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Antitumour activity of *Annona squamosa* seed extracts is through the generation of free radicals and induction of apoptosis

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The plant, *Annona squamosa* traditionally known as custard apple possesses potent bioactive principles in all its parts. The effect of aqueous and organic extracts from defatted seeds of *A. squamosa* was studied on a rat histiocytic tumour cell line, AK-5. Both the extracts caused significant apoptotic tumour cell death with enhanced caspase-3 activity, down regulation of antiapoptotic genes Bcl-2 and Bcl_{XL}, and enhanced the generation of intracellular ROS, which correlated well with the decreased levels of intracellular GSH. In addition, DNA fragmentation and annexin-V staining confirmed that the extracts induced apoptosis in tumour cells through the oxidative stress. Aqueous extracts of *A. squamosa* seeds possessed significant antitumor activity *in vivo* against AK-5 tumor.

Key words: *Annona squamosa*, reactive oxygen species (ROS), GSH, antitumour activity, apoptosis, Bcl-2, seed extract

Natural products from higher plants are being explored for possible anticancer activity. The alkaloids from *Catharanthus roseus* are reported to be effective against P-1534 leukemia in DBA/2 mice[1]. These alkaloids have been shown to arrest the cancer cell growth during metaphase, thereby leading to cell death[2]. An extract, paclitaxel obtained from the bark of pacific yew tree (*Taxus brevifolia*) showed significant activity against a number of murine tumours[3,4]. Resvertrol isolated from grapes induced apoptosis by triggering CD95 in HL-60 cells[5]. The plant, *Annona squamosa* traditionally known as custard apple possesses potent bioactive principles in all its parts. Acetogenins, a class of natural compounds, isolated from members of Annonaceae have potent anti-neoplastic, parasiticidal, pesticidal and anti-microbial activities[6,7]. Acetogenins belonging to a series of C-35/C-37, and derived from C-32/C-34 long chain fatty acids are known to be powerful inhibitors of complex I (NADH: ubiquinone oxidoreductase) in mammalian and insect mitochondrial electron transport systems[8,9]. Two Acetogenins squamocin and squamostatin isolated from *A. squamosa* seeds have shown cytotoxic activity[9,10]. Squamocin inhibited proliferation of HL-60 cells and induced apoptosis by the activation of caspase-3. Ascimicin, another acetogenin also exhibited cytotoxic activity against 9KB, A549, HT-29 and 9ASK tumour cells[11]. Recently, two more acetogenins, squamocin-O^1 and squamocin-O^2 were reported from the methanolic extracts of the seeds of *A. squamosa*[12].

The physiological form of cell death is induced by a variety of stimuli[13] and several genes have been implicated in the apoptotic cell death process[14]. Annonacin, a mono-tetrahydrofuran acetogenin isolated from *A. reticulata* is reported to arrest cancer cells at the G1 phase and cause cytotoxicity in a Bax- and caspase-3-related pathway[15]. In the present paper, the effect of aqueous and organic extracts obtained from defatted seeds of *A. squamosa* on BC-8 tumour cells[16] (a single cell clone of a rat histiocytic tumour, AK-5[17]) was studied to elucidate the mechanism(s) involved in the induction of apoptotic cellular death. Both organic and aqueous seed extracts induced apoptosis in BC-8 tumour cells. Two extracts induced typical apoptotic features, such as formation of apoptotic bodies, fragmentation of nuclear DNA as analysed by DNA ladder assay and staining with annexin-V. The induced apoptosis in BC-8 cells was associated with increased generation of intracellular reactive oxygen species (ROS), reduced levels of GSH, activation of caspase-3 and downregulation of antiapoptotic genes Bcl-2 and Bcl_{XL}. The antitumour activity of aqueous extract *in vivo* in i.p. tumour-

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Abbreviations: ROS, reactive oxygen species; DMEM, Dulbecco's modified essential medium; FCS, fetal calf serum

transplanted animals was also investigated. The animals (80%) transplanted with AK-5 cells did not develop ascites after treatment with aqueous extracts of *A. squamosa* seeds.

