

# ***Annona squamosa* Linn: Cytotoxic activity found in leaf extract against human tumor cell lines**

**De-Shen Wang<sup>1,2</sup>, Ghazala H Rizwani<sup>3\*</sup>, Huiqin Guo<sup>4</sup>, Mansoor Ahmed<sup>5</sup>, Maryam Ahmed<sup>3</sup>, Syed Zeeshan Hassan<sup>3</sup>, Amir Hassan<sup>5</sup>, Zhe-Sheng Chen<sup>2</sup> and Rui-Hua Xu<sup>1</sup>**

<sup>1</sup>Department of Medical Oncology, State Key Laboratory of Oncology in South China,

Collaborative Innovation Center for Cancer Medicine, Sun Yat-sen University Cancer Center, Guangzhou, China

<sup>2</sup>Department of Pharmaceutical Science, College of Pharmacy and Health Sciences, St. John's University, Queen, UY, USA

<sup>3</sup>Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, Karachi, Pakistan

<sup>4</sup>Department of Thoracic Surgery, Peking Union Medical College Hospital, Beijing, China

<sup>5</sup>Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Karachi, Karachi, Pakistan

**Abstract:** Cancer is a common cause of death in human populations. Surgery, chemotherapy and radiotherapy still remain the corner stone of treatment. However, herbal medicines are gaining popularity on account of their lesser harmful side effects on non-targeted human cells and biological environment. *Annona squamosa* Linn is a common delicious edible fruit and its leaf have been used for the treatment in various types of diseases. The objective of present study is to determine the anticancer potential of the organic and aqueous extracts of leaf of *Annona squamosa* L. MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-biphenyl tetrazolium bromide) assay against hepatocellular carcinoma cell line BEL-7404, lung cancer line H460, human epidermoid carcinoma cell line KB-3-1, prostatic cancer cell line DU145, breast carcinoma cell line MDA-MB-435, and colon cancer cell line HCT-116 Human primary embryonic kidney cell line HEK293 as control were used for the study. The crude extract (Zcd) and Ethyl acetate extract (ZE) were found significant anticancer activity only on human epidermoid carcinoma cell line KB-3-1 and colon cancer cell line HCT-116.

**Keywords:** *Annona squamosa* Linn, custard apple, leaf, cancer cell lines, cytotoxicity.

## **INTRODUCTION**

Cancer is the common cause of death in human worldwide. Currently, surgery, chemotherapy and radiotherapy, which are the main and the major therapies, are still unavailable to the populations of many third world and underdeveloped countries. However, herbal medicines are gaining popularity on account of their lesser harmful side effects even on non-targeted biological environment.

The nature has bestowed upon us a wide range of fauna and flora. One or the other types of different medicinal plants are found worldwide fulfilling the health related needs of mankind. Some commonly edible plants also possess compounds of medicinal importance in their various parts. Developments of novel medicinal products are made possible by the process of pharmacological screening of plants and the exploration of important chemical constituents through activity directed bioassays. Pharmacologically safe herbal compounds bearing the potential of inhibiting the growth and proliferation of tumor cells have a probable future as antineoplastic or anticancer agents (Dewick, 1989).

*Annona squamosa* Linn. (Annonaceae), popular, with common name of "custard apple or sugar apple", while in

Urdu it is called "Sharifa". Its origin from West Indies and Central America. The plant is also cultivated throughout the South Asia especially in tropical regions for edible purpose (Kirtikar and Basu, 2001).

*Annona squamosa* Linn. a common plant worldwide and mainly grown in gardens for its delicious fruit and its great ornamentals value. It is a small (about 5-6 m in height) deciduous tree with irregular branches. Young branches and leaf are sparsely hairy, bark is light brown, smoothish to slightly fissured while pale green leaf occur singly (Khare, 2007; Kirtikar and Basu, 2001). The plant has been used in traditional medicine of several tropical countries to treat epilepsy, dysentery, cardiac problems, worm infection, constipation, hemorrhage, antibacterial, dysurea, fever and ulcer. It also has anti-fertility and abortifacient properties (Soni *et al.*, 2012). It is effective remedy for various types of inflammatory diseases as well as tumorous growths. All parts of this plant i.e. leaf, bark, shoot and roots have various compounds of medicinal importance and hence were used in different kinds of health problems. Ripe fruits of plant are applied to malignant tumors to hasten suppuration while vermin are destroyed by the used of its dried unripe fruit powder. The seeds are acrid and poisonous and their powder served as fish poison and as insecticidal agent. Seeds paste has been used in eradication of head lice, for destroying worm in the wound of cattle's and also has shown antifertility

