

Antiproliferative Effect of *Lignosus rhinocerotis*, the Tiger Milk Mushroom on HCT 116 Human Colorectal Cancer Cells

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Abstract: Tiger Milk mushroom is a unique mushroom that has number of medicinal properties. Communities in Malaysia have been using this mushroom to treat different kinds of cancer. This study was conducted to elucidate the antiproliferation activity of this national treasure mushroom on human colorectal cancer cells (HCT 116). Colon cancer cells were cultured in the presence of *L. rhinocerotis* for aqueous or methanol pressurized liquid extraction (PLE) at various concentrations for 24 hours. The percentage of cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-di phenyl tetrazolium bromide (MTT) assay. Our results showed that methanol PLE of *Lignosus rhinocerotis* sclerotia was found to exhibit antiproliferative activity against HCT 116 cells, with an IC₅₀ of 600 µg/mL and 1200 µg/mL for aqueous PLE. In comparison, both extractions did not show significant cytotoxicity against normal human colon cell (CCD-18CO). The IC₅₀ values suggested that the solvent differences in pressurized liquid extraction greatly affected their antiproliferative activities. Additionally, weak antioxidant capacities were detected in both methanol and aqueous PLE of *L. rhinocerotis* by the FRAP (Ferric Reducing Antioxidant Power) Assay. However, this capacity was not significant when the aqueous extract was tested in the presence of HCT 116 cells. Preliminary phytochemical screening revealed that both methanol and aqueous pressurized liquid extraction of *L. rhinocerotis* contained alkaloids.

Keywords: Human Colorectal Cancer Cells, Pressurized liquid extraction, MTT, Antiproliferation activity, Ferric reducing ability of plasma.

1. INTRODUCTION

CANCERS of the digestive system can be potentially life-threatening. In Malaysia, colorectal cancer (cancer of the colon and rectum) makes up more than 12% of the total number of recorded cancers in Malaysia [1]. Although surgical resection is curative in early stage disease, currently used chemotherapies for advanced colon cancer are mainly palliative [2]. Drug toxicity and the development of resistance to chemotherapeutic agents are also potential obstacles to the successful treatment of malignancies. More effective cancer-preventive and chemotherapeutic strategies for the management of colon cancer are therefore required. One promising approach involves the administration of dietary phytochemicals that possess cancer-preventative activity and growth inhibitory activity against cancer cells.

Tiger's milk fungus, is a novel mushroom with sclerotium belonging to the Polyporaceae family and has miraculous medicinal properties. Burkill (1966) documented that local communities use fungi for the treatment of a variety of ailments. He is also noted that the sclerotium of a *Lignosus* species ("cendawan susu harimau" or "susu rimau" in Malay language or tiger's milk fungus in English) was

used to treat consumption and coughs and this mushroom species are still used by the Malays, Chinese and indigenous communities in Malaysia [3]. For many years ago, it is used by indigenous communities as a general tonic, to treat cancer and swollen breast as well as medicinal treatments for food poisoning. Furthermore, the local malay communities have been using tiger milk mushroom to treat different kinds of cancer including stomach and breast cancer and as medication for swellings all over the body [4]. Previous studies have shown that the sclerotium of *L. rhinocerotis* is a traditional Chinese medicine in which there has been some preliminary data showing their effectiveness in treating liver cancer, chronic hepatitis and gastric ulcer [5].

Antioxidant agents are closely associated to the prevention of degenerative diseases, such as cardiovascular and neurological illnesses, oxidative stress malfunctions and cancer [6]. In this paper, the Ferric Reducing Ability of Plasma (FRAP) activity of some *L. rhinocerotis*, has been evaluated. This assay is simple and comparatively cheap. It utilizes the reducing potential of the antioxidants to react with a ferric tripyridyltriazine (FeIII-TPTZ) complex and produce a colored ferrous tripyridyltriazine (FeII-TPTZ) [7].

2. MATERIALS AND METHODS

2.1. *Lignosus rhinocerotis*

L. rhinocerotis (Tiger Milk mushroom) were collected from indigenous people in Sg. Perak. The sequence of pre-

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treatment for Tiger Milk mushroom extract include: Peeling the sclerotia, slicing, followed by drying and grinding prior to solvent extraction.

