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# Different Polarity Extracts of *Polygonum minus* towards Cytotoxic Activities against Colon Cancer Cell Lines (HT-29, HCT-116, CT-26)

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author MAKR designed the study, methodology and manuscript review. Author NAH design the methodology and data acquisition. Author NR carried out the experimental, data acquisition and manuscript writing. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** To investigate the cytotoxic properties of different polarity solvents of *Polygonum minus* extracts towards colon cancer cell lines, HT-29, HCT-116 and CT-26.
**Study Design:** Experimental study.
**Place and Duration of Study:** Central Laboratory, Tissue Culture Laboratory, Universiti Sultan Zainal Abidin, Terengganu between September 2019 until December 2019.
**Methodology:** The different polarity solvents of *P. minus* extracts had been led to tetrazolium salt reduction (MTT) assay and an inhibition concentration of 50 ($IC_{50}$) value for their cytotoxic potential against colon cancer cells. Then, cell morphology observation and fluorescence double staining of treatment cells were determined using a light inverted microscope and acridine orange/propidium iodide staining.
**Results:** The results indicated that an extraction yields aligned from 0.01% for acetone and ethyl acetate to 0.45% for aqueous solution with decreasing order of aqueous solution > 70% aqueous ethanol > 50% aqueous ethanol > methanol > ethanol > acetone and ethyl acetate. Meanwhile, the

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ethyl acetate extract showed a higher cytotoxic effect at $IC_{50}$ values of 7.00 ± 0.06 µg/mL and 7.00 ± 0.30 µg/mL towards the HCT-116 and CT-26 cells; and 50% aqueous ethanol towards HT-29 cells (24.00 ± 0.01 µg/mL). The different solvent extracts of *P. minus* induced cytotoxic effects on the treated cell lines by altering their normal cell morphology and cell membrane integrity (except for acetone extract).

**Conclusion:** Therefore, the use of different polarity solvent extracts of *P. minus* as an anti-cancer agent is promising more on ethyl acetate and warrants further investigation.

*Keywords: Polygonum minus; colon cancer; cytotoxic; MTT assay; ethyl acetate.*

## 1. INTRODUCTION

Colorectal cancer is the fourth most common death in the world and this cancer normally originates in the cells inside the colon and rectum [1]. By contributes as an anti-cancer agents, the mechanism included the induction of apoptosis, cell cycle arrest, and inhibition of various signalling pathways [2]. In plants, the apoptosis-inducing capabilities induced by antioxidants are of point interest in cancer study due to cost efficiency and apparently have less aftereffects compared to synthetic antioxidants [3]. Due to its useful medicinal properties, *Polygonum minus* had traditionally been used as a postnatal tonic [4] and commonly used for sprains and alleviating body aches as well as removing dandruff [5].

*P. minus* is a tropical herb species from the Polygonaceae family that is commonly known as 'Kesum', 'Daun Laksa' or 'Cenohom' at Malaysia [5]. The plant is native to Southeast Asian nations such as Thailand, Malaysia, Indonesia, and Vietnam [6]. This herb is often used as a spice and flavouring enhancer in various dishes [7]. Several studies have shown that *P. minus* extracts exhibit anti-microbial [8], anti-inflammatory [9], and gastric cyto-protective activities [10] as well as represent source of antioxidants [11].

In the previous researches, Ahmad et al. [6] and Abdullah et al. [7] had revealed the cytotoxicity and anti-proliferative activities of *P. minus* extract against colon (HCT-116 and CCD841), breast (MCF-7), and leukaemia (K562) cancer cells. Also, the cytotoxicity of *P. minus* against the human colorectal carcinoma cell line, HCT-116 had well explain in the context of its usage to treat digestive disorders in the pharmaceutical industry [12]. However, there are no reports on the different polarity solvents of *P. minus* extract towards different colon cancer cell lines. As the previous study had emphasize cytotoxicity of several *P. minus* extract on HCT-116 cell line, this study further aim to investigate the selectivity of variables polarity solvents used against three types of colon cancer *in-vitro.* Therefore, the study purpose to examine the cytotoxic properties of different polarity solvents of *P. minus* extracts as a potential anti-cancer agent towards HT-29, HCT-116, and CT-26 cell lines.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection and Extraction Procedure of *P. minus*

*P. minus* leaves samples were obtained from Biotropics Malaysia Berhad through the research collaboration with Universiti Kebangsaan Malaysia, Malaysia. The *P. minus* leaf samples were labelled with the batch number, KE 180804.

In this study, several adjustments was done with the extraction method represent by Mohd Adzim Khalili et al. [13]. A total of 0.5 g of *P. minus* leaves were weighed prior to its soaking process (1:10; w/v) in 100% aqueous solution (distilled water), 100% methanol, 100% ethanol, 70% aqueous ethanol, 50% aqueous ethanol, 100% acetone, and 100% ethyl acetate at 25$^{o}$C for 24 hours. Subsequently, all the solvent extracts were filtered by using Whatmann filter paper (20-25 µm) and concentrated in a rotary evaporator at 40$^{o}$C. The concentrated extracts were placed in the oven at 40°C to allow for complete solvent evaporation and the dried extracts were kept in a -20$^{o}$C freezer prior to its use for further analysis.

