

RESEARCH ARTICLE

In Vitro Antitumor Properties of an Isolate from Leaves of *Cassia alata* L

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Abstract

Leaf extracts of *Cassia alata* L (akapulko), traditionally used for treatment of a variety of diseases, were evaluated for their potential antitumor properties *in vitro*. MTT assays were used to examine the cytotoxic effects of crude extracts on five human cancer cell lines, namely MCF-7, derived from a breast carcinoma, SK-BR-3, another breast carcinoma, T24 a bladder carcinoma, Col 2, a colorectal carcinoma, and A549, a non-small cell lung adenocarcinoma. Hexane extracts showed remarkable cytotoxicity against MCF-7, T24, and Col 2 in a dose-dependent manner. This observation was confirmed by morphological investigation using light microscopy. Further bioassay-directed fractionation of the cytotoxic extract led to the isolation of a TLC-pure isolate labeled as f6l. Isolate f6l was further evaluated using MTT assay and morphological and biochemical investigations, which likewise showed selectivity to MCF-7, T24, and Col 2 cells with IC₅₀ values of 16, 17, and 17 µg/ml, respectively. Isolate f6l, however, showed no cytotoxicity towards the non-cancer Chinese hamster ovarian cell line (CHO-AA8). Cytochemical investigation using DAPI staining and biochemical investigation using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-a method used to detect DNA fragmentation-together with caspase assay, demonstrated apoptotic cell death. Spectral characterization of isolate f6l revealed that it contained polyunsaturated fatty acid esters. Considering the cytotoxicity profile and its mode of action, f6l might represent a new promising compound with potential for development as an anticancer drug with low or no toxicity to non-cancer cells used in this study.

Keywords: *Cassia alata* L, *in vitro* antitumor property - MTT formazan assay - TUNEL - caspase assay

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Introduction

The desire to produce more effective but less toxic drugs fuels the continuous search for new bioactive compounds from plants. Many natural products of plant origin have been subjected to mass screening for bioactivity such as that conducted by the National Cancer Institute in the USA. The NCI uses a panel of 60 human cancer cell lines from 9 types of tumors, so called NCI-60 (Phillips, 2011). This has paved the way for the discovery of a number of candidate substances based on differential *in vitro* cytotoxicity (Pessoa, 2000; Matic, 2013). In the quest for new bioactive compounds, rapid screening of the world's plant species for cytotoxic activities is made possible with high throughput technologies.

In an attempt to explore Philippine plants traditionally used for therapeutic purposes, extracts from *Cassia alata* L were tested *in vitro* for cytotoxicity against selected cancer cell lines. *C. alata*, commonly called "akapulko" in the

Philippines, belongs to family Leguminosae. It has been studied for its antimicrobial, antifungal, anti-inflammatory, antimutagenic, and hypoglycemic activities (Ibrahim and Osman, 1995; Villaseñor et al., 2002). However, its therapeutic and preventive effects against cancer remain relatively unexplored.

A strong cancer chemopreventive potential was reported by Jacinto et al. (2005) when *C. alata* hexane leaf extract was observed to induce quinone reductase with specific activity comparable to bromoflavone, a known chemopreventive agent. Quinone reductase is a Phase II enzyme that helps to inactivate carcinogenic compounds *in vivo*. This enzyme mediates the conversion of quinones into enols, which are easily metabolized and excreted by the body.

This paper presents the results of a study on the antitumor activities of crude leaf extracts and a TLC-pure isolate from *C. alata*. *In vitro* cytotoxicity was evaluated using the following human cancer cell lines: two breast

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carcinomas, MCF-7 and SK-BR-3; a bladder carcinoma, T24; colorectal carcinoma, Col 2; non-small cell lung adenocarcinoma, A549; and the non-cancer Chinese hamster ovarian cell line, AA8. The apoptosis-inducing ability of the TLC-pure isolate was also assessed by noting specific cellular morphology typical of apoptosis, as well as testing for DNA fragmentation and for the activation of effector caspases.

