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# Biochemical characterisation and antioxidant activity of mycelium of *Ganoderma lucidum* from Central Italy

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ABSTRACT

*Ganoderma lucidum* species is currently popular and used in the formulation of nutraceuticals and as functional foods, but a broad biochemical characterisation of its mycelium has not yet been reported. In this study new Italian and Chinese isolates, both identified as *G. lucidum,* were molecularly and biochemically characterised and compared. The mycelia differ both in terms of the enzymatic activities and in protein content revealed by 2D-PAGE electrophoretograms. The ethanolic extracts were screened for their possible antioxidant activities using three different tests: chelating activity on $Fe^{2+}$, lipoxygenase assay and DPPH· free radical scavenging. Only a fraction containing the low molecular weight compounds (L) showed antioxidative properties, whereas the soluble intracellular polysaccharides fraction (P) was ineffective. The correlation between total phenol content and scavenging activity on DPPH· assay was also discussed. Increased interest in the identification of natural molecules with good antioxidant properties suggests further investigations into the use of Italian *G. lucidum* in the formulation of nutraceuticals and functional foods.



## 1. Introduction

The fungi of the genus *Ganoderma* are popular medicinal mushrooms, and they have been used widely in China, Japan and Korea over the past two millennia (Sliva, 2006). The most frequently cited species in research publications on the cultivation, chemical analysis, pharmacology, and medicinal effects of *Ganoderma* is the *Ganoderma lucidum* species, also known by the common names Reishi or Mannentake (Japanese) and Ling Zhi (Chinese) (Stamets & Yao, 2002). The major chemical constituents of *G. lucidum* and related species, such as polysaccharides, triterpenes, sterols, lectins and some proteins, have beneficial properties for the prevention and treatment of a variety of ailments. Remarkably, these include very important diseases such as hypertension, diabetes, hepatitis, cancers and AIDS (Paterson, 2006). In addition to its therapeutic effects, the methanolic extracts from *G. lucidum* and *Ganoderma tsugae* also possess antioxidant abilities (Mau, Lin, & Chen, 2002).

Normally, *G. lucidum* is available in the form of mature fruiting bodies, mycelia and fermentation filtrate. The production of fruiting bodies includes a long cultivation in a plastic bag whereas mycelia and fermentation filtrate require a brief submerged fermentation. Hence mycelia and their fermentation filtrate byproduct are alternative or substitute products of mature fruiting bodies. However, both the fruiting bodies and mycelia of *G. lucidum* are currently available in Asia and North America and are mainly prepared for use in the formulation of nutraceuticals and functional foods. Biomedical investigations have been conducted predominately in China, Korea, Japan and the United States and it is unclear why other countries have not got involved in this research (Paterson, 2006). However, in the last few years, several experiments demonstrating the medicinal properties of local *Ganoderma* have been performed in Europe as well. For example, new sesquiterpenoid hydroquinones produced by the European species *Ganoderma pfeifferi* Bres., called ganomycins, inhibit the growth of methicillin-resistant *Staphylococcus aureus* and other bacteria (Mothana, Jansen, Jülich, & Lindequist, 2000). *G. lucidum* isolated from Slovenian forests is able to produce polysaccharides with potential immunostimulatory effects on the induction cytokine (TNF-$\alpha$ and IFN-$\gamma$) synthesis in primary cultures of human mononuclear cells (Berovič et al., 2003).

Despite the continuing search for active principles from the extract of *G. lucidum*, the biochemical characterisation of its mycelium is not available. In this study *G. lucidum* from Central Italy was isolated, phylogenetically affiliated, biochemically characterised and compared to an isolate labelled as *G. lucidum* from China. To this aim ITS1-5.8S-ITS2 sequence analyses, 2D-PAGE technique and enzymatic activity assays were performed. Furthermore, to

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identify novel bioactive molecules, ethanolic extracts were fractionated and the correlation between total phenol content and scavenging activity was examined.

This study suggests that the Italian *G. lucidum* may be a valuable source to be used in the formulation of nutraceuticals and as functional foods.

## 2. Materials and methods

### 2.1. Fungal cultures

The Italian *G. lucidum* mycelium was isolated from Central Italy by Iotti and Zambonelli and stored in the herbarium of Dipartimento di Protezione e Valorizzazione, University of Bologna, Italy (CMI Unibo No. 5108). The Chinese *G. lucidum* mycelium strain No. 0537-8851472 comes from the Fungal Institute of Jinxiang (Shandong province, China).

