Research Article

Anti proliferative Effect of *Lignosus rhinocerus* extract on Colorectal Cancer Cells via Apoptosis and Cell Cycle Arrest

***Suziana Zaila Che Fauzi[1], Nor Fadilah Rajab[2,3], Lek Mun Leong[2], Kok Lun Pang[2], Norfazlina Mohd Nawi[1], Nurshahirah Nasir[1], Florinsiah Lorin[1], Farida Zuraina Mohd Yusof[1].**

[1]Faculty of Applied Sciences, Universiti Teknologi, MARA Shah Alam Selangor, Malaysia.

[2]Biomedical Science Programme, School of Diagnostic and Applied Health Science, Faculty of Health Sci, Universiti Kebangsaan, Kuala Lumpur, Malaysia.

[3]Toxicology Laboratory, Faculty of Health Sciences, Universiti Kebangsaan, Jalan Raja Muda Abdul Aziz, Kuala Lumpur, Malaysia.

***Corresponding author's E-mail:** zsuziana@gmail.com

Accepted on: 13-03-2015; **Finalized on:** 30-06-2015.

ABSTRACT

Lignosus rhinocerus, locally known in Malaysia as *cendawan susu rimau* or tiger milk mushroom, belongs to Polyporaceae family. The mushroom is traditionally used in Malaysia to improve health status as well as cure for cough, asthma, fever, chronic hepatitis and cancer. In this study, pressurized liquid extraction was performed to *L. rhinocerus* and cellular effects of *L. rhinocerus* methanol extract(*LRME*)and aqueous extract (*LRAE*)were assessed in HCT 116 human colorectal carcinoma cells. *LRME* exerted higher cytotoxic activity (IC_{50}: 600 µg/ml) compared to *LRAE* (IC_{50}: 1200 µg/ml) in HCT 116 cells as determined by MTT assay. Interestingly, both extracts did not exert cytotoxic activity on CCD-18co human normal colon fibroblast cells and V79-4 Chinese hamster lung fibroblast cells (IC_{50}> 2000 µg/ml). *LRME* and *LRAE* effect on cell cycle and mode of cell death were then investigated in HCT 116 cells as targeted cells. The mode of cell death induced by *LRME and LRAE* was primarily apoptosis as assessed by Annexin V-FITC/PI dual staining. Under low dose, both extracts arrested HCT 116 cells at G_0/G_1 phases with corresponding decrease of S-phase population. In conclusion, this is the first report of *Lignosus rhinocerus* possible mechanism of inducing anti-proliferation on HCT 116 cells.

Keywords: *Lignosus rhinocerus*; pressurized liquid extraction; apoptosis; HCT 116 cells; tiger milk mushroom.

INTRODUCTION

Lignosus rhinocerus (Tiger milk mushroom) is a type of mushroom native in tropical regions such as South China and Southeast Asia[1]. It is also known as *cendawan susu rimau* (in Malay) and regarded as important local medicine by indigenous communities of Malaysia[2]. The sclerotium of this mushroom is believed to posses medicinal properties. It had been used as a cure for fever, cough, asthma, food poisoning, liver related illness, cancer and as general tonic[2-5]. *L. rhinocerus* had been demonstrated to be safe up to 1000 mg/kg under subacute and subchronic toxicity testing[3,6]. It is also demonstrated to be non-genotoxic up to 50 mg/ml in reverse mutation Ames test[3]. Recent advancement in cultivation of *Lignosus rhinocerus* had shed light in overcoming the supply problem of *L. Rhinocerus*[4].

Colorectal cancer is common among developed and as well as developing countries. It ranked second in cancer related death worldwide and contributed 10-15 % of all forms of cancer[8]. A number of common drugs used for colorectal cancer included 5-flurouracil (5-FU), leucovorin, oxaliplatin and capecitabine[9]. Chemotherapy drugs like oxaliplatin and 5-flurouracil (5-FU) showed adverse side effects of peripheral neuropathy and leukopenia correspondingly[10-12]. To address these issues, there are increasing efforts done to investigate the potential of natural product in cancer treatment in hope to discover new classes of compound with better efficacy.

L. rhinocerus extract had been showed to exert cytotoxic effect against breast cancer cells and lung carcinoma cells with good selectivity compared to normal cells[1,13]. Previous studies performed aquoues extraction to *L. rhinocerus* and studied the cytotoxic action toward cancer cells[1,14]. In this study, we intend to compare the potency of *L. rhinocerus* extracted with different solvent systems. This study also aimed to investigate the anti-proliferation mechanism of *L. rhinocerus* on HCT 116 human colorectal carcinoma cells *in vitro*.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma (USA) excepted stated otherwise.