Materials and Methods

Preparation of extracts

The kernels from *A. squamosa* seeds were separated, powdered using mortar and pestle and the powder was defatted using petroleum ether at 50°C for 20 hr. The defatted powder was air dried and divided into two parts. One part was extracted with PBS at 4°C for 24 hr and the supernatant was collected by centrifugation and used as aqueous extract. Second part was extracted with dichloromethane at 35°C for 20 hr, and the supernatant was collected by centrifugation, dichloromethane evaporated to dryness (the traces of solvent removed by desiccation) and was used as organic extract.

Cell culture

AK-5 tumour is maintained as ascites in Wistar rats and was adapted to grow for several generations in DMEM supplemented with 10% FCS in the presence of penicillin (100 U/ml) and streptomycin (50 μg/ml). A single cell clone of AK-5 tumour called BC-8 was used in the study to avoid ambiguity in results due to tumour heterogeneity[16].

Treatment of tumour cells with extracts

BC-8 cells (1×10^6) were treated with organic extract (5%) or aqueous extract (25%) for different time periods. The concentration of extracts used was arrived at after a careful titration with different concentrations. Similarly, incubation time was also optimized to achieve a significant difference between controls and treated samples.

Propidium iodide staining and flow cytometry

After treatment with extracts, BC-8 cells were washed with PBS and fixed in 70% methanol. The fixed cells were then stained with PI reagent (propidium iodide, 50 μg/ml in 0.1% sodium citrate containing 0.1% Triton X-100) and analysed by flow cytometry.

DNA fragmentation analysis

Cells were fixed in methanol, washed with PBS, and suspended in citrate-phosphate buffer. DNA was extracted, as described[18], electrophoresed on 1% agarose gel at 2 v/cm for 16 hr, stained with 5 μg/ml ethidium bromide and visualized under UV light.

Annexin-V staining and quantitation of apoptosis

BC-8 cells (1×10^6) treated with the extracts were stained with annexin V-FITC (Boehringer-Mannheim, Germany) in binding buffer (10 m*M* HEPES, *p*H 7.4, 140 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 2.5 m*M* $CaCl_2$, *p*H 7.4) at 4°C for 15 min. The binding of annexin V-FITC to phosphatidylserine, exposed on the cell surface was analyzed by flow cytometry.

Estimation of intracellular ROS

Intracellular superoxide was estimated by flow cytometry, using the oxidation-sensitive fluorescent probe 2′, 7′-dichlorofluorescein diacetate (DCF; molecular probes)[19]. Before exposure to the organic and aqueous extracts of *A. squamosa*, the cells were washed with PBS and incubated with DCF (100 μ*M*) for 60 min at 37°C. Subsequently, the cells were washed, resuspended in culture medium and incubated with the extracts at 37°C. At different time intervals, the cells were collected and analysed by flowcytometry.

Estimation of intracellular GSH

Intracellular levels of GSH were estimated using fluorescent reagent ortho-phthaladehyde[20]. After incubation with *A. squamosa* seed extracts, the cells were treated with 25% phosphoric acid. The cell lysate was centrifuged to pellet the precipitated proteins and the supernatants were treated with 0.1 *M* sodium phosphate – 5 m*M* EDTA buffer, *p*H 8.0 and GSH fluorescence was measured after addition of OPT reagent, using spectrofluorometer (excitation 350 nm and emission 420 nm).

Immuno-staining and flow cytometry

BC-8 cells were treated with aqueous (25%) or organic extracts (5%) for different time periods and permeabilised. Thereafter, the cells were treated with anti caspase-3 (1:100; Santa Cruz Biotechnology Inc., USA) for 1 hr; the cells were washed and treated with FITC-conjugated anti-rabbit Ig (1:250, Amersham International, UK) for 30 min. The cells were washed thoroughly and the percentage positive cells were quantified by flow cytometry.

RT-PCR analysis

Total cellular RNA was isolated from BC-8 cells, with and without extract treatment using Trizol reagent (Gibco, BRL) and reverse transcribed using

random hexamer primers and AMV reverse transcriptase. The messages Bcl-2, and Bcl_{XL} were amplified by PCR, along with the internal control, GAPDH. PCR products were run on agarose gel, transferred to nylon membrane and the blots were hybridized with specific cDNA probes. The relative density of spots was normalized with GAPDH levels.

