

RESEARCH COMMUNICATION

Quantitative Assessment of the Relative Antineoplastic Potential of the n-butanolic Leaf Extract of *Annona Muricata* Linn. in Normal and Immortalized Human Cell Lines

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Abstract

Natural products have been the target for cancer therapy for several years but there is still a dearth of information on potent compounds that may protect normal cells and selectively destroy cancerous cells. The present study was aimed to evaluate the cytotoxic potential of n-butanolic leaf extract of *Annona muricata* L. on WRL-68 (normal human hepatic cells), MDA-MB-435S (human breast carcinoma cells) and HaCaT (human immortalized keratinocyte cells) lines by XTT assay. Prior to cytotoxicity testing, the extract was subjected to phytochemical screening for detecting the presence of compounds with therapeutic potential. Their relative antioxidant properties were evaluated using the reducing power and DPPH^{*} radical scavenging assay. Since most of the observed chemo-preventive potential invariably correlated with the amount of total phenolics present in the extract, their levels were quantified and identified by HPLC analysis. Correlation studies indicated a strong and significant ($P < 0.05$) positive correlation of phenolic compounds with free radical scavenging potential. The results revealed that the extract was moderately cytotoxic to normal cells with a mean IC₅₀ value of 52.4 μ g when compared with those obtained for cancerous cells (IC₅₀ values of 29.2 μ g for MDA-MB-435S and 30.1 μ g for HaCaT respectively). The study confirms the presence of therapeutically active antineoplastic compounds in the n-butanolic leaf extract of *Annona muricata*. Isolation of the active metabolites from the extract is in prospect.

Keywords: Antineoplastic - cytotoxicity *in vitro* - WRL 68 - MDA MB 435S - HaCaT - cell lines

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Introduction

Medicinal plants are considered to be the main sources of biologically active compounds that can be used for the treatment of various ailments including cancer. Out of the 250,000-500,000 plant species on the earth, only 1-10% have been studied chemically and pharmacologically for their potential medicinal value especially for chemotherapeutic effect (Borris, 1996). The era of chemotherapy began in 1940s with the first use of nitrogen mustards and antifolate drugs (Chabner and Roberts, 2005). Thereafter, cancer drug discovery and development have been the major research endeavour around the globe as evidenced by several peer-reviewed papers in the scientific literature (Suresh et al., 2006). Excessive free radical formation is one of the hallmarks of cancer cells. Several studies have shown that plant-derived antioxidant nutraceuticals scavenge free radicals and modulate oxidative stress-related effects (Thatte et al., 2000). Various compounds isolated from plants are known to be effective against proliferating cells. They exhibit cytotoxic effects either by damaging DNA or by

blocking the formation of mitotic spindle during stages of cell division (Gali-Muhtasib and Bakkar, 2002). However, most of the cytotoxic chemopreventive drugs exhibit side effects (Powis, 1983) at some point of time during therapy, and hence, there is a need to isolate compounds that are potent and selective with minimal side effects on normal cells.

Annona muricata L. is a naturally occurring plant, traditionally used to treat various ailments including cancer. It belongs to the family *Annonaceae* and is widely distributed in India and Central America. Fruits of *Annona muricata*, also known as Graviola in South America are taken internally for worms and parasites, for fever, to increase mother's milk after child birth and as an astringent for diarrhoea and dysentery. The plant is also reported to have good antioxidant property (Baskar et al., 2007). Furthermore, the leaves of the plant are found to be anti-spasmodic, hypotensive and are rich in annonaceous acetogenins (Gouemo et al., 2003; Yuan et al., 2003). Various other plants from this family have also been reported for their cytotoxic potential (Liaw et al., 2002; Ye et al., 2002; Pardhasaradhi et al., 2005; Magadula

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et al., 2009).