### 2.2 Extraction Yield Procedure of *P. minus* Extracts

The extraction yield was calculated according to the method of Zhang et al. [14] using the formula;

W2/W1 * 100% [14]

W1= original weight of sample= 0.5 g,
W2= weight of dried extract.

## 2.3 *In-vitro* Study

### 2.3.1 Preparation of *P. minus* extracts

The methods used previously by Khalili et al. [15] were adopted for this study with modifications. A total of 1 mg of different polarity solvents of *P. minus* extracts were dissolved in 1 mL of DMSO to prepare a stock solution of 1 mg/mL. The extract solutions were kept at 4□C before use. The stock solution for each extract was further diluted in completed RPMI-1640 and McCoy's 5A media added with foetal bovine serum (10%) and penicillin-streptomycin (1%) to obtain a working solution of 100 µg/mL.

### 2.3.2 Cell maintaining and harvesting

Colon cancer cell lines of human colorectal adenocarcinoma, HT-29 (ATCC® HTB-38™), human colorectal carcinoma, HCT-116 (ATCC® CCL-247™), and mouse colorectal carcinoma, CT-26 (ATCC® CRL-2638™) were used in this study. The cells were obtained at passage 3 (P3) from the Faculty of Bioresources and Food Industry, UniSZA. The cell lines were grown and maintained in completed RPMI-1640 for CT-26 cells and McCoy's 5A media for HT-29 and HCT-116 cells added with foetal bovine serum of 10% and penicillin-streptomycin of 1% at 37□C in an incubator humidified with 5% CO2 and relative humidity of 95%. The cell media was replaced twice weekly to replenish the nutrients required for cell growth.

### 2.3.3 Determination inhibition concentration of 50% ($IC_{50}$) by *P. minus* extracts

The inhibitory concentration ($IC_{50}$) of *P. minus* extracts was evaluated using a colorimetric micro-titration method known as the MTT assay or tetrazolium salt reduction assay [16]. The cells were harvested from the media, counted using a haemocytometer, and further diluted in a completed RPMI and McCoy's 5A medium (added with 10% foetal bovine serum and 1% penicillin-streptomycin). A total of 100 µL of cell suspension was seeded in triplicates using 96-well culture plates (SPL Life Sciences, Korea) at an optimized density of 1 x 10^5 cells/cm2 for each cell line. After 24 hours, triplicate serial dilutions of *P. minus* extracts (100 – 1.56 µg/mL) [17] and doxorubicin (drug) (1.00 - 0.016 µg/mL) were added into each well. Each 96-well plate was equipped with blank cells (blank) and untreated cells (positive control). After a 72-hour incubation period, 20 µL (5 µg/mL) of MTT assay was added into each well in 96-well plate and kept for an additional 4 hours. The medium was discarded and 100 µL of DMSO reagent was further into each well. Next, the absorbance at 570 nm with reference to 630 nm was measured using a microplate reader (TECAN, INFINITE M200, Switzerland). Appropriate controls for the determination of cell viability were also measured. The relative cell viability of the treated cells was described as %cell viability and calculated based on the following formula: (A570 of treated cells/ A570 of control cells) x 100% and calculated to depend on the non-linear regression of the response curves within the same region.

### 2.3.4 Cell morphology observation

The effects of *P. minus* extracts on the cellular morphological changes were determined using the method by Merlin et al. [18]. In this method, the effective dosage concentration of the extract is based on the inhibition concentration ($IC_{50}$) value determined using the MTT assay. The morphological observation was performed at 37°C for 24, 48, and 72 hours using a light inverted microscope (Nikon, Japan) at magnification 10x.

### 2.3.5 Fluorescence microscopy of apoptosis using AO/PI double staining

The steps used previously by Hajiaghaalipour et al. [19] were adopted for this study. The determination of the apoptotic effects of *P. minus* extracts on the cancer cell lines was done by applying stain of acridine orange (AO)/propidium iodide (PI) and then observed under the fluorescence microscope. A total of 1 x 10^5 cells/well cell density were seeded into 96-well plates and treated with the $IC_{50}$ dosage concentrations of each extract for 72 hours. Then, both untreated and treated cells were incubated with AO and PI staining at a concentration of 10 µg/mL, and apoptosis/cell viability was visualized at 10x magnification using an Olympus-BX51 fluorescence microscope (Olympus, Japan) fitted with a Nikon camera (Nikon, Japan).

## 2.4 Statistical Analysis

The data were explored using descriptive and inferential statistical analysis by using the Microsoft Excel Spreadsheet 2013 and Scientific Package for Social Sciences version 20.0 software (IBM Corp. US) with $p<0.05$ is

statistically significant. Descriptive statistics was applied to measure the extraction yield and an $IC_{50}$ value as means and standard deviation (SD). Based on the variables that were analyzed, this analysis was relating the One-way ANOVA test in percentages of extract yield and an $IC_{50}$ value among different cell lines tested.

## 3. RESULTS AND DISCUSSION

### 3.1 Extraction Yield of *P. minus* Extracts

In plants, there are variables of different compounds with chemical properties presence; therefore gives different polarities of solubility in particular organic solvents [20]. Generally, pure polar solvents are chosen to extract polyphenols from plant matrices with applicable solvents used are combination of aqueous with methanol, ethanol, ethyl acetate and acetone [21]. Based on Daj and Mumper [22], methanol as a polar solvent had been used due to its higher extractability of low molecular weight of polyphenols, whereas aqueous acetone due to the extraction of high molecular weight capability such as flavanols. Meanwhile, ethanol is known for its safety and a good solvent in extractability of polyphenols.

