Methanolic extract of *Anthocephalus cadamba* induces apoptosis in Ehrlich ascites carcinoma cells in experimental mice

Narayan Dolai, Aminul Islam¹, Pallab Kanti Haldar

**Abstract:**

Objective: *Anthocephalus cadamba* (Roxb.) Miq. (Family: Rubiaceae), a folk medicine commonly known as “Kadam” in Bengali, has been used for the treatment of tumor. The methanolic extract of *A. cadamba* (MEAC) showing antitumor activity on Ehrlich ascites carcinoma (EAC) cells treated mice was already reported. This study was designed to study the apoptosis-inducing property of MEAC and its mechanism in EAC cells in mice.

Materials and Methods: Apoptogenic morphology was determined by fluorescent DNA-binding double staining method using dyes acridine orange (AO)/ethidium bromide (EB). Comet assay was estimated to check the DNA damage. Flow cytometry (fluorescence-activated cell sorting [FACS]) was used to detect the apoptotic rate quantitatively by double labeling techniques using annexin V FITC/propidium iodide staining. Apoptotic protein expression was done using Western blotting assay method.

Statistical Analysis: Results are expressed as mean ± standard deviation. Statistical analysis was performed using ANOVA followed by Dunnett’s *post hoc* test of GraphPad Prism software. *P* < 0.05, **P** < 0.01 and ***P*** < 0.001 were considered statistically significant.

Results: Apoptosis-inducing effect of MEAC on EAC cells was confirmed from AO/EB staining and FACS analysis. MEAC treatment showed dose-dependent induction of DNA damage. Apoptosis was induced by increasing the expression of multiple downstream factors such as pro-apoptotic protein p53 and p21 in EAC. Bax was up-regulated and anti-apoptotic protein Bcl-2 was down-regulated resulting in decrease of the Bcl-2/Bax ratio by MEAC treatment.

Conclusion: Experimental results revealed that MEAC induces apoptosis by modulating the expression of some pro-apoptotic and anti-apoptotic proteins in EAC and thus exerts its anti-tumor activity.

Key words: Apoptosis, comet assay, flow cytometry, Kadamba, Western blots

Apoptosis is a highly coordinated programmed cell death for removing unwanted cells from the system during organ development, tissue remodeling, and immune responses. Cancer is specifically due to unconstrained proliferations of tumor cells and a loss of their apoptosis. Compounds that induce apoptosis can prevent or suppress the proliferation of tumor cells and thus are considered to have potential as antitumor agents. Recently, considerable focus has been on identifying naturally occurring chemopreventive compounds capable of inhibiting, retarding or reversing the process of multistage carcinogenesis. Hence, the primary objective of chemotherapy is to obliterate cancer cells along with a secondary goal to induce apoptosis for its wipe out from the system quickly and quietly by neighboring phagocytic cells.¹²

The folk medicine, *Anthocephalus cadamba* (Roxb.) Miq. (Family: Rubiaceae) is commonly known as “Kadam” in Bengal and is distributed throughout the greater part of India in the moist deciduous evergreen forests.¹¹ This medicinal plant has been used for the treatment of tumor, fever, hematological diseases, uterine complaints, skin diseases, hypoglycemic agent, reduces pain, and inflammation.¹⁴ Early reports from bioactivity determination provided evidence for its cytotoxic effect on human cancer cell lines,¹⁵ free radical scavenging and anti-inflammatory.¹⁶

**How to cite this article:** Dolai N, Islam A, Haldar PK. Methanolic extract of *Anthocephalus cadamba* induces apoptosis in Ehrlich ascites carcinoma cells in experimental mice. Indian J Pharmacol 2016;48:445-9.
antidiabetic,[9] antioxidant, antimicrobial, and wound healing activities.[10] The stem bark has a wide range of chemical constituents, namely, cadamine, isocadamine, cadambine, 3α-dihydrocadambine, isodihydrocadambine, and chlorogenic acid.[11] The antitumor activity of methanol extract of *A. cadamba* (MEAC) on Ehrlich ascites carcinoma (EAC) cells treated mice was already reported.[12] However, its mechanism is not clearly defined. Hence, in this study was performed to establish the apoptogenic effects of MEAC on EAC cells treated mice and its mechanism.

