

Induction of apoptosis in myelogenous leukemic K562 cells by ethanolic leaf extract of *Annona muricata*

Ezirim AU¹, Okochi VI¹, James AB¹, Adebeshi OA², Ogunnowo S¹ and Odeghe, OB³

¹Department of Biochemistry, University of Lagos, Akoka, Nigeria

² Department of Cell biology, University of Lagos, Akoka, Nigeria

³Department of Biochemistry, University of Port Harcourt, Rivers State, Nigeria.
bensandym@yahoo.com*; amanda_ezirim@yahoo.com

Abstract

Prevalence of cancer, especially in Nigeria, is silently growing at exponential rates due to its nature and numerous risk factors associated with its development. Most chemotherapeutic drugs used today are expensive and toxic to normal cells, hence the need for alternative treatment options. In this present study, the efficacy of ethanolic extracts of *Annona muricata* leaves for its cytotoxicity potential and induction of apoptosis in K562 cancer cells, was investigated. Phytochemical screening verified presence of alkaloids, tannins, flavonoids, saponins, anthraquinones and cardiac glycosides. Using Neutral red uptake assay, the ethanolic extract showed peak cytotoxicity levels ($P < 0.001$) at 2.5mg/ml which decreased with increased concentrations. Caspase-3 activity was significantly enhanced ($P < 0.001$) during apoptosis induced by the extract at low quantities, with the peak activity shown at 50µg/ml. Apoptosis was confirmed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay. Caspase-3 activity and TUNEL results suggested that the ethanolic extract of *Annona muricata* induced apoptosis in the myelogenous leukemic K562 cell line. This supports the therapeutic application of *Annona muricata* to be considered as a natural product source for the development of pro-apoptotic drugs.

KEYWORDS: APOPTOSIS, K562 CELLS, ANNONA MURICATA

1. Introduction

Cancer remains one of the most dreaded diseases causing an astonishingly high death rate, second only to cardiac arrest (Shafi *et al.*, 2009). Although overall cancer incidence rates in the developing world are half those seen in the developed world in both sexes, the overall cancer mortality rates are generally similar (Jemal *et al.*, 2011). Cancer survival tends to be poorer in developing countries, most likely because of a combination of a late stage at diagnosis and limited access to timely and standard treatment (Jemal *et al.*, 2011). In 2000, approximately 256,000 children and adults around the world developed some form of leukaemia, and 209,000 died from it (Mathers *et al.*, 2001). The myeloid leukaemia are a heterogeneous group of diseases characterized by infiltration of the blood, bone marrow and other tissues by neoplastic cells of the hematopoietic system (Kasper *et al.*, 2005).

Plant medicines are considered safer and better than synthetic drugs, since the ingredients in plants such as carbohydrates, fats, proteins, vitamins and minerals are also of body composition (Kilham, 1999). The extensive repertoire of traditional medicinal knowledge systems from various parts of the world are being re-investigated for their healing properties. The fact that conventional and newly emerging treatment procedures like chemotherapy, catalytic therapy, photodynamic therapy and radiotherapy have not succeeded in reverting the outcome of the disease to any drastic extent, has made researchers investigate alternative treatment options (Shafi *et al.*, 2009).

An alternative treatment approach is the induction of apoptosis in tumour cells using herbs. Apoptosis is characterised by distinct morphologic changes, including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies (Wyllie, 1997). Upstream initiator caspases including caspase-9 activate downstream effector caspases such as caspase-3, playing a pivotal role in the induction of apoptosis by various stimuli (Wyllie, 1997). The hallmark of apoptosis is DNA fragmentation into approximately 200bp by the action of Caspase activated Dnase (CAD) (Pirmia *et al.*, 2002). This is usually assayed for ISEL, TUNEL.

Annona muricata is the most tropical semi deciduous tree with the largest fruits of the *Annona* genus. It is also native to Sub-Saharan Africa countries that lie within the tropics (Zafra-Polo *et al.*, 2002; Alali *et al.*, 1999). Traditional ethnobotanical uses in the Peruvian Amazon include its actions as a hypotensive, cardiodepressant (Carbajal *et al.*, 1991), antispasmodic, anticonvulsant (N'Gouemo *et al.*, 1997) and sedative (Vasques, 1990) agent, besides other numerous documented properties as an emetic, febrifuge,

vermifuge, nervine, decongestant, lactagogue and poison antidote (de Feo, 1992).