Commonly, the efficient extractability is influenced by the chemistry of the polyphenols, methods of extraction used, the particle size of sample, solvent polarities and the existence of substance interference [23]. At the same time and at the same extraction temperature, the solvent and sample composition are recognized as the most crucial specifications [24]. In this study, *P. minus* extracts were attained by applying different polarity solvents of 100% aqueous solution, 100% methanol, aqueous ethanol (100%, 70%, 50%), 100% acetone, and 100% ethyl acetate. The polarity solvents increased as following: ethyl acetate < acetone < ethanol < methanol < 70% aqueous ethanol < 50% aqueous ethanol < aqueous solution. Based on Table 1 presented below, the yields of extraction aligned from 0.01% for acetone and ethyl acetate to 0.45% for aqueous solution.

By using different solvents, the extraction yields decreasing as follows: 100% aqueous solution > 70% aqueous ethanol > 50% aqueous ethanol > 100% methanol > 100% ethanol > 100% acetone and 100% ethyl acetate. The results shows that the pattern of extraction yields is based on the polarity solvents used in the study; which non-polar solvents has the lowest yield while polar solvents has the highest yields. The yield of extraction in pure methanol (0.17%) had been observed to be higher than that of pure ethanol (0.03%), pure acetone (0.01%) and pure ethyl acetate (0.01%). Additionally, it can also be seen that the aqueous solution (0.45%) and aqueous ethanol (70%, 50%) (0.39% and 0.28%) gives higher extract yield than pure solvent extracts used (from 0.01% to 0.17%).

Based on the results, it can be inferred that increasing of the extraction yield is due to the increasing aqueous in the solvents used. Quy et al. [21] had stated that this may due to the increasing solubility of carbohydrates and proteins in aqueous than in pure solvents as an ethanol, methanol and acetone; which have advantages in extraction of other compounds than phenol and gives higher yield. Furthermore, it was in compromise result with Chatha et al. [25], which the extraction yields of aqueous solvents higher than the pure solvents of methanol, ethanol and acetone. Therefore, the combination use of water and pure solvent can aid the extraction of water soluble and/or pure solvent compounds [21].

### 3.2 Cytotoxic Activities of *P. minus* Extracts

In this study, the cytotoxic activities of diverse polarity extracts of *P. minus* on HT-29, HCT-116 and CT-26 cells were assessed using MTT assay (Table 2). An ethyl acetate extract showed a higher cytotoxic effect at $IC_{50}$ values of 7.00 ± 0.06 µg/mL and 7.00 ± 0.30 µg/mL towards the HCT-116 and CT-26 cells; and 50% aqueous ethanol towards HT-29 cells, respectively at 72 hours treatment. However, no cytotoxic and anti-proliferative effect was observed by the acetone extract at the same treatment duration on three types of colon cancer cell lines. Meanwhile, results observed that aqueous solution of *P. minus* extract displayed the highest $IC_{50}$ values of 33.00 ± 0.04 µg/mL and 29.00 ± 0.05 µg/mL towards the HCT-116 and CT-26 cell lines, respectively. On the other hand, doxorubicin showed prominent anti-proliferative activity against HT-29, HCT-116 and CT-26 cells with $IC_{50}$ values 0.63 ± 0.02, 0.46 ± 0.19, and 0.14 ± 0.01 µg/mL ($p<0.05$). The $IC_{50}$ values obtained at 72 hours between different polarity extracts towards different colon cancer cell lines was found significantly difference ($p<0.05$).

**Table 1. Determination of percentages extract yield (%) of different solvents of *P. minus* extracts**

| Solvents | Crude extract weight (g) | Extraction yield (%) | F-statistics (df) | *P*-value |
|---|---|---|---|---|
| 100% aqueous solution | 0.222 ± 0.002 | 0.45 | 8.045 (6, 14) | <0.01* |
| 100% methanol | 0.097 ± 0.002 | 0.17 | | |
| 100% ethanol | 0.015 ± 0.002 | 0.03 | | |
| 70% aqueous ethanol | 0.198 ± 0.002 | 0.39 | | |
| 50% aqueous ethanol | 0.141 ± 0.001 | 0.28 | | |
| 100% acetone | 0.005 ± 0.001 | 0.01 | | |
| 100% ethyl acetate | 0.003 ± 0.001 | 0.01 | | |

*Data represent the mean ± SD of three independent experiments; One-way ANOVA test, *p<0.01; Extract yield present in percentages (%)*

