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Anti-proliferative Properties of New Variety Organic Rice MRQ74 Extracts against Colon Cancer Cells: *In-vitro* Study

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Authors' contributions

This work was carried out in collaboration among all authors. Author MAKR did the design the study, methodology and manuscript review. Author NAH did the 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 examine the anti-proliferative properties of different extracts of new variety an organic rice MRQ74 towards colon cancer cells: *in-vitro* study.
Study Design: Experimental study.
Place and Duration of Study: Central Laboratory, Tissue Culture Laboratory, University of Sultan Zainal Abidin, Terengganu from November 2019 until February 2020.
Methodology: The organic rice MRQ74 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. Meanwhile, cells morphology observation and fluorescence double staining of treatment cells were determined using a light inverted microscope and acridine orange/propidium iodide staining.
Results: Results showed that 50% aqueous ethanol of rice grains gave the lowest IC_{50} values towards HCT-116 and CT-26 cell lines, while an aqueous solution of rice grains gave the lowest

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IC_{50} values towards HT-29 cells ($p<0.05$). Thoroughly, the treated colon cancer cells had shown morphological alterations after treated with different solvent extracts of an organic rice MRQ74.
Conclusion: The outcomes had observed preliminary results on cancer study for better health by inspiring the consumption of an organic rice MRQ74 and future product developments.

Keywords: Organic rice MRQ74; colon cancer; cytotoxic; MTT assay; AO/PI staining.

1. INTRODUCTION

Fragrant rice is one of high quality and special rice other than Basmati and Ponni rice [1]. To meet the growing consumer demand for fragrant rice, varieties development was conducted at the Malaysian Agricultural Research and Development Institute (MARDI) more than a decade ago. To date, two fragrant rice varieties have been declared by MARDI namely MRQ76 and MRQ74. Varieties MRQ74 had been declared in 2005 has properties similar to Basmati rice [2]. Meanwhile, varieties MRQ76 was declared in 2012 by the quality of the rice fragrant, soft and slightly sticky and medium resistant to bacterial leaf blight, blast, and sheath blight. The characteristics are similar to Jasmine, a type of fragrant rice imported from Thailand [3]. Both of these varieties have a high potential for high yield [4].

Apart from agronomy, scientific field studies have been conducted to prove organic fragrant rice could give a positive value to consumer's health. Based on Abubakar et al. [2], the cultivation rice of MRQ74 in germinated brown forms revealed 85% scavenging abilities in antioxidant levels, and 25.7% of high content of amylose. Additionally, the MRQ74 rice had a moderate glycemic index and glycemic load by 67.5 and 32.3, respectively. While the modest capabilities of glucose uptake by 33.69% [2]. A white rice and glutinous rice has a high glycemic index with around 80 and 100, respectively [3]. Therefore, the study shows MRQ74 had a moderate glycemic index (67.6), health benefits as the consumer will not experience a sharp rise in glucose level after meals of a new variety an organic rice MRQ74 [5].

Additionally, emerging studies suggest that dietary rice bran variety may have beneficial effects on several cancer types. Bioactive components such as ferulic acid [6], g-oryzanol, phytic acid [7], coumaric acid [8], pectin [9], tricin, and tocotrienol-tocopherols have been identified to inhibit tumor growth and/or cause apoptosis on cancer cells [10]. Based on Angela et al. [11], protection of bioactive components of rice bran against tissue damage through the scavenging of free radicals and blocking of chronic inflammatory responses. However, it has also been shown that rice bran phytochemicals activate immune responses to cancer and affect the colonic tumor microenvironment in favour of improved colorectal cancer chemoprevention [12,13]. Results from a study conducted by Sun et al. [14] demonstrated the ability of g-tocotrienol to induce apoptosis in the gastric cancer cells of SGC-7901 by activating caspase-3 as well as causing cell cycle arrest in the phase G0/G1.

By maintaining the positive features and advantages in terms of health studies that have been done [4], it was needed to explore more on the pharmaceutical value of the organic fragrant rice varieties. The study on anti-proliferative properties against colon cancer had been chosen to be done as based on our knowledge, the results of MRQ74 samples have not yet been reported in any medical journal. Besides, colorectal cancer is the most common cancer that occurred among men and women in the world [15,16]. In conjunction with that, the apoptosis-inducing capabilities induced by plants are of point interest due to cost efficiency and have fewer aftereffects compared to synthetic antioxidants. Therefore, this study aimed to examine the anti-proliferative properties of a new variety of organic rice MRQ74 on HT-29, HCT-116 and CT-26 cell lines: *in-vitro* study.

2. MATERIALS AND METHODS

2.1 Sample Collection of Rice Samples

A new variety of organic rice MRQ74 was obtained from MARDI, Malaysia. The rice grains samples were cautiously separated from any physical residue and put aside in a closed containers.

