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Chemical composition and cellular toxicity of ethnobotanical-based hot and cold aqueous preparations of the tiger's milk mushroom (*Lignosus rhinocerotis*)

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ABSTRACT

Ethnopharmacological relevance: The sclerotium of the "tiger's milk mushroom" (*Lignosus rhinocerotis)* is used as tonic and folk medicine for the treatment of cancer, fever, cough and asthma by the local and indigenous communities. It is traditionally prepared by either boiling or maceration-like methods; however, there is no attempt to understand how different processing methods might affect their efficacies as anticancer agents.

Aim of the study: This investigation was undertaken to evaluate the cytotoxicity of the hot and cold aqueous extracts of *Lignosus rhinocerotis* and to deduce the nature of the chemical component(s) that might be responsible for differential cellular toxicity of the extracts.

Materials and methods: The hot (LR-HA) and cold (LR-CA) aqueous extracts of the sclerotium of *Lignosus rhinocerotis* were prepared. The levels of bioactive components in the extracts were determined and chemical profiling was performed using UPLC-ESI-MS, SDS-PAGE and SELDI-TOF MS. Cytotoxicity of LR-HA and LR-CA against a panel of human cancer and normal cell lines was assessed by the MTT and trypan blue exclusion assays. Changes in cell morphology upon treatment with the extracts were observed. The chemical composition and bioactivities data were correlated to explain the nature of the cytotoxic component(s).

Results: LR-HA and LR-CA were particularly abundant in polar components. Both extracts exhibited varying degree of cytotoxicity against the cancer cell lines with LR-CA showed significantly stronger cytotoxicity (IC_{50}: 37–355 μg/ml) than LR-HA ($IC_{50} > 500$ μg/ml); however, LR-CA lacked selectivity in that it also has cytotoxic effect on the normal cell lines. Based on the results of protein profiling of heat-treated LR-CA (40–100 °C) coupled to the MTT assay, the cytotoxic component(s) in LR-CA were deduced to be thermo-labile, water-soluble protein/peptide(s).

Conclusion: Our findings have shown that the use of different preparation methods (hot and cold aqueous extraction) for *Lignosus rhinocerotis* has resulted in extracts with distinctively different cellular toxicity in which the cytotoxic constituents were present only in LR-CA.

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1. Introduction

In the tropical region, especially Southeast Asia, *Lignosus rhinocerotis* (as '*rhinocerus*') (Cooke) Ryvarden, commonly known as the "tiger's milk mushroom", is used as tonic and folk medicine. Unlike other mushrooms, the part with medicinal value is not the sporophore (fruiting body) but the sclerotium (plural, sclerotia)—a compact mass of hardened mycelia buried underground. It is traditionally used to treat cancer, fever, cough and asthma by the local and indigenous communities in Malaysia (Chang and Lee, 2004).

According to Huang (1999), *Polyporus rhinocerus* (Cooke) or hurulingzhi (in Chinese) is taxonomically synonymous with *Lignosus rhinocerus* (Cooke) Ryvarden; however, in terms of scientific nomenclature, *Lignosus rhinocerus* is an orthographic variant (variant spelling of the same name) of *Lignosus rhinocerotis* as cited in MycoBank (http://www.mycobank.org) and Index Fungorum (http://www.indexfungorum.org). The Chinese physicians in Hong Kong utilised the sclerotium of *Polyporus rhinocerus* to treat liver cancer, chronic hepatitis and gastric ulcer (Wong and Cheung, 2008).

In ethnomedicine, herbal preparation is given due emphasis as processing methods serve to enhance the efficacy and/or reduce the toxicity of resulting preparations (Zhao et al., 2010). Several methods of preparing the sclerotium of the "tiger's milk mushroom" for medicinal purposes have been documented. Chan (1953) described

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a technique which mimics cold aqueous extraction whereby the sclerotium is grated on a hard surface, e.g. granite plate, with some water and the resulting mixture is further diluted with water before consumed. In more recent ethnobotanical surveys, it was mentioned that *Lignosus rhinocerus* is often consumed in the form of decoction; sliced sclerotium is boiled but usually in combination with other herbs (Chang and Lee, 2004; Azliza et al., 2012). There are also non-aqueous-based preparations that are associated with communities in certain locations or for specific uses as described by Burkill (1966).

Attempts to validate the medicinal properties of *Lignosus rhinocerotis* so far focused mainly on its anticancer effect; however, there is a lack of information pertaining to specific preparation methods in existing literature and ethnobotanical records. The anticancer potential of *Polyporus rhinocerus* via in vitro cytotoxicity against leukemic cells (Lai et al., 2008) and in vivo immunomodulatory effect (Wong et al., 2011) has been reported. The hot water-soluble polysaccharides of *Polyporus rhinocerus* moderately inhibited the growth of the human acute promyelocytic leukaemia cells (HL-60), human chronic myelogenous leukaemia cells (K562) and human acute monocytic leukaemia cells (THP-1) with approximate IC_{50} values of 100, 400 and >400 μg/ml, respectively (Lai et al., 2008). Recently, Lee et al. (2012) has shown that the cold water extract of a local cultivar of *Lignosus rhinocerus* (designated as TM02) exhibited poor cytotoxicity against the human breast (MCF7) and lung carcinoma (A549) with IC_{50} values of 96.7 and 466.7 μg/ml, respectively.