Materials and Methods

Bioassay-directed fractionation

Isolation and purification of the antitumor fraction Fresh, mature leaves of *Cassia alata* were collected from their natural habitat in Agoon, La Union, Philippines, and were authenticated at the Jose Vera Santos Herbarium of the Institute of Biology, University of the Philippines, Diliman, Quezon City with voucher specimen #14511. The leaves were washed, air dried, homogenized, and extracted with methanol. The methanol extract was concentrated in *vacuo* at 40°C using a rotary evaporator (Heidolph) yielding fraction A (FA, 96 g, 9.6%). FA was dissolved in 250 ml of distilled water and partitioned between hexane (6X) and water. The resulting aqueous layer was further partitioned using ethyl acetate (6X).

Both the hexane (FB, 18.8g, 1.88%) and EtOAc (FC, 4.7g, 0.47%) fractions were concentrated in *vacuo* at 40°C. Subsequently, the three crude fractions were subjected to cytotoxicity assay based on the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to purple formazan using human cancer cell lines, as well as non-cancer AA8 cells from Chinese hamster ovaries. Preliminary screening for the cytotoxic fraction showed that FB was the most active fraction.

FB was then subjected to repeated and sequential normal phase column chromatography using gradient ratios of EtOAc in hexane, resulting in fractions FB1-FB10, subfractions FB6a-FB6m, and isolate f6l (8.8 mg, 0.00088%) following bioactivity-directed fractionation.

MTT cytotoxicity assay

The cytotoxic effect of the plant extract was evaluated using the protocol for reduction of MTT into formazan crystals developed by Mosmann (1983). In detail, trypsinized cultures of MCF-7, SK-BR-3, T24, Col 2, A549 and AA8, were harvested and seeded at cell densities of $1-4 \times 10^4$ cells per well then incubated at 37°C for 24 h in a 5% CO₂ incubator. After incubation, the cells were treated with the extracts at the following concentrations: 3.75, 7.5, 15, 25, 50, and 100 µg/ml; TLC-pure f6l was tested at final concentrations of 6.25, 12.5, 25, and 50 µg/ml. Doxorubicin, an anticancer drug with major clinical applications in carcinomas, was used to treat cells as the positive control, and DMSO-treated cells were the negative control. Similar controls were used in all other assays. The absorbance was then measured at 570 nm. Cell survival was computed by using the following formula:

$$\% \text{Cell survival} = \frac{(\text{mean optical density of the sample} - \text{blank})}{(\text{mean optical density of the control} - \text{blank})} \times 100$$

The concentration at which cell proliferation is

inhibited by 50% (IC₅₀) was computed by linear regression of absorbance against concentrations tested. Three trials were done, where each sample concentration was replicated three times.

Apoptosis assay

To determine whether f6l modulates cell death by apoptosis, a combination of three techniques namely staining with 4',6-diamidino-2-phenylindole (DAPI), terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Alshatwi et al., 2011) and caspase assay were conducted. DAPI is a powerful tool in cytochemical analysis because it stains the double stranded DNA of dead cells. The TUNEL solution binds to the 3'OH nick ends of DNA and nuclear materials of non-viable cells that result to a yellow or green fluorescent signal indicating the presence of fragmented DNA. Conversely, TUNEL solution would not stain in viable cells. Caspases are cysteine-aspartic acid proteases that cleave different proteins in the cell during apoptosis. In these assays, cells against which f6l is cytotoxic were used.

Bright-field microscopy analysis

Treated cells were investigated for morphological features using a bright-field microscope (Nikon Diaphot 300, Japan) at 200X magnification. MCF-7, T24, and Col 2 cells treated with f6l at 50 µg/ml were observed after 72 h incubation with the isolate. The typical features of apoptosis that were monitored included formation of cytoplasmic membrane blebs, apoptotic bodies, cytoplasmic and nuclear shrinkage, chromatin condensation, and loss of cell-to-cell contact (Pereira and Amarante-Mendes, 2011).

