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Comparative Cytotoxicity Evaluation of Heat-Assisted *vs* Cold Water Extractions of Six Medicinal Fungi against Breast and Lung Cancer Cells

Running head: Comparative Cytotoxicity of Cold vs. Heat-assisted Water Fungi Extracts

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SUMMARY

Research background. Preparation of medicinal fungi for experimental purposes usually involves extraction and determination of both the quality and quantity of bioactive compounds prior to biological experimentation. Water, a common polar solvent, is typically used in traditional preparations for consumption. The application of high temperatures during water extraction can potentially impact the chemical composition and functional outcomes of the extracts. Hence, this study aims to compare the compositional differences between heat-assisted (HAWE) and cold water (CWE) extracts of six

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selected fungi species (*Lignosus rhinocerus*; *Ophiocordyceps sinensis*; *Inonotus obliquus*; *Antrodia camphorata*; *Phellinus linteus*; *Monascus purpureus*) and their cytotoxicity against human lung and breast cancer cells.

Experimental approach. CWE and HAWE of six fungi species were analysed to determine their protein, carbohydrate, and phenolic compositions. Their cytotoxicities were examined against lung (A549) and breast (MCF-7; MDA-MB-231) cancer cell lines. The most potent extract was further separated into its protein and non-protein fractions to determine their respective cytotoxicity.

Results and conclusions. The cytotoxicity varied between CWEs and HAWEs for the different fungi extracts. Comparing CWEs and HAWEs, CWEs provided a significantly higher yield of protein (except *M. purpureus)* and phenolic (except *A. camphorata*) compounds, while HAWE of *I. obliquus* and *M. purpureus* have significantly higher carbohydrates content. Notably, *I. obliquus*-CWE exhibited cytotoxicity (IC_{50}=700.95±34.67 μg/mL) that was one of the most potent of the tested extracts against A549 cells. *I. obliquus*-CWE was selected for further evaluations. Our results showed that CWEs generally have higher cytotoxicities against selected human cancer cell lines, with the exception of *O. sinensis* and *A. camphorata* extracts.

Novelty and scientific contribution. This study reported the advantage of fungi CWE over HAWE in terms of cytotoxicity towards human cancer cell lines, highlighting the role of extraction conditions, particularly heat, in influencing chemical composition and cytotoxic effects.

Keywords: fungal extract; heat-assisted water extraction; cold water extraction; protein/carbohydrate/phenolic compositions; cytotoxicity

INTRODUCTION

Medicinal fungi have a long history of use in traditional and complementary medicine due to their therapeutic properties such as antimicrobial, antioxidant, anti-inflammatory, and anticancer (*1*). These are attributed to the presence of various bioactive compounds such as polysaccharides, lectins, lanostanoids, alkaloids, and phenolics (*1*). Many fungi have gained considerable attention due to their rich and varied chemical composition.

In this study, we focused on six notable medicinal fungi species that are mainly found in Asia: *Lignosus rhinocerus*, *Ophiocordyceps sinensis*, *Inonotus obliquus*, *Antrodia camphorata*, *Phellinus linteus*, and *Monascus purpureus*. *Lignosus rhinocerus*, also known as Tiger Milk mushroom, is a rare

and highly sought-after medicinal fungus indigenous to Southeast Asia. It has been used by aboriginal tribes to treat respiratory conditions, arthritis, and cancers (*2,3*). Chinese caterpillar fungus, scientifically known as *O. sinensis*, possesses several bioactivities that have earned it immense recognition in traditional Chinese medicine. Its discovery was initially prompted by its parasitic relationship with moth caterpillars (*4*). *O. sinensis* is frequently used in the treatment of asthma, bronchial inflammation, lung ailments, and is traditionally regarded as a supplement for supporting longevity. The distribution of *O. sinensis* is mainly confined to the Tibetan Plateau and its adjacent areas.

Chaga, scientifically known as *I. obliquus*, grows in the frigid birch forests in Siberia (*5*). Its antioxidant and anti-inflammatory effects are enhanced by bioactive components such as polysaccharides and betulinic acid (*6*). *Antrodia camphorata* from Taiwan was found on decomposing *Cinnamomum kanehirai* wood (*7*). It is well-known for its hepatoprotective and potential anticancer properties (*7*). *Phellinus linteus*, also known as Sang Hwang, is a fungus that is commonly found in Asia, particularly in Korea and China (*8*). Traditionally, it is used for glycaemic regulation, blood circulation enhancement, hepatoprotection, and to strengthen the immune system (*8*). *Monascus purpureus*, or red yeast, is discovered in red yeast rice as well as in fermented foods and beverages made with red yeast rice, primarily in China, Taiwan, and Korea (*9*). It is known for producing vibrant red pigments that are commonly used as food colorants as well as possesses cholesterol-lowering properties due to the compound monacolin K (*10*).