During the experimental work, the isolates were kept on Petri dishes on Potato Dextrose Agar (PDA, Difco, Italy) at 30 °C and were re-inoculated every 3 weeks to maintain their viability and activity. Isolates were grown in a liquid medium containing: 50 g $l^{-1}$ of glucose, 2.0 g $l^{-1}$ of polypeptone, 2.0 g $l^{-1}$ of yeast extract, 5.0 g $l^{-1}$ of $KH_2PO_4$, 2.5 g $l^{-1}$ of $MgSO_4$ and 10 g $l^{-1}$ of maltose, pH 5.7. Mycelia were cultured in 100 ml flasks, each containing 30 ml of medium inoculated with 1 $cm^2$ cuts of a seven-day-old culture from PDA, and kept in a growth chamber at 30 °C with no agitation. In this growth condition the mycelia developed an aerial agglomerate consisting of a dense mass of white hyphae (90%) and a submerged agglomerate (10%) composed of a solid mucilaginous mass. The two mycelial phases were harvested after 20 days of growth and used for all the experiments reported below.

### 2.2. PCR amplification, DNA sequencing and phylogenetic affiliation

Primer pair ITS1F-ITS4 (Gardes & Bruns, 1993) were used to amplify ITS regions of the two *Ganoderma* isolates by direct PCR technique (Iotti & Zambonelli, 2006). As previously described by Bonuso, Iotti, Macrì, and Zambonelli (2006), 10–20 hyphae 1–2 mm length were removed from 2-weeks old pure culture in Petri dishes and transferred directly to the reaction tube containing 20 μl of sterile water. PCRs were performed in a 50 μl reaction volume consisting of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 200 mM for each dNTP and 300 nM for each primer. All reactions used 1.5 U of TaKaRa$^{TM}$ *Taq* DNA polymerase (Takara) and 20 μg of bovine serum albumin to relieve PCR interference. PCRs were conducted by a T gradient thermal cycler (Biometra) using the following parameters: 6 min of initial denaturation at 95 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min and a final extension step of 72 °C for 10 min. Amplified DNA fragments were run on 1% agarose gel and visualised in a GeneGenius Imaging System (SynGene). PCR products were first purified by the Gene Clean II kit (BIO 101, Vista, CA) and then sequenced in both directions using ITS1F and ITS4 primers. Sequencing reactions were performed using the ABI PRISM 3700 DNA Analyser (Applied Biosystems) and Big Dye Terminator v3.1 chemistry. The ITS sequences obtained from *Ganoderma* mycelia were submitted in GenBank database with the accession numbers EU498990 and EU498091 and compared with the other deposited sequences using the BLASTN search (Altschul et al., 1997).

To determine phylogenetic affiliations of Italian and Chinese mycelia their ITS1 and ITS2 sequences were used in conjunction with others from GenBank databases (http://www.ncbi.nlm.nih.gov/) obtained from *G. lucidum* basidiomata of known geographical origin. The 5.8S region was excluded from the analysis because it was missing from the sequences deposited in GenBank databases. DNA sequences were aligned by CLUSTAL W (Thompson, Higgins, & Gibson, 1994) using default settings and manually optimised with BioEdit version 5.0.9 (Hall, 1999). Phylogenetic analyses were conducted under neighbour-joining (NJ) and maximum parsimony (MP) as implemented in PAUP 4.0b (Swofford, 2000) where gaps were treated as missing data. The MP trees were found by using the tree-bisection–reconnection (TBR) branch swapping algorithm, with randomised stepwise addition of taxa under the heuristic search method. When more than one MP tree was found, the 50% major-rule consensus tree was calculated from all MP trees. For both NJ and MP internal branch support was assessed by bootstrap (BS) analysis of 1000 replicates with 10 random additions per replicate using FastStep algorithm (Felsenstein, 1985). *Amauroderma rude* was selected as outgroup in the phylogram.

### 2.3. Enzyme assay

The aerial and submerged mycelia were washed with distilled water to remove traces of the growth medium and homogenised using a Potter homogenizer with a glass pestle (Steroglass, Italy), in 100 mM $KH_2PO_4/Na_2HPO_4$ buffer, pH 6.7. The suspension obtained was then centrifuged for 15 min at 4 °C and 14,000 rpm. The supernatant was used for the activity assay of hexokinase (HK, EC 2.7.1.1), glucose phosphate isomerase (GPI, EC 5.3.1.9), phosphofructokinase (PFK, EC 2.7.1.11), pyruvate kinase (PK, EC 2.7.1.40), lactate dehydrogenase (LDH, EC 1.1.1.27), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6-PGDH, EC 1.1.1.44) and phoshoglucomutase (PGM, EC 5.4.2.2) as described in Saltarelli et al. (1998). Mannitol dehydrogenase (MDH, EC 1.1.1.138) was assayed using the method described in Ceccaroli et al. (2007). One unit (U) of enzymes is defined as the amount of enzyme which catalyses the formation of 1 μmol of product $min^{-1}$ at 37 °C. The values obtained were the means of six independent determinations. The evaluation of statistical significance was determined by a nonparametric analysis with the Mann-Whitney test. *P* values less than 0.05 were considered significant.