Nuclear staining with 4'-6-diamidino-2-phenylindole (DAPI)

To further assess the alterations in nuclear morphology with f6l treatment, the cells were plated at a density of 2.5×10^4 (MCF-7), 4×10^4 (T24), or 5×10^4 (Col 2) cells per well in 96-well plates (Costar) and incubated in 5% CO₂ at 37°C for 24 h. The cells were then treated with f6l at 50 µg/ml and incubated with 5% CO₂ at 37°C for 24 h, using a concentration of thrice its IC₅₀, as was done by Agarwal et al. (2002) with grape seeds, made it possible to observe apoptotic features at an even earlier time. Apoptosis was investigated 12 h, 24 h, 48 h, and 72 h after treatment. In detail, the medium was discarded and 50 µl of 1% DAPI (Sigma) was added to the cells. DAPI forms fluorescent complexes with the double-stranded DNA in dead cells. The apoptotic index was determined under a fluorescent microscope by counting the number of DAPI-stained cells and dividing by the total number of cells scored.

Detection of DNA fragmentation through the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method

Nuclear DNA fragmentation, a biochemical hallmark of apoptosis, was evaluated using the TUNEL assay kit (Boehringer Mannheim Catalog No. 1684795, Mannheim, Germany). Following the manufacturer's protocol, growing cancer cells were seeded at 2.5×10^4 (MCF-7)

or 4×10^4 (T24 and Col 2) cells per well, in 96-well, flat-bottomed microtiter plates (Costar) and incubated at 37°C for 24 h. Fifty (50) $\mu\text{g/ml}$ were added and the cells were incubated for 24 h. The TUNEL solution stains non-viable cells or cell fragments and binds to the free 3'-hydroxyl ends of the DNA of chromatin and nuclear materials. The nuclei of cells undergoing apoptosis exhibit green or yellow fluorescence indicating the presence of fragmented DNA. Viable cells, however, would not take up the stain.

Measurement of caspase-3 activity

Cells were grown in culture media and seeded at a density of 1×10^6 cells/ml following the instructions of the Caspase-3 assay kit (Catalogue No. G7220, Promega, Madison, WI, USA). To induce apoptosis, 50 $\mu\text{g/ml}$ f61 was added to the experimental set ups. For the inhibited apoptosis set-ups, Z-VAD-FMK (Z-Val-Ala-Asp-fluoromethyl ketone) inhibitor was added to the cells to a final concentration of 50 μM . The difference between the amounts of yellow color produced in the presence or absence of the caspase inhibitor indicates caspase-3 activity in the samples. The cells were incubated for 12 h when significant cell death was observed in previous assays. The cells were sonicated and the protein concentration of the lysates was determined using the bicinchoninic acid (BCA) protein quantification method. The cell lysates were collected to a total volume of 100 μL in a 96-well plate precoated with the caspase-3 substrate DEVD-pNA. Caspase-3 activity was determined from the liberated pNA measured at 405 nm.

Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate significance between the data. The data that were found to be significant were further analyzed using Tukey's comparison of means and a p-value less than 0.05 was accepted as significant.