**Table 2. The $IC_{50}$ values of HT-29, HCT-116 and CT-26 cell lines by *P. minus* extracts at 72 hours treatment**

| Samples | $IC_{50}$ values (µg/mL) | | | F-statistics (df) | *P*-value |
|---|---|---|---|---|---|
| | HT-29 | HCT-116 | CT-26 | | |
| 100% aqueous solution | 75.00 ± 0.01 | 33.00 ± 0.04 | 29.00 ± 0.05 | 5.85 (2, 6) | <0.01* |
| 100% methanol | 78.00 ± 0.01 | 30.00 ± 0.02 | 20.00 ± 0.02 | 8.66 (2, 6) | <0.01* |
| 100% ethanol | 73.00 ± 0.01 | 31.00 ± 0.03 | 26.00 ± 0.01 | 6.00 (2, 6) | <0.01* |
| 70% aqueous ethanol | 34.00 ± 0.07 | 13.00 ± 0.02 | 10.00 ± 0.10 | 7.64 (2, 6) | <0.01* |
| 50% aqueous ethanol | 24.00 ± 0.01 | 20.00 ± 0.19 | 23.00 ± 0.09 | 1.71 (2, 6) | <0.01* |
| 100% acetone | nil | nil | nil | NA | NA |
| 100% ethyl acetate | 33.00 ± 0.12 | 7.00 ± 0.06 | 7.00 ± 0.30 | 2.02 (2, 6) | <0.01* |
| Doxorubicin | 0.63 ± 0.02 | 0.46 ± 0.19 | 0.14 ± 0.01 | 6.20 (2, 6) | <0.01* |

*Data represent the mean ± SD of three independent experiments; One-way ANOVA test, *p<0.01*

In the previous study, the similar result was seen by ethyl acetate extract towards HCT-116 cells compared to methanol and aqueous solution but at much higher $IC_{50}$ values [26]. The differences may cause of the difference in range of the concentration or the cell density used. Similarly, Abdullah et al. [26] also shows the polar solvents such as aqueous solution and methanol gives higher $IC_{50}$ values compared to non-polar solvents of hexane and ethyl acetate. On the addition, Mohd Alfazari et al. [27] reported that the aqueous solution of P. minus extract had no anti-proliferative effect on HCT-116, displaying $IC_{50}$ > 250 µg/mL. Following, the present result were consistent with a study by Ghazali et al. [28] who observed that the *P. minus* ethyl acetate extract demonstrated cytotoxicity and had been observed in capability of inducing arrest of the cell cycle S-phase and HepG2 cells apoptosis.

Findings observed the cytotoxic potency of the acetone and ethyl acetate extracts as non-polar solvents towards three different cancer cell lines differed with the acetone extract observed no anti-proliferative effect compared to ethyl acetate. Based on Elzbieta et al. [29], this may be the result of the synergistic or antagonistic effects of some compounds, including the phenolic and flavonoids in the extract. An exogenous antioxidant contained in the extract can lead to both good and bad reactions in a redox reaction [30]. In addition, some studies have shown that external antioxidants have produce controversial results, especially at high or low concentrations. The type, concentration, and matrix of an external antioxidant from a natural compound are characteristics that influence its effectiveness [31].

Out of all extracts used in this study, ethyl acetate exhibited the most potent cytotoxic activity with a significantly higher cytotoxic effect than other polar solvents and acetone. This finding correlates with the previous study which reported that ethyl acetate extract from *P. minus* leaves displayed the highest cytotoxic effect towards colorectal cancer cell line, HCT-116 [26, 28]. It had been discussed that the cytotoxic activity of a plant against cancer cells is based on their phytochemical properties [32]. The

phytochemical screening of *P. minus* extract had identified tannins and other flavonoids, comprising hyperoside, miquelianin, quercitrin, isoquercetin, apigetrin, quercetin and astragalin [33,34].

Flavonoids had demonstrated an apoptosis mechanism of *P. minus* extract which selectively destroy cancer cells that often have higher levels of reactive oxygen species (ROS) than normal cells [35]. Consistently, elevated levels of ROS cause by an adaptive response to stress enables cancer cells to thrive in a high ROS environment to preserve cell viability [36]. Following, flavonoids are known as efficient ROS scavengers and are capable of modulating proteins that are involved in cell proliferation mainly through cell cycle regulation [37]. Flavonoids were widely used in plants to interrupt the cell cycle in human gastric, prostate and melanoma cancer cells at the G1 phase, and were an apoptotic inducer on both intrinsic and extrinsic pathways [28].

Follows, the potent cytotoxic effect exerted by ethyl acetate extract in this study may cause by non-polar flavonoid compound, quercetin-3-0-rhamnoside [33]. These had been supported by Hashim et al. [38]; which reported that quercetin and quercetin-3-0-rhamnoside in the ethyl acetate extract of *P. minus* had reported $IC_{50}$ less than 20 µg/mL in the DPPH scavenging assay, which gave a significant antioxidant activity compared to other polar solvents. Hence, an ethyl acetate extract from *P. minus* exhibited selective toxicity towards colorectal cancer cell lines, HCT-116 and CT-26.

### 3.3 Cell Morphology Observation and Fluorescence Staining of Apoptosis of *P. minus* Extracts on Colon Cancer Cell Lines

In anti-cancer development and progression, programmed cell death or known as an apoptosis shows a significant role as it is a profoundly regulated process or mechanism. The process described including the cleavage of proteins and revival of caspases in living cells, thereby rises in cellular shrinkage, reduction of chromatins, blebbing of cell membrane and DNA fragmentation [39]. The morphological changes of HT-29, HCT-116 and CT-26 treated cells with $IC_{50}$ were incubated for 72 hours and compared with the untreated cells shown in Fig. 1. Under control conditions at 0 hour, HT-29, HCT-116 and CT-26 cells appeared healthy with growth of up to 90% cell confluence.

