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ANTI-OXIDANT AND ANTI-CANCER ACTIVITY OF *ANNONAMURICATA* LEAF EXTRACT IN MCF 7 CELL LINE

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ABSTRACT

Cancer has been a constant battle globally with a lot of development in cures and preventative therapies. The disease is characterized by cells in the human body continually multiplying with the inability to be controlled or stopped. When the free radicals are balanced by the body's oxidative defence system, the body is in healthy condition, depletion or loss of antioxidant levels may lead to free radical caused oxidative stress which can cause cellular or tissue damage, DNA damage and cancer. The study is on the Anti-oxidant and Anti-cancer activity of *Annonamuricata*in MCF 7 cell line. The plant has significant antioxidant activity which is attributed to its high flavonoids content. Therefore *A.Muricata* is regarded as excellent source for bioactive compounds that can be further developed into a drug to combat oxidative stress. The antioxidant effect against DPPH, Hydroxyl Radical Scavenging Assay, and FRAP which resulted in IC_{50} values of (80 µl). There is a remarkable anticancer potential observed against cervical cancer cell lines in leaf extract of *A.Muricata.* There is a maximum cell growth inhibition observed in the concentration at 100µl of methanolic extract. The methanolic extract of *A.Muricata* showed 58.47% growth inhibition against breast cancer cells

KEYWORDS: ***Antioxidant, Anti-cancer, Annonamuricata, MCF 7 cell line and cell growth inhibition.***

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INTRODUCTION

Cancer is a complex family of diseases since there are as many tumour types as there are cell types in the human body. Therefore it is a group of heterogeneous diseases that share common biological properties [1].Breast cancer is not a single disease; research evidence continues to indicate that there are a number of subtypes of breast cancer. They happen at varying rates in different groups and respond differently to treatments. Some are more aggressive than others and have very different long-term survival rates. Breast cancer is the most common malignant tumour in women. It ranks second overall (10.9%) of all cancers. Estimates of 2008 indicate that 1.38 million (23% of all cancers) women were diagnosed and 0.46 million died from the disease worldwide. Currently, the incidence in the developed and developing nation is similar, whereas incidence rates are tending to plateau or decline in the west, and are increasing in the developing nation, presumably related to changes in dietary and reproductive behaviours and ageing populations. Total mortality for breast cancer is already higher in the developing nation[2] Antioxidants are radical scavengers which protect the human body against free radical that may cause pathological conditions such as ischaemia, asthma, arthritis, inflammation, neurodegeneration.Parkinson's disease, Mongolism n, aging process and dementia. Though synthetic antioxidants are available their activity is moderate, hence there has been mentioned the antioxidant activity of plants might be due to their phenolic compounds[9]. Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and antioxidant nzymes and anti-inflammatory and action[5].Medicinal plants are the backbone of traditional medicinal systems in developing countries. Even today plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries [1]. From the last few decades it has been the subject of very intense pharmacological studies. It has become a potential source of new compounds of therapeutic value and as a source of new compounds in drug development. The widespread use of herbal remedies and health care has been described in the ancient texts like the Bible and Vedas have been traced to the occurrence of natural products with medicinal properties. Infact, plants produce a wide range of bioactive molecules, making them rich source of different types of medicine[1] . Soursop is the fruit of *Annonamuricata*, a broad leaf, flowering, evergreen tree. The exact origin is unknown; it is native to the tropical regions of the Americas and the Caribbean and is widely propagated. It is in the same genus, *Annona*, as cherimoya and is in the Annonaceae family. The soursop is adapted to areas of high humidity and relatively warm winters; temperatures below 5 °C (41 °F) will cause damage to leaves and small branches, and temperatures below 3 °C (37 °F) can be fatal. The fruit becomes dry and is no longer good for concentrate. with aroma similar to pineapple, the flavor of the fruit has been described as a combination of strawberries and apples, and sour citrusflavor notes, contrasting with an underlying creamy texture reminiscent of coconut or bananas. Soursop is widely promoted as an alternative cancer treatment, but there is no medical evidence it is effective for treating cancer or any disease [1].

