

Screening of Anticancer and Anti-Oxidant Activity of Leaves of *Annona Reticulata* on Eac Induced Mammary Tumor

Kashikant Yadav^{1*}, Nagarathna P.K.M², Diana Mioria Martin Lou³,
Gyamcho Bhutia⁴

Department of Pharmacology, Karnataka College of Pharmacy #33/2, Thirumena Halli,

*Corresponding author: Kashikant Yadav

Abstract: *Annona reticulata* Linn is a highly versatile plant in medicine for the treatment of various diseases. The plant has many activities like anti-helminthic, analgesic, anti-inflammatory, anti-pyretic, wound healing, anti-tumor and abortifacient properties.

The present review article gives an overview on anticancer and antioxidant activity of leaves of *Annona reticulata* on EAC induce mammary tumor.

The methanolic extracts of *Annona reticulata* is used against Ehrlich ascites carcinoma (EAC) in mice. Tumor cells (2×10^6 cells/mice) are injected into the hind limb of mice subcutaneously and tumor is allowed to develop. The effect of methanolic extracts of *Annona reticulata* is checked on the growth of tumor.

Keywords: *Annona reticulata*, anti-tumor, antioxidant, EAC.

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I. INTRODUCTION

Cancer is the uncontrolled growth of the cell which is considered as the second life threatening disease after the cardiovascular diseases which is characterized by the uncontrolled growth of the cells, affects the local tissue and may spread to the other site which is called as the metastasis. Cancer causes more than 6 million death per year and it may reach up to the 11.5 millions in the coming years by 2030 in the world [1]. Since, the cancer affect the cells so, it is called as the cellular tumor that unlike benign tumor cells which can metastasize and invade to the neighboring and also to the distant tissues. In the cancer there is abnormal growth of the cells in our bodies so, it can lead to the death. Cancer cells are born due to the imbalance between the division and death of the cell in the body and it can be treated by the correcting this imbalance growth of the cells [2]. The number of cancer patient in our India is estimated between 7 to 9 lakhs. Cancers are of many types but all of they start because of abnormal growth of cells. In place of dying, the cancer cells continue to grow and form new one, abnormal cells. The cells become cancerous cells because it made the damage to the DNA. When in the normal cell, DNA gets damaged, the cell either repairs the damage cell or the cells die but in the cancer cells, the damaged DNA is not dying. This cell goes on making the new cells in the body which we do not need. These new cells will cause the same type of damage to the DNA as the first cell [3]. Cancer cells affect the all age group people, even the fetus but the risk is increases with the age. It causes 13% death of the all human beings. According to the American Cancer Society, around 7.6 million people dies every year from the Cancer [4]. The medicinal plants, due to their enormous propensity which synthesize a variety of structurally diverse bioactive compounds which can be used for the treatment of various types of diseases. The medicinal plant kingdom is a potential source of various chemical constituents that have antitumor and cytotoxic activities. Due to the diverse plant sources of India which can provide effective anticancer agents. Antioxidant agent which quench the free radicals so, act as the cancer chemo-preventive agents [5]. The phytochemicals which are obtained from the plant such as terpenoids, flavonoids, alkaloids, glycosides and phenolics exhibits the meticulous effects on the body physiology which have the desirable effects. In the comparison to the synthetic chemotherapeutic agents, plant based medicine are found to be safer due to the less toxicity, side effect and wide margin of error. The need of the present time is to develop the new anticancer agent with minimum or no side effects [6].

Annona reticulata Linn. (Custard apple, Bullock's heart, Ramphal) is traditionally very important medicinal plant for the treatment of various diseases. This plant belongs to the family Annonaceae. The synonyms of the plant are *Annona excels* kunth, *A. custescens* and *A. laevis* kunth [7]. The different phytoconstituents have been identified from the different part of this plant. The bark of stem which contains alkaloid, tannins and phenolic compounds. Similarly, the leaves of this plant contain wide range of chemicals like amino acids, carbohydrates, alkaloids, steroids, proteins, flavonoids, tannins, phenolics and glycosides.

Even the root has been identified to content of alkaloid, flavonoids proteins and tannins. The plant is rich in different types of minerals such as P, Ca, Na, Mg, S, Cl, Mn, Cu, Fe, Co, Se, Ni, and Cr [8,9,10]. One of the biologically important polyphenol constituents, (+)-catechin which was isolated from the fruit of *Annona reticulata* Linn. This isolated (+)-catechin was characterized by the IR, UV, H-NMR13, C-NMR and it was found that it has antioxidant and anticancer activities [11].

