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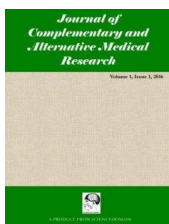
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Phytochemical Screening and *in vitro* Antiproliferative Activity of the Fruit of *Annona muricata* and *Abelmoschus esculentus* Pods against Selected Cancer Cell Lines

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MJ, CMN, JC, JWN, RWM, TA and PGM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MJ and CMN managed the analysis of the study. Authors TA and PGM managed the data presentation and literature for this work. All authors read and approved the final manuscript.

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ABSTRACT

Incorporation of fruits and vegetables in diet can successfully be used in prevention and treatment of cancer. *Annona muricata* and *Abelmoschus esculentus* which belongs to the annonaceae and malvaceae family respectively have commonly been used in traditional medicine to treat various ailments. This study evaluated the phytochemical components of both *A. muricata* and *A. esculentus* and their antiproliferative activity on the breast, cervical and prostate cancer cell lines. Both *A. muricata* and *A. esculentus* were extracted using methanol and dichloromethane in a ratio of 1:1. Phytochemical screening was done using standard analytical procedures. The MTT assay was used to evaluate the antiproliferative activity of *A. muricata* and *A. esculentus* extracts against breast cancer, cervical cancer, prostate cancer and Vero cell lines. Phytochemical screening confirmed that the fruit of *A. muricata* and the pods of *A. esculentus* are rich in saponins, tannins, alkaloids, terpenoids, glycosides, flavonoids and phenols. *A. muricata* had an IC_{50} of $23.632 \pm 1.3465 \mu\text{g/ml}$, $72.5860 \pm 1.9819 \mu\text{g/ml}$ and $93.6233 \pm 3.0570 \mu\text{g/ml}$ on Hela (cervical cancer cells), DU145 (Prostate cancer) and HCC 1395 (Breast cancer) cells respectively. *A. esculentus* demonstrated antiproliferative activity on Hela cells with an IC_{50} of $20.3840 \pm 1.2132 \mu\text{g/ml}$ on DU145 and HCC 1395 cells an IC_{50} of $50.013 \pm 0.2502 \mu\text{g/ml}$ and $171.6460 \pm 4.7642 \mu\text{g/ml}$ respectively. The standard drug used had an IC_{50} of $21.126 \mu\text{g/ml}$ on HCC and $24.850 \mu\text{g/ml}$ on Hela cells. Both plants selectively inhibited the growth of the cancerous cells tested ($SI > 3$) with the highest selectivity observed in HCC 1395 cells. This study authenticates traditional use and suggests potential use of these plants in cancer management and treatment.

Keywords: *Annona muricata*; *Abelmoschus esculentus*; antiproliferative; phytochemical.

1. INTRODUCTION

Cancer is among the leading causes of morbidity and mortality in the world. Worldwide, 32.6 million people are living with cancer [1]. Approximately 14 million new cases and 8.2 million cancer-related deaths were reported in 2012. The burden of cancer is increasing in Africa. This can be attributed to lifestyle changes and increased industrialized population as well as increased prevalence of risk factors associated with economic transition including smoking, obesity, lack of physical exercise and generative behaviors [2]. According to the United Nation's population estimates, the population of Africa between 2010 and 2030 is projected to increase by 50% and by 90% for those aged 60 years, the age bracket that is mostly affected by cancer [3]. In developing countries, the mortality rate is increasing probably due to the adoption of westernized lifestyle, delayed cancer screening and inadequate access to medication [4]. Breast cancer is the most diagnosed cancer in women [5]. It is the leading cause of death among females in the world, having an estimate of 1.7 million cases and 521,900 deaths in 2012 [6]. Cervical cancer is the second most diagnosed cancer with an estimate of 527,600 new cases. Approximately 265,700 deaths worldwide in 2012 were due to cervical cancer [7]. It is also the third leading cause of deaths among females in developing countries [8]. In men, prostate cancer

is the second most diagnosed cancer worldwide having an estimate of 1.1 million new cases in 2012. It is the fifth leading cause of cancer worldwide [6,9].

