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, anti-oxidant and anti-cancer activities of ethanolic and water leaves extracts of Annona 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 DPPH and reducing power methods whereas the in vitro anticancer activity was determined using three different cell lines. Results: Phytochemical screening of the extracts revealed them to be rich in secondary class metabolite compounds of 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 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 IC50 was 2.0456mg/ml and 0.9077mg/ml for ethanolic and water leaves extracts of Annona muricata respectively. The ethanolic leaves extract was found to be selectively cytotoxic in vitro to tumor cell lines (EACC, MDA and SKBR3) with IC50 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 Annona muricata had no anticancer effect at all tested concentrations. Conclusion: The results showed that Annona muricata is a promising new antioxidant and anticancer agent.

1. Introduction

Among the major causes of mortality and morbidity globally is cancer. According to recent estimates by the WHO[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[8]. The current available methods of treatment all induce significant side effects and therefore the need for alternate adjuvant therapies has arisen[9]. 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[10, 11]. Many synthetic drugs cause severe side effects that were not acceptable except as treatments of last resort for terminal diseases such as cancer and that the metabolites discovered in medicinal plants may avoid the side effect of synthetic drugs[13].

Antioxidants are a group of substances that are useful for fighting cancer and other processes that potentially lead to diseases such as atherosclerosis, Alzheimer’s, Parkinson’s, diabetes, and heart disease[13, 14, 15]. 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[14, 15, 16] 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[14, 15, 16].

Plant use in treating diseases is as old as civilization[8, 17] and traditional medicines are still a major part of habitual treatments of different maladies[8, 18]. 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[8]. Annona muricata is widely used in the traditional treatment of cancer in many countries. Annona muricata commonly known as graviola or soursop belongs to the family of Annonaceae[19, 20] and is the most tropical semi deciduous tree with the largest fruits of the Annona genus. 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, molluscidal, antiviral[22, 23] and most importantly, their anticancer properties[22, 24, 25].

Ancient herbal medicines may have some advantages over single purified chemicals[26, 27]. Often the different components in a herb have synergistic activities or buffer toxic effects. This study therefore aimed to determine the phytochemical composition, anti–oxidant activity as well as determining the in vitro anti–cancer potential of ethanolic and water leaves extracts of Annona 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 Annona 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 was identified and authenticated in the Makerere University Botanical Herbarium (MHU) 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 [28]](image)

2.2 Samples preparation and Extraction

The leaves of Annona muricata were washed with water and cut into small pieces, drying was done at room temperature, and the dried leaves were powdered. Equal amounts (350grams) of powdered leaves were extracted using ethanol and distilled water for three days by the plant tissue homogenization method as previously described[29]. The extracts were then concentrated using rotary evaporator and dry block heater respectively and kept at –20oC 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 (NCI) 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[29]. Samples of the ethanolic and water extracts of Annona 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 present

Following the identification of the different phytochemicals present in both ethanolic and water leaves extracts of Annona muricata, the relative abundance of the phytochemicals in each of the extracts was determined[30]. 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. Ho: The concentration of the phytochemical in the sample is neither high nor low, thus 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 Annona muricata was determined[31]. 20µl of the extract was taken from each of the extract and added to 1580µl of distilled water. This was followed by adding of 100µl of Folin reagent (1%) and left to stand for 2 minutes. To each of the samples was then added 300µl of Na2CO3 (7.5%), mixed thoroughly and left to stand for 2 hours 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 Annona muricata were determined[32]. Gallic acid was used as standard. 200µl of each of the samples per extract as well as the standard at different concentrations were taken separately and mixed with 500µl of 0.2M phosphate buffer (pH 6.6) and 500µl of Potassium ferricyanide. The samples were then incubated at 50°C for 20 min. Then 500µl of 10% Trichloroacetic acid were added and centrifuged at 6500 rpm for 16 min, 700µl of supernatant were added to 700µl distilled water, 140µl of freshly prepared ferric chloride, and left to stand for 10 minutes. Finally the absorbance was measured at 700nm. 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 DPPH method (2, 2-diphenyl-2-picyrylhydrazyl).

The free radical scavenging activity (RSA) was preceded. Different concentrations of the extracts (0, 250, 500, 750, 1000, and 1250 µg/ml) were used. 2.5ml of 0.04% DPPH solution (0.04%) was mixed with 0.5ml of all the concentrations of both extracts separately. After 30 minutes incubation at room temperature in the dark, the absorbance was read at 517 nm, in triplicates for each concentration[33]. Butylated Hydroxytoluene (BHT) and Butylated Hydroxylanisole (BHA) were used as positive control. The percent inhibition of free radical formation was calculated as follows: Radical Scavenging Activity (% E) = [(Acontrol - Asample) / Acontrol] x 100.

2.10 TLC Fractionation of the ethanolic leaves extracts of Annona muricata

The ethanolic leaves extracts of Annona muricata was fractionated using thin layer chromatography (TLC) technique. The extract was applied on silica gel 60 F254 TLC aluminum sheets (20 x20) (Merek, Darmstadt, Germany) at one of extremes to separate the different fractions. Mobile phase was Petroleum ether: Ethyl acetate: Glacial acetic acid (4:1:1), 11 fractions were scratched and named as EEAM1b – EEAM11. All The fractions were tested for anti-oxidant activity, reducing power and anti–cancer activity.

