*Article*

# Evaluation of Two Extraction Methods for the Analysis of Hydrophilic Low Molecular Weight Compounds from *Ganoderma lucidum* Spores and Antiproliferative Activity on Human Cell Lines

**Maria Michela Salvatore** [1,†] , **Vincenza De Gregorio** [2,3,†] , **Monica Gallo** [4] ,
**Maria Michela Corsaro** [1] , **Angela Casillo** [1] , **Raffaele Vecchione** [3,*] , **Anna Andolfi** [1,*] ,
**Daniele Naviglio** [1,‡] and **Paolo Antonio Netti** [2,3,5,‡]

1   Department of Chemical Sciences, University of Naples Federico II, 80126 Naples, Italy;
    mariamichela.salvatore@unina.it (M.M.S.); corsaro@unina.it (M.M.C.); angela.casillo@unina.it (A.C.);
    naviglio@unina.it (D.N.)
2   Interdisciplinary Research Centre on Biomaterials (CRIB), University of Naples Federico II, 80125 Naples,
    Italy; vincenza.degregorio@unina.it (V.D.G.); nettipa@unina.it (P.A.N.)
3   Center for Advanced Biomaterials for Health Care @ CRIB, Istituto Italiano di Tecnologia, 80125 Naples, Italy
4   Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II,
    80131 Naples, Italy; mongallo@unina.it
5   Department of Chemical, Materials and Industrial Production Engineering (DICMAPI) University of Naples
    Federico II, 80125 Naples, Italy
*   Correspondence: raffaele.vecchione@iit.it (R.V.); andolfi@unina.it (A.A.); Tel.: +39-081-2539179 (A.A.)
†   These authors contributed equally to this work.
‡   These authors contributed equally to this work.

check for updates

**Featured Application: In this study two extraction methods for *Ganoderma lucidum* spores were evaluated for the potential use of extracted compounds in cancer treatments. Our findings showed that the innovative Rapid Solid Liquid Dynamic Extraction (RSLDE) permits the successful extraction of compounds with antiproliferative activity against human cell lines**

**Abstract:** Background: The genus Ganoderma includes about 80 species of mushrooms. *Ganoderma lucidum* is the best-known fungal species in mycotherapy and likely has the highest number of studies. Numerous bioactive compounds seem to be responsible for its beneficial effects; in particular, triterpenes, peptidoglycans and polysaccharides are the main physiologically active constituents. The aim of the present work is to identify the main bioactive components in aqueous extracts of *G. lucidum* spores obtained by two different extraction processes. Methods: The spores were at first extracted by a Soxhlet apparatus with *n*-hexane and the aqueous solutions were submitted to two different procedures: hot water extraction (HWE) and rapid solid-liquid dynamic extraction (RSLDE) using the Naviglio extractor. The extracts were then dialyzed to separate the compounds with higher molecular weight from polar compounds with lower molecular weight. The aqueous extracts and dialyzed fractions were tested on two human cell lines: human colonic epithelial cells (Caco-2) and human colorectal carcinoma cells (HTC-116). Results: GC-MS and NMR data revealed the presence of a mixture of glucose and mannitol in ratio 3.7:1 in the most active fraction. Conclusions: The outside dialysis phase of RSLDE extract seems to be particularly cytotoxic for HTC-116 and, interestingly, solutions with different concentrations of pure mannitol showed cytotoxic activity against this cell line too, although to a lesser extent.

**Keywords:** spores; extraction techniques; RSLDE; mannitol; NMR; antiproliferative activity; GC-MS

## 1. Introduction

Since the seventeenth century, the growing interest in mushrooms and their use for medicinal purposes has led to the birth of a new science, mycotherapy. Recent research has highlighted the potential activity of mushrooms for the treatment of various human diseases due to their valuable proprieties, such as antioxidant, antimicrobial, anti-inflammatory and immunomodulatory activities [1,2]. In particular, the data from recent in vitro and in vivo studies demonstrate promising anti-cancer effects on many forms of cancer, both in supportive therapies, to reduce side effects, and directly involved as curative agents [3,4].

*Ganoderma lucidum* is a saprophytic mushroom, belonging to the phylum of the basidiomycetes, which grows on oak and chestnut trees. It has been used since ancient times in traditional Chinese medicine and it is currently increasingly known in the West for its beneficial effects on health [5]. *G. lucidum*, like all basidiomycetes, consists of three main components: spores, mycelium and fruiting body. Numerous studies have shown that *G. lucidum* contains various pharmacologically active substances, such as proteins, polysaccharides, triterpenes, nucleosides, triglycerides and mineral salts, which regulate numerous physiological functions and contrast diseases such as hypertension, thrombosis, allergies, as well as liver and cardiovascular problems [6–9]. Currently, most therapeutic formulations mainly involve the use of the *G. lucidum* fruiting body and mycelium. Emphasis on *G. lucidum* spores became more pronounced when findings showed that they may be the best source of bioactive compounds [10–12]. In this respect, in recent years the scientific community has focused on the improvement of strategies to extract and identify compounds from spores.

The spores of *G. lucidum*, unlike the cells in the other parts of the fungus, are protected by a double wall of chitin, which prevents correct digestion by the human being and correct absorption of the macro and the micronutrients present in the spores. For this reason, it is necessary to break the capsule before marketing and using spores for the extraction of bioactive metabolites [13]. In particular, the present work focuses on the development of a strategy to obtain extracts with antiproliferative activity against human cancer cells and on the study of the main hydrophilic bioactive components of *G. lucidum* spores. To achieve these goals, two different extraction procedures, based on hot water extraction (HWE) and rapid solid-liquid dynamic extraction (RSLDE) at room temperature, were compared. The aqueous extracts and dialyzed fractions were tested on two human cell lines: human colonic epithelial cells (Caco-2) and human colorectal carcinoma cells (HTC-116). The main components of the most active fraction were identified by GC-MS and chemical and spectroscopic techniques. Further investigations showed that the extract is more active on cancer cells than the pure main component within the extract, identified as mannitol, at the same concentration.