Currently, chemotherapy, radiotherapy and surgery are the main techniques used in cancer treatment and management. However, these techniques are expensive and have been associated with detrimental side effects. Drug resistance against the currently used anticancer drugs has been reported. Drawbacks being experienced with the current techniques necessitate the need for alternative leads in cancer treatment and management [10].

Plant-derived products are being used as alternative therapies because they are thought to be cheap, effective, safe and accessible. Regular consumption of fruits and vegetables has been associated with reduced risk of cancer and other chronic diseases [11]. High cost and side effects associated with the current techniques have compelled many people to shift from conventional medication to traditional medicine because they are not only readily available and affordable but are also perceived to have less harmful effects [12]. Research has revealed that plants possess secondary metabolites which can be used in the treatment of various diseases including cancer [13]. Many edible plants

possess therapeutic compounds and therefore are used as nutraceuticals [14,15]. Utilization of food such as fruits and vegetables has the potential to have a 7-31% reduction of all cancers worldwide [16]. Dietary phytochemicals are believed to induce apoptotic cell death in preneoplastic or neoplastic cells through different growth inhibitory mechanisms [17].

Abelmoschus esculentus also known as finger's lady in English speaking countries is a plant that belongs to the Malvaceae family. Commonly, it is referred as okra, gumbo and kingombo. In Kenya, okra is widely distributed in Kitui and Machakos Counties. Its geographical origin is believed to be West Africa, Ethiopia and South Africa. The plant grows well in tropical, subtropical and warm temperate regions [18]. Traditionally, *A. esculentus* has been used to treat various diseases. The decoction of the fruit is used to cure inflammation of mucous membrane especially respiratory tract [19]. The juice is used to treat a sore throat as a result of coughing. Its decoction is also used to treat fever, headache and arthritis [20]. The decoction is also used to ease the menstrual pain [21]. In 2010, a research conducted by French and Dutch researchers found that okra contains pectin which is effective in destroying highly metastatic mouse melanoma cells. The antioxidants present in okra are rich in beta-carotene which is important in cancer prevention [22]. Okra seeds are a good source of unsaturated oil and proteins. Seeds can also be used to obtain caffeine-free coffee [20]. It is rich in fiber, vitamin C and folate and because of this many people have incorporated it into their diet [23]. It is also known to be rich in antioxidants. Also, it contains appreciable amounts of calcium and potassium [24]. It is rich in phenolic compounds. Epidemiological studies suggest that consumption of foods rich in phenolic compounds reduce the risks associated with cardiovascular diseases, stroke and certain forms of cancer [25,26,27].

Annona muricata belongs to the family Annonaceae. The evergreen plant can grow to a height 4M tall. It is commonly found in tropical regions. The plant is commonly known as soursop in English, Mstafeli in Kenya, Ekitafeli in Uganda and Dukume in West Africa. Its medicinal use can be traced to many years ago and since then; there is a lot of research because of the bioactivity and toxicity it contains. The leaves, bark, fruit and seeds of *A. muricata* have been used for many traditional cultures for a

variety of physical ailments. Traditionally leaves, roots, fruits and seeds have been used to treat various diseases such as, skin rashes, stomach pain, malaria and prostate cancer [28]. It has also been used in respiratory illness [29] and gastrointestinal problems [30]. *A. muricata* is rich in acetogenins which is the principal bioactive compound isolated [31,32]. Recent studies have suggested that *A. muricata* expresses analgesics effect, anti-inflammation effects, promotes apoptosis and cytotoxicity on cancer cells that may result from the presence of alkaloids, essential oils and acetogenins [33]. Acetogenins are active compounds capable of preventing abnormal or cancer type cells from proliferating. It is important to understand the existing relationship between phytochemical and the bioactivity of these plants [34]. Hence, this study aimed to determine the antiproliferative potential of *A. muricata* and *A. esculentus* on selected cancer and normal cell lines. Results provide preliminary affirmation that *A. muricata* and *A. esculentus* extracts could be used in the treatment and management of breast, prostate and cervical cancer.

