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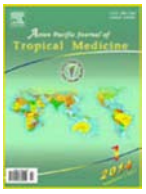

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Phytochemical screening, anti-oxidant activity and *in vitro* anticancer potential of ethanolic and water leaves extracts of *Annona muricata* (Graviola)

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ABSTRACT

Objective: To determine the phytochemical composition, antioxidant and anticancer activities of ethanolic and water leaves extracts of *Annona muricata* (*A. muricata*) from the Eastern Uganda.

Methods: Phytochemical screening was conducted using standard qualitative methods and a *Chi*-square goodness of fit test was used to assign the relative abundance of the different phytochemicals. The antioxidant activity was determined using the 2, 2-diphenyl-2-picrylhydrazyl and reducing power methods whereas the *in vitro* anticancer activity was determined using three different cell lines.

Results: Phytochemical screening of the extracts revealed that they were rich in secondary class metabolite compounds such as alkaloids, saponins, terpenoids, flavonoids, coumarins and lactones, anthraquinones, tannins, cardiac glycosides, phenols and phytosterols. Total phenolics in the water extract were (683.69±0.09) μg/mL gallic acid equivalents (GAE) while it was (372.92 ±0.15) μg/mL GAE in the ethanolic extract. The reducing power was 216.41 μg/mL in the water extract and 470.51 μg/mL GAE in the ethanolic extract. *In vitro* antioxidant activity IC_{50} was 2.0456 mg/mL and 0.9077 mg/mL for ethanolic and water leaves extracts of *A. muricata* respectively. The ethanolic leaves extract was found to be selectively cytotoxic *in vitro* to tumor cell lines (EACC, MDA and SKBR3) with IC_{50} values of 335.85 μg/mL, 248.77 μg/mL, 202.33 μg/mL respectively, while it had no cytotoxic effect on normal spleen cells. The data also showed that water leaves extract of *A. muricata* had no anticancer effect at all tested concentrations.

Conclusions: The results showed that *A. muricata* was a promising new antioxidant and anticancer agent.

1. Introduction

Cancer is the major cause of mortality and morbidity globally. According to recent estimates by the World Health Organization[1,2], annual cancer incidence in sub-Saharan Africa is 551 200 with a mortality of 421 000[3,4]. About 70% of all cancer deaths occurred in low- and middle-income countries[3,4].

Molecular targeted agents are currently being studied in all treatment settings including that of chemoprevention, which is defined as the use of natural or synthetic non-essential dietary agents to interrupt the process of carcinogenesis and to prevent or delay tumor growth[5,6]. The available treatment methods include surgery, chemotherapy, and radiation[7]. The current available methods of treatment all induce significant side effects and therefore the need for alternate adjuvant therapies

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has arisen[8]. Natural products are extremely an important source of medicinal agents. Although there are some new approaches to drug discovery, such as combinatorial chemistry and computer based molecular modeling design, none of them can replace the importance of natural products in drug discovery and development[9,10]. Many synthetic drugs cause severe side effects that are not acceptable except as treatments of last resort for terminal diseases such as cancer and the metabolites discovered in medicinal plants may avoid the side effect of synthetic drugs[11].

Antioxidants are a group of substances that are useful for fighting cancer and other processes that potentially lead to diseases such as atherosclerosis, Alzheimer, Parkinson, diabetes, and heart disease[11-13]. Antioxidants act by preventing the onset of cancer during carcinogenesis, and they are generally beneficial to cells. Oxidants damage macromolecules such as proteins, lipids, enzymes, and DNA and to combat these radicals, living organisms produce enzymes or rely on non-enzymatic molecules such as cysteine, ascorbic acid, flavonoids, and vitamin K for protection[12-14].

Plant used in treating diseases is as old as civilization and traditional medicines are still a major part of habitual treatments of different maladies[7,15,16]. In recent times, folk medicine has taken an important place especially in developing countries where limited health services are available. The absence of scientific evaluation of medicinal plants to validate their use may cause serious adverse effects[7]. *Annona muricata* (*A. muricata*) is widely used in the traditional treatment of cancer in many countries. *A. muricata* commonly known as Graviola or soursop belongs to the family of Annonaceae and is the most tropical semi deciduous tree with the largest fruits of the *Annona* genus[17,18]. It is widely distributed and native to sub-Saharan Africa countries. Earlier studies have demonstrated its anti-hyperglycemic, anti-hyperlipidemic, antimalarial, anti-parasitic, antibacterial, insecticidal, molluscicidal, antiviral and most importantly, their anticancer properties[19-23].

