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Total Phenolic Content and *in vitro* Antiproliferative Activity of *Tragia brevipes* (Pax) and *Tetradenia riparia* (Hochst) Leaves Extract

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JC, CN, MJ, RM, DKN, JWN, DK and PM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors JC and CN managed the analyses of the study. Authors DK and PM managed the data presentation and literature for this work. All authors read and approved the final manuscript.

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ABSTRACT

Tragia brevipes and *Tetradenia riparia* have been widely used in traditional medicine. *T. brevipes* relief stomach pain and in treatment of rheumatism while *T. riparia;* heal chest pains, stomach-ache, malaria and act as antioxidant. However, scanty data exist on their potential anticancer activity. The total phenolic content was determined and anti-proliferative activity of the Methanol-Dichloromethane extract from the leaves evaluated against cancerous cell lines. The total phenolic content of the plants extract was determined using an UV visual spectrophotometer at 765 nm. The 3- (4-5-dimethyl-2-thiazoly)-2, 5-diphenyltetrazolium bromide (MTT) cell proliferation bioassay was used to test for anti-proliferative activity on Hela (cervical), DU145 (prostrate) and HCC (breast) cancer cell lines and Vero (normal) cell. *T. brevipes* and *T. riparia* had a phenolic concentration of 30.44 and 20.25 µg/ml, respectively. Both plants showed anti-proliferative activity on cancer cells with the most inhibited being DU145 with an IC_{50} of 29.67 ± 4.60 µg/ml for *T. brevipes* and 11.45 ± 0.87 µg/ml for *T. riparia*. The standard 5 Fluorouracil had an IC_{50} of 5.04 ± 4.12 µg/ml on DU145. *T. riparia* had the highest antiproliferative activity of 65.54 ± 16.85 µg/ml on Hela cells and 62.84 ± 1.10 µg/ml on HCC. *T. brevipes* had an IC_{50} of 661.11 ± 15. 12 µg/ml on Hela cells and 703.09 ± 18.35 µg/ml on HCC. Extracts from both plants were highly selective on DU145 cancer cells with a selectivity index (SI) of 21.62 for *T. brevipes* and 7.88 for *T. riparia*. Methanol-Dichloromethane extracts from *T. brevipes* and *T. riparia* exert anti-proliferative activity, however, the active compound (s) and the mechanisms of anti-proliferative action need to be investigated further.

Keywords: Tetradenia riparia; tragia brevipes; antiproliferative activity; phenolic compounds; selectivity index.

1. INTRODUCTION

Cancer is a leading cause of death worldwide [1,2]. In 2013, 14.9 million cancer cases were reported; 8.2 million deaths and 196.3 million disability-adjusted life years (DALYs) were recorded worldwide. Prostate cancer is the leading type of cancer among men, affecting 1.4 million men globally while breast cancer is the leading type of cancer among women [3]. The rising burden of cancer and other non-communicable diseases (NCDs) places enormous strain on the health care systems of developing countries, of which many are ill-equipped to cope with the escalating numbers of people with cancer [4]. According to World Health Organization (2014), a 70% rise in the number of people with cancer is expected over the next 2 decades. Approximately one third of all cancer deaths have been associated with five behavioral and dietary risks: high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco and alcohol use. Tobacco causes 20% of the global cancer deaths and around 70% of the global lung cancer deaths. More than 60% of world´s total new annual cases occur in Africa, Asia and South and Central America [4,2].

The burden of cancer is increasing in Africa because of aging, population growth as well as increased prevalence of risk factors. In Kenya, cancer is the third highest causative agent of mortality and morbidity, after infectious disease and cardiovascular diseases [5,6]. Around 39,000 new cancer cases occur each year in Kenya with more than 28,000 deaths per annum [7]. The most common types of cancer treatment techniques are surgery, chemotherapy and radiation. These methods have been associated with various side effects including higher risk of infection, serious bleeding after cut and extreme fatigue. Additionally, these methods of treatment are expensive [8]. The high cost of conventional cancer treatment has made many people turn to herbal medicine for treatment [1,2,5]. More than 80% of the population in developing countries relies on the use of herbal medicine for their primary healthcare [2,9].