2.2 Extraction Procedure of Rice Samples

The method introduced by Melini & Acquistucci [17] with slight adjustments was used in the

extraction process. Two samples were used in this study which is; rice grains and cooked rice samples. Samples of rice grains were prepared immediately before analyses by grinding rice grains into a fine flour at maximum speed using an electronic blender (Warring 2-Speed Blender, 240 VAC-Standard Motor) for about 30 sec which confirmed that the grains were thoroughly ground. Then, cooked rice samples were prepared by using a similar cooking treatment. In detail, rice grains was placed in a rice cooker, and water was added with ratio 1:2 (w/v). Samples were cooked until complete absorption water by grains was achieved. Afterward, cooked rice samples were cooled down and being dried in the drying oven at 40°C until samples dried. Next, the dried cooked rice samples were grinded into a fine flour at a maximum speed using electronic blender (Warring 2-Speed Blender, 240 VAC-Standard Motor) for about 30 sec which confirmed that the samples were thoroughly grinded. Then, the fine flour of both rice grains and cooked rice samples was passed through a mesh sieve prior to use for analyses.

A 40 g of fine flour rice grains and cooked rice samples in those two different methods applied were continued with a soaking process (1:10; w/v) using 50% aqueous ethanol and aqueous solution (deionized water) at 25°C for 24 hours. Subsequently, all the solvent extracts were filtered by using Whatman filter paper (20-25 µm) and concentrated in a rotary evaporator at 40°C [18]. The concentrated extracts were then placed in the oven at 40°C to allow for complete solvents evaporation. Finally, all the extracts were kept in -20°C freezer until they were used for the next analysis.

2.3 Extraction Yield Procedure of Rice Extracts

The extraction yield was calculated according to the method of Zhang et al. [19] using the formula;

W2/W1 * 100% [19]
W1= original weight of sample= 40 g,
W2= weight of dried extract.

2.4 *In-vitro* Study

2.4.1 Preparation of rice extracts

The methods used previously by Khalili et al. [20] were adopted for this study with modifications. A total of 10 mg of rice grains and cooked rice 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 of 10% and penicillin-streptomycin of 1% to obtain a working solution of 1 mg/mL.

2.4.2 Cells 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. 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 cell lines 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.

2.4.3 Determination inhibition concentration of 50% (IC_{50}) by rice extracts

The inhibitory concentration (IC_{50}) of rice extracts was evaluated using a colorimetric micro-titration method known as the MTT assay or tetrazolium salt reduction assay [21]. 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 cells 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 cells. After 24 hours, triplicate serial dilutions of the rice grains and cooked rice extracts (1.00 - 0.016 mg/mL) [22] 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 cells viability were also measured. The relative cells viability of the treated cells was described as % of cells viability and calculated based on the following formula: (A570 of treated cells/ A570 of control cells) x 100%. The dosage concentration of IC_{50} was calculated to depend on the non-linear regression of the response curves within the same region.

2.4.4 Cells morphology observation

The effects of rice extracts on the cellular morphological changes were determined using the method by Merlin et al. [23]. 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.

2.4.5 Fluorescence microscopy of apoptosis using AO/PI doubles staining

The steps used previously by Hajiaghaalipour et al. [24] were adopted for this study. The determination of the apoptotic effects of rice extracts on the different 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/cells viability was visualized at 10x magnification using an Olympus-BX51 fluorescence microscope (Olympus, Japan) fitted with a Nikon camera (Nikon, Japan).

2.5 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 Independent T-test in percentages of extract yield. Other than that, a One-way ANOVA test was used to compare an IC_{50} value among different cell lines tested.

3. RESULTS AND DISCUSSION

3.1 Extraction Yield of Rice Grains and Cooked Rice Extracts

Based on Table 1, it is observed that a higher percentage of the extract yield was removed by an aqueous solution of cooked rice which gave 1.55 ± 0.01%, while the second higher extract yield gave 0.73 ± 0.01% by an aqueous solution of rice grains. Meanwhile, the lowest percentage of the extract yield was removed by 50% aqueous ethanol of cooked rice with 0.20 ± 0.01%. Moreover, the percentage of the extract yield by an aqueous solution of cooked rice and rice grains gave significantly higher yield compared with 50% aqueous ethanol by 87% and 18%, respectively ($p<0.05$).