Sample Collection

Samples of wild *L. rhinocerus* were obtained from indigenous people in Sungai Perak (geographical coordinate: 100° 51' 50.70" E, 4°00' 46.56" N) and identified by Dr. Liew Gee Moi (Universiti Teknologi MARA, Samarahan Campus, Sarawak, Malaysia).

Species identification was made based on the morphological criteria and geographical origin as described by Dr. Lee Su See (Forest Health and Conservation Programme, Forest Biodiversity Division, Forest Research Institute Malaysia (FRIM), Malaysia).

Pressurized Liquid Extraction (PLE)

PLE was performed in a Dionex ASE 150 system (Dionex Corp., Sunnyvale, CA, USA) using two solvents under optimized condition.

Dried powder of *L. rhinocerus* sclerotium (approximately 15 g) was placed into a 66 ml stainless steel extraction

cell, which further extracted with 70% methanol and distilled water respectively (100 °C, 1.034 × 10^4 kPa, 15 min of static time for 1 cycle). Extracts were then purged out by nitrogen and transferred into a 25 ml volumetric flask, which were then filled up to its volume with the same solvent. Freeze dry was performed for 72 hours to completely lyophilize the extracts. Both *L. rhinocerus* methanol extract (*LRME*) and *L. rhinocerus* aquoues extract (*LRAE*) were stored at 4°C for prior usage.

Cell Culture

HCT 116 human colorectal carcinoma cells, CCD-18co human normal colon fibroblast cells and V79-4 Chinese hamster lung fibroblast cells were obtained from American Type Culture Collection (ATCC). HCT 116, CCD-80co and V79-4 cells were maintained in McCoy's 5A medium, Eagle's minimal essential medium (EMEM) and Dulbecco's modified Eagle's medium (DMEM) respectively. All mediums were supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin Streptomycin. All cultures were maintained under controlled condition (37°C, humidified air with 5% carbon dioxide). Testing was performed on cells with 70 – 80% confluent.

Anti-proliferation Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to determine anti-proliferative activities of *LRME* and *LRAE in vitro* as described by Yen[15]. In brief, 5 × 10^3 HCT 116, CCD-18co and V79-4 cells were plated in each well under 96 well plates and incubated overnight for cell attachment. Cells were then treated with *LRME* and *LRAE* at various concentrations (0 – 2000 µg/ml) for 24 hours. After incubation period, MTT solution (5 mg/ml) was added to the plate at the final concentration of 0.5 mg/ml and further incubated for 4 hours. The resulting formazan was dissolved by adding 200 µl of DMSO. Absorbance of wells was measured at wavelength 570 nm using ELISA plate reader (Bio-Rad, USA).

Cell Cycle Analysis

HCT 116 cells were seeded and treated with 500, 1000 and 2000 µg/ml *of LRME and LRAE* for 24 hours. Following treatment, cells were trypsinized and fixed overnight with 70% ethanol.

After fixation, ethanol was discarded and cells were washed twice with phosphate buffer solution (PBS) before incubated in staining solution [propidium iodide (50 µg/ml), sodium citrate (0.1%), Triton X-100 (0.1%) and DNase-free RNase (20 µg/ml)] for 30 min at room temperature.

Stained cells were then analyzed for DNA content using FAS Canto II flow cytometer (BD Bioscience, USA) installed with Modfit LT (Verity Software House).

Apoptosis Assessment

Annexin V-FITC/PI staining was performed to investigate the mode of cell death induced by *LRME* and *LRAE* in HCT 116 cells[16]. In brief, HCT 116 cells were seeded and treated with *LRME* and *LRAE* for various concentrations (500, 1000 and 2000 µg/ml). Following 24 hours treatment, cells were collected via trypsinization and washed twice with chilled PBS. Thereafter, cells were suspended in annexin binding buffer at a concentration of 1 × 10^6 cells/ml. One hundred microliter of cell suspension was transferred to polystyrene round bottom tube. Cells were then stained by adding 5 µl of Annexin V-FITC (BD Bioscience, USA).

After 15 minutes incubation at room temperature in the dark, 5 µl of propidium iodide were added and further incubated for 2 minutes. Four hundred microliter annexin binding buffer was added to each tube and cells were analysed using FACS Canto II flow cytometer (BD Bioscience, USA) within 1 hour.