The protein concentration was spectrophotometrically determined at 595 nm using the Protein Assay Dye Reagent Concentrate (BioRad) according to Bradford's method (1976). Bovine serum albumin was used as standard reference.

### 2.4. 2D-electrophoresis

The aerial and submerged mycelia were ground with liquid nitrogen and homogenised in 8 M urea, 4% 3-CHAPS, 65 mM DTE, 40 mM Tris base using the Sample Grinding Kit (GE Healthcare). After centrifugation at 14,000 rpm, protein concentration in the supernatant was determined by Bradford's assay (1976).

One hundred micrograms of total proteins were used for each electrophoretic run. Isoelectric focusing was carried out on Immobiline strips providing a non linear pH 3–10 gradient (GE Healthcare) using a IPGphore system (GE Healthcare) and applying an increasing voltage from 200 V to 3500 during the first 3 h, later stabilised at 5000 V for 20 h. After isoelectric focusing, IPG strips were equilibrated by soaking in a buffer containing 50 mM Tris–HCl pH 6.8, 6 M urea, 2% SDS, 30% glycerol and 2% DTE for 15 min, and then 50 mM Tris–HCl pH 6.8, 6 M urea, 2% SDS, 30% glycerol with 2.5% iodacetamide and trace amount of bromophenol blue for 15 min more. The second dimension was carried out in a Laemmli system on 9–16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm) at 40 mA/gel constant current, until the dye front reached the bottom of the gel. Analytical gels were stained with silver nitrate (Sinha, Poland, Schnölzer, & Rabilloud, 2001). Gel images were acquired by Fluor-S MAX multi-imaging system (BioRad) and the data were analysed, including spots detec-

tion, quantification and normalisation, using ImageMaster 2D Platinum version 5.0 software (GE Healthcare), in particular, protein quantification values were calculated as relative volume (%). The results were confirmed by three independent analyses.

### 2.5. Preparation of mycelial extracts

The aerial mycelia were harvested and dried at 60 °C for 8 h and weighed (10 g). They were then ground with a pestle potter in liquid nitrogen and extracted with 80% ethanol/water (80:20, v/v) at 4 °C. The fungal extract was subsequently centrifuged at 1,500 rpm for 15 min and treated with two consecutive extractions in ethanol/water. The supernatants from the centrifugation step of three extractions were recovered, dried and stored at 4 °C. This fraction contained the low molecular weight compounds (L) and the dry weight was 1.92 g for Chinese L fraction (LC) and 2.1 g for Italian L fraction (LI). For the intracellular polysaccharide determination, the remaining pellets were extracted with hot water ($T$ = 100 °C, 3 h), centrifuged at 1,500 rpm for 15 min and then precipitated by 96% ethanol. The crude polysaccharides were suspended in water with stirring, and the insoluble fraction was removed by filtration whereas the soluble fraction (P) was separated by chromatography. The polysaccharide content was determined by the anthrone colorimetric method (Wang et al., 2002). Total polyphenol content was determined using Folin-Ciocalteau method described by Singleton, Orthofer, and Lamuela-Raventos (1999). The amount of total phenolics was expressed as caffeic acid equivalents through the calibration curve of caffeic acid. The calibration curve ranged from 1 to 15 μg ml$^{-1}$ ($R^2$ = 0.9973).

### 2.6. Fractionation of polysaccharides

The water soluble fraction of polysaccharides was fractionated by ion-exchange chromatography using a Gold liquid chromatographic system from Beckman (Beckman Coulter Inc., Fullerton, CA, USA). The HPLC apparatus consisted of two Model 126 pumps and a Model 168 diode array detector. The chromatographic separations were performed using a Toyopearl DEAE 650S, 15 cm × 4.6 cm I.D. The sugars were separated at a flow rate of 1.0 ml min$^{-1}$ using the following isocratic steps of 10 min each: (a) $H_2O$, (b) 0.1 M $NaHCO_3$, (c) 0.3 M $NaHCO_3$, (d) 0.5 M $NaHCO_3$, (e) 0.1 M NaOH and (f) $H_2O$. Fractions of 1.0 ml were collected and assayed using the anthrone method. The pooled fractions containing polysaccharides were precipitated with 96% ethanol, suspended in water and charged on TSK gel 3000 (Toyopearl Progel G3000SWXL, Supelco Park, Bellefonte, PA, USA) equipped with a guard column and using the same above-mentioned HPLC apparatus. The polysaccarides were separated in isocratic water at a flow rate of 0.5 ml min$^{-1}$ and collected in fractions of 0.5 ml.