**Materials and Methods**

**Plant Materials and Preparation of Extracts**

We collected the stem bark of *A. cadamba* from middle hill region of Sikkim (in the month of September) which was authenticated by the Botanical Survey of India, Gangtok, India (Authenticated No: SHRC–5/5/2010/Tech. 47A). The stem bark was shade dried at room temperature for 7 days and then powdered in a mechanical grinder. The extraction of powdered plant material (1 kg) was performed using a Soxhlet extraction apparatus in petroleum ether (60–80°C) succeeded by methanol. In a rotary evaporator, the solvent was completely evaporated in reduced pressure. The petroleum ether (PEAC; 80 g; 8% w/w) and methanol extract (MEAC; 200 g; 20% w/w) were collected separately. The concentrated extracts were sealed in a glass beaker and stored at 20°C for further use.

**Animals**

Swiss albino mice (20–25 g) of 8 weeks of age were used for the experiment and were kept in polycrylic cages (38 cm × 23 cm × 10 cm). The animals (mice) were grouped with not more than six animals per cage. In standard laboratory conditions with the temperature of 25–30°C, relative humidity of 55–60% and with the dark/light cycle of 14/10 h, the animals were maintained. The free access of standard dry pellet diet and water *ad libitum* was provided. The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All the described procedures were reviewed and approved by University Animal Ethics Committee (367001/C/CPCSEA).

**Acute Toxicity and Dose Calculation**

The OECD guideline 425 (2008) was followed to evaluate the acute toxicity of MEAC in Swiss albino mice. The extract was safe up to the dose of 2 g/kg b.w. per oral for mice.[13]

**Cell Culture**

We obtained EAC cells from Chittaranjan National Cancer Institute, Kolkata, India. The EAC cells were maintained in *vivo* in Swiss albino mice by intraperitoneal transplantation of 2 × 10⁶ cells per mouse after every 10 days and it is used for the present experiment.[10]

**Cell Viability Assay**

Cell viability of MEAC was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[11] In brief, 0.1 ml of EAC cell suspension was seeded in 96-well plates (Greiner, Frickenhausen, Germany) with a seeding density of 1 × 10⁴ cells/well. The cells were treated with MEAC (25–200 µg/ml), incubated for 24 h at 37°C, 5% CO₂ with 98% relative humidity. After incubation, 20 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) were added to each well and again incubated continuously at 37°C for 4 h. The colored formazan crystals which were produced from MTT were dissolved in 150 µl of dimethyl sulfoxide, and the absorbance was measured at a wavelength of 570 nm by ELISA plate reader. Concentrations of MEAC showing a 50% reduction in cell viability (i.e., IC₅₀) values were calculated.

**Tumor Model**

Swiss albino mice were divided into three groups (*n* = 12). Group I served as EAC control and Group II–III served as treated group. Mice in each group were being injected EAC cells (2 × 10⁶ cells/mouse, i.p.) which were marked as day “0.” After 24 h, EAC transplanted Group II and III were being injected MEAC (200 and 400 mg/kg b.w., i.p.) once daily for 14 consecutive days.[12] After administration of the last dose, the mice were kept fasting for 18 h and then sacrificed for collection of EAC cells from the peritoneal cavity to check the apoptogenic properties of MEAC.

**Fluorescence Morphological Study**

The morphology of apoptotic and necrotic cells was detected using acridine orange (AO) and ethidium bromide (EB) staining method.[13] EAC cells (1 × 10⁶) were collected from sacrificed mice. The cells were detached, washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 5 min. The stained cells were observed with a fluorescence microscope (Leica DM 3000, Germany) at 40X magnifications.