Medicinal herbs and their derivative phytocompounds are increasingly being recognized as useful complementary therapies for cancer management. A large number of clinical studies have reported the beneficial effects of herbal medicine on the survival, immune modulation, and quality of life of cancer patients when used in combination with conventional therapeutics [10]. Natural phenolic compounds have been considered as one of the most interesting secondary metabolites due to their chemopreventive and chemotherapeutic effects in cancer treatment and management [11].

Phenolics belong to a large and diverse family of phytochemicals which are classified into several subgroups such as simple phenols, lignans, phenylpropanoids, flavonoids and coumarins among others [12]. They contain an aromatic benzene ring with one or more hydroxyl groups and are synthesized from phenylalanine through the action of phenylalanine ammonia lyase pathway [13]. Phenols are produced and accumulated in the sub-epidermal layers of plant tissues exposed to stress and pathogen attack [14]. They act as protective agents, inhibitors, natural animal toxicants, pesticides against invading organisms and antibacterial agents against pathogens [15]. Phenolic compounds represent a host of natural antioxidants used to combat cancer. They modulate cellular mechanisms that initiate anticancer activities by regulating growth factor-receptor interactions and cell signaling cascades [16]. Furthermore, phenolic compounds have been studied for their antitumor, proapoptotic and antiangiogenic effects [17]. Phenolics obtained from mushrooms have also shown to possess anticancer properties [18]. Hence the importance of determining the total phenolic content of the leaves extracts of these plants.

Tragia brevipes is a shrub in Euphorbiaceae family [19]. It grows in river line banks, thickets and high rainfall woodlands in Kenya [20]. The plant roots are used as purgative and during labor to increase uterine contraction. The plant is also ethnobotanically used as an analgesic and treatment of rheumatism [21]. It is also used traditionally in the treatment of stomach problems in the lower eastern part of Kenya [22]. Previous studies on a methanolic-aqua extract of the *T. brevipes* leaves have shown that the plant has antibacterial activity which has been associated to the presence of pharmacologically important phytochemicals in the plant [23]. *Tetradenia riparia* belongs to the Lamiaceae family. It is a plant that grows mainly along river banks, forest margins and hilly sides in many parts of Kenya. It is mainly referred to as ginger bush and grows approximately up to a height of 3 m [24]. The plant is used to relief chain pains, stomachache and malaria. Inhaling the scent of the crushed leaves is said to relieve headaches [25]. *T. riparia* possesses other medicinal properties such as a cough, dropsy, diarrhea, fever, headache and toothache remedies [26]. The essential oil from *T. riparia* leaves has been proved to have repellency potential [27]. Studies conducted on essential oils of *T. riparia* have reported some antioxidant activity of the isolated compounds [28]. However, little information existed on its potential use in cancer treatment. Hence the aim of this study was to determine the total phenolic content and antiproliferative potential of the leaves extract of the two plants.

2. MATERIALS AND METHODS

2.1 Plants Collection

The plant leaves were collected from Baraton village, Nandi County, Kenya with the help of a botanist. The plants were transported to the Centre for Traditional Medicine and Drug Research, Kenya Medical Research Institute laboratories. A botanical sample voucher number Jean/01/2016 is stored at CTMDR, KEMRI. The samples were washed under running tap water to remove soil particles and other particulate matter. The leaves were then dried under a shade and ground into fine powder using a laboratory mill. The fine powder was stored in polythene bags in a well-aerated room until use.

2.2 Extraction Procedure

Maceration extraction techniques were used. Briefly, 100 g of the plant's leaves were weighed using an electrical top balance and put in a 500 ml flat-bottomed flask. Methanol and dichloromethane (DCM) in a ratio of 1:1 was added until the plant material was completely submerged-200ml. The mixtures were then agitated for thorough mixing then left to extract for 24 h with frequent shaking to ensure effective extraction. After the 24 h the mixture contents were filtered using Büchner funnel with Whatman No. 1 filter paper. The residues were then re-soaked for additional 24 h using the same solvent and filtered. The filtrates were concentrated using a rotary evaporator (Büchi Labortechnik AG, Switzerland) in a water bath at 40°C. The concentrated extracts were packed in airtight vials and stored at 4°C until use.