MATERIALS AND METHODS

Collection of plant and extract preparation

Thoroughly washed leaf were allowed for shade drying under room temperature in the laboratory. The leaves were ground to find powder using a blender. The powder was preserved in an airtight bottle for further studies. Thoroughly grounded 10 g of the powder which was soaked in 100ml of organic solvent (methanol) in a conical flask, plugged with cotton and kept on a rotary shaker at 190-220 rpm for 24hrs at 40°c. Whatman filter paper No 1 was used for filteration after 24 hours and the supernatant was collected and the solvent was evaporated to make final volume one fourth of the original volume.

Dpph radical scavenging activity

The antioxidant activity of the sample was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of [2]. Silver nanoparticles at various concentrations (20 - 100µL) were taken and 100µL 0.1 mM methanolic solution of DPPH was added and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Percentage radical scavenging activity of the sample
was calculated as follows:

% DPPH radical scavenging activity = (control OD-sample OD / control OD) × 100 the constant used was vitamin C

The analysis was performed in triplicate. The graph of inhibition percentage against sample concentration was calculated under the assay condition and the sample concentration provided 50% inhibition.

Hydroxyl radical scavenging activity

The scavenging activity of the sample on hydroxyl radical was measured according to the method of [7] . Different concentrations of the extract (200 - 100µL) were added with 10µL of 7.5mM ferrous sulphate, 10 µL of 7.5mM phenanthroline, 50µL 0.2M phosphate buffer, and 5µL of hydrogen peroxide. The reaction mixture was allowed to stand for 5min at room temperature. The absorbance of the sample was measured at 536 nm. The % hydroxyl radical scavenging activity was calculated as follows:

% Hydroxyl radical scavenging activity = (control OD-sample OD / control OD) × 100 the constant used was vitamin C

The analysis was performed in triplicate. The graph of inhibition percentage against sample concentration was calculated under the assay condition and the sample concentration provided 50% inhibition.

Ferrous ion chelating activity

The ferrous ion chelating activity assay was used to estimate the reducing capacity of the sample, according to the method of [3]. Different concentrations of Silver nanoparticles (200-100µL) were added with 50µL of 2mM ferrous sulphate, 30 µL of 5mM Ferrozine. The reaction mixture was allowed to stand for 10min at room temperature. The absorbance of the sample was measured at 562nm.The % ferrous ion chelating activity was calculated as follows:

% ferrous ion chelating activity = (control OD-sample OD / control OD) × 100 the constant used was vitamin C

The analysis was performed in triplicate. The graph of inhibition percentage against sample concentration was calculated under the assay condition and the sample concentration provided 50% inhibition.

Anticancer activity

Cell lines and culture

MCF7 (Human breast adenocarcinoma cell line) cell line was cultured in DMEM supplemented with 10% inactivated FBS penicillin (100 IU/ml), streptomycin (100µg/ml) and amphotericin B (5µg/ml) in a humidified atmosphere of 5% CO2 at 37ºc C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures, were grown in 25cm^2 20 culture flask, and all experiments were carried out in 96 microtiter plates (Tarsons India pvt Ltd., Kolkata, India.) [11].

Cell inhibition by MTT Assay

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1mg/ml concentration and sterilized by filtration. Serial two field dilutions were prepared from this carrying out cytotoxic studies. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×105 cells/ml using DMEM containing 10% FBS. The standard drug was added and to each well of the 96 well microtiter plate, and the suspension of Soursop extracts were added at a concentration (12, 25 and 55µg respectively). After 24, hours when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with the medium and 100µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37ºc C for 3days in 5%$CO_2$2atmospheres, and microscopic examination was carried out and observations were noted every 24hrs interval. After 72hrs, the drug solution in the wells were discarded and 50µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3hrs at 37ºCin 5% $CO_2$2 atmosphere. The supernatant was removed, and 150µl of MTT solvent was added and the plates were gently shaken. Read The plate was read under the absorbance of 575nm.

