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Goat's Milk as a Potential Anti-proliferative against Colon Cancer Cell Lines

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AZAL, CAAB and MAKR designed the study concept and design. Authors RA and NAH design the methodology and authors ND, JKB, NR, RA and MNJ carried out the experimental, data acquisition and data interpretation. Authors ND, JKB and NR drafting the manuscript and authors AAB, AZAL, CAAB and MAKR revised critically on the manuscript written. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To investigate anti-proliferative effect of fresh and pasteurized goat milk against colon cancer cell lines (HT-29, HCT-116, CT-26).
Study Design: Experimental study.
Place and Duration of Study: Central Laboratory, Tissue Culture Laboratory, Universiti Sultan Zainal Abidin, Terengganu between January 2020 and April 2020.
Methodology: Samples comprised of two types goat milk, which were fresh and pasteurized in

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powder form. The samples were analysed for the anti-proliferative effect by MTT assay, and IC_{50} value was determined. Then, cell apoptotic changes were observed by light inverted microscope by 24, 48 and 72 hours.
Results: Experimental data showed that the fresh sample produce the highest yield (9.40%) than the pasteurized sample (7.17%). The fresh sample yielded the most potent cytotoxic value (0.28 ± 0.03), followed by pasteurized sample with value IC_{50} 0.32 ± 0.02 against HCT-116 cells. Then, the anti-proliferative effect was observed on cell apoptotic changes by reduction of cell volume, cell densed, and presence of fragmentation and apoptotic bodies at 24, 48 and 72 hours treatment.
Conclusion: In conclusion, the fresh sample of goat milk yielded the potent anti-proliferative effect than pasteurized sample.

Keywords: Anti-proliferative; goat milk; colon cancer; MTT assay.

1. INTRODUCTION

Colorectal or colon cancer is regarded as the third most common cancer in women and the second most common cancer in men in Malaysia [1]. Sajesh et al. [2] and Syiful et al. [3] reported that over 90% of Malaysians diagnosed with colorectal cancer are over 40 years of age, regardless of their ethnicity. The highest proportion of incidents is observed among those aged 60 to 69 [3]. According to the National Cancer Registry Report 2012–2016, the growing prevalence of colorectal cancer is due to socioeconomic class and western lifestyle habits [4]. As a result, alternative bases are of special importance and are now being explored extensively as anticancer medicines due to their generally low toxicity profiles.

The value of goats for human nourishment, particularly their milk, has most likely been known since their domestication.The goat was once regarded as the "poor man's cow" because it was more cheap and easy to reproduce in any situation [5]. Goat milk and its processed products as functional foods have received increasing attention from the food industry and have been used in the current trend of healthy eating patterns in developing countries, sustaining nourishment and health for people of all ages, particularly those who are allergic to cow's milk [6]. Smaller fat globules in goat milk are responsible for a finer texture in derived products, as well as greater digestion [5,7,8]. Aside from that, goat milk has been noted for having fewer allergenic characteristics than cow milk, with an as1-casein protein concentration of 89 percent lower [5,9]. When compared to cow milk, goat milk contains less pesticides and has a different microbial composition, making it a healthier alternative [5].

Despite the numerous health advantages of goat milk, only a few research have looked at its possible anti-proliferative properties. Nandhini & Palaniswany [10] previously proposed that goat milk hydrolysates fermented by Lactobacillus plantarum and Lactobacillus paracasei might be a more promising dietary component for cancer prevention. Rahmat et al. [11] discovered that giving goat milk to rats reduced the activity of tumor-marker enzymes during hepatocarcino-genesis.

Shariatikia et al. [12] found that goat milk had no cytotoxic action on the MCF-7 cancer cell line, but they did find a strong link between anti-cancer activity of milk caseins and their physicochemical characteristics. Because more research is needed to fully understand the study of goat milk, the efficacy of fresh and pasteurized goat milk has yet to be shown clinically or experimentally. As a result, the goal of this study was to see if fresh and pasteurized goat milk had an anti-proliferative impact on colon cancer cell lines HT-29, HCT-116, and CT-26.

2. MATERIALS AND METHODS

2.1 Sample Preparation of Goat Milk

The goat milk used in this study was from *Saanen* goat that was obtained from Ladang Pasir Akar, Universiti Sultan Zainal Abidin (UniSZA) at Kampus Besut, Terengganu. A total of 1.5 L of milk was collected from a healthy female goat from the farm in a seamless steel bucket. The collected milk then were filtered to remove dirt such as grass and bugs using milk filter and a stainless steel strainer. Then, were stored into the glass jar and at 4^{o}C in Food Biotechnology Lab in Kampus Besut, Terengganu prior to analysis.