The HT-29 and HCT-116 cells were round and intact as opposed to the CT-26 cells which displayed a polygonal and branching shape, thus reflecting the normal growth patterns for each of the colon cancer cell lines [Fig. 1 a (i), b (i), c (i)]. After a 24- hour treatment with the respective $IC_{50}$ dosage of *P. minus* extracts, all three cell lines displayed cellular shrinkage, cell rounding, partial detachment, and presence of lobulated apoptotic cells [Fig. 1 a (ii), b (ii), c (ii)]. As there is no cytotoxic effect of acetone extract towards HT-29, HCT-116 and CT-26 cells, morphological observation from these extract was not done accordingly.

Subsequently, after 48- hours of treatment, cytoplasmic protrusion features known as cellular blebs were observed in several cells that led to the formation of small cytoplasmic vesicles, also known as apoptotic bodies. At this stage, prominent features of cellular shrinkage, cell rounding, partial detachment, and lobulated apoptotic cells were observed in all three cell lines [Fig. 1 a (iii), b (iii), c (iii)]. After a 72-hour treatment, the colon cancer cell lines appeared to be smaller, possibly due to the significant reduction in cellular mass. Several cells were observed to be in the final stages of apoptosis with condensed cytoplasm, aggregation of the nuclear chromatin and the occurrence of fragmented cell bodies [Fig. 1 a (iv), b (iv), c (iv)].

Based on these observations, the fluorescence double staining procedure was performed to analyse the membrane integrity of the cell lines following treatment with different extracts of *P. minus* at the concentration of $IC_{50}$ dosage (Fig. 2, 3, 4). The AO/PI stains are nuclei acid-binding dyes that emit a strong fluorescence when bound to DNA, whereby AO is penetrable to living and dead cells, while PI is only permeable to dead cells [40]. Red-stained cells indicated apoptotic or necrotic cells that have lost their membrane integrity, while the green-stained cells indicated cells that are still viable [41]. The negative control cells were stained green and displayed round and green nuclei as the PI stain was resistant to the cell membrane of the untreated cell [Fig. 2 (g), 3 (g), and 4 (g)]. However, several HT-29, HCT-116, and CT-26 cells treated with different solvents of *P. minus* extracts were stained red and orange as the cells lost their shape within 72 hours due to the anti-proliferative and cytotoxic properties of the extract [42].