Ancient herbal medicines may have some advantages over single purified chemicals[24,25]. Often the different components in an herb have synergistic activities or buffer toxic effects. This study therefore aimed to determine the phytochemical composition, anti-oxidant activity as well as determine the *in vitro* anti-cancer potential of ethanolic and water leaves extracts of *A. muricata* from Eastern Uganda, as an alternative medicine in the prevention and treatment of cancer and other oxidative stress related diseases.

2. Materials and methods

2.1. Sample collection and authentication

Fresh leaves of *A. muricata* L. were collected from the wild in Eastern Uganda in the districts of Kaliro and Iganga Municipality (Figure 1), during the month of August 2013. The plant (Figure 2) was identified and authenticated in the Makerere University Botanical Herbarium by Ms. Olivia Wanyana Mangeni. A voucher specimen was deposited in the herbarium under the collection number GY 021- 10/13-MB 300-0007/12-001.

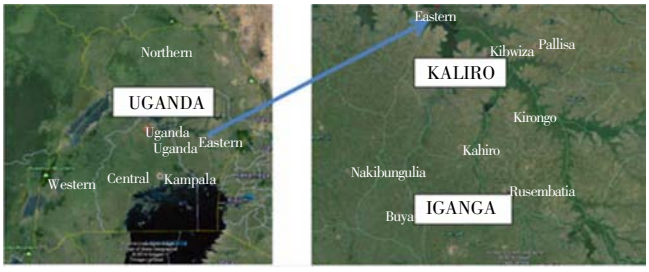

Figure 1. Map of Uganda showing the study areas of Kaliro and Iganga.

Figure 2. *A. muricata* leaves.

2.2. Samples preparation and extraction

The leaves of *A. muricata* were washed with water and cut into small pieces, drying was done at room temperature, and the dried leaves were powdered. Equal amounts (350 g) of powdered leaves were extracted using ethanol and distilled water for 3 d by the plant tissue homogenization method as previously described[26]. The extracts were then concentrated using rotary evaporator and dry block heater respectively and kept at -20 °C until used.

2.3. Chemicals, reagents and cell lines

All chemicals and reagents were procured from certified suppliers and were of the highest analytical standard. The Ehrlich ascites carcinoma cells (EACC) had been obtained from the National Cancer Institute, Cairo, Egypt. The breast cancer cell lines MDA and SKBR3 were obtained from the Physiology and Cancer Biology Laboratory in the Zoology Department of the Faculty of Science at Cairo University.

2.4. Phytochemical screening of the extracts

Phytochemical screening was done using standard procedures as previously described[26]. Samples of the ethanolic and water extracts of *A. muricata* were

screened for the following phyto constituents: alkaloids, saponins, terpenoids, flavonoids, coumarins and lactones, anthraquinones, tannins, cardiac glycosides, phenols and phytosterols.

2.5. Determination of relative abundance of the phytochemicals

Following the identification of the different phytochemicals present in both ethanolic and water leaves extracts of *A. muricata*, the relative abundance of the phytochemicals in each of the extracts was determined[27]. The results were analyzed using the *Chi*-square goodness of fit test between low and high abundance. For each of the nine runs per phytochemical, we allocated it as either high or low upon which the final allocation of the relative abundance would be based. H_0: The concentration of the phytochemical in the sample is neither high nor low, thus No preference (average); H1: There is a difference in the concentration of the phytochemical in the sample; α=0.1, Expected value (E)=4.5, Degrees of freedom=1 and $\chi^2_{critical}$=2.7055. All conditions of the *Chi*-square test were met, except the standard minimum expected value of 5, for which our expected value was 4.5, as the total data set for each test was 9 values.

2.6. Determination of total phenolics

The phenolic content of the *A. muricata* was determined[28]. Exactly 20 μL of the extract was taken from each of the extract and added to 1 580 μL of distilled water. This was followed by adding of 100 μL of Folin reagent (1%) and left to stand for 2 min. Then 300 μL of Na_2CO_3 (7.5%) was added to each of the samples, mixed thoroughly and left to stand for 2 h at 20 °C. All results were expressed as gallic using a standard curve of gallic acid and a linear equation was used to calculate the total phenols of the extracts.

2.7. Determination of reducing power

The reducing power of the ethanolic and water leaves extracts of *A. muricata* were determined[29]. Gallic acid was used as standard. A volume of 200 μL of each of the samples per extract as well as the standard at different concentrations were taken separately and mixed with 500 μL of 0.2 mol/L phosphate buffer (pH 6.6) and 500 μL of potassium ferricyanide. The samples were then incubated at 50 °C for 20 min. Then 500 μL of 10% trichloroacetic acid were added and centrifuged at 6 500 r/min for 16 min. About 700 μL of supernatant were added to 700 μL distilled water, 140 μL of freshly prepared ferric chloride, and left to stand for 10 min. Finally, the absorbance was measured at 700 nm. A standard curve for gallic acid was generated and the linear equation was used to calculate the reducing power of the extracts.