Tumour growth

Two groups of 10 animals each were transplanted with 2×10^6 AK-5 cells i.p. Aqueous extract group was administered with 250 µl extract per rat/injection i.p. on days 0, 2, 4, 6 and 8 after tumour transplantation and the control rats were given PBS. Animals were regularly monitored for the appearance of peritoneal bulge/ascites and their survival was evaluated upto 40 days. Control animals start dying by day 6.

Results

Induction of apoptosis in tumour cells by *A. squamosa* extracts

Aqueous and organic extracts from defatted seeds of *A. squamosa* induced apoptosis in BC-8 cells. BC-8 cells were incubated with the extracts (organic 5% and aqueous 25%) for 24 hr and apoptosis was measured by flow cytometry after propidium iodide staining. Fig. 1A shows morphological features of the cells after treatment with extracts, wherein the formation of apoptotic bodies is seen. The number of apoptotic cells quantified by flow cytometry are shown in Fig. 1B. The results indicate that both the extracts induced apoptotic death in BC-8 tumour cells, which was further confirmed by annexin-V staining, wherein only apoptotic cells are stained. Fig. 2B shows the percentage of annexin-V stained cells, as scored by flowcytometry.

Induction of DNA fragmentation

Extracts-treated tumour cells were analyzed for DNA fragmentation, which is a typical hallmark of apoptotic cell death. Both extracts induced DNA fragmentation in BC-8 cells (Fig. 2A), confirming that the cell death caused by the seed extracts is through apoptosis.

Induction of superoxide generation

As the oxidative stress is one of the mediators of apoptosis[21], we examined the effect of two extracts on the intracellular levels of ROS. BC-8 cells were incubated with the extracts for 10 hr and the intracellular ROS was measured with DCF using flowcytometer[19]. Both extracts significantly enhanced the intracellular ROS levels in treated cells as compared to the untreated control cells (Fig. 3A).

Cells are protected from undergoing apoptosis by GSH, an antioxidant and a decrease in intracellular levels of GSH is associated with enhanced susceptibility to apoptosis[22]. We also measured intracellular levels of GSH in BC-8 cells after treatment with aqueous and organic extracts and observed a slight reduction in GSH levels in aqueous extract treated cells and about 60% reduction in organic extract-treated BC-8 tumour cells (Fig. 3B). These observations suggest that the tumour cells are sensitized to apoptotic death due to the reduction in

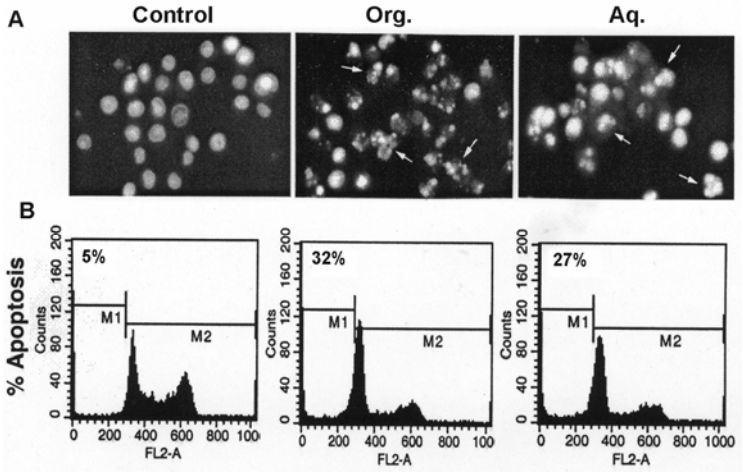

Fig. 1—Induction of apoptosis in BC-8 tumour cells by *Annona squamosa* seed extracts [Extract treated cells were stained with propidium iodide and analysed. (A): Formation of apoptotic bodies in organic and aqueous seed extracts-treated tumour cells after PI staining; and (B): Flowcytometric quantitation of apoptotic cells after treatment with extracts for 24 hr]