## 2. Materials and Methods

### 2.1. General Experimental Procedures

NMR spectra were recorded at 600 MHz in $D_2O$ at 298 K on a Bruker AVANCE 600 MHz spectrometer (Milan, Italy), by using external acetone ($\delta_H$ 2.225 ppm) as references. All reagents and solvents were analytical grade and purchased from Carlo Erba (Milan, Italy), Sigma-Aldrich (Saint Louis, MO, USA), and Fluka (Buchs, Switzerland).

The phenol assay, used for the qualitative analysis of the presence of sugars in the aqueous phase extracts, was carried out based on the method of Dubois et al. [14].

### 2.2. Fungal Material

The spores of *G. lucidum* were supplied by the Italian Way (San Bernardino Verbano, Italy) and cultivated using the traditional method within the Da Xing An Ling natural park region, in China. The manufacturing company guaranteed 95% spore opening.

### 2.3. Extraction of the Hydrophilic Component

#### 2.3.1. Defatting of *Ganoderma lucidum* Spores

Spores of *G. lucidum* were defatted by *n*-hexane in a Soxhlet apparatus. In particular, 100 g of spores were placed in a thimble of filter paper and extracted with 450 mL of *n*-hexane for 18 h for a total of 720 cycles. At the end, the organic phase was filtered and evaporated under reduced pressure. The experiment was carried out in duplicate. The extract weight thus obtained was 32.650 ± 0.236 g with a yield of 32.65%.

#### 2.3.2. Hot Water Extraction (HWE)

First, 20 g of the defatted spores (Section 2.3.1) were subjected to extraction in 100 mL of demineralized $H_2O$ at 90 °C for 2 h under magnetic stirring. Subsequently the mixture was cooled, centrifuged at 6000 rpm for 20 min at room temperature to the separate powders and, finally the water phase lyophilized. The experiment was carried out in duplicate. The extract weight thus obtained was 0.6293 ± 0.0365 g with a yield of 3.15%.

#### 2.3.3. Rapid Solid-Liquid Dynamic Extraction (RSLDE) by Naviglio Extractor

Then, 40 g of the defatted spores (Section 2.3.1) were submitted to rapid solid-liquid dynamic extraction (RSLDE) with demineralized $H_2O$ for 2 h. The extraction was carried out using a Naviglio extractor series LAB mod. 500 (Atlas Filtri Engineering s.r.l, Padova, Italy), with a static phase of 2 min and a dynamic phase of 2 min (20 s $cycle^{-1}$), pressure 8 bar, at room temperature. To overcome problems caused by compacting samples, Pyrex® solid glass beads (Sigma-Aldrich, Saint Louis, MO, USA) were employed during the RSLDE. The extract was centrifuged at 6000 rpm for 20 min at room temperature and finally lyophilized. The experiment was carried out in duplicate. The extract weight thus obtained was 0.7466 ± 0.0598 g with a yield of 1.87%.

#### 2.3.4. Dialysis of Aqueous Extracts

Aqueous extracts (10 mL) obtained by HWE and via RSLDE were dialyzed. For dialysis, tubes with a cut-off of 3500 Da were used (Spectra/Por Dialysis Membrane, Spectrum Laboratories, Rancho Dominguez, CA, USA) against deionized water, performing 5 changes of 100 mL each. The outside aqueous phases (OUT) of dialysis were collected and lyophilized, as well as the internal phase (IN) of the dialysis tube for both HWE and RSLDE extracts. The weights of samples from dialysis OUT ($HWE_{OUT}$ and $RSLDE_{OUT}$) and IN ($HWE_{IN}$ and $RSLDE_{IN}$) were $HWE_{OUT}$ = 0.049 g ± 0.012 g (yield = 0.049%), $HWE_{IN}$ = 0.208 g ± 0.035 g (yield = 0.208%), $RSLDE_{OUT}$ = 0.350 g ± 0.047 g (yield = 0.350%), and $RSLDE_{IN}$ = 0.080 g ± 0.016 g (yield = 0.080%).

#### 2.3.5. Sugar Analysis

The glycosyl analysis was performed as already reported [15]. Briefly, sample (0.5 mg) was mixed with 1 mL of 2M $HCl/CH_3OH$, subjected to methanolysis for 16 h at 80 °C, and then acetylated. The obtained acetylated methyl glycosides were analyzed by GC-MS using an Agilent Technologies gas chromatograph 7820A equipped with a mass selective detector 5977 and a HP-5ms capillary column (Agilent, Italy 30 m × 0.25 mm i.d., flow rate 1 mL $min^{-1}$, He as carrier gas). The acetylated methyl glycosides were analyzed using the following temperature program: 140 °C for 3 min, and 140 °C → 240 °C at 3 °C $min^{-1}$.

### 2.4. Assays of Anticancer Activity

Human colonic epithelial cell line (Caco-2, non-invasive phenotype) was obtained from American Type Cell Culture (ATCC, Manassas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Dublin, Ireland) with the addition of 10% fetal bovine serum (FBS, Sigma Aldrich),

100 µg mL$^{-1}$ of L-glutamine (Gibco), and 100 IU mL$^{-1}$ of streptomycin/penicillin (Gibco) at 37 °C with 5% $CO_2$. Human colorectal carcinoma cell lines (HTC-116, invasive phenotype) were grown in Roswell Park Memorial Institute (RPMI) 1640 Medium (ATCC, USA) and supplemented with FBS (Sigma Aldrich), 100 µg mL$^{-1}$ of L-glutamine (Gibco), and 100 IU mL$^{-1}$ of streptomycin/penicillin (Gibco) at 37 °C with 5% $CO_2$. To assess cell viability, the cells were seeded in 96-well micro-plates, starved in 2% FBS-media, briefly rinsed and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) test was performed according to the manufacturer's instructions (Dojindo Molecular Technologies Inc., Rockville, MD, USA). The optical density of the samples in each well was measured by reading at 570 nm with a microplate spectrophotometer (Perkin Elmer 2300 Enspireplate reader, Waltham, MA, USA). Tests were performed at a concentration of 1.0 mg mL$^{-1}$, 2.5 mg mL$^{-1}$, and 5.0 mg mL$^{-1}$ and with exposure for 24 and 48 h. For a subset of experiments, both Caco-2 and HCT116 were loaded with RSLDE$_{OUT}$ or RSLDE$_{IN}$ and HWE$_{OUT}$ and HWE$_{IN}$ at a concentration of 5 mg mL$^{-1}$ or with mannitol solution (Sigma-Aldrich) at different concentrations (0, 0.6, 1, 1.2, 2.43, 4.5, 9.0 mg mL$^{-1}$) with exposure for 24 and 48 h, and the cell viability was assayed. All the analyses were carried out in triplicate.