2.8. Quantification of antioxidant activity using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method

The free radical scavenging activity (RSA) was preceded. Different concentrations of the extracts (0, 250, 500, 750, 1 000, and 1 250 μg/mL) were used. A volume of 2.5 mL of 0.04% DPPH solution was mixed with 0.5 mL of all the concentrations of both extracts separately. After 30 min incubation at room temperature in the dark, the absorbance was read at 517 nm in triplicates for each concentration[30]. Butylated hydroxytoluene (BHT) and butylated hydroxylanisole (BHA) were used as positive control. The percent inhibition of free radical formation was calculated as follows:

$$RSA\ (\%)=[(A_{control}-A_{sample})/(A_{control})]\times 100$$

2.9. TLC fractionation of the ethanolic leaves extracts of A. muricata

The ethanolic leaves extracts of *A. muricata* were fractionated using thin layer chromatography (TLC) technique. The extract was applied on silica gel 60 F254 TLC aluminum sheets (20×20) (Merck, Darmstadt, Germany) at one of extremes to separate the different fractions. Mobile phase was petroleum ether: ethyl acetate: glacial acetic acid (4:1:1). Eleven fractions were scratched and named as EEAM1b-EEAM11. All the fractions were tested for anti-oxidant activity, reducing power and anti-cancer activity.

2.10. In vitro anti-cancer activity of the extracts on EACC tumor cell-lines

The culture medium was prepared using RPMI1640 media (Gibco, Grand Island, USA), 10% inactivated fetal bovine serum (Gibco), and 100 units/mL penicillin and 100 mg/mL streptomycin were added. A line of Ehrlich ascites carcinoma has been used. About 2 mL of media containing EACC (2×10^4 cells) were transferred into a set of tubes each, then different concentrations of the extracts both water and ethanol (0, 250, 500, 750, 1 000, and 1 250 μg/mL) were added. The tubes were incubated at 37 °C in the presence of 5% (v/v) CO_2 for 2 h[31]. For each examined material (and control), a new clean, dry small test tube was used and 10 μL of cell suspension, 80 μL saline and 10 μL trypan blue (0.4%) were added and mixed. Then the number of living cells (non-stained) was calculated using a hemocytometer slide by microscope (Nikon, TMS). The extracts concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentages against logarithm of concentration after transforming the concentrations.

2.11. MTT assay for breast cancer cell lines MDA and SKBR3

The culture medium was prepared using modified Earle's salt with 1.2 g/L sodium carbonate and L-glutamine (Gibco, Grand Island, USA), 10% inactivated fetal bovine serum (Gibco), and 100 units/mL penicillin and 100 mg/mL streptomycin were added. The anticancer effect of the ethanolic leaves extracts on the MDA and SKBR3 cell lines was determined by the MTT assay[32]. The cell count was adjusted to 1×10^5 cells/0.1 mL and plated in 100 μL of medium/well in 96-well plates (Costar Corning, Rochester, NY). The cells were then incubated in the presence of

various concentrations of the ethanolic extract for 72 h at 37 °C in triplicates per concentration (750, 500, 250, and 0 μg/mL). The sample solutions were then removed and washed with phosphate buffer solution (pH 7.4). A volume of 20 μL/well of 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-- tetrazolium bromide (MTT 0.5%) was added. The samples were then incubated for 4 h. Amount of formazan was determined by measuring the absorbance at 570 nm using an ELISA plate reader (ELx800 universal micro plate reader, Biotech, USA). Concentration required for an inhibition concentration (IC_{50}) was determined graphically. % Cell death was calculated using the following formula:

% Cell death=(Control OD − Sample OD)/Control OD×100

2.12. Cytotoxicity effect of ethanolic leaves extracts of A. muricata on normal spleen cells

Spleen cells were isolated from normal healthy mice[33]. Spleen cells viability after and before incubation with different concentrations of ethanol at 37 °C in the presence of 5% (v/v) CO_2 for 2 h was calculated using trypan blue technique[31]. All concentrations were assayed in triplicates.

2.13. Data analysis

Quantitative and graphical data was analyzed using Microsoft Excel Package. The results of each series of experiments (performed in triplicates) were expressed as the mean±standard deviation. Qualitative data for phytochemical analysis was analyzed using the χ^2 goodness of fit test.

