Differential cytotoxic effects of *Annona squamosa* seed extracts on human tumour cell lines: Role of reactive oxygen species and glutathione

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Annonaceous acetogenins are a new class of compounds that have been reported to have potent pesticidal, parasiticidal, anti-microbial, cell growth inhibitory activities. In this study, organic and aqueous extracts from the defatted seeds of *Annona squamosa* (custard apple) were tested on different human tumour cell lines for antitumoural activity. While organic and aqueous extracts induced apoptosis in MCF-7 and K-562 cells, they failed to do so in COLO-205 cells. Treatment of MCF-7 and K-562 cells with organic and aqueous extracts resulted in nuclear condensation, DNA fragmentation, induction of reactive oxygen species (ROS) generation and reduced intracellular glutathione levels. In addition downregulation of Bcl-2 and PS externalization by Annexin-V staining suggested induction of apoptosis in MCF-7 and K-562 cells by both the extracts through oxidative stress. On the contrary, COLO-205 cells showed only PS externalization but no change in ROS and glutathione levels. These observations suggest that the induction of apoptosis by *A. squamosa* extracts can be selective for certain types of cancerous cells.

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1. Introduction

The Annonaceous acetogenins are a new class of natural compounds, whose potent biological activity and special structures have attracted considerable attention. Since the initial discovery of uvaricin as a new, *in vivo*, antitumour agent (Jolad *et al* 1982), over 350 natural Annonaceous acetogenins have been reported (Zafra-Polo *et al* 1998; Alali *et al* 1999). Numerous Annonaceous acetogenins have been shown to possess cytotoxic, pesticidal, antimalarial, cell growth inhibitory, antiparasitic and antimicrobial activities (Oberlies *et al* 1997; Chih *et al* 2001). Bullatacin is one such compound that possessed antitumoural and pesticidal activity *in vivo* (Ahmmadsahib *et al* 1993). Methanolic extracts of *Annona muricata* and *A. cherimola* seeds have been shown to have antiparasitic activity (Bories *et al* 1991). Six more Annonaceous acetogenins isolated from the seeds of *Annona atemoya* exhibited potent cytotoxicity against HepG2, KB, CCM2 and CEM cancer cell lines (Fong-Rong *et al* 1999). Squamocin, another Annonaceous acetogenin has been reported to exert antiproliferative effects on HL-60 cancer cells via activation of caspase-3 (Xiao-Feng *et al* 2002).

Oxidative stress induced by reactive oxygen intermediates including superoxide, hydrogen peroxide are known to cause apoptotic cell death in the pathogenesis of diverse human diseases, including cancer, diabetes and neurodegenerative disorders. The protooncogene Bcl-2 is known to inhibit apoptotic and necrotic cell death (Kane *et al* 1993). On the other hand, loss of Bcl-2 expression is known to sensitize the cells to apoptotic death (Robertson *et al* 1997). We have studied the effect of organic and aqueous extracts obtained from the defatted seeds of *Annona squamosa* on different human tumour cell lines MCF-7,

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Abbreviations used: DCF, 2′,7′-dichlorofluorescein; GSH, glutathione; OPT, orthophthaladehyde; PI, propidium iodide; PS, phosphatidyl serine; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction.

K-562 and COLO-205 and have elucidated the mechanisms involved in the cell death process. Our results showed that the two extracts from *A. squamosa* seeds induced apoptotic features like formation of apoptotic bodies, DNA fragmentation and phosphotidyl serine externalization by Annexin-V staining in MCF-7 breast carcinoma and K-562 erythroleukemic cells. However, these extracts induced no changes in cellular morphology or DNA fragmentation and showed only Annexin-V staining in COLO-205 colon carcinoma cells. Increased levels of intracellular reactive oxygen species (ROS), reduced glutathione (GSH) content concomitant with the downregulation or loss of Bcl-2 expression were observed in MCF-7 and K-562 cells but not in COLO-205 cells after treatment with *A. squamosa* extracts.

2. Materials and methods

2.1 *Tumour cell lines*

Human tumour cell lines MCF-7, COLO-205, K-562 were from the National Cancer Institute/American Type Culture Collection and were maintained in DMEM containing 10% fetal calf serum (FCS) and a mixture of penicillin (100 U/ml) and streptomycin (50 μg/ml). MCF-7 cells were subcultured after mild trypsinization.