**DNA Damage by Comet Assay**

The DNA damage was quantified by alkaline single cell gel electrophoresis, also known as comet assay.[14] EAC cells were suspended in 0.5% (w/v) low melting agarose followed by layered over a frosted microscopic slide coated with a layer of 1% normal melting agarose. The slides were soaked in a lysing solution overnight at 4°C. Electrophoresis was performed for 30 min (280 mA, 20 V) at 4°C. The slides were washed thrice with neutralizing buffer (Tris 0.4 M, pH 7.5), stained with EB, examined under a fluorescence microscope (Leica DM3000, Germany) and subjected to image analysis using CometScore software.

**Flow Cytometric Analysis (Fluorescence-activated Cell Sorting)**

Apoptotic and necrotic cells were differentiated, in a double labeling system, EAC cells from tumor-bearing mice were washed twice with cold PBS and then re-suspended in 1X binding buffer at a concentration of 1 × 10⁶ cells/ml. Then, 100 µl of the cell suspension was transferred to the 5 ml culture tube and 25 µl of annexin V-FITC and/or propidium iodide (PI) solution added to the cell suspension. The cells were gently mixed by vortexing and incubated for 15 min at 37°C. Then, 400 µl of 1X binding buffer was added to each tube and analyzed by fluorescence-activated cell sorting (FACS) within 1 h using flowcytometer (BD LSRFortessa™ Cell analyzer, USA).[15]

**Apoptotic of Protein Expression by Western Blotting**

EAC lysate was weighed into a 10% sodium dodecyl sulphate-polyacrylamide gel. After electrophoresis, the gel was transferred to nitrocellulose membrane and blocked with 5% bovine serum albumin in 1X Tris-buffered saline. The
membrane was then incubated with specific primary antibodies of p53, p21, Bax, Bcl-2 and β-actin (1:1000) for overnight at 4°C. The protein of interest was visualized by treating with alkaline phosphatase conjugated specific secondary antibody. Chromogenic substrate bromochloroindolyl phosphate and nitroblue tetrazolium were used for the detection of protein expression level. The protein in each lane was equally loaded for β-actin antibody probing.[20]

**Statistical Analysis**

Results are expressed as mean ± standard deviation. Statistical analysis was performed using ANOVA, followed by Dunnett’s post hoc test of GraphPad Prism software (Version 5.0, GraphPad Prism Software Inc., San Diego, CA). *P < 0.05, **P < 0.01 and ***P < 0.001 were considered statistically significant.

**Results**

**Cell Viability Assay**

The mortality of EAC cells by MEAC was dose-dependent and the IC_{50} value was found to be 82.65 ± 2.54 µg/mL.

**Fluorescence Morphological Study**

The morphological changes associated with apoptosis were observed in EAC cells after MEAC treatment by AO/EB double staining method. AO is taken up by both viable and nonviable cells. Green fluorescence is emitted when the dye is intercalated double-stranded nucleic acid (DNA) or red fluorescence when bound to single-stranded nucleic acid (RNA). EB is taken up only by non-viable cells, and red fluorescence is emitted when intercalated into DNA. Figure 1a shows that, green nuclei and uniform chromatin with intact cell membrane in live EAC control cells. When treated with MEAC at doses 200 and 400 mg/kg, it was seen that the number of apoptotic cells 39.3% ± 2.52% and 43.6% ± 1.82% after MEAC treatment and was compared with that of untreated control (2.05 ± 0.45 µm) group [Figure 2d].

**DNA Damage by Comet Assay**

Treatment dependent DNA damage in EAC cells was observed after MEAC treatment and was compared with that of untreated EAC control [Figure 2a]. MEAC-induced DNA damage even at a low concentration (200 mg/kg), is indicated by the presence of the DNA tail [Figure 2b]. The tail length (distance from DNA head to the end of DNA tail) indicates the DNA damage which was treated with 400 mg/kg doses [Figure 2c]. Experimental results revealed that MEAC induced substantial DNA damage which was evident from the appearance of comet length 9.0 ± 1.50 and 12.1 ± 2.93 µm, respectively, in MEAC at the doses 200 and 400 mg/kg, which was more than that in the untreated control (2.05 ± 0.45 µm) group [Figure 2d].