Phytochemically, *Annona muricata* is rich in miscellaneous lactones and isoquinoline alkaloids (Alali *et al.*, 1999). It contains many active compounds and chemicals; these are the natural phytochemicals known as *annonaceous acetogenins* (Alali *et al.*, 1999; Kojima & Tanaka, 2009). Studies have demonstrated their antihyperglycemic (Adeyemi *et al.*, 2009a), antihyperlipidemic (Adeyemi *et al.*, 2009b), antimalarial (Antoun *et al.*, 1993; Gbeassor *et al.*, 1990), antiparasitic (de Feo, 1992; Jaramilo *et al.*, 2000; Borries *et al.*, 1991), antibacterial (Khan, 1998), insecticidal (Alali *et al.*, 1998; Guadano *et al.*, 2000), molluscidal (Santos & Sant'Ana, 2001), antiviral (Antoun *et al.*, 1999) and most importantly, their anticancer properties (Zafra-Polo *et al.*, 2002; Alali *et al.*, 1999; Yang *et al.*, 2009).

Specific acetogenins in extracts of *Annona muricata* have been reported to be selectively toxic *in vitro* to certain types of tumour cells including: lung carcinoma cell lines; human breast solid tumour lines; prostate adenocarcinoma; pancreatic carcinoma cell lines; colon adenocarcinoma cell lines; mammary adenocarcinoma cell lines; liver cancer cell lines; human lymphoma cell lines; and multi-drug resistant human breast adenocarcinoma (Alali *et al.*, 1999; Yang *et al.*, 2009; Liaw, 2002).

It is generally accepted that the mode of action of acetogenins is the inhibition of NADH-ubiquinone oxidoreductase (complex I) in mitochondria. Inhibition suppresses ATP production, especially for cancer cells with high metabolic levels, leading to apoptosis (Morré *et al.*, 1995; Zeng & McLaughlin, 1996; Oberlies *et al.*, 1997).

Considering the significance of this cancer sub-type, the objective of this study was to investigate, using K562 cells, the *in vitro* apoptotic activity of the ethanolic extracts of *Annona muricata* leaves. K562 cells were the first human immortalised myelogenous leukaemia line to be established (Lozzio *et al.*, 1981). To determine the plant's antiproliferative activity, we examined the effects of the varying concentrations of ethanolic extracts of *Annona muricata* on cell viability using the neutral red uptake assay. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay and Caspase-3 activity assay.

2. Materials and method

2.1 Plant material

Fresh leaves of *Annona muricata* leaves were collected in May 2010, from Ibeme, Isiala-Mbano in Imo State of Nigeria. They were identified and authenticated by Mr. T. I. Adeleke of Pharmacognosy Department, College of Medicine, University of Lagos, Nigeria.

2.2 Preparation of organic extract

Annona muricata leaves were oven-dried for five days. The dried leaves were powdered in a warring blender and extracted with 85% ethanol using the Soxlet apparatus. The organic phase was later evaporated under reduced pressure to obtain a dried extract.

2.3 Phytochemical analysis

The crude ethanolic extract of *A. muricata* was subjected to qualitative phytochemical screening for identification of basic classes of active chemical constituents. The phytochemical analysis was done using standard methods (Trease & Evans, 1989).

2.4 Maintenance of K562 cell line

K562 cells were graciously given by Dr. Wolfgang Fisher of Ludwig-Maximilians University, Munchen, Germany. Cell lines were maintained and propagated in RPMI medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured and maintained at 37°C in a humidified atmosphere of 5% CO₂.

2.5 Cell viability assay

Cell viability was assessed using neutral red uptake assay (Borenfreund & Puerner, 1984). Neutral red, a weak cationic dye that penetrates cell membranes by non-ionic diffusion, accumulates at the anionic sites in the lysosomal matrix. Briefly, cells were seeded in 96-well tissue culture plates and tested for 6hrs with varying concentrations of 0.625mg/ml, 1.25mg/ml, 2.5mg/ml and 5.0mg/ml of the extract in triplicates. The plates were then incubated for 2hrs at with serum-free medium containing neutral red. The cells were subsequently washed, the neutral red dye was extracted from each plate and optical densities were determined at 540nm on a multi-well spectrophotometer.