\*Corresponding author: e-mail: deanpharmacy@hotmail.com

activity or being used as valuable contraceptive. The crushed leaf are sniffed to overcome hysteria and fainting spells, they are also applied on ulcer and wounds (Parvin *et al.*, 2003; Sobiya *et al.*, 2009). The plant aerial parts are used as antibacterial, anti-diabetic, anti-hyperlipidemic, anti-microbial, antioxidant, anti-head lice effect, antitumor, cytotoxic, hepatoprotective, insecticidal, chemopreventive & anti-lipidperoxidative, mosquitocidal, pesticidal, molluscicidal, anti-plasmodial, vasorelaxant, anti-thyroidic, anti-fertility, antiviral and anthelmintic activity (Neha Pandey and Dushyant Barve, 2011).

The present study focus on the cell growth inhibitory activity of *Annona squamosa* L. leaf. In this study, organic and aqueous extracts of *Annona squamosa* L. leaf were tested on different tumor cell lines of human origin for detection of its antitumor activity, which might make it, enable to be a source of developing novel antitumor medicinal product from nature.

## MATERIAL AND METHODS

### *Collection and authentication of plant materials*

The plant materials fresh leaf (2kg) were collected locally collected from a garden during the month of November-January 2007. Chemotaxonomic identification and botanical enumeration of the plant materials was authenticated by Professor from Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, Pakistan.

### *Chemicals and reagents*

The purified chemicals used during research work were commercially purchased from Oxoid (England) and Merck (Germany).

### *Extraction and fractionation*

The leaf of *Annona squamosa* L. were shed dried under normal condition and homogenized to coarse powder. The dried powder was macerated with ethanol and stored for 72 hours in ice cold condition. After that the miscella was filtered off by using Whatmann No.1 filter paper and organic layer was allowed to evaporate. The resulted dark green extract was concentrated on rota vapour under controlled temperature and reduced pressure to get Crude extract (Zcd). The concentrated crude extract was charged into separating funnel and fractionation was carried out with distilled water and ethyl acetate (Merck) to obtained two extract, Water extract (ZH) and Ethyl acetate extract (ZE).

### *Method (cytotoxicity assays)*

Three extracts as Crude extract (Zcd) Water extract (ZH) and Ethyl acetate extract (ZE) of plant were assayed on six different cell lines i.e. hepatocellular carcinoma cell line BEL-7404, lung cancer line H460, human epidermoid carcinoma cell line KB-3-1, prostatic cancer cell line

DU145, breast carcinoma cell line MDA-MB-435, and colon cancer cell line HCT-116 were used to determine cytotoxicity of these extracts. Human primary embryonic kidney cell line HEK293 was used as non-cancer cell line (control). MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-biphenyl tetrazolium bromide) assay was used for this study. Cells ( $5 \times 10^3$ /well) were seeded in 96-well plates and cultured overnight then various concentrations (0, 0.3, 1, 3, 10, 30, 100 and 300 mg/ml) of each plant extracts (Zcd, ZH and ZE) were added respectively. After 72 hours of incubation, 20 ml MTT solution (4 mg/ml) was added to each well and incubated for an additional 4 hours and then the supernatant was discarded. Subsequently, each well was dissolved in 100  $\mu$ l of dimethylsulfoxide (DMSO). The absorbance was determined using an OPSYS microplate Reader from DYNEX Technologies, Inc. (Chantilly, VA) at a wave length of 570 nm. All the experiments of each extracts were repeated thrice and mean were calculated (HuiqinGuo *et al.*, 2013).

