ORIGINAL ARTICLE

Anti-proliferative Activity of *Annona muricata* Seeds Extracts against Esophageal Carcinoma within SKG Cell Line

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ABSTRACT

Esophageal cancer is very aggressive and the 6th most prominent cause of cancer death throughout the world. It had an extremely poor prognosis, and current chemotherapy treatments like 5-fluorouracil (5-FU) often have limited success and fatal outcomes. Recently, plenty of studies report the significant anti-proliferative effects of different extracts of *Annona muricata* and their isolated acetogenins (AGEs) towards various cancer cell lines, so it may be a candidate as an alternative or adjuvant to chemotherapies. In this study, the cytotoxic effect of *A. muricata* seeds extract (AMSE) showed concentration-dependent growth inhibition for SKG cell line by MTT assay. Chou-Talalay analysis showed a synergistic effect between extracts and 5-FU on the SKG cell line. Hematoxylin and eosin staining after 72 hours showed different morphological changes. The AO/PI double staining assay after 72 h showed that the percent of the dead cell significantly elevated in 5-FU, chloroform, ethanol, and their combinations. Analysis of RT-qPCR after 24h showed that the Bax gene was up-regulated by 5-FU, chloroform and its combination, while downregulated by ethanol and its combination. The Bcl2 was up-regulated by 5-FU while downregulated by chloroform, ethanol, and their combinations. Human apoptosis antibody array analysis showed that AMSE and their combination after 24 hours could induce cell apoptosis through the activation of caspase-8 and modulation of apoptosis-related proteins (such as Fas, CD40, TNF-α, TNF-β, and TNFR), which confirmed the contribution of the extrinsic pathway. Also, overexpression of cytochrome-c, Bax, and bad proteins, along with the suppression of Bcl-2, confirmed that mitochondrial-dependent pathway also contributed to AMSE-induced cell death. In conclusion, chloroform and ethanol extract of *A. muricata* have a powerful cytotoxic activity and synergistic effect with 5-FU against SKG cell lines. The proposed underlying mechanism involves the induction of apoptotic proteins.

Keywords: Annona muricata, Apoptosis, Cytotoxicity, Esophageal cancer, SKG cells.

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INTRODUCTION

Esophageal cancer is highly aggressive and the sixth leading cause of cancer death worldwide and in the US. Both the incidence and mortality of esophageal cancer have increased over the past two decades. Esophageal squamous cell carcinoma (ESCC) accounts for most of the cases of esophageal cancer worldwide; however, trends now indicate that there has been a dramatic increase in the incidence of esophageal adenocarcinoma (EAC) in the US. Although esophageal adenocarcinoma and esophageal squamous cell carcinoma differ in their epidemiologic distribution and histology, some of their risk factors and underlying mechanisms of carcinogenesis are the same. Esophageal cancer continues to have a poor prognosis, with 5 years survival rates between 10–13% despite many improvements in both diagnostic and therapeutic techniques over the past three decades.[1] The ESCC and EAC incidence rates in men are 3–8 fold higher than in women. Tobacco smoking, alcohol consumption and abdominal obesity are an etiological factors which are more prevalent in men. Also increases gastroesophageal reflux leads to an increased incidence of EAC.[2] Evidence from animal studies support a biological basis for this male predominance, in which estrogen is an inhibitor of esophageal carcinogenesis.[3]

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The 5-flurouracil (5-FU) is commonly used to treat a variety of tumors, involving colorectal cancer, breast cancer, and aerodigestive tract cancers (esophagus and liver). Although many recent researches has been conducted into cancer treatment, and control advances, significant work and improvement efforts remain unsatisfied. The key disadvantages to synthetic drugs are related to adverse effects.[4]

Annona muricata, commonly known as Graviola, Soursop, or Guanabana, is a member of the *Annonaceae* family, comprising approximately 130 genera and 2300 species.[5] It is native to the warmest tropical areas in South and North America, and is now widely distributed throughout tropical and sub-tropical parts of the world, including Nigeria, Malaysia, and India.[6] *A. muricata* is extensively used as traditional medicines against an array of human ailments and diseases, especially cancer and parasitic infections. The fruit is used as a natural medicine for arthritic pain, neuralgia, diarrhea, dysentery, fever, malaria, parasites, rheumatism, skin rushes and, worms.[7] Plenty of studies report the significant anti-proliferative effects of different extracts of *A. muricata* and isolated acetogenins (AGEs) towards various cancer cell lines. Ethyl acetate extract of *A. muricata* leaves showed chemopreventive properties on azoxymethane-induced colonic aberrant crypt foci in rats.[8] and breast cancer cell line (T47D).[9] Meanwhile, *A. muricata* seeds extract (AMSE) show toxicity against brine shrimp, lung A549, breast MCF-7, and colon HT-29 cancer cells.[10] Also, Graviola fruit peel extract (GFPE) proved to be toxic to HT-29 colorectal adenocarcinoma while not affecting non-malignant fibroblastic MRC-5 and epithelial CCD-1074 cell lines. Such results suggest that low doses of the extract could specifically target cancer Cell Lines while not affecting normal cells.[11]

This study was proposed to evaluate the anti-proliferative effect of AMSE alone and in combination with 5-fluorouracil on esophageal cancer cell line (SKG), mechanisms by which AMSE can produce anti-proliferative effects.

