Original Research Article

Phytochemical screening and *in vitro* antiproliferative activity of the fruit of *Annona muricata* and *Abelmoschus esculentus* pods against selected cancer cell lines.

Abstract

Incorporation of fruits and vegetables in diet can successfully be used in prevention and treatment of cancer. *Annona muricata* and *Abelmoschus esculentus* plants 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, terpernoids, glycosides, flavonoids and phenols. *A. muricata* had an IC_{50} of 23.632±1.3465µg/ml, 72.5860±1.9819µg/ml and 93.6233±3.0570µg/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±1.2132µg/ml on DU145 and HCC 1395 cells an IC_{50} of 50.013±0.2502 µg/ml and 171.6460±4.7642µg/ml respectively. The standard drug used had an IC_{50} of 21.126µg/ml on HCC and 24.850µg/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.

Key words: *Annona muricata*, *Abelmoschus esculentus*, anticancer, antiproliferative, phytochemical.

INTRODUCTION

Cancer is among the leading causes of morbidity and mortality in the world. Worldwide, 32.6 million people are living with cancer (Siegel *et al.,* 2018 *et al.,* 2018). Approximately 14 million new cases and 8.2 million cancer related deaths were reported in 2012 (WHO, 2014). The burden of cancer is increasing in Africa. This can be attributed to the 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 (WHO, 2014). 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 (IARC, 2014).

In developing countries, the mortality rate is increasing probably due to adoption of westernized lifestyle, delayed cancer screening and inadequate access to medication (Kanavos, *2006*). Breast cancer is the most diagnosed cancer in women (Jemal *et al*., 2007). 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 (Globocan, 2012). 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 (Peng *et al.*, 2016). It is also the third leading cause of deaths among females in developing countries (Ferlay *et al*., 2010). 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 (Bashir, 2015; Torre *et al.,* 2015). In Kenya, between 2010 and 2014 the rate of cancer death escalated from 31 to 33 deaths per 100 000 people. Cancer cases are expected to increase from 3% to 6% annually by 2026 hence doubling to 66 cancer deaths per 100000 people (Institute of Economic Affairs, 2014)

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.

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 (Hung H *et al*., 2004). 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 (Molassiotis *et al.,* 2005) Research has revealed that, plants possess secondary metabolites which can be used in the treatment of various diseases including cancer (Lai *et al*., 2004). Many edible plants possess therapeutic compounds and therefore are used as nutraceuticals (Zhao, J, 2007; Ravindranath *et al*., 2004). Utilization of food such as fruits and vegetables has the potential to have a 7-31% reduction of all cancers worldwide (Surh, 2003). Dietary phytochemicals are believed to induce apoptotic cell death in pre- neoplastic or neoplastic cells through different growth inhibitory mechanisms (Tan & Konczak, 2011).

Abelmoschus esculentus is a perennial plant widely spread in Africa. It is commonly known as finger's lady, okra, mbinda and gumbo. Okra seeds are a good source of unsaturated oil and proteins. Seeds can also be used to obtain caffeine free coffee (Gemede H. F *et al*., 2014). It is rich in fiber, vitamin C and folate and because of this many people have incorporated it in their diet (American Dietetic Association, 2003). It is also known to be rich in antioxidants. In addition, it contains appreciable amounts of calcium and potassium (Gemede H *et al*, 2015). 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 (Hollman & Katan, 1999; Klimczak *et al*., 2007; Kubola & Siriamornpun, 2008*)*.

Annona muricata belongs to the family Annonaceae. The plant is commonly known as Graviola and is widely used by many traditional cultures for a variety of physical ailments. 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 (De Sousa *et al.,* 2010). Acetogenins are active compounds capable of preventing abnormal or cancer type cells from proliferating. However, there is limited number of published data that illustrate the anti-proliferative potential of *A. muricata* extracts on cancer cells. 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 of *A. muricata* and *A. esculentus* extracts could be used in the treatment and management of breast, prostate and cervical cancer.

MATERIAL AND METHODS

Plant collection

The fruits of *Annona muricata* and pods of *Abelmoschus esculentus* were collected from Kitui County 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.