STATISTICAL ANALYSIS

The results were expressed as mean ± standard deviation. Descriptive statistics was used to analyze the mean, standard deviation, variation, and level of statistical significance between groups. When $p < 0.05$ and $p < 0.01$, it was considered statistically significant for analysis of percent inhibition of cell growth.

RESULTS AND DISCUSSION

In the present study, a fresh leaf of Annonamuricata were collected from the Nilgiris and was washed under running tap water and shade dried for a week. The dried plant samples were homogenized to a fine powder and further subjected to extraction. Thoroughly washed leaf was allowed for shade drying under room temperature in the laboratory. The leaves were ground to a fine powder using a blender. The powder was preserved in an airtight bottle for further studies.10 grams of thoroughly grounded powder were then soaked in 100ml of organic solvent (methanol) in a conical flask, plugged with cotton and kept on a rotary shaker at 190-220 rpm for 24hrs at 40°c. After 24hrs, it was filtered through Whatman filter paper No 1 and the supernatant was collected and the solvent was evaporated to make final volume one-fourth of the original volume.

Antioxidant activity

Dpph radical scavenging activity

DPPH is one of the free radicals widely used for testing preliminary radical scavenging activity of the plant extract. Scavenging of DPPH radical is related to the inhibition of lipid peroxidation. DPPH is usually used as a substance to evaluate antioxidant activity. Antioxidants either transfer an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character. DPPH test, which is based on the ability of DPPH, a stable free radical, to decolourize in the presence of antioxidants, is a direct and reliable method for determining radical scavenging action. The reducing capacity of compounds could serve as an indicator of potential antioxidant property [12].In the present study, the percentage of scavenging effect on DPPH radical was concomitantly increased with the increase in the concentration of leaf methanolic extracts from 10 to 50µg/ml. The decolourization from purple DPPH radical by the sample in a dose-dependent manner with an IC50 value of 75.47/80 µg/ml indicated the sample's high radical scavenging activity as shown in Fig 1. From the results, it is known that the species, *Annonamuricata* possess hydrogen donating capabilities for methanolic leaf extract and does scavenging free radicals. Furthermore, it was noticed that the leaf extract has more pronounced scavenging activity.