MATERIALS AND METHODS

Chemicals, Reagents and Kits

All chemicals and reagents were from the good origin, Rosswell Park Memorial Media -1640 (RPMI) and Fetal bovine serum (FBS) were purchased from Euroclone/Italy. MTT (3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) and acridine orange were purchased from Bioworld/ USA. Propidium iodide solution, Hematoxylin, and Eosin stain were purchased from Santa Cruz, USA. Automated total RNA extraction kits were purchased from Anatalia gene work, Turkey, RayBio ® C-Series Human Apoptosis Antibody Array Kit C1 (AAH-APO-1-8) from Ray Biotech, USA, while One-Step Bright Green qRT-PCR-Low ROX was from Abm Good, Canada.

Cell Culture

The SKG-T4 cell line was supplied by Experimental Therapy Department/ Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), AL-Mustansiriyah University (Baghdad, Iraq). The cells were cultured in its specific medium (RPMI) with L-glutamine,10% fetal bovine serum (FBS), in addition to 100 mg/mL streptomycin and 100 IU/mL penicillin, was added to those medium to prepare a complete growth medium under standard conditions (37°C, 5% CO_2, humidified atmosphere).

Preparation of Plant Extract

Annona muricata were seeds collected from the local market in Baghdad. Before being ground into a fine powder, the seeds were pooled together and air-dried at room temperature. Approximately 100 g of the powder was weighed and extracted successively with chloroform three times after agitation for 48 hours on an orbital shaker. Then the chloroform extracts were filtered, combined, and then evaporated to be dried under low pressure in a rotary evaporator at 40°C.[12] The chloroform extract was dark brownish colored-oily residue, weighing 6.3g. Then the filtered seed residues were extracted again by ethanol using the same procedure. The ethanol extract was also dark brownish colored-oily residue with a yield of 6.8g. The yield of extract for chloroform and ethanol solvents were calculated according to the following equation:[13]

$$\text{The yield of extract} = (\text{weight of material obtained})/(\text{weight of start material}) \times 100$$

MTT Assay

The SKG cell line was seeded in 96 well microplates with 200µL RPMI containing 10% FBS in each well and incubated 24 hours in a humidified atmosphere at 37°C. The whole media was then removed, and the culture was checked to ensure adding 1×10^4 cells/well by an inverted microscope magnification (X40). Then ethanol and chloroform extracts were added to seeded cells at a final concentration of 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/mL from a stock of 100 mg/mL and incubated for 72 hours in a CO_2 incubator at 37°C. The untreated cells received 5-FU as positive control while DMSO as a negative control (124). Then 100 µL of MTT solution was added to the culture (MTT, 5 mg/mL dissolved in PBS). It was incubated at 37°C for 3 hours. The MTT was removed, and 50 µL of DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cells got lysed and color was obtained. Optical density was read at 580 nm by using ELISA technology (Enzyme-linked immunosorbent assay), and the percent of cell viability and growth of inhibition was calculated.[14]

Potential Interaction of the Extracts with 5-Fluorouracil

The median effective doses (ED_{50}) were calculated for 5-FU, extracts, and their combination for each cell line. For determination of synergism, extracts and 5 fluorouracils were studied as constant ratio. Chou-Talalay combination indices (CI) were calculated using CompuSyn® software (Combo Syn, Inc., Paramus, NJ, USA). To analyze the combination of extract and 5-FU, fixed ratio of extracts and 5-FU, with mutually exclusive equations, was used to determine the CI. A combination index between 0.9–1.1 is considered additive, whereas $CI < 0.9$ and $CI > 1.1$ indicate synergism and antagonism, respectively.[15]

Morphological Analysis by Cytopathological Staining (Hematoxylin and Eosin)

The cells were cultured on tissue culture flasks (5×10^4 cells/flask) with serum media until the confluence become monolayer. The old media was removed and replaced by serum-free media, and the cells were exposed to maximal inhibitory concentration (IC_{50}) of ethanol and chloroform extracts, 5-FU, and their combinations and incubated for 72 hours, at 37°C under a humidified atmosphere with 5% CO_2. Then, 4% formaldehyde, diluted in PBS, was added up to 5 minutes for fixation, and then Hematoxylin and Eosin were added to stain the morphological changes.. The cells were examined under an inverted microscope and pictured by camera under 40X.[16]

Acridine Orange/Propidium Iodide (AO/PI) Double Staining Assay

The SKG cells were seeded in 96 well microplates for 24 hours, followed by treatment with extracts (IC_{50} concentration) and combined with chemotherapy for 72 hours. After incubation time, untreated and treated SKG cells were washed with PBS and then, stained with a mixture of 1 µL propidium iodide + 1 µL acridine orange in 1-mL PBS and further incubated for 30 minutes. The stained cells were taken to view under a fluorescent microscope with 40X magnification power.[17]