A total of 250 mL milk was pasteurized at 65°C for 30 minutes and then rapidly cooled for 4 seconds [13] before being spray dried to generate a powdered pasteurized goat milk

sample. At temperatures of 150°C (inlet) and 70°C (outlet), the spray drying procedure followed Ian and Richard's approach [14]. Then, a total of 250 mL of milk was spray dried to create a powdered sample of fresh goat milk. Fresh and pasteurized goat milk powder samples were prepared in triplicates and kept at -20°C until utilized for analysis.

The extraction yield (Y) was calculated using the formula below [15].

Y (%) = W2/W1 * 100%

Where W1 = the original weight of the sample (250 mL), and W2 = weight of the powder form.

2.2 *In-vitro* Study

2.2.1 Preparation of goat milk

The method depicted by Khalili et al., [16] was used in this study with several adjustments. A total of 10 mg goat milk samples were dissolved in 1 mL of DMSO to prepare a stock solution of 10 mg/mL. The sample solutions were kept at 4^0 C prior use. The stock solutions for each sample was further diluted in completed RPMI-1640 media added with foetal bovine serum (10%) and penicillin-streptomycin (1%) to obtain a working solution of 1 mg/mL for treated into the colon cancer cell lines.

2.2.2 Cell maintenance 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 added with foetal bovine serum of 10% and penicillin-streptomycin of 1% at 37°C in an incubator humidified with 5% CO_2 and relative humidity of 95%. The cell media was replaced twice weekly to replenish the nutrients required for cell growth.

2.2.3 Anti-proliferative assay

Anti-proliferative effects by goat milk samples were evaluated using a colorimetric micro-titration method known as the tetrazolium salt reduction method or MTT assay [17]. The cells were harvested from the media, counted using a haemocytometer, and further diluted in a completed RPMI-1640 medium (added with 10% foetal bovine serum and 1% penicillin-streptomycin). A total of 100 µL cell suspension was seeded in triplicates using 96-well culture plates (SPL Life Sciences, Korea) at an optimized density of 1.0 x 10^5 cells/mL for each cell line. After 24 hours, triplicate serial dilutions of goat milk samples (1.0 - 0.0313 mg/mL) [18] and doxorubicin as positive 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 (negative control). After a 72-hour incubation period, 20 µL of MTT (Fisher Scientific, USA) (5 µg/mL) 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 micro-plate 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: [A_{570} of treated cells] / [A_{570} of control cells] x 100% and calculated to depend on the non-linear regression of the response curves within the same region.

2.2.4 Cell morphology observation

The effects of goat milk samples on the cellular morphological changes were determined using the method by Merlin et al. [19]. In this method, the effective dosage concentration of the sample 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 20x [20].

2.3 Statistical Analysis

Both descriptive and inferential statistical analyses were used to analyze the data. The software Statistical Package for Social Sciences (SPSS, version 20.0, IBM, Armonk, USA) was used to perform two-tailed tests at the significance level of 0.05.

3. RESULTS AND DISCUSSION

3.1 Extraction Yield of Goat Milk Samples

Table 1 shows the yield of fresh and pasteurized goat milk samples. Result shows that fresh goat milk sample gave the higher extraction yield of 9.4%, while pasteurized goat milk sample gave 7.15% extraction yield ($p<0.05$).

Table 1. Extraction yield of fresh and pasteurized goat milk

Samples	Original Weight (mL)	Weight of Dried Extract (g)	Extraction Yield (%)	p value
Fresh	250.00 ± 0.0	23.51 ± 0.04	9.40 ± 0.02	0.65
Pasteurized	250.00 ± 0.0	17.89 ± 0.03	7.17 ± 0.02	

Data are expressed as mean ± standard deviation
Values shown are means of 3 independent experiments
Post hoc analysis: the extraction yield is statistically not different from each other

Table 2. Anti-proliferative effect of goat milk samples towards HCT-116, HT-29 and CT-26 cells

Samples	IC50 ±SD (mg/mL)		
	HCT-116	HT-29	CT-26
Fresh	0.28 ± 0.03	0.34 ± 0.04	0.32 ± 0.10
Pasteurized	0.32 ± 0.02	0.34 ± 0.04	0.52 ± 0.10