Spectrophotometric characterization

Isolate f61: m.pt. $55-70^\circ\text{C}$; FT-IR (KBr, cm^{-1}) 3414 (O-H str), 2928 and 2856 (C-H str), 1712 (C=O, C=C str), 1464 (C-O str), 1261 , 909 , 735 , 650 ; ^{13}C -NMR (100 MHz, CDCl_3) [DEPT] $\delta 177.4$ [acyl C], 135.2 [sp^2CH], 134.8 [sp^2CH], 132.9 [sp^2C], 127.8 [sp^2CH], 125.9 [sp^2CH], 123.7 [sp^2CH], 72.1 [CH], 70.3 [CH], 64.9 [C], 63.0 [c], 35.2 [CH_2], 33.7 [CH_2], 32.0 [CH_2], 29.7 [CH_2], 29.4 [CH_2], 29.3 [CH_2], 28.8 [CH_2], 27.6 [CH_2], 24.6 [CH_2], 22.7 [CH_2], 20.8 [CH_2], 14.2 (CH/ CH_3), 14.1 (CH/ CH_3); ^1H -NMR (400 MHz, CDCl_3) δ 0.81 (4H, m), 0.90 (t, 3H, $J=7.0$ Hz), 1.25 (br s, 43H), 1.40 (s, 12H), 1.61 (br, 11H), 1.71 (s, 6H), 1.99 (q, 6H, $J=7.6$ Hz), 2.11 (m, 2H, $J=5.4$ Hz), 2.27 (t, 8H, $J=7$ Hz), 2.57 (3, 12H), 4.09 (m), 4.15 (d, 1H, $J=6$ Hz), 4.73 (s, 10H), 5.29 (q, 1H, $J=10.7$ Hz), 5.37 (q, 1H, $J=10.0$ Hz), 5.48 (t, 1H, $J=7.0$ Hz), 5.62 (dd, 1H, $J=6$ Hz), 5.90 (t, 1H, $J=11.0$ Hz), 6.43 (d, $J=11$ Hz), 6.47 (d, $J=11.5$ Hz, 1H); GC-MS (m/z , % abundance): Rt $6.826-155$ (2), 154 (M^+ , 5), 143 (15), 135 (8), 129 (18), 129 (19), 111 (60), 101 (27), 98 (28), 95 (18), 83 (70), 73 (90), 69 (60), 68 (27), 67 (29), 60 (51), 57 (30), 55 (100); Rt $7.78-211$ (2), 210 (M^+ , 19), 167 (2), 152 (8), 123 (11), 111 (5), 96 (8), 95 (10), 94 (54), 82 (25), 81 (100), 55

(23); Rt $9.278-257$ (5), 256 (M^+ , 38), 236 (1), 213 (32), 185 (19), 171 (19), 157 (20), 129 (47), 116 (2), 115 (18), 113 (6), 111 (18), 97 (38), 83 (40), 81 (10), 73 (100), 71 (40), 69 (52), 60 (79), 57 (69), 55 (93); Rt $10.981-312$ (M^+ , 8), 280 (9), 264 (5), 241 (5), 213 (5), 185 (9), 167 (5), 151 (10), 126 (19), 114 (15), 111 (25), 109 (27), 99 (95), 97 (39), 95 (49), 83 (45), 81 (60), 79 (41), 73 (34), 71 (33), 69 (64), 67 (65), 60 (30), 57 (52), 55 (100).

Results

Isolation of the *C. alata* compound

The hexane fraction (FB) showed cytotoxicity to MCF-7, T24, and Col 2 at concentrations of 100 , 50 , and 25 $\mu\text{g/ml}$ (Figures. 1A-F). The toxicities of FB on the different human cancer cell lines were found to be in the following order: MCF-7>T24>Col 2>SK-BR-3>A549 cells. This indicates selective toxicity. At lower concentrations, FB showed weak toxicity to MCF-7, T24, and Col 2 cells. SK-BR-3 and A549 cells, on the other hand, were not sensitive to FB, showing almost 100% cell survival in all concentrations used, comparable to the non-cancer Chinese hamster ovary cells, AA8.

The TLC-pure f61, isolated from active FB, demonstrated cytotoxicity against MCF-7, T 24, and Col 2 cells in a dose-dependent manner (Figures 2A-C) with IC_{50} values of 16 , 17.13 , and 17.04 $\mu\text{g/ml}$ respectively. Although f61's induction of cell death did not occur in a very low concentration range, like other potential anticancer drugs, it can be a good candidate chemotherapeutic agent because it may not pose toxicity problems for normal cells, as seen from the absence of toxicity on the AA8 non-cancer cells. In contrast, doxorubicin was highly toxic to both cancer and non-cancer cells. However, further experiments with f61 on