2.3 Quantitative Determination of the Total Phenolic Composition

The total concentration of phenolics in the plant extracts were determined using spectrophotometric method [29]. Methanolic solution of the extracts in the concentration of 0.5 mg/ml was used in the analysis. The reaction mixtures were prepared by mixing 0.5 ml of a methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.0 ml of 7.5% sodium carbonate (Na_2CO_3). A

blank containing 0.5 ml methanol, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.0 ml of 7.5% of Na_2CO_3 was also prepared. The tubes were then allowed to stand at room temperature for 60 min before absorbance being read using UV visual spectrophotometer (Labtech, India) at 765 nm. The samples were prepared in triplicates for each analysis and the mean value of absorbance obtained. Tannic acid was used as a standard in concentrations of 50,100, 150, 200 and 250 µg/ml. The standard concentrations were read in constructing a calibration line. Based on the measured absorbance, the concentrations of phenolics was read (µg/ml) from the calibration line [30].

2.4 Antiproliferative Assay

2.4.1 Sample preparation

Briefly, 10 mg of the plant extracts in a 1.5 ml Eppendorf tube was weighed using a top balance and 100 µl Dimethylsulfoxide (DMSO) added, and the mixture vortexed. Phosphate Buffer Saline (PBS) was added to make 1 ml of the solution and storage done at -20°C until use.

2.4.2 Cell culturing

Vero cell line (normal) and selected cancer cell lines (American Type Culture Collection: ATCC) were obtained from CTMDR (KEMRI) biological laboratory. The cells' vials were thawed in a water bath at 37°C and cultured in T-75 flasks with Minimum Essential Medium (MEM, SIGMA USA) supplemented with 10% Fetal Bovine Serum (FBS) and 100 µg/ml streptomycin then incubated in 5% CO_2 at 37°C until confluence was attained.

2.4.3 Anti-proliferation assay

Upon attainment of confluence, cells were washed using PBS and harvested by trypsinization. The number of viable cells was determined by Trypan blue exclusion test (cell density counting). A hemocytometer was used in counting the number of viable cells. An aliquot of 2.0×10^4 cells/ml suspension were seeded in 96-well plate and incubated at 37°C for 24 h at 5% CO_2 for the cells to attach to the plate. Briefly, 15 µl of the test sample extracts was then added to the wells in rows H and topped up to 50 µl using media. Serial dilution was made from raw H to B. Row A acted as the negative control. Threefold serial dilutions were performed and the plates incubated at 37°C in 5% CO_2 for 48 h. The viability of the cells after extract addition and incubation was done using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The growth of the cells was then quantified by the ability of the living cells to reduce the yellow 3- (4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan product [31]. After 48 h, 10 µl MTT dye was added to the cells and incubated for 4 h in 5% CO_2 at 37°C. The overlaying media was removed and the formazan crystals formed solubilized using 50 µl of 100% DMSO [32]. The absorbance was read using an ELISA reader at 576 nm and a reference wavelength at 620 nm respectively. The percentage cell viability at different extracts concentration was calculated using the formula:

$$\text{Percentage cell viability} = 100 - \frac{A_t - A_b}{A_c - A_b} \times 100$$

Where: A_t = Absorbance value of test compound; A_b = Absorbance value of blank; A_c = Absorbance value of control.

The effect of *T. riparia* and *T. brevipes* extracts on Vero cells was expressed in CC_{50} values (the extracts concentration which kills 50% of the treated cells) [33]. Effects of extracts on cancer cells were represented in IC_{50}values. The CC_{50} and IC_{50} values were calculated using linear regression curves. Selectivity index (SI = CC_{50}/IC_{50}) of the three cancer cell lines was calculated from the CC_{50} ratio of the Vero cells and IC_{50} of the cancer cells.

2.4.4 Data management and analysis

All activities related to this study were recorded. Raw and processed data was entered into excel data sheet. Concentration required to inhibit the growth of 50% of the cells was calculated using CalcuSyn software version. The differences between the control and the treatments in these experiments were tested for statistical significance by unpaired Student's t-test. A value of $p \leq 0.05$ was considered to indicate statistical significance. Values are expressed as mean ±S.E.

3. RESULTS AND DISCUSSION

3.1 Total Phenolic Content

The bar graph above illustrates the total phenolic content of the leaf extract. *T. brevipes* and *T. riparia* had a phenolic concentration of 30.44 µg/ml and 20.25 µg/ml respectively.