3. Results

3.1. Phytochemical analysis of ethanolic and water leaves extracts of A. muricata

All phytochemicals tested were present in both types of *A. muricata* leaves extracts. The χ^2 goodness of fit test has been used to allocate the relative abundance of each of the phytochemicals. Phytochemicals with computed χ^2 values (blue region) higher than the $\chi^2_{critical}$ (red area) were designated as high or low, depending on the initial count while those that had χ^2 values less than the $\chi^2_{critical}$ were assigned average abundance as shown in Figures 3 and 4 below.

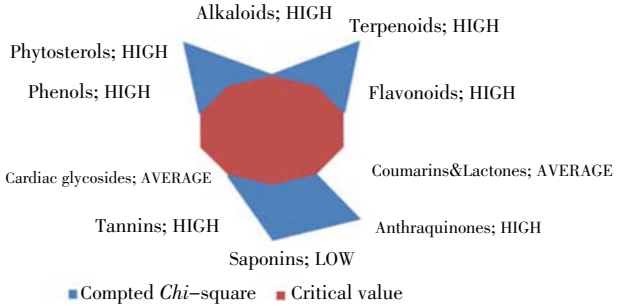

Figure 3. Phytochemicals present in ethanolic leaves extracts of *A. muricata* with relative abundance computed from the χ^2 test.

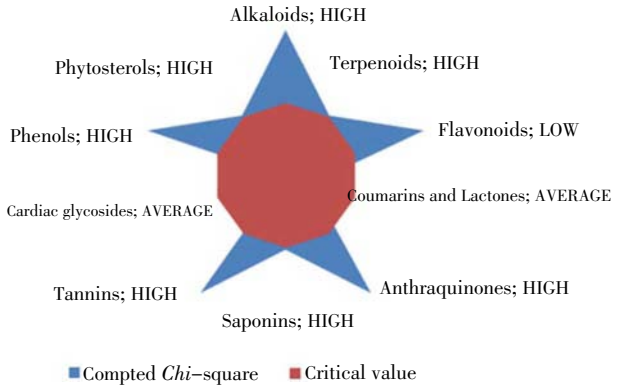

Figure 4. Phytochemicals present in water leaf extracts of *A. muricata* with relative abundance computed from the χ^2 test.

3.2. Total phenolic compounds (y=0.0026x+0.0044)

Total phenolics in the water extract were computed to be (683.69±0.09) μg/mL gallic acid equivalents (GAE) while it was (372.92±0.15) μg/mL GAE in the ethanolic extract. These values indicate a higher level of phenolics in the water extract as compared to the ethanolic extracts. These results give an indication on the potential effect of the roles played by phenolic compounds in the activity of this plant with expectation of higher effect in water extracts as compared to ethanolic extracts.

3.3. Reducing power of the extracts and fractions (y=0.0039x)

The reducing power of both the ethanolic and water leaves extracts of *A. muricata* were determined by relation to that of the gallic acid from the standard curve with the linear equation y=0.0039x. The reducing power was 216.41 μg/mL in the water extract and 470.51 μg/mL GAE in the ethanolic extract. It was evident that the water extract had a higher reducing power than the ethanolic extract.

Similarly, the reducing power of the TLC fractions of the ethanolic leaves extract was determined as above. The results expressed as μg/mL GAE were recorded as follows: EEAM1b (15.77), EEAM2 (1.54), EEAM3 (8.72), EEAM4 (37.69), EEAM5 (0.77), EEAM6 (3.33), EEAM7 (7.44), EEAM8 (5.77), EEAM9 (9.36), EEAM10 (4.36) and EEAM11 (0.00). It was evident that the TLC fraction EEAM4 had the highest reducing power whereas fraction EEAM11 registered no reducing power activity. Although some fractions had a reducing power of more than 15 μg/ml GAE, most of them registered a very low value of less than 10 μg/mL GAE.

3.4. Quantification of antioxidant activity using the DPPH method

Figure 5 shows a decrease in the concentration of DPPH radical due to the scavenging ability of the soluble constituents in the ethanolic and water leaves extracts of *A. muricata*. There was a direct positive relationship between antioxidant activity and increasing concentration of the extracts. The relationship was more pronounced in the water extract than in the ethanolic extract. There was ultimately

a higher antioxidant power registered by the water extracts as compared to the ethanolic extracts as represented by the calculated IC_{50} values of 0.9077 mg/mL and 2.0456 mg/mL respectively.