2.6 Caspase 3 assay

Apoptosis was induced for 12hrs and a negative control set at the same time. The cells were collected, washed twice with PBS

(phosphate buffer saline) and to a required number of collected cells; a fresh cold prepared lysis buffer was added. The cells were incubated on ice for 20-60 minutes, and vortexed three to four times for 10 seconds each time following centrifugation at 10,000 rpm at 4°C for one minute. A small quantity of supernatant was obtained to assay the protein concentration by the Bradford method. To assess Caspase-3 activity, the Caspase-3 Colorimetric Assay Kit (Genscript, USA) was used following the instructions of the manufacturer. The Caspase-3 Colorimetric Assay Kit is based on the spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labelled substrate DEVD-*p*NA. The *p*NA was quantified at 405nm.

2.7 TUNEL assay

To evaluate apoptosis in the cells, the TUNEL Apoptosis Detection Kit (Genscript, USA) was used following the instructions of the manufacturer. Cells were exposed to the organic extract on chamber slides for 12hrs and then fixed with a fixation solution (4% paraformaldehyde in PBS, pH 7.4, freshly prepared) for 1hr at 15-25°C. The slides were rinsed thrice with PBS, and incubated subsequently on ice following procedure with blocking solution (3% H₂O₂ in methanol) and permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate in water, freshly prepared). Before beginning the labelling procedures, the fixed and permeabilized cells were incubated with 100 µl DNase I Solution (500U/ml DNase I [grade 1] in 1X DNase buffer) for 10 minutes at 15-25°C to induce DNA strand degradation. During the labelling procedure, biotinylated nucleotide is labelled at the DNA 3'-OH ends using the recombinant Terminal Deoxynucleotidyl Transferase (rTDT); then horseradish peroxidase-labelled Streptavidin (Streptavidin-HRP) is bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide and 3,3'-Diaminobenzidine (DAB), a stable chromogen. Using this procedure, apoptotic nuclei are stained dark brown.

2.8 Statistical analysis

Graphpad Prism 5.0 was used for data analysis. The results of each series of experiments (performed in triplicates) are expressed as the mean ± standard error of mean (SEM). The significance of difference in the means of all parameters reported was determined using ANOVA and Bonferroni Post Hoc test.

3. Results

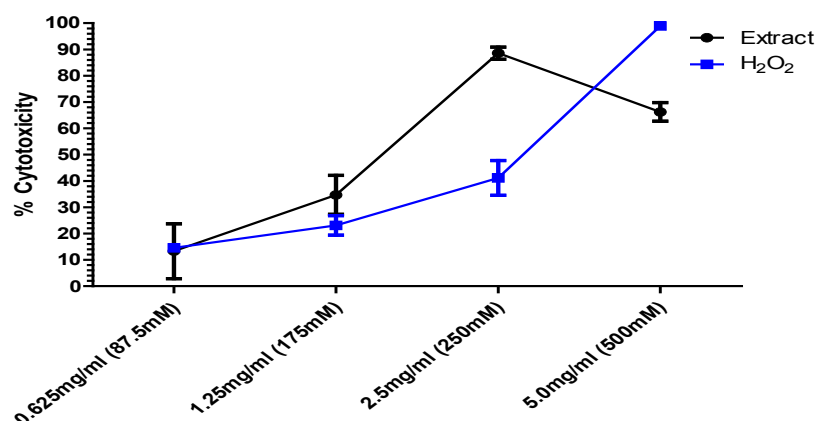
3.1 Phytochemical analysis

The phytochemical screening of the ethanolic leaf extract of *Annona muricata* revealed strong presence of alkaloids, flavonoids, tannins – hydrolysable and condensing tannins – anthraquinone glycosides and saponins; and weak traces of cyanogenic glycosides and cardiac glycosides.