## RESULTS

Results of this study ZE (table 1 and fig. 1A) had the cytotoxicity in most of cancer cell lines except for hepatocellular carcinoma cancer cell line BEL-7404. Interestingly, the IC<sub>50</sub> values of ZE extract were  $13.6629 \pm 0.7334 \mu\text{g/ml}$  and  $1.3740 \pm 0.6382 \mu\text{g/ml}$  for KB-3-1 cells and HCT-116 cells, respectively, whereas the IC<sub>50</sub> values were  $74.5183 \pm 0.9188 \mu\text{g/ml}$  for HEK293 (Table 1 and Fig 1A). Moreover, the KB-3-1 cells and HCT-116 cells were also sensitive to the crude extract (Zcd), the IC<sub>50</sub> values were  $53.8659 \pm 3.3301 \mu\text{g/ml}$  and  $15.0616 \pm 1.3153 \mu\text{g/ml}$  respectively (Table 1 and Fig 1B).

## DISCUSSION

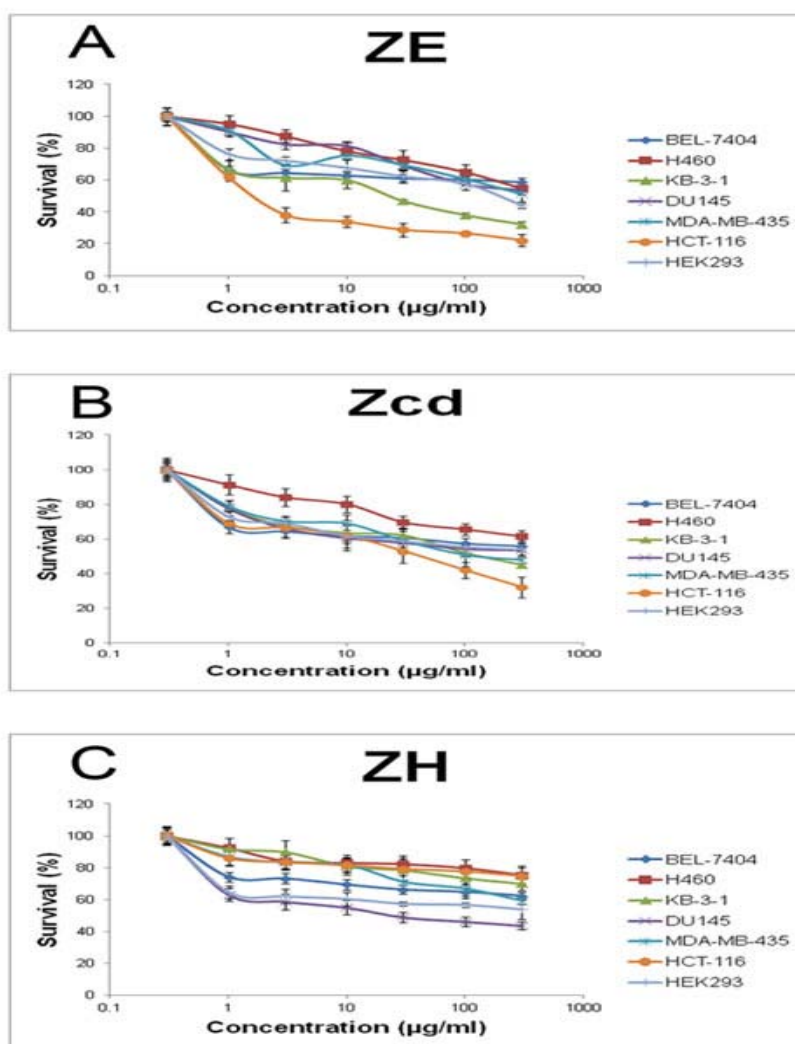
Abundantly cultivated South Asia plant, *Annona squamosa* L (Annonaceae family), having high content of nutritional compounds such as vitamin C, thiamine, niacin, niacinamide, dietary fiber, potassium and iron in considerably significant amounts (Annonymous, 2014). Acetogenins are the toxic agent found rich in seeds (Chen *et al.*, 2012).

Various parts of the plant have been used as a source of medicinal agents worldwide. The root was long been known as a powerful purgative while the root bark scraping relieve the toothache. Seeds are anthelmintic and anti-pendincolide along with anti-fertility activity. The unripe fruit is a useful astringent. Leaf extracts of *Annona squamosa* L widely used in the treatment and healing of abscesses, insect bites, allergic skin conditions and malignant growth as well as being a vermifugal agent (Indian Materia Medica, 2000; Khare, 2007). While leaf extract of this plant has thyroid inhibitory and anti-peroxidative in nature (Panda & Kar, 2003) which also effective in proper functioning of carbohydrate as well as lipid metabolism (Ganong, 1994).