1.1 Plant Profile

Scientific classification

Domain: Eukaryota

Kingdom: Plantae

Class: Angiosperms

Division: Magnolids

Order: Magnoliales

Family: Annonaceae

Genus: *Annona*

Species: *Reticulata*

Botanical name: *Annona reticulata*

Synonyms: Shubha, Sitaphala

Common names: Custerd apple

Vernacular names

Hindi: Sitaphal, Sharifa

Assamese: Atlas, Ata

Gujrat: Sitaphal

Punjab: Sharifa

Oriya: Ato [12].

1.2 Geographical distribution of plant

A. reticulata is widely distributed plant which is found in tropical and subtropical regions of the world [13]. This plant is indigenous to the West Indies. In India this plant is widely cultivated and naturalized for its fruit consuming plant and deciduous tree. It is distributed in Burma, Bengal and Southern regions of India. The plant is native to tropical regions of America, mostly in West Indies and South America. The plant is widely cultivated in Bangladesh, Pakistan and other part of the world [14,15,16].

1.2 Morphology of plant

The height of *Annona reticulata* plant is about 6.0-7.5m. It has various numerous lateral type of branch. It is small tree which has glabrous branches. The stem of the plant are cylindrical which is having lenticels and very small coffee colored hairs[9]. The leaves of plant are lanceolate, oblong, membranous, ante and curate or rounded at the base. The upper surface of the leaves is glabrous while on the lower surface it has few spreading hairs. There may be two to four flowers present on the lateral pedicel side. The fruit of this plant is edible which heart shaped, rough to touch and yellow in color which finally changes to yellowish red on ripening. The seeds of plant are smooth and black in color [17]. The fruits have sweet and astringent properties so, it is useful in blood complaints [18].

1.3 Phytoconstituents

Various chemical constituents have been identified from the different parts of *Annona reticulata*. Leaves have found to contain wide range of chemicals like alkaloids, carbohydrates, amino acids, flavonoids, steroids, tannins, proteins, phenolics and glycosides. The stem contains alkaloid, tannins and phenolic compounds. Also, roots contain acetogenin, carbohydrate, alkaloid, flavonoids, proteins, tannins. The plant is found to be rich in various minerals like K, Ca, Mg, P, Cl, Na, , Zn, S, Mn, Se, Ni, Cu, Cr and Fe[14,19,20].

1.4 Anti-proliferative activity

The antiproliferative activity of aporphine alkaloids liriodenine, reticuline, norushinsunine and one acetogenin neoannoninin which was isolated from the roots of *Annona reticulata* was investigated against A-549, HeLa, K-562, MDA-MB cancer cell lines and also the normal cell lines(vero cells) by MTT assay. The chemical compounds were identified structurally by ¹HNMR, ¹³CNMR and also mass spectroscopy methods. From the ethanolic extract of roots aporphine alkaloids were obtained by the column chromatography using toluene: ethyl acetate: diethyl amine (70:20:10) as solvent system. Acetogenin which was isolated by partitioning of ethanol extract with ethyl acetate and column chromatography using n-hexane, ethyl acetate and

methanol as solvent system. By using the 100 μ l of isolated compounds, the activity was carried out and each at the concentration 5, 10 and 20 μ g respectively.

The untreated micro titre plates of cell lines containing DMSO (0.3 % v/v in water) were considered as proliferative control. Neoannonin showed potent cytotoxicity (IC₅₀ value from 5.8 to 6.9mg/ml) against all cancer cell lines where as norushinsunine exhibited moderate cytotoxicity (IC₅₀ value from 7.4 to 8.8mg/ml). Test compound showed less cytotoxicity (IC₅₀ value from 13.8 to 26.0mg/ml) on normal cell line (Vero cells) as compared to cancer cell lines. The study concluded that prominent cytotoxicity of isolated aporphine alkaloids is because of isoquinoline moiety, presence of hydroxyl group and apoptosis inducing ability of these isolated compounds in cancer cell lines [21,22].