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The complementary DNA (cDNA) was synthesized with the master mixed of amplification reaction (one-step Evagreen qRT-PCR kit, Canada). It was applied in a final volume of 20 µL using a master amplification reaction kit with specific primers. The RNA from cells treated with chloroform, ethanol, 5-flurouracil and their combinations was studied for important genes (P53, CASP3, Bax, Bcl2, and GABDH) for esophageal cancer cells with presence of One-Step Bright Green qRT-PCR Kit (Cat#G471-LR) for real-time PCR by the amplification reaction and values were normalized to the housekeeping gene GAPDH and calculated (Table 1).

Human Apoptosis Protein Study

We performed this assay to identify the potential apoptotic pathways induced by AMSE and determine apoptosis-related proteins' involvement using the Human Apoptosis Antibody Array according to the manufacturer's instructions. In brief, the SKG cells here, were treated with AMSE at IC_{50} concentration for 24 hours. Around 1000 µg of extracted protein from each sample was incubated with the human apoptosis antibody array overnight. The membranes were used to quantify the apoptosis array data via chemiluminescence imaging.

Statistical Analysis

The present study's results were expressed by Mean ± standard deviation (M ± SD) and percentage of growth inhibition (GI %) for SKG cell lines. Graph Pad Prism® 7.0 software was used to show the difference among treatment groups and measuring IC_{50}. It would be significant if $p \leq 0.05$ where least significant difference (LSD) test was used to compare between means in this study statistically. For synergy analysis, CompuSyn® software program algorithm was used to estimate the combination index. Image J (USA-2019) was used for analyzing acridine orange/propidium iodide stain results.

RESULTS

Anti-proliferative Activity (MTT assay)

The 5-flurouracil, chloroform, and ethanol extracts of *A. muricata* caused growth inhibition for SKG cell line in concentration-dependent manner after 72 hours. The half-maximal inhibitory concentration (IC_{50}) was 26.54 µg/mL, 3.8 µg/mL and 2.6 µg/mL, respectively (Figure 1). Microscopic images under crystal violet stain is shown in Figure 2.

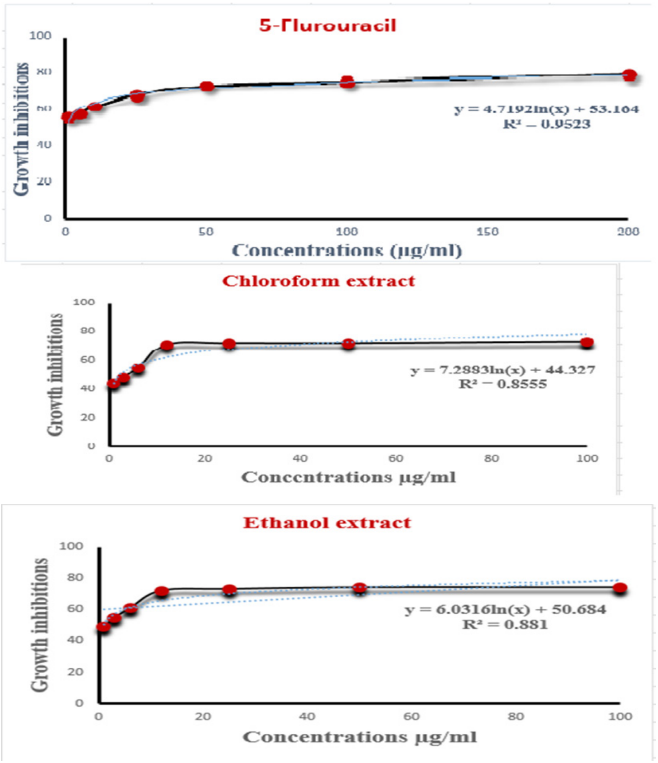

Figure 1: Dose-response curve of 5-FU, chloroform, and ethanol extract for *A. muricata* seeds against SKG cell line after 72 hours exposure period.

Table 1: Primer sequences used for real-time PCR.

Gene	*Forward primer*	*Reverse primer*
Bax	GTT TGC CCT CGG ATC TCT GG	GCT TCC AAC AGC GTA AAT CCA A
Bcl-2	GGT GGG GTC ATG TGT GTG G	CGG TTC AGG TAC TCA GTC ATC C
P53	GAG GTT GGC TCT GAC TGT ACC	TCC GTC CCA GTA GAT TAC CAC
Caspase 3	GAA ATT GTG GAA TTG ATG CGT GA	CTA CAA CGA TCC CCT CTG AAA AA
GAPDH	CTG GGC TAC ACT GAG CAC C	AAG TGG TCG TTG AGG GCA ATG

Potential Interaction of the Extracts with 5-fluorouracil (Chou–Talalay Analysis)

To study the potential interaction between AMSE and 5-FU, the studied concentrations for AME and 5-FU were select depending on IC_{50} for each i.e., we take three concentrations (below, at, and above IC_{50}) and tested on SKG cell line. The cell viability was determined after 72 hours by MTT assay. Adding the chloroform and ethanol extract of *A. muricata* to 5-FU produced a moderate to strong synergic anti-proliferative effect (concentration-dependent manner) against SKG cell line after an exposure period of 72 hours compared with a single treatment, according to the values of combination index (Figure 3).