2.11 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. Two ml of media containing EACC (2 x104 cells) were transferred into a set of tubes each, then Different concentrations of the extracts both water and ethanolic (0, 250, 500, 750, 1000, and 1250µg/ml) were added. The tubes were incubated at 37°C in the presence of 5% (v/v) CO2 for 2 hours.[34]. For each examined materials (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 (IC50) was calculated from the graph plotting inhibition percentages against logarithm of concentration after transforming the concentrations.

2.12 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[35]. The cell count was adjusted to 1x10^5 cells/0.1 ml and plated in 100µl 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 72h at 37°C in triplicates per concentration (750, 500, 250, and 0µg /ml). The sample solutions were then removed and washed with PBS (pH 7.4). 20µl/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 hours. 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 (IC50) was determined graphically.

% Cell death was calculated using the following formula: % Cell death = (Control OD -Sample OD)/Control OD x 100.

2.13 Cytotoxicity effect of ethanolic leaves extracts of Annona muricata on normal spleen cells

Spleen cells were isolated from normal healthy mice[36]. Spleen cells viability after and before incubation with different concentrations of ethanolic at 37°C in the presence of 5% (v/v) CO2 for 2hours was calculated using trypan blue technique[34]. All concentrations were assayed in triplicates.

2.14 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 χ² goodness of fit test.

3. Results

3.1 Phytochemical analysis of ethanolic and water leaves extracts of Annona muricata

All phytochemicals tested were present in both types of Annona muricata leaves extracts. The χ² goodness of fit test has been used to allocate the relative abundance of each of the phytochemicals. Phytochemicals with computed χ² values (blue region) higher than the χ²critical (red area)were designated as high or low, depending on the initial count while those that had χ² values less than the χ²critical were assigned average abundance as shown in figures 3 and 4 above.

Figure 3, Phytochemicals present in Ethanol leaves extracts of Annona muricata with relative abundance computed from the χ² Test

Figure 4, Phytochemicals present in water leaf extracts of Annona muricata with relative abundance computed from the χ² 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 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 Annona 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 216.41µg/ml in the water extract and 470.51µg/ml GAE in the ethanolic extract.
extract. It is 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), EEAM11 (0.00). It is 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 15µg/ml GAE, most of them registered a very low value of less than 10µg/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 Annona 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 IC50 values of 0.9077µg/ml and 2.0456µg/ml respectively.

![Figure 5](image)

The anti-oxidant activities of each of the 11 fractions isolated from the ethanolic leaves extracts of Annona muricata by TLC technique was 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%), EEAM11 (15.85%). The results show relatively low antioxidant activity recorded by most of the fractions.

3.5 In vitro Anti–cancer activity of leaves extracts of Annona muricata

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

Figure 6 shows the anticancer activity of ethanolic and water leaves extracts of Annona muricata on EACC. The minimum detectable anticancer activity on EACC cell line was observed in the ethanolic leaves extract of Annona muricata at a concentration of 250µg/ml, with an inhibition of 32.9% Cell death, and reaching a maximum inhibition of 100% cell death at a concentration of 750µg/ml (Figure 10). IC50 of ethanolic extracts was determined to be 335.85µg/ml. On the other hand, however, the water leaves extracts of Annona muricata had no effect across all concentrations tested.

Figure 7 shows the Anticancer activity of ethanolic leaves extracts of Annona muricata on MDA Cell line while figure 8 shows the anticancer activity of ethanolic leaves extracts of Annona 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 750µg/ml for 72hours, 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 extract 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 IC50 of 248.77µg/ml and 202.33µg/ml respectively.
The anticancer activity of the ethanolic leaves extract TLC fractions of Annona muricata was determined. Three fractions EEAM2, EEAM6, and EEAM8 showed no activity. The remaining fractions had some anticancer activity 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%), EEAM11 (64%). The results show 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 above shows a comparison between Antioxidant and anticancer activity of Ethanolic leaves extracts TLC Fractions of Annona muricata. It reveals a general trend in the relationship between the two aspects of the study where all fractions which showed high anticancer activity have high antioxidant activity (as measured by the Reducing power and DPPH Radical scavenging assay), while the opposite trend is not.

4. Discussion

4.1 Phytochemical screening

Phytochemical screening conducted on leaves extracts of Annona 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 in both as shown in the figures 3 and 4. These results are in line with earlier studies that carried out on the ethanolic seeds extract of Annona muricata, and the phytochemical tests showed that ethanol soursop seeds extract contains secondary metabolites compounds group of saponins, alkaloids and triterpenoids, flavonoids, anthraquinones, tannins, and cardiac glycosides, which he noted that they are defense chemical compounds of plants produced in the plant tissue.