Natural compounds from living organisms, like plants, fungi, and microbes, form the basis for the health benefits found in many natural remedies that support overall well-being (*11*). The preparation of these natural remedies and medicinal concoctions dates back to ancient times. Water-based preparation methods, including decoction and hot infusion, are the two most commonly used approaches. Decoction involves vigorously boiling tougher parts of raw ingredients in water, while hot infusion involves gently steeping their softer parts in hot water. These approaches aim to extract beneficial compounds for therapeutic purposes. While the practice of preparing decoctions using hot water have been extensively studied, the potential effects of hot (steeped in hot water) and cold (soaked in cold water for 12 to 24 h) water extractions as well as their compositional and functional differences have not been thoroughly investigated, particularly for the selected fungi used in this study. Hence, our study used two types of water extraction methods: heat-assisted water extraction and cold water extraction, as models for hot and cold water infusions.

Different extraction temperatures have the potential to yield distinct functional compounds with varied bioactivities (*12*). This study aims to evaluate and compare the compositional differences

(carbohydrate, protein, phenolic compound) between cold water (CWE) and heat-assisted water (HAWE) extracts of six selected fungi. Our study also aimed to assess the cytotoxic effects of these extracts *in vitro* using various lung and breast cancer cell lines. Of the selected medicinal fungi, those exhibiting higher cytotoxicity were further separated to identify the specific fraction that contains potential cytotoxic compounds of interest, which may be exploited for future drug development or prophylactic regimens.

MATERIALS AND METHODS

Fungi samples

L. rhinocerus TM02® (Batch no: PL/1107/020), *O. sinensis* OCS02® (Batch no: OC/21/001-1122), *I. obliquus* (Batch no: CG/20/003-1122), *A. camphorata* (Batch no: AC/21/001-1122), *P. linteus* (Batch no: SH/21/002-1122) and *M. purpureus* (batch number not applicable, single cultivar) were obtained from LiGNO Biotech Sdn Bhd (Selangor, Malaysia). These fungi were cultivated using their proprietary solid stage fermentation technology with rice-based medium as substrate. PCR amplification, coupled with sequence analysis of their respective internal transcribed spacer (ITS) region, was employed to verify their identity (*13,14*).

Cell lines and cell culture

Human lung carcinoma epithelial cells A549 (CRM-CCL-185™, ATCC, Manassas, Virginia, United States) and human breast adenocarcinoma MCF-7 (HTB-22™, ATCC, Manassas, Virginia, United States) were cultured in HyClone RPMI-1640 (Cytiva, Marlborough, Massachusetts, United States) supplemented with 10 % foetal bovine serum (FBS) (Tico Europe Ltd., Amstelveen, Netherlands). Human breast adenocarcinoma MDA-MB-231 (CRM-HTB-26™, ATCC, Manassas, Virginia, United States) was cultured in DMEM (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10 % (by volume) FBS. Nontumorigenic human lung epithelial cell line NL20 (CRL-2503, ATCC, Manassas, Virginia, United States) was cultured in Ham's F-12 Medium (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10 % (by volume) FBS. All cell lines were incubated at 37 °C in a 5 % CO_2 incubator (Heracell 150, Marshall Scientific, Hampton, Virginia, United States). Cells were subcultured every 3 –4 days, with 0.25 % (*m*/*V*) trypsin-EDTA solution (Biosera, Cholet, France).

Extraction methods

Fungal extracts were prepared using two different water extraction methods: cold water and heat-assisted water extractions.

Cold water extraction

Dried crude fungi powder was dissolved in a concentration of 50 kg/m^3 (30 g of powder in 0.6 L of distilled water). The solution was stirred for 24 h at 4 °C in a cold room. The mixture was then centrifuged at 8000×*g* for 30 min at 4 °C with Sorvall Biofuge PRIMO R refrigerated centrifuge (Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States) to remove any insoluble compounds. The resulting supernatant was filtered through Whatman filter paper (Grade 1, pore size=1.1×10^{-5} m, GE Healthcare, Chicago, Illinois, United States) and subsequently freeze-dried. The CWE was stored at -20 °C.

Heat-assisted water extraction

A total of 100 g crude fungi powder was dissolved in 0.5 L of warm water (50–60 °C). After all fungi crude powder was thoroughly hydrated, the volume was topped up with boiled distilled water to a final volume of 1 L. The final temperature of the solution was 80 °C. The mixture was agitated periodically at room temperature for 3 h. The mixture was transferred to a centrifuge tube and spun at 5000×*g* for 30 min. The supernatant named HAWE was then collected, freeze dried and stored at -20 °C.

For both *L. rhinocerus* and *O. sinensis*, the frozen solutions were removed and thawed at room temperature (25 °C) after 12 h. Precipitates were observed upon thawing, and the mixture was centrifuged at 5000×*g* for 30 min. The supernatant was subsequently transferred to a new tube. The remaining precipitate was washed with distilled water and spun at 5000×*g* for 30 min. The water used for rinsing was removed, and the rinsed precipitate (the obtained precipitate was only soluble up to 80 °C) was resuspended in 0.2 L distilled water. The precipitate was freeze dried and stored at -20 °C until further use.

Quantification of protein, carbohydrate, and phenolic content of extracts

To quantify the protein content in samples, 2-D Quant kit (GE Healthcare, Chicago, Illinois, United States) was used following the manufacturer's instruction.