2.2. Pressurized Liquid Extraction

PLE was performed on a Dionex ASE 150 system (Dionex Corp., Sunnyvale, CA, USA) in two solvent under optimized conditions. Dried powder of *Lignosus rhinocerotis* sclerotium (~15 g) was placed into an 66 mL stainless steel extraction cell, then extracted with two different solvent 70% aqueous methanol and aqueous at 100 °C for 15 min of static time for 1 cycle with pressure at 1.034×10^4 kPa. The extract purged out by nitrogen was transferred into a 25 mL volumetric flask, which was made up to its volume with the same solvent. The extract will be completely lyophilized by continuous freeze drying operation for 72 hours, yielding a certain amount of crude extract. A dark semi-solid material will be stored at 4°C to be used later.

2.3. Cell Lines and Cell Culture Condition

The human colorectal cancer (HCT 116) and normal human colon cell (CCD-18CO) cell lines were obtained from American Type Culture Collection (ATCC), Maryland, USA. HCT 116 cells were maintained in McCoy's 5A Medium (modified) (1X), and CCD-18CO cells were maintained in Eagle's minimal essential medium (EMEM) , McCoy's 5A Medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin Streptomycin while 2mM glutamine and 1% Non-Essential Amino Acids 10% FBS in EMEM. Both type of cells were maintained in 75 cm² flask at 37°C in a humidified atmosphere with 5% CO₂.

2.4. Cell Viability Assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Assay

For cell viability assay, 5×10^4 cells (HCT 116 and V79-4) per well were plated in 100 µl of McCoy's and DMEM media each. HCT 116 and CCD-18CO cells were incubated overnight at 37°C in humidified atmosphere of 5% CO₂ for cells attachment. Tiger milk mushroom extract was added at various concentrations ranging from 0, 125, 250, 500, 1000, and 2000 µg/mL after 24 h incubation. After 24 h incubation, MTT solution (2 mg/mL) was added to the plate at the final concentration of 0.5 mg/mL. The resulting MTT- products were determined by measuring the absorbance at 570 nm with ELISA reader. Each point represents the mean of triplicate experiments.

$$\% \text{ viability} = \frac{\text{Optical density of sample}}{\text{Optical density of control}} \times 100$$

2.5. Ferric Reducing/Antioxidant Power (FRAP) Assay

Briefly, the FRAP reagent was prepared by mixing acetate buffer (300 IM, pH 3.6), a solution of 10 IM TPTZ in 40 IM HCl, and 20 IM FeCl₃ at 10:1:1 (v/v/v). The reagent (175µl) and sample solutions (50 µl) were added to each well and mixed thoroughly. The absorbance was taken at 593 nm after 5 min. Standard curve was prepared using different concentrations of ascorbic. All solutions were used on the day of preparation. The results were corrected for dilution (e.g. to 1000 mL). The reducing ability of biological sample was determined by FRAP assay of Benzie and Strain (1996). FRAP assay was based on the reducing power of a

compound that has antioxidant properties. A potential antioxidant will reduce the ferric ion (Fe^{III}) to the ferrous ion (Fe^{II}) resulting a blue coloured Fe^{II}-tripyridyltriazine compound and the absorbance values were measured at 595 nm. The oxidised Fe^{III} color form by the action of electron donating antioxidants.

2.6. Phytochemical Screening

Phytochemical screening was performed on the extracts using standard procedures to identify chemical constituents as described by Trease and Evans (1989), Harborne (1973) and Sofowora (1993) [8-10].

Screening for Alkaloid

0.5 g of the extract was stirred in 5 ml of 1% HCl on a steam bath and filtered while hot. Distilled water was added to the residue and 1 ml of the filtrate was treated with a few drops of Wagner's reagent. A reddish brown precipitate indicates the presence of alkaloids.

Screening for Flavonoids

Two millilitres of dilute sodium hydroxide was added to 2 ml of the extract. The appearance of a yellow color indicates the presence of flavonoids.

