

## EVALUATION OF *ALOE VERA* LEAF EXTRACTS AND ALOE EMODIN ON SEVERAL CANCER CELL LINES

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### Abstract

Fresh leaf skin aqueous and methanolic extracts as well as shade-dried leaf skin methanolic extracts prepared separately from the leaves of *Aloe vera* and aloe emodin (AE) were assayed on human gastric (AGS), colon (HT-29, HCT116) and hepatocellular (HEPG2) cancer cell lines relative to human umbilical vein endothelial cells (HUVEC). AE, 5-fluorouracil (5-FU) and imatinib (IM) were tested as positive controls. Among the four extracts studied, *A. vera* gel extract (AVG) had the highest cytotoxic effect on cancer cells, with the highest effect on HCT116 cells, while no cytotoxic effect on HUVEC cells was detected. AE has also selective cytotoxic and apoptotic effects on the cancer cells and was ineffective on normal cells. AVG treatment in HCT116 cells induced the apoptosis by the activation of caspase-9 and caspase-3. According to our results, AVG and AE could be proposed as promising cytotoxic drugs from natural origin.

### Rezumat

Extractele apoase și metanolice ale frunzelor proaspete, precum și extractele metanolice ale frunzelor uscate de *Aloe vera* și aloe emodin (AE) au fost analizate pe linii celulare canceroase gastrice (AGS), de colon (HT-29, HCT116) și hepatocelulare (HEPG2) față de celulele endoteliale ale venei ombilicale umane (HUVEC). AE, 5-fluorouracil (5-FU) și imatinib (IM) au fost martori pozitivi. Dintre cele patru extracte studiate, extractul de *A. vera* gel (AVG) a prezentat efect citotoxic superior asupra celulelor canceroase, în special asupra celulelor HCT116, și nici un efect citotoxic asupra celulelor HUVEC. AE are, de asemenea, efecte selective citotoxice și apoptotice asupra celulelor canceroase. Tratatamentul cu AVG asupra celulelor HCT116 a indus apoptoza prin activarea caspazei-9 și a caspazei-3. În concluzie, AVG și AE prezintă potențial chimioterapic promițător pentru terapia cancerului.

**Keywords:** AGS, aloe emodin, *Aloe vera*, annexin V, caspase, HCT116, HEPG2, HT-29, HUVEC, MTT

### Introduction

As it is known, conventional chemotherapeutic drugs have deleterious side effects, by killing normal cells along with cancer cells. Therefore, plants are widely researched in the hope of finding anti-cancer and cytotoxic compounds [35, 40]. Many studies were conducted on the antitumour effects of *Aloe vera* since 1981 [9, 39, 41]. In our laboratory, *A. vera* leaf skin aqueous extract was proved to be effective as prophylactic against *Ehrlich ascites* tumours *in vivo* [4]. Antitumour and cytotoxic potential of *A. vera* extracts continues to be the interest of scientific research until recent years [6, 13, 28]. The success of *A. vera* in the treatment of multiple diseases has been attributed to the presence of several compounds [3, 8, 13, 34]. The inner gel or pulp has been shown to contain compounds with immunomodulatory activity [16], nevertheless some researchers claim that the synergistic activity of all chemicals contained in the whole leaf extracts could be responsible for the healing properties of the “wonder plant” [15]. Aloe emodin (AE), which is one of the major anthraquinone derivatives of *A. vera*, was reported to have

anti-tumour and angiogenic properties [5, 20, 22]. Therefore, in recent years, aloe emodin has been investigated for its cytotoxic and anti-cancer effects in various types of cancer [7, 25, 38].

*A. vera* has also been proved beneficial for most digestive problems including constipation, colitis, peptic ulcers [19, 34] as well as hepatoprotective [29]. The present study evaluates the cytotoxic effect of different aqueous and methanolic extracts prepared from the plant leaves and Aloe emodin on different gastric and hepatic tumour and normal cell lines.

### Materials and Methods

#### *Cell lines and cell culture*

Human gastric (AGS), colon (HT-29 and HCT116), hepatocellular (HEPG2) tumour cell lines and human umbilical vein endothelial cells (HUVEC) were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in DMEM (Dulbecco's Modified Eagle's medium; Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS; Capricorn FBS-12A), 100 units/mL penicillin and

100 µg/mL of streptomycin (Gibco 15140-122), in a humidified incubator containing 5% CO<sub>2</sub> at 37°C. In order to reach the sufficient cell number for tests, cells were passaged after reaching 80% monolayer confluency. Cells were harvested gently by 0.25% trypsin (Merck)/EDTA (Chem Cruz) solution. Cells were sub-cultured every 2 or 3 days.

#### *Plant material*

Fresh leaves of the *A. vera* (L.) Burm. f. (*Aloaceae*; *Xanthoraceae*) plant which was cultivated in the Greenhouse of Istanbul University Alfred Heilbronn Botanical Garden, Turkey, were used in the study. A voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy, ISTE No. 65118.

#### *Preparation of extracts*

Four types of extract were prepared separately: *A. vera* fresh leaf skin aqueous extract, *A. vera* gel extract (AVG), *A. vera* fresh leaf skin methanolic extract and “*A. vera* dried leaf skin methanolic extract”.

#### *Preparation of fresh leaves skin aqueous extract and gel extracts*

*A. vera* fresh leaves were washed carefully with water and dried with filter paper (Whatmann 41). Then the leaves were longitudinally split in two, the gel (198.74 g) was separated by scraping and homogenized in a Waring blender. The gel was filtered through cloth and then filtrate was centrifuged at 4°C, 10,000 rpm, for 30 min. The supernatant was lyophilised (4.63 g) and considered to be AVG. The remaining leaves (leaf skins 171 g) were cut in small pieces, homogenized in a Waring blender with 855 mL distilled water, filtered through cloth and then the filtrate was centrifuged (Thermo) at 4°C, 12,000 rpm, 15 min. The supernatant was lyophilised (11.57 g) and considered to be “*A. vera* fresh leaf skin aqueous extract”.

#### *Preparation of fresh leaves skin and dried leaf skin methanolic extracts*

Fresh leaf skins (33.67 g) were dried in ventilated oven at 60°C, 2 hours. The dried leaf skins were powdered and stored at room temperature in the dark until extraction. The obtained dried leaf skin (4.25 g) and fresh leaf skin (73.4 g) were extracted using Soxhlet extractor with methanol for 3 days. After extraction and filtration through paper, 260 mL of fresh leaves filtrate and 143 mL of dried leaves filtrate were obtained. The methanol solvent was evaporated for 30 min in a rotary evaporator, giving rise to a semi solid crude extract (3.32 g) named *A. vera* fresh leaf skin methanolic extract. The same procedure was performed after drying leaf skins at 60°C for 2 hours in an oven and considered to be (1.17 g) “*A. vera* dried leaf skin methanolic extract”. These four different extracts prepared as described above were conserved at -20°C until further use.