For quantification of total carbohydrate content, the phenol-sulphuric acid method was used (*15*). Briefly, 200 µL of 5 % phenol (Merck KGaA, Darmstadt, Germany) was added to 200 µL of sample, followed by the addition of 1 mL of 95 % sulphuric acid (H_2SO_4; Friendemann Schmidt Chemical, Kuala Lumpur, Malaysia). After thorough mixing, the mixture was incubated at room temperature for 20–30 min. The absorbance of the mixture was measured at 490 nm using a UV–Vis spectrophotometer (SpectraMax® ABS Plus, Molecular Devices, San Jose, California, United States).

For quantification of total phenolic content, the Folin-Ciocalteu assay was used (*16*). A 10 µL of extract was mixed with 500 µL Folin-Ciocalteu phenol reagent (Merck KGaA, Darmstadt, Germany) and incubated for 5 min at room temperature. Next, 350 µL of sodium carbonate (Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany) (115 µg/mL) was added to the mixture and incubated for 2 h. Gallic acid (Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany) was used as a standard at concentrations ranging from 20 to 200 µg/mL. Their absorbance was measured using a UV–Vis spectrophotometer (SpectraMax® ABS Plus, Molecular Devices, San Jose, California, United States) at 765 nm.

Ammonium sulphate precipitation of I. obliquus-CWE

Ammonium sulphate (Friendemann Schmidt Chemical, Kuala Lumpur, Malaysia) at 100 % saturation was used to precipitate the protein and non-protein compounds of the *I. obliquus*-CWE, with stirring for 1 h at 4 °C. Centrifugation was used to separate the precipitated protein pellet from the supernatant (containing non-protein compounds). Desalting was subsequently performed for both protein and non-protein compounds using the Vivaspin® Centrifugal Concentrator 15R (MWCO-5 kDa) (Sartorius AG, Göttingen, Germany).

Cytotoxicity assay

The MTT (2,5-diphenyl-2H-tetrazolium bromide) assay (Merck KGaA, Darmstadt, Germany) was used to examine the cytotoxicity of extracts against a panel of human cancer cell lines, including MCF-7 (breast cancer; ER^+; PR^+), MDA-MB-231 (breast cancer; ER/PR^-, $HER2^-$) and A549 (lung cancer). The cytotoxicity of both *I. obliquus*-CWE protein and non-protein components were tested against lung carcinoma A549 and its respective non-tumorigenic cell line, NL20. On Day 1, each cell line was seeded into a 96-well plate. The cells were treated with both extracts after 24 h and incubated for 72 h in a CO_2 incubator. Then, 20 µL of 5 mg/mL MTT reagent (Sigma-Aldrich®, Merck KGaA, Darmstadt, Germany) was added into each well and incubated for 3 h at 37 °C. Following incubation, the MTT reagent was removed and 200 µL of DMSO (Friendemann Schmidt Chemical, Kuala Lumpur, Malaysia) was added to each well. The 96-well plate was shaken for 15 min using an orbital shaker. The absorbance was then measured at wavelength 570 nm for 1 h. IC_{50} was obtained through plotted survival curve of cells in each treatment. Selectivity index is equal to the IC_{50} of non-tumorigenic cell lines divided by the IC_{50} of cancer cell lines (*17*).

Statistical analysis

Shapiro-Wilk test was used to assess data normality, and Levene's test was used to determine the equality of variances. The statistical significance between the protein, carbohydrate and phenolic content of CWE, HAWE-supernatant and HAWE-precipitate of *L. rhinocerus* and *O. sinensis* were evaluated using analysis of variance (ANOVA) with Tukey's HSD post-hoc analysis. As the dataset of *M. purpureus*-CWE and -HAWE protein content did not satisfy the normality assumption test, comparison between two groups were analysed using the non-parametric Mann-Whitney U-test. For the remaining groups, determination of the protein, carbohydrate and phenolic content between CWE and HAWE were evaluated using parametric Student's T test. All data are presented as mean±standard deviation (SD). A *p*-value less than 0.05 was considered statistically significant. The data was analysed using Microsoft® Excel (Redmond, Washington, United States) (*18*), IBM® SPSS® Statistics software (version 26.0) (Armonk, New York, United State) (*19*) and GraphPad Prism® (version 6.01) (Dotmatics, Boston, Massachusetts, United States) (*20*).

RESULTS AND DISCUSSION

Protein, carbohydrate, and phenolic contents of fungal extracts

Our analysis revealed significant differences between CWEs and HAWEs in protein and phenolic content (Table 1). Most CWEs possessed significantly higher protein content than HAWEs. An exception was observed in *M. purpureus* extracts, where both CWE and HAWE demonstrated similarly low protein contents, likely due to inherently low protein content or measurements falling below the detection limit. As the sensitivity of most proteins to high temperatures, the cold water extraction proves advantageous in preserving protein integrity (*21*). It is crucial to note that the heat-assisted water extraction used in this study does not involve boiling, unlike previously reported hot water extraction (*22*). Nonetheless, both methods involve high temperatures that may potentially damage thermolabile compounds.

CWE demonstrated significantly higher phenolic content than HAWE, except *A. camphorata* (Table 1). Phenolic compounds have the ability to undergo redox reactions as hydrogen atom donors or reducing agents serve to neutralize electrons of reactive oxygen species (ROS) (*23*). It is worth noting that *I. obliquus*-HAWE and *M. purpureus*-HAWE have significantly higher levels of carbohydrates than their CWEs. Elevated temperature in heat-assisted water extraction likely increased carbohydrate extraction by reducing solvent viscosity, enhancing carbohydrate solubility, and promoting facilitated diffusion in water (*24*).