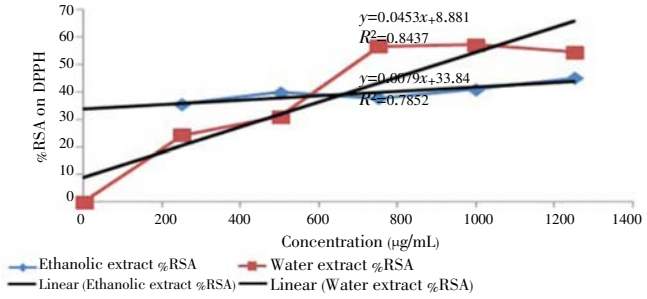

Figure 5. Antioxidant activity of leaves extracts of *A. muricata* on DPPH.

The antioxidant activities of each of the 11 fractions isolated from the ethanolic leaves extracts of *A. muricata* by TLC technique were also determined. The highest activity was recorded in fraction EEAM8 and the lowest activity was registered by fraction EEAM2. The percentage radical scavenging assay on DPPH of each of the fractions was as follows: negative control (0%), EEAM1b (3.95%), EEAM2 (1.09%), EEAM3 (1.91%), EEAM4 (7.28%), EEAM5 (30.34%), EEAM6 (9.52%), EEAM7 (5.2%), EEAM8 (31.16%), EEAM9 (28.98%), EEAM10 (29.52%) and EEAM11 (15.85%). The results showed relatively low antioxidant activity recorded by most of the fractions.

3.5. In vitro anti-cancer activity of leaves extracts of A. muricata

Trypan blue-exclusion assay (TBEA) was used for the evaluation of anticancer activity of ethanolic and water leaves extracts of *A. muricata* against EACC, and for cytotoxicity against normal spleen cells. While the MTT assay was used for the evaluation of anticancer activity of ethanolic leaves extracts of *A. muricata* against two human breast cancer cell lines MDA and SKBR3.

Figure 6 shows the anticancer activity of ethanolic and water leaves extracts of *A. muricata* on EACC. The minimum detectable anticancer activity on EACC cell line was observed in the ethanolic leaves extract of *A. muricata* at a concentration of 250 μg/mL, with an inhibition of 32.9% cell death, and reaching a maximum inhibition of 100% cell death at a concentration of 750 μg/mL. IC_{50} of ethanolic extracts was determined to be 335.85 μg/mL. On the other hand, however, the water leaves extracts of *A. muricata* had no effect across all concentrations tested.

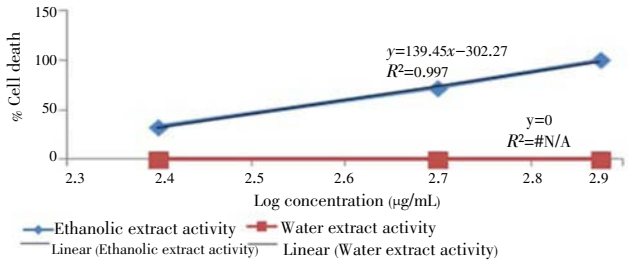

Figure 6. Effect of ethanolic and water leaves extracts of *A. muricata* on EACC.

Figure 7 shows the anticancer activity of ethanolic leaves extracts of *A. muricata* on MDA cell line while Figure 8 shows the anticancer activity of ethanolic leaves extracts of *A. muricata* on SKBR3 cell line. There is a general increase in percentage cell death with increase in concentration of the ethanolic extracts. The effect of ethanolic extract on two human breast cancer cell lines MDA and SKBR3 was tested at concentrations ranging from 250 to 750 μg/mL for 72 h, and % of cell death was measured by the MTT assay. The results demonstrated a strong dose-dependent inhibition in treated cell lines. The ethanolic leaves extracts were thus found to be highly cytotoxic *in vitro* against the two human breast cancer cell lines MDA and SKBR3 (Figures 7 and 8) with IC_{50} of 248.77 μg/mL and 202.33 μg/mL respectively.

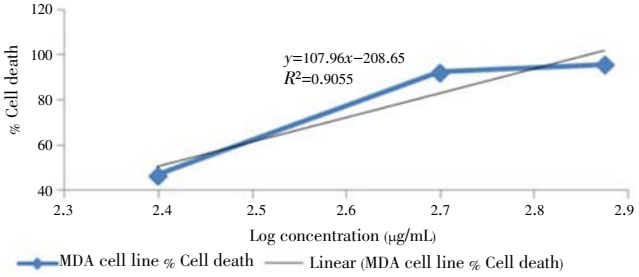

Figure 7. Effect of ethanolic leaves extracts of *A. muricata* on MDA cell line.