2.2 *Preparation of seed extracts*

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

2.3 *Treatment with extracts*

Different tumour cell lines (1×10^6) were incubated with different concentrations of organic (2–5%) dissolved in DMSO and aqueous (10–25%) extracts for 24 and 48 h. The cells were washed, fixed in ethanol and stained with propidium iodide (PI) reagent.

2.4 *Propidium iodide staining and flow cytometry*

Tumour cell lines after treatment with extracts were washed with PBS, and fixed in 70% ethanol and stained with PI reagent (propidium iodide, calbiochem, 50 μg/ml in 0·1% sodium citrate containing 0·1% Triton X-100) and analysed by flow cytometry.

2.5 *DNA extraction and electrophoresis*

Extract treated cells were washed with phosphate buffered saline fixed in ethanol, and suspended in citrate-phosphate buffer. DNA was extracted following the previously published procedure (Gong *et al* 1994), electrophoresed on 1% agarose gel at 2 v/cm for 16 h, stained with 5 μg/ml ethidium bromide and visualized under UV light.

2.6 *Quantitation of apoptosis using Annexin-V*

Extract treated human tumour cell lines (1×10^6) were stained with Annexin-V-FITC (Boehringer-Mannheim, Germany) in binding buffer (10 mM HEPES, pH 7·4, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2·5 mM $CaCl_2$, pH 7·4) at 4°C for 15 min. The binding of Annexin-V-FITC to phosphatidylserine, exposed on the cell surface was analysed by laser scanning confocal microscopy. Annexin-V-FITC stained apoptotic cells were also quantified by flow cytometry.

2.7 *Intracellular ROS estimation*

Intracellular ROS was estimated by flow cytometry using the oxidation sensitive fluorescent probe 2′,7′-dichlorofluorescein (DCF) diacetate (Molecular Probes) (Royall and Ischiropoulos 1993). Human tumour cells (1×10^6) were incubated with DCF (5 μM) for 30 min at 37°C and then washed with PBS. Stained cells were treated with the extracts for different time intervals, washed and analysed by flow cytometry.

2.8 *GSH estimation*

Intracellular levels of GSH were estimated using fluorescent reagent orthophthaladehyde (OPT) as described previously (Hissin and Hilf 1976). Extracts treated tumour cells were treated with 25% phosphoric acid, precipitated proteins were centrifuged and the supernatants were treated with 0·1 M sodium phosphate – 5 mM EDTA buffer pH 8·0 and the fluorescence was measured after the addition of OPT reagent using spectrofluorometer (excitation 350 nm and emission 420 nm).

2.9 *Reverse transcriptase-polymerase chain reaction analysis*

Total cellular RNA was isolated with Trizol reagent (Gibco, BRL) and reverse transcribed using random hexamer primers. Primer sets for Bcl-2 and Bax were synthesized

according to the published procedure (Sawada *et al* 2000). Caspase-3 and Bcl-XL primers were previously designed in the laboratory. The primer set for GAPDH was synthesized as previously reported (Hockenbery *et al* 1993). The PCR products along with GAPDH amplicons were run on agarose gel, transferred to nylon membrane and the blots were hybridized with specific cDNA probes for Bcl-2, Bax, Bcl_{XL} and caspase-3. The relative densities of the spots were normalized with corresponding GAPDH controls.

3. Results

3.1 *Extract induced apoptotic death in tumour cells*

Both organic and aqueous extracts of defatted seeds of *A. squamosa* induced apoptosis in MCF-7 and K-562 cells. Tumour cells were incubated with various concentrations of the organic (2–5%) and aqueous (5–25%) extracts for 24 h and apoptosis was measured by flow cytometry after PI staining. The percentage of apoptotic cells was scored by flow cytometry as shown in figure 1A. COLO-205 cells did not show any apoptotic cell death with either organic or aqueous extracts. Annexin-V, which specifically binds to the apoptotic cells was used in confocal microscopy. All the 3 cell lines stained positive with Annexin-V-FITC as shown in figure 1B.

3.2 *DNA fragmentation analysis*

DNA fragmentation, which is a typical hallmark of the apoptotic cell death was analysed. Aqueous and organic extracts induced apoptosis in MCF-7 and K-562 cells. However, there was no fragmentation of DNA in COLO-205 cells as shown in figure 2A. These observations confirm that the cell death caused by *A. squamosa* seed extracts is through apoptosis.