**Flow Cytometric Analysis (Fluorescence-activated Cell Sorting)**

Cells stained with annexin V-FITC and PI were classified as necrotic cells (the upper left quadrant; annexin−/PI+), late apoptotic cells (the upper right quadrant; annexin+/PI+), intact cells (the lower left quadrant; annexin−/PI−) or early apoptotic cells (the lower right quadrant; annexin+/PI−). The results showed quantitatively induce apoptosis by MEAC on EAC cells [Figure 3a–c]. Apoptosis ratios by MEAC at the doses 200 and 400 mg/kg were found to be 41.9% and 47.7%, respectively.

**Apoptotic of Protein Expression by Western Blotting**

Expression of p53, p21, Bax (pro-apoptotic protein), and Bcl-2 (anti-apoptotic protein) in our mice model was examined to find out the mechanism behind the apoptosis of MEAC on EAC cells. The significant up-regulation in the levels of p53, p21, and Bax protein expression and down-regulation of Bcl-2 were observed after MEAC treatment group [Figure 4a] resulting in Bcl-2/Bax ratio to be significantly lowered following MEAC treatment [Figure 4b].

**Discussion**

*A. cadamba* have been used for the antitumor purpose in folklore medicine, possesses cytotoxic effect on the EAC cells in a concentration-dependent manner. The EAC cell tumor implantation induces per se a local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration, progressive ascitic

![Figure 1: Methanolic extract of A. cadamba induces apoptotic morphological changes on Ehrlich ascites carcinoma cells. (a) Ehrlich ascites carcinoma control, (b) methanolic extract of A. cadamba 200 mg/kg and (c) methanolic extract of A. cadamba 400 mg/kg. The cells were stained with acridine orange/ethidium bromide (100 µg/mL). Blue arrows next to “L” point to live cells; yellow arrows next to “A” indicate apoptotic cells; and red arrows next to “N” indicate necrotic cells (40X)](image)

![Figure 2: The DNA damage was measured by comet assay after methanolic extract of A. cadamba treatment on Ehrlich ascites carcinoma cells. (a) Ehrlich ascites carcinoma control, (b) methanolic extract of A. cadamba 200 mg/kg and (c) methanolic extract of A. cadamba 400 mg/kg. (d) The extent of DNA damage was expressed in terms of comet % tail length. Data are the mean ± standard deviation from three replicate measurements. Treated groups versus Ehrlich ascites carcinoma control group, ***P < 0.001)](image)
Dolai, et al.: Methanolic extract of A. cadamba induces apoptosis on EAC cells

Fluid formation on EAC cells treated mice. The experimental results of which presumably indicate either a direct cytotoxic effect or an indirect local effect, involving activation of macrophage and inhibition of vascular permeability. Accordingly, to the Singh et al., the cytotoxic effect of the chloroform extract of A. cadamba on different human cancer cell line indicated that anti-proliferation pathway leading to cancer cell death may be due to the presence of indole-based phytoconstituents. However, the detailed mechanism by which it possesses the cytotoxic activity is still elusive and need to be elucidated. Hence, in this study was conducted to understand the mechanism by which MEAC-induced apoptosis in the EAC cells.

Alteration in morphological features of apoptosis after MEAC treatment was analyzed using fluorescence microscopic analysis. The discernment among healthy, apoptotic and necrotic cells are made by the color change of nuclei with chromatin integrity ranging from green nuclei and uniform chromatin in intact cell membrane in healthy cells, orange or green nuclei with condensed chromatin in apoptotic cells and red nuclei with damaged cell membrane in necrotic cells. As shown in Figure 1, MEAC (200 and 400 mg/kg) treated cells were mostly apoptotic with green or orange fragmented nuclei (39.3% ±2.52% and 43.6% ±1.82%) which were also in accordance with relatively low cell viability.