The therapeutic potentials of the n-butanolic extract of *Annona muricata* were studied on WRL-68, MDA-MB-435S and HaCaT cell lines. Since most of the chemotherapeutic drugs affect normal cells as well, WRL-68 cells were analysed for the relative cytotoxic response in with comparison that quantified in MDA-MB-435S and HaCaT cell lines. n- butanolic leaf extract of several medicinal plant posses significant anticancer potentials in human cancerous cells (Marcia et al., 2009; Pintusorn et al., 2009). However, reports on antineoplastic activities of *Annona muricata* have not been evaluated thus far. Hence, the present study aims to investigate the *in vitro* antineoplastic potential of the n-butanolic leaf extract of *Annona muricata* in cancer cells and in normal cells, after evaluating the therapeutically active components in the extract by semi-quantitative phytochemical screening and estimation of its antioxidant potentials.

Materials and Methods

Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH^{*}), ascorbic acid, trichloroacetic acid (TCA), phenazine methosulfate (PMS) (also known as N-methylphenazonium methosulfate), dimethyl sulfoxide (DMSO), L-15 (Leibovitz's) cell culture medium (with L-glutamine), DMEM & MEM cell culture medium (with Earle's salt, NEAA and L-glutamine), ferric chloride (FeCl₃) were purchased from Himedia Laboratories Pvt. Ltd. (India). XTT {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu reagent was procured from Sisco Research Lab (India). The remaining chemicals and solvents used were of standard analytical and HPLC grade, respectively. MDA-MB-435S (human breast carcinoma), HaCaT (human immortalized keratinocyte) and WRL-68 (normal human hepatic) cell lines were obtained from National Centre for Cell Science (NCCS), Pune, India.

Plant collection, extraction and processing

Healthy leaves of *Annona muricata* were collected from Teeose nursery, Thrissur district, Kerala, India and identified by Dr. E. M. Muralidharan, Scientist E-II, Kerala Forest Research Institute, Peechi, Kerala, India. Voucher specimens are maintained at our laboratory for future references (VIT/SBST/CCL/10/October/08).

The collected leaves were screened for contamination and thoroughly washed. The specimens were then shade dried, ground in a mechanical mixer-grinder, and extracted with n-butanol by maintaining the powder: solvent ratio as 1:6 using a Soxhlet apparatus. The crude extract obtained was concentrated at 40°C under reduced pressure (72 mbar) with a Rotavapor R-215 (BUCHI Labortechnik AG, Switzerland). The dried extract was weighed to determine the yield of soluble constituents and stored in a vacuum desiccator at room temperature until further use.

Phytochemical screening

Semi-quantitative phytochemical screening of the

extract was done as described by Dohou et al. (2003) and Bekro et al. (2007) with little modifications. The extract was analysed for the presence of flavonoids, saponins, terpenoids, reducing sugars, cardiac glycosides, steroids, tannins, phlobatannins, anthraquinones and oil.

Determination of antioxidant potential

Reducing power of extract: The ability of the extract to reduce Fe³⁺– Fe²⁺ was analysed by the method of Yildirim et al. (2001) with slight modifications. 25, 50, 100, 200 and 400 µg of the extract were mixed with 2.5 ml of 0.2 M phosphate buffer and 2.5 ml of 1% potassium ferricyanide followed by incubation at 50°C for 30 min. 2.5 ml of 10% trichloroacetic acid was later added and the tubes were centrifuged at 3000 rpm for 10 min. 2.5 ml of the upper layer solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated increased reducing power.