3.2 Neutral red uptake analysis

Cytotoxicity test was carried out on the ethanolic extract of *Annona muricata* leaves via neutral red uptake method in a dose-dependent manner, shown in Fig.1. Maximum cytotoxicity of the extract on K562 cells were observed at 2.5mg/ml, which revealed a significant increase ($P<0.001$) compared to the positive control (H₂O₂). At higher concentrations of the control, a significant decrease ($P<0.01$) in cytotoxicity was detected of the ethanolic extract.

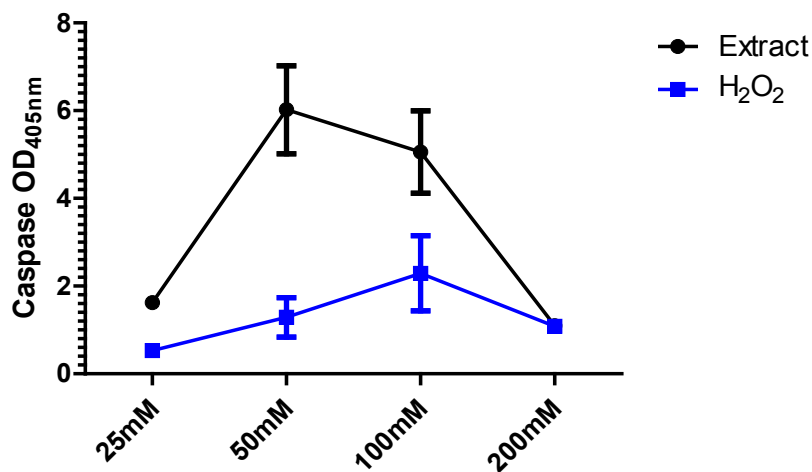
Fig.1. Neutral red uptake cytotoxicity assay of ethanolic leaf extract of *A.muricata*



3.3 Caspase-3 assay

The results, shown in Fig.2, showed a steady increase in Caspase-3 activity, attaining its peak at 50 μ g/ml which was highly significant ($P<0.001$) in comparison to the positive control. A decline in caspase-3 activity was evident with increased concentrations but still showed significant ($P<0.05$) values.

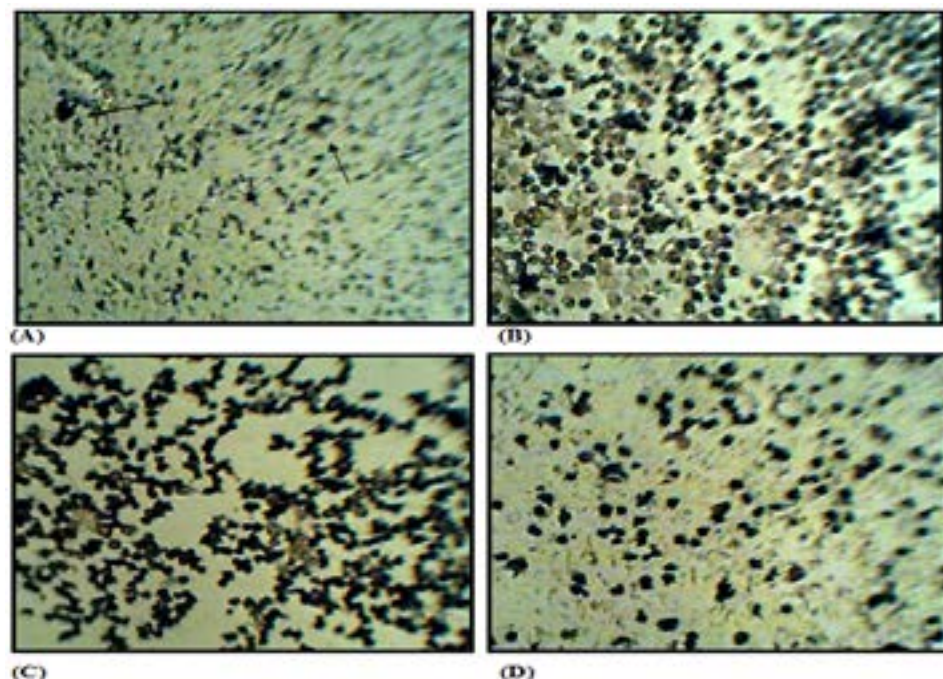
Fig.2. Plot of absorbance against concentration of Caspase-3 activity during apoptosis of K562 cells induced by leaf ethanolic extract of *Annona muricata*.



