts and the *A. gummifera* leaf aqueous extract had CC₅₀ values greater than 100µg/ml. Stem bark MeOH: DCM extract of *A. gummifera* was the most cytotoxic with a CC₅₀ value of 15.68±0.08µg/ml. This was then followed by the root bark MeOH: DCM and the stem bark aqueous extracts of the same plant with CC₅₀ values of 52.27±0.37µg/ml and of 63.00±0.01µg/ml. *R. staddo* root bark MeOH: DCM extract displayed a CC₅₀ value of 77.95±0.14µg/ml. The lowest cytotoxicity was observed in leaf

MeOH: DCM and root aqueous extracts of *A. gummifera* with CC_{50} values of $85.64 \pm 0.07 \mu\text{g/ml}$ and $84.43 \pm 0.49 \mu\text{g/ml}$ respectively. The doxorubicin was the most toxic towards the normal cells with a CC_{50} value of $0.98 \pm 0.01 \mu\text{g/ml}$ (Table 1). All Aqueous and MeOH: DCM leaf extracts of *S. didymobotrya* exhibited no cytotoxicity. There was significant difference amongst the cytotoxicity of all the extracts ($p \leq 0.05$).

Table 1: IC₅₀ and CC₅₀ values of the plant extracts on the selected cell lines

Plant Sample	Part Used	Solvent	DU145 IC ₅₀ (µg/ml)	HCC 1395 IC ₅₀ (µg/ml)	Hep 2 IC ₅₀ (µg/ml)	CT 26 IC ₅₀ (µg/ml)	VERO CC ₅₀ (µg/ml)
<i>S. didymobotrya</i>	Stem bark	Aqueous	>100	>100	>100	>100	>100
	Leaves	Aqueous	>100	>100	>100	>100	>100
	Root bark	Aqueous	>100	>100	>100	>100	>100
	Stem bark	MeOH: DCM	>100	58.67±0.02 ^b	66.66±0.35 ^a	>100	>100
	Leaves	MeOH: DCM	65.72±0.01 ^c	32.32± 0.03 ^e	39.17±0.29 ^c	>100	>100
	Root bark	MeOH: DCM	>100	65.06±0.07 ^a	62.10±0.11 ^b	15.13±0.03 ^d	>100
<i>A. gummifera</i>	Stem bark	Aqueous	18.29±0.02 ^f	21.38±0.03 ^f	23.41±0.21 ^f	27.00±0.12 ^c	63.00±0.0 ^d
	Leaves	Aqueous	66.26±0.04 ^b	>100	>100	>100	>100
	Root bark	Aqueous	25.29±0.09 ^e	35.58±0.25 ^d	55.38±0.38 ^c	64.77±0.13 ^b	84.43±0.4 ^b
	Stem bark	MeOH: DCM	3.34±0.05 ^h	6.07±0.04 ^h	7.02±0.01 ⁱ	5.78±0.08 ^g	15.68±0.0 ^f
	Leaves	MeOH: DCM	64.48±0.24 ^d	53.77±0.06 ^c	49.21±0.33 ^d	79.86±0.14 ^a	85.64±0.0 ^e
	Root bark	MeOH: DCM	79.71±0.10 ^a	2.38±0.019 ⁱ	12.15±0.07 ^g	6.04±0.17 ^f	52.27±0.3 ^e
<i>R. staddo</i>	Root bark	MeOH: DCM	9.36±0.10 ^g	15.71±0.04 ^g	9.81±0.09 ^h	11.14±0.39 ^e	77.95±0.1 ^c
Doxorubicin			0.24±0.03 ⁱ	0.54±0.30 ^j	0.26±0.01 ^j	2.95±0.04 ^h	0.98±0.01 ^g

Values are expressed as Mean±SEM. Values that do not share a letter are significantly different (p≤0.05).