2. MATERIALS AND METHODS

2.1 Plant Collection

The fruits of *A. muricata* and pods of *A. esculentus* were collected from Mwingi, Kitui County, 200km east of Nairobi with latitude 0° 56' 3.66" N and longitude 38° 03' 36.18" E in 2016 from a farmer's land after permission had been sought. This region was selected because the plants are abundant. The plant samples were picked, labelled and transported to Centre for Traditional Medicine and Drug Research (CTMDR) KEMRI, Nairobi. Identification of the collected samples was conducted by a specialist botanist and voucher specimens stored at the University of Nairobi Herbarium.

2.1.1 Sample preparation

The samples were washed under running tap water to remove soil particles and other particulate matter. The pods of *A. esculentus* were chopped into small pieces, and the fruits of *A. muricata* cut into quarters to ensure proper drying of the plant materials. The samples were then dried at room temperature under indirect sunlight exposure with good air ventilation. The dried fruits and pods were grounded into fine powder using a Gibbons electric miller (Wood-Rolfe Road, Tollesbury Essex, UK), packed,

labelled and stored in a well-aerated room until use.

2.1.2 Extraction

Briefly, 200 g of the fine powder was weighed using an electric top balance and put in a 500 ml flat-bottomed conical flask. Methanol and dichloromethane (DCM) in the ratio of 1:1 was added until the sample was completely submerged. The mixture was then agitated by shaking for thorough mixing then left to extract for 24 hours with frequent shaking. The mixture was then filtered using a Butchner funnel and Whatman No. 1 filter paper. The residue was then re-soaked and left to extract for another 24 hours and filtered. The filtrate was concentrated using a rotary evaporator (Buchi water bath 8-480, Butch laboratechn IK AG, Switzerland) in a water bath at 40°C [33]. The concentrated extracts were then weighed, labelled and stored at 4°C until use.

2.1.3 Qualitative phytochemical screening

Qualitative phytochemical screening of *A. muricata* and *A. esculentus* was done using standard procedures [35]. These phytochemical components analysed included saponins, alkaloids, phenols, flavonoids, glycosides, terpenoids and tannins

2.2 Antiproliferative Assay

2.2.1 Cell culturing

DU145 (prostate cancer, HTB-81), HCC 1395 (breast cancer, CRL-2324), Hela (cervical cancer, CCL-2) and Vero Cells (Normal cell from monkey kidney) obtained from ATCC (Manassas, VA, USA) were used. The cells initially stored in liquid nitrogen were speedily thawed in a water bath at 37°C and cultured in T-75 flasks with Eagle's Minimum Essential Media (MEM, SIGMA USA) supplemented with 100µl/ml streptomycin and 10% Fetal Bovine serum (FBS) then incubated at 5% CO₂ at 37°C until 70% confluence was attained.

2.2.2 Anti-proliferation assay

Upon attainment of confluence, the cells were washed using Phosphate Buffer Saline (PBS) and harvested by trypsinization. The number of viable cells was determined by Trypan blue exclusion test (cell density counting) using a hemocytometer. An aliquot of 2.0×10^4 cells/ml suspension were seeded in a 96-well plate and

incubated at 37°C for 24h at 5% CO₂. After 24 hrs, 15 µl of the test sample extracts at seven different concentrations each serially diluted was added to the wells in rows H to B. Row A acted as the negative control. The plates were then incubated at 37°C in 5% CO₂ for 48 hrs. The viability of the cells after extract addition and incubation was done using 3-(4-5-dimethyl-2-thiazoly)-2, 5-diphenyltetrazolium bromide (MTT) assay. The growth of the cells was then quantified by ability of the living cells to reduce the yellow 3-(4-5-dimethyl-2-thiazoly)-2, 5-diphenyltetrazolium bromide (MTT) to a purple formazan product [36,37]. After 48 h, 10 µl MTT dye was added to the cells and incubated for 2hrs in 5% CO₂ at 37°C. The formazan crystals formed were then solubilized using 50 µl of 100% DMSO. The absorbance was read using Multiskan EX Elisa reader (Bio Tek, US) at 576 nm and a reference wavelength at 620 nm. The percentage cell viability at different extracts concentration was calculated using the formula [38].