Data are expressed as mean ± standard deviation

Both fresh and pasteurized goat milk samples were spray dried to generate powder in this investigation. Due to its connection to the drying chamber's wall and exhaust air exposed to an intake temperature of 150°C and an exit temperature of 70°C, the sample will reduced in volume during spray drying [14]. Because the fresh goat milk sample only went through one heat treatment, the higher output was predicted (spray drying). On the other hand, pasteurized goat milk had a reduced yield due to the additional heat treatments (pasteurization and spray drying). Aside from the spray drying process, pasteurization reduces the volume of the milk owing to the high temperature [21]. The milk is often sprayed on surfaces and fixtures above the milk level during the pasteurizer's vat filling, including the bottom of the vat cover [22]. The milk is continually spilling over while the pasteurization process continues, resulting in a decrease in milk volume.

3.2 Anti-proliferative Effect of Goat Milk Samples

In Table 2, the anti-proliferative activities of goat milk samples on HT-29, HCT-116 and CT-26 cells were assessed using MTT assay. Fresh and pasteurized goat milk samples gives high anti-proliferative effect with IC_{50} 0.28 ± 0.03 mg/mL and 0.32 ± 0.02 mg/mL to HCT-116 cells, respectively. Meanwhile, low anti-proliferative effect was observed on HT-29 cells (0.34 ± 0.04) by fresh sample and CT-26 cells (0.52 ± 0.10) by pasteurized sample. The 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 ($p<0.05$). Thoroughly, fresh goat milk sample was more promising as an anti-proliferation agent towards colon cancer cells.

Both samples of goat milk exhibited a possible anti-proliferative impact against colon cancer cell lines, according to the study. Fatty acid (FA) in milk fat has been proven in animal experiments to decrease the occurrence of chemically induced cancers [23]. Some functional FA in goat milk, such as short chain FA (SCFA) (e.g. butyric acid, caproic acid, caprylic acid), mono-unsaturated FA (MUFA), and poly-unsaturated FA (PUFA) (e.g. conjugated linoleic acids, linolenic acid), have been studied due to reported positive outcomes against various cancer risks [24,25]. Butyric acid is a SCFA that is a significant regulator of genetic control in colonocyte proliferation and death, as well as immune-regulatory and anti-inflammatory actions [26,27], prompting a number of researchers to focus on this mechanism in the hopes of lowering colon cancer risk [28].

Amoolya et al. [29], on the other hand, looked at the capacity of capric, caprylic, and caproic acid to kill HCT-116, A-431, and CCD-33Co cells. The results showed that these three FA decreased cell viability by 70% to 90% ($p<0.05$), implying that they had anticancer effects via down-regulating cell-cycle regulatory genes and up-regulating apoptosis-related genes. Conjugated linoleic acids (CLA) found in dairy fat have also piqued the interest of researchers interested in cancer prevention [30,31].