**Table:** The cytotoxic effects of various plant extracts (ZE, Zcd, and ZH) in the BEL-7404, H460, KB-3-1, DU145, MDA-MB-435, HCT-116 and HEK293 cell lines.

IC50 ± SD (µg/ml)			
	ZE	Zcd	ZH
BEL-7404	>300	>300	>300
H460	184.5867±6.0899 *	> 300	>300
KB-3-1	13.6629±0.7334 *	53.8659±3.3301**	>300
DU145	84.8312±6.3101**	93.4347±6.3174**	145.0150±12.0265
MDA-MB-435	148.4360±9.9028**	85.0000±3.6062**	165.8606±2.9806
HCT-116	1.3740±0.6382*	15.0616±1.3153**	>300
HEK293	74.5183±0.9188*	>300	>300

The cytotoxic effects of various plant extracts (ZE, Zcd, and ZH) in the BEL-7404, H460, KB-3-1, DU145, MDA-MB-435, HCT-116 and HEK293 cell lines. A serial concentrations used in various plant extracts were 0, 0.3, 1, 3, 10, 30, 100, 300 µg/ml, respectively. \*: The IC50 values of ZE were significantly different (smaller,  $p < 0.05$ ) compared with those in both Zcd and ZH. \*\*: The IC50 values of ZE or Zcd were significantly different (smaller,  $P < 0.05$ ) compared with those in ZH.



**Fig:** The cytotoxic effects of various plant extracts (A) ZE, (B) Zcd (C) ZH, in the BEL-7404, H460, KB-3-1, DU145, MDA-MB-435, HCT-116 and HEK293 cell lines as determined by MTT assays. A serial concentrations used in various plant extracts were 0, 0.3, 1, 3, 10, 30, 100, 300 µg/ml, respectively.

Previous studies showed that the hot aqueous extract of *Annona squamosa* L. leaf have significant hypoglycemic and thus anti-diabetic activity in experimental animals (Gupta *et al.*, 2008). Much research work has been done on the seed and stem bark of *Annona squamosa* and it is still waiting to be carried out on the leaf. According to the previous studies, compounds isolated from seeds of plant have showed remarkable anti-microbial and cytotoxic activities (Rakesh & Mahendra, 2009), whereas ethanolic extract of leaf and stem has reported anticancer activity (Sobiya *et al.*, 2009). However, alkaloids, proteins and amino acids were found considerably negligible in leaf extract (Jayshree & Vipin 2008). In the present study, three extracts as Crude extract (Zcd), Water extract (ZH) and Ethyl acetate extract (ZE) were obtained from the *Annona squamosa* L. leaf, respectively. The anticancer activity of extracts was tested on different tumor cell lines of human origin. The results found that Ethyl acetate extract (ZE) and crude extract (Zcd) have the potential anticancer activity in human epidermoid carcinoma cell line KB-3-1 and colon cancer cell line HCT-116. The results implied that Ethyl acetate extract and crude extract might be able to become a source of developing novel antitumor activity.

Acetogenins were found abundant among the members of *Annonaceae* family which known to have potent anti-neoplastic, as well as anti-parasitocidal, pesticidal and anti-microbial activities (Oberlies *et al.*, 1997 and Alali *et al.*, 1999). Two acetogenins, named ANNORETICUIN and ISOANNORETICUIN, present in the leaf of *Annona* species were selectively toxic to certain tumors of human origin. However, the leaf and stem were found to have alkaloids, dofamine, salsolinol and coclaurine (Kirtikar & Basu 2001; Indian Materia Medica 2000). Seeds aqueous extracts of *A. squamosa* possessed significant antitumor activity *in vivo* against AK-5 tumor (Khar Ashok, 2004). Also the seed extract showed significant anti-tumor activity against four human hepatoma cells lines *in vitro* and *in vivo* (Chen *et al.*, 2012).

So the pure chemical components, which have pronounced anti-cancer activity from these three extracts needed to isolate which will be proceeds further in future. Although, activity directed bioassay is also required for the selective identification of components, which are active against cell lines from the leaf of this plant.