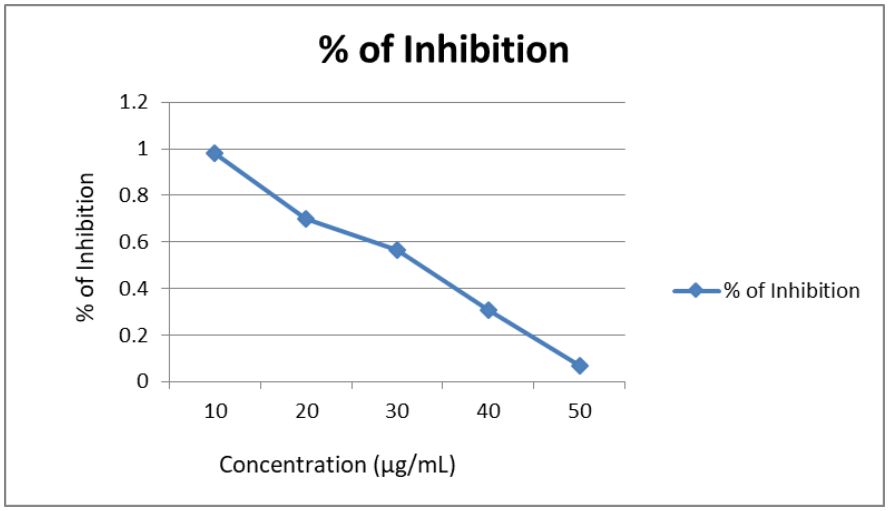

Figure 1
DPPH radical scavenging activity of Annonamuricata

Hydroxyl radical scavenging activity

Hydroxyl radical can be formed by the Fenton reaction in the presence of reduced transition metals (such as Fe^{2+}) and H_2O_2, which is known to be the most reactive of all the reduced forms of dioxygen and is thought to initiate cell damage *in vivo*. Scavenging of hydroxyl radical is an important antioxidant activity because of the very high reactivity of the OH radical, enabling it to react with a wide range of molecules found in living cells, such as sugars, amino acids, lipids, and nucleotides [13]. Thus, removing $OH^{\bullet}$ is very important for the protection of living systems. At a high concentration of 100µg/ml showed 57% hydroxyl radical scavenging activity. The IC_{50} value of 87.7/100 µg/ml indicated the sample's high radical scavenging activity is shown in Fig. 2. Each extract showing hydroxyl radical scavenging activity was increased with increasing concentration of sample extracts.

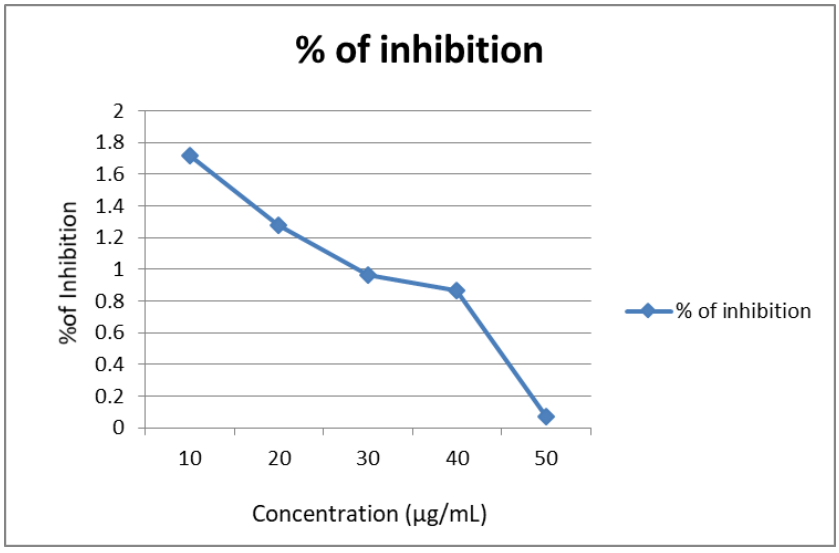

Figure 2
Hydroxyl radical scavenging activity Annonamuricata

Ferrous ion radical scavenging activity

Iron is essential for life because it is required for oxygen transport, respiration and activity of many enzymes. However, it is an extremely reactive metal and catalyzes oxidative changes in lipids, proteins and other cellular components. The metal chelating ability of the methanolic leaf extracts was measured by the formation of ferrous ion ferrozine complex. Ferrozine combines with ferrous ions forming a red coloured complex which absorbs at 562 nm (Yamaguchi *et al.,* 2000). It was reported that the chelating agents which form σ bond with metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion. The scavenging capacity of *A.muricata* was shown at a high concentration of 100 µg/ml showed 50% ferrous ion radical scavenging activity. The IC50 value of 54.10/50 µg/ml indicated the sample's high radical scavenging activity as shown in Fig 3.