Cytotoxicity of fungal extracts towards lung and breast cancer cells

Carbohydrates, proteins, and phenolic compounds in fungi, including beta-glucans, serine proteases, and resveratrol, have known cytotoxic bioactivities (*25-27*). We hypothesized that varying compositions in the extracts, due to different extraction conditions (Table 1), might influence the cytotoxicity of selected fungi extracts. Fig. 1 illustrates the overall half maximal inhibitory concentration (IC_{50}) of the extracts against A549, MCF7 and MDA-MB-231 cell lines.

Our present findings (Fig. 1) for *L. rhinocerus* are aligned with previous studies (*12,28*), where *L. rhinocerus*-CWE demonstrated higher cytotoxicity in comparison to its hot water extract. This suggests that the cytotoxicity of *L. rhinocerus* may be attributed to thermolabile water-soluble compounds that are altered or degraded during heat-assisted extraction. Cold water extraction appears to be the most effective method for extracting bioactive (anticancer, anti-inflammation) compounds from *L. rhinocerus*, based on the results of numerous studies (*12,29*).

O. sinensis water extracts had varying cytotoxicities against different human cancer cell lines. *O. sinensis*-CWE is more cytotoxic against A549 cells than *O. sinensis*-HAWE, while *O. sinensis*-HAWE supernatant exhibited stronger cytotoxicity against MCF-7 cells (Fig. 1). *O. sinensis*-CWE contained higher levels of protein and phenolic compounds compared to both *O. sinensis*-HAWE supernatant and precipitate (Table 1), consistent with previous studies (*30*). This finding suggests that thermolabile compounds, including the proteins and phenolic compounds in *O. sinensis*-CWE, are possibly the main contributors to its observed cytotoxicity against A549 cells. Meanwhile, considering that both the supernatant and precipitate of *O. sinensis*-HAWE have similarly high carbohydrate content and minute amounts of proteins and phenolics, the cytotoxic compounds against MCF-7 cells are likely to be carbohydrates and possibly other water-soluble thermostable compounds. Cordycepin, a nucleoside with anticancer properties, could be a candidate as it can be extracted at higher temperatures (up to 70 °C) (*31*). Our findings indicate that the cytotoxicity of the *O. sinensis* extracts may depend on the specific cancer cell type and extraction temperature. The differences in cytotoxic activities of *O. sinensis* extracts are likely due to the presence of specific compounds in each extract that exhibit selective toxicity towards specific cancer cell lines, potentially targeting different signalling pathways or factors. These variations arise from the physiological characteristics and signalling mechanisms of cancer cells from different organs.

Both *A. camphorata*-CWE and *A. camphorata*-HAWE demonstrated similar cytotoxicity against A549 cell lines, suggesting that the activity of *A. camphorata* is attributed to thermostable water-soluble compounds present in the fungi that are retained even after heat-assisted extraction. These cytotoxic components in *A. camphorata* water extracts are likely carbohydrates or phenolic

compounds, as both *A. camphorata*-CWE and *A. camphorata*-HAWE exhibited similar content of these metabolites.

Both *M. purpureus*-CWE and *M. purpureus*-HAWE did not exhibit cytotoxicity (IC_{50}>2000 μg/mL) against any of the tested cancer cell lines (Fig. 1). Despite the water extracts containing high carbohydrate levels, we hypothesize that the cytotoxic compounds in *M. purpureus* may be non-polar. Previous studies have demonstrated that *M. purpureus* extracts using different solvents, such as petroleum ether-soluble portion of the EtOH extract, contain non-polar monapurones A-C, which exhibit strong selectively cytotoxic effects on lung cancer cell lines A549 (*32*). The extraction of these cytotoxic compounds can be achieved using solvents with low polarity, such as petroleum ether, suggesting bioactive compounds in *M. purpureus* with anticancer properties may either be insoluble in water or present in low concentrations in the water extract.

I. obliquus-CWE demonstrated one of the lowest IC_{50} values against A549 cells, with no significant difference compared to *L. rhinocerus*-CWE and *O. sinensis*-CWE, yet it outperformed *I. obliquus*-HAWE and other tested fungal extracts (Fig. 1, Table S1). Our result is of interest as this is the only reported aqueous extract from *I. obliquus* that resulted with a low IC_{50} value (*33*). It is also lower when compared with other organic solvent extract methods, such as methanol (IC_{50}=2.03±0.20 mg/mL) (*34*). Water is a solvent with greater polarity than methanol. Hence, it can extract a higher amount of hydrophilic compounds. We postulate that the highly cytotoxic compound to the A549 cell lines is most likely hydrophilic. Another study also reported the cytotoxicity of a room temperature *I. obliquus* aqueous extract against A549 lung cancer cells (IC_{50}=Not reported) (*33*). In agreement with our results, the bioactive compounds in *I. obliquus* may be extracted effectively under aqueous conditions and are potentially thermolabile. Importantly, none of the extracts from previous *I. obliquus* studies were obtained through cold water extraction. Therefore, our findings prompt further investigation into the specific cytotoxic components responsible for the observed cytotoxicity in *I. obliquus*-CWE.