DNA damage in proliferating cells activates a pathway that arrests cell division to allow either DNA repair or induction of cell death by apoptosis. Under alkaline conditions, necrotic or apoptotic cells can result in comets with small or nonexistent head and large diffuse tails (called a “hedgehog” comet) as observed on EAC cells after treatment with MEAC. The presence of the DNA tail, which indicated DNA damage induced by MEAC, was evident from the appearance of comet tail length (9.0 ± 1.50 and 12.1 ± 2.93 µm) at the doses 200 and 400 mg/kg. Double labeling techniques were performed using annexin V FITC/PI staining followed by FACS to distinguish between apoptotic and necrotic cells and to investigate the percentage of cells undergoing apoptosis by MEAC. Annexin V binds to phosphatidylserine (PS) in a calcium-dependent manner. On the intracellular leaflet of the plasma membrane abundance of PS were usually found in healthy cells. The lost in the membrane asymmetry during early apoptosis phase which induces the PS to translocate in the external leaflet. Annexin V labeled with fluorochrome targets apoptotic cells and necrotic cells were excluded as PI unable to bind. The intercalation of the dyes to DNA resulted in positive staining at the late stage of apoptotic and necrotic cells. The flowcytometric data revealed that treatment with different doses of MEAC-induced cell death by two distinct modes – apoptosis and necrosis, which can be distinguished by morphological and biochemical features. Annexin V-FITC/PI staining of MEAC treated EAC cells resulted in an increase in annexin⁻/PI⁻ and annexin⁻/PI⁺ cells compared to the control (annexin⁻/PI⁻), indicating apoptosis as a possible mode of cell death.

Figure 3: Flowcytometric detection of Ehrlich ascites carcinoma apoptosis after methanolic extract of A. cadamba treatment to detect increases the apoptotic rates of cells. (a) Ehrlich ascites carcinoma control, (b) methanolic extract of A. cadamba 200 mg/kg and (c) methanolic extract of A. cadamba 400 mg/kg. Quadrants: Lower left, live cells; lower right, apoptotic cells; upper right, necrotic cells.

Figure 4: (a) Western blots analysis of pro-apoptotic proteins p53, p21, Bax and anti-apoptotic protein Bcl-2 and visualized by alkaline phosphatase-conjugated secondary antibody. The β-actin band confirmed equal protein loading. (b) Quantitative expression and ratio of Western blots, data are the mean ± standard deviation from three replicate measurements. Treated groups versus Ehrlich ascites carcinoma control group, *P < 0.05 and **P < 0.01.
The commencement of apoptosis depends on the balance between proteins that mediate growth arrest and cell death, for example, p53, p21, Bax and proteins that promote cell viability, for example, Bcl-2. Our data clearly revealed that MEAC treatment to EAC cells resulted in a dose-dependent increased in the level of Bax with a concomitant decrease in Bcl-2 levels and decreased in Bax/Bcl-2 ratio.[13] The significant up-regulation of p53 indicates its role to facilitate apoptosis in the EAC cell. The up-regulation of Bax might be associated with p53 activation. p53-dependent induction of p21 restricts entry of cell in S phase, which is indicated by an increase in p21 level in MEAC treated group. p53, the tumor suppressor gene, act as a transcription factor by binding with p53-specific DNA consensus sequence of responsive genes, which would be expected to increase the synthesis of p21.[21] It was evident from various studies, that up-regulation of cell growth-regulating genes, on p53 induction, may block the cell cycle but increased expression of pro-apoptotic factors can override the growth-arresting message and thereby ultimately lead to apoptosis.

**Conclusion**

The earlier study as well as present investigation indicates that the MEAC exhibit potential antitumor activity. The tumor suppressing mechanism involved the induction of apoptosis and/or cell death followed due to DNA damage property of the MEAC. Further, we proved that MEAC could induce apoptosis in EAC cells through modulation of balance between pro- and anti-apoptotic proteins. The apoptosis inducing property of MEAC on EAC cells in animal model suggest the development of a chemopreventive agent active in cancer therapy.

**Acknowledgments**

The authors greatly acknowledge Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India for technical support.

**Financial Support and Sponsorship**

This research work was financially supported by the University Grant Commission – Rajiv Gandhi National Fellowship under grant (number: F1-17.1/2011-12/RGNF-SC-WES-1705/ [SA-III/Website]), Government of India.

**Conflicts of Interest**

There are no conflicts of interest.