Free radical scavenging activity: The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH^{*}) was used for the determination of free radical scavenging activity of the extract following the method of Koleva et al. (2002) with slight modifications. Different concentrations (25, 50, 100, 200 and 400 µg) of the leaf extract were added, at an equal volume, to the ethanolic solution of 3 mL of DPPH^{*} (0.1mM). After 30 min of incubation in the dark in room temperature, the absorbance was recorded at 517 nm using a Cary 50 UV-Vis Spectrophotometer (Varian Inc., Australia). Ascorbic acid was used as a standard. The percentage inhibition (I %) was calculated using the formula,

$$I \% = \frac{[\text{Abs (Control)} - \text{Abs (Sample)}]}{\text{Abs (Control)}} \times 100.$$

In vitro cytotoxicity assay

Cell culture: Human breast carcinoma cells (MDA-MB-435S), human immortalized keratinocyte (HaCaT) and normal human hepatic cells (WRL-68) cultures were initiated and propagated in L-15 (Leibovitz's), DMEM (Dulbecco's Modified Eagle Medium) and MEM (Minimum Essential Medium) respectively. The cells were supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO₂ (except for MDA-MB-435S) at 37°C. Cells were grown in polystyrene-coated T25 (25 cm²) cell culture flasks, and were harvested in logarithmic phase of growth. The cells were maintained at the above-mentioned culture conditions for all experiments.

Cell proliferation assay: XTT assay was performed on MDA-MB-435S, HaCaT and WRL-68 cell lines as described by Weislow et al. (1989) with minor modifications. 6 x 10³ cells were seeded in each well of a 96-well plate and the cells were added with 200 µl of the respective culture medium and incubated at 37°C for a period of 24 h. The media were then replaced with 200 µl of fresh media containing varying concentrations of the extract (5, 10, 20, 40 and 80 µg). The plate was then re-incubated maintaining the same conditions for 24 h, after which, medium containing extract was substituted

by 200 μ l of fresh medium. 50 μ l of XTT reagent prepared in medium (0.6 mg/ml) containing 25 μ M of PMS was then added to all the wells and the plate was incubated in dark humid conditions at 37°C for 4 h. After incubation, the orange colored complex formed was read at 450 nm using a Dynex Opsys MRTM Microplate Reader (Dynex Technologies, VA, USA) with a 630 nm reference filter. Wells containing cells without the extract served as the control and wells containing only culture medium and XTT reagent served as the blank. Percentage cytotoxicity of the extracts was calculated by using the formula:

$$\% \text{ Cytotoxicity} = \frac{(\text{OD of control} - \text{OD of treated cells}) \times 100}{\text{OD of control}}$$

Estimation of total phenolics

Quantification of polyphenolics: Total phenolic content of the extract was determined using the Folin-Ciocalteu reagent method described by Lister and Wilson (2001). To the 50 μ l of each extract concentrations (25, 50, 100, 200 and 400 μ g), 2.5 ml of Folin-Ciocalteu reagent (1/10 dilution) and 2 ml of 7.5% Na_2CO_3 (w/v) were added and mixed well. The mixture was incubated at 45°C for 15 min. The absorbance were measured at 765 nm using a Cary 50 UV-Vis spectrophotometer (Varian, Inc., CA, USA) with Na_2CO_3 solution (2 ml of 7.5% Na_2CO_3 in 2.55 ml of distilled water) as blank. Gallic acid was used as a standard, and results were expressed as GAE (gallic acid equivalence) in μ g.

HPLC analysis of polyphenolics: HPLC analysis was performed using a Waters 2487 HPLC system consisting of a dual λ detector and a Waters 1525 binary pump, and equipped with a Waters Symmetry® C18 column (5 mm, 4.6 \times 50 mm) with Waters Sentry™ universal guard column (5 mm, 4.6 \times 20 mm) (Waters Corporation, Milford, MA, USA). Phenolic compounds in the n-butanolic extract of *Annona muricata* were studied using the reference HPLC method by comparing experimental retention times with reported reference values (Sakakibara et al., 2003). Gradient elution was performed at 35°C with solution A (50mM sodium phosphate in 10% methanol; pH 3.3) and solution B (70% methanol) in the following gradient elution program: 0–15 min—100% of Solution A; 15–45 min—70% of Solution A; 45–65 min—65% of Solution A; 65–70 min—60% of Solution A; 70–95 min—50% of Solution A; 95–100min—0% of Solution A. Flow rate was 1 ml min^{-1} and injection volume was 20 μ l. Detection was monitored at diverse wavelengths (around λ max) for various phenolic compounds, i.e. 250 nm for benzoic acids, isoflavones and most anthraquinones; 280 nm for some flavones, flavanones, catechins, theaflavins and some anthraquinones; 320 nm for cinnamic acids, most flavones and chalcones; 370 nm for flavonols; 510 nm for anthocyanins.