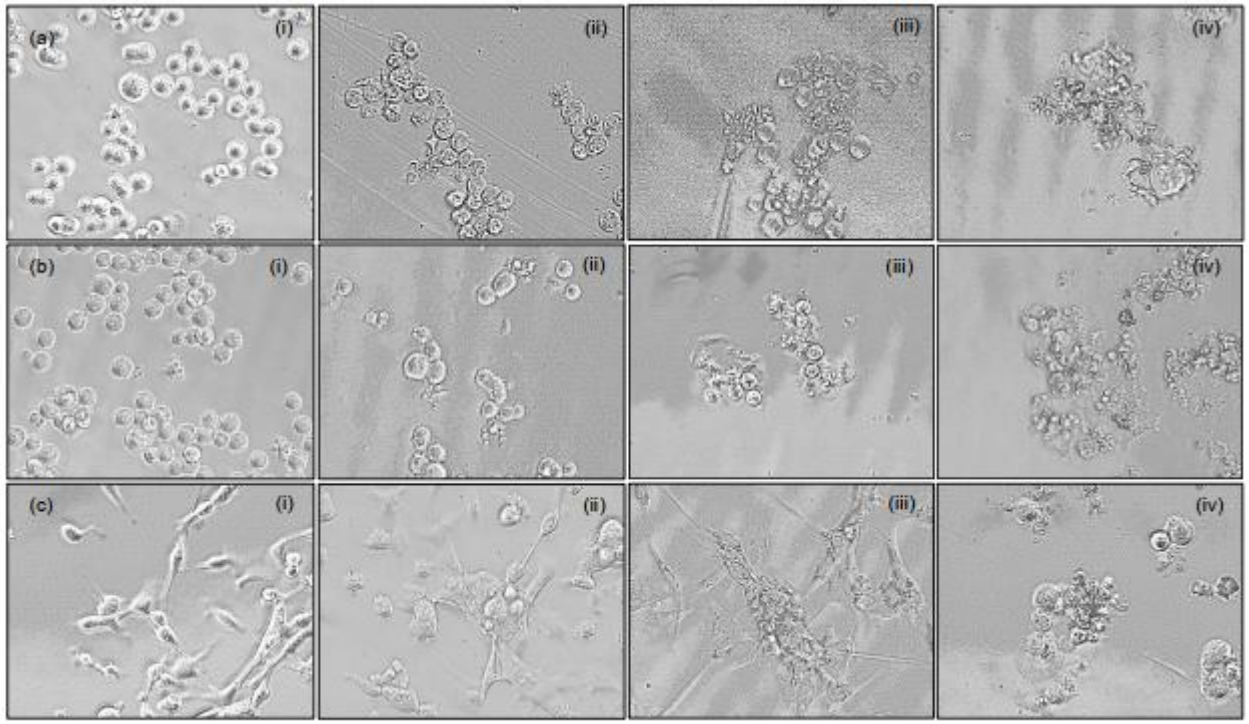

Fig. 1. Inhibition of (a) HT-29, (b) HCT-116 and (c) CT-26 by fresh goat milk for 3 days. Cell morphology of HT-29, HCT-116 and CT-26 were examined after being treated with IC_{50} at (i) 0 hour, (ii) 24 hours (iii) 48 hours and (v) 72 hours. The photographs were taken at 20x magnification with inverted microscope (Nikon, Japan)