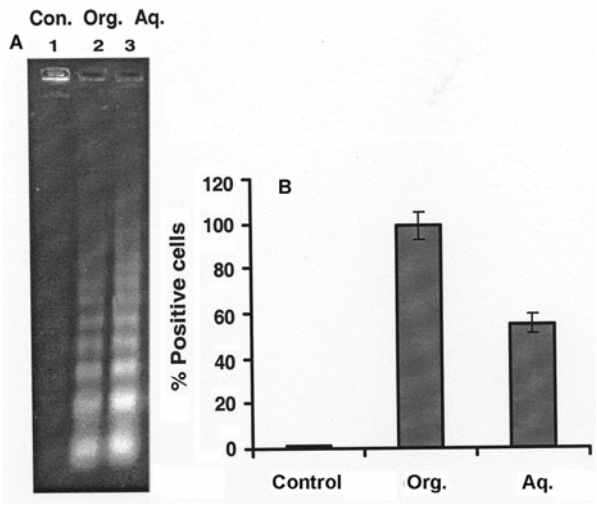

Fig. 2—*A. squamosa* extract-treated cells undergo DNA fragmentation after 24 hr [Fragmented DNA was resolved on Agarose gel and stained with ethidium bromide. (A): Lane 1, control; lane 2, organic extract; lane 3, aqueous extract. Annexin-V stained positive cells were quantified by flowcytometry (B)]

intracellular GSH levels, which is known to scavenge the generated free oxygen radicals[23].

Activation of caspases during apoptosis

The effect of both extracts on the activation of different caspases was examined by using specific caspase inhibitors and anti-caspase-3 antibody staining[24]. The anti-caspase-3 staining of extracts-treated tumour cells and the effect of different caspase inhibitors on tumour cell apoptosis are shown in Fig. 4A & B, respectively. Caspase-3 inhibitor (DEVD) significantly inhibited apoptosis in extracts-treated BC-8 cells. Inhibition of apoptosis was also observed with tetrapeptide pan caspase inhibitor ZVAD, suggesting that the activation of caspase-3 is important in the extract-induced apoptosis of BC-8 cells. Other tetrapeptide inhibitors, like YVAD, IETD and LEHD showed lower inhibition of apoptosis.

Downregulation of Bcl-2 and Bcl_{XL} by seed extracts

Bcl-2 is known to serve as an antioxidant[25]. Increased intracellular ROS levels were observed when BC-8 cells were treated with the seed extracts. We also examined the effect of extracts on the expression of Bcl-2 and Bcl_{XL}. Bcl-2 gene expression showed significant down-regulation when treated with the organic extract. Similarly, Bcl_{XL} was downregulated upon treatment with both the extracts (Fig. 5). These observations indicate that downregulation of Bcl-2 and Bcl_{XL} plays an important role in the induction of apoptosis in BC-8 cells treated with either aqueous or organic seed extracts.

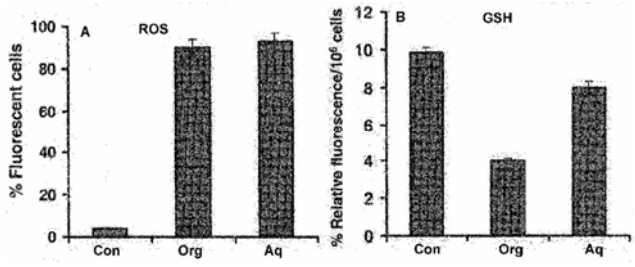

Fig. 3—Levels of ROS and GSH in *A. squamosa* extract-treated cells. [ROS levels were quantified using DCF pretreated cells after treatment with organic and aqueous seed extracts for 10 hr (A). Similarly, GSH levels were measured spectrofluorometrically; and (B) after treatment with seed extracts for 10 hr]

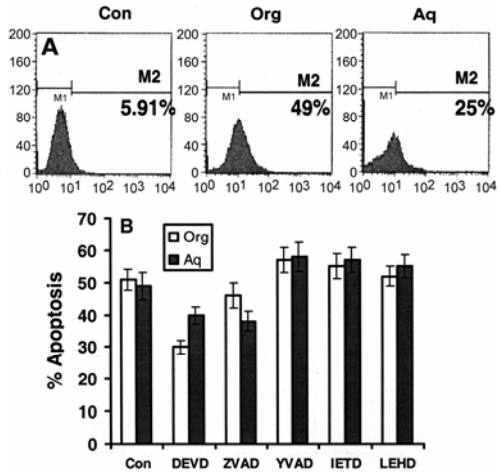

Fig. 4—Activation of caspases by *A. squamosa* extracts.[BC-8 cells were incubated with seed extracts and caspase-3 activity was measured by flowcytometry after antibody staining (A): Apoptotic death in BC-8 cells after organic and aqueous seed extracts treatment in the presence of different tetrapeptide caspase inhibitors after 24 hr of incubation; and (B). Con, control; org, organic extract; Aq. Aqueous extract]