Morphological Analysis by Cytopathological Staining (Hematoxylin and Eosin)

Morphological changes of SKG cell line by H and E staining after exposure to IC_{50} of 5-FU, chloroform and ethanol extract of *A. muricata* seeds with their combinations after 72 hours incubation showed cell rounding, nuclear condensation, cytoplasmic vaculation and blebbing with cell necrosis, while untreated SKG cells showed a confluent monolayer and was apparently healthy. The 5-FU, chloroform and ethanol show more hydropic degeneration which represent a sign of cell injury due to cytotoxic activity in which cells looked enlarged, while in combination groups we see more pyknotic cells (condensation of chromatin in the nucleus of a cell undergoing necrosis or apoptosis) in addition to cytoplasmic vaculation (Figure 4).

Acridine Orange-Propidium Iodide (AO-PI) Double Staining Assay

Double staining was used to indicate apoptosis in the esophageal cancer cell line, acridine orange-propidium iodide (AO-PI). The SKG cell line was exposed to IC_{50} of 5-FU, chloroform and ethanol extracts of *A. muricata* seeds with their combinations for 72 hours, as shown in (Figure 5). Under a fluorescent microscope, the percent of the dead cell was significantly elevated in 5-FU, chloroform, ethanol, and their combinations compared to control ($p > 0.05$). Also chloroform combination with 5-FU increased dead cell percent significantly when compared to 5-FU, chloroform and ethanol alone ($p > 0.05$), while ethanol combination with 5-FU elevated dead cell percent significantly just when compared to 5-FU alone ($p > 0.05$) (Figure 6).

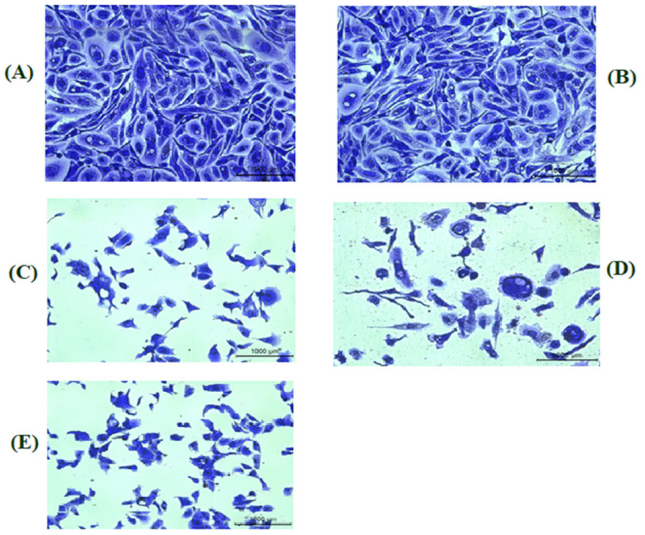

Figure 2: Microscopic image for cytotoxicity of 5-FU, chloroform and ethanol extract of *Annona muricata* seeds extract against SKG cell line after 72 hr. exposure period with (A) Control, (B) DMSO, (C) 5-fluorouracil, (D) Chloroform extract (E) Ethanol extract, under crystal violet stain (10X).

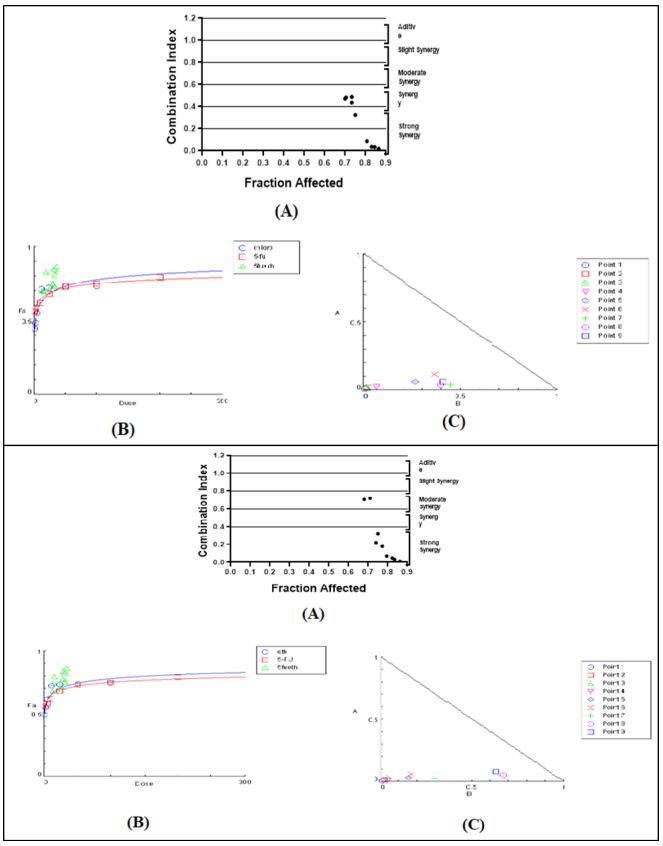

Figure 3: Potential interaction of 5-FU with chloroform (top) and ethanol (bottom) on SKG cell line after 72h exposure period (a) combination index (b) dose-effect curve (c) isobologram analysis.

