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Phytochemical screening and assessment of *in vitro* anticancer potential of ethanolic extract of *Annona muricata* Leaves

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Abstract
Liver cancer is one of the most dreadful cancers with an incidence of one million cases every year in human beings. The currently available methods of treatment like surgery, chemotherapy and radiation induce significant side effects. So there is a need to explore an effective alternate therapies with minimal side effects. Hence, the present study was aimed to assess the *in vitro* anticancer effect of ethanolic extract of *Annona muricata* leaves which is being widely used in the traditional treatment of cancer in many countries. Phytochemical screening of ethanolic extract of *Annona muricata* leaves was carried out by using standard qualitative and quantitative methods. The *in vitro* anticancerous activity on HepG2 cell line was determined by MTT assay. Cells were treated with different concentration of the extract (0.01, 0.1, 0.3, 0.7, 0.9, 1, 3, 5 µg/ml) for 72 hours. Qualitative screening showed the presence of saponins, tannins, phenols, alkaloids, terpenoids, flavonoids, hydrolysable tannins and cardiac glycosides while amino acids, proteins, carbohydrates, phlobatannins, volatile oils, glycosides and vitamin C were absent. The quantitative screening revealed 3 mg of total alkaloid, 9 mg of phenol, 15 mg of tannin, 2.5 mg of flavonoid per gram of ethanolic extract. The IC_{50} value of ethanolic extract of *Annona muricata* leaves was determined as1.116 µg/ml at 72 hours.

Keywords: liver cancer, *Annona muricata*, phytochemicals, HepG2 and IC_{50}

Introduction
Cancer is one of the leading causes of morbidity and mortality in human beings and it is the second major cause of death globally. Liver cancer is one of the most common malignancies in the world especially in Asia. Among this, Hepatocellular carcinoma (HCC) accounts for about 80-90 per cent of all liver cancers and it is the second leading cause of cancer mortality throughout the world. The prognosis of liver cancer is very poor as the overall ratio of mortality to incidence due to this cancer is 0.95 (95:100). Owing to its extremely aggressive nature and poor survival rate, it remains an important public health issue worldwide. Major risk factors like change in life style, viral hepatitis, food additives, alcohol, aflatoxins, environmental toxins, industrial chemicals, air and water pollutants contribute to higher occurrence of liver cancer (Al-Rejaie *et al*., 2009; Ferlay *et al*., 2014) [1, 4]. This increase in magnitude of liver cancer problem with respect to incidence, morbidity and mortality warrants further studies to identify novel and safe approaches to evade the disease.
The currently available therapeutic protocols like surgery, chemotherapy and radiation will induce significant side effects. So there is a need for an alternate therapy of which exploring the traditionally used anticancer plants is envisaged to give effective remedy. Now-a-days folk medicines have taken a pivotal role in the treatment of various diseases because of multidrug resistance and other causes. One such medicinally important plant is Annona muricata, commonly known as “Graviola” or “soursop” and popularly called as “the cancer killer” which belongs to the family of Annonaceae. Though the earlier studies have demonstrated its therapeutic efficacy against hyperglycaemia, hyperlipidaemia, virus, bacteria, insects and malarial parasites (Cheng, 2001) [3], its anticancerous effect against liver cancer is not yet elucidated.
Hence, the present study was carried out to determine the phytochemical composition and in vitro anti-cancer effect of ethanolic extract of Annona muricata leaves on HepG2 cell line (liver cancer cell line).

Materials and Methods
Annona muricata
Fresh leaves of Annona muricata were collected from Bhavani, Erode district, Tamil Nadu between November’ 2017 and March’ 2018. It was authenticated taxonomically at the

Botanical Survey of India, Southern Regional Centre, TNAU campus, Coimbatore and identified as *Annona muricata* L. (Family: Annonaceae).

Preparation of plant extract

Leaves of *Annona muricata* were collected and washed by rubbing the surface gently under running tape water. The leaves were shade-dried and powdered using blender. Ten gram of powder was soaked in 100 ml of 70 per cent ethanol and kept in rotatory shaker for 72 hrs. Then it was filtered with Whatman filter paper No.3 and kept in an incubator at 37 º C to obtain semi solid substance. The dried extract was weighed and stored at 4 º C in an air tight container.