3.3 *Labelling with Annexin-V-FITC*

Translocation of phosphatidyl serine (PS) from the inner face of plasma membrane to the outer cell surface occurs during the early stages of apoptosis. Apoptosis induced by organic and aqueous extracts was quantified using Annexin-V-FITC and the cells were scored by flow cytometry. Annexin-V-FITC binds to the cell surface expressed PS and therefore includes only apoptotic cells and not the necrotic cells. All the 3 cell lines used in this study stain positive for Annexin-V binding (figure 2B) after treatment with either organic or aqueous extracts of *A. squamosa*.

3.4 *Induction of ROS generation*

Oxidative stress is well known to be one of the mediators of apoptosis. Intracellular ROS was measured with DCF diacetate using flow cytometer. Extracts treated MCF-7 and K-562 cells showed significantly higher levels of ROS, whereas COLO-205 cells do not show significant increase in ROS levels as compared to the untreated control cells (figure 3A). This probably explains the differential response of the 3 cell lines towards *A. squamosa* seed extracts.

3.5 *Estimation of GSH*

GSH is an anti-oxidant and decreased intracellular levels of GSH are associated with enhanced susceptibility to ROS mediated apoptosis (Beaver and Waring 1995). GSH protects cells from undergoing apoptosis by neutralizing free radicals. In our present study we have measured intracellular levels of GSH in MCF-7, COLO-205 and K-562 cells after treatment with the two extracts. We have observed a significant reduction in the intracellular GSH levels in MCF-7 and K-562 cells after treatment with extracts. On the other hand, COLO-205 cells showed only slight reduction in GSH on treatment with the extracts (figure 3B). These observations suggest that, at reduced intracellular GSH levels, which are known to scavenge the generated free oxygen radicals, tumour cells are sensitized to apoptotic death.

3.6 *Role of caspases in apoptosis*

In order to understand the mechanisms involved in the organic extracts induced apoptotic death, we have studied the effect of various inhibitors of caspases. Different tetrapeptide inhibitors like DEVD, YVAD, IETD and LEHD which are specific to different caspases were used in the assays. The tetrapeptide DEVD which is a specific inhibitor of caspase-3 showed a significant inhibition of apoptosis in K-562 cells. MCF-7 cells, which are negative for caspase-3, showed no effect (figure 4A). COLO-205 cells had no significant effect with any of the inhibitors tested as they did not undergo apoptosis.

3.7 *Downregulation of Bcl-2 expression*

We also examined the effect of extracts on the expression of Bcl-2, an apoptosis inhibitor protein. Upon treatment with the extracts, we observed downregulation or loss of Bcl-2 gene expression in K-562 and MCF-7 cells whereas, no change in the expression of Bcl-2 gene in COLO-205 cells was observed (figure 4B). The reverse