Selectivity index (SI) of *Senna didymobotrya*, *Albizia gummifera* and *Rhamnus staddo*

In *A. gummifera* the greatest SI was observed on the root bark MeOH: DCM extract on the breast cancer cell line (SI = 21.68) and on the colorectal cancer cell line (SI = 8.57). A SI was also recorded on the stem bark MeOH: DCM and aqueous extracts, and root bark MeOH: DCM extract on the prostate cancer cell line (SI = 4.79, 3.44, 3.28), respectively. The stem bark MeOH: DCM and aqueous extracts had a SI of 3.16 and 2.94 on breast and colorectal cancer cell lines respectively. The least selective extracts on the prostate cancer cell line was the root bark MeOH: DCM extract (SI = 0.65), followed by the leaf MeOH: DCM extracts (SI = 1.32) and then the leaf aqueous extract (SI = 1.51). On the breast cancer cell line, the leaf MeOH: DCM extract was the least selective with a SI of 1.50. And finally, on colorectal cancer cell line, the least selective extracts were the root bark aqueous extract (SI = 1.28) and leaf MeOH: DCM extract (SI = 1.07). In *S. didymobotrya*, the root bark MeOH: DCM extract was the most selective with a SI of 6.59 on the colorectal cancer cell line. The least selective extract was the stem bark MeOH: DCM extract on throat cancer cell line with a SI of 1.49. For *R. staddo* root bark MeOH: DCM extract, a high selectivity ($SI \geq 3$) was observed on all the cancer cell lines tested (Table 2)

Plant Sample	Part Used	Solvent	DU 145	HCC 1395	Hep 2	CT 26
<i>S. didymobotrya</i>	Leaf	Aqueous	N/A	N/A	N/A	N/A
	Stem bark	Aqueous	N/A	N/A	N/A	N/A
	Root bark	Aqueous	N/A	N/A	N/A	N/A
	Leaf	MeOH: DCM	1.52	3.09	2.52	N/A
	Stem bark	MeOH: DCM	N/A	1.70	1.49	N/A
	Root bark	MeOH: DCM	N/A	1.53	1.61	6.58
<i>A. gummifera</i>	Leaf	Aqueous	1.51	N/A	N/A	N/A
	Stem bark	Aqueous	3.44	2.94	2.66	2.33
	Root bark	Aqueous	3.28	2.33	1.49	1.28
	Leaf	MeOH: DCM	1.32	1.59	1.72	1.07
	Stem bark	MeOH: DCM	4.79	2.60	2.25	3.16
	Root bark	MeOH: DCM	0.65	21.68	4.22	8.57
<i>R. staddo</i>	Root bark	MeOH: DCM	5.15	3.03	4.81	4.54

Table 2: Selectivity index of *Senna didymobotrya*, *Albizia gummifera* and *Rhamnus staddo*

Key: N/A: Not applicable because the test drug did not inhibit the growth of the cell

KEY:

MeOH: DCM- Methanol: Dichloromethane (1:1)

Assessment of expression of apoptotic (*p53*) and angiogenic (*VEGF*) genes

This study investigated the changes in *p53* and *VEGF* gene expressions in DU145 prostate cancer cells. The relative mRNA expression levels of the genes were determined using a real time PCR. The fold increase or decrease in the expression of the genes was evaluated, relative to the calibrator (Relative Quantification=1). It was observed that all the extracts showed a significant fold increase in the *p53* expression (Table 3). The MeOH: DCM extracts of *S. didymobotrya*, *A. gummifera*, *R. staddo* increased *p53* expression in DU145 by a fold change of 15.990, 16.066 and 15.985 respectively. A downregulation of the *VEGF* gene was noted in cells treated with *S. didymobotrya*, *A. gummifera* and *R. staddo* MeOH: DCM extracts with a fold change of 0.015±0.007, - 0.070±0.015 and 0.045 respectively. (Table 3).