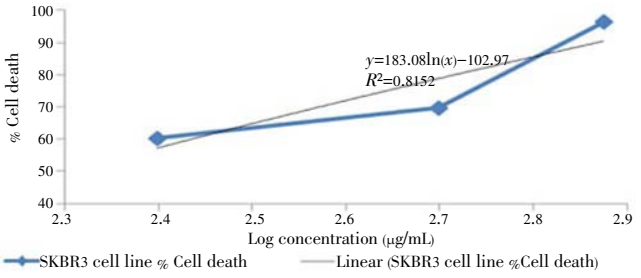

Figure 8. Effect of ethanolic leaves extracts of *A. muricata* on SKBR3 cell line.

Figure 9 below shows results for the cytotoxicity test for the activity of ethanolic leaves extracts of *A. muricata* on normal spleen cells using the TBEA. There was no cytotoxicity effect registered across the whole range of concentrations used, implying that the extracts had no effect on the normal cells, and an indication of the high selectivity for the target cell lines.

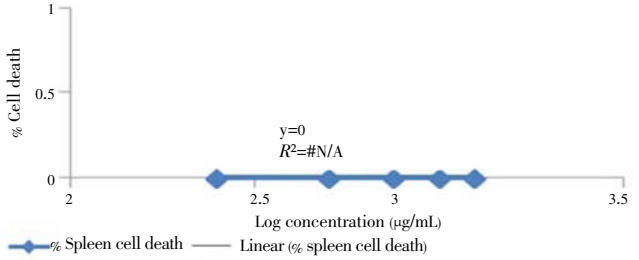

Figure 9. Effect of ethanolic leaves extracts of *A. muricata* on normal spleen cells.

The anticancer activity of the ethanolic leaves extract TLC fractions of *A. muricata* was determined. Three fractions EEAM1b, EEAM2, and EEAM4 showed no activity. The

remaining fractions had some anticancer activities and four fractions showed more than 50% cell death. The percentage cell death registered by the different fractions were as follows: negative control (0%), EEAM1b (0%), EEAM2 (0%), EEAM3 (8.5%), EEAM4 (0%), EEAM5 (10%), EEAM6 (40.2%), EEAM7 (5%), EEAM8 (53.75%), EEAM9 (76%), EEAM10 (84.5%) and EEAM11 (64%). The results showed that the net effect of the extracts would be contributed upon by only a few of the fractions in the extract as revealed above. This contribution would be either synergistic or inhibitory affecting the final effect registered by the entire extract as a whole.

Figure 10 below shows a comparison between antioxidant activity (reducing power and %RSA) and cytotoxic activity (% cell death) of ethanolic leaves extracts TLC fractions of *A. muricata*. It revealed a general trend in the relationship between the two aspects of the study where all fractions which showed high anticancer activity had high antioxidant activity (as measured by the reducing power and DPPH radical scavenging assay), while the opposite trend was not.

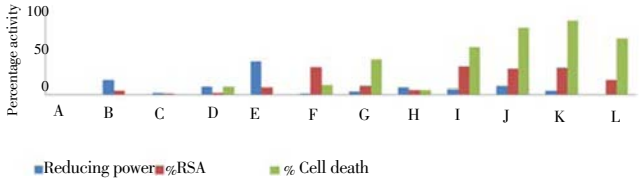

Figure 10. Comparison between antioxidant and anticancer activity of ethanolic leaves extracts TLC fractions of *A. muricata*.

A: Negative control; B: EEAM1b; C: EEAM2; D: EEAM3; E: EEAM4; F: EEAM5; G: EEAM6; H: EEAM7; I: EEAM8; J: EEAM9; K: EEAM10; L: EEAM11.

4. Discussion

Phytochemical screening conducted on leaves extracts of *A. muricata* revealed the presence of following classes of compounds: alkaloids, flavonoids, terpenoids, coumarins and lactones, anthraquinones, tannins, cardiac glycosides, phenols, phytosterols, and saponins. They were present in both the ethanolic and water leaves extracts, but with noticeable differences in relative abundance ranging from low, average and high. These results are in line with earlier studies that carried out on the ethanolic seeds extract of *A. muricata*, and the phytochemical tests showed that ethanol soursop seeds extract contained secondary metabolites compounds: saponins, alkaloids and triterpenoids, flavonoids, anthraquinones, tannins, and cardiac glycosides. They are defense chemical compounds of plants produced in the plant tissue[17,34].

The extracts were found to be rich in alkaloids which have wide pharmacological effects and thus have been used extensively as drugs in medical field. The detection of high levels of alkaloids in the leaves extracts of *A. muricata* further reinforces the presence of alkaloid in this species as already outlined by other independent studies that showed that among the chemical constituents found in *A. muricata*, the alkaloids and essential oils stood out[8]. Cardiac glycosides are molecules used in treatment of heart failure[35], hence the present findings are suitable for use in treatment of heart diseases.