With the exception of some cases mentioned above, our overall findings showed that CWEs exhibited higher cytotoxicity across most tested fungi (Fig. 1, Table S1). Our results demonstrate the importance of understanding the potency, stability and solubility of specific bioactive compounds to determine the best extraction method for different species of medicinal fungi. The varied cytotoxicity observed between the extracts of different fungi also highlights the complex nature and potential selectivity of their bioactive compounds towards specific cancer cell lines.

Cytotoxic activity of I. obliquus-CWE protein and non-protein components

I. obliquus-CWE contained significantly higher levels of protein and phenolic compounds compared to *I. obliquus*-HAWE, indicating the potential cytotoxic compounds present in the *I. obliquus*-CWE. Therefore, we decided to delve deeper by separating it further into protein (*I. obliquus*-CWE-P) and non-protein (*I. obliquus*-CWE-NP) fractions via ammonium sulphate precipitation. *I. obliquus*-CWE-P contained higher amount of protein (76.26±2.31 %) with lower amount of carbohydrates (18.95±5.61 %) compared to *I. obliquus*-CWE-NP, which had no detectable protein and high amount of carbohydrates (69.20±3.54 %).

The analysis showed that the *I. obliquus*-CWE exhibits higher cytotoxicity against A549 cells compared to the protein and non-protein fractions. *I. obliquus*-CWE-NP exhibited no detectable cytotoxicity (IC_{50}>1000 μg/mL). Surprisingly, both the *I. obliquus*-CWE and its protein fraction exhibited significantly higher cytotoxic selectivity towards non-tumorigenic human lung NL20 cells compared to A549 (Table 2, Fig. 2). Nevertheless, the *I. obliquus*-CWE exhibited lower cytotoxicity to NL20 cells (IC_{50}=549.44±44.10 μg/mL)$^{\#}$ compared to the protein fraction (IC_{50}=31.36±0.65 μg/mL)$^{¥}$ (refer to Table 2 for the descriptions of the symbols, $^{\#}$ $^{¥}$). In the *I. obliquus*-CWE, the non-protein compounds may have counteracted the cytotoxicity exerted by the proteins towards NL20 cells, thus reducing its cytotoxic selectivity towards NL20 (selectivity index=0.784). Overall, this suggests that consuming *I. obliquus* in its entirety could provide a more balanced effect than consuming only the protein fraction. Further research is needed to understand the synergistic interactions between its protein and non-protein compounds by identifying the specific bioactive compounds present in *I. obliquus*-CWE and *I. obliquus*-CWE-P that affect lung non-tumorigenic cells.

It is important to note that the protein fraction of the *O. sinensis* CWE has been reported to exhibit greater cytotoxicity towards NL20 *in vitro (30)*. In addition, the CWE of *L. tigris* was also reported to be cytotoxic to the NL20 cell line. However, intraperitoneal treatment of *L. tigris* CWE protein fraction in a mouse xenograft tumour model did not result in any structural damage to the lungs of the mice (*35*). Therefore, the NL20 may be more sensitive to the cytotoxic compounds derived from the fungi. Hence, future studies should include other non-tumorigenic lung cell lines, such as BEAS-2B or HULEC-5a, to confirm the cytotoxic selectiveness of *I. obliquus*-CWE-P. Moreover, the synergistic interactions between fungi proteins and polysaccharides in a biological system could potentially explain the observed discrepancy between *in vitro* and *in vivo* results for fungi CWE extract. Therefore, future *in vivo* toxicity assessments of *I. obliquus*-CWE and *I. obliquus*-CWE-P and their effects on the lungs are warranted.

Generally, in this study, we found that most of the fungi CWEs are cytotoxic to human cancer cell lines, though there are some exceptions. We hypothesize that the native environment in which the fungi grow may play a deciding role in determining the characteristics of the bioactive molecules present in them, thus accounting for the observed exceptions or inconsistencies in trends. For instance, *I. obliquus* is native to cold circumboreal regions, while *O. sinensis* can be found in meadows above 3,500 m in the Tibetan region. These fungi thrive in cold or relatively cool temperatures, implying that their proteins that provide essential homeostasis are adapted to low temperatures and may not be stable in higher temperatures. This may explain why cold water extraction is more appropriate for these fungi to produce more potent cytotoxic compounds against A549 cells. The same theory could apply to *L. rhinocerus*, which typically grows underground in a shaded area under the canopy. Exposure to higher temperatures may degrade their bioactive compounds. On the other hand, fungi such as *A. camphorata* parasitizes endemic camphor trees in Taiwan at altitudes of 400-2000 m above sea level and are more susceptible to temperature fluctuations due to seasonal changes. This may be the reason that the cytotoxic components in *A. camphorata* are retained at high temperatures. Given that our theories are derived from the observations and results obtained, it is necessary to conduct physiochemical characterization and chemical composition identification of the fungal molecules to validate such claims.