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 fruit of *A. muricata* cut into quarters to ensure proper drying of the plant materials. The samples were then dried at room temperature. 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.

Extraction

Briefly, 200g of the fine powder was weighed using an electric top balance and put in a 500ml flat bottomed 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 resoaked 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 40^0C. The concentrated extracts were then weighed, labelled and stored at 4^0C until use.

Qualitative Phytochemical screening

Qualitative phytochemical screening of *A. muricata* and *A. esculentus* was done using standard procedures.

Test for alkaloids

Three drops of Mayer's reagent were added to 2ml of the extract. Formation of a yellow colored precipitate indicates the presence of alkaloids.

Test for saponins

Five milliliters of the extract were diluted with distilled water to 10 ml in a graduated cylinder and shaken for 10 minutes. Formation of a persistent layer of foam indicates the presence of saponins.

Test for phenols

Three to four drops of ferric chloride solution were added to the extract. Formation of a blue-black color indicates the presence of phenols.

Test for flavonoids

Two milliliters of dilute ammonia and two milliliters of concentrated sulphuric acid were added to the extract. Formation of intense yellow color indicates the presence of flavonoids.

Test for glycosides

One milliliter of glacial acetic acid was added to the 0.5ml of the extract. One drop of iron chloride was added and the mixture shaken. One milliliter of concentrated sulphuric acid was then added to the mixture. Formation of a brown ring indicates the presence of glycosides.

Test for terpernoids

Two milliliters of chloroform were added to one milliliter of the plant extract and shaken vigorously. Two milliliters of concentrated sulphuric acid was added and heated for 2 minutes. Formation of grey color indicates the presence of terpernoids.

Test for tannins

Five milliliters of distilled water were added to two milliliters of the plant extracts and heated to boil. Two percent of iron chloride was then added. Blue black colour indicates the presence of tannins.

Antiproliferative assay

Cell culturing

DU145 (prostate cancer), HCC 1395(breast cancer), Hela (cervical cancer) 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 37^0C 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% CO_2 at 37^0C until 70% confluence was attained.

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 2.0×10^4cells/ml suspension were seeded in a 96-well plate and incubated at 37°C for 24h at 5% CO_2. After 24hrs, 15µl 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% CO_2 for 48hrs. 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-2thiazoly)-2, 5-diphenyltetrazolium bromide (MTT) to a purple formazan product (Tim, 1998). After 48h, 10µl MTT dye was added to the cells and incubated for 2hrs in 5% CO_2 at 37°C. The formazan crystals formed were then solubilized using 50µl of 100% DMSO. The absorbance was read using multiplex Elisa reader at 576nm and a reference wavelength at 620nm. The percentage cell viability at different extracts concentration was calculated using the formula:

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

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

$$Percentage\ inhibition = 100 - \frac{At - Ab}{Ac - Ab} X100$$

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 CC_{50} values (the concentration of extracts required to kill 50% of the treated cells). The effects of the 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 = CC50/IC50) of the three cancer cell lines was calculated from the CC_{50} ratio of the Vero cells and IC_{50} of the cancer cells.

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 Cruzi 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 ±Standard Error of Mean (S.E.M). Tables and graphs were used for clear presentation of the results.

RESULTS AND DISCUSSION

Compounds	*Abelmoschus esculentus*	*Annona muricata*
Alkaloids	_	+
Flavonoids	+	+
Terpernoids	+	+
Glycosides	+	+
Tannins	+	_
Saponins	+	+
Phenols	+	+

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

Key: + (Present), (-) Absent

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, terpernoids, phenols and glycosides compounds. Flavonoids, terpernoids, 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 above.

Saponins are secondary metabolites that are natural glycosides possessing a wide range of pharmacological activities including cytotoxic and chemo preventive properties (Podolak *et al*., 2008). Flavonoids in recent research have been shown to inhibit initiation, promotion and progression of cancerous cells (Ramos S, 2007). Studies have shown that flavonoids have a potent dietary chemo preventive activity (Walle *et al*., 2007). 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 (Koleckar *et al.,* 2008)

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 terpernoids may be advantageous in prevention and management of cancer such as prostate, breast and colon (Gupta *et al*., (2014). Phenols also play a role in anticancer activity through processes such as apoptosis and inhibition of DNA binding (Sas *et al*., 2008).