#### *Preparation of test materials and reference drugs*

Aloe emodin (AE; 1,8-dihydroxy-3-[hydroxymethyl]-anthraquinone) was purchased from Sigma-Aldrich (St Louis, MO, cat no. A7687). *A. vera* extracts (10 mg/mL) and AE (20 mM) stock solutions were prepared

in dimethyl sulfoxide (DMSO was purchased from Sigma-Aldrich D2650). The reference chemotherapeutic drugs, 5-fluorouracil (5-FU; 50 mg/mL in physiological water, ready for use, kind present of Clinical Oncology Department, Istanbul Faculty of Medicine, Istanbul University), and imatinib (purchased from Santa Cruz) 10 mM stock solution was prepared in DMSO; All solutions were stored at -20°C and diluted with medium when used.

#### *Trypan blue exclusion assay*

The total number of viable cells was determined at each time point by the trypan blue exclusion test [37]. Exactly 10 µl of cell suspensions were stained with an equal volume of trypan blue (0.4% in 10 mM in phosphate buffer saline (PBS)) for 1 min. Then the numbers of viable cells were counted with Neubauer Chamber by light microscopy (Olympus). Cells that retained a blue colour were considered as dead cells.

#### *MTT colorimetric assay*

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to screen for cytotoxic activity [27]. For this purpose 96-well plates were used and the assay was done in a total volume of 100 µL. Briefly, 10 µL/well of varying concentrations of *A. vera* extracts and AE (1.25 - 200 µM), IM (0.1 - 50 µM), and 5-FU (0.15 - 5 mg/mL), as positive controls were added and subsequently the cells (90 µL/well; 10<sup>5</sup> cells/mL culture medium) were seeded to treat for 72 h. In addition, 90 µL cell suspension and 10 µL medium were added to control wells. The supernatant was aspirated (50 µL/well), subsequently the cells were treated with MTT (Sigma-Aldrich) solution (10 µL of 5 mg/mL PBS) at 37°C for 3 h. Finally, cells were lysed with 100 µL DMSO. Absorbance was measured at 570 nm using a ELISA microplate reader (Rayto RT-2100C). The percentage of viable cells (VI) determined with the equation (1):

$$VI = (\text{Absorbance of the treated cells} / \text{Absorbance of the control cells}) \times 100 \quad (1)$$

The IC<sub>50</sub> which is the amount of sample that provides 50% inhibition of cell growth was calculated from a dose-response curve. The cytotoxic effect of *A. vera* extracts and controls were evaluated by comparing the IC<sub>50</sub> values of the samples for all cell lines.

#### *Flow cytometry analysis*

Annexin V-FITC/propidium iodide (PI) assay kit (Millipore) was used to distinguish normal, apoptotic, and necrotic cells. For this purpose a 6-well plate was used and the assay was done in a total volume of 2 mL. The three groups of cells (two untreated control cells group: to apply and unapplied Annexin V-FITC/PI for one test group; 1800 µL/well; 10<sup>5</sup> cells/mL culture medium) were seeded in a final concentration of IC<sub>50</sub> (200 µL/well) of AVG and AE. Subsequent to culture at 37°C with 5% CO<sub>2</sub> for 72 h, the cells were harvested by trypsinization. Trypsinized and loose cells were then combined and pelleted by centrifuging at 2000 rpm

for 10 min. The pellets were resuspended and washed with PBS, then resuspended in 100  $\mu$ L of Annexin Binding Buffer (4X) and stained with 3  $\mu$ L Annexin V-FITC, 2  $\mu$ L PI. The cell suspension was incubated for 45 min at room temperature in the dark. The cell suspension was then immediately analysed by flow cytometry. Cell Quest software was used to analyse  $10^4$  cells. Acquisition of samples were determined with a FACS Calibur flow cytometer and analysed with CELLQUEST software (BD Biosciences).

#### Western Blot Analysis

For apoptic protein expression analysis, cells (45 mL;  $10^5$  cells/mL culture medium) were seeded in 75 cm<sup>2</sup> cell culture treated flasks. After 12, 24 and 72 h of the *A. vera* gel treatment, cells were harvested and pelleted. Pellets washed with PBS, then resuspended in 100  $\mu$ L lysis buffer (1 mM EDTA, 10 mM Tris-HCL, 0.5% Triton X-100, pH 8) with addition (1:1000) of 100 nM phenylmethylsulfonyl fluoride (PMSF) followed by incubation on ice for 60 min by vortexing every 10 min. The cell lysate was centrifuged at 14,000 rpm for 10 min and the supernatant was collected as total protein extract. The protein concentration was detected using the Bradford method. All the samples were mixed with Laemli sample buffer (2X) (Biorad), then transferred to a 80°C water bath for 15 min and stored at -80°C for later use. 10 - 20  $\mu$ g of protein was separated on 15% SDS-PAGE gel performed at 200 mA. PVDF blotting membranes (0.45 micron), activated in methanol for a few seconds, after electrophoresis. Blotting were performed overnight at 40 mA at 4°C

using Towbin reagent, Wet/Tank Blotting Systems, BIORAD. The membranes were blocked with 5% skimmed milk powder in TBS (Sigma) containing 0.05% Tween-20 (Santa Cruz Biotech., Dallas, Texas, USA) for 1 h, at RT and then incubated with primary antibodies overnight at 4°C on a shaker at low speed. Antibodies directed against the following proteins were used in this study: actin (1:1000, Santa Cruz Biotech., sc-1616, goat polyclonal IgG), caspase-3 (1:200, Santa Cruz Biotechnology, sc-7148, rabbit polyclonal IgG), caspase-9 (1:200, Santa Cruz Biotech., sc-7885, rabbit polyclonal IgG). The secondary antibodies were used goat anti-rabbit IgG-HRP (HRP: Horseradish Peroxidase, Santa Cruz Biotech., sc-2030) for caspase-3 and caspase-9. The fluorescent bands were visualized with KETA Wealtec Chemiluminescence Imaging System with Magic-Chemi software and Novex® AP Chromogenic Substrate (BCIP/NBT, cat no. WP20001, invitrogen).

*Statistical analysis.* The results were statistically analysed using the independent Student's t-test. Data were represented as means  $\pm$  standard deviation (S.D.) and at least in triplicate. Results were considered significant for  $p < 0.05$ .