3.4 TUNEL assay

The TUNEL assay (TdT-mediated dUTP Nick End Labelling) was developed as a method to identify individual cells that are undergoing apoptosis by labelling the ends of the degrading DNA with the polymerase terminal deoxynucleotidyl transferase (TdT) (Heatwole, 1999). After the induction with 0.625mg/ml, 1.25mg/ml, 2.5mg/ml and 5.0mg/ml ethanolic extracts of *Annona muricata*, K562 cells were stained dark brown under the light microscope. This observation, as shown in fig.3, was assessed in comparison with results obtained from the positive control (DNase 1 solution). The results confirm that apoptosis had taken place in K562 cells when induced with the leaf ethanolic extract of *Annona muricata*.

Fig.3. TUNEL assay results after the induction of apoptosis in K562 cell line (x100). Arrows indicate artefacts.



- (A) Negative Control
 (B) Hydrogen Peroxide (Positive inducer)
 (C) DNase I (Positive control)
 (D) 1.25mg/ml ethanolic extract of *Annona muricata*

4. Discussion and conclusion

The phytochemical investigation on ethanolic leaf extract of *Annona muricata* confirmed its diverse traditional ethnobotanical uses derived from its long recorded indigenous use and recent documented medicinal properties (de Feo, 1992).

Cytotoxic drugs that exert their maximum cytotoxicity on cancer cells during the S-phase of the cycle prevent cells from progressing through the cell cycle to the S-phase; this is accomplished by sublethal inhibition of RNA and protein synthesis (Craig & Stitzel, 2003). Studies have shown that cancer cells at the S phase of their cell cycle are more vulnerable to the compounds that have been, and are still being, isolated from *Annona muricata* called *Annonaceous acetogenins* (Yuan, 2003).

In this present study, it is clearly shown that *Annona muricata* has cytotoxic effects on the myelogenous leukemic K562 cell line. Maximum cytotoxicity of the extract on K562 cells were observed at 2.5mg/ml, which revealed a significant difference ($P < 0.001$) to the corresponding molarity of the positive control. This cytotoxic property decreased significantly ($P < 0.01$) at higher concentrations of the control. This observation concurs with some clinical findings which have suggested that at very low dosages, *annonaceous acetogenins* of *Annona muricata* exhibited highly toxic effects particularly to ovarian, breast, cervical, bladder and skin cancer cell lines (Alali, et al., 1999). Another study observed that some of the derivatives within the different structural types and some positional isomers showed remarkable selectivities among certain cell lines (Yang, et al., 2009). Lack of selective toxicity is the major limiting factor in the chemotherapy of cancer (Craig & Stitzel, 2003).

The TUNEL assay results clearly shows that apoptosis had been induced by the ethanolic leaf extract of *Annona muricata* on the myelogenous leukemic K562 cell line. This outcome was compared with results obtained from the positive control (DNase 1 solution), as apoptotic nuclei was stained dark brown.

Furthermore, the results of the Caspase-3 activity further substantiated that apoptosis had taken place. The activation of Caspase-3 is an important downstream event in apoptosis (Crow, et al., 2004). Increased caspase-3 activity was noted to be directly proportional to increased concentration of the ethanolic extract of *Annona muricata* reaching a peak at 50µg/ml. The least (25mM) and the highest (200mM) concentrations of protein obtained during the apoptosis induced by the extract, showed no significant difference ($P > 0.05$) to the control.

Great progress has been made in the understanding of the basic mechanisms of apoptosis and the gene products involved (Wyllie, 1997). The regulation of apoptosis in normal and malignant cells has become an area of intensive study in cancer research (Johnstone, et al., 2000). Agents that suppress the proliferation of malignant cells by inducing apoptosis may represent a useful mechanistic approach to both cancer chemoprevention and chemotherapy (Khan & Mlugwana, 1999).