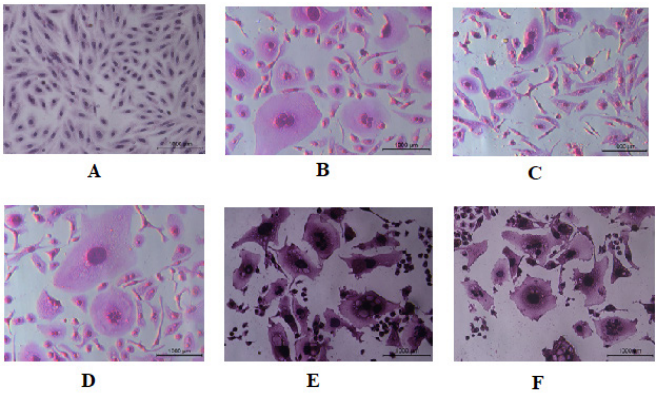

Figure 4: Hematoxylin and Eosin staining showing the morphological changes of SKG cell line after 72h incubation period (A) control, (B) 5-FU, (C) chloroform extract, (D) ethanol extract, (E) chloroform+5-FU, (F) ethanol+5-FU.

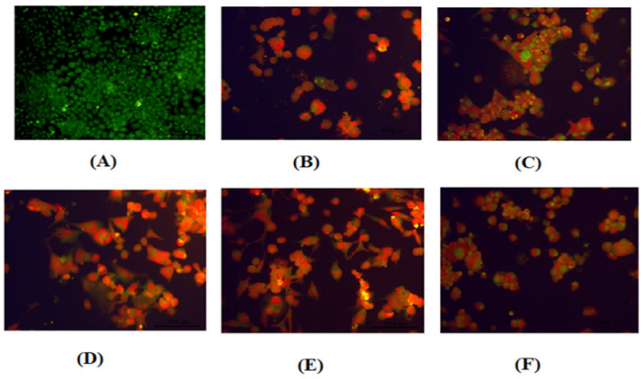

Figure 5: Acridine orange –propidium iodide (AO-PI) double staining of SKG cell line after 72 hours incubation (A) control, (B) 5-FU, (C) chloroform extract, (D) ethanol extract, (E) chloroform+5-FU, (F) ethanol+5-FU, under flouresent microscope (20X).

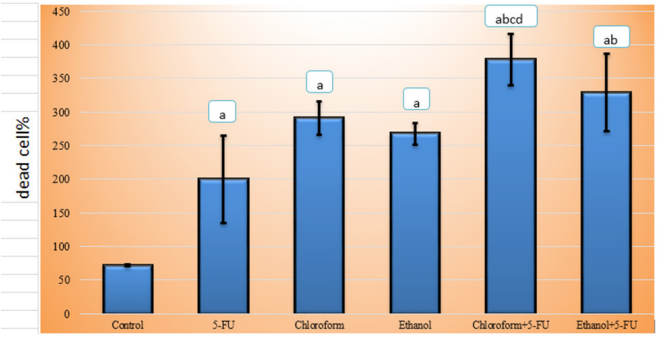

Figure 6: Percent of dead cells (apoptotic SKG cells) after 72 hours exposure to different treatment groups, according to AO-PI stain. Data represent as percent of mean ±SD. a = significant difference from control using LSD test at 0.05 level. b = significant difference from 5-FU using LSD test at 0.05 level. c = significant difference from chloroform using LSD test at 0.05 level. d = significant difference from ethanol using LSD test at 0.05 level.

Real Time Polymerase Chain Reaction (RT-PCR)

Gene expression analysis was evaluated for different four proteins (Bax, Bcl2, P53, and CASP3) by using Ct (cycle threshold) values of qRT-PCR analysis in esophageal cancer cell line (SKG) using IC_{50} for each of 5-FU, chloroform and ethanol extract with their combinations after 24 hr incubation time. The Bax was up-regulated by 5-FU, chloroform, and combination, while downregulated by ethanol and its combination. The Bcl2 was up-regulated by 5-FU, while down regulated by chloroform, ethanol, and their combinations. Meanwhile, P53 was up-regulated by ethanol and chloroform combinations while downregulated by 5-FU, chloroform, and ethanol. The CASP3 was up-regulated by chloroform, ethanol, and their combination with 5-FU, while downregulated by 5-FU (Figure 7).