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

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

Where,

At= Absorbance value of test compound

Ab= Absorbance value of blank

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

The effect of *A. esculentus* and *A. muricata* on Vero cells was expressed as CC₅₀ values (the concentration of extracts required to kill 50% of the treated cells). The effects of the extracts on cancer cells were represented in IC₅₀ values. The CC₅₀ and IC₅₀ values were calculated using linear regression curves. Selectivity index (SI = CC₅₀/IC₅₀) of the three cancer cell lines was calculated from the CC₅₀ ratio of the Vero cells and IC₅₀ of the cancer cells.

2.3 Data Management and Analysis

All the activities were recorded in a laboratory notebook. Raw and processed data was entered in excel data sheets and analysis done using Statistical Package of Social Science (SPSS Version 20). The concentration required to inhibit 50% of the cells was calculated using Cruci cytotoxicity software. The differences between the control and the treatments were tested for statistical significance using One-way Analysis of

Variance (ANOVA). A value of $p \leq 0.05$ was considered to indicate statistical significance. The IC_{50} and CC_{50} values were expressed as Mean \pm Standard Error of Mean (S.E.M). Tables and graphs were used for clear presentation of the results.

3. RESULTS AND DISCUSSION

Generally, the potential medicinal value of any plant is usually attributed to the phytochemicals present which have a certain biological importance defined by the biotic activities. These important phytochemicals are alkaloids, tannins, saponins, flavonoids, terpenoids, phenols and glycosides compounds. Flavonoids, terpenoids, glycosides, saponins and phenols were found in both plants. Alkaloids were present in *A. muricata* but absent in *A. esculentus*. Similarly, tannins were present in *A. esculentus* and absent in *A. muricata* as illustrated in Table 1 below.

Saponins are secondary metabolites that are natural glycosides possessing a wide range of pharmacological activities including cytotoxic and chemo preventive properties [39]. Flavonoids in recent research have been shown to inhibit initiation, promotion and progression of cancerous cells [40]. Flavonoids are reported to have a potent dietary chemo preventive activity [41]. Tannins are water soluble polyphenols usually found in various edible plants. A number of anti-mutagenic activities have been shown to be reduced by tannin molecules [42].

Terpenoids, also referred to as isoprenoid, are phytochemical components found in most plants. Several studies experimentally carried out have shown that monoterpenes, a class of terpenoids may be advantageous in prevention and management of cancer such as prostate, breast and colon [43]. Phenols also play a role in anticancer activity through processes such as apoptosis and inhibition of DNA binding [44].

This study focused to determine antiproliferative activity of organic extract of *A. muricata* and *A. esculentus* on cervical (Hela), prostate (DU145) and Breast (HCC) cancer cell lines. Both plant extracts exhibited a growth inhibitory effect on Hela, HCC and DU145 cancer cell lines (Fig. 3 below). A plant extract with an $IC_{50} \leq 20 \mu\text{g/ml}$ is considered active, $20 \mu\text{g/ml}$ - $100 \mu\text{g/ml}$ moderately active, weakly active when it ranges between $100 \mu\text{g/ml}$ \rightarrow $1000 \mu\text{g/ml}$ and inactive at above $1000 \mu\text{g/ml}$ [45]. From the extracts tested, *A. esculentus* had the highest antiproliferative activity on Hela with an IC_{50} of 20.384 ± 1.2132 as