Commonly, the efficient extractability yield is influenced by methods of extraction and polarities of solvents used [25-28]. Based on the results, it can be inferred that increasing the extraction yield is due to the increasing aqueous in the solvents used; in which water had a higher yield compare to 50% aqueous ethanol. Dailey & Vuong [29] also reported that extraction yields of aqueous solvents higher than the pure and aqueous solvents used. Additionally, the higher yield from cooked rice sample was expected as the rice grains absorb water through boiling or steaming process [3]. The absorption process was done by cooking the rice grains in ratio 1:2 with the water. Overall, this eventually softens the cooked rice texture compared to rice grains itself. Even though the cooked rice undergoes drying and ground processed into a fine flour, the highest yield observed shows that the expansion during cooked had increased its content during the extraction process compared to the rice grains.

The findings confirm the methods of extraction and solvents polarities used had a crucial role in extractability of yield from plant samples [30]. Therefore, results showed an aqueous solution is a more suitable solvent for rice samples extraction.

Table 1. Determination of percentages extract yield (%) of rice and cooked rice extracts

Sample Preparations		Crude extract weight (g)	Extraction yield (%)	F-statistics (df)	*P*-value
Rice	50% aqueous ethanol	0.237 ± 0.01	0.60	-12.02 (4)	<0.01*
	Aqueous solution	0.293 ± 0.01	0.73		
Cooked rice	50% aqueous ethanol	0.087 ± 0.01	0.20	-81.50 (4)	<0.01*
	Aqueous solution	0.630 ± 0.01	1.55		

*Data represent the mean ± SD of three independent experiments. Independent T-test, *p<0.01. Extract yield present in percentages (%)*

3.2 Anti-proliferative Activities of Rice Grains and Cooked Rice Extracts

In this study, the cytotoxic activity of different colon cancer cell lines using solvents of 50% aqueous ethanol and an aqueous solution of rice grains and cooked rice extracts from a new variety an organic rice MRQ74 (0 – 1 mg/mL) was investigated by using the MTT assay. The MTT assay is the source for various *in-vitro* assays that comprise quantitatively cells viability and cells proliferation. Generally, the reduced number of viability cancer cells is due to the toxicity of a particular agent by cells killing and/or interference proliferation of cells. In this study, an IC_{50} value was analyzed as a cytotoxic parameter due to the concentration of a drug or particular agents that caused 50% suppression of tumor cells [21].

The MTT assay indicated that 50% aqueous ethanol and an aqueous solution of rice grains extracts displayed potent anti-proliferative activity, with an IC_{50} value of 0.14 ± 0.01 mg/mL, 0.12 ± 0.03 mg/mL and 0.22 ± 0.06 mg/mL against HT-29, HCT-116 and CT-26 cell lines, compared to cooked rice extracts (Table 2). The biological properties and reactions of certain compounds in each sample differ between different colon cancer cell lines. In this study, HCT-116 cells were the most sensitive to treat with 50% aqueous ethanol of rice grains, cooked rice and an aqueous solution of cooked rice followed by HT-29 and CT-26 cell lines. The cooked rice extracts of 50% aqueous ethanol at 24 hours treatment did not exert toxic effect against HT-29 and CT-26 cell lines, with viability more than 50%, compared to other extracts.

Under similar conditions, an IC_{50} value of doxorubicin after 72 hours on treated HT-29 is 0.63 ± 0.02 µg/mL, HCT-116 is 0.46 ± 0.19 µg/mL and CT-26 is 0.14 ± 0.01 µg/mL (P<0.01) (Table 3). As doxorubicin is known as commercial drug used in treated cells, the drug is more effective in inhibiting the proliferation of treated cancer cells at low concentration compared to the rice extracts. Additionally, the outcomes demonstrated there were a significant difference between an IC_{50} value (mg/mL) of 50% aqueous ethanol cooked rice extract among different colon cancer cell lines used (p<0.05) after 72 hours treatment, but not in other sample extracts (Table 3). Previously, an abundant

Table 2. The IC_{50} values of HT-29, HCT-116 and CT-26 cell lines by rice and cooked rice extracts

Samples	Incubation periods	IC_{50} values (mg/mL)		
		HT-29	HCT-116	CT-26
50% aqueous ethanol of rice	24 hours	0.34 ± 0.01	0.20 ± 0.03	0.34 ± 0.01
	48 hours	0.20 ± 0.03	0.12 ± 0.03	0.22 ± 0.06
	72 hours	0.31 ± 0.01	0.25 ± 0.06	0.29 ± 0.02
Aqueous solution of rice	24 hours	0.14 ± 0.01	0.03 ± 0.03	0.31 ± 0.02
	48 hours	0.14 ± 0.01	0.25 ± 0.01	0.26 ± 0.02
	72 hours	0.31 ± 0.02	0.32 ± 0.04	0.35 ± 0.04
50% aqueous ethanol of cooked rice	24 hours	nil	0.25 ± 0.08	nil
	48 hours	0.46 ± 0.01	0.30 ± 0.02	0.43 ± 0.04
	72 hours	0.59 ± 0.01	0.25 ± 0.01	0.35 ± 0.01
Aqueous solution of cooked rice	24 hours	0.48 ± 0.03	0.21 ± 0.05	0.25 ± 0.01
	48 hours	0.31 ± 0.01	0.32 ± 0.13	0.26 ± 0.01
	72 hours	0.42 ± 0.01	0.32 ± 0.02	0.36 ± 0.06