Table 3: Fold change in expression of mRNA apoptotic (*p53*) and angiogenic (*VEGF*) genes

Sample	P53 gene expression	VEGF gene expression
<i>S. didymobotrya</i> leaf MeOH: DCM	15.990±0.014	0.015±0.007
<i>A. gummifera</i> stem bark MeOH: DCM	16.066±1.965	0.070±0.015
<i>R. staddo</i> root bark MeOH: DCM	15.985±0.015	0.045±0.017

Key: MeOH: DCM-Methanol dichloromethane (1:1)

Qualitative Phytochemical Screening

Phytochemical screening demonstrated the presence of different types of phytochemicals including alkaloids, saponins, flavonoids, phenols, glycosides, tannins and terpenoids which could be responsible for the various pharmacological properties. Saponins, flavonoids, glycosides, terpenoids and tannins were found across all the plant extracts. Phenols were also found present in all extracts apart from the root and stem aqueous extracts of *A. gummifera*. Alkaloids were present in *A. gummifera* extracts except in the leaf aqueous extract. Alkaloids were also exhibited in *R. staddo* and *S. didymobotrya* roots methanol dichloromethane extracts (Table 4)

Table 4: Phytochemical constituents of aqueous and methanol dichloromethane extracts of *S. didymobotrya*, *A. gummifera* and *R. staddo*

Plant			Classes of Phytochemicals						
	Part	Type of extract	Alkaloids	Saponins	Phenols	Flavonoids	Glycosides	Terpenoids	Tannins
<i>Senna didymobotrya</i>	Leaf	Aqueous	-	+	+	+	+	+	+
	Stem Bark	Aqueous	-	+	+	+	+	+	+
	Root Bark	Aqueous	-	+	+	+	+	+	+
	Leaf	MeOH: DCM	-	+	+	+	+	+	+
	Stem Bark	MeOH: DCM	-	+	+	+	+	+	+
	Root Bark	MeOH: DCM	+	+	+	+	+	+	+
<i>Albizia gummifera</i>	Leaf	Aqueous	-	+	+	+	+	+	+
	Stem Bark	Aqueous	+	+	-	+	+	+	+
	Root Bark	Aqueous	+	+	-	+	+	+	+
	Leaf	MeOH: DCM	+	+	+	+	+	+	+
	Stem Bark	MeOH: DCM	+	+	+	+	+	+	+
	Root Bark	MeOH: DCM	+	+	+	+	+	+	+
<i>Rhamnus staddo</i>	Root Bark	MeOH: DCM	+	+	+	+	+	+	+

(+) = Presence, (-) = Absence, MeOH: DCM- Methanol: Dichloromethane

Discussion

Antiproliferative activity of *Senna didymobotrya*, *Albizia gummifera* and *Rhamnus staddo*

Generally, the plant extracts inhibited the proliferation of the cancer cells. The antiproliferative activities of the extracts were categorized based on median inhibitory concentration (IC₅₀) [38]. Based on the National Cancer Institute (NCI) criterion, *A. gummifera* stem bark MeOH: DCM extracts showed the most potent cytotoxic effect on all the cancer cells tested, with an inhibitory concentration of less than 20µg/ml against all cell lines. The stem bark aqueous extracts also exhibited high cytotoxicity on the prostate cancer cells with an IC₅₀ value of 18.29±0.02µg /ml. Notably, the IC₅₀ values of *A. gummifera* stem bark MeOH: DCM extract on prostate cancer cells (3.34µg /ml) and *A. gummifera* root bark MeOH: DCM extract on breast cancer cells (2.38µg /ml) were found to be lower than those specified by the NCI, USA for categorization of a pure compound (IC₅₀ < 4µg/ml). The *R. staddo* root bark MeOH: DCM extract was classified as highly antiproliferative against all the cell lines with an IC₅₀ value less than 20µg /ml, with the highest antiproliferative activity was observed against DU145 cells with an IC₅₀ value of 9.36±0.10µg/ml. The MeOH: DCM leaf extracts of *S. didymobotrya* was categorized as highly antiproliferative against colon CT26 cancer cell line with an IC₅₀ of 15.13±0.03 µg/ml. The extract reported a moderate antiproliferative activity against DU145 cells (IC₅₀ of 65.72± 0.01 µg/ml), HCC 1395 (IC₅₀ of 32.32± 0.03 µg/ml) and Hep2 cells (IC₅₀ of 39.17±0.29 µg/ml). Doxorubicin was used as the positive control. It showed the highest antiproliferative activity, but it was the most toxic to normal cells.