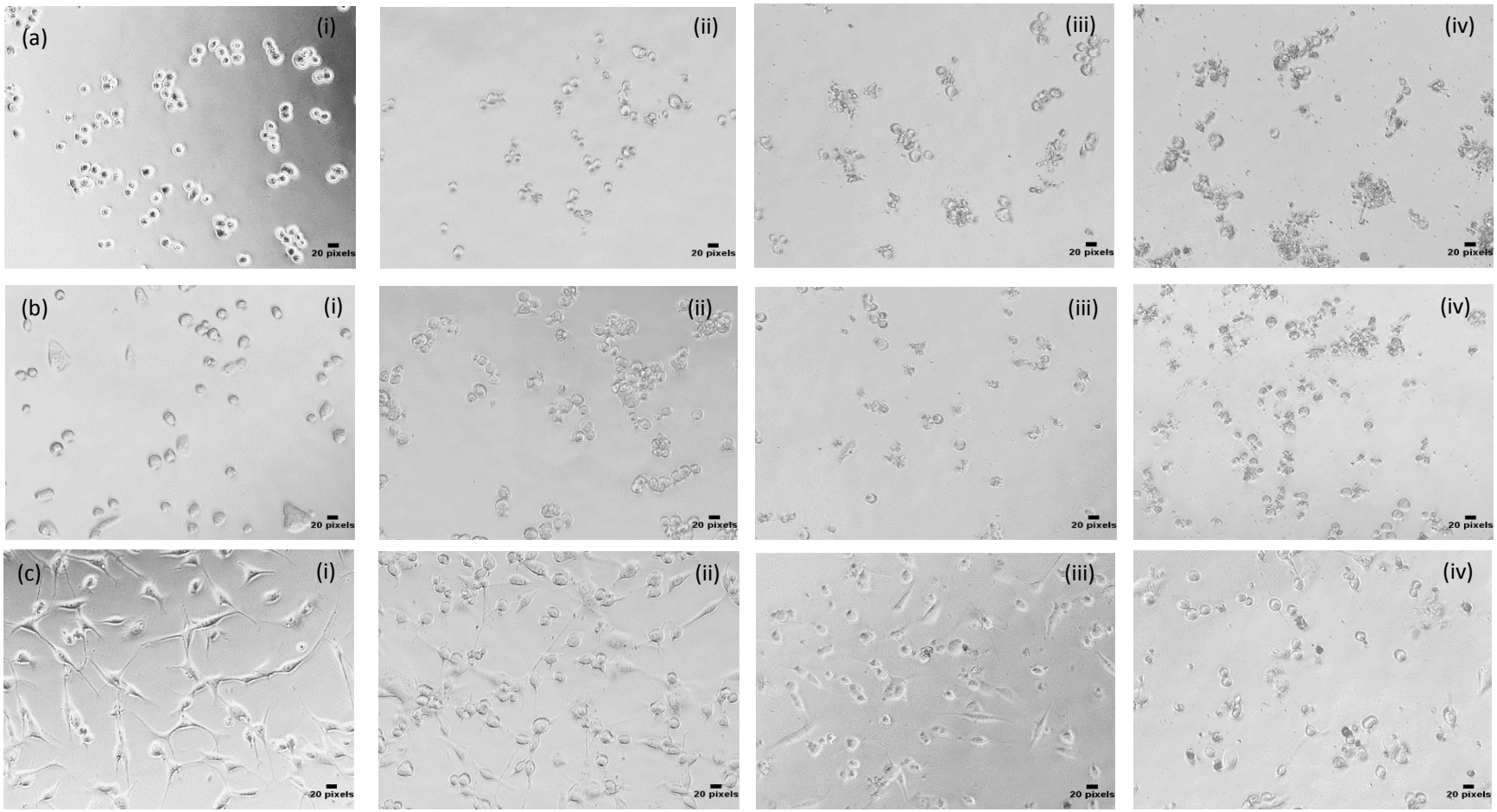


**Fig. 1. Inhibition of (a) HT-29, (b) HCT-116 and (c) CT-26 by *P. minus* extracts for 3 days (except for acetone). Cell morphology of HT-29, HCT-116 and CT-26 were examined after being treated with $IC_{50}$ at (i) 0 hour, (ii) 24 hours (iii) 48 hours and (v) 72 hours. The photographs were taken at 10x magnification with inverted microscope (Nikon, Japan)**

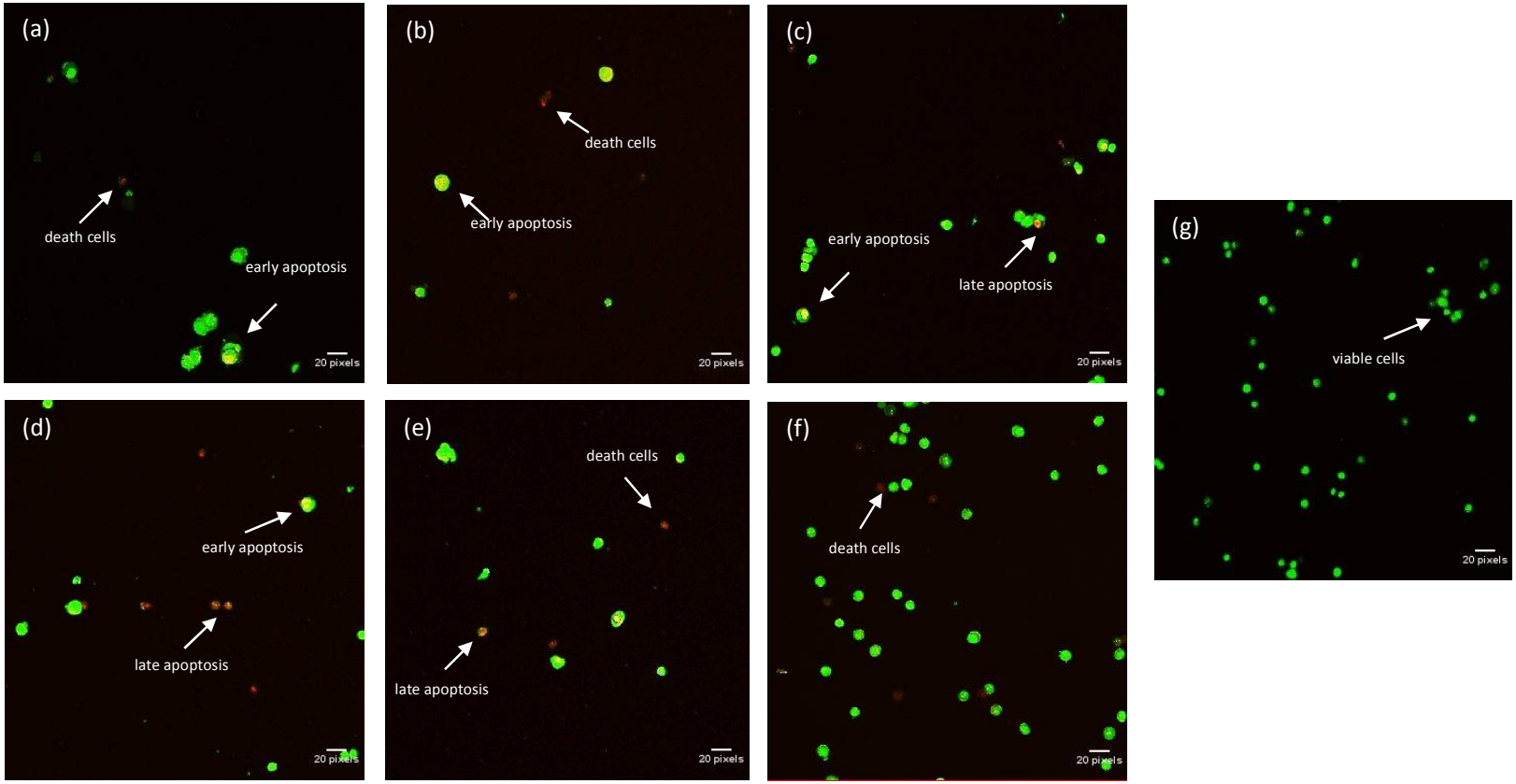


**Fig. 2. Fluorescence micrographs of *P. minus* extracts stained with AO/PI. The micrographs of HT-29 was examined after treated with $IC_{50}$ values each extract at 72 hours. (a) 100% aqueous solution; (b) 100% methanol; (c) 100% ethanol; (d) 70% aqueous ethanol; (e) 50% aqueous ethanol; (f) 100% ethyl acetate; (g) untreated cells. *Magnification 10x***