Phytochemical screening

Qualitative analysis

Ethanolic extracts of *Annona muricata* leaves were analyzed for the presence of saponins, terpenoids, flavonoids, cardiac glycosides, tannins, phenols, phlobatannins, alkaloids, carbohydrates, glycosides, hydrolysable tannins, amino acids, proteins, volatile oil and vitamin C by Harborne's method, (1998) [5].

Quantitative assay

The phenols, alkaloids, flavonoids and tannins were quantified by using the standard procedure described by Tiwari *et al*. (2011) [11].

Cell culture

Human hepatoma (HepG2) cell line was procured from the National Centre for Cell Sciences, Pune and cultured in Dulbecco's Modified Eagle Medium (DMEM). Then it was supplemented with 10 per cent of fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (Himedia) and kept in a CO_2 incubator at 37˚C.

Cytotoxicity assay

The cytotoxic action of *Annona muricata* leaves extract on HepG2 cells was measured by using 3-(4, 5-dimethylthiazol-2-ly) 2, 5-diphenyltetrazolium bromide (MTT) assay. Cells (1×10^5) were seeded in 100 µl of DMEM/well into a 96-well culture plate and incubated for 48 hrs to reach the confluence state of cells. Then the cells were incubated with different concentration of the plant extract (0.01, 0.1, 0.3, 0.7, 0.9, 1, 3, 5 µg/ml) for 72 hrs. Then each well was replaced with fresh media and MTT (0.5 mg/ml) was added and incubated at 37˚C for 4 hrs. At the end of incubation, medium was removed and 150 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the purple-blue MTT formazan precipitate. Finally, the absorbance was noted with the ELISA reader at 570 nm and calculated the percentage of viable and non-viable cells (Liu *et al*., 2016 and Banerjee *et al*., 2017) [7, 2]. Inhibition Concentration (IC_{50}) value was calculated graphically based on the percentage of viable and non-viable cells.

$$\text{Percentage of cell viability} = \frac{\text{Sample OD - Blank OD}}{\text{Control OD - Blank OD}} \times 100$$

Percentage of cell inhibition = 100 - Percentage of cell viability 6-

Results and Discussion

The result obtained from the qualitative phytochemical screening of the ethanolic extract of *Annona muricata* is depicted in Table 1. In qualitative phytochemical screening, the extract was positive for eight phytochemicals *viz*. saponins, tannins, phenols, alkaloids, terpenoids, flavonoids, hydrolysable tannins and cardiac glycosides. While amino acids, proteins, carbohydrates, phlobatannins, volatile oils, glycosides and vitamin C were absent. This finding is in agreement with the observation of Mat Daud *et al*. (2016) who also analyzed phytochemical properties of *Annona muricata* leaves for anti-cancer effect.

Quantitative estimation of selected phytochemicals like alkaloids, phenols, tannins and flavonoids are presented in Table 2. Phenols was found to be more when compared to other phytochemicals and is in agreement with the findings of Minari *et al*. (2014) [9] who also detected highest level of phenols.

Cytotoxic effects of ethanolic extract of *Annona muricata* leaves on HepG2 cells measured by MTT assay was shown in Figure 1. Cell viability reduced from 100 per cent to 99, 69, 68, 66, 62, 60, 56, 54, 49, 45 and 36 per cent in response to dose dependent increase in concentration of extract from 0 µg/ml to 0.01, 0.1, 0.3, 0.5, 0.7, 0.9, 1, 2, 3, 4 and 5µg/ml respectively. Thus, IC_{50} value of ethanolic extract was determined as 1.116 µg/ml at 72 hours.

The *in vitro* anticancer activity of *Annona muricata* leaves on HepG2 cell line might be due to the presence of the various bioactive compounds which are very well exemplified by qualitative and quantitative assay. These bioactive compounds present in the plant extract have the potential to be used as medications in the form of novel and safe products for the prevention and treatment of various diseases. Such medications are now emerging as target for the researchers to find a cure for diseases including various cancers (Sharmila and Padma, 2013) [10].