## Results and Discussion

#### Cytotoxic Activity

The results of cytotoxicity studies are summarized in Table I.

**Table I**

IC<sub>50</sub> values of different *A. vera* leaf extracts and reference drugs on cell lines

Cell line	IC <sub>50</sub> * $\pm$ S.D.						
	Fresh leaf skin aqueous extract ( $\mu$ g/mL)	AVG ( $\mu$ g/mL)	Fresh leaf skin methanolic extract ( $\mu$ g/mL)	Dried leaf skin methanolic extract ( $\mu$ g/mL)	AE ( $\mu$ M)	IM ( $\mu$ M)	5-FU (mg/mL)
AGS	493.11 $\pm$ 143.43	528.07 $\pm$ 88.46	443.55 $\pm$ 51.18	427.06 $\pm$ 40.69	19.03 $\pm$ 0.25	65 $\pm$ 1.31	> 5
HT-29	1803.97 $\pm$ 1.80	334.18 $\pm$ 2.67	1026.72 $\pm$ 1.13	3204.85 $\pm$ 15.07	> 100	56 $\pm$ 1.37	> 5
HCT116	275.77 $\pm$ 26.03	206.88 $\pm$ 6.03	241.33 $\pm$ 26.02	292.42 $\pm$ 35.11	> 150	50 $\pm$ 2.60	0.40 $\pm$ 0.03
HEPG2	967.62 $\pm$ 247.42	697.14 $\pm$ 154.71	602.52 $\pm$ 131.28	595.17 $\pm$ 380.65	166.97 $\pm$ 5.41	106 $\pm$ 3.40	0.47 $\pm$ 0.02
HUVEC	1445.09 $\pm$ 35.37	927.79 $\pm$ 57.22	868.87 $\pm$ 44.82	574.12 $\pm$ 20.69	> 100	25 $\pm$ 1.65	> 5

\*IC<sub>50</sub> is the effective concentration for which the cell alive was 50% and was obtained from dose response curve.

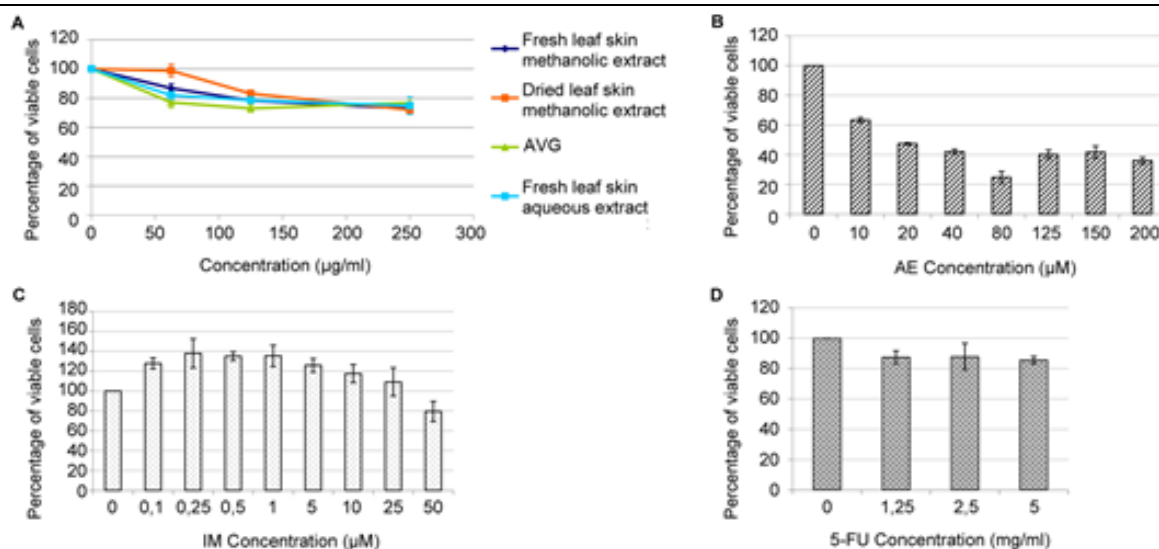
Data are presented as the mean of three replicates  $\pm$  standard deviation.

#### Effect of *A. vera* extracts and reference drugs on AGS cells

The cytotoxic effects of *A. vera* extracts (62.5, 125 and 250  $\mu$ g/mL) on AGS cells given as percentage of viable cells are shown in Figure 1A. The cytotoxic efficacy was as follows: *A. vera* dried leaf skin methanolic extract > *A. vera* fresh leaf skin methanolic extract > *A. vera* fresh leaf skin aqueous extract > AVG. The cytotoxic effects of AE on AGS cells given as percentage of viable cells are shown in Figure 1B. The best result was obtained at 80  $\mu$ M AE concentration. Whereas low cytotoxic effects were observed for IM (Figure 1C) and 5-FU (Figure 1D) on AGS cells.

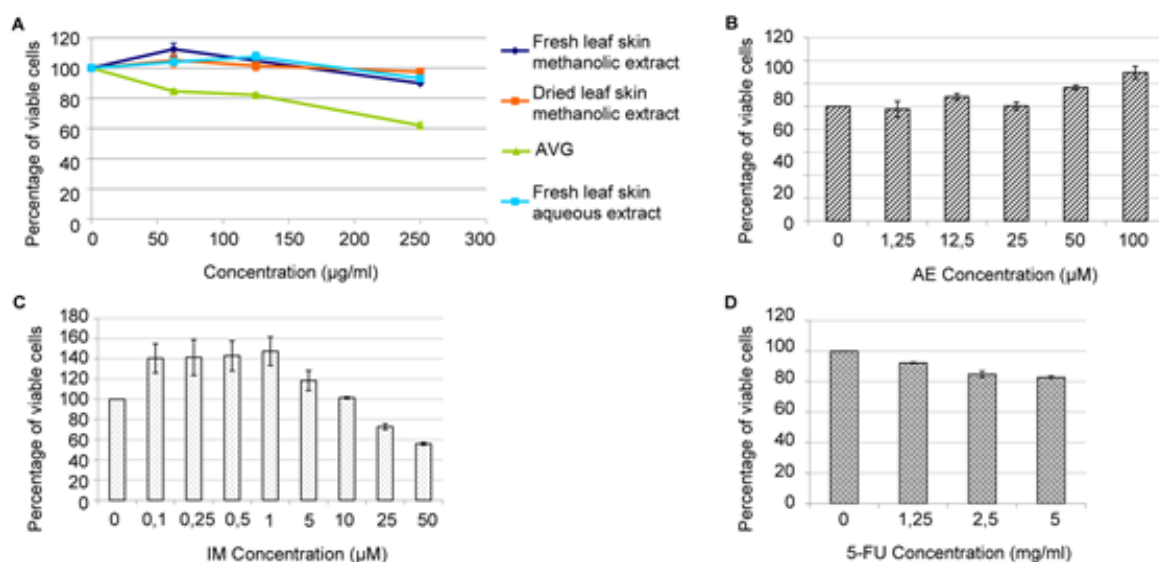
#### Effect of *A. vera* extracts and reference drugs on HT-29 cells