Water is an abundant, cost-effective, non-toxic, and environmentally-friendly solvent, making it well-suited for extraction purposes (*36*). Water-based extraction methods have gained interest as eco-friendly alternatives for efficiently extracting natural compounds, saving time and energy (*37*). The process of heating water reduces its permittivity, viscosity, and surface tension, while increasing its diffusivity (*38, 39*). However, the application of heat during extraction plays a critical role as it can influence extraction rates and concentration of compounds, which can potentially impact the chemical composition of the final extract (*40*). Interestingly, recent studies have shown that cold water extraction of certain plants and fungi can yield extracts with greater bioactivities compared to hot water extraction (*12,28,41*). This suggests that the temperature used during the extraction process can significantly impact the stability and effectiveness of the extracted compounds.

Water-based extraction methods are prominently employed to extract bioactive compounds from medicinal fungi, such as bioactive polysaccharides and triterpenoids from *Ganoderma lucidum* and bioactive polysaccharide (CP2-S) from *Cordyceps militaris* (*42,43*). Extracting these bioactive compounds from fungi is a critical step in drug development, requiring the selection of appropriate and efficient extraction methods (*40*). The product of extraction can be impacted by various factors, including but not limited to temperature and duration of extraction, pressure, pH and polarity of the

solvent used (*44*). Adjusting the duration of extraction and increasing temperature are effective modifications that can enhance extraction processes, potentially improving the yield and functionality of the extracted compounds (*45,46*). However, the resulting product can also be impacted by variables such as extended exposure to atmospheric oxygen, oxidative or enzymatic degradation, and intermolecular reactions among mycochemicals (*47*). A noteworthy limitation of our present investigation, which could serve as a basis for future research, is that the observed outcomes are not solely influenced by heat application. Factors such as extraction duration and agitation methods can also impact the extraction process and its resulting products. The use of water as the sole extraction solvent may have restricted our ability to capture less hydrophilic compounds, potentially overlooking additional bioactive substances that could be extracted with alternative solvents. Moving forward, it is essential to delve deeper into these variables. Incorporating additional extraction techniques, such as organic solvent extraction, ultrasonication, and supercritical fluid extraction, will broaden our understanding and enhance the effectiveness of the extraction processes.

Limitations of study

This study, while providing valuable insights into the cytotoxic properties of fungal water extracts, has several other limitations. The stability of these compounds over the 72-hour treatment period was not evaluated. Without these stability tests, it is uncertain whether the observed cytotoxic effects are attributable to the initially prepared extracts or potentially unstable by-products formed during extended exposure. The focus of this study on a limited set of cancer and non-tumorigenic cell lines also restricts the generalizability of the findings, as different cell lines may exhibit varying responses to the same treatments. Moreover, while the results are promising *in vitro*, they may not directly translate to *in vivo* systems, where complex interactions with the immune system, metabolism, and bioavailability can alter the effects observed. Finally, the absence of hormonal analysis, particularly in breast cancer cell lines with varying hormone receptor statuses (MCF-7: ER^+; PR^+; MDA-MB-231: ER/PR^-, $HER2^-$), may limit our understanding of the mechanisms through which the extracts exert their effects. By recognizing these issues, future studies are proposed to address these limitations by incorporating a broader range of extraction solvents, stability testing, more diverse cell lines, *in vivo* testing, and detailed hormonal influence assessments to comprehensively elucidate the therapeutic potential of these fungal extracts.

CONCLUSIONS

In summary, our study revealed that CWEs generally exhibited enhanced cytotoxicities towards specific human cancer cell lines. It is worth noting that bioactivities can vary due to the isolation of different compounds with distinct properties, emphasizing that each method of extraction has its unique advantages. This study has highlighted the potential of CWE as a good source of protein and phenolic compounds, while HAWE can be used to extract carbohydrates. Our study also emphasizes the importance of extraction conditions, especially heat application, in influencing the bioactivities of fungal extracts and the complexity of extracting their bioactive compounds in order to harness their full therapeutic potential. Therefore, additional studies are warranted to validate our findings and further investigations into various factors and conditions that can affect the bioactivities of these fungal extracts are needed. Further research will contribute to a deeper understanding of the optimal extraction methodologies and conditions needed to isolate specific bioactive compounds for future drug development from natural products.

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CONFLICT OF INTEREST

CST is employed by LiGNO Biotech Sdn. Bhd. (Malaysia). The remaining authors have no conflicts of interest to declare.

SUPPLEMENTARY MATERIALS

Supplementary materials are available at: www.ftb.com.hr.

AUTHORS' CONTRIBUTION

M.J. Ng compiled all the data and prepared the original draft of the manuscript. N.Y. Goh, M.F. Mohamad Razif, H.Y.Y. Yap, B.H. Kong, and S.Y. Fung provided critical revisions and proofread the manuscript. C.S. Tan cultivated and prepared the freeze-dried fungi sample. M.J. Ng and N.Y. Goh performed the experiments, sample preparation, analysed and interpreted the data. M.F. Mohamad Razif, H.Y.Y. Yap, B.H. Kong, and S.Y. Fung supervised the study. All authors were involved in the conception, design of the study and reviewed the final manuscript.