Data represent the mean ± SD of three independent experiments

amounts of bioactive compounds in rice bran varietal as vitamin E isoforms (α-, γ-, δ-tocotrienols, and tocopherols) and γ-oryzanol have been shown to suppress the growth of colorectal cancer cells; however the outcomes may be diverse among different rice varieties [31].

A study by Tan et al. [32,33] had observed the toxicity of brewers rice on HT-29 cells and reported that water extract had inhibited proliferation. Meanwhile, rice husk extract of methanol also had been found to give an anti-proliferation effect on colon cancer cells with an IC_{50} value of 0.5 µg/mL [34]. Previously, the chemo-prevention activity of colorectal cancer had been emphasized by the role of bioactive compounds in rice samples such as tricin, phytic acid, and flavonoids [35,36]. Follows, the phenolic compound of cycloartenyl ferulate in rice bran had been found to suppress the proliferation of colorectal adenocarcinoma, SW480 cells [37]. Meanwhile, Forster et al. [31] also observed that the growth of colorectal cancer cells had been reduced after treated with γ-tocotrienol and total phenolics from rice bran samples ($P<0.01$).

Subsequently, the anti-proliferative activity of different extracts of varieties MRQ74 on different colon cancer cell lines might be attributed strongly by its polyphenols content [38], which known could display effective antioxidant activities against cancer cells [39-41]. These were supported by Li et al. [42], which abundant of phenolic acids are present in whole grain included rice comprises of p-coumaric acid, ferulic acid, vanillic acid, and caffeic acid. Therefore, the anti-proliferative activity of new variety of organic rice MRQ74 extracts against different colon cancer cell lines was observed in this study.

3.3 Cells Morphology Observation and Fluorescence Staining Apoptosis of Rice Grains and Cooked Rice Extracts on Colon Cancer Cells

Apoptosis or cell death programmed plays a vital role within the tissue maintenance, organ physiological state, and hereditarily controlled cell death towards the stability of cell proliferation [43]. Apoptosis is a common process or mechanism which accompanied by multifaceted physiological processes such as cell turnover and chemical-induced cell death [44]. Accordingly, the therapeutic agent in plant matrixes has the potential to modulate cell death in cancer cells *in-vitro* [45,46]. Previous studies had observed that bioactive compounds in whole grains such as tocotrienol-tocopherols had been observed to induce apoptosis in HeLa cells [46].

Consequently, the preliminary apoptotic effect had been studied using morphology observation and fluorescence AO/PI staining methods. AO is a membrane-penetrable cationic colour that gets to the nucleic acids of living cells and diverts a green fluorescence. Then, PI is not permeable to intact membranes, yet penetrable the membranes of non-viable cells, causing orange fluorescence. Therefore, AO/PI staining can be feasibly utilized to analyze apoptotic-associated changes of the cell membranes during the process of cell death [47]. In this study, a new variety organic rice grains and cooked rice MRQ74 extracts had distinctive morphological changes that incriminate apoptosis markers such as membrane blebbing, fragmentation of DNA and formation of the apoptotic body after 72 hours treatment in HT-29, HCT-116 and CT-26 cell lines (Fig. 1 a (iv), b (iv) & c (iv)).

Table 3. The IC_{50} values of HT-29, HCT-116 and CT-26 cell lines by rice and cooked rice extracts at 72 hours treatment

Samples	IC_{50} values (mg/mL)			F-statistics (df)	*P*-value
	HT-29	HCT-116	CT-26		
50% aqueous ethanol of rice	0.31 ± 0.01	0.25 ± 0.06	0.29 ± 0.02	1.14 (2, 6)	0.38
Aqueous solution of rice	0.31 ± 0.02	0.32 ± 0.04	0.35 ± 0.04	0.23 (2, 6)	0.80
50% aqueous ethanol of cooked rice	0.59 ± 0.01	0.25 ± 0.01	0.35 ± 0.01	0.01 (2, 6)	<0.01*
Aqueous solution of cooked rice	0.42 ± 0.01	0.32 ± 0.02	0.36 ± 0.06	1.67 (2, 6)	0.27
Doxorubicin (µg/mL)	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*