1.5 Antioxidant activity

The extract obtained from the roots of *Annona reticulata* investigated for the antioxidant activity. For the antioxidant screening and DPPH free radical scavenging and hydrogen peroxide assay was performed. The DPPH free radical scavenging assay and hydrogen peroxide assay for the antioxidant activity was determined at 20, 40, 60, 80 and 100 μ g/ml concentration of extract and absorbance was measured at 517nm and 230nm respectively[23].

II. MATERIALS AND METHODS

2.1 Collection and authentication of plant material

The leaves of *Annona reticulata* was identified and authenticated by Dr. Gachande B.D., Botanist, Associate Professor of Botany department, N.E.S. Sciences college, Nanded, India[6].

2.2 Extraction and Isolation

Collected *Annona reticulata* Linn. Leaves was converted to small pieces and dried under the shade at room temperature. Powder of dried leaves (200g) were prepared using grinder. Then, the methanolic extract was obtained by Soxhlet extractor using 1 L methanol for 8 h at 64⁰c and sample was concentrated with the help of rotary evaporator [6].

2.3 Drugs and Chemical

All the drugs & chemicals are of pure analytical grade was obtained from the Rankem (India)

2.4 Acute oral toxicity

The acute oral toxicity study was performed by using up and down procedure (OECD/OCDE GUIDELINE) 425 Adopted on 17th December 2001[24]. It was found to be that a dose of 2000mg/kg did not show any effect on respiratory rate, salivation, heart rate, body temperature corneal reflex, locomotors activity, abdominal tone, tremors, piloerection, tail elevation, body tone, skin tone, grip strength twitches, and convulsions was also not observed. No any mortality was observed after 14 days of observation period in tested mice [25].

2.5 Animals

Healthy male adult Swiss Albino mice (16-25gm) were used for the study. The animals were housed in microloan boxes in a controlled environment (temperature 25 $\pm$ 2⁰c) and 12 hr dark/light cycle) with standard laboratory diet (Sai Durga feeds and foods, Bangalore) and water and libitum. All the mice were segregated based on their gender and quarantined for 15 days before the commencement of the experiment then they were fed on healthy diet and maintained in hygienic environment in our animal house [26].

2.6 Tumor cells

Ehrlich Ascites Carcinoma (EAC) cells were obtained from Amala cancer Research Institute, Thrissur, kerala India. The freshly drawn ascetic fluid is diluted in phosphate buffer solution pH (6.8) and aliquot of (2 x 10⁶ cells 0.25 mL) of the diluted solution was injected subcutaneously inoculation to mice belonging to age group of 5 to 6 weeks and weight (20 to 25 gms).

2.7 Experimental protocol

The cancer animal's randomly divided into 5 groups of 6 mice each.

Group I - vehicle control (normal saline 5ml/kg/po)

Group II- EAC control (0.1ml/10gm BW, SC containing 2 $\times$ 10⁶ cells/mice)

Group III- EAC (0.1ml/10gm BW, SC containing 2 $\times$ 10⁶ cells/mice) + 5 Fluorouracil (20mg/kg IP for 14 days)

Group IV – EAC (0.1ml/10gm BW, SC containing 2 $\times$ 10⁶ cells/mice) + Methanolic extract of *Annona*

reticulata (100mg/kg IP for 14 days)

Group V- EAC (0.1ml/10gm BW, SC containing 2×10^6 cells/mice) + Methanolic extract of Annona reticulata(200mg/kg IP for 14 days)

The test and standard drugs (5 fluorouracil) were suspended in water for injection solution as vehicle and administered IP for 14 hours after the inoculation of EAC. After administration of last dose, 3 mice from each group were kept fasting for 18 hours and sacrificed for the study of antitumor activity, hematological parameters, biochemical estimations and liver antioxidant studies. The remaining animals in each of the group were kept aside to determine the median survival time (MST) and the body weight analysis of the tumor bearing mice [27].

2.8 Effect on survival time

Animal was inoculated with EAC cells (2×10^6 cells/ mouse) on day '0' and the median survival time (MST) of each group, consisting of 6 mice was noted [28].

$$MST = \frac{(\text{Day of first death} + \text{Day of last death})}{2}$$

2.9 Percentage increase life span (%ILS)

The effect of the drugs on tumor growth was monitored by recording the mortality daily for a period of 6 weeks and percentage in life span (%ILS) was calculated [29].

$$\%ILS = [(T-C)/T] \times 100$$

Where, T = number of days the treated animal survived.

C = number of days the control animal survived.