The cytotoxic effects of *A. vera* extracts (62.5, 125 and 250  $\mu$ g/mL) on HT-29 cells given as percentage of viable cells are shown in Figure 2A. The cytotoxic efficacy was as follows: AVG > *A. vera* fresh leaf skin aqueous extract > *A. vera* fresh leaf skin methanolic extract = *A. vera* dried leaf skin methanolic extract. No cytotoxic effect was observed for AE (Figure 2B) and 5-FU (Figure 2D). The best result was obtained at 50  $\mu$ M IM concentration. on HT-29 cells (Figure 2C).



**Figure 1.**

The cytotoxic effects of *A. vera* extracts (A), Aloe emodin (B), imatinib (C) and 5-fluorourasil (D) on AGS cell line



**Figure 2.**

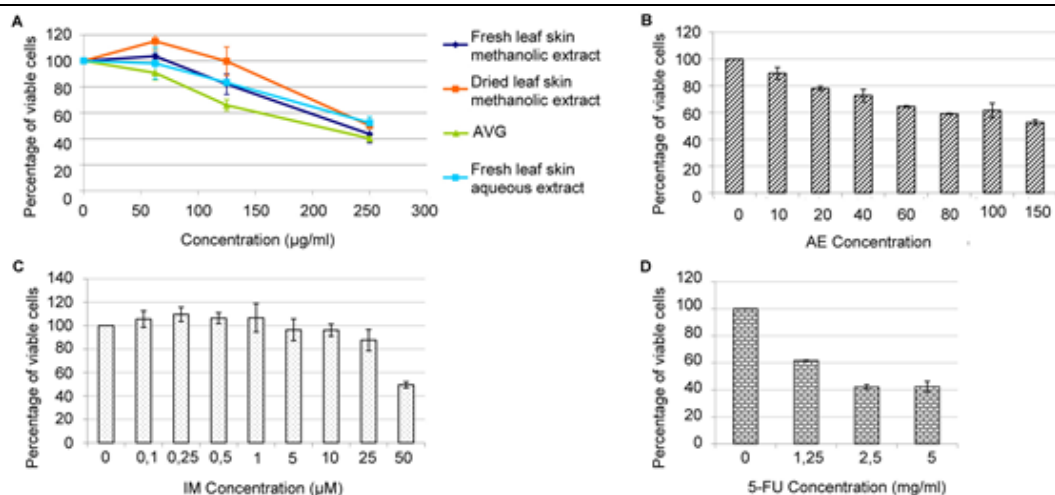
The cytotoxic effects of *A. vera* extracts (A), Aloe emodin (B), imatinib (C) and 5-fluorourasil (D) on HT-29 cell line

*Effect of A. vera extracts and reference drugs on HCT116 cells*

The cytotoxic effects of *A. vera* extracts (62.5, 125 and 250 µg/ml) on HCT116 cells given as percentage of viable cells are shown in Figure 3A. The cytotoxic efficacy was as follows: AVG > *A. vera* fresh leaf skin methanolic extract > *A. vera* fresh leaf skin aqueous extract > *A. vera* dried leaf skin methanolic extract. The cytotoxic effects of AE on HCT116 cells given as percentage of viable cells are shown in Figure 3B. The best result was obtained at 150 µM AE concentration. The best cytotoxic effect of IM (Figure 3C) was found at 50 µM and of 5-FU (Figure 3D) at 0.5 mg/mL concentration on HCT116 cells.

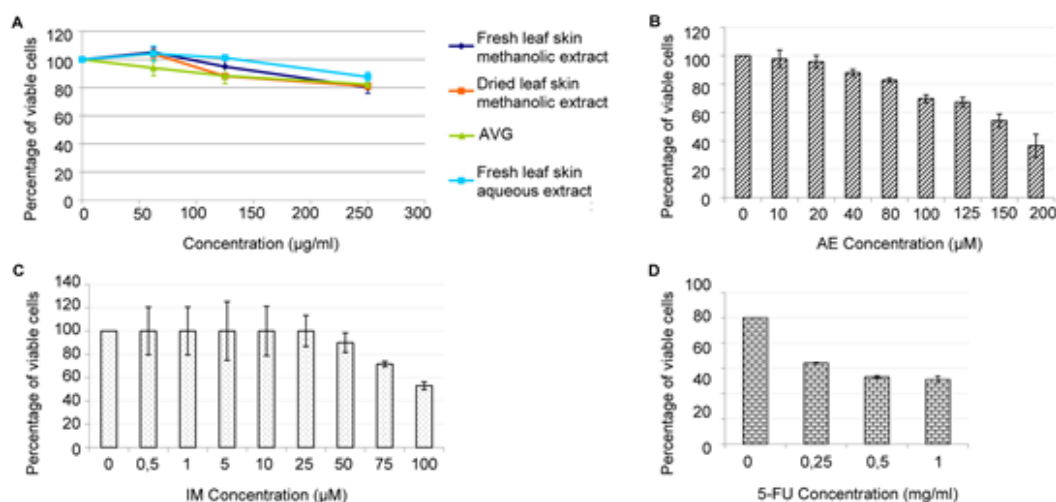
*Effect of A. vera extracts and reference drugs on HEPG2 cells*

The cytotoxic effects of *A. vera* extracts (62.5, 125 and 250 µg/mL) on HEPG2 cells given as percentage of viable cells are shown in Figure 4A. The cytotoxic efficacy was as follows: AVG > *A. vera* dried leaf skin methanolic extract > *A. vera* fresh leaf skin methanolic extract > *A. vera* fresh leaf skin aqueous extract. The cytotoxic effects of AE on HEPG2 are shown in Figure 4B. The best result was obtained at 200 µM AE concentration. The best cytotoxic effect of IM (Figure 4C) was found at 100 µM and of 5-FU (Figure 4D) at 0.5 mg/mL concentration on HEPG2 cells.