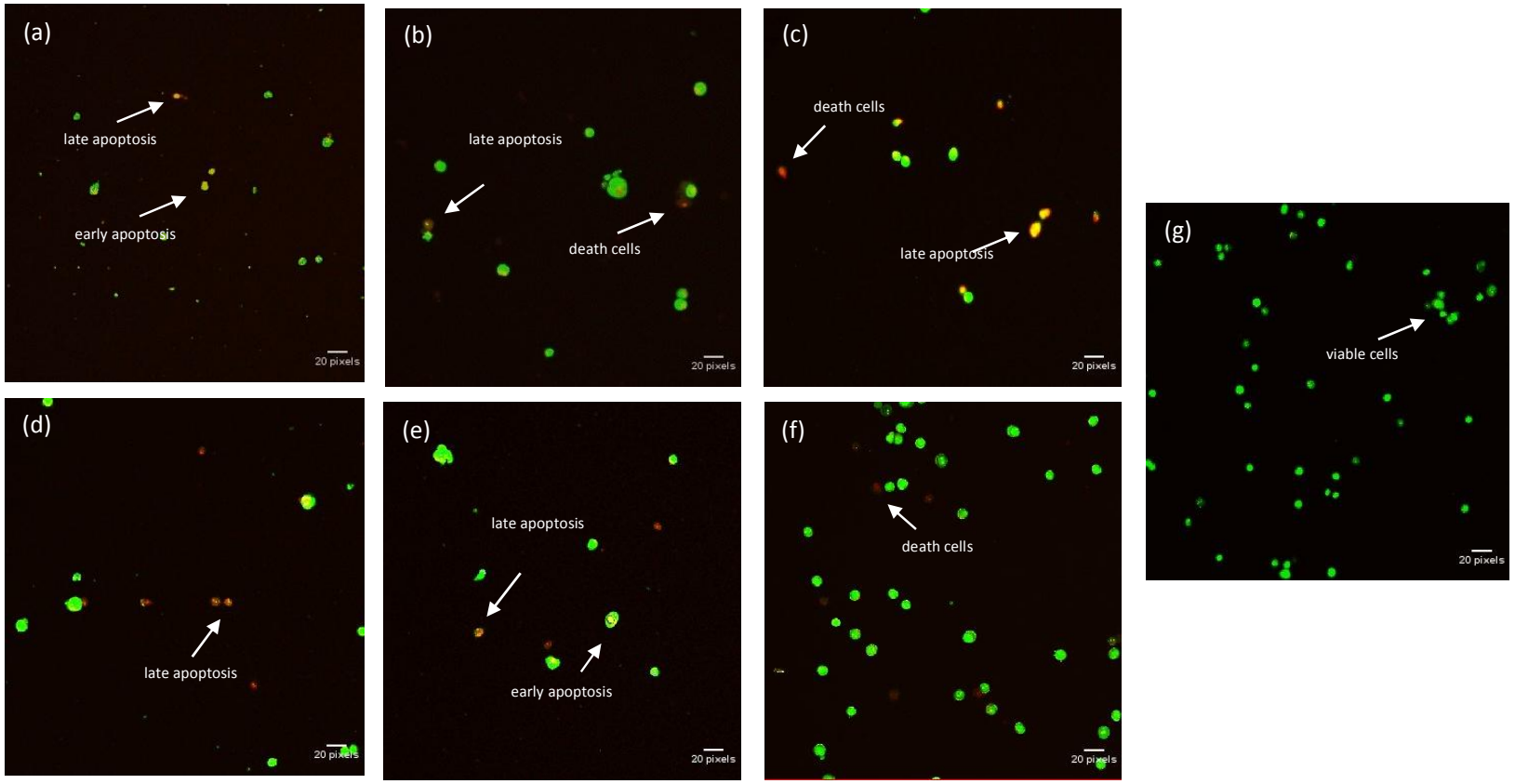


**Fig. 3. Fluorescence micrographs of *P. minus* extracts stained with AO/PI. The micrographs of HCT-116 was examined after treated with $IC_{50}$ values each extract at 72 hours. (a) 100[ aqueous solution; (b) 100% methanol; (c) 100% ethanol; (d) 70% aqueous ethanol; (e) 50% aqueous ethanol; (f) 100% ethyl acetate; (g) untreated cells. *Magnification 10x***