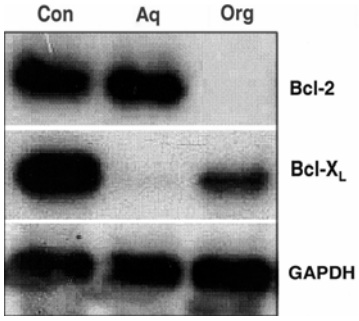

Fig. 5—RT-PCR analysis of the expression of Bcl-2 and Bcl_{XL} by BC-8 cells after treatment with organic and aqueous seed extracts of *A. squamosa* [GAPDH level is also shown as control. Lane 1, control; lane 2, aqueous extract treated cells and lane 3, organic extract treatment]

Inhibition of *in vivo* tumour growth

The effect of aqueous extract on the growth of AK-5 tumour as ascites in rats was examined. A significant arrest of tumour growth by the aqueous extract was observed. All the control animals developed ascites and 90% animals died by day 10. However, 80% of the extract injected animals survived without any development of ascites up to 40 days. These observations strongly suggest antitumour properties of *A. squamosa* seed extracts.

Discussion

Both organic and aqueous extracts from defatted *A. squamosa* seeds induced apoptosis in BC-8 cells with typical apoptotic features, like DNA fragmentation, formation of apoptotic bodies and annexin-V staining. Extracts-treated BC-8 tumour cells generate free radicals, such as superoxide ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH$) and non-radical derivatives of oxygen like H_2O_2. These radicals are known to cause damage in most of the biomolecules, including DNA, protein and lipid membrane, etc.[26]. The major source of ROS in many cell types is the electron leakage from mitochondrial electron transport chain that reduces molecular oxygen to superoxide ion. Elevated levels of intracellular ROS are sufficient to trigger apoptosis, and it is well documented that ROS are the biochemical mediators of apoptosis[26]. In this study, we observed enhanced intracellular ROS levels in BC-8 cells, after treatment with seed extracts.

GSH, a physiological antioxidant protects cells from oxidative stress-induced apoptosis[27]. We analyzed the intracellular GSH levels in BC-8 cells after treatment with two extracts. Interestingly, the treated cells were found to be low in GSH levels, as compared to control cells. GSH produced by the cells after the treatment with extracts is not sufficient to neutralize the ROS generated by these cells. These observations suggest that ROS play a key role in the induction of apoptosis. Bcl-2 has also been reported to alter GSH compartmentalization[28]. In the present study, downregulation of Bcl-2 and Bcl_{XL} expression after treatment of the cells with the seed extracts has been demonstrated. The loss of Bcl-2 expression is shown to sensitize the cells to apoptotic death[29] via regulation of ROS generation. Thus, we could correlate downregulation of Bcl-2, Bcl_{XL} and ROS burst accompanied by reduced GSH levels, which ultimately led to the apoptotic death of tumour cells.

The role of caspases in *A. squamosa* seed extract induced apoptosis has also been studied. Caspase activity was assessed by using caspase-specific tetrapeptide inhibitors for caspases 1, 3, 8 and 9. DEVD-CHO, a specific inhibitor for caspase-3 showed a marked reduction in apoptotic cell death. Downregulation of Bcl-2 in BC-8 cells is a key event in the apoptotic cascade, prior to caspase activation[24]. These observations suggest that caspase-3 plays an important role in the apoptotic cascade in our system. Aqueous seed extracts are also highly effective *in vivo* in inhibiting intraperitoneal tumour growth. Thus, present studies suggest that *A. squamosa* seed extracts induce apoptosis in BC-8 tumour cells by inducing oxidative stress. It would be of interest to identify the active component(s) present in the seed extracts showing such promising anti-cancer activity.

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