Statistical analysis

All the experiments were carried out in triplicates. Data were presented as mean \pm standard deviation (SD). Statistical analyses were performed by one-way

ANOVA. To evaluate relationships between experimental parameters, results were analyzed for correlation and tested for significance by Student's t-test. Significant differences between groups were determined at $P < 0.05$. MATLAB ver. 7.0 (Natick, MA, USA), Graphpad Prism 5.0 (San Diego, CA, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations.

Results

Yield of the Extract

50 grams of leaf powder yielded 5.4 g of n-butanolic crude extract.

Phytochemical screening

The phytochemical analysis of the n-butanolic leaf extract of *Annona muricata* revealed the presence of flavonoids, terpenoids, tannins, cardiac glycosides and reducing sugars. Whereas, the extract showed the absence of saponins, steroids, phlobatannins, oil and anthraquinones tested which is presented in Table 1.

Reducing power

Reducing power is evaluated by the transformation of Fe^{3+} to Fe^{2+} in the presence of the extract (Gulcin et al., 2003). Various studies have indicated that the electron donation capacity of the bioactive compound is associated with their antioxidant activity (Siddhuraju et al., 2002; Yen et al., 1993). The reducing power of the extract was determined and compared with butylated hydroxytoluene (BHT) as standard (Figure 1). The extract showed concentration dependent reducing effect.

Table 1. Phytochemical Screening of n-butanolic Leaf Extract of *Annona muricata*

Phytochemical tests	Leaves
1. Saponins	-
2. Flavonoids	+
3. Terpenoids	+
4. Tannins	+
5. Steroids	-
6. Phlobatannins	-
7. Oil	-
8. Cardiac glycosides	+
9. Reducing sugars	+
10. Anthraquinones	-

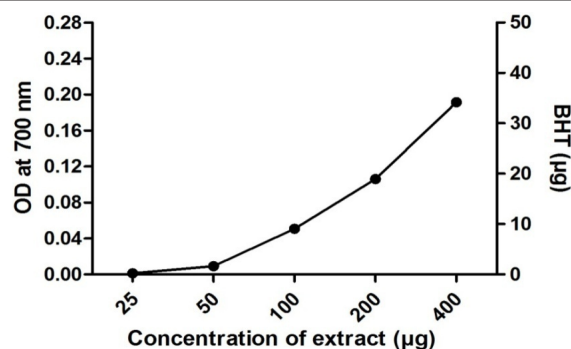


Figure 1. Reducing Power of n-butanolic Leaf Extract of with BHT Equivalence. Data expressed as mean \pm SD of n = 3 samples ($P < 0.05$)

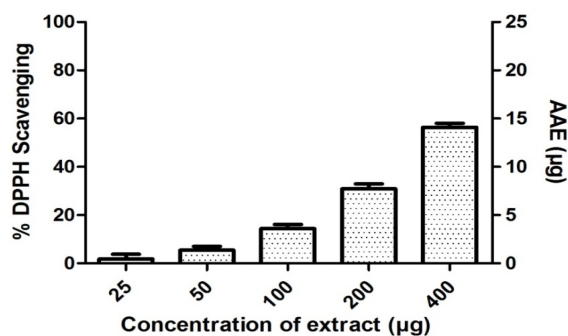


Figure 2. DPPH* Scavenging Activities of n-butanolic Leaf Extract of *Annona muricata* along with AAE. Data expressed as mean \pm SD of n = 3 samples (P < 0.05)