3.2 Antiproliferative Activity

In the several epidemiological studies conducted, dietary phenolic compounds have been shown to possess cancer preventive potential [34]. A number of cohort studies have demonstrated the existence of an inverse relationship between consumption of dietary phenolics and cancer risk [35,36]. This has been further demonstrated by the existence of a direct relationship between total phenolic content and antiproliferative effect of mushroom extracts [8]. In this study, *T. brevipes* had a higher concentration of total phenolics as compared to *T. riparia*.

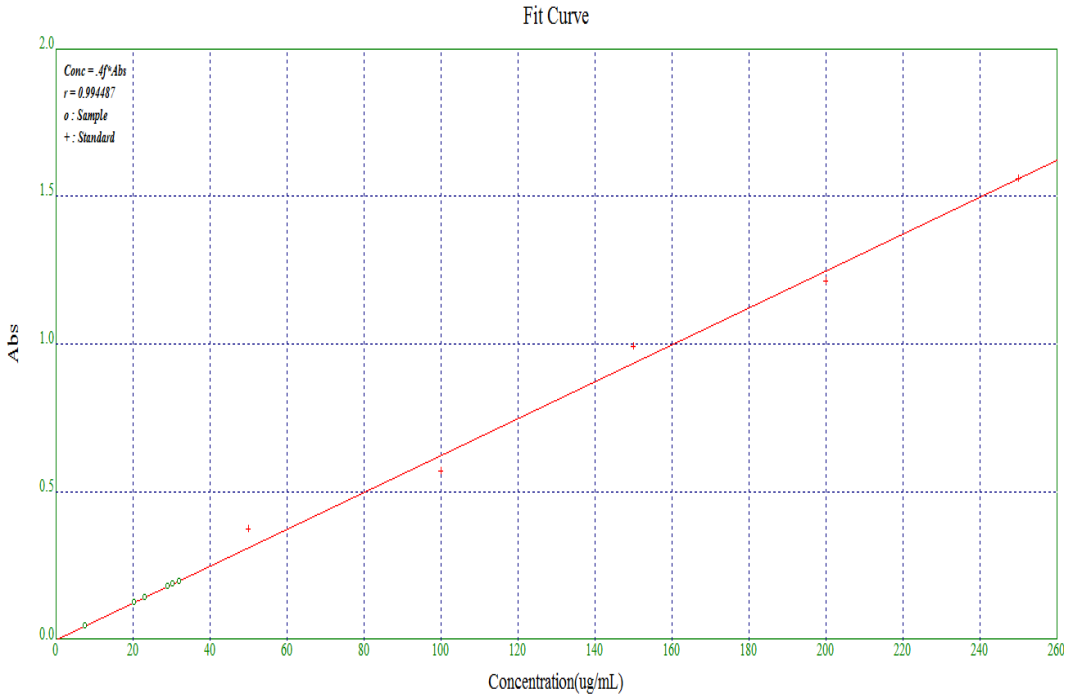

Fig. 1. A linear standard curve for different concentrations of tannic acid (Standard) used during the determination of the total phenolic content of the plant's leaf extracts

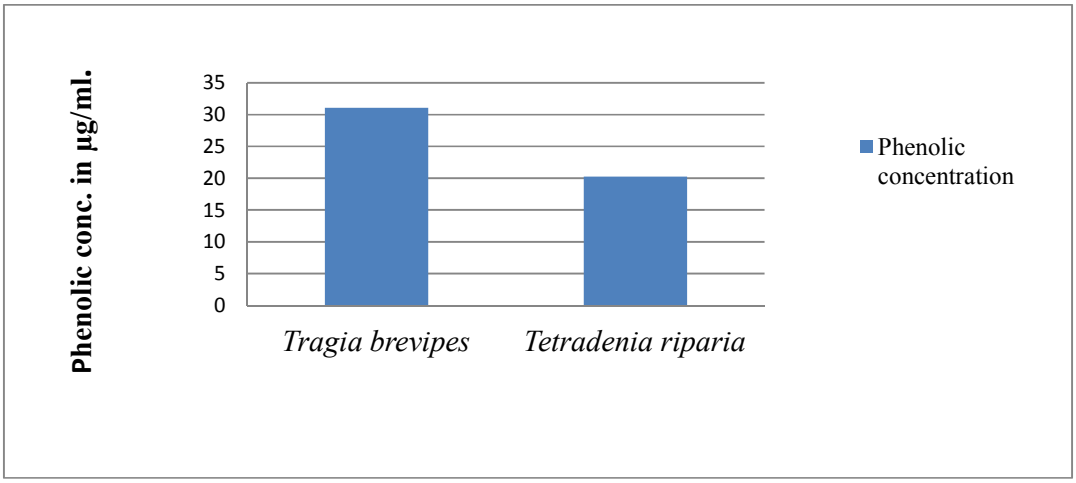

Fig. 2. Phenolic concentration of *T. brevipes* and *T. riparia* leaf extracts in µg/ml