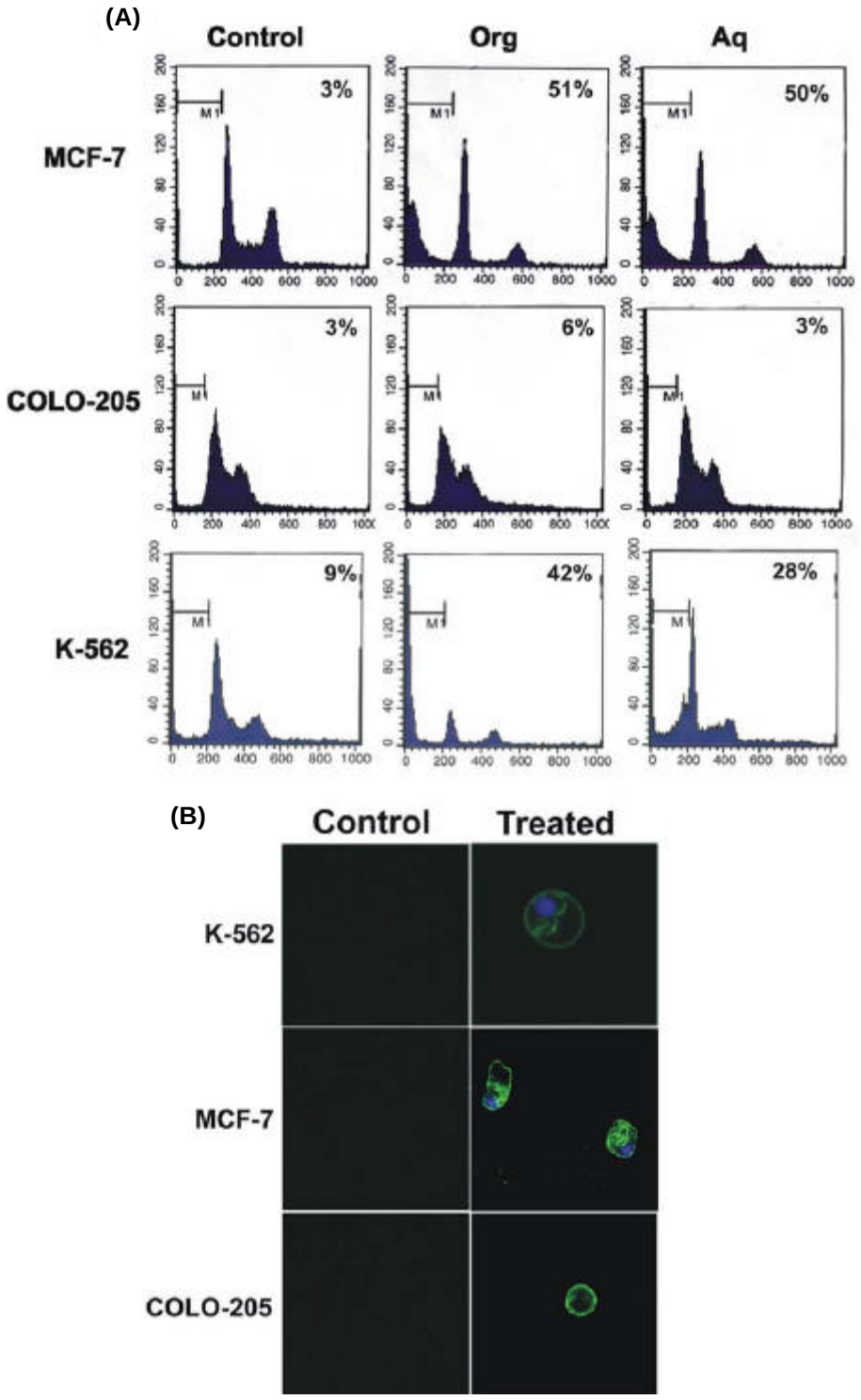

Figure 1. Induction of apoptosis in MCF-7, COLO-205 and K-562 cells by *A. squamosa* seed extracts (organic 5% and aqueous fractions 25%) after 24 h. (**A**) Percent apoptotic cells after PI staining and flow cytometry. (**B**) Annexin-V-FITC staining of apoptotic cells analysed by laser scanning confocal microscopy. The data shown is representative of 3 similar experiments.

transcriptase-polymerase chain reaction (RT-PCR) results were confirmed at the protein level by Western blotting (figure 4C). These observations shown in figure 4(B,C) clearly indicate that Bcl-2 may play an important role in the induction of apoptosis in MCF-7 and K-562 cells (Hockenbery *et al* 1993) and the lack of any effect on Bcl-2 expression by COLO-205 cells might explain their resistance to apoptotic death induced by the extracts. RT-PCR analysis of Bax, caspase-3 and Bcl_{XL} did not show any significant differences upon treatment of cells with *A. squamosa* extracts (data not shown).

4. Discussion

Annonaceous acetogenins have been reported to possess insecticidal, pesticidal and cytotoxic activities (Fujimoto