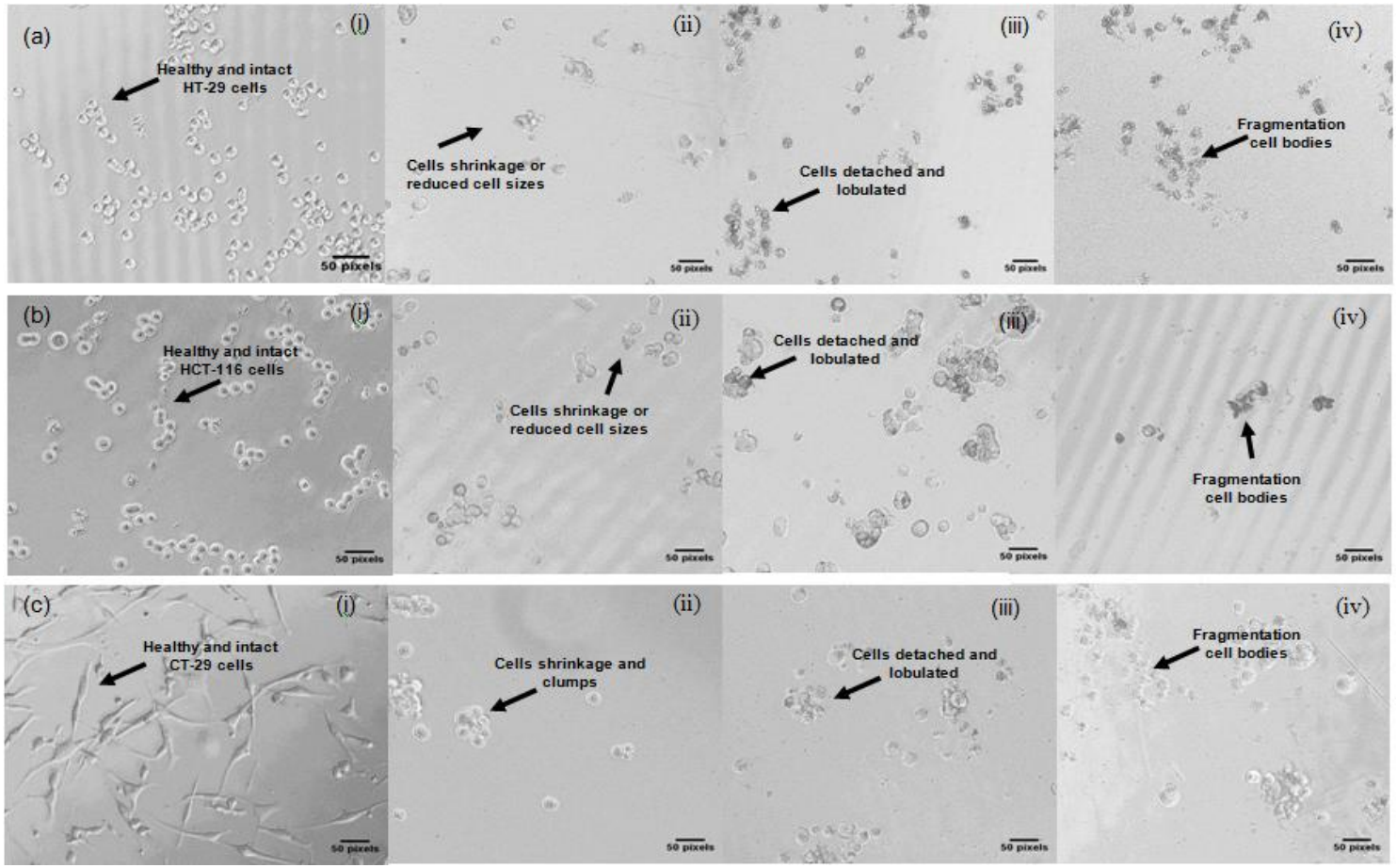

Fig. 1. Inhibition of (a) HT-29, (b) HCT-116 and (c) CT-26 by rice extracts for 3 days. Cells morphology of HT-29, HCT-116 and CT-26 were examined after being treated with IC_{50} at (i) untreated, (ii) 24 hours (iii) 48 hours and (v) 72 hours. The photographs were taken at 10x magnification with inverted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