shown in Fig. 1 below. Research done on flavones from *A. esculentus* exhibited pro-angiogenic activity by activating the VEGF-A/VEGFR2-P13/Akt Signaling axis [46]. Therefore, these results can be attributed to the same pathway of activity (Table 1). DU 145 had an IC_{50} of 50.013 ± 0.2502 , and IC_{50} 171.646 ± 4.7642 on HCC 1395 respectively. On the other hand, *A. muricata* inhibited the growth of the cancer cell lines with highest activity on Hela which had an IC_{50} of 23.632 ± 1.3465 . Crude leaf extract of *A. muricata* inhibited proliferation of 4T1 breast cancer cell line [47]. This was similar to HCC1395 breast cancer cell line as shown in Figure 1 and 2 below. Similarly, the fruit extract reduced the size of breast tumor in a 5week treatment in rats [48]. The cytotoxic mechanism suggests the inhibition of multiple signaling pathways which regulate metastasis, metabolism, induction of necrosis and cell cycle [29]. Crude leaf extract of *A. muricata* demonstrated the *in vitro* inhibition of prostate cancer proliferation and a tremendous effect on growth inhibition of the tumor compared to isolated flavonoid compound [32]. This illustrates interaction of the different phytochemicals present and the bioactive acetogenins.

Table 1. Phytochemical composition of *A. esculentus* and *A. muricata*

Compounds	<i>Abelmoschus esculentus</i>	<i>Annona muricata</i>
Alkaloids	-	+
Flavonoids	+	+
Terpenoids	+	+
Glycosides	+	+
Tannins	+	-
Saponins	+	+
Phenols	+	+

Key: + (Present), (-) Absent

Both *A. muricata* and *A. esculentus* were non-toxic to normal cells. They both showed selective toxicity to cancer cell lines ($SI > 3$) with the highest selectivity index on Hela 1395 (SI of 23.575 and 28.939 respectively) as illustrated in Table 2 below. These results are consistent with the study carried out on lectin isolated from *A. esculentus* promoted selective antitumor effects on MCF-7 breast cancer cells [49]. As illustrated in Table 3, all the extracts except *A. muricata* on Hela cells ($P=0.05$) showed a significant difference from 5-Fluoro uracil ($P=0.05$).

This study reports the anti-proliferative potential of *A. muricata* and *A. esculentus*

on prostate, cervical and breast cancer cells. This activity could be attributed to the phytochemical components present. The study reveals that these plants could act as potent agents in treatment and management of breast, cervical and prostate cancer.