Statistical Analysis

Results were expressed as mean ± standard error mean (SEM). Experimental groups and control group were subjected to statistical analysis using one-way ANOVA. Difference was considered significant at $p< 0.05$.

RESULTS AND DISCUSSION

LRME and LRAE exerted anti-proliferative effect on HCT 116 cells

We evaluated anti-proliferative effect of *LRME* and *LRAE* on HCT 116 human colorectal carcinoma cells, CCD-18co human normal colon fibroblast cells and V79-4 Chinese hamster lung fibroblast cells.

Various concentrations of *LRME* and *LRAE* were added to cells for 24 hours incubation and cell viability was evaluated using MTT assay.

Figure 1 demonstrates cell viability curve of three cell lines treated with both extracts. Both *LRME* and *LRAE* exhibited cytotoxic activity on HCT 116 cells with IC_{50} value 600 ± 0.09 µg/ml and 1200 ± 0.05 µg/ml respectively. Interestingly, both extracts did not exert anti-proliferative activity on normal cell lines such as CCD-18co and V79-4 up to 2000 µg/ml.

Table 1: Inhibitory concentration (IC_{50}) of LRME and LRAE on HCT 116 human colorectal carcinoma cells, CCD-18co human normal colon fibroblast cells and V79-4 Chinese hamster lung fibroblast cells. Cells were treated with various concentrations of extracts for 24 hours. Cytotoxicity activity was evaluated using MTT assay.

Cell lines	IC_{50} value (µg/ml)	
	LRME	*LRAE*
Cancerous cell model		
HCT 116	600 ± 0.09	1200 ± 0.05
Normal cell models		
CCD-18Co	>2000	>2000
V79-4	>2000	>2000

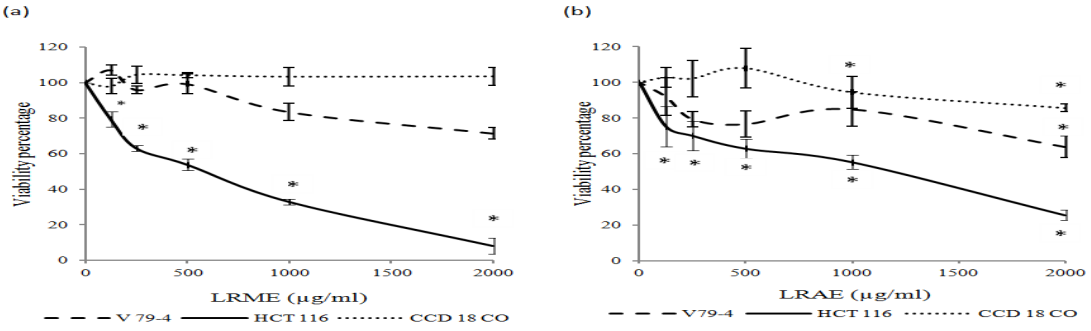
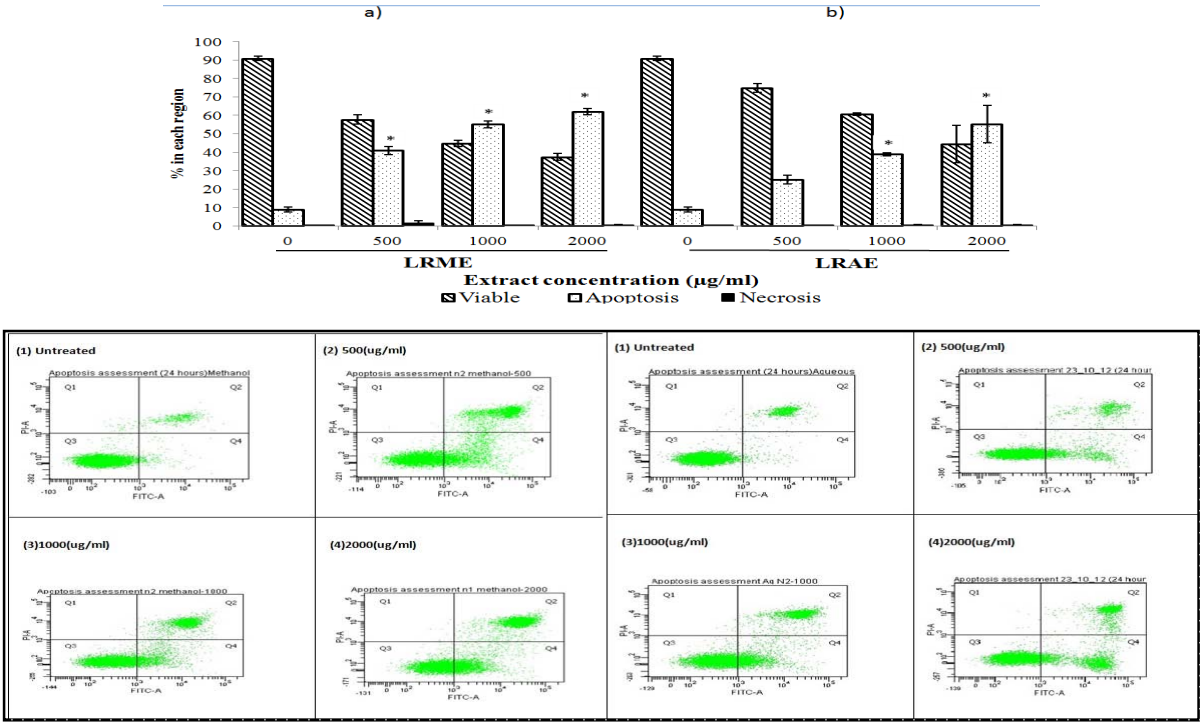
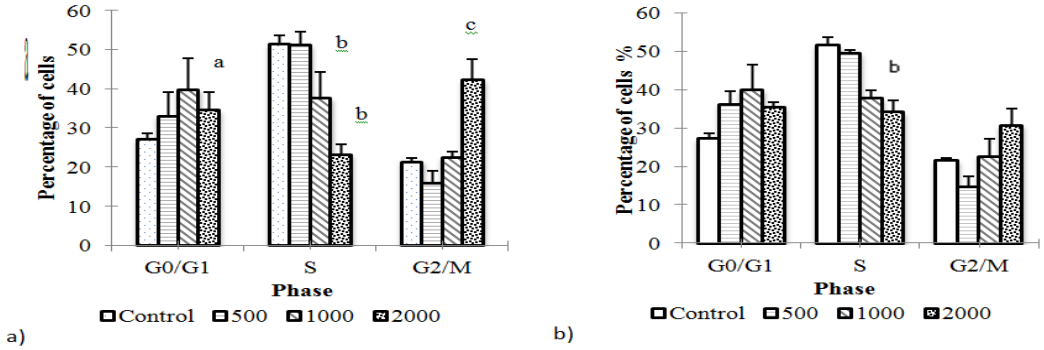