Several studies have demonstrated the antiproliferative activity of *Albizia* species. The methanolic bark extract of *A. adinocephala* showed antiproliferative activity on breast cancer expressing receptor HER-2+, estrogen dependent breast cancer cells (ER+) and leukemia CCRF-CEM cells

with IC₅₀ values of 4.8µg/ml, 5.9µg/ml and 1.45µg/ml, respectively [39]. *A. adinocephala* root extract also exhibited excellent cytotoxic effects on leukemia CCRF-CEM cells with an IC₅₀ value of 0.98µg/ml [40]. The aqueous and methanolic extracts of *A. amara* exhibited cytotoxic effects to the MCF-7 breast cancer cells with IC₅₀ values of 83.16µg/ml and 57.53µg/ml, respectively. A stronger cytotoxicity was found in the ethyl acetate extract with an IC₅₀ value of 36.31µg/ml [41]. The cytotoxicity of *A. zygia* hydroethanolic and curcumin extracts has also been reported with IC₅₀ values of 3.37µg/ml on (Jurkat) human T-lymphoblast-like leukemia, 6.08µg/ml on (LNCap) prostate cancer cells and 2.56µg/ml on (MCF-7) breast cancer cells [42]. These previous studies demonstrate the potential of Albizia species to inhibit the growth of several cancer cell lines.

A number of species belonging to the Rhamnaceae family have been shown to possess antiproliferative properties on cancer cell lines. *Ziziphus spina Christi* demonstrated antiproliferative potential on MCF-7 breast cancer cells by inducing apoptosis with an IC₅₀ value of 20µg/ml [43]. *Rhamnus davunica* exhibited antiproliferative effect on human cancer cells HT-29 (colon carcinoma) and SGC-79 (gastric carcinoma) with IC₅₀ values of 24.96µg/ml and 89.53µg/ml, respectively. *Rhamnus nepalensis* is also reported to be cytotoxic to the human oral carcinoma cell line with an IC₅₀ value of 35.69µg/ml [44].

A number of *Senna* species have been shown to have antiproliferative activity against several cancer cell lines. The aqueous root bark extract of *Cassia abbreviata* inhibited the growth of Hep2 throat cancer cell line with an IC₅₀ value of 1.49µg/ml [45]. *Senna alata* methanolic leaf extract with 22.46µg/ml exhibited growth inhibitory potential on HepG2 liver cancer cell line [46]. The leaf extract of *T. cacao* (*Senna* spp) exhibited antiproliferative activity against MCF-7 breast cancer cells with an IC₅₀ value of 41.4µg/ml. The husk fermented shell of *T. cacao* had IC₅₀ values

of 71.4µg/ml against HeLa and 68.9µg/ml on HepG2 [47]. On the contrary, *Senna covensis* leaf methanolic was reported to have no antiproliferative activity on HeLa cell line [48].

The anti-proliferation potential of these plants could be attributed to the phytochemicals present. Natural products have been an important source of pharmacologically active molecules that are effective and have a low occurrence of side effects [49]. Phytochemical screening demonstrated the presence of different types of phytocompounds including alkaloids, saponins, flavonoids, phenols, glycosides tannins and terpenoids. Phytochemical studies of different species belonging to the *Albizia* genus has revealed different classes of secondary metabolites such as saponins, terpenes, alkaloids and flavonoids [10]. In this study, all the methanol dichloromethane extracts of *A. gummifera* contained alkaloids, saponins, terpenoids, flavonoids and phenols, results that correlate with the findings done by Nigussie *et al* [50]. Phenols were absent in the root and stem aqueous extracts of *A. gummifera*.