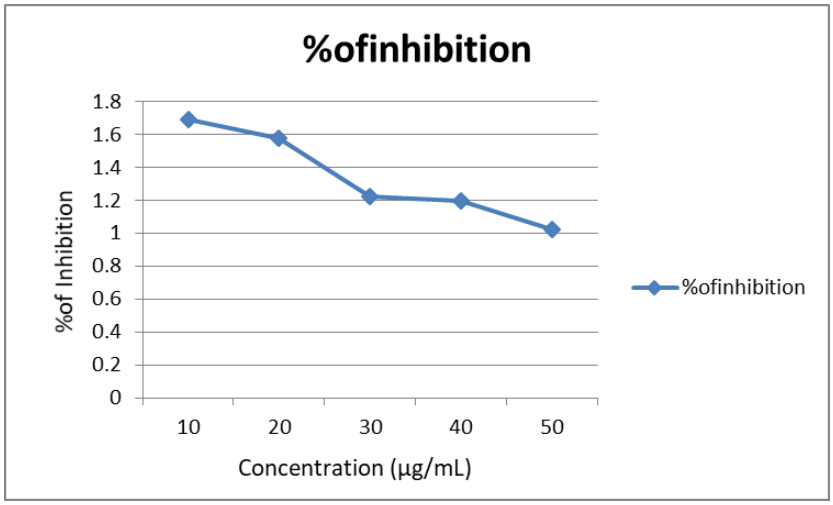

Figure 3
Ferrous ion radical scavenging activity *Annonamuricata*

Cell inhibition activity by MTT assay

Interest in the pharmacological effects of bioactive compounds on cancer treatments and prevention has increased dramatically over the past twenty years. It has been shown to possess numerous anti-cancer activities in various cancer cells through different forms of cytotoxic effects without exhibiting considerable damage to normal cells. Breast cancer is the second leading cause of cancer-related deaths in women. Current anti-estrogen medicine, tamoxifen, is widely used in the prevention and treatment of estrogen receptor-positive breast cancer. However, a significant number of patients develop tamoxifen resistance and experience severe side effects [8]. Thus, it is imperative to search for new alternatives to breast cancer prevention agents.Medicinal plants are able to act through several mechanisms to provide protection against cancer.[9] The percentage cytotoxicity of MCF-7 cells exposed to the polysaccharide to wide concentration at 12, 25, 55µg/ml was found to be certain concentrations respectively. Dose-dependent anti-proliferative effect on the cell viability of MCF-7 was observed as shown in fig 4 and 5. The results have been summarized in the table 1. inhibitory concentration 50 (IC_{50})(55) value indicated that the maximum cytotoxic effect of polysaccharide isolated fractions of *A.muricata* showed 50% reduction in cell viability upon treatment with a higher dose (1 mg/ml).The results of the present study have demonstrated that a methanol extract of the Soursop leaves arrested growth of proliferating cells from 2 breast cancer cell lines, which was associated with induction of apoptosis as measured by cell cycle profiling, and induction 21 of caspase activity. This cytotoxic apoptotic response was not dependent on the estrogen receptor, as similar results were obtained in cells possessing (MCF7) and lacking (MDA-MB- 231) this receptor. Flow cytometric analysis demonstrated that Soursop methanol extract caused an increase in the proportion of cells in a sub G1 phase, which reflects the induction of apoptosis. The methanol extract strongly inhibited the proliferation of MCF-7, MDA-MB- 231 and HEK-293 cell lines with IC50 values of 12, 25, 55µg/ml respectively. The extract showed an increase in the apoptotic cell cycle in the G1/S phase in MCF7 and G0/G1 phase in MDAMB- 231 cells. There are also increase in the activity of caspase-2,-3,-6,-8 and -9 activity by extract and Soursop extract was also found to decrease the intracellular ROS generation and mitochondrial membrane potential (Horiuchi, 1988). The methanolic leaf extract of *A.muricata* was tested for their anticancer potential against MCF 7 breast cancer cell line. It was found that the methanolic extract possessed anticancer activity. The absorbance value 58.47% at the concentration of 55µl as shown in table 1.

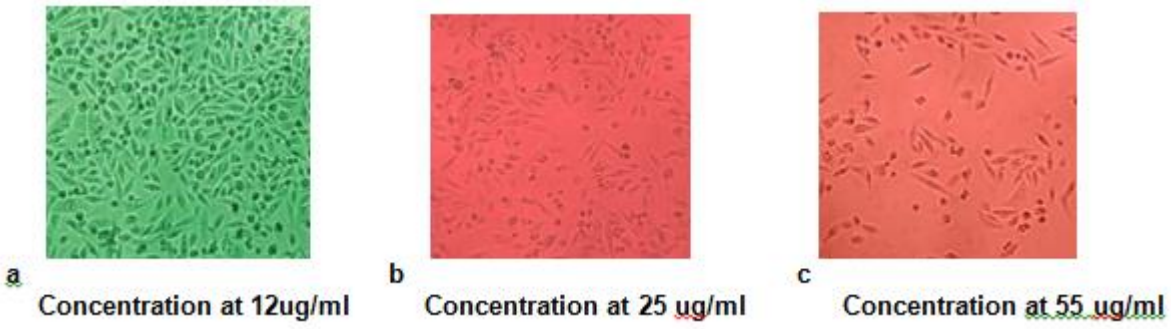

Figure 4
Cell inhibition activity by MTT assay of the leaf extract of Annonamuricata