Generally, presence of alkaloids, flavonoids, terpenoids, coumarins and lactones, anthraquinones, tannins, cardiac glycosides, phenols, phytosterols, and saponins confirms that *A. muricata* leaves extracts contain molecules known for extensive use in the medical field both traditionally and pharmaceutically. This would be an indication for its potential use in anti-inflammatory, anti-allergic, antibacterial, and antiviral, heart failure, antioxidant and anticancer activity among others. These findings emphasize the value of traditional knowledge in the use of plants for medicinal use as well as pharmaceutical development. The use of *A. muricata* in traditional medicine is validated by presence of these phytochemicals of known health benefits and thus further studies on this species are needed.

The phenolic content of the *A. muricata* was determined and all results were expressed as GAE. Typical phenolics that possess antioxidant activity have been characterized as phenolic acids and flavonoids[36,37]. Phenols are among the non-enzymatic compounds obtained from natural sources, which have received high attention due to their proven antioxidant capabilities. Although phenolic compounds have been related to antioxidant activity, some studies have emphasized specific classes such as flavonoids and tannins[12]. Our results revealed that the water leaves extract had higher total phenolic content as compared to the ethanolic leaves extract of *A. muricata*. The higher phenolic content in the water extract would partly contribute to its higher antioxidant activity.

Several methods have been developed to measure the efficiency of antioxidants as pure compounds or in extract. These methods focus on different mechanisms of the oxidant defense system that is scavenging active oxygen species and hydroxyle radicals, inhibiting lipid peroxidation, or chelating metal ions[29]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. It was found that in general, the reducing power of the water leaves extracts was higher than that of the ethanolic leaves extracts, giving an indication in the potential higher antioxidant activity of the extracts.

The 11 fractions had a very low reducing power of less than 50 μg/mL GAE each. This may be translated into the observation that perhaps the final reducing power of the extract is as a result of the combined effect of each of the compounds in the fractions.

Free RSA of the leaves extracts was determined. The data showed that water leaves extracts of *A. muricata* had a higher free radical inhibition with an IC_{50} of 0.9077 mg/mL as compared to the ethanolic leaves extract with an IC_{50} of 2.0456 mg/mL. The standard antioxidants BHA and BHT were used as positive controls. This free RSA of the leaves

extracts was far lower than the standard positive controls, implying that the extracts, at similar concentrations may not be competitively strong antioxidants. It is however likely that the leaves extract's antioxidant activity of *A. muricata* may be as strong as standard BHA and BHT, given that the samples assayed in this study were crude extracts, while the standard controls are usually very purified compounds.

It is not surprising that the water leaves extracts of *A. muricata* had a stronger antioxidant activity as compared to the ethanolic leaves extract. This revealed that the total phenolics were two fold higher in the water leaves extracts than the ethanolic leaves extracts, and phenolics had long been associated with antioxidant activity. Similarly, the water leaves extracts had reducing power almost two times higher than that of the ethanolic leaves extracts. In general however, the relatively strong antioxidant activity makes this plant efficient in managing oxidative stress related diseases; this could be the reason why it is used in traditional medicine to manage such diseases where the water extracts are mostly applied.

Earlier studies revealed the antioxidant activity of methanolic bark extract of *A. muricata* with an IC_{50} of (0.22150±0.01652) mg/mL, which is far higher than our current study[12]. Also in another study, the ethanolic bark extracts of *A. muricata* registered the IC_{50} values as 0.109 mg/mL[38]. The difference in antioxidant power in results recorded can be partly attributed to the fact that different parts of the plant were used in the current study and the previous studies attributed to the difference in geographical locations, as both studies were conducted in different areas. The results however agree with Mishra *et al.* who noted that *A. muricata* leaves extracts had antioxidant and molluscicidal properties[8].

The ethanolic extract fractions showed relatively low reducing power less than 50% inhibition even in the fraction showing the highest activity. This suggests that probably the overall antioxidant activity of the extract is as a result of the synergistic combination of the activity of all the compounds in the fractions, especially fractions EEAM5, EEAM8, EEAM9, EEAM10, and EEAM11, which registered activity of higher than 15%. These results provide a possible lead towards the further studies and development of pharmaceutical products with antioxidant properties by targeting the fractions showing the highest activity.

The results for anticancer activity studies showed that the ethanolic leaves extract had a very high anticancer activity on three cell lines of EACC, MDA and SKBR3 with IC_{50} values which are low and very close to each other, despite the difference in the method used and source of the cells.