Screening for Saponins

One milliliter of distilled water was added to 1 ml of the extract and shaken vigorously. A stable persistent froth indicated the presence of saponins.

Screening for Phenols

Equal volumes (1 ml) of extract and Iron (III) chloride were mixed. A deep bluish green solution gave an indication of the presence of phenols.

Screening for Tannins

A portion of the extract was dissolved in water, after which the solution was clarified by filtration. 10% ferric chloride solution was then added to the resulting filtrate. The appearance of a bluish black colour indicates the presence of tannins.

Screening for Anthraquinones

0.5 g of the extract was shaken with 10 ml of benzene and filtered. 10% of ammonia solution was added to filtrate and the mixture was shaken. The formation of a pink, red or violet color on the ammoniacal phase indicates the presence of anthraquinones.

Screening for Terpenoids

0.5 ml of acetic anhydride was mixed with 1 ml of sample extract and a few drops of concentrated H₂SO₄. A bluish green precipitate indicates the presence of terpenes.

3. RESULT

3.1. Cell Viability Assay: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Assay

To determine optimal conditions for cytotoxicity in cultures of HCT-116 colon cancer cells, cell viability at 24 hours was then assessed by MTT assay. The HCT-116 human colon cancer cells were treated with various

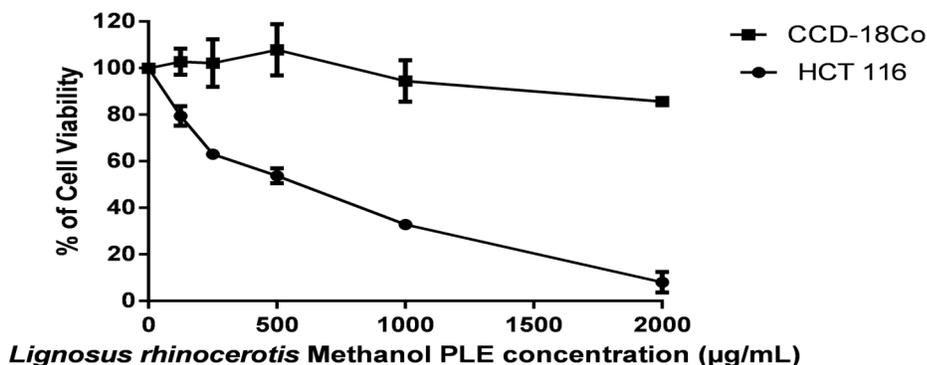


Fig. (1). Effect of Tiger Milk mushroom extract on the proliferation of HCT 116 & CCD-18Co cells. Cells were treated with different concentration of methanol PLE Tiger Milk mushroom PLE, incubated at 37°C and harvested after 24 hour. Cell viability was determined by the MTT assay. Data represent the mean ± S.E.M (n = 3).

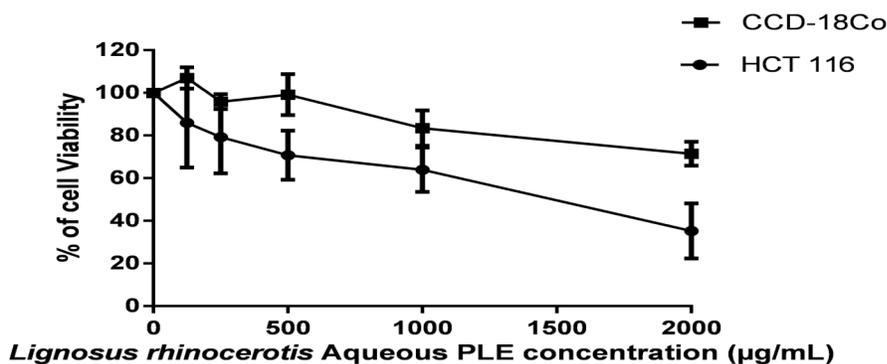


Fig. (2). Effect of Tiger Milk mushroom PLE on the proliferation of HCT 116 & CCD-18Co cells. Cells were treated with different concentration of aqueous PLE Tiger Milk mushroom PLE, incubated at 37°C and harvested after 24 hour. Cell viability was determined by the MTT assay. Data represent the mean ± S.E.M (n = 3).

concentrations of tiger milk mushroom PLE at different concentrations in 10% FBS.