Even though reports on the cytotoxicity of *Lignosus rhinocerotis* exist in the literature, this study investigates a number of aspects which have not been systematically studied before. First, there was no attempt to compare the activities of extracts from different preparation methods, e.g. hot and cold aqueous extractions—which are thought to be relevant from the ethnomedicine point of view. Second, previous cytotoxicity screening, as described above, was limited to only two solid tumours and three leukemic cell lines. Surprisingly, no work has been done against liver cancer in which *Polyporus rhinocerus* was claimed to be an effective remedy. Third, the hot aqueous extract of *Lignosus rhinocerotis* has not been screened for cytotoxicity against any solid tumour cell lines and the cold aqueous extract, on the other hand, was not tested on any leukemic cell lines. This represents a gap in the existing literature. Fourth, the chemical composition of the hot water extract of *Polyporus rhinocerus* was characterized but Lee et al. (2012) only estimated the carbohydrate and protein contents of the cold water extract of *Lignosus rhinocerus* TM02. Other chemical constituents in the aqueous extract of *Lignosus rhinocerotis*, e.g. secondary metabolites, have not been profiled. Last and most importantly, is the fact that the exact cytotoxic compound from cold aqueous extract of the sclerotium of *Lignosus rhinocerotis* has yet to be identified.

In accordance with the traditional practices mentioned in ethnobotanical records, the hot and cold aqueous extracts of the sclerotium of *Lignosus rhinocerotis* were prepared and chemically profiled. The objectives of the present investigation are to substantiate the claimed anticancer effect of the aqueous extracts of *Lignosus rhinocerotis* via cytotoxicity across a panel of human cancer and normal cell lines and to deduce the nature of chemical components in the sclerotium that might be responsible for the cytotoxicity.

2. Materials and methods

2.1. Mushroom samples

Specimens of *Lignosus rhinocerotis* (sporophore and sclerotium) were collected from their natural habitat in the Kenaboi Forest Reserve, Negeri Sembilan, Malaysia in 2008 and authenticated by mycologists from the Mushroom Research Centre (MRC), University of Malaya. Axenic cultures, derived from tissue culture of the sclerotium, were maintained by periodic subculturing on malt extract agar (Oxoid) and stored at 4 °C. Voucher specimen and stock cultures (KUM61075) were deposited in the MRC culture collections.

2.2. Mushroom cultivation

The sclerotium of *Lignosus rhinocerotis* (KUM61075) was induced from solid-substrate fermentation of the mycelia on agro-residues (Abdullah et al., 2013). Briefly, optimised formulation of selected agro-residues (sawdust, paddy straw and spent yeast) at suitable pH and moisture were filled into transparent plastic polypropylene bags. Open ends of the bags were covered with cotton plugs prior to sterilization in an autoclave. After the bags have cooled down, each was aseptically inoculated with 10 mycelia plugs (diameter: 10 mm). The substrate bags were then incubated for 50–60 days under dark condition at room temperature (25 ± 2 °C). After the mycelia had fully colonized the substrates, the substrate blocks were buried under the soil at suitable depth for sclerotia development. Following 10–12 months of burial, mature sclerotia were harvested, cleaned and dried in the oven at 40 °C for 3–4 days. Harvested sclerotia of variable sizes (diameter: 30–60 mm) were mostly round with rough and wrinkly outer surface, and light yellow to white internal tissue.

2.3. Preparation of hot and cold aqueous extracts

The sclerotium of *Lignosus rhinocerotis* were cut into smaller pieces and ground to fine powder in a Waring blender before subjected to extraction procedures as follows: for the hot aqueous extraction, powdered sclerotium were boiled in distilled water at the ratio of 1:20 (w/v) at 90–95 °C for 60 min, whereas for the cold aqueous extraction, mixture at similar ratio were subjected to continuous stirring at 4 °C for 24 h. Resulting mixtures from the hot and cold aqueous extraction were filtered with Whatman No. 1 filter paper and residues were re-extracted twice. Combined mixtures from three rounds of the hot and cold aqueous extraction were separately centrifuged at 10 000 rpm and freeze-dried (Labconco). The resulting hot (LR-HA) and cold (LR-CA) aqueous extracts of *Lignosus rhinocerotis* were kept at −20 °C prior to analysis. Both extracts were brown and fluffy.

2.4. Determination of chemical composition of aqueous extracts

The aqueous extracts of *Lignosus rhinocerotis* were dissolved in distilled water and further diluted to desired concentrations for the following assays: The phenol–sulfuric acid assay in microplate format (Masuko et al., 2005) was used for estimating total carbohydrate content. D-glucose (0–2 mg/ml) (Sigma) was used as a standard. Protein content was determined using the Pierce® Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) according to recommended protocols. Protein content was estimated from the standard curve of bovine serum albumin (0–2 mg/ml). Total phenolic content was assayed using the Folin–Ciocalteu reagent (Sigma) and gallic acid (Sigma) as standard according to the method of Slinkard and Singleton (1977) with some modifications. Results were expressed as gallic acid equivalents (mg GAE/g of extract). The levels of α- and β-glucans were measured using the Mushroom and Yeast β-glucan Assay Kit (Megazyme International Ireland) according to manufacturer's protocol.

2.5. Chemical profiling of aqueous extracts

2.5.1. Chromatographic analysis via UPLC-ESI-MS

The aqueous extracts of *Lignosus rhinocerotis* were dissolved in 50% (v/v) aqueous methanol at a final concentration of 20 mg/ml and filtered through a membrane filter (0.45 μm). Analysis was carried out using the Acquity™ Ultra Performance Liquid

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