Table 1: Qualitative analysis of ethanolic extract of *Annona muricata* leaves

S.No	Phytochemicals	Ethanolic extract of *Annona muricata*
1	Saponins	+
2	Tannins	+
3	Phenols	+
4	Alkaloids	+
5	Terpenoids	+
6	Flavonoids	+
7	Amino acids and proteins	-
8	Carbohydrates	-
9	Phlobatannins	-
10	Volatile oils	-
11	Hydrolysable tannins	+
12	Glycosides	-
13	Cardiac glycosides	+
14	Vitamin C	-

Key: (+): Presence of phytochemicals; (-): Absence of phytochemicals

Table 2: Quantitative analysis of ethanolic extract of *Annona muricata* leaves

S. No	Phytochemicals	Concentration (mg/g)
1	Alkaloids	3
2	Phenols	9
3	Tannins	0.7
4	Flavonoids	2.3

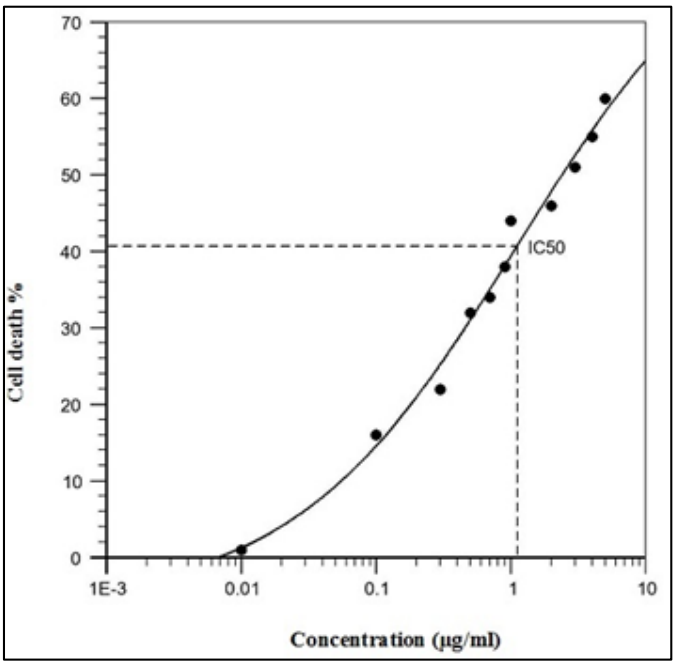

Fig 1: Effect of ethanolic extract of *Annona muricata* leaves on HepG2 cell line

Ethanolic extract of *Annona muricata* leaves were found to be rich in various phytochemicals which have wide pharmacological effects. Cytotoxic activity of *Annona muricata* on HepG2 might be due to the presence of higher concentration of phenols which contribute anticancer activity by various pathways *viz*. cell cycle arrest, induction of apoptosis, anti-oxidant activity and suppression of angiogenesis. Likewise presence of flavonoids in this plant also attributes antioxidant property and thereby protects the cells from the oxidative stress (Kuno *et al.*, 2012) [6].

Earlier researches have reported that *Annona muricata* leaves extract is selectively toxic against various types of cancerous cells without harming healthy cells (Banerjee *et al*., 2017) [2]. In the present study, the inhibitory concentration of ethanolic extract on HepG2 cell line was found to be 1.116 µg/ml at 72 hours. The IC_{50} value of this ethanolic extract was also recorded as 180 and 80 µg/ml after 24 and 48 hours respectively by Yang *et al.* (2016) [7], He also reported that the *Annona muricata* had anti-proliferation effects on HepG2 cells by promoting apoptosis and used as an alternative or complementary treatment for liver cancer.

This study shows that *Annona muricata* leaves extract exhibit cytotoxic activity in HepG2 liver cancer cell line in a dose dependent manner. Hence the present study suggest that *Annona muricata* leaves can be used as a source of anticancer drug. However, further studies have to be conducted with *in vivo* model to correlate the findings of *in vitro* studies.

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