Table 1. Cytotoxic Activities (IC₅₀) of Methanol Pressurized Liquid Extraction of Sclerotia of Tiger Milk Mushroom and Aqueous Pressurized Liquid Extraction. Treatment time was 24 Hours

Extracts	HCT 116 (µg/mL)	CCD-18Co (µg/mL)
Methanol pressurized liquid extraction	600 ± 0.09	ND
Aqueous pressurized liquid extraction	1200 ± 0.05	ND

ND= Not Determine

Table 2. Percentage Increase of FRAP Values of Methanol PLE. Each Value Represents the mean ± S.E.M. of Triplicate from 3 Different Experiment

Tiger Milk Mushroom ple (Methanol)(µg/mL)	Percentage Increase in Frap Value mean ± sem
0	0±0
125	6.95 ± 1.04
250	9.33 ± 1.78
500	28.31 ± 3.82

1000	41.34 ± 4.98
2000	76.59 ± 2.55

Table 3. Percentage Increase of frap Values of Aqueous PLE. Each value Represents the mean ± s.e.m. of Triplicate from 3 Different Experiment

Tiger Milk Mushroom ple (Aqueous)(µg/mL)	Percentage Increase in Frap value mean ± sem
0	0±0
125	4.55 ± 1.83
250	4.40± 2.63
500	12.87 ± 4.99
1000	25.48± 8.34
2000	48.91 ± 7.08

4. DISCUSSION

We have selected *L. rhinocerotis* in this study is to investigated the effects of *Lignosus rhinocerotis* sclerotium extracted by using PLE method on HCT 116 cell lines. Our results indicated that both aqueous and methanol PLE exhibit significant antiproliferative activity against human colorectal cancer cells HCT 116. The antiproliferative active against HCT 116 provides a basis for asserting scientific plausibility for traditional use of *L. rhinocerotis* in colon cancer treatment by Malaysian natives. Proliferation of HCT 116

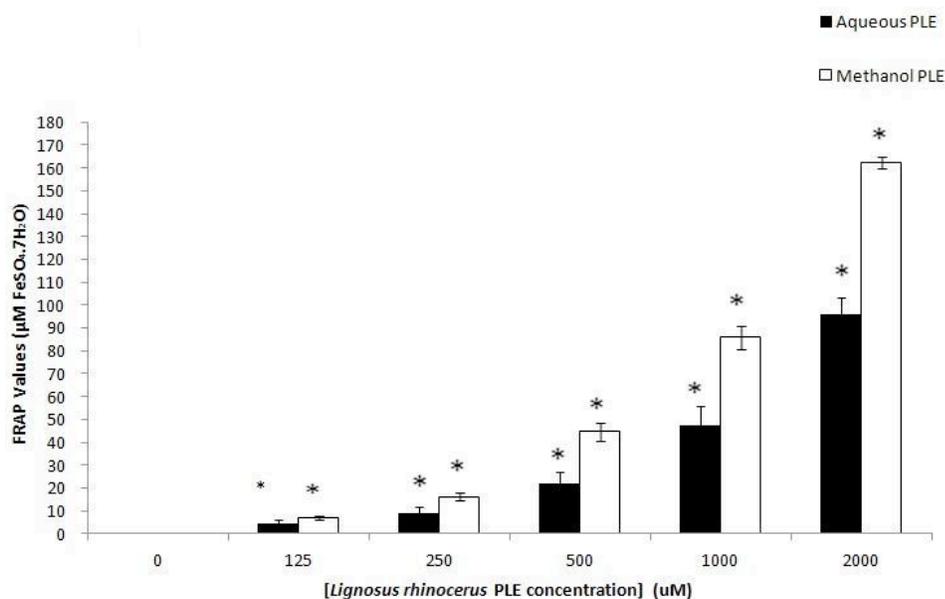


Fig. (3). FRAP values (Total Antioxidant Content) versus concentration of aqueous and methanol PLE graph. Note: * indicated significant differences ($p < 0.05$) between control and treatment groups. Data was expressed as mean \pm SEM ($n = 3$).