### 2.5. Statistical Analysis

The results of the viability assay were expressed as the mean ± standard deviation (SD) from three independent experiments ($n = 3$). All results were then statistically analyzed by the Student's *t*-test. The differences between two or more groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post-test. Statistical significance was set at a value of $p < 0.05$.

## 3. Results

The spores of *Ganoderma lucidum* were at first defatted in a Soxhlet apparatus with *n*-hexane. The overall strategy employed in this work has been schematically reported in Figure 1.
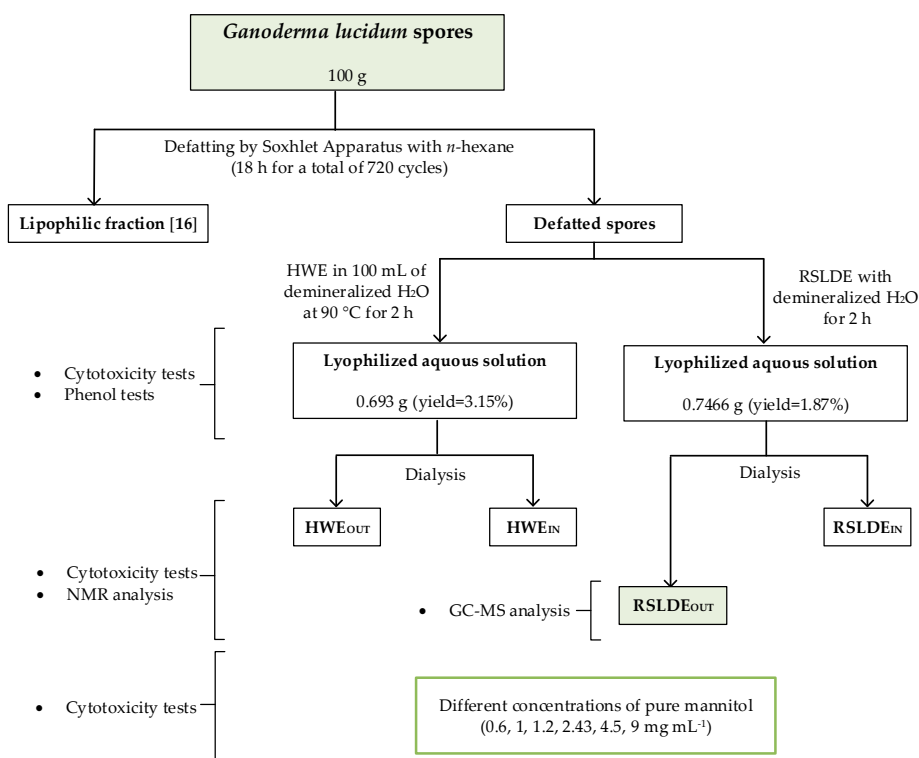


**Figure 1.** Schematic representation of the analysis performed on the defatted spores of *G. lucidum*.

The lipophilic fraction was analyzed via NMR and GC-MS, and the results of an accurate identification and quantification of the lipid content were previously reported [16]. Defatted spores were split into two fractions: one fraction was subjected to extraction with hot water (HWE), and the other fraction was extracted by rapid solid-liquid dynamic extraction (RSLDE). The phenol test was carried out on each of these extracts to qualitatively highlight the presence of sugars, obtaining a positive response in both cases.

Since *G. lucidum* preparations have been used in the treatment of diverse human diseases [17], showing beneficial influences on cancer patients [18], the effect of the extracts on cell viability was evaluated on two different human cell lines. In detail, the HWE and RSLDE extracts (Figure 2) did not evidence the ability to interfere with Caco-2 cells (non-invasive) viability at different concentrations and at two different time points tested. On the other hand, a strong reduction in cell viability was reported for HTC-116 (invasive), indicating the aptitude, for both HWE and RSLDE extracts, to affect the cancer cell viability with more pronounced activity using 5.0 mg mL$^{-1}$ of lyophilized extract at 48 h of incubation time (Figure 2B,D). Furthermore, viability assays also revealed that the extracts from RSLDE strongly reduced the cell viability of HCT-116 cells in a dose- and time-dependent manner (Figure 2C,D). At last, by comparing the treatment of HWE and RSLDE methods on HCT-116 cells, the best results were highlighted after extract treatment from RSLDE, with a reduction of 59% in cell viability at 5 mg mL$^{-1}$ after 48 h exposure (Figure 2D).
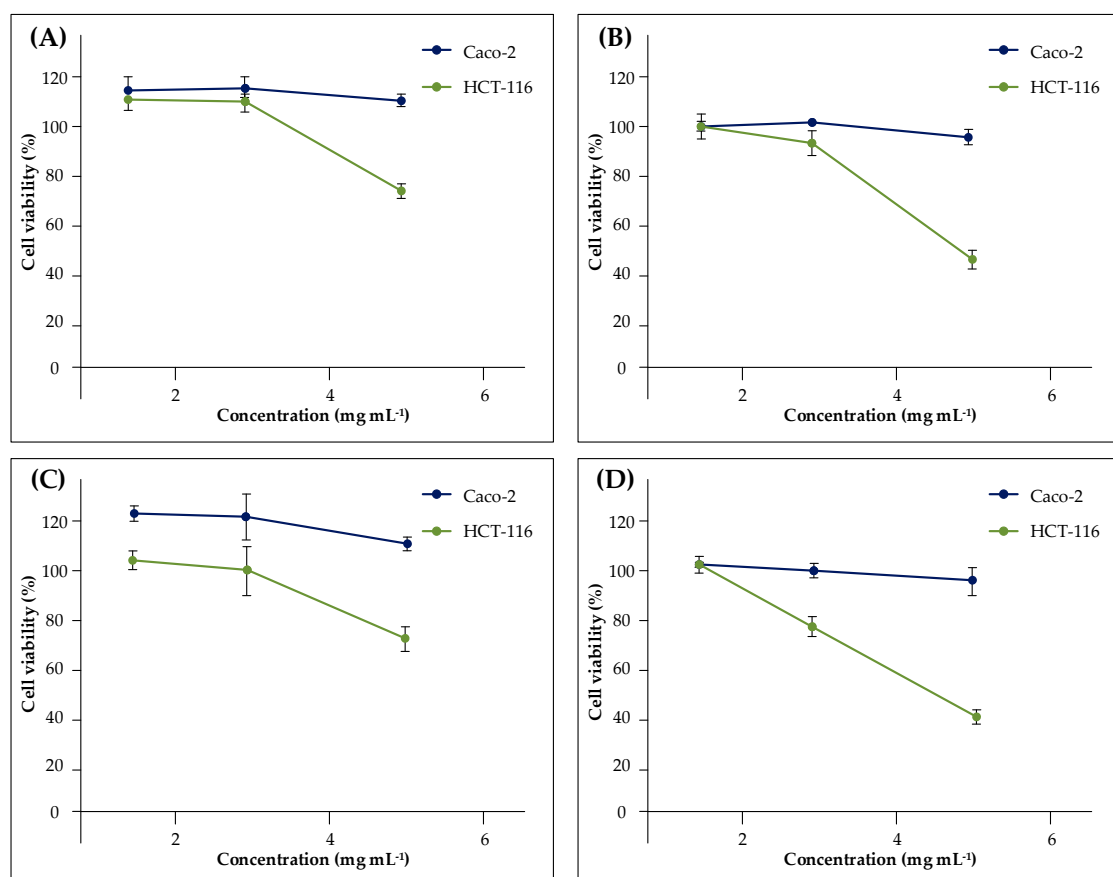