Saponins, flavonoids, sterols, alkaloids, phenol, tannins, terpenoids and glycosides were found in leaf, stem bark and root bark aqueous and MeOH: DCM extracts of *S. didymobotrya*. According to reports on pharmacological activity of Senna species, it has been suggested that the genus is a potential source of enormous bioactive compounds. Phytochemicals such as tannins, alkaloids, saponins, steroids, flavonoids, and terpenoids are constituents of *S. didymobotrya* [51].

Similarly, the methanol dichloromethane roots extracts of *S. didymobotrya* had all these phytochemicals. This correlated with a study that reported the presence of tannins, alkaloids, saponins, steroids, flavonoids, and terpenoids in the roots extracts of *S. didymobotrya*. [52]. Another study also reported the presence of saponins, terpenoids and flavonoids in the stem and root extracts of *S. didymobotrya* [53]. The leaf extract also revealed the presence of tannins, saponins, flavonoids, glycosides, sterols and phenols [53]. The leaf however did not have

alkaloids. This was in line with a study conducted by Ngule *et al* [54]. Likewise, all these phytochemicals were found in *S. didymobotrya* extracts in the present study.

Saponins, flavonoids, sterols, alkaloids, terpenoids and glycosides were found present in the *R. staddo* root methanol dichloromethane extract. The root extract of *R. staddo* has similarly been reported to be rich in saponins, flavonoids, sterols, alkaloids, terpenoids and glycosides [55]. Additionally, *Ziziphus lotus* (*Rhamnaceae*) was observed to contain similar phytochemical profiles including alkaloids, saponins, terpenoids, flavonoids and coumarins. However, the extract did not contain anthraquinones [56].

A number of studies have been conducted to prove the protective effect of flavonoids against cancer. It has been linked to reduced incidence of estrogen- related cancers [57,58]. A correlation has been observed between the flavonoids in *R. davunica* with its antiproliferative activities against colon and gastric cancer cells [59]. *S. didymobotrya* was found to have flavonoids [44]. These are secondary metabolites that are potent water-soluble antioxidants and free radical scavengers that prevent oxidative cell damage and have strong anticancer activity [60]. Increased consumption of isoflavones is directly proportional to decreased risk of cancer and vascular diseases [61], with a 50% reduction in the risk of being susceptible to stomach and lung cancer [62]. *In vitro*, isoflavonoids inhibited the proliferation of breast cancer cells in a concentration dependent manner [63]. Flavonoids have also been pointed out as enzyme inhibitors and ligands of receptors involved in signal transductions [64,65].

Terpenoids have been demonstrated to possess antitumor properties, which has attracted more attention and these compounds have been screened in multiple cultured cancer cells. Several terpenoids have been isolated from plants and have been proved to have antiproliferative potential [66]. Terpenoids isolated from *Eurphobia kansui* exerted an antiproliferative effect on MDA-MB-

435 breast cancer cells and Colo205 colorectal cancer cells [67]. The terpenoids extracted from *Ferulago macrocarpa* fruits exerted cytotoxic effects on MCF-7 breast cancer cells and HT-27 colon cancer cells [68].