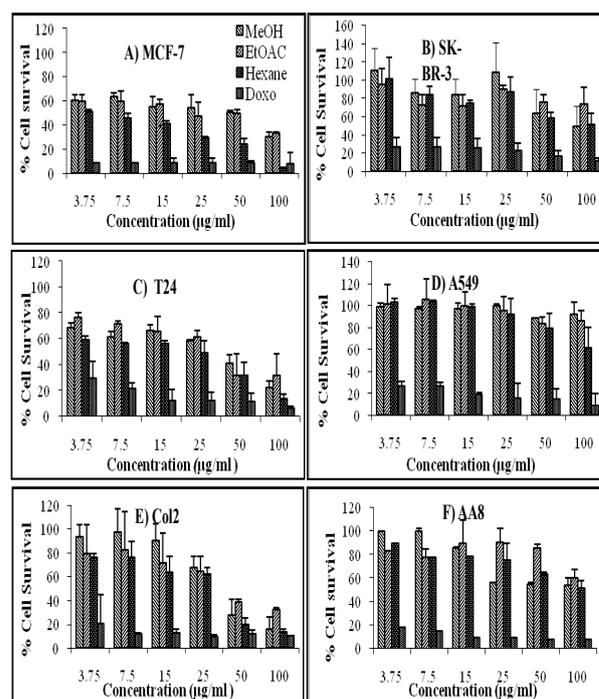


Figure 1. Cytotoxicity of *Cassia alata* Leaf Fractions to Selected Human Cancer Cells and Non-Cancer Chinese Hamster Ovary cells, AA8

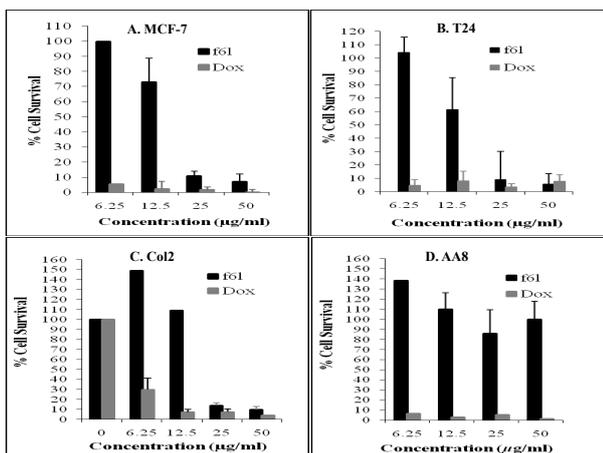


Figure 2. Cytotoxic dose Response of MCF-7. (A) T24 (B), Col 2 (C), and AA8 (D) Cells Treated with f6l and Doxorubicin

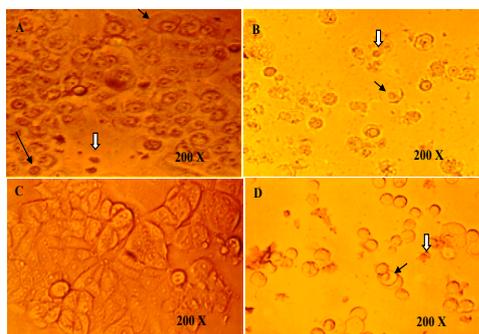


Figure 3. Morphological Features in MCF-7 Cells Treated with Hexane Extract and Isolates

normal human cell lines must be conducted.

Microscopic examination

Bright-field microscopy showed that after 12-24 h exposure to f6l, MCF-7, T24, and Col 2 cells rounded up and lost contact with neighboring cells and the substratum of the wells. The cells also exhibited chromatin condensation, formation of membrane blebs (short black arrows) and apoptotic bodies (white arrows) and reduction in overall size (long black arrows). These are morphological features associated with apoptosis that persisted until the 72nd h of observation, but were not observed with untreated counterparts (Figure 3 A-D).

The cells that showed remarkable cell death were further evaluated by DAPI staining (Figure 4 A-I). The apoptotic indices (AI) of MCF-7, T24, and Col 2 cells treated with 50 µg/ml f6l for 72 h were generated by counting cells that were positively stained with DAPI using a fluorescence microscope (Figure 5). Treatment with f6l resulted in 76% cell death (apoptotic index=0.76) in Col 2, followed by T24 and MCF-7 cells, both with 75% stained nuclei after 12 h of incubation. After 72 h, T24 showed the highest apoptotic index of 0.78. Apoptotic indices from the 12th to 72nd h culture were not significantly different from each other (p=0.304). Cellular and nuclear condensation observed with DAPI staining were not evident in their untreated counterparts.