Extracts	**Vero**	**DU145**	**Selectivity index**	**Hela**	**Selectivity index**	**HCC**	**Selectivity index**
A. muricata	615.4587±3.9957	73.3390±1.8651	8.245	26.4340±1.9317	23.575	93.6233±3.0570	6.4422
A. esculentus	663.2197±7.8646	48.2210±2210	12.920	22.7257±1.1834	28.939	171.646±4.7642	3.652

Table 2: IC_{50} values and selectivity indices of *A. muricata* and *A. esculentus* extracts on prostate, cervical, breast and Vero cells used.

Comparison	**P value**
HCC 1395 *A.muricata* vs. HCC1395 -5-fluoro uracil	0.001[a]
HCC 1395 *A.esculentus* vs HCC1395 -5-fluoro uracil	0.001[a]
DU145 *A.muricata* vs DU145 -5-fluoro uracil	0.001[a]
DU145 *A.esculentus* vs DU145 5-fluoro uracil	0.001[a]

Hela *A.muricata* vs Hela 5-fluoro uracil	0.460[b]
Hela *A.esculentus* vs Hela 5-fluoro uracil	0.01[a]
Vero *A.muricata* vs Vero 5-fluoro uracil	0.01[a]

Table 3: Turkey's multiple comparisons of the IC_{50} values of selected cell lines treated with the plant extracts and the conventional drug (5-fluorouracil).

Key: a- Significance, b- no significance

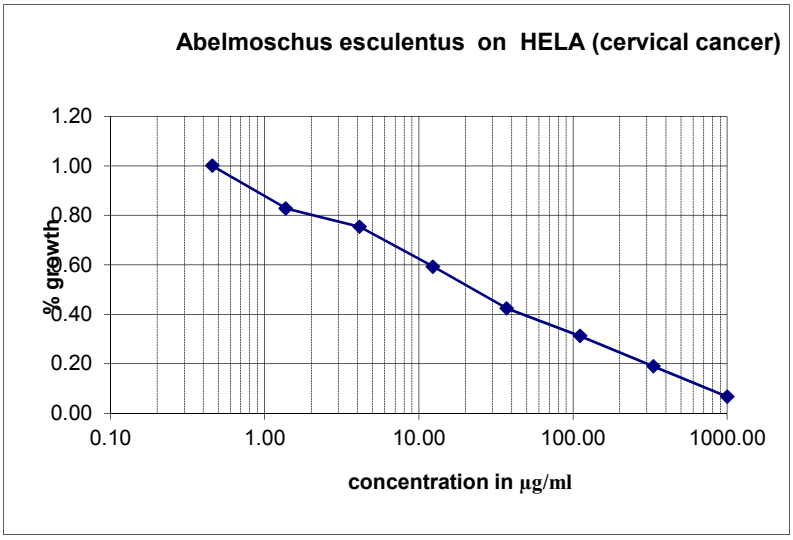

Figure 1: Shows the growth inhibition percentage of cancer cells against concentration in µg/ml of the extracts. The plant extracts inhibited the growth of the selected cancerous cells in a concentration dependent manner.