It is evident in this present study that the significant increase in Caspase-3 activity and TUNEL assay results suggest that the ethanolic leaf extract of *Annona muricata* had induced apoptosis in myelogenous leukemic K562 cell line.

5. References

- 1• Adeyemi OD, Komolafe OA, Adewole OS, Obuotor EM and Adenowo KT (2009a) Anti Hyperglycemic activities of *Annona muricata*. *Afr. J. Tradit. Complement Altern. Med.* 6(1), 62-69
- 2• Adeyemi OD, Komolafe OA, Adewole OS and Obuotor EM (2009b) Anti Hyperlipidemic activities of *Annona muricata*. *The Internet J. Alternative Med.* 7, 1
- 3• Alali FQ, Kaakeh W, Bennett GW and McLaughlin JL (1998) Annonaceous acetogenins as natural pesticides; potent toxicity against insecticide-susceptible and resistant german cockroaches (Dictyoptera: blattellidae). *J. Econ. Entomol.* 91(3), 641-649
- 4• Alali FQ, Liu XX and McLaughlin JL (1999) Annonaceous acetogenins: recent progress. *J. Nat. Prod.* 62(3), 504-540
- 5• Antoun MD, Gerena L, and Milhous WK (1993) Screening of the flora of Puerto Rico for potential antimalarial bioactives. *Int. J. Pharmacog.* 31(4), 255-259
- 6• Antoun MD, Martinez E, Caballero R, Oquendo I, Proctor GR, Weislow OS, McCloud TG, Kiser R, Staley P, and Clanton D (1999) Evaluation of the flora of Puerto Rico for *in vitro* cytotoxic and anti-HIV activities. *Pharmaceutical Biol.* 37(4), 277-280
- 7• Borenfreund E and Puerner JA (1984) A simple quantitative procedure using monolayer culture for toxicity assays. *J. Tissue Cult. Meth.* 9,7-9
- 8• Bories C, Loiseau P, Cortes D, Myint SH, Hocquemiller R, Gayral P, Cavé A and Laurens A (1991) Antiparasitic activity of *Annona muricata* and *Annona cherimolia* seeds. *Planta Med.* 57(5), 434-436
- 9• Carbajal D, Casaco A, Arruzazabala L, Gonzalez R and Fuentes V (1991) Pharmacological screening of plant decoctions commonly used in Cuban folk medicine. *J. Ethnopharmacol.* 33(1/2), 21-24