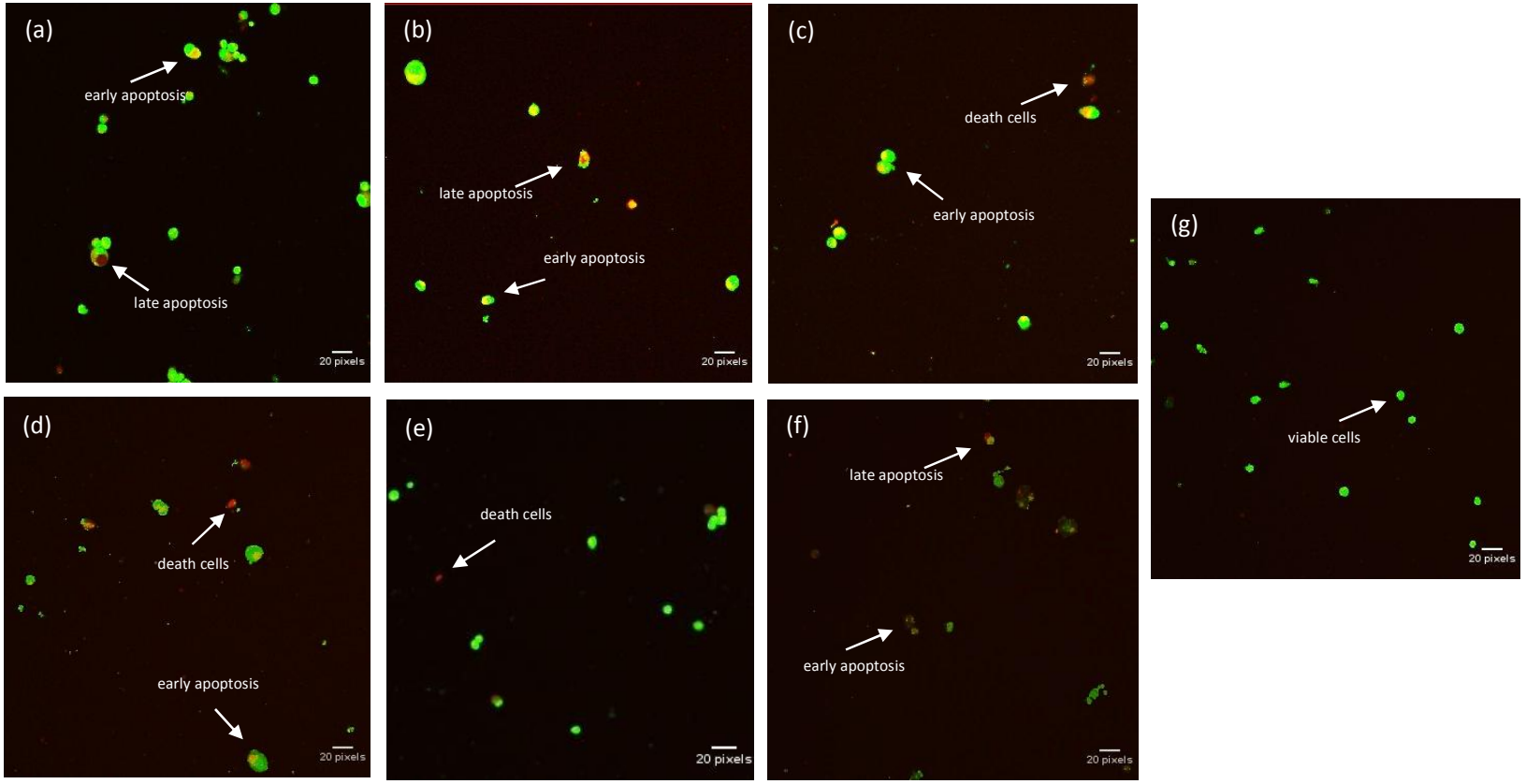


**Fig. 4. Fluorescence micrographs of *P. minus* extracts stained with AO/PI. The micrographs of CT-26 was examined after treated with $IC_{50}$ values each extract at 72 hours. (a) 100% aqueous solution; (b) 100% methanol; (c) 100% ethanol; (d) 70% aqueous ethanol; (e) 50% aqueous ethanol; (f) 100% ethyl acetate; (g) untreated cells. *Magnification 10x***

In this study, the different extracts of *P. minus* exhibited cells that were stained red and green. The cell nuclei stained with orange and light red colours displayed cells that still had an intact shape, thereby indicating that the cells were at the late apoptosis stage but not dead [38]. Meanwhile, cell nuclei stained with fluorescence red indicated dead nucleated cells. Hence, the membrane integrity of the colorectal cancer cell lines was lost after treated with different solvents of *P. minus* extracts at $IC_{50}$ concentrations after 72 hours of treatment; as there were penetrated of red and orange colour of cells. The different solvents of *P. minus* extracts were able to induce apoptosis, thus indicating that these extracts had anti-proliferative and cytotoxicity activities against all three colorectal cancer cell lines.

## 4. CONCLUSION

In conclusion, an ethyl acetate extract from *P. minus* exerts the most potent cytotoxic effect towards HCT-116 and CT-26 cell lines; while 50% aqueous ethanol towards HT-29 cell lines. However, no cytotoxic effect was observed by the acetone extract at the same treatment duration on three types of colon cancer cell lines. Each of solvent extract of *P. minus* (except for acetone) demonstrated cell death through apoptosis in cells morphology and membrane integrity in HT-29, HCT-116 and CT-26 cell lines, either polar or non-polar solvents. Therefore, more study should be conducted in investigating the effects of *P. minus* action and mechanism of apoptosis and regulations of gene expression against colon cancer cell lines.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## ACKNOWLEDGEMENTS

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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