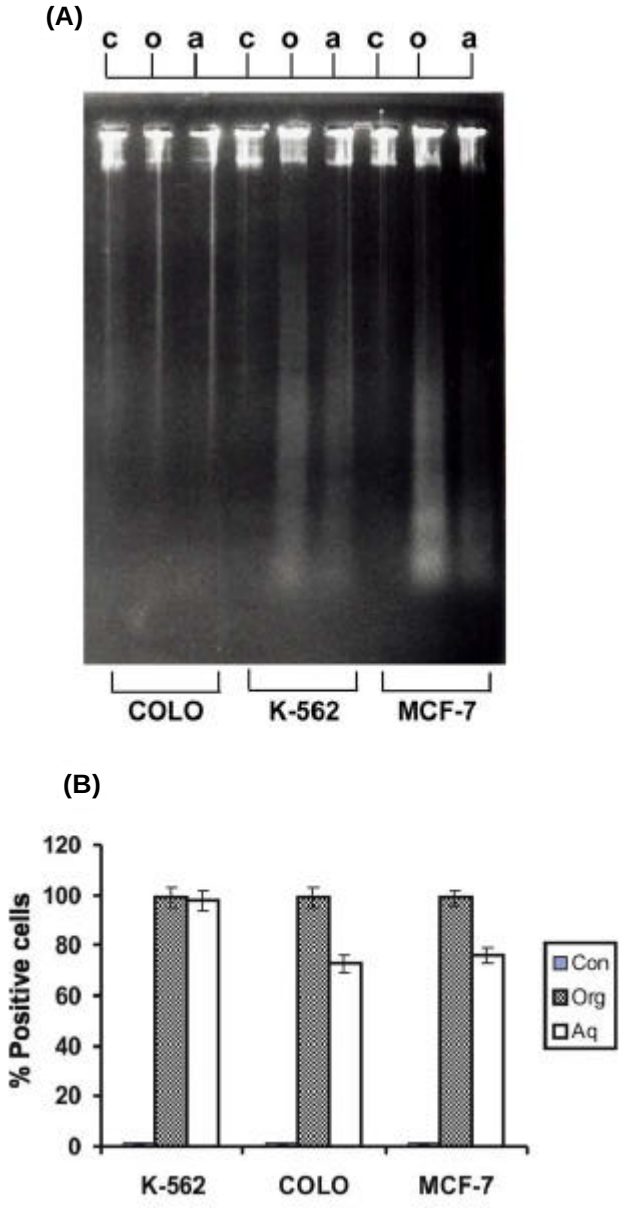

Figure 2. (**A**) DNA fragmentation induced by *A. squamosa* extracts in human tumour cell lines. Lanes c, control; o, organic extract 5%; and a, aqueous extract 25% treated cells. (**B**) Annexin-V-FITC stained positive cells after treatment with organic and aqueous extracts of *A. squamosa* for 24 h and analysed by FACS. Bars represent ± SD. The data shown is representative of 3 similar experiments.

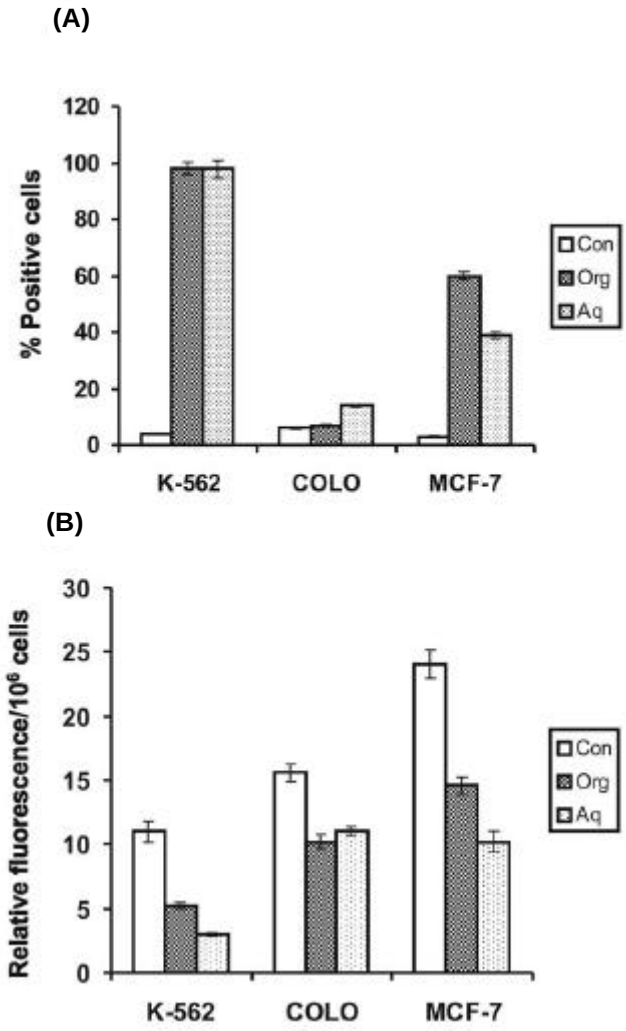

Figure 3. (**A**) Intracellular ROS levels generated by *A. squamosa* extract treated K-562, COLO-205 and MCF-7 cells. COLO-205 produced significantly lower levels of ROS as compared to K-562 and MCF-7. Intracellular ROS levels were estimated using DCF. (**B**) GSH levels in K-562, COLO-205 and MCF-7 after treatment with *A. squamosa* extracts. Significantly lower levels of GSH were observed in K-562 and MCF-7 cells as compared to COLO-205 cells. Bars represent ± SD. The data shown is representative of 3 similar experiments.