**Figure 2.** Cell viability assay on Caco-2 (non-invasive) blue line and HCT-116 (invasive) green line after treatment with aqueous extracts obtained by hot water extraction (HWE; **A**,**B**) and rapid solid-liquid dynamic extraction (RSLDE; **C**,**D**) at different concentrations. The response was observed after (**A**,**C**) 24 and (**B**,**D**) 48 h exposure.

The extracts obtained from HWE and RSLDE were then dialyzed in order to separate the compounds with higher molecular weight, such as the polysaccharides, from compounds with lower molecular weight, as described in Section 2 (called "$HWE_N$, $HWE_{OUT}$, $RSLDE_{IN}$ and $RSLDE_{OUT}$" from this point forward). The samples were freeze-dried and tested to determine the carbohydrates content. All the extracts resulted to contain sugars. According to the positive phenol assay response, the $^1$H NMR spectra revealed in all the samples the presence of signals attributable to carbohydrates. However, the sharpness of signals in the $HWE_{IN}$ and $RSLDE_{IN}$ were attributable to free monosaccharides (Figure 3B,D), whereas the broadening of both anomeric and carbinolic signals in the $HWE_{IN}$ and $RSLDE_{IN}$ suggested the presence of polysaccharides (Figure 3A,C).
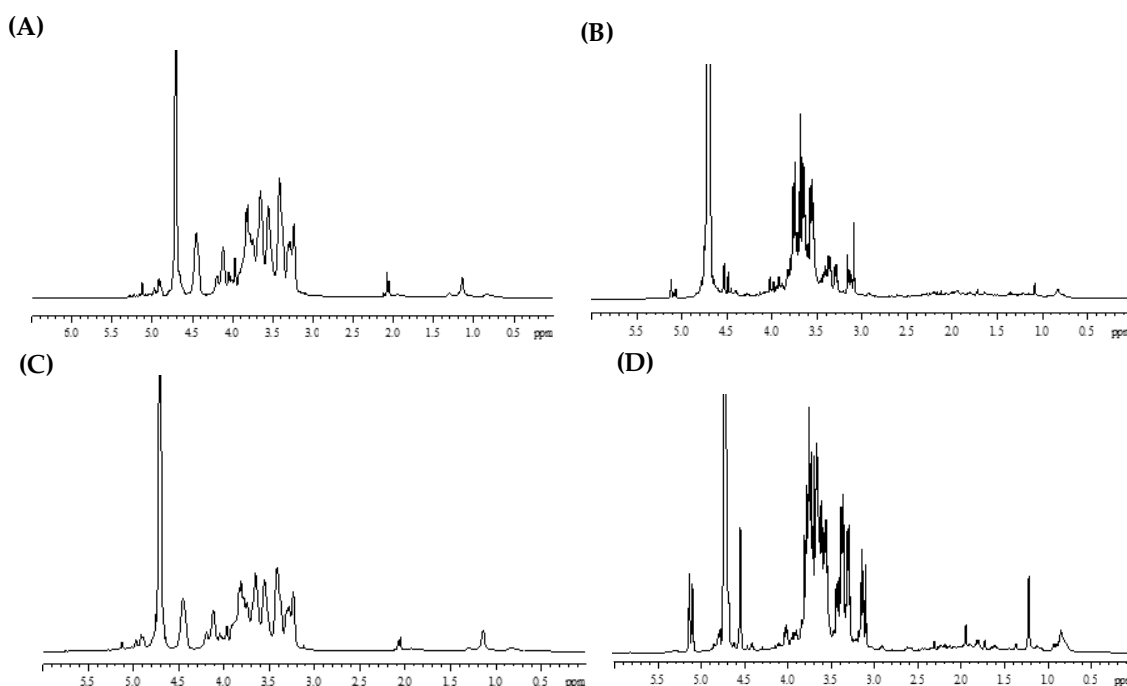


**Figure 3.** $^1$H NMR spectra of dialyzed extracts from hot water extraction (HWE) and rapid solid-liquid dynamic extraction (RSLDE): $HWE_{IN}$ (**A**), $HWE_{OUT}$ (**B**), $RSLDE_{IN}$ (**C**), and $RSLDE_{OUT}$ (**D**).