Figure 1: Shows cytotoxic activity of (a) *LRME* (b) *LRAE* on various cell lines. Data were shown as mean ± S.E.M. *significantly different compare to control group (p<0.05).



* Significantly different compare to control group (p<0.05).

Figure 2: (a) shows flow cytometry analysis of mode of cell death induced by *LRME* and *LRAE* in HCT 116 human colorectal carcinoma cells. Representative Cytograms for (b) *LRME* and (c) *LRAE* were shown. Data were represented as mean ± S.E.M.



[a]significantly different compare to G_0/G_1 phase of control group

[b]significantly different compare to S phase of control group

[c]siginificantly different compare to G_2/M phase of control group

Figure 3: Shows distribition of cells in cell cycle of HCT 116 colon cancer cells treated with (a) *LRME* (b) *LRAE*. After 24 hours treatment of *LRME* and *LRAE*, cell cycle distribution was evaluated using flow cytometer. Data were shown as mean ± S.E.M.

LRME and _LRAE_ induced apoptosis in dose-dependent manner.

Apoptosis assessment was carried out using Annexin V-FITC/PI dual staining method to investigate the mode of cell death induced by *LRME* and *LRAE*. As shown in Figure 2, both *LRME* and *LRAE* induced apoptosis in HCT 116 cells. The increase of apoptotic population occurred in dose dependent manner.

Low doses _LRME_ and _LRAE_ arrested HCT 116 cells at G_0/G_1 phase.

As shown in Figures 3, control group contained 27.13 ± 1.45% cell population in G_2/M phase. Treatment with 500 µg/ml LRME and LRAE increased cell population in G_0/G_1 phase to 32.92 ± 6.25% and 36.07 ± 3.60% respectively ($p< 0.05$). Increase of cell population at G_2/G_1 phase were accompanied by corresponding reduction in the percentages of cells in S phase as shown in Figure 2 ($p< 0.05$).