- 10• Craig R and Stitzel H (2003) *Modern pharmacology with clinical applications*. 5th ed. Lippincott Williams and Wilkins edition. pp: 631-633
- 11• Crow MT, Mani K, Nam YJ and Kitsis RN (2004) The mitochondrial death pathway and cardiac myocyte apoptosis. *Circ. Res.* 95(10), 957-970
- 12• de Feo V (1992) Medicinal plants of the Andes Peru. *Fitoterapia* 63,417-440
- 13• Gbeassor M, Kedjagni AY, Koumaglo K, de Souza C, Agbo K, Aklikokou K, and Amegbo KA (1990) *In vitro* antimalarial activity of six medicinal plants.” *Phytother. Res.* 4(3), 115-117
- 14• Guadano A, Carmen G, Eduardo P, Cortes D and González-Coloma A (2000) “Insecticidal and mutagenic evaluation of two annonaceous acetogenins.” *J. Nat. Prod.* 63(6), 773-776
- 15• Heatwole VM (1999) TUNEL Assay for apoptotic cells. *Mol.Biol.* 115 (111), 141-148.
- 16• Jaramillo MC, Arango GJ, González MC, Robledo SM and Velez ID (2000) Cytotoxicity and antileishmanial activity of *Annona muricata* pericarp *Fitoterapia.* 71(2), 183-186
- 17• Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D (2011) Global cancer statistics. *CA Cancer J. for clinicians* 61 (2), 134
- 18• Johnstone RW, Ruefli AA and Lowe SW (2000) Apoptosis: A link between cancer genetics and chemotherapy. *Cell* 108, 153-164
- 19• Kasper DL, Braunwald E, Fauci AS, Hauser SL, Longo DL and Jameson JL (2005) *Harrison's Principle of Internal Medicine*. 16th ed. McGraw-Hill companies, Inc. USA. pp: 630
- 20• Khan MR (1998) Antibacterial activity of some annonaceae Part I. *Fitoterapia* 69(4), 367-369
- 21• Khan MR and Mlungwana SM (1999) C-sitosterol, a cytotoxic sterol from *Markhamia zanzibarica* and *Kigelia africana*. *Fitoterapia* 70, 96-97
- 22• Kilham EL (1999) Evaluating A Health Service at a primary care clinic in Chilamanca, Bolivia. *Social Sci. Med.* 49, 663-678
- 23• Kojima N and Tanaka T (2009) Medicinal chemistry of *Annonaceous* Acetogenins: Design, synthesis, and biological evaluation of novel analogues *Molecules* 14, 3621-3661
- 24• Liaw CC (2002) New cytotoxic monotetrahydrofuran Annonaceous acetogenins from *Annona muricata*. *J. Nat. Prod.* 65(4), 470-475
- 25• Lozzio BB, Lozzio CB, Bamberger EG, Feliu AS (1981) A multipotential leukaemia cell line (K-562) of human origin. *Proc. Society for Experimental Biol. & Med.* 166, 546-550
- 26• Mathers CD, Boschi-Pinto C, Lopez AD and Murray CJL (2001) Cancer incidence, mortality and survival by site for 14 regions of the world. World Health Organization. <http://www.who.int/entity/healthinfo/paper13.pdf>
- 27• Morré DJ, de Cabo R, Farley C, Oberlies NH and McLaughlin JL (1995) Mode of action of bullatacin, a potent antitumor acetogenin: Inhibition of NADH oxidase activity of Hela and HL-60, but not liver, plasma membranes. *Life Sci.* 56, 343-348
- 28• N’Gouemo P, Koudogbo B, Tchivounda PH, Akono-Nguema C and Etoua MM (1997) Effects of ethanol extract of *Annona muricata* on pentylenetetrazol-induced convulsive seizures in mice. *Phytother. Res.* 11(3), 243-245
- 29• Oberlies NH, Croy VL, Harrison ML and McLaughlin JL (1997) The annonaceous acetogenin bullatacin is cytotoxic against multidrug-resistant human mammary adenocarcinoma cells. *Cancer Lett.* 115, 73-79
- 30• Pirnia F, Schneider E, Betticher DC and Borner MM (2002) Mitomycin C induces apoptosis and caspase-8 and -9 processing through a caspase-3 and Fas-independent pathway. *Cell Death Differ.* 9(9),905-914
- 31• Santos AF and Sant’Ana AEG (2001) Molluscicidal properties of some species of *Annona*. *Phytomedicine* 8(2), 115-120
- 32• Shafi G, Anjana M, Hasan T, Alshatwi A and Jyothy A and Lei DK (2009) Induction of apoptosis in HeLa cells by chloroform fraction of seed extracts of *Nigella Sativa*. *Cancer cell Int.* 9,29
- 33• Trease and Evans (1989) *Pharmacognosy*. 14th ed. Evans edition, WB Saunder company Ltd. London.
- 34• Vasques MR (1990) Useful plants of Amazonian Peru. Second Draft Field with USDA’s National Agricultural Library. USA
- 35• Wyllie AH (1997) Apoptosis: An overview. *British Medical Bulletin* 53, 451-465

- 36•** Yang H, Zhang N, Li X, Chen J and Cai B (2009) Structure-activity relationships of diverse annonaceous acetogenins against human tumor cells. *Bioorg. Med. Chem. Lett.* 19 (8), 2199-2202
- 37•** Yuan SF (2003) Annonacin, a mono-tetrahydrofuran acetogenin, arrests cancer cells at the G1 phase and causes cytotoxicity in a Bax- and caspase-3-related pathway. *Life Sci.* 72, 2853-2861
- 38•** Zafra-Polo MC, Figadère B, Gallardo T, Tormo JR and Cortes D (1998) Natural acetogenins from annonaceae, synthesis and mechanisms of action. *Phytochem.* 48: 1087-1117
- 39•** Zeng L and McLaughlin JL (1996) Recent advances in Annonaceous acetogenins. *Nat. Prod. Rep.* 13(4), 275-306