### 2.7. Chelating activity on $Fe^{2+}$

The chelating activity on $Fe^{2+}$ was measured as reported by Yen and Wu (1999) with slight modifications. Two hundred microliters of extract (0.6–6.0 mg ml$^{-1}$) were mixed with 740 μl of deionised water; the mixture was reacted with 20 μl $FeSO_4$ (2 mM) and 40 μl ferrozine (5 mM) for 10 min and then the absorbance at 562 nm determined spectrophotometrically. Chelating activity was calculated as % = [($A_{562\ nm}$ of blank − $A_{562\ nm}$ of sample)/$A_{562\ nm}$ of blank] × 100. The effective concentration value ($EC_{50}$) is the plot extrapolated concentration at which ferrous ions were chelated by 50%.

### 2.8. Lipoxygenase assay

Lipoxygenase (E.C. 1.13.11.12) was assayed according Sestili et al. (2007). One milliliter of reaction mixture contained 100 μM linoleic acid, the extract at different concentrations or Tris/HCl, pH 7.0 as blank and 50 mM sodium phosphate, pH 6.8. The reaction mixture was pre-equilibrated at 20 °C for 20 min and then was spectrophotometrically assayed at 235 nm until stability. The 5-lipoxygenase (0.18 μg ml$^{-1}$) was added and the formation of hydroperoxides from linoleic acid was observed spectrophotometrically at 235 nm at 20 °C. The lipoxygenase activity was calculated as % = 100−{[($\Delta_{235\ nm}$ of blank − $\Delta_{235\ nm}$ of sample)/$\Delta_{235\ nm}$ of blank] × 100}. The inhibition concentration value ($IC_{50}$) was determined by plotting the graph with concentration of extracts versus percentage of inhibition of linoleic acid peroxidation.

### 2.9. Scavenging DPPH· radicals

The antioxidant capacity was evaluated using the DPPH· assay as reported by Mau et al. (2002) with slight modifications: 100 μM DPPH· ethanol solution was prepared and 0.85 ml were added to 0.15 ml of sample diluted in 50 mM Tris–HCl, pH 7.4. The range of concentrations used was 0.011–1.65 mg ml$^{-1}$. The absorbance decrease at 517 nm was recorded after 10 min at room temperature. The scavenger effect was calculate as % = [($A_{517\ nm}$ of blank − $A_{517\ nm}$ of sample)/$A_{517\ nm}$ of blank] × 100. The $EC_{50}$ value was calculated from the plots as concentration extracts required to provide 50% free radical scavenging activity.

## 3. Results and discussion

### 3.1. General

Though *G. lucidum* is a very important medicinal mushroom widely used in China, Japan and Korea over the past two millennia (Sliva, 2006), previous studies have only focused on its active ingredients and healing mechanisms. A broad biochemical characterisation of *G. lucidum* with respect to its protein pattern and the enzymatic activities of its major metabolic pathways has not yet been reported. In this study Italian and Chinese isolates, both identified as *G. lucidum*, were molecularly and biochemically characterised and compared. Furthermore, soluble polysaccharide and the ethanolic extracts containing low molecular weight compounds were tested for their antioxidant properties.

### 3.2. Molecular characterisation and phylogenetic analyses

*Ganoderma* is one of the largest genera of polypore fungi. Their macro- and micromorphological characters are extensively variable, and more than 290 taxonomic names have been published in the genus (Moncalvo & Ryvarden, 1997). In Europe *G. lucidum* has been described 13 times as a new species (Ryvarden, 2000) because different authors were unaware of the work of others or because of the macroscopic variability of its morphological characteristics. These aspects led many taxonomists to explore chemical and molecular methods to distinguish between the *Ganoderma* species. The systematic affinities of *Ganoderma* have largely been resolved in the extensive publications of Moncalvo and coworkers where the model, proposed to circumscribe the species, uses both phylogenetic analysis (using sequences of ITS and 26S rDNA and RAPD–PCR) and morphological, ecological, cultural and mating studies (Hseu, Wang, Wang, & Moncalvo, 1996; Moncalvo, Wang, & Hseu, 1995a, 1995b).

ITS amplification of Italian and Chinese mycelia both labelled as *G. lucidum* produced amplicons of 681 bp and 674 bp, respectively and the resulting sequences showed 95% similarity. The two isolates diverged 11% in the ITS1 and 6% in the ITS2 region.