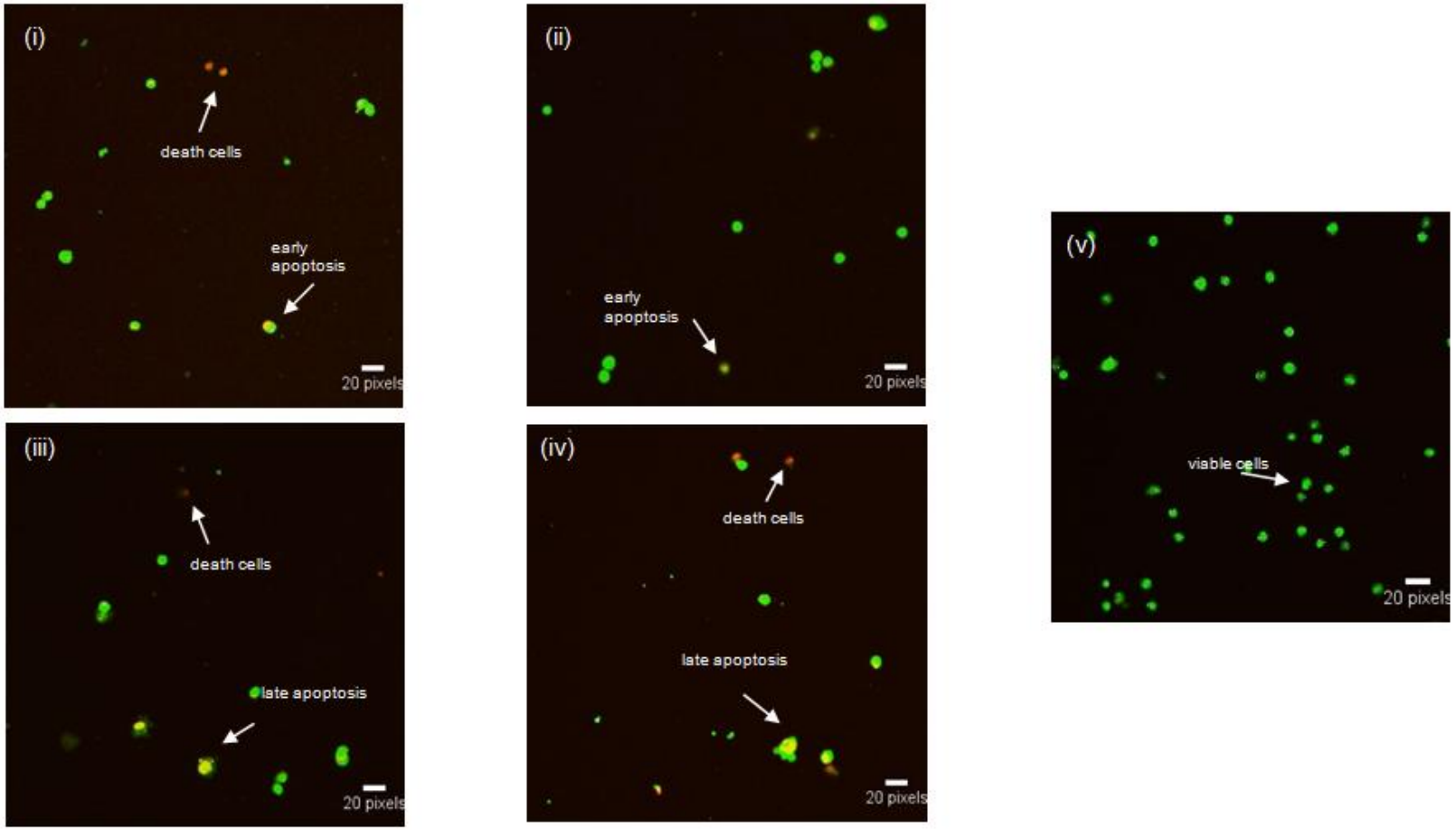

Fig. 2. Fluorescence micrographs of rice and cooked rice extracts stained with AO/PI. The micrographs of HT-29 were examined after treated with IC_{50} values each extract at 72 hours. (i) 50% aqueous ethanol of rice; (ii) aqueous solution of rice; (iii) 50% aqueous ethanol of cooked rice; (iv) aqueous solution of cooked rice; (v) untreated cells. *Magnification 10x*