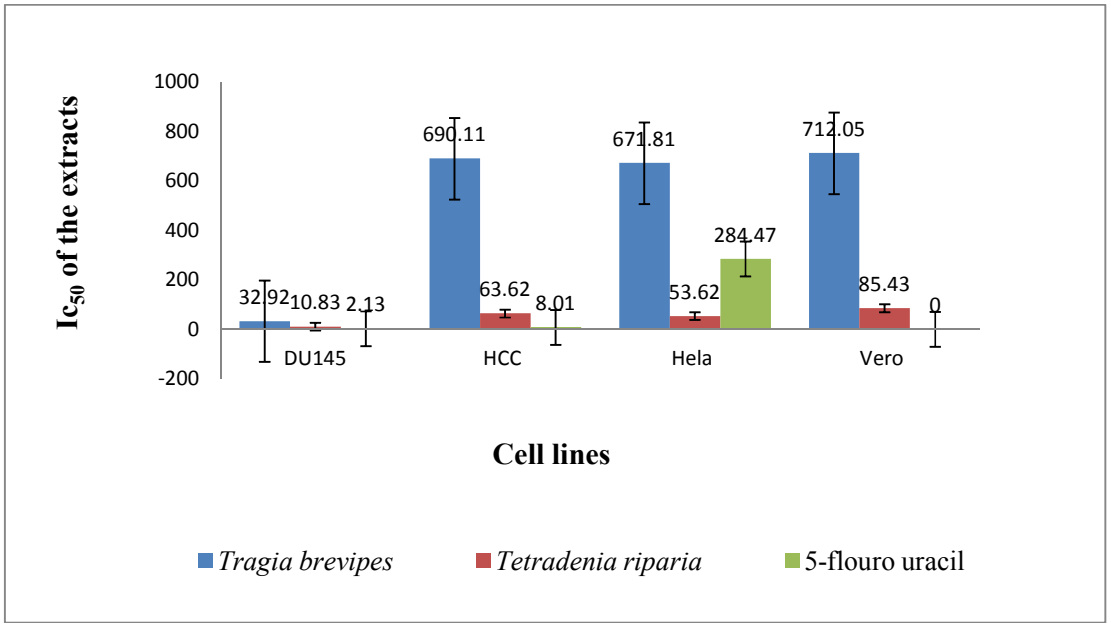

Fig. 3. The effects of *T. brevipes* and *T. riparia* extracts on the selected normal and cancerous cells in µg/ml

Key: DU145- Prostrate cancer cell lines, Vero-Normal monkey kidney cells, HCC- Breast cancer cell lines and Hela-Cervical cancer cells. Fig. 3 illustrates the IC_{50} of the leaves against the selected cancer cell lines. It shows the amount of the plants extracts required to inhibit the growth of cells by 50%.

Table 1. Tukey's multiple comparisons of the IC_{50} values of different normal and cancerous cells treated with plants' extracts and 5-flourouracil (conventional drug)

Comparison	P-value	Significance
DU 145-*T. brevipes* vs DU 145-5-Flourouracil	0.589	NS
DU 145- *T. riparia* vs DU 145-5-Flourouracil	1.000	NS
Hela- *T. brevipes* vs Hela-5-Flourouracil	0.001	S
Hela- *T. riparia* vs Hela-5-Flourouracil	0.997	NS
HCC- *T. brevipes* vs HCC-5-Flourouracil	0.989	NS
HCC- *T. brevipes* vs HCC-5-Flourouracil	0.001	S
Vero- *T. brevipes* vs Vero-5-Flourouracil	0.001	S
Vero- *T. riparia* vs Vero-5-Flourouracil	0.001	S