Cytotoxicity tests of $RSLDE_{IN}$ and $RSLDE_{OUT}$ showed no inhibitory or proliferative activity for non-invasive cell lines (Caco-2) and slight inhibition of cell viability especially for the $RSLDE_{OUT}$ after 48 h. The results of the MTT test for the $RSLDE_{OUT}$ after 48 h showed a significant reduction in cell viability (78%) for invasive cell line (HTC-116). HCT-116 displayed a reduction in cell viability of 66% when treated for 48 h with $RSLDE_{IN+OUT}$ which is a combination of the $RSLDE_{OUT}$ and $RSLDE_{IN}$, as reported in Figure S1. For $HWE_{OUT}$ a strong reduction in Caco-2 cell viability after 48 h was found, indicating that the fractions analyzed induce the epithelial cell injury. Comparing RSLDE and HWE dialyzed samples the best results on HCT-116 viability inhibition are shown on $RSLDE_{OUT}$ (Figure 4).

$RSLDE_{OUT}$ was identified via GC-MS as a mixture of glucose and mannitol (ratio 3.7:1), from the typical electron ionization (EI) patterns and from the retention times of authentic standards (Figure 5).

The $^1$H NMR confirmed the occurrence of only one free reducing monosaccharide together with an alditol sugar, since only two anomeric signals at δ 5.10 ppm (α-glucose) and at δ 4.65 ppm (β-glucose) were found (Figure 3D).

Based on the concept that glucose per se does not significantly interfere with cell viability, except for concentrations beyond 20 mg mL$^{-1}$ [19], and considering the ratio between glucose and mannitol in $RSLDE_{OUT}$ (ratio 3.7: 1), additional MTT tests on both cell lines treated with different concentrations (0, 0.6, 1.0, 1.2, 2.43, 4.5, 9.0 mg mL$^{-1}$) of commercially available mannitol were performed at 24 and 48 h. The cell viability assays had the same trend on both cell samples after 24 and 48 h exposure.

In fact, significant cytotoxic activity was observed on Caco-2 cells up to 1.2 mg mL$^{-1}$ of mannitol whereas, between 1.0 and 1.2 mg mL$^{-1}$ of mannitol, there was a significant reduction in HCT-116 cell viability (around 40%) after 48 h exposure compared to the viability of Caco-2 treated with the same concentration of mannitol (Figure 6). This range 1.0–1.2 mg mL$^{-1}$ is of interest since it corresponds to the amount of mannitol contained (1.1 mg mL$^{-1}$) in the RSLDE$_{OUT}$.
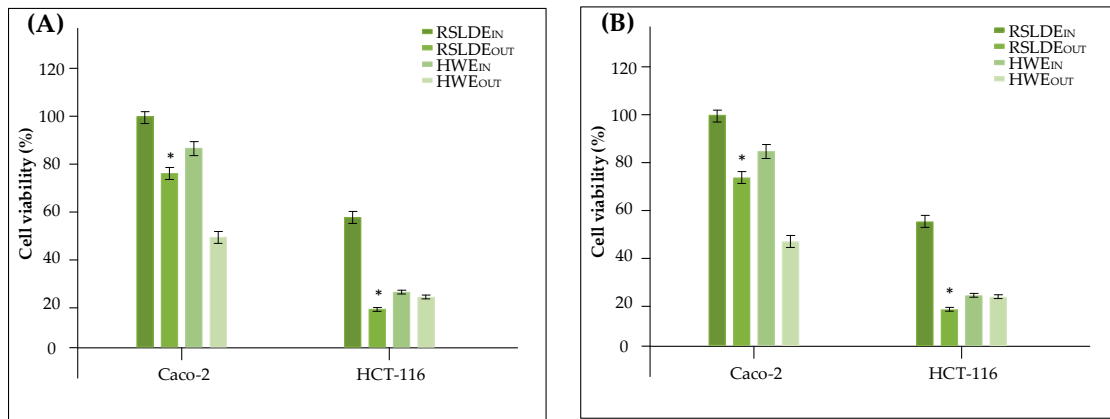


**Figure 4.** Comparison of proliferative activities on Caco-2 (non-invasive) and HCT-116 (invasive) cell lines of the dialyzed extracts from hot water extraction (HWE$_{IN}$ and HWE$_{OUT}$) and rapid solid-liquid dynamic extraction (RSLDE$_{IN}$ and RSLDE$_{OUT}$) after 24 (**A**) and 48 h(**B**) exposure (* $p < 0.05$).
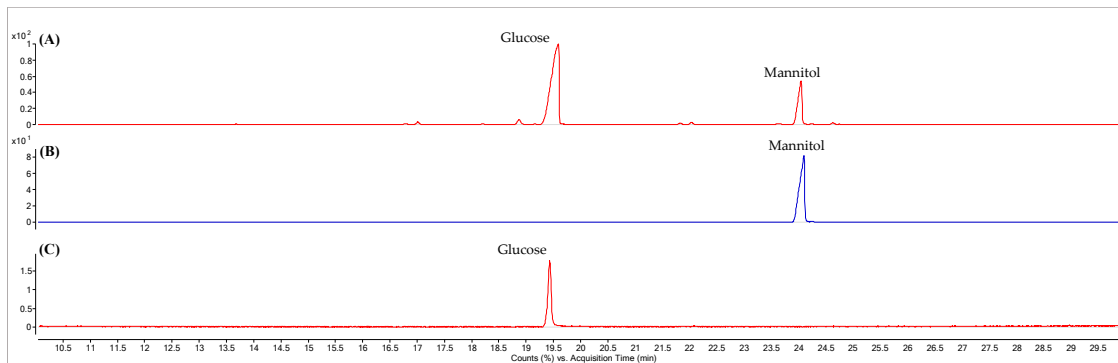