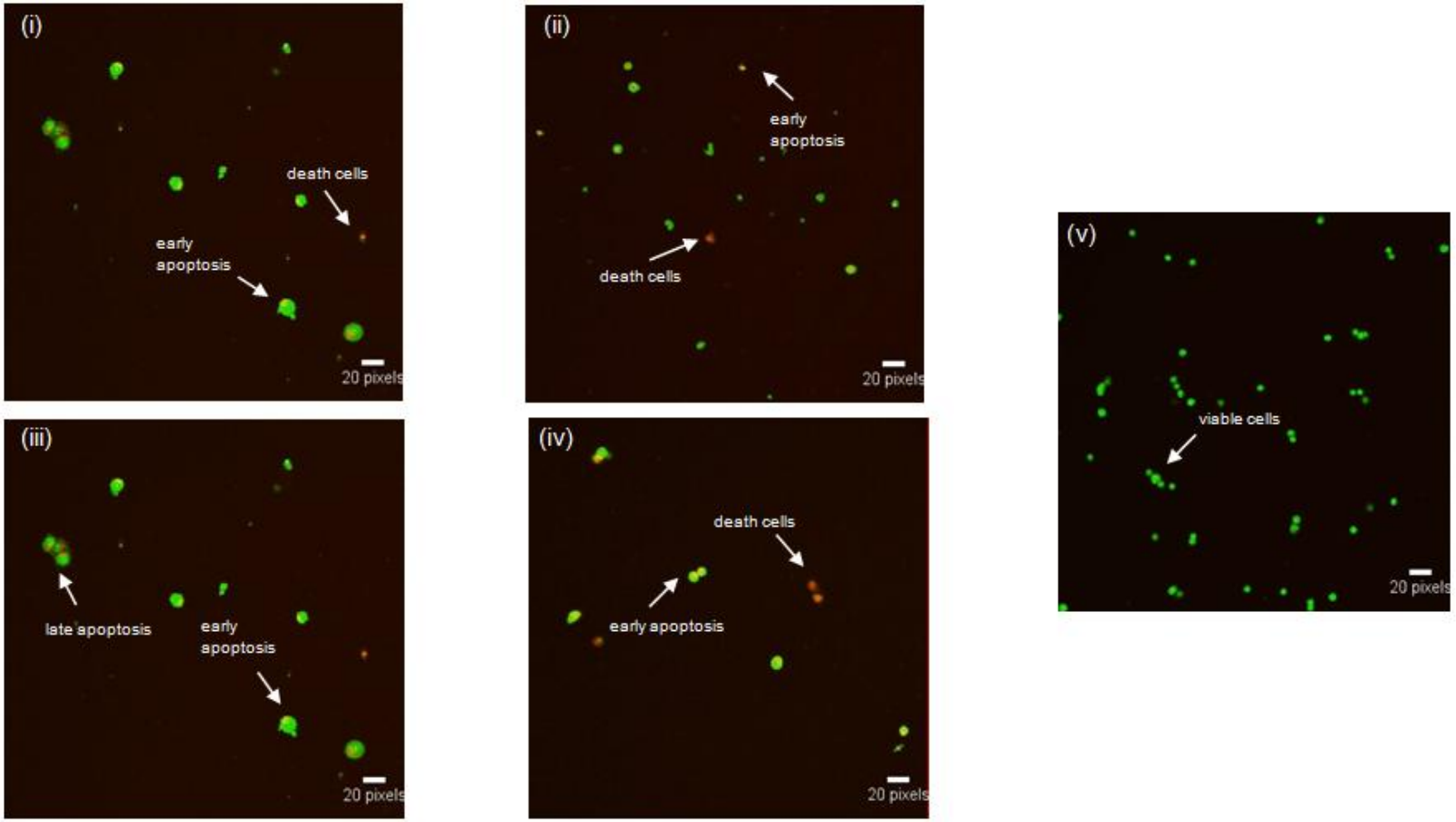

Fig. 3. Fluorescence micrographs of rice and cooked rice extracts stained with AO/PI. The micrographs of HCT-116 was examined after treated with IC_{50} values each extract at 72 hours. (i) 50% aqueous ethanol of rice; (ii) aqueous solution of rice; (iii) 50% aqueous ethanol of cooked rice; (iv) aqueous solution of cooked rice; (v) untreated cells. *Magnification 10x*