cells decreased when treated with *L. rhinocerotis* methanol PLE extract resulting in a 50% reduction at 600 ± 0.09 $\mu\text{g}/\text{mL}$, while for aqueous PLE extract showed significant decrease in proliferation of HCT 116 cells with 50% reduction at 1200 ± 0.05 $\mu\text{g}/\text{mL}$ of *L. rhinocerotis* extract (Table 1), suggesting that methanol PLE IC_{50} much more lower than aqueous PLE because many organic molecules are soluble in methanol. The presence of water in aqueous methanol (70:30 v/v) will almost certainly allow water soluble material in the mushroom to be extracted more readily that caused the IC_{50} value of methanol PLE lower than aqueous PLE. Statistical analysis indicated that both methanol and aqueous PLE possess significant ($*p < 0.05$) reduction in total cell numbers. Our results demonstrated that antiproliferative activity by *L. rhinocerotis* sclerotia was found to exhibit against HCT 116 cells were included in Fig. (1 and 2). The IC_{50} values suggested that the solvent differences in pressurized liquid extraction greatly affected their antiproliferative activities. Besides, *Lignosus rhinocerotis* powder was extracted using Accelerated Solvent Extraction (ASE 150) machine which is a single-cell system for the extraction of solid and semisolid samples at elevated

temperatures and pressures that runs faster than Soxhlet, sonication, and other extraction methods, besides requires less solvent and labor. PLE is also a rapid and effective method for sample preparation of carbohydrates [11, 12].

A recent study of mushroom sclerotium had indicated that various fractions of sclerotial polysaccharides including water-soluble polysaccharide-protein complex (PSP) and alkali-soluble β -glucan isolated from *L. rhinocerotis* possessed remarkable host-mediated antitumor activity on the Sarcoma 180 implanted BALB/c mice. Besides, polysaccharides isolated from the fruit bodies and mycelia of *L. rhinocerotis* have been found to exert immunomodulatory function and antitumor activity in numerous *in vivo* and *in vitro* studies [13]. The antiproliferative agent may either be a type of protein-carbohydrate complex and proteins that target metabolic processes or signal transduction in cancer cells [14].

Although there has been no research about the anti-tumor effects of *L. rhinocerotis* on the HCT-116 cell line, some research has already demonstrated the inhibitory effects of *L. rhinocerotis* on cancer cells. In the previous study shown that *L. rhinocerotis* inhibits both breast and lung cancer cell proliferation in a concentration-dependent and time-dependent manner both in 24 hour [15]. Extensive investigation over the past few years has confirmed that Tiger Milk mushroom has an anticarcinogenic and cancer-killing activity. A few articles also demonstrated the significant growth inhibition of *L. rhinocerotis* on various human leukemic cell lines including HL-60, K562 and THP-1. It is also revealed that the onset of apoptosis of the HL-60 cells by the cell cycle arrest at G1 phase using flow cytometric analysis [16]. Additionally, a recent study also discovered that *L. rhinocerotis* has an effective antimicrobial activity against the selected pathogenic bacteria and fungi which are responsible for wound [17].

The therapeutic value of any drugs depends not only on its clinical efficacy, but also in its lack of toxic side-effects

Table 4. Phytochemical Analysis of Pressurized Liquid Extraction of Tiger Milk Mushroom

Phytochemical	Methanol PLE	Aqueous PLE
Alkaloids	++++	++++
Triterpenoid/ Steroid	-	-
Saponin	-	-
Phenols	-	-
Antraquinones	-	-
Tannins	-	-
Flavonoids	-	-

+ = present, - = absent, ++++ highly present.