**Figure 3.**

The cytotoxic effects of *A. vera* extracts (A), Aloe emodin (B), imatinib (C) and 5-fluorourasil (D) on HCT116 cell line



**Figure 4.**

The cytotoxic effects of *A. vera* extracts (A), Aloe emodin (B), imatinib (C) and 5-fluorourasil (D) on HEPG2 cell line

*Effect of A. vera extracts and reference drugs on HUVEC cells*

The cytotoxic effects of *A. vera* extracts (62.5, 125 and 250 µg/mL) on HUVEC cells given as percentage of viable cells are shown in Figure 5A. It could be sad that the extracts did not show cytotoxic effect on normal cells. The negligible cytotoxic efficacy was as follows: *A. vera* dried leaf skin methanolic extract > *A. vera* fresh leaf skin methanolic extract > AVG > *A. vera* fresh leaf skin aqueous extract. AE did not show cytotoxic effects on HUVEC cells (Figure 5B). The best cytotoxic effect of IM was found at 50 µM (Figure 5C) and at 1.25 mg/mL for 5-FU (Figure 5D) on HUVEC cells.

*Annexin-V/PI apoptosis assay*

Annexin-V-FITC/PI double-staining assay was used to evaluate the percentage of apoptotic and necrotic cells in order to confirm that the cytotoxic effect on AGS, HT-29, HCT116 and HEPG2 cells, with their

own IC<sub>50</sub> doses of AE and 250 µg/mL AVG for 72 h, was associated with apoptosis (Table II).

**Table II**

Rates of apoptosis (%) and necrosis (%) in cells

Cells	Apoptosis (%) / Necrosis (%)			
	AGS	HT-29	HCT116	HEPG2
AE	26.11/0	0.81/0.05	15.46/3.07	31.53/4.5
AVG	0.17/0.22	0.53/0.09	12.41/0	1.51/0

Cells were treated with their own IC<sub>50</sub> doses of AE and 250 µg/mL AVG for 72 h, stained with FITC-annexin V/PI and analysed by FACSscan flow cytometer marked for apoptosis/necrosis. Apoptosis induction was monitored by flow cytometry using the Annexin V-FITC/PI kit. Apoptosis values (apoptosis %) were considered as the total values of gate percentages of early and late apoptosis.

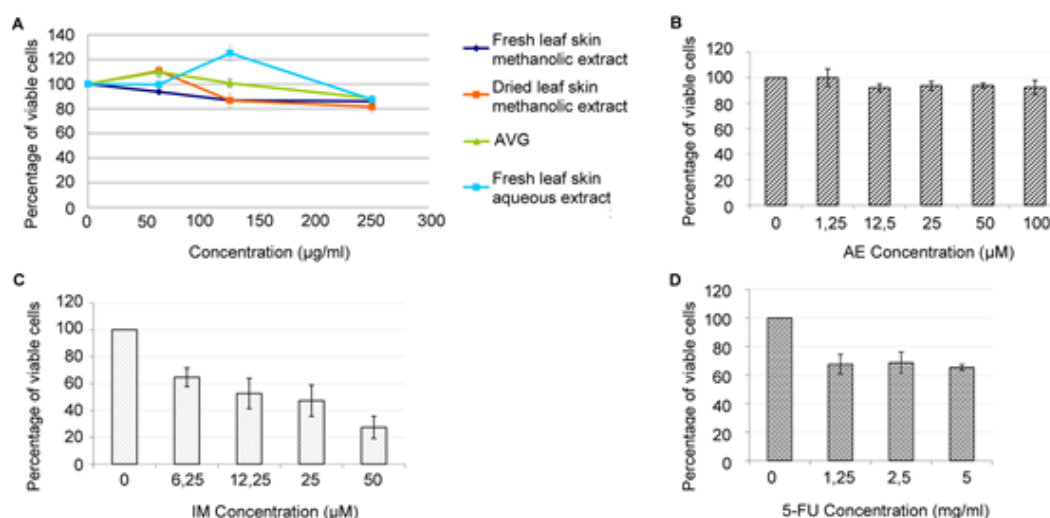
The cells with the most change in apoptotic cell percentage were identified as HCT116 cell. Treatment with the IC<sub>50</sub> value of 150 µM AE and 250 µg/mL AVG in HCT116 cell increased the percentage of early-late apoptotic cells from 11.81% - 8.43% to 15.50% -

20.20% (Figures 8A and 8B) and 46.92% - 29.26% to 39.81% - 48.78% (Figures 8C and 8D) respectively, indicating that AVG treatment induces apoptosis in HCT116 (Table II). In addition, treatment with the IC<sub>50</sub> values of AE induces apoptosis at the rates of 26.11% and 31.53% apoptosis in AGS (Figures 6A and 6B) and HEPG2 cells (Figures 9A and 9B), respectively. Referring to the results in Figure 7, no apoptosis or necrosis was observed in HT-29 cells.

*Expression of proteins related to apoptotic pathways in AVG treated HCT116 cells*

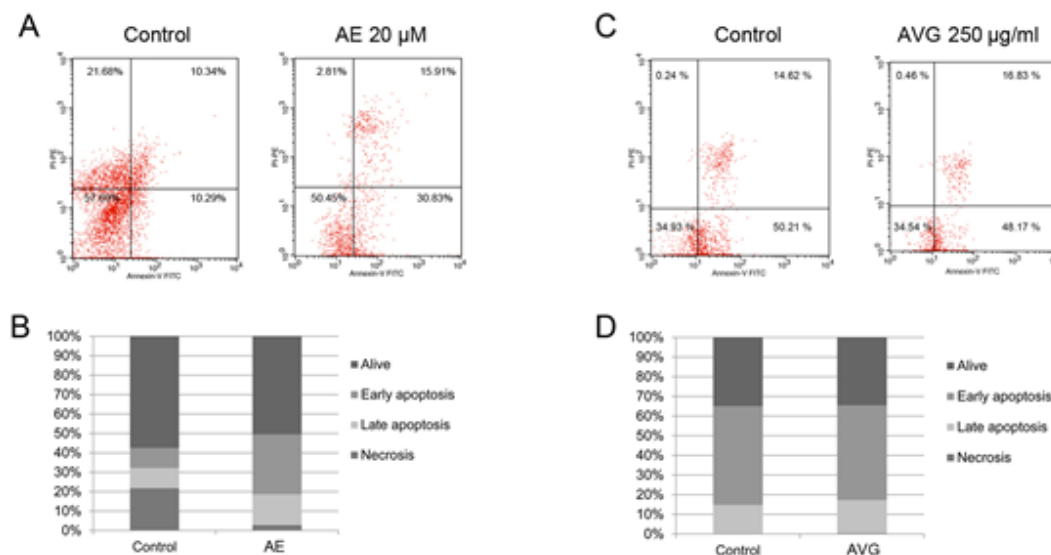
Since AVG showed apoptotic activity in HCT116 cells at the lowest dose of the IC<sub>50</sub>, we decided to perform

western blot analyses in order to reveal the underlying molecular mechanism. After cells were exposed to 200 µg/mL AVG for 12, 24 and 72 h, the expression of caspase-3 and caspase-9 proteins increased compared with the internal actin expression. As shown in Figure 10, while procaspase-3 expression increased procaspase-3 proteins were cleaved to the corresponding active forms in the first 12 hours and procaspase-9 was cleaved in the 24 hours. According to these results we can postulate that the apoptotic effect of *A. vera* gel treatment in HCT116 cells is mediated by a caspase-dependent pathway.