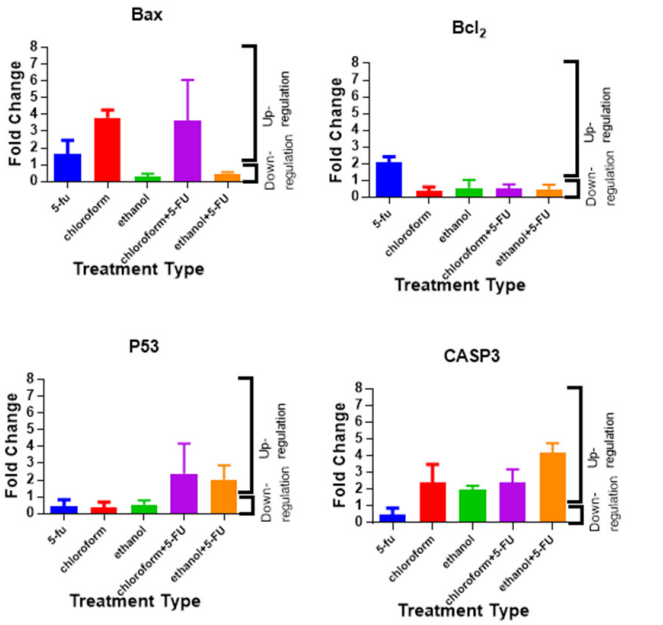

Figure 7: Gene expression analysis for SKG cell line after 24 hours incubation period with 5-FU, *Annona muricata* seeds extracts, and their combination.

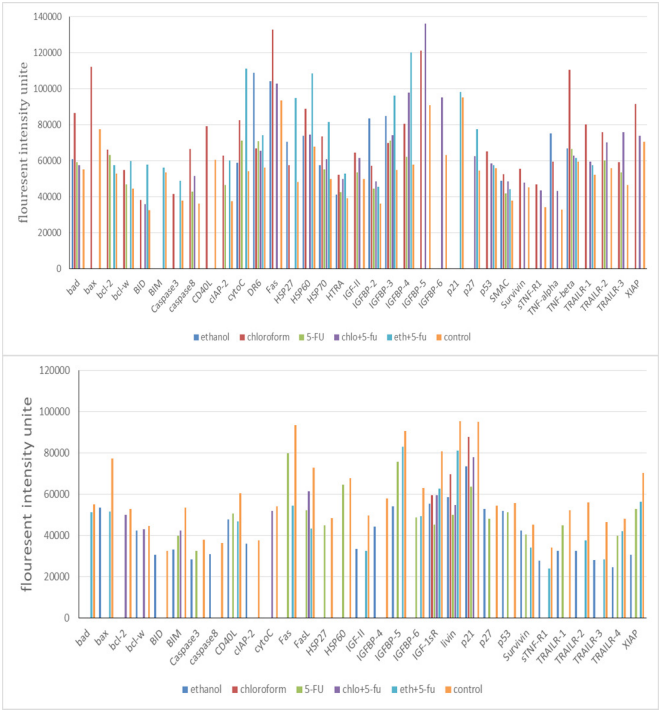

Figure 8: Quantitative analysis of human apoptosis antibody array of SKG cells after 24 hours incubation period for 5-FU, chloroform, ethanol and their combinations (A) up-regulated proteins, (B) down-regulated proteins.

Human Apoptosis Antibody Array Analysis

Human apoptosis proteins array was used to investigate the molecular profile of cell growth inhibition and cytotoxicity by studying the apoptosis proteins expression in treated and untreated esophageal cancer cell line (Figures 8 A and B). After exposure of SKG cells to AMSE for 24 hours, the critical proteins were implicated with cell death and apoptotic pathways were observed via human apoptosis protein array. Many proteins were observed to be up- or downregulated according to their role in apoptotic pathways. As shown in Figure 8A, the important proteins that play a critical role in the intrinsic apoptosis, such as Bax, Bim, cytochrome c, SMAC, and HtrA-2 were significantly upregulated after 24 hours of

treatment, while figure 8B showed that expressions of anti-apoptotic proteins including Bcl-2, Bcl-w, cIAP-2, XIAP, and LIVIN were inhibited.