Phylogenetic reconstructions of the obtained ITS1 and ITS2 sequences in conjunction with other *G. lucidum* sequences selected

from GenBank gave congruent topology using NJ and MP methods. The dataset included 22 taxa and 435 characters, 62 of which were parsimony informative. As regards monophyly, branch length and bootstrap support, *G. lucidum* accessions were divided into six monophyletic groups which matched to as many geographic clusters (Fig. 1). Only the *G. lucidum* from North America were found to be polyphyletic and distributed in three different groups. The Italian isolate and all the other *G. lucidum* from Europe formed a monophyletic cluster (Group VI) with a strong bootstrap support of 100%. The Chinese isolate was separated into an independent lineage with the others labelled as *G. lucidum* from China (Group I) phylogenetically distant from the European clade. The same results were previously obtained by other authors using different molecular markers. In particular, Hong and Jung (2004) using nearly full sequences of mitochondrial small-subunit ribosomal DNA (mt SSU rDNAs), demonstrated that five strains labelled *G. lucidum* from Asia were monophyletic and distinguishable from *G. lucidum* from Europe and North America. More recently, Sun et al. (2006), using the Sequence Related Amplified Polymorphism (SRAP) marker, found that *G. lucidum* strains from China and Korea were also different from the *G. lucidum* strain from Yugoslavia.

Our results reinforce the hypothesis that *G. lucidum* is a complex of species sharing macroscopical morphological traits where monophyletic groups correlate fairly well with geographic origin (Moncalvo et al., 1995a). The European members (Group VI) should be considered *G. lucidum sensu stricto* because this species was firstly described in Europe (Moncalvo et al., 1995a; Buchanann, 2001).

### 3.3. Enzymatic determinations

The activity levels of some enzymes of the carbohydrate metabolism were evaluated in Chinese and Italian *G. lucidum* isolates. Table 1 reports the activity of the main glycolytic and pentose phosphate enzymes and the level of lactate dehydrogenase, phosphoglucomutase and mannitol dehydrogenase. Glycolysis is a nearly universal pathway for energy generation in living cells and usually gives an indication of the functional state of mycelial hyphe. The enzymatic activities, evaluated in aerial and submerged agglomerates of each mycelium, are not significantly different. On the contrary, the comparison of glycolytic enzyme activities showed that there were significant differences between the two mycelia examined. In particular, in Italian *G. lucidum* the specific activity of the hexokinase and phosphoglucose isomerase, the first and second steps of the glycolytic pathway, were about twice as high as that of the Chinese *G. lucidum*. On the contrary, the phosphofructokinase and piruvate kinase activities, the main irreversible and regulatory steps in glycolysis, were about three and two times higher in Chinese *G. lucidum,* respectively. PFK, which catalyses the phosphorylation of fructose 6-phosphate to fructose 1,6-biphosphate in the presence of magnesium and adenosine triphosphate, is controlled by many allosteric effectors (activators and inhibitors) and plays a key role in the regulation of the glycolytic flux. The highest specific activity of this enzyme, together with PK activity in Chinese *G. lucidum* suggests that the anaerobic glucose catabolism processes faster in this mycelium than in the Ital-