**Figure 5.**

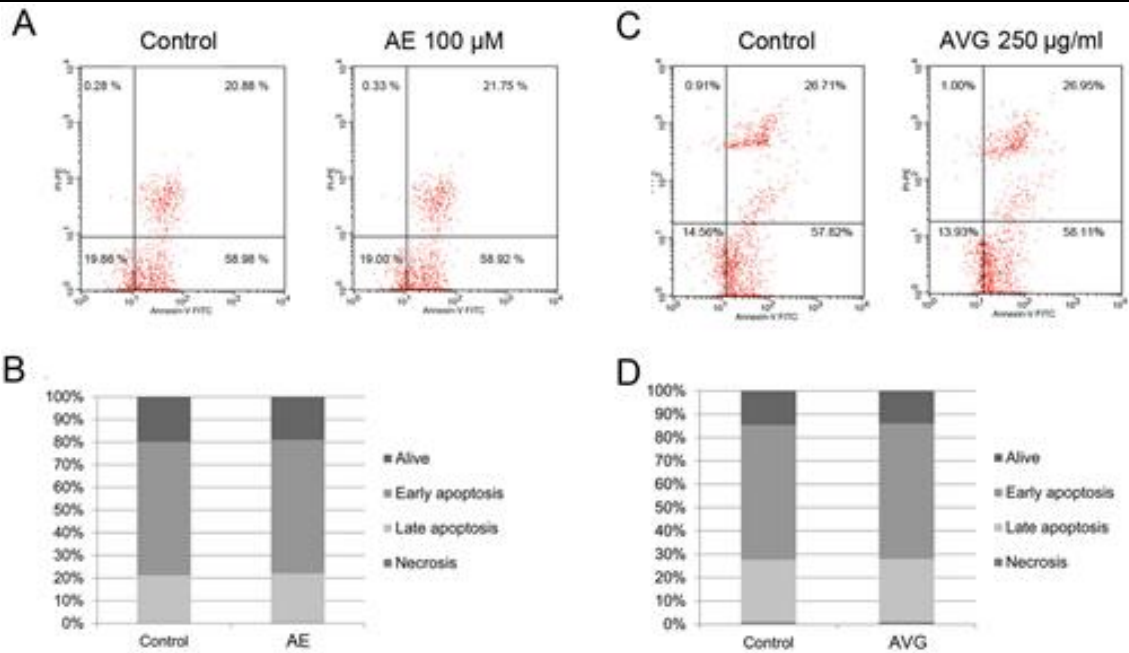
The cytotoxic effects of *A. vera* extracts (A), Aloe emodin (B), imatinib (C) and 5-fluorourasil (D) on HUVEC cell line



**Figure 6.**

Flow Cytometry of AGS cells treated with Annexin V-FITC/PI. The cells were maintained with the IC<sub>50</sub> value of 20 µM AE (A) and 250 µg/mL AVG (C) for 72 h, stained with FITC-annexin V/PI and analysed by FACScan flow cytometer marked for apoptosis/necrosis

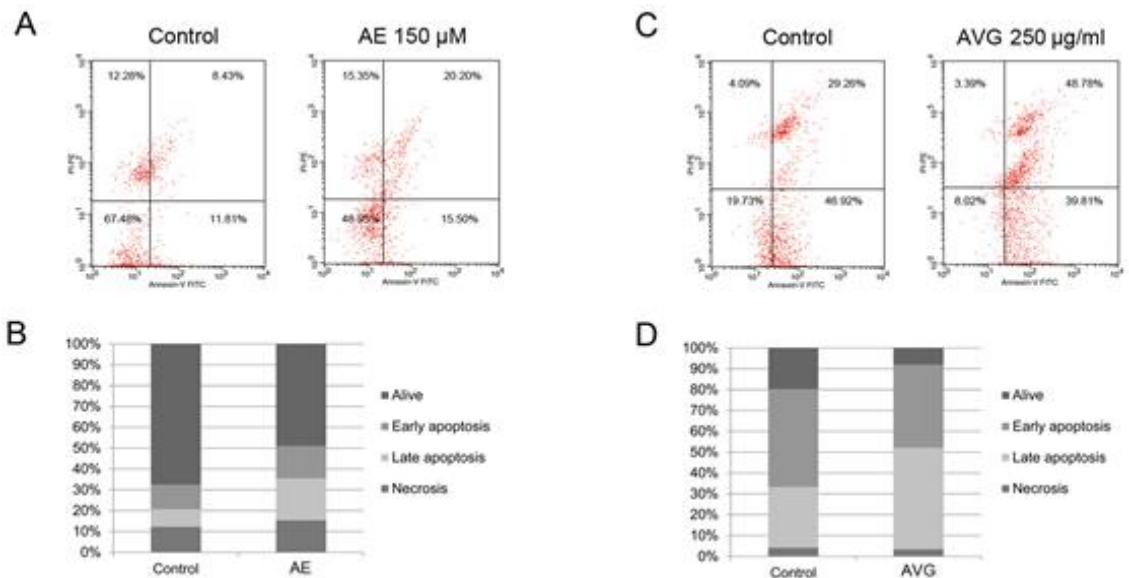
Q1: Annexin V negative/PI positive; Q2: Annexin V/PI positive; Q3: Annexin V positive/PI negative; Q4: Annexin V/PI negative. Control cells without the presence of AE and AVG. The analysis of data from flow Cytometry was performed using the FlowJo software. (B, D) Alive %, early apoptosis %, late apoptosis and necrosis % were calculated comparing with treatment and non-treatment groups



**Figure 7.**

Flow Cytometry of HT-29 cells treated with Annexin V-FITC/PI. The cells were maintained with the IC<sub>50</sub> value of 100 µM AE (A) and 250 µg/mL AVG (C) for 72 h, stained with FITC-annexin V/PI and analysed by FACScan flow cytometer marked for apoptosis/necrosis