DNA fragmentation

TUNEL positive cells displayed condensed and

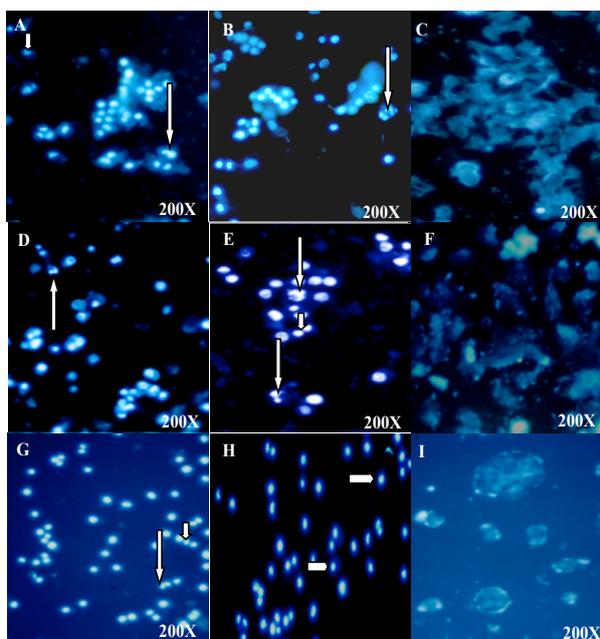


Figure 4. Cell Death Visualized with DAPI Staining. Incubating 50 µg/ml f6l with MCF-7 (A, B, C), T24 (D, E, F), and Col 2 (G, H, I) Long arrows point at apoptotic bodies; short arrows at condensed nuclei

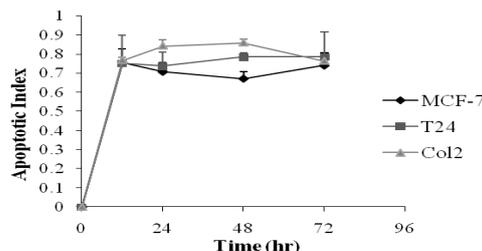


Figure 5. Apoptotic Indices of MCF 7, T24, and Col 2 Cells Incubated Up to 72 h with 50 µg/ml f6l

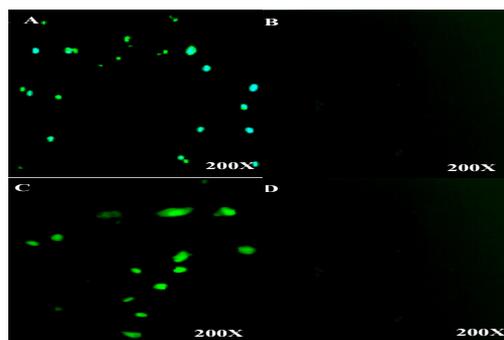


Figure 6. DNA Fragmentation Seen in the TUNEL Assay under Fluorescence Microscopy

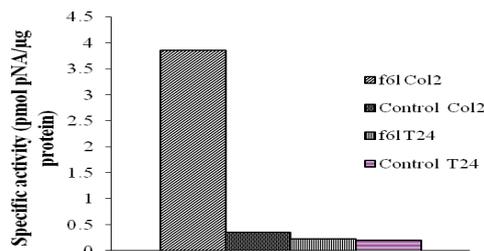


Figure 7. Caspase-3 Activity in Col 2 and T24 cells Treated with 50 µg/ml f6l for 4 h. pNA/µg protein was higher in f6l-treated Col 2 cells than f6l-treated T24 Cells

fragmented DNA in f6l treated Col 2 and MCF-7 cells (Figure 6). Similar results were observed with T24 stained with TUNEL (data not shown). Fragmented DNA were indicated by the intense green fluorescence after 24 h of exposure to the isolate. Under bright field microscopy, however, untreated cells did not exhibit fluorescent labeling; rather, they retained their size and cytoplasmic membrane integrity.