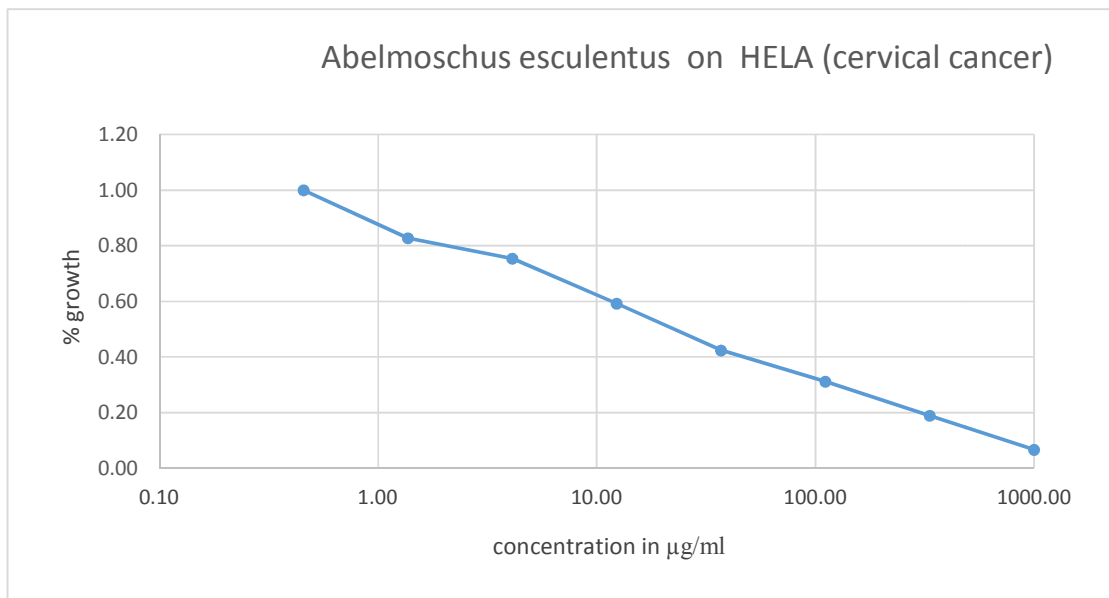


Fig. 1

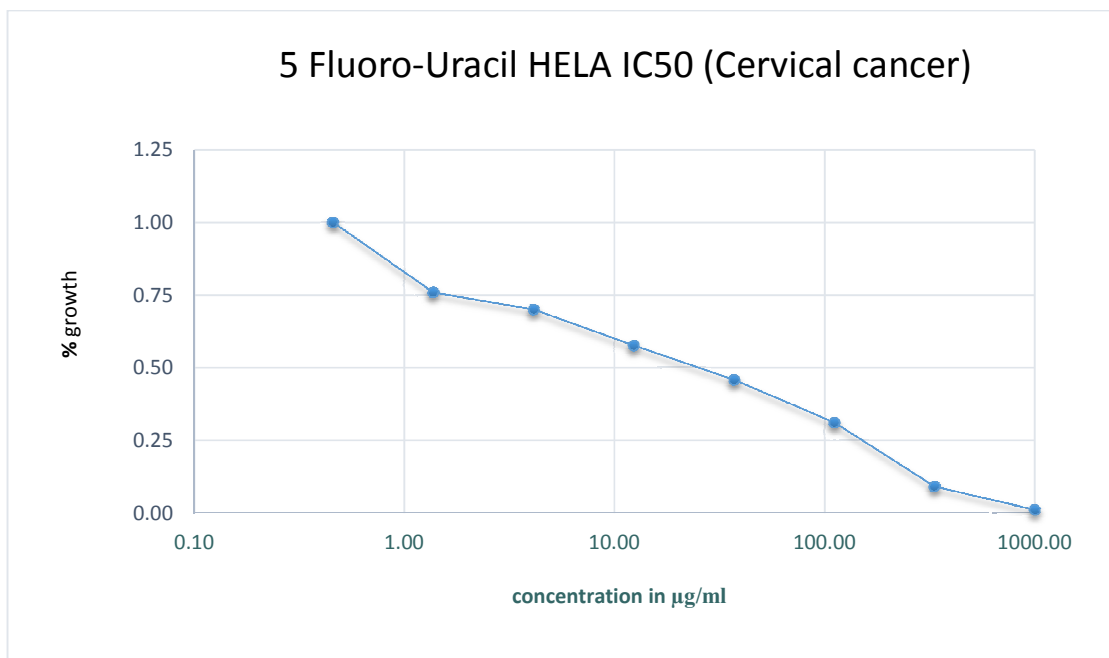


Fig. 2

Fig. 1 and 2. Show the growth inhibition percentage of cancer cells against concentration in µg/ml of the extracts and standard drug. The plant extracts of *A. esculentus* and 5 Fluoro-Uracil inhibited the growth of the selected cancerous cells in a concentration dependent manner

Table 2. Antiproliferative activity (Mean ±SEM) and selectivity indices of *A. muricata* and *A. esculentus* extracts on prostate, cervical, breast and Vero cells used

Extracts	Vero	DU145	Selectivity index	Hela	Selectivity index	HCC	Selectivity index
<i>A. muricata</i>	615.4587±3.9957	73.3390±1.8651	8.245	26.4340±1.9317	23.575	93.6233±3.0570	6.4422
<i>A. esculentus</i>	663.2197±7.864	48.2210±2210	12.920	22.7257±1.1834	28.939	171.646±4.7642	3.652

Table 3. Tukey's multiple comparisons of the IC₅₀ values of selected cell lines treated with the plant extracts and the conventional drug (5-fluorouracil)

Comparison	P value
HCC 1395 <i>A. muricata</i> vs. HCC1395 -5-fluoro uracil	0.001
HCC 1395 <i>A. esculentus</i> vs HCC1395 -5-fluoro uracil	0.001
DU145 <i>A. muricata</i> vs DU145 -5-fluoro uracil	0.001
DU145 <i>A. esculentus</i> vs DU145 5-fluoro uracil	0.001
Hela <i>A. muricata</i> vs Hela 5-fluoro uracil	0.460
Hela <i>A. esculentus</i> vs Hela 5-fluoro uracil	0.01
Vero <i>A. muricata</i> vs Vero 5-fluoro uracil	0.01