**Figure 5.** GC-MS chromatograms of acetylated methyl glycosides from RSLDE$_{OUT}$ (**A**); acetylated mannitol (**B**); acetylated methyl glycoside of glucose (**C**).
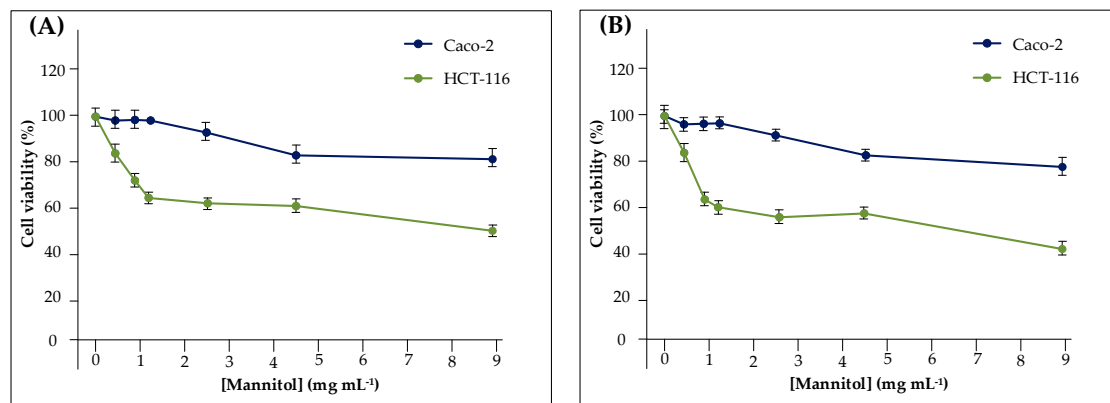


**Figure 6.** Caco-2 and HCT-116 cytotoxicity under different mannitol concentrations (0, 1.0, 1.2, 2.43, 4.5, 9.0 mg mL$^{-1}$). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) cell viability after 24 (**A**) and 48 h (**B**) exposure.

From Figure 7 it can be seen that $RSLDE_{OUT}$ is more effective on cancer cells than pure mannitol at the same concentration.
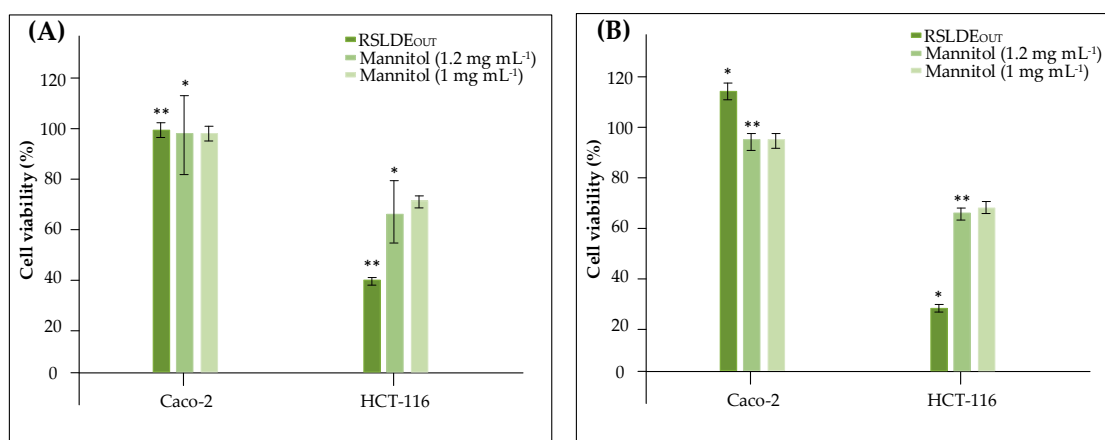


**Figure 7.** Comparison of proliferative activities on Caco-2 (non-proliferative) and HCT-116 (proliferative) cell lines of $RSLDE_{OUT}$ (containing 1.1 mg mL$^{-1}$ of mannitol) and the mannitol concentrations at 1 and 1.2 mg mL$^{-1}$ after 24 (**A**) and 48 h (**B**) exposure (* $p < 0.05$, ** $p < 0.01$).

## 4. Discussion

*Ganoderma lucidum* spores are a rich source of bioactive compounds which are known to help in improving human health [20,21]. In the last decades, their possible implication for cancer prevention and treatment has stimulated a huge research activity concerning the exploitation of this part of *G. lucidum* for medical purposes [11,22–24]. For this reason, many extraction techniques were employed to obtain the maximum yield of bioactive compounds from *G. lucidum* [25].

In the present study, two strategies were compared to determine the best way to obtain an extract with antiproliferative activity against human cancer cells from *G. lucidum* spores (Figure 1). Hence, the sporoderm-broken spores of *G. lucidum* were extracted with a Soxhlet apparatus in *n*-hexane. The lipophilic fraction was analyzed via NMR and GC-MS [16], while the exhausted material was further processed. In particular, it was split into two fractions, one of which was extracted by hot water extraction (HWE) and the other fraction by rapid solid-liquid dynamic extraction (RSLDE) in order to compare a common with an innovative extraction technique. As it is known, this latter method exploits an extraction principle that allows the optimization of diffusion and osmosis phenomena, increasing the yield and reducing the extraction times compared to the classic extraction phenomena [26]. Using this principle, RSLDE allows to extract at room temperature without the diffusion effect due to temperature [27]. Furthermore, in this study RSLDE was optimized to extract the spores of *G. lucidum* adding Pyrex® solid glass beads in order to avoid the excessive sample compaction during the extraction. Cytotoxicity tests on HWE and RSLDE extracts were performed on two human cancer cell lines: Caco-2 cells show poor tumorigenic or non-tumorigenic features with a non-invasive phenotype [28] and HCT-116 cells display a high invasive ability in vitro [29]. The Caco-2 cells viability was not significantly affected by the exposure of the HWE and RSLDE extracts, but a strong dose- and time-dependent reduction in cell viability was observed for HTC-116. These interesting results triggered us to study the composition of the extracts.

The extracts were dialyzed to separate compounds with higher molecular weight from hydrophilic compounds with lower molecular weight. The outside aqueous phases (OUT) of dialysis, as well as the internal phases (IN) of the dialysis tube for both HWE and RSLDE extracts were analyzed via NMR and also evaluated for toxicity on Caco-2 and HCT-116. In particular, among the fractions analyzed, a high reduction in cell viability for Caco-2 cells was shown by the exposure of $HWE_{OUT}$, probably due to the presence of some low molecular weight products that were extracted from thermal degradation. On the other hand, the most active fraction on HTC-116 is the outside dialysis phase of RSLDE extract

which was found to be a mixture of glucose and mannitol in ratio 3.7:1. Mannitol is responsible of several functions in many organisms, such as protection from osmotic, saline, and oxidative stress [30]. In fact, a few in vivo studies have investigated the effects of mannitol as a free radical scavenger [31].