However, *T. riparia* had better antiproliferative activity. Therefore, based on these results there was no direct correlation between high phenolic content and antiproliferative activity *in vitro*. However, this could be attributed to the presence of pharmacologically important compounds; saponins, flavanoids, tannins, phenols and alkaloids in both plants leaves [22,23]. These compounds for instance saponins have been found to treat hypercholesterolemia, hyperglycemia, to have antioxidant, anti-inflammatory, central nervous system activities, anticancer and weight loss properties [37]. Flavanoids have also been found to play an important role in preventing oxidative stress which has been associated with inflammation, autoimmune diseases, cataract, cancer, Parkinson's disease, aging and arteriosclerosis. It also plays a role in heart diseases and neurodegenerative diseases; this is due to presence of antioxidants [38]. Also in a study by Jia et al. [39], a tannin compound known as corilagin, has been isolated from a number of medicinal plants including *Phyllanthus niruri* and has been shown to have antiproliferative effects against ovarian cancer cell lines.

Both plants showed anti-proliferative activity on cancer cells. The antiproliferative activity was categorized based in their median inhibitory concentration (IC_{50}) with most anti-proliferation being on DU145 with an IC_{50} of 29.67± 4.60

µg/ml for *T. brevipes* and 11.45 ± 0.87 µg/ml for *T. riparia.* The standard 5-Fluorouracil had an IC_{50} of 5.04 ± 4.12 µg/ml in DU145. *T. riparia* had the highest antiproliferative activity of 64.54 ± 16.85 µg/ml in Hela cells and 62.84 ± 1.10 µg/ml in HCC. *T. brevipes* had an IC_{50} of 661.11 ± 15. 12 µg/ml in Hela cells and 703.09±18.35 µg/ml in HCC. According to the U.S National Cancer Institute, for a crude extract to be considered to be cytotoxic it should have an IC_{50}<20 g/mL in the preliminary assay [40]. In the U.S National Cancer Institute Plant Screening Program, a crude extract is generally considered to have an *in vitro* cytotoxic activity if the IC_{50} value in carcinoma cells following incubation between 48 and 72 hours, should be less than or equal to 20 µg/ml while pure compounds should have an IC_{50} less than 4 µg/ml [41]. However, the American National Cancer Institute set the limit for crude extracts after 72 hours time of exposure of the extracts to the cells to be <30 g/mL with high toxicity being set at <20 g/mL. Therefore, following this criteria *T. riparia* showed better antiproliferative potential as compared to *T. brevipes*.

The plants and the used standard (5-Flourouracil) gave comparable IC_{50} values ($P>0.05$) except for those of the plants extracts against Vero, *T. brevipes* against Hela and HCC which were significantly lower compared to the standard drug ($P<0.001$). Although the p values are significantly different, this does not correlate with the antiproliferative activity.

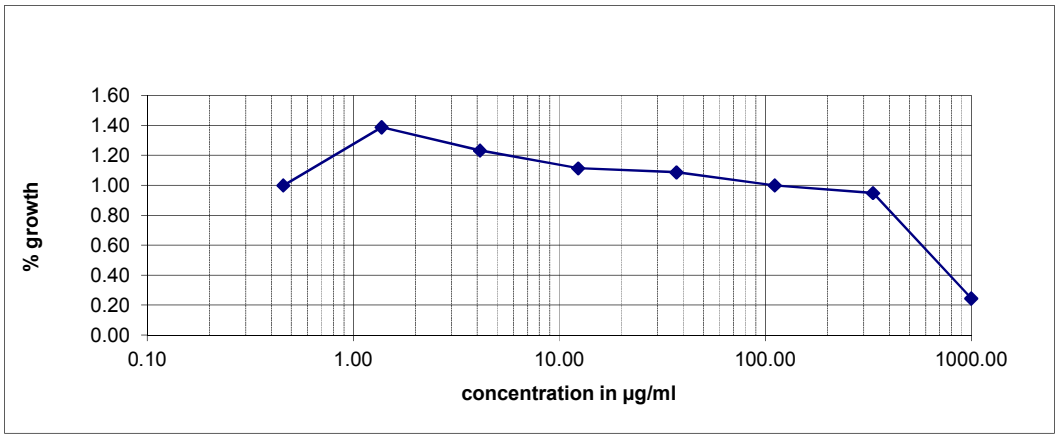

Fig. 4. Concentration dependent growth inhibition of *Tragia brevipes* against DU 145 (prostate) cancer cells