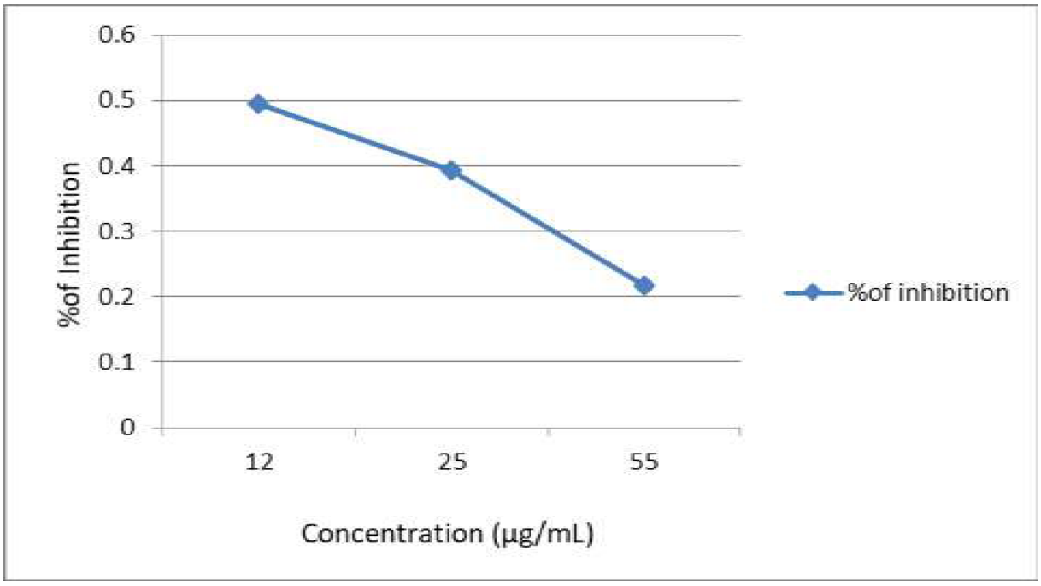

Figure 5
Cytotoxicity induction

Table 1
Cytotoxicity induction

Sample concentration (µg/mL)	OD value at 570nm	% of inhibition
Control	0.525±0.003	
12	0.494± 0.04	5.90%
25	0.393±0.02	25.14%
55	0.218±0.03	58.47%

Values are mean ±SD; (n=3)
****p< 0.01 when compared with control***

CONCLUSION

Using methanol 50% of the *A.Muricata* leaf extract was prepared. . The plant has significant antioxidant activity which is attributed to its high flavonoids content. Therefore *A.Muricata*is regarded as excellent source for a bioactive compound that can be further developed into a drug to combat oxidative stress. The antioxidant effect against DPPH, Hydroxyl Radical Scavenging Assay, and FRAP which resulted in IC50 values of (80µl). The *A.Muricata*leaf extract was found more toxic for cancer cells than normal cell line. In this regards,' therapeutic index' is an important parameter to select samples for developing drugs. This value is the ratio of the concentration of the extract at which 50% of the normal cell line survived. to that of the extract at which 50% of cancer cell death occurred in cancer cell lines. A drug is considered to be worthy of further testing if it has a therapeutic index value of 16 or greater. The present study found a therapeutic index value of 16 in MCF-7 cases of cancer lines for the *A.Muricata*leaf extract. These species are promising for its further development as an anticancer drug. Conclusion of our study states that the antioxidant potential and anticancer studies of *A.muricata* showed better activity in methanolic extract.

CONFLICT OF INTEREST

Conflict of interest declared none.

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