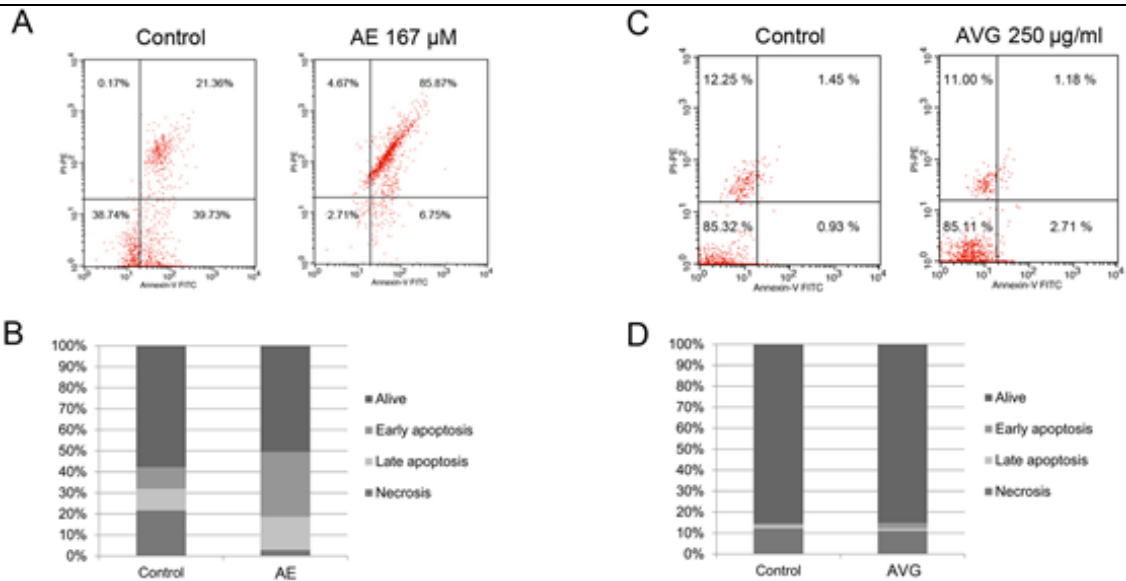
Q1: Annexin V negative/PI positive; Q2: Annexin V/PI positive; Q3: Annexin V positive/PI negative; Q4: Annexin V/PI negative. Control cells without the presence of AE and AVG. The analysis of data from flow Cytometry was performed using the FlowJo software. (B, D) Alive %, early apoptosis %, late apoptosis and necrosis % were calculated comparing with treatment and non-treatment groups



**Figure 8.**

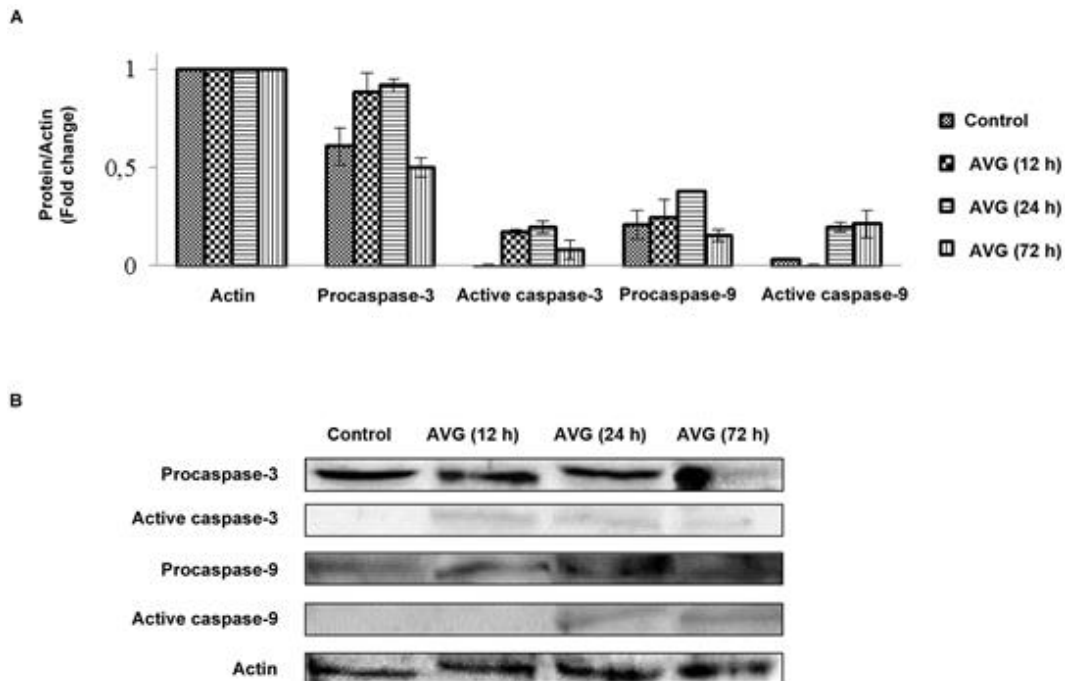
Flow Cytometry of HCT116 cells treated with Annexin V-FITC/PI. The cells were maintained with the IC<sub>50</sub> value of 150 µM AE (A) and 250 µg/mL AVG (C) for 72 h, stained with FITC-annexin V/PI and analyzed by FACScan flow cytometer marked for apoptosis/necrosis

Q1: Annexin V negative/PI positive; Q2: Annexin V/PI positive; Q3: Annexin V positive/PI negative; Q4: Annexin V/PI negative. Control cells without the presence of AE and AVG. The analysis of data from flow Cytometry was performed using the FlowJo software. (B, D) Alive %, early apoptosis %, late apoptosis and necrosis % were calculated comparing with treatment and non-treatment groups



**Figure 9.**

Flow Cytometry of HEPG2 cells treated with Annexin V-FITC/PI. The cells were maintained with the IC<sub>50</sub> value of 167 µM AE (A) and 250 µg/mL AVG (C) for 72 h, stained with FITC-annexin V/PI and analyzed by FACScan flow cytometer marked for apoptosis/necrosis  
 Q1: Annexin V negative/PI positive; Q2: Annexin V/PI positive; Q3: Annexin V positive/PI negative; Q4: Annexin V/PI negative. Control cells without the presence of AE and AVG. The analysis of data from flow Cytometry was performed using the FlowJo software. (B, D) Alive %, early apoptosis %, late apoptosis and necrosis % were calculated comparing with treatment and non-treatment groups



**Figure 10.**

Western blot analysis of caspase proteins expression levels after 250 µg/mlAVG treatment of HCT116 cells. Band intensity was analyzed by densitometry. Fold change of protein expression levels was calculated after bands were normalized to Actin

There are several data concerning the antitumour potential of *A. vera* extracts. *Aloe* species are cited between medicinal plants used as antitumour agents in Brazil [12]. Recent studies have demonstrated the *in vivo* anti-angiogenic effect of another species, *Aloe*

*arborescens* on squamous-cell carcinoma and kidney tumours [17]. Several *in vivo* tests have been performed on different tumour cells and all of them gave positive results concerning antitumour activity of both *A. vera* gel and aloe extracts [32, 36]. There is also one study



undertaken on patients with colorectal cancer which have received 5-FU therapy alone and in combination with *Aloe arborescens* suggesting that the disease regressed significantly in the patients treated with Aloe alone [23]. The present study is the first to our knowledge undertaken *in vitro* on gastric, colon and hepatocellular cell cultures and comparing aqueous, methanolic leaf extracts, the gel extract and a purified substance from *A. vera*, AE.