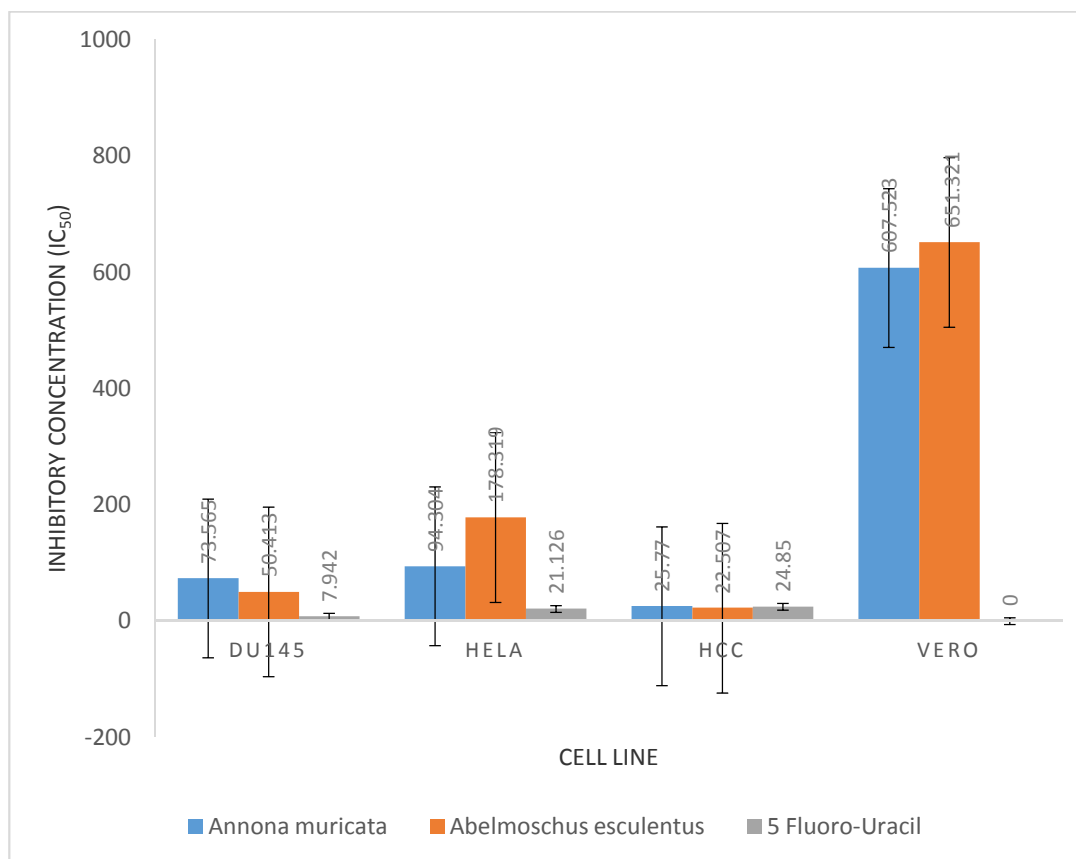


Fig. 3. Effects of *A. muricata* and *A. esculentus* extracts on selected cancer cell lines

4. CONCLUSIONS

The antiproliferative activity of *A. muricata* and *A. esculentus* has been well established in this study. The antiproliferative potential could be attributed to the essential phytochemicals exhibited by both plant extracts. This can be attributed to the synergistic interaction of the various phytochemicals enriched in the crude extracts. The extracts also selectively inhibited the cancerous cells. In vivo studies are envisaged to make a milestone in determining these extracts efficacy and safety. Moreover, isolation and identification of pure compounds and determination of different growth inhibitory mechanisms of action is needed to shed more light on these findings.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Authority to conduct this study was sought and granted from Kenya Medical Research Institute

(KEMRI), CTMDR Centre Scientific Committee (CSC) and Scientific and Ethics Review Unit (SERU) through study approval number KEMRI/SERU/CTMDR/035/3346.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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