An integrated part of cancer cell development is the resistance to programmed cell death (apoptosis) and therefore re-establishment of apoptosis in cancer cells is a target mechanism for anticancer agents[39]. Some plant-derived products are known to selectively induce apoptosis in cancer cells, which represent the ideal property for successful anticancer agents[7,39]. The current study showed the highly effective action of the ethanolic leaves extract of *A. muricata* and can be used in the management and treatment of cancer. This is in line with a study which showed that any extract had anticancer and cytotoxic activity if it had an IC_{50} value less than 1 000 μg/mL after 24 h contact time, and that the smaller the IC_{50} value of a test compound, the more toxic the compound was[40].

The results of the cytotoxicity test on normal spleen cells of the ethanolic leaves extracts of *A. muricata* indicated a very high selectivity of the extracts for cancer cells, as they showed no effect on the normal spleen cells throughout the range of concentrations tested. 100% spleen cell viability was observed at all tested concentrations. The high selectivity of the extract for cancer cells is a very important aspect for its use in treatment of cancer as normal cells would not be targeted.

The current study confirms earlier studies which showed that extracts of *A. muricata* had been reported to be selectively toxic *in vitro* to certain types of tumour cells including: lung carcinoma cell lines, human breast solid tumour lines, prostate adenocarcinoma, pancreatic carcinoma cell lines, colon adenocarcinoma cell lines, mammary adenocarcinoma cell lines, liver cancer cell lines, human lymphoma cell lines and multi-drug resistant human breast adenocarcinoma[20]. Other earlier studies also demonstrated it to be selectively toxic against various types of the cancerous cells without harming healthy cells[23,41,42].

The water extracts however showed no effect throughout the range of tested concentrations. This conspicuous lack of anticancer activity of the water leaves extract despite its having a high antioxidant activity and reducing power compared to the ethanolic extract may elicit a number of theories pertaining the mechanism of action of the anticancer agents in this plant which may be different from the commonly generalized idea that anticancer activity is directly related to antioxidant activity. Our results are in line with earlier preliminary studies which showed a good relationship between antioxidant efficacy of plant extracts and anticancer potency. All of the extracts which gave high anticancer potency have high antioxidant activity while the opposite trend is not[13].

In this case, we propose that the anticancer agents present in the ethanolic leaves extracts may be acting in a very different mechanism from that of the antioxidant mechanism. These compounds related to the anticancer activity may also be absent from the water extract and not easily detected by the common phytochemical screening methods. Earlier studies showed that *A. muricata* contained many active compounds and chemicals which were the natural phytochemicals known as annonaceous acetogenins[22,43], yet there are no readily available methods for identifying them. Some of these may have been present in very high quantities

in the ethanolic extract, yet absent in the water extract, leading to the difference in anticancer activity. However, more studies need to be conducted to elucidate the root cause of this difference.

The anticancer activity of the ethanolic leaves extracts fractions showed the highest single activity to be caused by the EEAM10 fraction at a cytotoxic level of more than 80% cell death. Generally, four fractions showed very good promising anticancer activity with cytotoxicity levels of more than 50% cell death, and these fractions were EEAM8, EEAM9, EEAM10 and EEAM11. These fractions may be responsible for the highest anticancer activity of ethanolic extract. These compounds may be not present in water extract, specifying that the fractions were in medium in polarity (nature of mobile phase). The encouraging results obtained from this work on anticancer activity of ethanolic leaves extracts of *A. muricata* and isolation of the most active fractions represent an important step towards the effective purification, characterization of the active principles in this extract and to understand the mechanism of cytotoxicity of these extracts. This study showed *A. muricata* was a promising new antioxidant and anticancer agent.

The *in vitro* antioxidant activity of ethanolic and water leaves extracts of *A. muricata* revealed a significant antioxidant activity in water extract and thus its potential use in oxidative stress related diseases management. Our study has also proved that ethanolic leaves extracts of *A. muricata* have a direct potential inhibitory action on three cell lines (EACC, MDA and SKBR3).

Hence, it is anticipated that *A. muricata* would be a useful pharmaceutical material to treat breast cancer. Four TLC fractions have anticancer activity more than 50%. There is also hope that this plant would be equally cytotoxic on other types of cancers, however, further studies have to be extended for other cell lines and the molecular levels are required to identify specific mechanism that could induce growth inhibition. Our results also suggest that inclusion of antioxidant and anticancer –rich extract or fractions of *A. muricata* as a dietary supplementary has beneficial effects for human health. The data of the current work appear useful for further research aiming to chemically identify the specific compounds responsible for the antioxidant and anticancer activities of *A. muricata*.

Conflict of interest statement

We declare that we have no conflict of interest.

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