Caspase-3-activity

The activation of effector caspase-3 in f6l-induced cell death was evident in Col 2 cells (Figure 7). The isolate showed a higher specific activity (SA) of 3.85 pmol pNA/ μ g protein on Col 2, but the untreated Col 2 cells showed an SA of only 0.35 pmol pNA/ μ g protein. However, in T24, caspase-3 activation was not evident upon treatment with f6l, as reflected by a comparable SA of the treated and untreated T24 cells of 0.22 and 0.20 pmol/ μ g protein, respectively. Another apoptotic pathway that is not caspase-3 dependent may be involved in the f6l-induced cell death. Samejima et al. (1998) also suggested that there are apoptosis pathways that do not involve caspase-3. Co-treatment of Col 2 and T24 cells with f6l and benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone (Z-VAD-FMK), a generalized caspase inhibitor, registered a low absorbance comparable to the negative control. MCF-7 cells were not tested for caspase-3 activation as this cell line follows caspase 3-independent apoptosis due to a functional deletion in its caspase-3 gene (Janicke, 2009).

TLC-pure isolate f6l was eluted at 25% EtOAc in hexane. It is a yellowish, sticky solid mass. GC-MS showed that isolate f6l is an intractable mixture of four compounds with Rt 6.826, 7.78, 9.278, and 10.981 min. Evaluation of the mass fragmentation patterns for each compound showed the presence of long chain moieties with differences of m/z 14 (-CH₂-). Further spectrophotometric analyses characterized isolate f6l as a mixture of polyunsaturated fatty acid esters.

Discussion

The cytotoxic property of polyunsaturated fatty acids (PUFA) is well documented. In a review by Serini et al (2009), the ability of dietary PUFAs to induce apoptosis is presented as well as its significant role in the development of diseases including inflammatory and neurodegenerative diseases and cancer. PUFAs have been documented to be toxic to cancer cells. Cytotoxic action of Dihomo-gamma-linolenic acid (DGLA) and AA, EPA and DHA were observed *in vitro* on vincristine-sensitive (KB-3-1) and resistant (KB-Ch^R-8-5) cancer cells (Undurti and Madhavi, 2011). Hanada et al (2011) reported that DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid) induced growth arrest and apoptosis in AML-I cells at concentrations between 250 and 500 μ M demonstrated by Annexin V-FITC staining. Chiu et al. (2000) showed that PUFA-, AA-, and EPA-induced apoptosis involved endonuclease activity. Similarly, Siddiqui et al., (2005) observed that 25 μ M DHA and EPA induced apoptosis by 5-15% and resulted in 2-5% caspase-positive cells.

Caspases are implicated in the degradation of cytoskeleton causing fragmentation of cells into apoptotic bodies. Caspase-3 plays a vital role starting with a cascade of events involving the release of cytochrome c, which binds to apoptotic protease activating factor 1 (Apaf-1), in turn triggering the activation of the activator caspase-9 (Huai et al., 2010). Activated caspase-9 mobilizes a sequential activation of effector caspases such as caspase-3, leading to the biochemical and morphological changes associated with apoptosis (Pereira and Amarante-Mendes, 2011). Activation of caspase-3 may also follow the extrinsic pathway involving the binding of FAS ligand to a surface cell receptor, which activates caspase-8; this, in turn, activates caspase-3.

In conclusion, this study showed that FB and f6l exerted a cytotoxic effect on MCF-7, T24, and Col 2 cells in a dose dependent manner, but were not effective against A549 and SK-BR-3 cells. It is worth noting that isolate f6l is not toxic to a non-cancer Chinese hamster cell line, AA8. Morphological observations showed membrane blebbing, chromatin condensation, formation of apoptotic bodies, and reduction in size, which are indicators of apoptosis. DNA fragmentation with DAPI and TUNEL assays demonstrated that the mode of cell death is by apoptosis. Caspase activity is involved in the apoptosis induced in Col 2. Spectral characterization of f6l using FT-IR, GC-MS, and NMR showed that it contained a mixture polyunsaturated fatty acid esters. The isolate may have potential for development as a cancer chemotherapeutic agent.

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