2.10 Body weight

Body weights of the experimental mice was recorded both in treated and control group at the beginning of the experiment (day 0) and sequentially on every 5th day during the treatment period [2].

2.11 Solid tumor volume

Tumor mass was measured from 15th day of tumor induction. The measurement was carried out every 5th day for a period of 30 days. The volume of tumor mass was calculated by using the formula [30].

$V = 4/3 (\pi r^2)$, where 'r' is the mean of 'r₁' and 'r₂' which are the two independent radii of the tumor mass.

2.12 Effect on hematological parameters

At the end of the experimental period, 6 mice of each group was sacrificed, the next day after an overnight fast by cervical dislocation. Blood was collected by Retro-orbital route and used for the estimation Hemoglobin (Hb%) content, red blood cell count (RBC) [31] and white blood cell count (WBC) [32].

2.13 Histopathological studies

A portion of Liver and Kidney of animals in all groups was stored in container for 12 h in 10% formalin solution and subjected to histopathological studies [33].

III. IN-VITRO ANTI OXIDANT STUDIES

3.1 Hydroxyl Radical Scavenging activity

About 60µl of ferrous chloride (1mM), was added to 90µl of 1,10-henanthroline (1mM). About 2.4ml of phosphate buffer saline (0.2 M, pH 7.4) was added to the mixture, followed by the addition of 150µl of hydrogen peroxide (0.17 M) and 1.5 ml of different concentrations of the extracts (10µg/ml - 100µg/ml). The mixture was incubated for 5 min at room temperature then all tests were performed in triplicate. The absorbance of the mixture was observed at 560 nm in a Double beam UV-visible Spectrophotometer (SYSTRONICS 2201) against blank (distilled water) [34].

3.2 Nitric oxide radical scavenging

Sodium nitroprusside 5 mM were prepared in phosphate buffer having pH 7.4. To 1 ml of various concentrations of test compound, sodium nitroprusside 0.3 ml was added. The test tubes were incubated at 25 °C for 5 hrs after which, 0.5 ml of Griess reagent was added. The absorbance of the chromophore was observed at 546 nm. The experiment was performed in triplicate (Sreejayan, 1996) [35].

3.3 Superoxide scavenging

Alkaline DMSO was used as a super oxide generating system. To 0.5 ml of different concentrations of the test compound, 1 ml of alkaline DMSO and 0.2 ml of NBT 20mM in phosphate buffer pH 7.4 was added. The experiment was performed in triplicate (Govindarajan, 2003) [36].

3.4 DPPH-radical scavenging activity

The DPPH assay is based on the measurement of the scavenging ability of an antioxidant using the stable DPPH free radical. The free radical DPPH is purple in color in ethanol and is reduced to the corresponding hydrazine, which is yellow in color, when it reacts with a hydrogen donor. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol and the decrease in absorbance is measured at 490 nm.

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0 \times 100]$$

Where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the extract [37].

IV. IN-VITRO STUDIES

4.1 CELL LINES

The cell viability, cell growth as well as cell cytotoxicity of Ehrlich Ascites Carcinoma cell lines was determined by performing the following assays

4.2 MTT ASSAY

Cell proliferation activity of the drugs was carried out by MTT assay which estimate the effect of the various drugs on the growth of cell in vitro. Measurement of the cell in viability and proliferation forms should be used as basis for this in vitro assay. The reduction of terazolium salt now widely accepted to examine cell proliferation, the yellow colored terazolium, MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide)], was reduce metabolically active cell in part by the action of dehydrogenase enzymes which generate the reducing equivalents such as NADH and NADPH. The resulting intracellular purple color zones were solubilised and quantify by spectrophotometric method. When metabolic event leads to necrosis or apoptosis in cell, the MTT method was measure the cell viability. The assay gives low background absorbance values in the absence of necrosis of the cell. MTT should be dissolved in PBS at a concentration of 5mg/ml then, 50 μ g of the MTT solution was added to each well of the 96-well culture plate, containing the 100 liter medium, and incubated at 37°C 4 h. This medium should be removed carefully without disturbing the purple colored formazan crystals; 50ml of dimethylsulphoxide was added to each well and mixed thoroughly to dissolve the crystals of formazan. Then these plates should be seen on a micro plate reader at a wavelength of 670nm. The reading should be presented as optical density. The growth inhibition of the cells by the drugs was identified [38].