Considering that glucose is not a cytotoxic compound [19], some solutions of different concentrations of commercially available mannitol were tested on both cell lines. In addition, HTC-116 cells seem to be more sensitive than Caco-2 and, interestingly, the outside dialysis phase of RSLDE extract was more active on cancer cells than pure mannitol at the same concentration. In fact, this fraction might also contain a series of minor compounds which in very little amount are important in providing a cumulative or synergistic effect which determine the inhibition of HCT-116 cells.

Our findings show that hydrophilic compounds with low molecular weight play a crucial role in the bioactivity of *G. lucidum*. The strategy employed for the extraction of *G. lucidum* is a decisive factor to obtain a mixture of not altered bioactive components and, not surprisingly, the best extraction conditions were provided by RSLDE. In fact, the application of this technique permits the extraction without using high temperature and it has already been successfully applied in food, cosmetic, and resource recovery fields [32–35].

## 5. Conclusions

This work allowed to highlight the selective antiproliferative activity on the HCT-116 cell line of the most polar component with low molecular weight present in the spores of *G. lucidum* and obtained by RSLDE. In particular, this extract resulted essentially in a mixture of glucose:mannitol (3.7:1). Therefore, the results obtained confirm that *G. lucidum* extracts have beneficial effects against various pathologies, including cancer. However, most of the known data on the benefits of *G. lucidum* are based on laboratory and preclinical studies, while few clinical studies have been conducted. Consequently, further studies are needed to evaluate the real potential of this fungus and its components and to allow their use to be addressed as potential chemopreventive and/or therapeutic agents.

## References

1.  Wasser, S.P. Medicinal mushrooms in human clinical studies. Part I. Anticancer, oncoimmunological, and immunomodulatory activities: A review. *Int. J. Med. Mushrooms* **2017**, *19*, 279–317. [CrossRef] [PubMed]

2.  Thu, Z.M.; Myo, K.K.; Aung, H.T.; Clericuzio, M.; Armijos, C.; Vidari, G. Bioactive phytochemical constituents of wild edible mushrooms from southeast Asia. *Molecules* **2020**, *25*, 1972. [CrossRef] [PubMed]

3.  Ivanova, T.S.; Krupodorova, T.A.; Barshteyn, V.Y.; Artamonova, A.B.; Shlyakhovenko, V.A. Anticancer substances of mushroom origin. *Exp. Oncol.* **2014**, *36*, 58–66. [PubMed]

4.  Badalyan, S.M.; Barkhudaryan, A.; Rapior, S. Recent progress in research on the pharmacological potential of mushrooms and prospects for their clinical application. In *Medicinal Mushrooms*; Springer: Singapore, 2019; pp. 1–70. ISBN 978-981-13-6381-8.

5.  Wang, P.Y.; Zhu, X.L.; Lin, Z.B. Antitumor and immunomodulatory effects of polysaccharides from broken-spore of *Ganoderma lucidum. Front. Pharmacol.* **2012**, *3*, 135. [CrossRef]

6.    Atay, S.; Ak, H.; Kalmis, E.; Kayalar, H.; Aydin, H.H. Diverse effects of the lingzhi or reishi medicinal mushroom, *Ganoderma lucidum* (Agaricomycetes), in combination with tamoxifen citrate and doxorubicin in MCF-7 breast cancer cells. *Int. J. Med. Mushrooms* **2016**, *18*. [CrossRef]

7.    Yang, Y.; Nirmagustina, D.E.; Kumrungsee, T.; Okazaki, Y.; Tomotake, H.; Kato, N. Feeding of the water extract from *Ganoderma lingzhi* to rats modulates secondary bile acids, intestinal microflora, mucins, and propionate important to colon cancer. *Biosci. Biotechnol. Biochem.* **2017**, *81*, 1796–1804. [CrossRef]

8.    Veena, R.K.; Ajith, T.A.; Janardhanan, K.K. Lingzhi or reishi medicinal mushroom, *Ganoderma lucidum* (Agaricomycetes), prevents doxorubicin-induced cardiotoxicity in rats. *Int. J. Med. Mushrooms* **2018**, *20*. [CrossRef]

9.    Sohretoglu, D.; Huang, S. *Ganoderma lucidum* polysaccharides as an anti-cancer agent. *Anticancer Agents Med. Chem.* **2018**, *18*, 667–674. [CrossRef]

10.   Min, B.S.; Nakamura, N.; Miyashiro, H.; Bae, K.W.; Hattori, M. Triterpenes from the spores of *Ganoderma lucidum* and their inhibitory activity against HIV-1 protease. *Chem. Pharm. Bull.* **1998**, *46*, 1607–1612. [CrossRef]

11.   Liu, X.; Yuan, J.P.; Chung, C.K.; Chen, X.J. Antitumor activity of the sporoderm-broken germinating spores of Ganoderma lucidum. *Cancer Lett.* **2002**, *182*, 155–161. [CrossRef]

12.   Liu, X.; Xu, S.P.; Wang, J.H.; Yuan, J.P.; Guo, L.X.; Li, X.; Huang, X.N. Characterization of ganoderma spore lipid by stable carbon isotope analysis: Implications for authentication. *Anal. Bioanal. Chem.* **2007**, *388*, 723–731. [CrossRef]

13.   Xu, J.; Li, P. Researches and Application of *Ganoderma* Spores Powder. In *Ganoderma and Health*; Springer: Singapore, 2019; pp. 157–186. ISBN 978-981-13-9866-7.