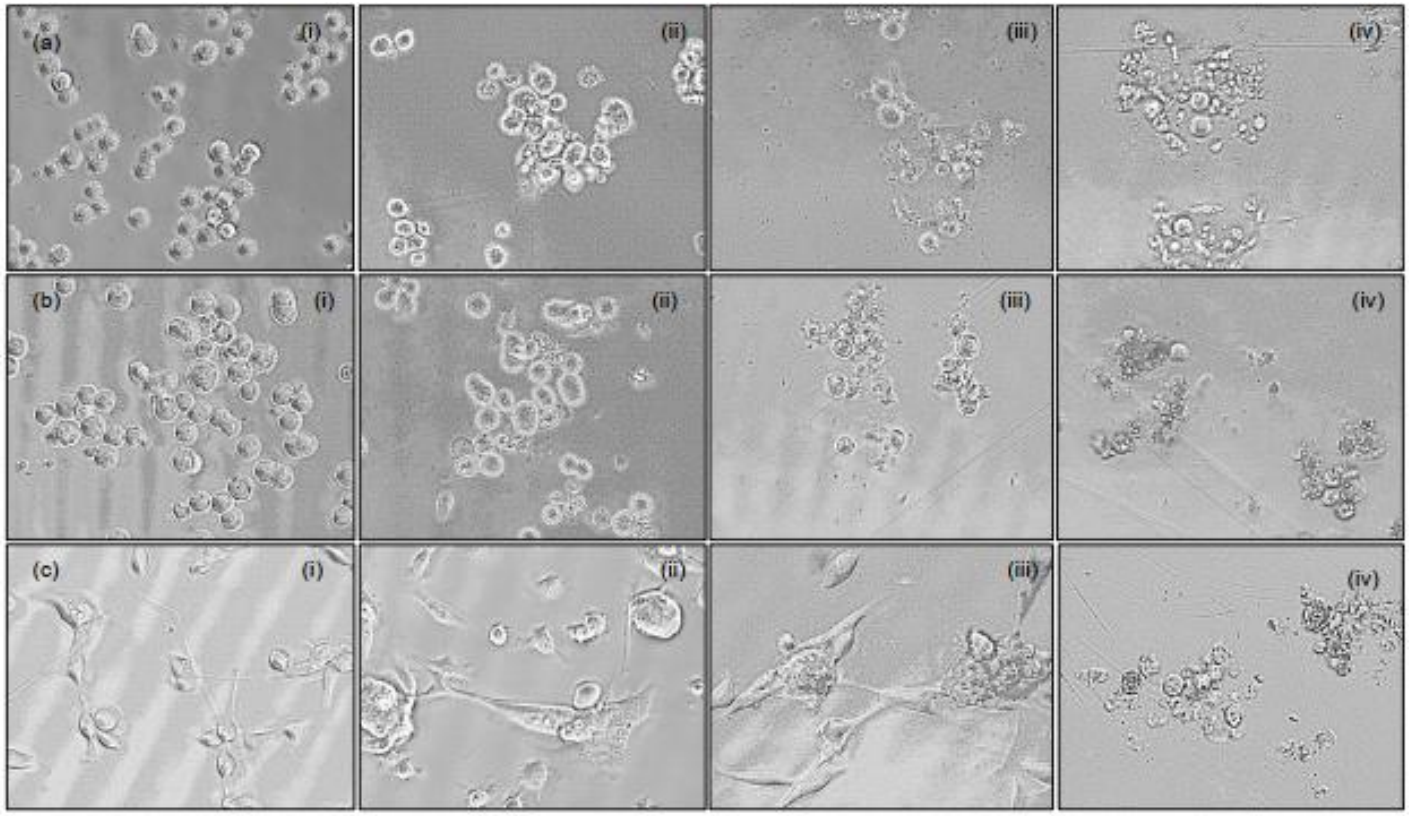

Fig. 2. Inhibition of (a) HT-29, (b) HCT-116 and (c) CT-26 by pasteurized goat milk for 3 days. Cell morphology of HT-29, HCT-116 and CT-26 were examined after being treated with IC_{50} at (i) 0 hour, (ii) 24 hours (iii) 48 hours and (v) 72 hours. The photographs were taken at 20x magnification with inverted microscope (Nikon, Japan)

CLAs have been shown to increase the production of tumor suppressive proteins such as p53, p27, and p21, which disrupt the cell cycle's G1-S phase [32]. Cho et al. [33] and Kim et al. [34] found that CLAs and its isomer inhibited cell growth in the DU-145 and HCT-116 cell lines, respectively. Rahmat et al. [11] also suggested that goat milk might lessen the severity of carcinogenesis, as evidenced by a decrease in the activity of plasma tumor marker enzymes glutamyl transpeptidase and alkaline phosphatase during rat hepatocarcinogenesis.

3.3 Cell Morphology Observation on Colon Cancer Cells by Goat Milk Samples

The treated cells of HT-29, HCT-116 and CT-26 with IC_{50} values goat milk samples were incubated for 72 h and the morphological changes effect were compared with the untreated cells, as been depicted in Fig 1 and 2 below. 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. Based on Latifah et al. [35], apoptotic changes of cells can be observed by characterization of nuclear condensation, reduction of cell volume, cells appeared condensed and round off, membrane blebbing, nuclear fragmentation, and detachment of cells from the surface, and presence of apoptotic bodies.

At 24 h, treated HT-29, HCT-116 and CT-26 with goat milk samples observed cells became condensed and shrink in size and some of the cells undergone partial detachment from the surface and there was a decline in the cells volume due to round off the cells [Fig. 1, 2 – (a, ii) (b, ii) (c, ii)]. Then at 48 h, there were presence of membrane blebbing (cytoplasmic protrusions) with some small cytoplasmic package (also called apoptotic bodies) and some of the organelles became too compact and appeared very condensed [Fig. 1, 2 – (a, iii) (b, iii) (c, iii)]. During 72 hours, the cells became lobulated due to loss of normal cells shape, cells fragmentation, lot of debris formation, some of the cell lysed, formation of apoptotic bodies, and some of the cells were crescent shaped indicating final stage of apoptosis [Fig. 1, 2 – (a, iv) (b, iv) (c, iv)].

4. CONCLUSION

Overall, the fresh sample goat milk gave the higher yield (9.40%) and followed by pasteurized sample goat milk (7.17%). Meanwhile, the fresh sample yielded the most potent cytotoxic value (0.28 ± 0.03), followed by pasteurized sample with value IC_{50} 0.32 ± 0.02 against HCT-116 cells. The most cytotoxic cells for goat milk samples was HCT-116 cells, while the least cytotoxic cell was CT-26 cells. Thoroughly, the anti-proliferative effect had been follows with cell morphological changes which are characterized by reduction of cell volume, dense of cell membrane, presence of apoptotic bodies and cell debris by 24, 48 and 72 hours. The results obtained proposed that goat milk may work as potential anti-cancer agents although further studies are required to explicate this aspect.

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.

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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