AE is a hydroxyanthraquinone found in *Aloe vera* which antitumour [18, 20, 26] and anti-angiogenic [5] properties were studied in recent years. AE was found to have specific cytotoxic activity *in vitro* and *in vivo* on neuroectodermal tumours, but was ineffective on normal fibroblasts [2, 30]. In a recent review, AE was presented as a new anti-cancer agent [42]. It has been shown that AE could be a potent anticancer agent for multiple tumour cells, suppressing their proliferation *via* p53 and its downstream p21 pathway and inducing in them cell cycle arrest and apoptosis [22]. Anti-tumour properties of AE isolated from *A. vera* leaves were evaluated against some different colon cancer lines (DLD-1 and HT-2) with IC<sub>50</sub> values of 8.94 and 10.78 µM respectively [14]. AE was assayed on HT-29 human colon cancer cells in a study undertaken by Yuenyongsawad *et al.* and an IC<sub>50</sub> value of 0.296 ± 0.010 µg/mL was reported [43]. Contrarily to this finding in the present study, AE had no cytotoxic effect on HT-29 cells. The cytotoxic effect of AE on HCT 116 human colon cancer lines was evaluated in a study of Cui *et al.* with an IC<sub>50</sub> value of 8.7 µM [10]. Accordingly, in this study AE was also found cytotoxic for the same cell line with an IC<sub>50</sub> value of > 150 µM. AE have been reported to exhibit cytotoxic effect on HEPG2 human hepatocellular carcinoma cells with an IC<sub>50</sub> value of 10 µM [10] whereas our study revealed a lower cytotoxicity of AE with an IC<sub>50</sub> value of 166.97 ± 5.41 µM on HEPG2 cells. The anticancer properties of AE were attributed primarily to its apoptotic effect [42]. Accordingly, in the present study AE has shown its cytotoxic effect by inducing apoptosis in AGS, HCT116 and HEPG2 cells.

Imatinib (IM) (imatinib mesylate, commercially available as Gleevec or Glivec, Novartis, Basel, Switzerland), is an inhibitor of certain tyrosine kinases and is used as a chemotherapeutic drug mostly in cancers like leukaemia. Nevertheless IM is also approved for gastrointestinal stromal tumours (GIST) [11]. IM was assayed on HCT116 cells at 10 - 50 µM doses and IC<sub>50</sub> of 21.2 µM was reported after 72 h exposure [1]. In the present study, similar results with an IC<sub>50</sub> of 50 ± 2.60 µM was found for IM on HCT116 cells after 72 h of exposure. However, no report on IC<sub>50</sub> values of IM on AGS and HT-29 cells was found in the literature. In our study IM showed cytotoxic effect with an IC<sub>50</sub> value of 65 ± 1.31 µM on AGS cells and of 56 ± 1.37 µM on HT-29 cells.

Fluorouracil (5-FU), a pyrimidine analogue, belongs to the family of drugs called antimetabolites and shows its cytotoxic effect by inhibiting irreversibly thymidylate synthase. 5-FU is commonly used in the chemotherapy of colorectal cancers and also as reference drug in cytotoxicity studies in cell cultures. Zhou *et al.* have reported IC<sub>50</sub> values of 27.52 ± 0.59 µM/L for 5-FU on AGS cell lines [44]. In our study IC<sub>50</sub> values of > 5 mg/mL was observed after 72 h of exposure. IC<sub>50</sub> value of 2.3 g/mL was reported for 5-FU on HT-29 colon cancer cells [31]. In our study, IC<sub>50</sub> values of > 5 mg/mL was observed for 5-FU on HT-29 cells. Literature survey revealed some studies undertaken on HCT116 colon cancer cell cultures for 5-FU with reported IC<sub>50</sub> values of 33.17 µg/mL, 27.76 µM and 6 µg/mL, respectively [21, 33]. In our study, in accordance, with these studies a high IC<sub>50</sub> of 0.4 ± 0.03 mg/mL was found for 5-FU on the HCT116 colon cancer cell line. Ma *et al.* found an IC<sub>50</sub> value of 46.83 µM for 5-FU on HEPG2 cells [24] whereas our finding was 0.47 ± 0.02 mg/mL on the same cells.

In the present study, no significant difference was seen between the cytotoxic activities of the fresh leaves and dried leaves methanolic extracts. Therefore, we can suggest that there is no need to dry the leaves and that fresh leaves could be used as usual.

Nevertheless, no significant difference was also seen between fresh leaves aqueous and methanolic extracts in view of cytotoxicity. Only for AGS cells, the effect of *A. vera* fresh leaf skin and dried leaf skin methanolic extracts were better than AVG. Among the extracts assayed the best cytotoxic effect was seen with AVG on HCT116 colon cancer, whereas another hepato-cellular carcinoma cell line HEPG2, was resistant to Aloe extracts even at higher doses. This finding is contradictory with Du Plessis and Hamman [13], which reported an IC<sub>50</sub> of 269.3 mg/mL as even higher dose for *A. vera* gel effect on HEPG2 cells. The most important finding is that the extracts have shown very low or no cytotoxic activity towards HUVEC cells, similar to the reference drugs AE and 5-FU.

Another important finding is that no literature report for the apoptotic pathway of *A. vera* gel on HCT116 cells. In our present study, in time course experiments of *A. vera* gel in HCT116 to investigate apoptotic pathway, we have demonstrated that, for HCT116 cells, caspase-3 activation was first initiated for it occurred as early as 12 h after AVG treatment, whereas caspase-9 showed activation after 24 h (Figure 10).

## Conclusions

As a conclusion we can assume that the selective cytotoxic and apoptotic effects exerted by *A. vera* gel and aloe emodin may represent an alternative cancer therapy.

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## Conflict of interest

The authors declare no conflict of interest.

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