**References**

1. Chatterjee S, Biswas G, Chandra S, Saha GK, Acharya K. Apoptogenic effects of *Tricholoma giganteum* on Ehrlich’s ascites carcinoma cell. Bioprocess Biosyst Eng 2013;36:101-7.
2. Brunelle JK, Zhang B. Apoptosis assays for quantifying the bioactivity of anticancer drug products. Drug Resist Updat 2010;13:172-9.
3. Umachigl SP, Kumar GS, Jayaveera K, Kishore KD, Ashok GC, Dhanapal R. Antimicrobial, wound healing and antioxidant activities of *Anthocephalus cadamba*. Afr J Tradit Complement Altern Med 2007;4:481-7.
4. Alam MA, Subhan N, Chowdhury SA, Awal MA, Mostofa M, Rashid MA, et al. *Anthocephalus cadamba* extract shows hypoglycemic effect and eases oxidative stress in alloxan-induced diabetic rats. J Ethnopharmacol 2011;121:155-64.
5. Dolai N, Karmakar I, Suresh Kumar RB, Kar B, Bala A, Haldar PK. Evaluation of antitumor activity and *in vivo* antioxidant status of *Anthocephalus cadamba* on Ehrlich ascites carcinoma treated mice. J Ethnopharmacol 2012;142:865-70.
6. Singh S, Ishar MP, Saxena AK, Kaur A. Cytotoxic effect of *Anthocephalus cadamba* Miq. leaves on human cancer cell lines. Pharmacog J 2013;5:127-9.
7. Dolai N, Karmari U, Islam A, Haldar PK. Inhibitory effects of *Anthocephalus cadamba* stem bark fractions intercede anti-inflammatory and carbon tetrachloride induced hepatotoxicity in rats. Orient Pharm Exp Med 2015;15:123-34.
8. Acharyya S, Dash GK, Mondal S, Dash SK. Studies on glucose lowering efficacy of the *Anthocephalus cadamba* (Roxb.) Miq. roots. Int J Pharm Bio Sci 2010;1:1-9.
9. Kapil A, Koul I, Suri OP. Antihepatotoxic effects of chlorogenic acid from *Anthocephalus cadamba*. Phytother Res 1995;9:189-93.
10. Dolai N, Karmakar I, Kumar RB, Bala A, Mazumder UK, Haldar PK. Antitumor potential of *Castanopsis indica* (Roxb. ex Lindl.) A. DC. leaf extract against Ehrlich’s ascites carcinoma cell. Indian J Exp Biol 2012;50:359-65.
11. Bhattacharyya S, Prasanna A, Haldar PK. Evaluation of antiproliferative activity and apoptosis-inducing mechanism of *Castanopsis indica* leaves. Nat Prod Res 2016;30:482-5.
12. Dolai N, Kumar A, Islam A, Haldar PK. Apoptogenic effects of β-sitosterol glucoside from *Castanopsis indica* leaves. Nat Prod Res 2016;30:482-5.
13. Attari F, Sepehri H, Delphi L, Goliaei B. Apoptotic and necrotic effects of pectic acid on rat pituitary GH3/B6 tumor cells. Iran Biomed J 2012;15:11-9.
14. Das B, Mandal S, Chaudhuri K. Role of arginine, a component of aqueous garlic extract, in remediation of sodium arsenite induced toxicity in A375 cells. Toxicol Res 2014;3:191-6.
15. Liao W, McNutt MA, Zhu WG. The comet assay: A sensitive method for detecting DNA damage in individual cells. Methods 2009;48:46-53.
16. Yang S, Zhao Q, Xiang H, Liu M, Zhang Q, Xue W, et al. Antiproliferative activity and apoptosis-inducing mechanism of constituents from *Toona sinensis* on human cancer cells. Cancer Cell Int 2013;13:12.
17. Bhattacharyya A, Choudhuri T, Pal S, Chattopadhyay S, Datta GK, Sa G, et al. Apoptogenic effects of black tea on Ehrlich’s ascites carcinoma cell. Carcinogenesis 2003;24:75-80.