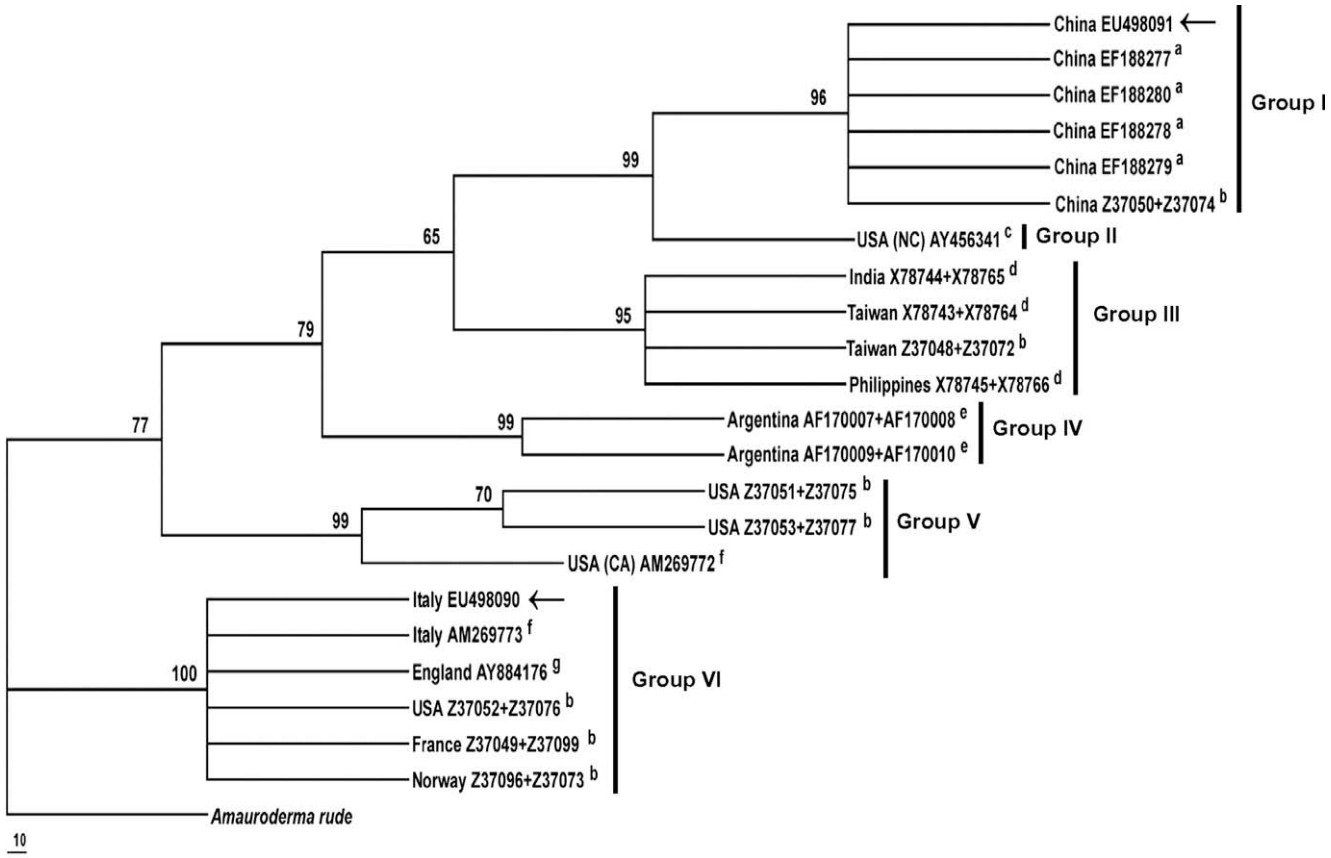


**Fig. 1.** Maximum parsimony tree based on ITS1-ITS2 rDNA for 22 taxa of *Ganoderma lucidum*. Taxa are labelled by geographic origin and GenBank accession number. The arrows indicate the Chinese and Italian mycelial isolates analysed in this study. References of the accessions from GeneBank are indicated as letters: [a]Jia, Zheng, and Gan (2007). published only in GeneBank database; [b]Moncalvo et al. (1995a) *Mycological Res*earch *99*, 1489–1499; [c]Edwards et al. (2004). *New Phytologist 162*, 755–770; [d]Moncalvo et al. (1995b) *Mycologia 87*, 223–238; [e]Gottlieb et al. (2000). *Mycological Research 104*, 1033–1045; [f]Guglielmo, Bergemann, Gonthier, Nicolotti, and Garbelotto (2007). *Journal of Applied Microbiology 103*, 1490–1507; [g]Wang and Yao (2006). published only in GeneBank database. Bootstrap values are indicated above branches. The tree is rooted with *Amauroderma rude*, GenBank accession number X78753 + X78774 [Moncalvo et al. (1995b) *Mycologia 87*, 223–238].

**Table 1**
Activity of different enzymes from Chinese and Italian *Ganoderma lucidum* mycelia.

| Enzymes | Chinese *G. lucidum* | Italian *G. lucidum* |
|---|---|---|
| Hexokinase | 0.30 ± 0.06* | 0.55 ± 0.05* |
| Glucose phosphate isomerase | 1.43 ± 0.63** | 2.28 ± 0.58** |
| Phosphofructokinase | 0.28 ± 0.13** | 0.094 ± 0.022** |
| Pyruvate kinase | 2.25 ± 0.77** | 1.53 ± 0.50** |
| Lactate dehydrogenase | 0.012 ± 0.002 | 0.023 ± 0.014 |
| Glucose-6-phosphate dehydrogenase | 0.43 ± 0.14 | 0.35 ± 0.11 |
| 6-Phosphogluconate dehydrogenase | 0.33 ± 0.12 | 0.32 ± 0.13 |
| Phosphoglucomutase | 2.8 ± 0.65* | 2.12 ± 0.59* |
| Mannitol dehydrogenase | 0.014 ± 0.005 | 0.028 ± 0.005 |

The enzymatic activities were evaluated in aerial and submerged mycelial agglomerates of Chinese and Italian *G. lucidum* strains as reported in Section 2. The values of enzymatic activities are expressed as Unit/mg of total proteins. Each value represents the mean of six independent measurements.
* Significant differences at $P < 0.05$.
** Significant differences at $P < 0.005$.

ian one. In the literature it has been reported that glyceraldehyde-3-phosphate dehydrogenase is the only enzyme characterised in *G. lucidum*. A *G. lucidum* cDNA library has been constructed and the fungal *gpd* gene and its adjacent regulatory elements have been characterised for the construction of transformation vectors (Fei, Zhao, & Li, 2006). An alternative to glycolysis, the pentose phosphate pathway is present in the higher fungi and its role is to supply both reduced nicotinamide adenine dinucleotide phosphate (NADPH) to the cell for biosynthetic reactions and pentose phosphates for the production of aromatic amino acids and nucleic acid synthesis (Wood, 1986). The activity levels of glucose-6-phosphate and 6-phosphogluconate dehydrogenase did not show any significant differences in Italian and Chinese *G. lucidum*.