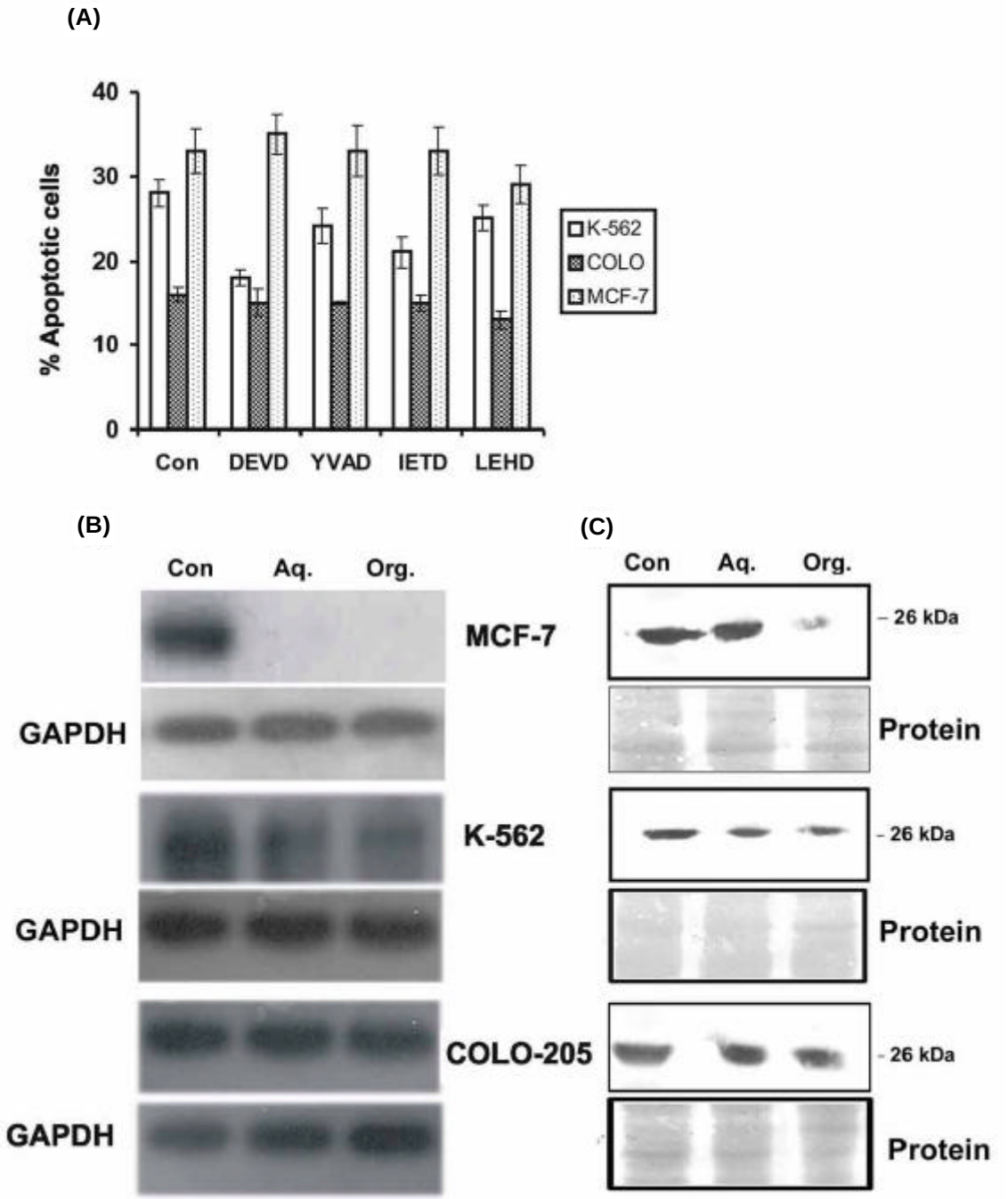

Figure 4. (**A**) Effect of various inhibitors of caspases on organic 5% *A. squamosa* seed extract induced apoptosis. Caspase-3 inhibitor, DEVD showed significant inhibition of apoptosis in K-562 cells. Apoptotic cells were scored by FACS after PI staining. Bars represent ± SD. (**B**) RT-PCR analysis for the expression of Bcl-2 by MCF-7, K-562 and COLO-205 cells after treatment with *A. squamosa* seed extracts. Blots were hybridized with specific cDNA probes. GAPDH is shown as loading control for each blot. The data shown is representative of 3 similar experiments. (**C**) Western blotting for Bcl-2 protein. Ponceau S stained blot is shown as the loading control.

et al 1998; Colman-Saizarbitoria *et al* 1995). Recently, these compounds have attracted increasing attention as potential antineoplastic agents due to their ability to kill tumour cells (Fang *et al* 1993).

In the present study, we used two extracts, organic and aqueous, obtained from the defatted *A. squamosa* seeds, and studied their effect on three human tumour cell lines MCF-7 breast carcinoma, K-562 erythroleukemia and COLO-205 colon carcinoma. Our observations demonstrate that both the organic and aqueous extracts induced apoptosis in MCF-7 and K-562 cells as assessed by Annexin-V-FITC staining which is used as a typical marker to estimate apoptosis by flow cytometry. COLO-205 cells also showed Annexin-V staining however, without any apoptotic death. Annexin-V-FITC binding is based on the transposition of PS from the inner to the outer leaflet of cell membrane during the early stages of apoptosis (Vermes *et al* 1995). In addition, DNA fragmentation was observed in MCF-7 and K-562 cells but not in COLO-205 cells, which showed no nuclear damage. The apoptotic activity of annonaccin, an acetogenin from *Annona reticulata* as reported earlier (Yuan *et al* 2003) involves activation of caspase-3 and Bax related pathway. Higher levels of intracellular ROS are sufficient to trigger apoptosis, and it has been well known that ROS are the biochemical mediators of apoptosis (Buttke and Sandstrom 1994). We also observed enhanced levels of intracellular ROS in MCF-7 and K-562 cells after treatment with *A. squamosa* seed extracts. Interestingly, COLO-205 cells showed neither superoxide generation nor apoptotic body formation after PI staining. These observations indicate that the two extracts used in this study exhibit varied and cell type specific actions.