DISCUSSION

Previous studies demonstrated cytotoxicity of *L. rhinocerus* cold water extract toward A549 human lung carcinoma cells, HepG_2 human hepatocellular carcinoma cells, HL-60 human acute promyelocytic cells, HCT 116 human colorectal carcinoma cells, PC-3 human prostate cancer cells, HSC2 human squamous carcinoma cells and HK1 human nasopharyngeal carcinoma cells[1, 14]. However, *L. rhinocerus* hot water extract was demonstrated not to possess cytotoxic activity on these cell lines up to 500 µg/ml[14]. On the other hand, study by Lai et al. (2008) demonstrated *Polyporus rhinocerus* hot aquoues extract cytotoxic activity on HL-60 cells, K562 human chronic myelogenous leukemic cells and THP-1 human acute monocytic leukemic cells[13].

In our study, we employed liquid pressurized extraction (PLE) which applied heat to plant samples during extraction, making *LRAE* a type of hot aquoues extract. Concurrently, we extrapolated the dose in anti-proliferative assay up to 2000 µg/ml. Our data demonstrated *L. rhinocerus* aqueous extract (*LRAE*) exerted cytotoxic activity on HCT 116 cells with IC_{50} value 1200 µg/ml. Both *LRAE* and *LRME* did not exert prominent cytotoxicity on CCD-18co and V79 normal cell lines. This is in agreement with previous findings stating *L. rhinocerus* hot water extract did not exert cytotoxicity activity normal cell lines such as WRL-68 human embryonic liver cells and MRC-5 human lung fibroblast cells[14]. *L. rhinocerus* cold water extract was also proven not to show cytotoxicity on normal cells 184B5 (human breast cells) and NL 20 (human lung cells)[1]. A good candidate of anti-cancer agent should be selective with regards to its cellular toxicity. PLE extraction method produced extracts with selective cytotoxic activity toward colon cancer cells thus highlighted this method as a better extraction method compare to maceration. However, both LRME and LRAE are not considered as strong cytotoxic agent on the basis of high IC_{50} value (< 20 µg/ml) according to the criteria set by National Cancer Institute, USA[20].

Apoptosis is a type of programmed cell death genrally characterized as by distinct morphological characteristic such as cell shrinkage, pyknosis, membrane blebbing etc[17]. It is belived that the cytotoxic action of many anti cancer agents is meadiated by apoptosis[1]. Previous studies demonstrated cytotoxic action of *L. rhinocerus* extract though apoptosis in HL-60, MCF-7 and A549 cells[1,13]. Our study is the first report to demonstrate LRME and LRAE's ability to induce apoptosis in HCT 116 cells. Our data also demonstrated that under low dose, LRAE increased cell population at G_0/G1 check point. This finding is parallel with Lai et al. (2008) study showing *Polyporus rhinocerus* Cooke ability to induce G_0/G_1 cell cycle arrest in HL-60 human acute promyelocytic leukemic cells[2,13].

In our study, we demonstrated that LRME imposed higher cytotoxic effect compared to LRAE, suggesting that methanol might serve as a better solvent system to isolate cytotoxic compound from*L. rhinocerus.*Cytotoxic compound present in both extract might be same, but suspected to be more abundant in methanol solvent. Previous finding documented high level of protein content in *L. rhinocerus* extract[1,14]. Interestingly, upon heating, the protein profile changes was accompanied by loss of cytotoxic activity[14]. Hence, Lau et al. (2013) speculated that compounds responsible for cytotoxic activity were therma-labile proteins. In PLE method, heat was applied to the raw material during processing. Hence, introduction of heat during PLE method explain the reason why both LRME and LRAE possess high IC_{50} value. Our data suggested that cold extraction is better than hot extraction method in isolating cytotoxic compound in *L. rhinocerus*.

CONCLUSION

The above data suggested that pressurized liquid extraction (PLE) extract of the sclerotia of *L. rhinocerus* possesses cytotoxicity to human colorectal cancer cells but were non-toxic to the corresponding normal cells. Anti-proliferative effect of *L. rhinocerus* is mediated by apoptosis and cell cycle arrest.

Acknowledgement: We thank Universiti Teknologi MARA and Ministry of Science, Technology and Innovation (MOSTI), Malaysia and Research Management Institute, RMI for providing research grant. The authors also express their sincere appreciations and gratitude to Dr Lee Su See from Forest Health and Conservation Programme, Forest Biodiversity Division, Forest Research Institute Malaysia (FRIM), Malaysia. We also thank Centre of Research and Instrument (CRIM), UKM for providing flow cytometry facilities.

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Source of Support: Nil, **Conflict of Interest:** None.