Alkaloids have been reported to be important active components in medicinal plants with significant biological activities. Some of these compounds have been developed successfully into chemotherapeutic drugs such as vinblastine, camptothecin and topoimerase 1 [69]. *Albizia amara* 's seeds methanolic extract was found to contain macrocyclic pithecolobine alkaloids which rendered its high cytotoxic potential towards human breast cancer, colon cancer, lung cancer and melanoma cell lines [70]. Noscapine, an alkaloid isolated from the opium flower *Papaver somniferum L.* induced G2/M arrest in colorectal cancer [71]. The activity of the root extract of *S. didymobotrya* could therefore be attributed to the alkaloids present. Saponins are natural glycosides, possessing a wide range of pharmacological properties including cytotoxicity, antitumor, immunomodulatory, antifungal and antiparasitic [72]. Oleanane-type triterpenoid saponins isolated from the roots of *A. gummifera* after a bio-assay guided fractionation showed cytotoxicity against the A2780 human ovarian cancer cell lines [12]. A triterpene saponin enriched fraction from *Bupleurum kaoi* inhibited the growth of lung cancer A549 cells through the apoptotic pathways with an enhancement in Fas ligand [73]. Cardiac glycosides tend to exert potent antineoplastic effects by increasing the immunogenicity of dying cancer cells [74].

The selective inhibitory activity of the extracts was determined and expressed as selectivity index (SI). The SI values demonstrated the differential activity of the extracts on normal cells compared to cancerous cell lines. A high SI value depicts high selectivity. Medicinal plants with SI values of 2 or greater than 2 are considered to be highly selective. Selectivity index of less than 2 indicates less selectivity [75].

A. gummifera showed selective toxicity to the cancer cells while sparing the Vero (normal) cells. However, the leaf MeOH: DCM extract exhibited non-selective inhibitory activity across all the cell lines tested with a selectivity index less than 2. This is similar to the findings in a study where the leaf of *A. adianthifolia* displayed toxicity to normal AML12 hepatocytes [76]. The root aqueous extracts were also found to be toxic to the normal cells with SI of 1.49 and 1.28 on the throat and colorectal cancer cell lines, respectively. The lowest selectivity was exhibited by the root bark MeOH: DCM extract with a SI of 0.65 on the prostate cancer cells. A study on *A. zygia* root exhibited toxicity on breast cancer cells with SI of 0.72 on the aqueous extract, 0.75 on the hydroethanolic extract and 1.86 on the curcumin extract. This was however different in *A. gummifera* root MeOH: DCM extract that had the highest selectivity on the breast cancer cells with a SI of 21.68. Nevertheless, the toxicity of these extracts towards cancer cells makes them potential candidates as anticancer therapeutic agents.

R. staddo MeOH: DCM root extract also demonstrated great selectivity to the cancer cells while sparing the normal cells ($SI \geq 3$). The highest selectivity was observed on the prostate cancer cells with a SI of 5.15. This is parallel to the findings of Chen *et al.*, where *R. davunica* showed no toxicity on the normal human hepatic cells (L-O2) [55].

S. didymobotrya extracts moderately inhibited the growth of cancer cells and were non-toxic to normal cells. The leaf MeOH: DCM extracts showed selectivity on both breast and throat cancer cells with SI of 3.09 and 2.52, respectively. The root bark MeOH: DCM extract had the best activity which was expressed on colorectal cancer cells an activity that was not exhibited by the other *Senna* extracts. It also expressed high selectivity to the colorectal cancer cells ($SI = 6.58$). This potentiates its use in colorectal cancer management because it showed strong cytotoxicity to

the cancer cells while being non-toxic to the normal cells. The finding of the preset study concurs with the results showing that *Cassia abbreviata* selectively inhibited Hep 2 cancer cell lines while sparing the normal cell line [77]. This study reports for the first time the anti-proliferative potential of *S. didymobotrya*, *A. gummifera* and *R. staddo* on breast, prostate, colorectal and throat cancer cells.