[18]. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damages to normal cells. In this investigation, MTT assay showed that the *L. rhinocerotis* methanol and aqueous PLE did not toxic to normal human colon cell CCD-18CO. In addition, previous studies have shown that there was no treatment-related sub-acute toxicity in rats following 28 days at the highest tested dose of 1,000 mg/kg of wild type *Lignosus rhinocerotis* sclerotial powder [19]. Earlier, Lee *et al.* also reported that cold water extraction of *L. rhinocerotis* did not show significant cytotoxicity against human normal cells, 184B5 (human breast cell) and NL 20 (human lung cell) [20]. These results indicate that the extracts are relatively safe to use for therapeutic purpose.

Whereas for the FRAP assay, the higher the concentration of *L. rhinocerotis* PLE extraction, the ability to reduce the ferric ion (Fe^{III}) to the ferrous ion (Fe^{II}) were increased (Table 2 and 3). The FRAP values of aqueous PLE were, however, much lower than the values for methanol PLE, suggested that the solvent differences in pressurized liquid extraction greatly affected their total FRAP values. The value of methanol/water (70:30 v/v), which was much higher than aqueous PLE demonstrated the influence of the solvent, as previously reported [21]. From statistical analysis, our results indicated that there is a significant different in the means of concentration among different concentration level. Also, there is a significant different in the means of concentration between different type of solvent that is aqueous and methanol (Fig. 3). However, the ferric reducing antioxidant power (FRAP) method is based on the reduction of a ferrioxal analogue, the Fe^{3+} complex of tripyridyltriazine $\text{Fe}(\text{TPTZ})^{3+}$ to the intensely blue-coloured Fe^{2+} complex $\text{Fe}(\text{TPTZ})^{2+}$ by antioxidants in acidic medium. However, the reducing capacity does not necessarily reflect antioxidant activity [22, 23]. FRAP values of both methanol and aqueous PLEs was significant as compared to the negative control but this value was low than L-ascorbic acid at the same concentration. This showed that *Lignosus rhinocerotis* PLEs had weak antioxidant capacities.

Preliminary phytochemical screening revealed that both methanol and aqueous pressurized liquid extraction of *L. rhinocerotis* contained alkaloids (Table 4). The highly trace of alkaloid might be responsible for the anticancer activity. The earlier studies stated that several alkaloids isolated from natural herbs exhibit antiproliferation and antimetastasis effects on various types of cancers both *in vitro* and *in vivo* [24]. One of the most common biological properties of alkaloids is their toxicity against cells of foreign organisms. These activities have been widely studied for their potential use in the elimination and reduction of human cancer cell lines [25]. Alkaloids which are one of the largest groups of phytochemicals in plants have amazing effects on humans and this has led to the development of powerful pain killer medications [26].

STATISTICAL ANALYSIS

Each experiment were repeated at least 3 times and the results were expressed as mean \pm S.E.M. The significance of difference between the experimental group and control was assessed by Student's *t* test. For the Frap assay the difference is considered significant if the *p* value is < 0.05 .

CONCLUSION

The above data suggested that pressurized liquid extraction (PLE) extract of the sclerotia of *L. rhinocerotis* possesses cytotoxicity to human colorectal cancer cells but were non-toxic to the corresponding normal cells. Here, we only presented the therapeutic results. Therefore, further study is suggested to investigate the mode of action and the mechanism of antitumor effects of tiger milk mushroom extract by evaluating apoptosis rate and cell cycle progression status in colon cancer cell lines HCT 116.

CONFLICT OF INTERESTS

To our knowledge, previous studies have shown the antitumor effect of *L. rhinocerotis* in other cancer cells but no report evidence on colon cancer cells. Thus, our study represent among the first to show the antiproliferative effect of *L. rhinocerotis* in colon cancer cells HCT 116.

ACKNOWLEDGEMENTS

The authors wish to thanks Universiti Teknologi MARA for providing research grant. Likewise, the authors are grateful with Universiti Kebangsaan Malaysia, KL for their kind cooperation and for granting permission to utilize their Biocompatibility & Toxicology Laboratory, Faculty of Health Sciences, Universiti Kebangsaan Malaysia.

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Received: May 29, 2013

Revised: September 24, 2013

Accepted: October 10, 2013

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