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Table 1. Protein, carbohydrate, and phenolic content of various fungi extracts

Fungi species	Extract	Protein content, *w* (protein)/(mg/g)	Carbohydrate content, *w* (carbohydrate)/(mg/g)	Total phenolic content, GAE/(mg/g)
Lignosus rhinocerus	CWE	4.13±0.79 [a]	708.05±41.7 [a]	1.08±0.04 [a]
	HAWE-supernatant	0.50±0.27 [b]	855.54±107.3 [a]	0.91±0.05 [b]
	HAWE-precipitate	0.53±0.10 [b]	764.04±126.30 [a]	0.16±0.02 [c]
Ophiocordyceps sinensis	CWE	2.27±0.24 [a]	445.67±70.10 [a]	1.92±0.50 [a]
	HAWE-supernatant	0.18±0.02 [b]	760.32±165.49 [a]	0.46±0.07 [b]
	HAWE-precipitate	0.28±0.09 [b]	739.20±126.30 [a]	0.08±0.01 [b]
Antrodia camphorata	CWE	0.70±0.03 *	626.28±20.50 [#]	10.11±0.21 [#]
	HAWE	0.24±0.06	634.60±26.70	10.96±0.39
Phellinus linteus	CWE	4.61±1.14 *	512.95±18.30 *	2.04±0.08 *
	HAWE	1.42±0.66	741.50±177.50	0.41±0.01
Inonotus obliquus	CWE	3.34±0.11 *	431.36±6.40 *	4.90±0.09 *
	HAWE	1.52±0.33	734.76±142.5	1.41±0.10
Monascus purpureus	CWE	0.01±0.06 [#]	515.56±41.80 *	10.21±0.16
	HAWE	0.18±0.03	836.64±92.80	NA

Protein and carbohydrate contents were estimated on a dry weight basis. Total phenolic content was reported in gallic acid equivalents (GAE) per milligram of sample (mg/g). The data are represented as mean±SD (n=9). Different superscript letters (a to c) denote significant difference ($p < 0.05$) among the respective *L. rhinocerus* and *O. sinensis* extracts; Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Tukey's HSD post-hoc analysis. The statistical significance of the remaining species was determined using Student's T test. * symbol is used to denote statistical significance, while [#] symbol indicates no statistical significance between both extraction methods (CWE & HAWE) for the respective fungal species. NA=not available, CWE=cold water extract, HAWE=heat-assisted water extract

Table 2. Half maximum inhibitory concentration (IC_{50}) of *Inonotus obliquus* CWE, CWE-P and CWE-NP against lung carcinoma A549 and non-tumorigenic NL20 cells after 72 h treatment. IC_{50} (μg/mL) was determined via MTT assay.

	IC_{50}/(μg/mL)		Selectivity index
	A549	NL20	
I. obliquus-CWE	700.95±34.67 *	549.44±44.10 #	0.784
I. obliquus-CWE-P	931.48±69.80 £	31.36±0.65 ¥	0.034
I. obliquus-CWE-NP	>1000	>1000	-

The data are represented as mean±SD (n=9). Different superscript symbols denote significant difference ($p<0.05$). Student's T test was used to compare IC_{50} values between *I. obliquus*-CWE and its CWE-P fraction within the same cell line; between the same extract/fraction across different cell lines (A549 & NL20). CWE=cold water extract, P=protein, NP=non-protein.