4.3 SULFORHODAMINE- B ASSAY

Sulforhodamine B (SRB) can be used to test the effects of active compounds on cell growth and viability, based on the method described by vichai and kirtikara, compound 1-8 should be dissolved in dimethylsulfoxide (DMSO) before diluting with growth medium to a final DMSO concentration of <0.05%. The cancer cells were seeded into 96 well plates in the growth medium at 3000 cells/well. After 24 h of incubation, the medium was replaced with a fresh growth medium containing the test compounds 1-8(0, 25, 50, 100 and 200 ml). The cells were incubated for another 48 h. The cells should be fixed with TCA by gently adding 50 μ l TCA (50%) to each well to a final TCA concentration of 10% with subsequent incubation for 1 h at 40°C. The plates should be washed 5 times with deionised water and air dried. The dried plates should be stained with 100 μ l of 0.4% (w/v) SRB prepared in 1% (v/v) acetic acid for 10 min at room temperature. The plates were rinsed quickly four times with 1% acetic acid to remove unbound dye, followed by air-drying until no moisture should be visible. The bound dye was solubilised in 2mM Tries base (100 μ l/well) for 5 min on shaker. Optical densities should read on a micro plate reader at 562nm [39].

V. STATISTICAL ANALYSIS

The statistical significance of the results was analyzed by ANOVA. $P < 0.05$ which indicate the significance of the result.

VI. RESULTS AND DISCUSSION

Since, the cancer is the multi-mechanistic second largest disease in the world because of this it needs the multidimensional approach for its treatment, prevention and even the control. Tumors are of various types such as carcinoma, sarcoma, leukemia and lymphoma. In the present study, Ehrlich Ascites Carcinoma is used

to induce the cancer cell in mice. The Ehrlich tumor is initially described as a spontaneous murine mammary adenocarcinoma. It is very rapidly growing carcinoma with very aggressive behavior and which is able to grow almost all strains of mice. In the ascites form it has been used as transplantable tumor model for the investigation of antitumor effect of several substances [40].

The extract inhibited percentage control growth of all the cell lines in dose dependent manner. The effect was found to be more in Human colon cancer cell line (HCT15) and Human hepatoma cell line (HEPG2) at 80µg/ml. While, the effect was least in Human lung cancer cell line (Hop65). The lethal concentration value (LC50) and total growth inhibition (TGI) for all the cell lines were >80µg/ml. The median growth inhibition (GI50) concentration for extract was found to be <10µg/ml against Human lung cancer and hepatoma cell line, showing anticancer efficacy of methanolic extract. However, median growth inhibition (GI50) concentration against Human colon cancer cell line was 39.1µg/ml which indicated no anticancer effect of extract.

Several previous studies showed that plant extract contains abundant number of phytochemicals which possess anticancer properties and might be responsible for anticancer effect of these plants extract [41]. Plants of Annonaceous family are rich in acetogenins, an anticancer property phytochemical [42]. The presence of acetogenin may be attributed to anticancer effect of methanolic extract of *Annona reticulata* Linn.

VII. CONCLUSION

From the very beginning the plants have been recognized as the most imperative source of the medicine. The different phytochemicals derived from the different parts of plant provide the potential bioactive agent for various disease treatment strategies. The present is conducted to attempts the anticancer and antioxidant activity of *A. reticulata* Linn. Traditionally this plant has used for the treatment of several diseases. Since, it contains wide range of secondary metabolites and minerals that are the responsible for the different therapeutic activity of the plant. The present review study ascertains the value of *A. reticulata* Linn. Plant which could be considerable interest in the development of plant based new drugs [43].

In the conclusion, in vitro anticancer and antioxidant activity of the methanolic extract and isolated constituent of catechin from the *A. reticulata*, the isolated catechin was characterized by the IR, UV, ¹H_NMR, and ¹³C_NMR. (+) catechin and MAR showed excellent free radical scavenging activity. Reports from the Literature evident that the reducing power of various bioactive compounds is associated with the antioxidant activity [44].

People who consume the fruits and vegetables have the lower risk of heart disease and also the neurological disease. There is also the evidence that some types of vegetables and fruits in general, protect against some cancer. This thought was occur because the environment of cancer cells has high level of oxidative stress, which makes the cancer cells more susceptible to the further oxidative stress induced by the cancer treatment [45].

Conflict of Interest statement

The authors declare there is no conflict of interest.

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