DISCUSSION

In the present study, the seeds of *A. muricata* were extracted sequentially to detect the amount of analogous compounds in the desired yield and separate them depending on the solvent's polarity.[18] The cold method was used in the present study (maceration method) to avoid any damage or loss of the composites inside the seeds when exposed to high temperatures.[19] Various factors affecting the extraction process of plants, for instance, shaking, time, type of maceration, solvents concentration and heat of the water that warm container having powder of the extracted part. These aspects explain the alterations in the percentage of yield of the extracts.[20] The extraction yield of *A. muricata* seeds obtained by using a rotary evaporator was 6.8% w/w for ethanol, which was more than that of chloroform (6.3%). This result was better if compared with a study demonstrated by Mohammad Zahid *et al*. (2017), who reported that ethanol extract yield just (3.6%) while n-hexane gave 14.85% for each 100g of *Annona squamosa* seeds.[21]

During this study, the half-maximal inhibition concentration (IC_{50}) for 5-FU, chloroform, and ethanol extract against the SKG cell line was 26.54 µg/mL, 3.8 µg/mL 2.6 µg/mL, respectively (Figure 1). It was found that both extracts of *A. muricata* seeds reduced SKG cell line's viability in a concentration-dependent manner and this was confirmed by crystal violet stain (Figure 2) but ethanol extract showed more growth inhibition effect than chloroform. This study was in line with a study conducted by Subin Varghese Thomas *et al* (2019) who found that IC50 of chloroform extract of *A. muricata* fruit (CHL-AMF) was 53.7 µg/mL compared to 292 µg/mL for 5-FU against HepG2-cell proliferation (hepatocellular carcinoma).[22] According to Unites States National Cancer Institute (NCI) plant screening program, if the IC_{50} value following exposure period between 48 and 72 hours is less than 20 µg/mL, a crude extract is considered to be cytotoxic.[23] This fact is agree with our results, where AMSE had cytotoxic effect against SKG cell line.

In our study, a combination of each extract of *A. muricata* seeds with 5-FU after 72 hours incubation period reduced the viability of SKG cancer cells in a concentration-dependent manner and showed a moderate to strong synergism, compared with extract or 5-FU alone, where 5-FU and *A. muricata* extract combination yield a synergistic effect with CI values ranging from 0.7 to 0.1 (Figure 3). The expected advantage of this combination is reducing the incidence of resistance to 5-FU and diminishing its required dose and, consequently, its adverse reactions.

As seen in the current study and after H and E staining, the SKG cells that were exposed to 72 hours of seeds extracts, 5-FU, or their combination revealed cell rounding, nuclear condensation, cytoplasmic vacuolation and hydropic changes, compared with control (untreated) cells. Combination of chloroform and ethanol with 5-FU showed more pyknotic cells, which mean chromatin condensation and necrosis (Figure 4). This result was in line with a study conducted by María P. Torres *et al.* (2012), who used Graviola leaf/stem powder against metastatic pancreatic cells. The H&E stained tumor sections showed necrotic cells in 20–50% of the pancreatic tumor tissues from graviola extract-treated mice, as compared with tumors from untreated mice.[24]

The AO/PI stain showed that treated cells with IC_{50} of AMSE, 5-FU, and their combinations caused a moderate apoptosis which clarified by nuclear chromatin condensation, blebbing, and presence of a reddish-orange color (Figure 5), because the binding of PI to denatured DNA indicate the late stages of apoptosis, while untreated cells showed a green intact nuclear structure after 72 hours incubation period.[25] In the current study, calculation of apoptotic index was achieved by dividing the number of cells undergoing apoptosis on the number of living cells,[26] where combination of chloroform with 5-FU gave highest significant index value than each treatment alone (Figure 6). This result was in agreement with a study proposed by Subin Varghese Thomas *et al* (2019) who found that chloroform extract of *A. muricata* fruit (CHL-AMF) at dose of 55 µg/mL show significant apoptotic index when compared to control after 48 hours incubation on HepG2 cell line by using AO/EB staining.[22]

In the RT-PCR study and as shown in Figure 7, 5-FU up regulated the anti-apoptotic protein Bcl2 and pro-apoptotic protein Bax, while downregulated P53 and CASP3 them important for apoptosis stimulation, this mean that 5-FU as single therapy have a weak apoptotic effect on SKG cell line. Regarding *A. muricata*, chloroform extract up-regulated Bax, which act as the main pro-apoptotic protein that cause release of small pro-apoptotic molecules such as cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF, and endonuclease G that induced cell death via mitochondrial membrane permeabilization (MMP), and CASP3 that mediate the apoptosis.[27] Ethanol extract up-regulated CASP3 and down-regulated Bcl2, Bax and P53. Meanwhile, chloroform plus 5-FU up-regulated Bax, P53 and CASP3 involved apoptotic stimulation and downregulated Bcl2, which represent the anti-apoptotic protein, meaning that combination of chloroform with 5-FU gave the highest apoptotic effect. Ethanol plus 5-FU up-regulated P53 and CASP3, while downregulated Bcl2 and Bax. This result was in line with study conducted by Soheil Zorofchian Moghadamtousi *et al.* (2014) who tested the expression of Bax and Bcl2 in treated A549 (human lung cancer cell) with *A. muricata* ethyl acetate extract (AMEAE) at different concentrations for 24 hours by quantitative PCR analysis. The results revealed that the pro-apoptotic Bax expression increased significantly when treated with 20 and 40 µg/mL of AMEAE, while Bcl-2 expression decreased significantly when A549 cells were treated with the same concentrations after the same incubation period, as compared with the control cells. Therefore, downregulation of Bcl-2 and upregulation of Bax upon AMEAE treatment could lead to loss of MMP, which facilitated cytochrome-c release and activation of the caspase cascade. These changes in the

gene expression of Bcl-2 and Bax confirmed the induction of apoptosis via mitochondrial-mediated intrinsic pathway.[28]