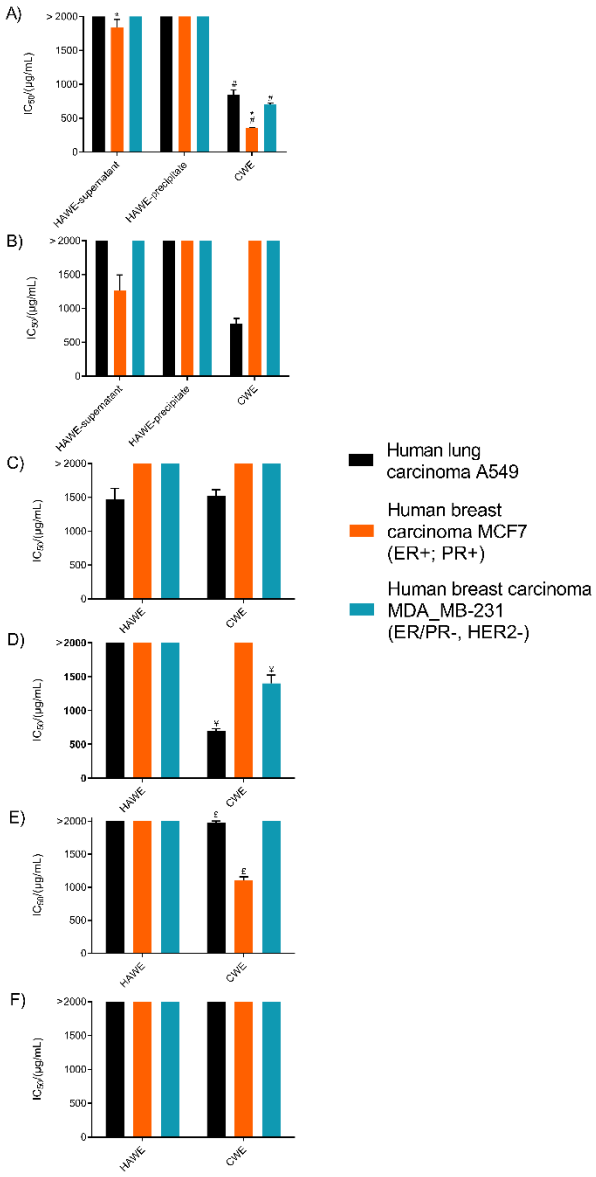

Fig. 1. Graphical representations of the IC_{50} values for each fungal extract: A) *L. rhinocerus*, B) *O. sinensis*, C) *A. camphorata*, D) *I. obliquus*, E) *P. linteus*, F) *M. purpureus*, with respect to the tested cancer cell lines. The data are represented as mean±SD (n=9). All IC_{50} values exceeding 2000 μg/mL are represented as >2000 μg/mL in the graphs, with no error bars displayed. # symbol indicates significant differences ($p<0.05$) of *L. rhinocerus* CWE IC_{50} values between the tested cell lines based on one-way ANOVA followed by Tukey's HSD post hoc test. The remaining statistical analysis was determined using Student's T test. * symbol denotes significant differences ($p<0.05$) of IC_{50} values between *L. rhinocerus* CWE and HAWE against MCF-7 cell line. ¥ and £ symbols indicate significant

differences ($p<0.05$) of IC_{50} of *I. obliquus* and *P. linteus* respectively, between the cell lines with IC_{50} < 2000 μg/mL. CWE=cold water extract, HAWE=heat-assisted water extract

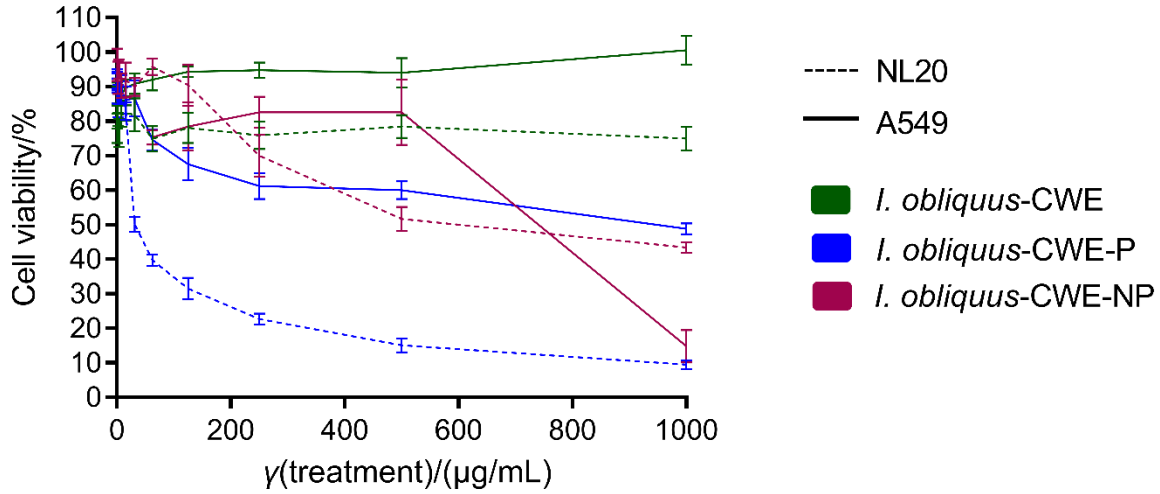

Fig. 2. Growth inhibition of lung carcinoma A549 and non-tumorigenic NL20 cell lines following 72 h treatment with *I. obliquus*-CWE and its protein (P) and non-protein (NP) fractions. Growth inhibition was determined via MTT assay. CWE=cold water extract

SUPPLEMENTARY MATERIAL

Table S1. Half maximum inhibitory concentration (IC_{50}) of fungi extracts against lung carcinoma A549, breast carcinoma MCF-7, and MDA-MB-231 after 72 h treatment

		IC_{50}/(μg/mL)		
		A549	MCF-7	MDA-MB-231
L. rhinocerus	CWE	847.66±71.35 [a]	351.98±10.35 [a]	702.82±20.07 [a]
	HAWE-supernatant	>2000	1837.74±121.93 [b]	>2000
	HAWE-precipitate	>2000	>2000	>2000
O. sinensis	CWE	773.5±80.6 [a]	>2000	>2000
	HAWE-supernatant	>2000	1269.25±224.17 [c]	>2000
	HAWE-precipitate	>2000	>2000	>2000
A. camphorata	CWE	1527.42±82.67 [b]	>2000	>2000
	HAWE	1467.76±165.98 [b]	>2000	>2000
I. obliquus	CWE	700.95±28.30 [a]	>2000	1401.32±119.57 [b]
	HAWE	>2000	>2000	>2000
P. linteus	CWE	1980.12±21.41 [c]	1110.21±48.2 [c]	>2000
	HAWE	>2000	>2000	>2000
M. purpureus	CWE	>2000	>2000	>2000
	HAWE	>2000	>2000	>2000

IC_{50} (μg/mL) was determined using MTT assay. The data are presented as mean±SD (n=9). Different superscript letters (a-c) in the same column denote significant differences ($p<0.05$). The comparison of IC_{50} values between the fungal extracts among the same cell line was analysed using one-way ANOVA followed by Tukey's HSD post hoc test (A549, MCF-7) and student's T test (MDA-MB-23). CWE=cold water extract, HAWE=heat-assisted water extract