In conclusion, crude ethanolic extract (Zcd) and Ethyl acetate extract (ZE), of the leaf of *Annona squamosa* L. have potential anticancer activity against human epidermoid carcinoma cell line KB-3-1 and colon cancer cell line HCT-116 as a result of preliminary research exercise.

## REFERENCES

- Alali FQ, Liu XX and McLaughlin JL (1999). Annonaceous acetogenins: Recent progress. *J. Nat. Prod.*, **62**(3): 504-540.
- Anonymous (2014). USDA national nutrient database for standard reference release 26 basic report 09321, Sugar-apples, (sweetsop).
- Chen Y and Xu SS *et al.* (2012). Anti-tumor activity of *Annona squamosa* seeds extract containing annonaceous acetogenin compounds. *J. Ethnopharmacol.*, **142**(2): 462-466.
- Dewick PM (1989) Tumor inhibitors from plants, in trease and evans' Pharmacognosy (13<sup>th</sup>. ed.), WC. Evans, Baillière Tindall, London.
- Ganong WF (1994). The thyroid gland in Review of medical Physiology. 17<sup>th</sup> Edn., Appleton and Lange, Philadelphia, pp.290-305
- Gupta RK, Kesari AN, Diwakarc S, Tyagia A, Tandon V, Chandraa R and Watal G (2008). *In vivo* evaluation of Anti-oxidant and Anti-lipidimic potential of annona squamosal extract in type 2 diabetic models. *Journal of Ethnopharmacology*, **118**: 21-25.
- Huiqin Guo, De-Shen Wang, Ghazala H Rizwani, Mansoor Ahmed, Maryam Ahmed, Amir Hassan, Rui-HuaXu, Najia Mansoor, Amit K Tiwari and Zhe-Sheng Chen (2013). Anti-neoplastic activity of *Holoptelea integrifolia* (Roxb.) Planch bark extracts (*in vitro*). *Pak. J. Pharm. Sci.*, **26**(6): 1151-1156.
- Indian materia medica (2000). By Dr. K.M. Nadkarni, Publisher: Bombay popular prakashan, reprinted.
- Jayshree PD and Vipin K (2008). *Annona squamosa* L: Phytochemical analysis and Anti-microbial screening. *Journal of Pharmacy Research*, **1**(1): 34-38.
- Khar Ashok (2004). Anti-tumour activity of *Annona squamosa* seed extracts is through the generation of free radicals and induction of apoptosis, Indian Journal of Biochemistry & Biophysics Vol. 41, pp. 167-172.
- Khare CP (2007). Indian medicinal plants, An Illustrated Dictionary, Springer Publication.
- Kirtikar KR and Basu BD (2001). *In: Indian Medicinal Plant*, 2<sup>nd</sup> edition, International Book Distributors, Dehradun, India.
- Neha Pandey and Dushyant Barve (2011). Phytochemical and Pharmacological review on *Annona squamosa* Linn. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, **2**(4): 1404-1412.
- Oberlies NH, Chang CJ and McLaughlin JL (1997). Structure-activity relationships of diverse annonaceous acetogenins against multi-drug resistant human mammary adenocarcinoma (MCF-7/Adr) cells. *Journal Medical Chemistry*, **40**: 2102-2106.
- Panda S and Kar A (2003). Squamosa leaf extract causing possible ammetioration of hyperthyroidism, *Current Sci.*, **84**: 1402-1404.

- Parvin Shahnaj Mst, Islam Ekramul M, Rahman Motiur Md and Haque Ekramul Md (2003). Toxicological evaluation of Annotemoyin-1 isolated from *Annona squamosa* from long evans Rats. *Pakistan Journal of Biological Sciences*, **6**(18): 1593-1596.
- Rakesh R and Mahendra S (2009). Coumarinolignans from the Seeds of *Annona squamosa* Linn. *E-journal of Chemistry*, **6**(2): 518-522.
- Sobiya R, Jannet DV, Aiyavu J and Panneerselvam C (2009). The hepatoprotective effect of alcoholic extract of *Annona squamosa* leaf on experimentally induced liver injury in Swiss albino mice. *International Journal of Integrative Biology*, **5**(3): 182-186.
- Soni H, Malik SS and Singhai AK (2012). Evaluation of leaf of aqueous extract of MeOH extract of *Annona squamosa* on cell viability American. *J. Pharm. Tech. Res.*, **2**(4): 936-944.