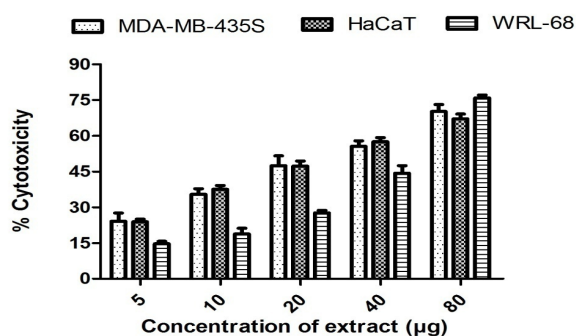


Figure 3. Cytotoxicity of n-butanolic Leaf Extract of *Annona Muricata* on MDA-MB-435S, HaCaT and WRL-68 cells as Estimated by XTT Assay. Values given are as mean \pm SD of n=3 samples (P < 0.05)

Free radical scavenging activity

Radical scavenging efficiency of the extract was examined using the stable free radical, DPPH*. It has a maximum absorbance at 517 nm. Absorbance decreases when antioxidants donate protons to DPPH*. The extract showed significant free radical scavenging ability in a dosage dependent manner and the results are depicted in Figure 2 along with its ascorbic acid equivalence (AAE) in μ g.

In vitro cytotoxicity of the extract

Cytotoxicity of the extract was tested in MDA-MB-435S, HaCaT and in WRL-68 cells by XTT assay. Results of which are shown in Figure 3. Cells were treated with different concentrations of the extract and the respective IC50 values were found to be 52.4 μ g (WRL-68), 29.2 μ g (MDA-MB-435S) and 30.1 μ g (HaCaT). The extract was found to destruct the cancer cells comparatively at lower doses than the normal cells. However, at the highest dose of 80 μ g, the extract exhibited more or less similar cytotoxic effect on all the cell lines tested.

Total phenolic estimation

Polyphenols, being secondary metabolites, present in large quantities in several plants. Many of them possess antioxidant, anti-inflammatory and several other therapeutic properties (Shalini and Rachana, 2009). According to Velioglu et al. (1998), phenolic compounds are one of the most effective kinds of antioxidants present in plants. Hence, the total phenolic content in the n-butanolic leaf extract of *Annona muricata* was quantified

Table 2. Total Phenolic Contents of n-butanolic Extract of *Annona Muricata*

Concentration (μ g)	GAE \pm SD (μ g) (P < 0.05) n-butanolic extract
25	06.60 \pm 0.09
50	07.37 \pm 0.13
100	08.31 \pm 0.31
200	11.54 \pm 0.18
400	37.42 \pm 0.36

Table 3. Correlations Between Total Phenolic Estimation and DPPH* Assay were Tested for Significance at P < 0.05. R² Denotes Coefficient of Determination

Correlation	Extract	R2 (<0.05)	Result
	n-butanolic	0.952493	Significant

Table 4. Major Phenolic Compounds Present in n-butanolic Extract of *Annona Muricata* by HPLC

Phenolic compounds	λ^a (nm)	EtR ^b (min)	RtR ^c (min)
A. Flavonols			
1. Quercetin	370	39.8	40.6
2. Robinetin	370	35.6	36.4
B. Polyphenols			
1. Gallic acid	250	06.1	05.8
C. Flavones			
1. Apigenin-6-C-glucoside	320	39.1	38.6
2. Vitexin	320	35.3	35.2
3. Luteolin 3',7-di-O-glucoside	320	30.8	31.6

^awavelength for determination, ^bexperimental retention time, ^cstandard retention time.

and expressed in GAE (gallic acid equivalence) (Table 2). A dosage-dependent increase in the amount of total phenolic content was observed. Total phenolic content of the extract illustrated significant and strong positive correlations (P < 0.05) with DPPH free-radical scavenging efficiency which is shown in Table 3.