Finally, fermentative, glycogen and mannitol metabolisms were investigated evaluating the lactate dehydrogenase, phosphoglucomutase and mannitol dehydrogenase activity. The level of lactate dehydrogenase was very low in both mycelia indicating that the fermentative carbohydrate metabolism is limited under these growth conditions. On the other hand, the phosphoglucomutase activity was considerable in both *Ganoderma*. This enzyme is an ubiquitous metal-protein expressed in all organisms and catalyses the inter-conversion of glucose-1-phosphate and glucose-6-phosphate in the presence of glucose-1,6-diphosphate and $Mg^{2+}$ and plays a pivotal role in the synthesis and breakdown of glycogen. In fungi, mannitol is also a carbon storage compound; this sugar alcohol has different roles such as carbon source, storage of reducing power and carbohydrate translocation (Jennings, 1984). We therefore evaluated the mannitol dehydrogenase activity which forms mannitol by direct reduction of fructose. This enzyme was present in low amounts both in Italian and Chinese *G. lucidum* suggesting that other carbohydrates are able to substitute the mannitol as a storage compound (e.g. glycogen and/or trehalose).

### 3.4. 2D-electrophoresis analyses

Using 2-DE, the proteome of the Italian and Chinese *G. lucidum* mycelia was analysed in both aerial and submerged agglomerates. The comparison among the electrophoretograms showed minor differences in protein expression of two different *Ganoderma* grown as aerial agglomerate (Fig. 2). In particular, in Chinese isolate no more than 21 proteins were up-regulated; whereas in Italian *G. lucidum* no more than eight proteins were up-regulated (Fig. 2, red arrows). There was not a protein that was found exclusively in one or the other mycelium. When we compared protein patterns of the two submerged grown mycelia the situation was completely different. Under this condition, Italian and Chinese isolates showed relevant differences in protein expression, from a

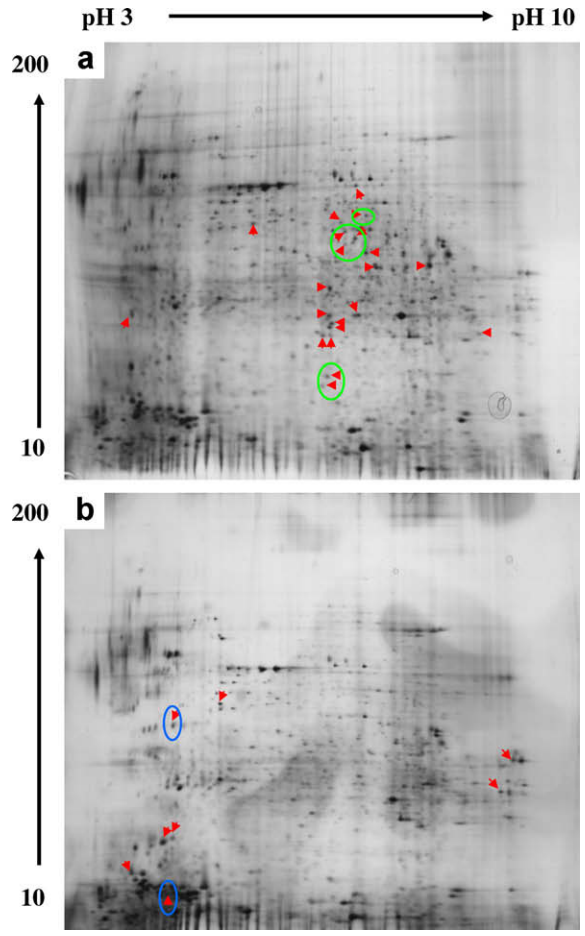


**Fig. 2.** 2-DE of 100 μg of total proteins from *G. lucidum* grown as aereal agglomerate. (a) Chinese strain; (b) Italian isolate. Gels were stained with silver nitrate.

qualitative and quantitative standpoint. As marked in Fig. 3, many proteins were exclusively present in one mycelium (yellow arrows), while many other proteins were expressed in both mycelia showing different expression levels (red arrows). Specifically, in the Chinese isolate there were at least 37 proteins more expressed, and 26 exclusively expressed compared with the Italian mycelium; whereas in Italian *G. lucidum* there were at least 35 proteins up-regulated, and 13 exclusively expressed. Moreover, we have found some proteins (Figs. 2 and 3, blue circled) whose increase was typical of the Italian *G. lucidum*, since their up-regulation was present both in submerged and aerial growth. The same thing happened for the Chinese isolate (Figs. 2 and 3, green circled proteins).