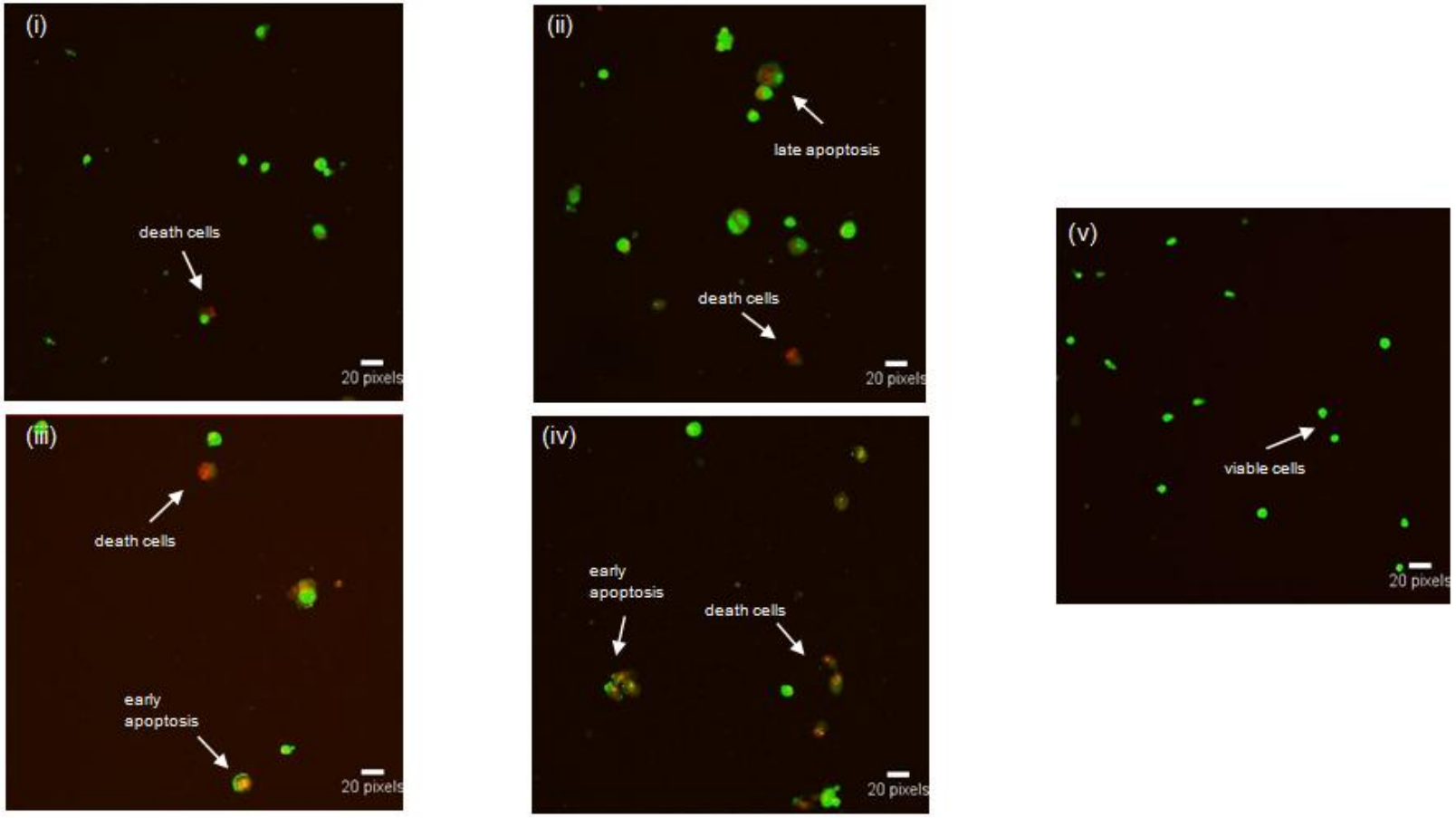

Fig. 4. Fluorescence micrographs of rice and cooked rice extracts stained with AO/PI. The micrographs of CT-26 were examined after treated with IC_{50} values each extract at 72 hours. (i) 50% aqueous ethanol of rice; (ii) aqueous solution of rice; (iii) 50% aqueous ethanol of cooked rice; (iv) aqueous solution of cooked rice; (v) untreated cells. *Magnification 10x*

Additionally, treated HT-29, HCT-116 and CT-26 cell lines were in the stages of final apoptosis and necrosis with chromatin shortened made fragmentation occurred after treated 72 hours. Then, these features are evinced under fluorescence microscopy by the bright-green, orange and red colour from the AO/PI dye stained. The negative control of cells was stained green and displayed round and green nuclei as the PI stain was resistant to the cell membrane of the untreated cells (Figs. 2 (v), 3 (v), 4 (v)). Whereas, each extract of rice grains and cooked rice demonstrated death and necrotic cells at concentration IC_{50} after 72 hours of treatment. This may probably due to the toxicity effect of the extract resulting in the damage of the plasma membrane of HCT-116, CT-26 and HT-29 cell lines, thus causing the PI dye to enter and make the cells appear red (necrosis) (Figs. 2, 3, 4).

Therefore, additional studies on MRQ74 extracts on physiological and biological mechanisms might aid in the development of anti-proliferative compounds in new variety of organic rice MRQ74 extracts for the colon cancer chemo-prevention.

4. CONCLUSION

The result above concludes there has been an observed study in the natural products as an alternative medicine for their therapeutic properties in the treatment. Based on the study using different colon cancer cell lines, it can be affirmed that new variety of organic rice MRQ74 extracts possess anti-proliferative activity against HCT-116, CT-26 and HT-29. Rice grains extracts of 50% aqueous ethanol are the most potent towards HCT-116 and CT-26 cell lines. Meanwhile, an aqueous solution rice grains extract is the most potent towards HT-29 cells. Moreover, each HCT-116, CT-26 and HT-29 cell lines had showed morphological alterations after treated with each of the solvent extracts of rice grains and cooked rice of MRQ74 by the formation of apoptotic bodies and reduction in cells viability in AO/PI staining thoroughly.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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