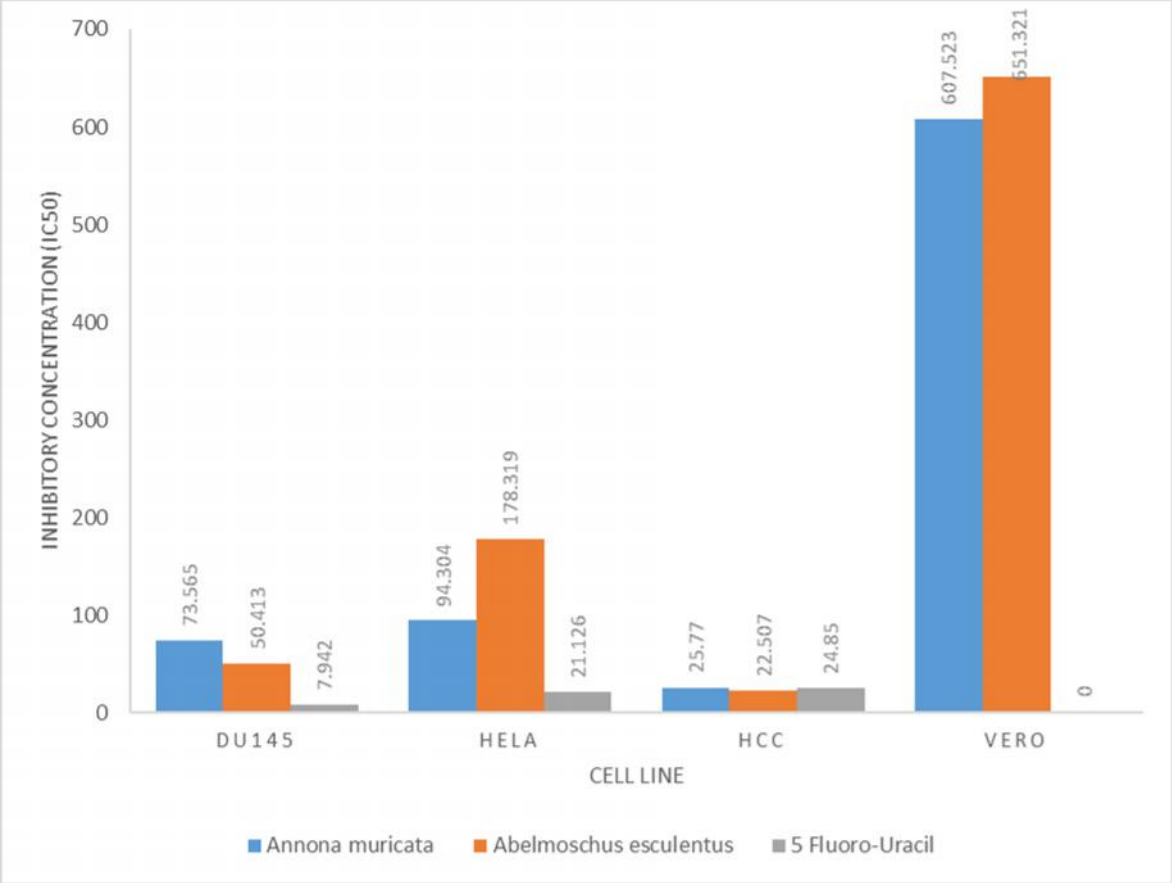

Figure 2: Effects of *A.muricata* and *A.esculentus* extracts on selected cancer cell lines.

Key words: DU145-prostate cancer, Hela-cervical cancer, HCC 1395-cervical cancer, Vero-normal kidney cells of monkey.

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 (Figure 2). According to Siti *et al*., 2011, a plant extract with an IC_{50} ≤20µg/ml is considered active, 20µg/ml-100µg/ml moderately active, weakly active when it ranges between 100µg/ml→1000µg/ml and inactive at above 1000µg/ml. 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 Figure 1 above. 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. In a study done by George *et al*.,

2012, the aqueous leaf extract of *A. muricata* inhibited proliferation of 4T1 breast cancer cell line. This was similar to HCC1395 breast cancer cell line as shown in Figure above 1.

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 above. As illustrated in table 3, all the extracts except *A.muricata* on Hela cells ($P \geq 0.05$) 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 cancer treatment and management of breast, cervical and prostate cancer.

Conclusions and recommendations

The antiproliferative activity of *A. muricata* and *A. esculentus* has been well established in this study. The antiproliferative potential could be attributed to the important phytochemicals exhibited by both plant extracts. However, isolation and identification of pure compounds and determination of different growth inhibitory mechanisms of action is needed to shed more light on these findings.

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.

Data Access

Data associated with this manuscript can be obtained freely at CTMDR, KEMRI.

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