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 Annona muricata further reinforces the presence of alkaloid in this species as already outlined by other independent studies[9] showed that among the chemical constituents found in Annona muricata, the alkaloids and essential oils stand out. Cardiac glycosides are molecules used in treatment of heart failure [38], hence the present findings with, finds leaves of extracts 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 Annona 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 Annona muricata in traditional medicine is validated by presence of these phytochemicals of known health benefits and thus the interest in further studies on this species.

4.2 Total Phenolic compounds
The phenolic content of the Annona muricata was determined and all results were expressed as Gallic acid equivalents (GAE). Typical phenolics that possess antioxidant activity have been characterized as phenolic acids and flavonoids\cite{39, 40}. 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\cite{14}. Our results revealed that the water leaves extract having higher total phenolic content as compared to the ethanolic leaves extract of Annona muricata. The higher phenolic content in the water extract would partly contribute to its higher antioxidant activity.

4.3 Reducing power

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 of lipid peroxidation, or chelating of metal ions\cite{32}. 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 anti-oxidant activity of the extracts.

The 11 fractions had a very low reducing power of less than 50\% inhibition even in the fraction showing the highest activity. This suggests that probably the overall anti-oxidant 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.

4.4 Antioxidant activity

Free radical scavenging activity of the leaves extracts was determined. The Data showed that water leaves extracts of Annona muricata had a higher free radical inhibition with an IC50 of 0.9077mg/ml as compared to the ethanolic leaves extract with an IC50 of 2.0456mg/ml (Figure 5). The standard antioxidants BHA and BHT were used as positive controls. This free radical scavenging activity of the leaves extracts were 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 Annona 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 Annona muricata had a stronger antioxidant activity as compared to the ethanolic leaves extract. This is expected as the early tests in this study revealed the total phenolics to be higher in the water leaves extracts two fold to the ethanolic leaves extracts, and phenolics have 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 as to why it is used in traditional medicine to manage such diseases where the water extracts are mostly applied.

Earlier studies\cite{14} revealed the antioxidant activity of methanolic bark extract of Annona muricata with an IC50 of 0.2215±0.01652 mg/ml, which is far higher than our current study. Also in another study, the ethanolic bark extracts of Annona muricata in registered the IC50 values as 0.109mg/ml\cite{41}. 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 as well as being attributed to the difference in geographical locations, as both studies were conducted in different areas. The results however agree with Mishra et al.,\cite{9} who noted that Annona muricata leaves extracts have antioxidant and molluscicidal properties.

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 anti-oxidant 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.

4.5 In vitro Anti-cancer 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 (figures 6, 7, and 8 respectively) with IC50 values which are low and very close to each other, despite the difference in the method used and source of the cells as shown in table 1.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Assay</th>
<th>IC50/ µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>EACC</td>
<td>TBEA</td>
<td>335.85</td>
</tr>
<tr>
<td>MDA</td>
<td>MTT</td>
<td>248.77</td>
</tr>
<tr>
<td>SKBR3</td>
<td>MTT</td>
<td>202.53</td>
</tr>
<tr>
<td>Spleen Cells (Normal)</td>
<td>TBEA</td>
<td>#N/A</td>
</tr>
</tbody>
</table>

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\[^{44}\]. Some plant-derived products are known to selectively induce apoptosis in cancer cells, which represent the ideal property for successful anticancer agents\[^{8, 44}\]. The current study showed the highly effective action of the ethanolic leaves extract of Annona muricata and can be used in the management and treatment of cancer. This is in line with a study which showed that any extract has anticancer and cytotoxic activity if it has an IC\(_50\) value less than 1000 \(\mu\)g/ml after 24 hours contact time, and that the smaller the IC\(_50\) value of a test compound the more toxic compound\[^{46}\].

The results of the cytotoxicity test on normal spleen cells of the ethanolic leaves extracts of Annona muricata indicate 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 (Figure 9). 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 Annona muricata have 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\[^{22}\]. Other earlier studies also demonstrated it to be selectively toxic against various types of the cancerous cells without harming healthy cells\[^{25, 42, 43}\].

The water extracts however showed no effect throughout the range of tested concentrations (Figure 6). 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 as shown in figure 10 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 Annona muricata contains many active compounds and chemicals which are the natural phytochemicals known as annonaceous acetogenins\[^{24, 45}\] yet there are no readily available methods of 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, specific 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 Annona muricata and isolation of the most active fractions represent is 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 Annona muricata to be a promising new antioxidant and anticancer agent.

5. Conclusion

The objective of this study was to determine the phytochemical composition, anti-oxidant activity and in vitro anti-cancer potential of ethanolic and water leaves extracts of Annona muricata. Phytochemical screening of leaves extracts revealed it to be rich in secondary class metabolite compounds. The use of Annona muricata in traditional medicine is validated by presence of these phytochemicals of known health benefits and thus the interest in further studies on this species. The in vitro antioxidant activity of ethanolic and water leaves extracts of Annona 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 Annona muricata has a direct potential inhibitory action on three cell lines EACC MDA and SKBR3.

Hence, it is anticipated that Annona 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 level 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 Annona 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 Annona muricata.

**Conflict of interest statement**

We declare that we have no conflict of interest.

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