Regarding microarray analysis, Figures 8 A and B showed that chloroform extract gave more apoptotic protein induction than other groups, while chloroform and ethanol combination with 5-FU gave nearly same effect. According to the current study, ethanol extract cause up-regulation of death receptors that involved within extrinsic apoptotic pathway (like TNF-α, TNF-β, and Fas). On the other hand, ethanol extract cause up regulation of pro-apoptotic proteins that mediate intrinsic apoptotic pathway, like cytochrome-c, SMAC (second mitochondrial activator of caspases), and HTRA (high-temperature requirement protein A). These proteins activate the caspase-dependent mitochondrial pathway, leading to activation of procaspase-9, also they inhibit IAP family as an inhibitors of apoptosis proteins (cIAP-1, XIAP, livin, and survivin). In addition, Bad (Bcl2-antagonist of cell death) was up regulated by ethanol, where it's the pro-apoptotic protein that responsible for alteration of mitochondrial membrane permeability and regulation of cytochrome-c release from the mitochondria, also cause down regulation to anti-apoptotic protein Bcl-w.[29]

In our study, chloroform extract caused up regulation of TNF-α, TNF-β, Fas, sTNF-R1, caspase-8 and caspase-3, this mean that chloroform extract mediate the extrinsic apoptotic pathway. Also, chloroform caused up regulation of cyto-c, SMAC, HTRA, Bad, and Bax that involved in intrinsic apoptotic pathway and Bid (BH3 interacting domain death agonist) which act as "cross-talk" between the death-receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway.[30] Another protein activated by chloroform was p53 (tumor suppressor gene), which initiates apoptosis through p53-dependent and independent mechanisms.[31] Also, chloroform extract causes up-regulation of death receptors (DR6 and TRAILR-1-2-3), these receptors possess a death domain and a number of molecules which attracted to the death domain when triggered by a death signal resulting in the activation of a signaling cascade.[32] In addition, chloroform cause downregulation of livin, as a member of IAP family.

The 5-FU caused up-regulation of TNF-β and caspase-8 involved in extrinsic apoptotic pathway, while up-regulated cyto-c, SMAC, bad and HTRA which involved intrinsic pathway; contrary, 5-FU inhibit IAP (survivin, livin, and XIAP) that inhibit the activity of caspase-3 and 9.[29] Also, cause up-regulation of death receptors (DR6 and TRAILR-2-3).[32]

Combination of chloroform plus 5-FU caused up-regulation of Fas, TNF-α, TNF-β, sTNF-R1 and caspase-8 that involved in the extrinsic apoptotic pathway and also caused downregulation of anti-apoptotic proteins (Bcl2 and Bcl-w) and up-regulation of pro-apoptotic proteins (Bad and BID), also cause up-regulation of SMAC and HTRA that involved in the intrinsic pathway, and induced death receptors like DR-6 and TRAILR-1-2-3. This combination mediates the caspase-independent apoptosis by inducing P53 and P27 as tumor suppressor genes.

Meanwhile, the combination of ethanol plus 5-FU induced TNF-β and caspase-3 levels means aggravating the extrinsic pathway of apoptosis, while induced cyto-c, HTRA, SMAC, and pro-apoptotic proteins (BID and BIM) that involved in the extrinsic pathway of apoptosis. Also induced tumor suppressor genes (P53, P27, and P21) that regulate DNA repairing and cell cycle arrest and inhibit IAP family member (XIAP, livin, and survivin). These results were in line with a study conducted by Elham Bagheri *et al*. (2018), who studied the effect of *Brucea javananica* fruit extract, which contains tetracyclic triterpene compounds like that of *Annona muricata*, on HT29 colon cancer cell line, where treatment with IC_{50} of this plant extract for 24 hours showed up-regulation of Bax, BIM, cytochrome-c, SMAC and HtrA-2 that play critical role in intrinsic apoptosis, while expressions of anti-apoptotic proteins including Bcl-2, Bcl-w, cIAP-2, XIAP, and LIVIN were inhibited. This study showed over expression of P53 and P27, which considered markers that suppress cell proliferation by inducing apoptosis and arrest of cell cycle. The same study also showed overexpression of caspase-8, Fas, CD40, DR6, TNF-receptors and related ligands that contribute to an extrinsic pathway of apoptosis in HT29.[33]

CONCLUSION

This study concludes that chloroform and ethanol extract of *A. muricata* have a powerful cytotoxic activity and synergistic effect with 5-FU against SKG cell line after 72 hours exposure period in a concentration-dependent manner. The proposed underlying mechanism of *A. muricata* anticancer activity was confirmed by H&E stain, AO/PI stain, RT-PCR technique, and microarray analysis represented by over-expression of the major apoptotic proteins.

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