HPLC analysis of extract

Major types of phenolic compounds present in the n-butanolic extract of *Annona muricata* leaves were determined by HPLC analysis. A library of the analytical characteristics (λ_{max} , retention time, determining λ , slope and limit calibration) of more than 100 phenolic standards established by Sakakibara et al. (2003) was used as reference for compound identification and our values were compared with the reported standard retention time values. Table 4 shows the phenolic compounds identified in the extract along with the respective retention times (Rt). The extract also contained unknown compounds evident from the HPLC data whose characterization is in prospect.

Discussion

Plants have been screened for centuries to explore their various therapeutic properties which might be effective in prevention, cure and management of various diseases.

The n-butanolic *Annona muricata* leaf extract has shown significant therapeutic potentials in all the analytical experiments employed in this study.

The medicinal value of a plant depends on the biological activities of the phytochemicals that possess a definite physiological function. The most important of these phytochemicals are alkaloids, flavonoids, tannins and phenolic compounds (Hill, 1952). The qualitative tests in the study have revealed the presence of some important phytochemicals (Table 1) that supports the resourcefulness of the extract. Plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers (Polterait, 1997). The amounts of total phenolics in the extract was quantified and found to be significantly higher with increasing dosage of the extract. Flavonoids and tannins are phenolic compounds and the presence of these compounds in the extract might be responsible for the free radical scavenging effects observed.

The previous study by Baskar et al. (2007) shows ethanolic leaf extract of this plant exhibits significant antioxidant property. Our study is consistent with earlier findings in that the n-butanolic extract (not reported before in the literature) also showed a dose-dependent increase in antioxidant activity for two assays. The differential scavenging activities of the extracts against DPPH* and Fe³⁺ radicals may be due to the different mechanisms of the radical-antioxidant reactions in these assays. The stoichiometry of reactions between the antioxidant compounds in the extract, and the DPPH* and Fe³⁺ radicals is distinctively dissimilar, which may be referred to as a reason for the difference in scavenging potentials. The diversity in radical scavenging shown in these assays may also be due to factors like stereoselectivity of the radicals or the differential solubility of the extract (Yu et al., 2002). Supportive evidence for this aspect is provided by the strength of the significant statistical correlation between the phenolic content of the extract and the free-radical scavenging activity.

Plant-derived compounds have played an important role in the development of several clinically useful anticancer agents. Lesser side effects may make naturally occurring compounds a better choice than synthetic compounds. In many cases, the actual compounds isolated from the plants might not serve as the drug, but they serve as leads to the development of potential anticancer agents (Sameer et al., 2011). Anticancer activity of *Annona muricata* leaf extract was studied on a cancerous (MDA-MB-435S, HaCaT) as well as on a normal (WRL-68) cell line. The extract at its lower doses exhibited a significant cytotoxicity on MDA-MB-435S and HaCaT cells with the IC₅₀ values of 29.2 and 30.1 µg respectively, while it exhibited only a moderate cytotoxicity towards WRL-68 with a comparatively higher IC₅₀ value of 52.4 µg, clearly indicating differential cytotoxicity, at least at the lower doses tested. However, at the highest tested dose, the cytotoxic effect of the extract was similar on both normal and cancer cell lines. This might be due to the influence of this particular extract over the normal cell turnover mechanisms which ultimately results in cell death (Fortunato et al., 2000). Furthermore, HPLC

analysis of the extract, also reported in this study, revealed the presence of a variety of phenolic compounds like flavonols, polyphenols and flavones, which could have been responsible for its observed antineoplastic potential.

In conclusion, the study concludes that the n-butanolic leaf extract of *Annona muricata* might have potential for the development of therapeutically active compounds, which could serve as precursors and/or chemical templates for the design of an effective, more potent and safe antineoplastic drug which may be more potent than existing drugs of its class. These encouraging preliminary results provide a scientific basis for further characterization of individual compounds from this extract.

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