It is well known that apoptosis is induced either by depletion of endogenous antioxidants or by generation of free radicals (Buttke and Sandstrom 1994). We have also analysed the intracellular GSH levels in MCF-7, COLO-205 and K-562 cells after treatment with the extracts. The results showed significantly lower levels of GSH in MCF-7 and K-562 cells. The GSH produced by MCF-7 and K-562 cells upon treatment with extracts may not be sufficient to neutralize the ROS generated by these cells. On the other hand, GSH levels produced by COLO-205 cells after treatment with the extracts may be sufficient to neutralize the ROS generated by these cells and therefore remain protected from apoptotic death. These observations suggest that ROS generation plays a key role in the induction of apoptosis in MCF-7 and K-562 cells.

We have also analysed the expression levels of Bcl-2 in MCF-7, COLO-205 and K-562 cells after treatment with the extracts. We observed loss of Bcl-2 expression in MCF-7 cells and downregulation of Bcl-2 expression in K-562 cells. However, there was no change in the expression levels of Bcl-2 in COLO-205 cells. Loss of Bcl-2 expression is known to sensitize the cells to apoptotic death (Robertson *et al* 1997). ROS generation is also known to be regulated by Bcl-2 (Khar *et al* 2003). Thus our results suggest that downregulation or loss of Bcl-2 expression leads to increased ROS generation which is concomitant with the reduction in GSH levels thereby culminating in apoptotic cell death.

Our studies suggest *A. squamosa* seeds to possess potent proapoptotic characteristics for several human tumour cells, however, there could be certain types of tumour cells which show resistance towards *A. squamosa* seed extracts. These studies also suggest *A. squamosa* seeds to be of significant potential in cancer therapy and therefore needs more detailed study.

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