The MeOH: DCM extracts of *S. didymobotrya*, *A. gummifera*, *R. staddo* increased *p53* expression in DU145 by a fold change of 15.99, 16.07 and 15.98 respectively. A downregulation of the *VEGF* gene was also noted in the cells treated with *S. didymobotrya* and *A. gummifera* and *R. staddo* extracts with a fold change of 0.015, 0.070 and 0.045, respectively. The medicinal bioactivity of these plants could be related to the identified phytochemicals. A phytochemical can suppress malignant transformation of an initiated pre-neoplastic cell or can block the metabolic conversion of the pro-carcinogen. These mechanisms of action could also be attributed to the vast range of phytochemicals present in the extracts. Several studies have shown the impact of these isolated phytochemicals to the various mechanisms of plants in inhibition of growth of cancer cells. Polyphyllin D is a steroidal saponin isolated from *Paridis rhizome* and has been shown to inhibit cell proliferation of cancer cells by inhibiting the expression of *VEGF* genes and by upregulating p21 [78]. Ellagic acid of pomegranate induces apoptosis in prostate and breast cancer cells and inhibits metastasis process of various cancer types [79]. Asiatic acid, a pentacyclic triterpene isolated from *Centella asiatica* and oridonin, a diterpene, both inhibited the growth of HepG2 liver cancer cells by increasing the expression of *p53* gene [80]. Apigenin, a flavone found in parsley, celery and chamomile targets the *p53* pathway and induces apoptosis in the lung adenocarcinoma cells. It induces caspase dependent extrinsic apoptosis in human epidermal growth factor receptor 2 (HER-2) over expressing BT-474 breast cancer cells [81]. The proapoptotic effects of Eupatilin,

an active flavone derived from *A. asiatica* on human gastric cancer cells has been reported. It was found that the treatment of the human gastric cancer cells with eupatilin resulted in elevated expression of the p53 gene [82].

Flavonoids also act as anti-angiogenic compounds by downregulating the expression of *VEGF* gene. Crocetin, a caretonoid in *Crocus sativus* and *Gardenia jasminoides* suppresses the production of the *VEGF* gene in the head and neck carcinoma cells [83]. The diterpene cafestrol from *Coffea arabita* and phenols such as carnosic acid and carnosol have been shown to inhibit angiogenesis hence preventing the process of carcinogenesis [84]. Curcumin, a polyphenol of *curcuma longa* inhibits the growth of human glioblastoma cells by upregulating the expression of p53 gene [85]. Triterpenoids that enhance cytotoxicity to cancer cells causes an increase in the level of reactive oxygen species leading to increased apoptosis [86]. Studies have shown that a combination of phytochemicals can be more effective against cancer than individual components [86]. The probable mechanism of action of *S. didymobotrya*, *A. gummifera*, *R. staddo* could be attributed to the phytochemicals present.

Conclusion

The plant extracts could be potential candidates for development of drugs for the management of breast, prostate, colorectal and throat cancer. Induction of apoptosis and anti-angiogenesis could be proposed as their probable mechanisms of action. The growth inhibitory potential of the plant extracts on the cancer cells and the probable mechanism of action could be attributed to the presence of pharmacologically important phytochemicals. This study confirms that amidst the many traditional and pharmacological uses of these plants, they could also be used in the fight against cancer menace.

Conflict of Interest

The authors declare no conflicts of interests

Ethical Consideration

There were no humans involved in this study. The animals and cell lines used were handled with a lot of care and professionalism and all protocols followed to the letter. All the safety standards in the place of study were observed and all measures considered to make sure that standard operating procedures were carried out maximally. Ethical approval was sought from Kenyatta University Graduate School Committee, Kenya Medical Research Institute (KEMRI), CTMDR Centre Scientific Committee (CSC) and Scientific and Ethics Review Unit (SERU) approval number KEMRI/SERU/CTMDR/O55/3535 before conducting the study

Acknowledgements

We are grateful to Kenya Medical Research Institute, Centre for Traditional Medicine and Drug Research fraternity for their unending support, guidance and co-operation in actualizing this work. We acknowledge the KEMRI's Internal Research Grant (IRG) for funding Dr. Peter Mwitari through SSC 2902 who supplied us with laboratory consumables and the cancer cell lines. We are also thankful to the Pyrethrum project funding, SSC 2969 that facilitated the purchase of the q-PCR and CO₂ incubator, equipment's that aided in the success of this work.

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