14.   Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.T.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]

15.   Restaino, O.F.; Finamore, R.; Diana, P.; Marseglia, M.; Vitiello, M.; Casillo, A.; Bedini, E.; Corsaro, M.M.; Parrilli, M.; Trifuoggi, M.; et al. A multi-analytical approach to better assess the keratan sulfate contamination in animal origin chondroitin sulfate. *Anal. Chim. Acta* **2017**, *958*, 59–70. [CrossRef]

16.   Salvatore, M.M.; Elvetico, A.; Gallo, M.; Salvatore, F.; DellaGreca, M.; Naviglio, D.; Andolfi, A. Fatty acids from *Ganoderma lucidum* spores: Extraction, identification and quantification. *Appl. Sci.* **2020**, *10*, 3907. [CrossRef]

17.   Bishop, K.S.; Kao, C.H.; Xu, Y.; Glucina, M.P.; Paterson, R.R.M.; Ferguson, L.R. From 2000 years of *Ganoderma lucidum* to recent developments in nutraceuticals. *Phytochemistry* **2015**, *114*, 56–65. [CrossRef]

18.   Boh, B. *Ganoderma lucidum*: A potential for biotechnological production of anti-cancer and immunomodulatory drugs. *Recent Pat. Anticancer Drug Discov.* **2013**, *8*, 255–287. [CrossRef]

19.   Shahveisi, K.; Mousavi, S.H.; Hosseini, M.; Rad, A.K.; Jalali, S.A.; Rajaei, Z.; Sadeghnia, H.R.; Hadjzadeh, M.A.R. The role of local renin-angiotensin system on high glucose-induced cell toxicity, apoptosis and reactive oxygen species production in PC12 cells. *Iran. J. Basic Med. Sci.* **2014**, *17*, 613.

20.   Baby, S.; Johnson, A.J.; Govindan, B. Secondary metabolites from Ganoderma. *Phytochemistry* **2015**, *114*, 66–101. [CrossRef]

21.   Ahmad, M.F. *Ganoderma lucidum*: Persuasive biologically active constituents and their health endorsement. *Biomedicine* **2018**, *107*, 507–519. [CrossRef]

22.   Gao, Y.; Gao, H.; Chan, E.; Tang, W.; Xu, A.; Yang, H.; Huang, M.; Lan, J.; Li, X.; Duan, W.; et al. Antitumor activity and underlying mechanisms of ganopoly, the refined polysaccharides extracted from *Ganoderma lucidum*, in mice. *Immunol. Investig.* **2005**, *34*, 171–198. [CrossRef]

23.   Wang, X.C.; Xi, R.J.; Li, Y.; Wang, D.M.; Yao, Y.J. The species identity of the widely cultivated Ganoderma, 'G. lucidum' (Ling-zhi), in China. *PLoS ONE* **2012**, *7*, e40857. [CrossRef]

24.   Sharma, C.; Bhardwaj, N.; Sharma, A.; Tuli, H.S.; Katyal, P.; Beniwal, V.; Gupta, G.K.; Sharma, A.K. Bioactive metabolites of *Ganoderma lucidum*: Factors, mechanism and broad spectrum therapeutic potential. *J. Herb. Med.* **2019**, *17–18*, 100268. [CrossRef]

25.   Huang, S.Q.; Li, J.W.; Wang, Z.; Pan, H.X.; Chen, J.X.; Ning, Z.X. Optimization of alkaline extraction of polysaccharides from *Ganoderma lucidum* and their effect on immune function in mice. *Molecules* **2010**, *15*, 3694–3708. [CrossRef] [PubMed]

26.   Naviglio, D. Naviglio's principle and presentation of an innovative solid–liquid extraction technology: Extractor Naviglio®. *Anal. Lett.* **2003**, *36*, 1647–1659. [CrossRef]

27.   Naviglio, D.; Scarano, P.; Ciaravolo, M.; Gallo, M. Rapid Solid-Liquid Dynamic Extraction (RSLDE): A powerful and greener alternative to the latest solid-liquid extraction techniques. *Foods* **2019**, *8*, 245. [CrossRef] [PubMed]

28. De Vries, J.E.; Dinjens, W.N.M.; De Bruyne, G.K.; Verspaget, H.W.; Van Der Linden, E.P.M.; De Bruine, A.P.; Mareel, M.M.; Bosman, F.T.; Ten Kate, J. In vivo and in vitro invasion in relation to phenotypic characteristics of human colorectal carcinoma cells. *Br. J. Cancer* **1995**, *71*, 271–277. [CrossRef] [PubMed]

29. Rajput, A.; San Martin, I.D.; Rose, R.; Beko, A.; LeVea, C.; Sharratt, E.; Mazurchuk, R.; Hoffman, R.M.; Brattain, M.G.; Wang, J. Characterization of HCT116 human colon cancer cells in an orthotopic model. *J. Surg. Res.* **2008**, *147*, 276–281. [CrossRef]

30. Patel, T.K.; Williamson, J.D. Mannitol in plants, fungi, and plant–fungal interactions. *Trends Plant Sci.* **2016**, *21*, 486–497. [CrossRef]

31. André, P.; Villain, F. Free radical scavenging properties of mannitol and its role as a constituent of hyaluronic acid fillers: A literature review. *Int. J. Cosmet. Sci.* **2017**, *39*, 355–360. [CrossRef]

32. Naviglio, D.; Formato, A.; Vitulano, M.; Cozzolino, I.; Ferrara, L.; Zanoelo, E.F.; Gallo, M. Comparison between the kinetics of conventional maceration and a cyclic pressurization extraction process for the production of lemon liqueur using a numerical model. *J. Food Process Eng.* **2017**, *40*, e12350. [CrossRef]

33. Gallo, M.; Vitulano, M.; Andolfi, A.; DellaGreca, M.; Naviglio, D. Rapid solid-liquid dynamic extraction (RSLDE), a new rapid and greener method to extract two steviol glycosides (stevioside and rebaudioside a) from stevia leaves. *Plant Foods Hum. Nutr.* **2017**, *72*, 141–148. [CrossRef] [PubMed]

34. Salvatore, M.M.; Ciaravolo, M.; Cirino, P.; Toscano, A.; Salvatore, F.; Gallo, M.; Naviglio, D.; Andolfi, A. Fatty Acids from *Paracentrotus lividus* Sea Urchin Shells Obtained via Rapid Solid Liquid Dynamic Extraction (RSLDE). *Separations* **2019**, *6*, 50. [CrossRef]

35. Scarano, P.; Naviglio, D.; Prigioniero, A.; Tartaglia, M.; Postiglione, A.; Sciarrillo, R.; Guarino, C. Sustainability: Obtaining natural dyes from waste matrices using the prickly pear peels of *Opuntia ficus-indica* (L.) Miller. *Agronomy* **2020**, *10*, 528. [CrossRef]