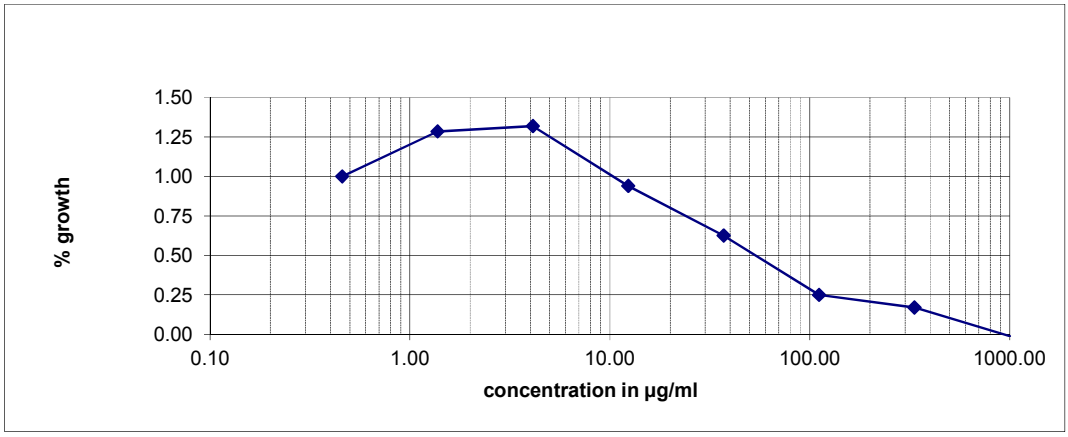

Fig. 5. Concentration dependent growth inhibition of *Tetradenia riparia* against DU 145 cancer cells

Figs. 4 and 5 show the growth inhibition percentage of cancer cells against concentration of the extracts. The rate of inhibition of the cancerous cells by the leaf extracts is directly proportional to the concentration. This correlates with a study which demonstrated a concentration dependent inhibition of five cell lines; human leukemia (HL-60) cell line, human colon cancer (HT-29) cell line, human breast cancer (MCF-7) cell line, human skin cancer (A 431) cell line and human lung cancer (A 549) cell lines by oleanane-type of triterpenoid saponin from stem bark extract of *Manilkara zapota* linn [42]. Another study also demonstrated a concentration dependent inhibition on colon and breast cancer cell lines by methanolic extract of *Origanum vulgare in vitro* [43].

From the results, the two plants showed varied ant-proliferative activity on the selected cancer cells. The cytotoxicity effect of the extracts on Vero (normal) cell lines was also investigated and from the results, the extracts were not toxic to normal cells. *T. brevipes* had the highest concentration of phenolics although its inhibition was lower as compared to that of *T. riparia*. When compared to normal cells, the extracts were highly selective against DU 145 with a selectivity index of 21.62 for *T. brevipes* and 7.88 for *T. riparia,* respectively. The selectivity index of both plants ranged between 1.00- 21.62. A selectivity index greater than three indicates the potential of the plant extract use in cancer management. Previous studies on *T. riparia* essential oils have also shown that the plant has antitumor properties [44]. Further studies have also shown that the essential oils of *T. riparia* have antioxidants properties [28]. Although the plant extracts were able to inhibit growth of cancer cells, the mode of action is unclear and may even involve multiple compounds in the extracts. We report for the first time the ability of *T. brevipes* to inhibit the growth of the three selected cancer cells Prostate, Cervical and Breast. Phenolics concentration and anti-proliferative activity seem to be inversely proportional for *T. brevipes* and *T. riparia*.

4. CONCLUSION AND RECOMMENDA-TIONS

Methanol-Dichloromethane extracts from *T. brevipes* and *T. riparia* exert anti-proliferative activity on breast, cervical and prostate cancer cells. *T. brevipes* and *T. riparia* leaves could be probable candidates in cancer treatment and management. The plants extract depicted low toxicity to normal cells as shown by high selectivity index values. If the current results are anything to go by, continued use of the plant extracts in the treatment of various diseases including cancer-associated symptoms is recommended. Isolation of the active ingredients which could shed more light to the current findings needs to be done. The mechanisms of action of the plants extracts is however unclear and need to be deduced. *In vivo* studies and interaction potential of the plants' extracts, bioactive components with conventional drugs would be prudent to widen the scope on the probable use of *T. riparia* and *T. brevipes* in cancer treatment and management.

DISCLAIMER

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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