It should be noted that the electrophoretograms of the two submerged mycelia showed significant differences in some areas so that it was difficult to compare them. It must also be noted that proteome changes considerably both in Italian and Chinese isolates depending on different growth conditions (aerial or submerged).

### 3.5. Isolation and separation of polysaccharides

For polysaccharide isolation, the aerial agglomerates of Chinese and Italian isolates were used. Crude polysaccharides were suspended in water with stirring and the insoluble polysaccharides were removed by filtration. The soluble polysaccharide content was higher in Italian than in Chinese mycelium (14.37 and 7.74 mg $g^{-1}$ of dry weight, respectively) whereas the insoluble fraction was comparable in both fungi (2.10 and 2.91 mg $g^{-1}$ of

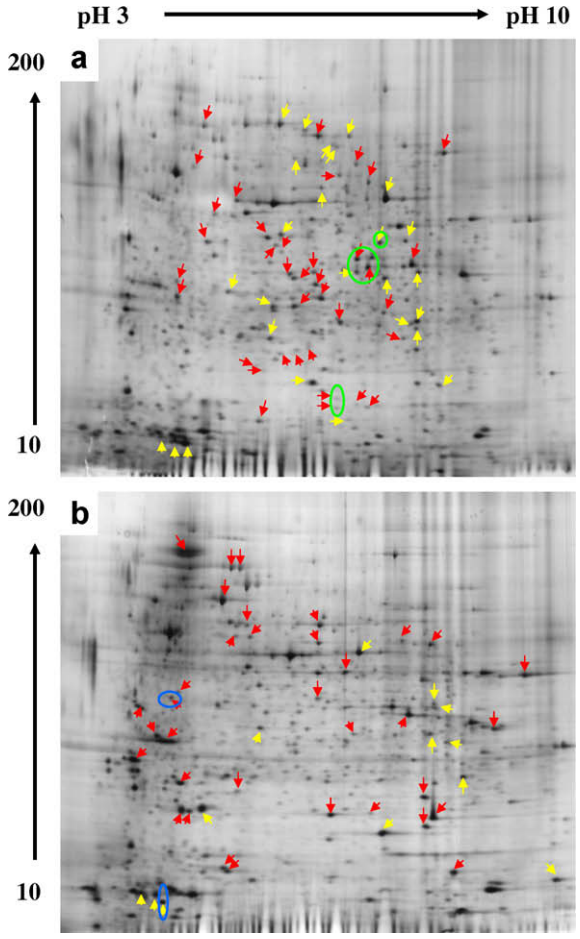


**Fig. 3.** 2-DE of 100 μg of total proteins from *G. lucidum* grown submerged. (a) Chinese isolate; (b) Italian isolate. Yellow arrows mark the proteins exclusively expressed in that isolate, red arrows mark the proteins whose expression is higher than the other strain. Gels were stained with silver nitrate.

dry weight, respectively). Furthermore, when the soluble fractions were separated by ion-exchange chromatography, the chromatographic profile showed that in Chinese isolate two peaks were present, C1 and C2 (Fig. 4a): the C1 peak eluted in isocratic condition with $H_2O$ and C2 with 0.1 M $NaHCO_3$ whereas the increasing of salt concentrations do not determine any peak elution. On the contrary, in Italian mycelium only one peak appeared (I) which eluted in the presence of 0.1 M $NaHCO_3$ (Fig 4b). This result suggests that the neutral or positive portion of soluble polysaccharide is nearly absent in Italian *G. lucidum*, whereas the anionic portion is twice as high. However, the chromatographic profile of polysaccharides extracted from Chinese isolate is comparable with that reported for the *G. lucidum* stain MZKI G97 isolated from a Slovenian forest (Berovič et al., 2003). The polysaccharide fractions were further separated by gel filtration and the C1 and C2 peaks presented two peaks, respectively (Fig. 4c) whereas I peak showed only one peak (Fig. 4d), which probably presents the same molecular weight of the first Chinese peak eluting in the same fraction numbers. The polysaccharides separated by gel filtration have a high molecular weight. The major bioactive *Ganoderma* polysaccharide species are β-1-3 and β-1-6-D glucans. They have high molecular weights as a common feature, which tends to increase water solubilities and results in more effective antitumor activity. It is known that the water soluble polysaccharides exert antitumor activities through the host-mediated immunity enhancing the interleukin, interferon and antibody production and stimulating the cytotoxic T lymphocytes (Paterson, 2006). Although it is difficult to correlate

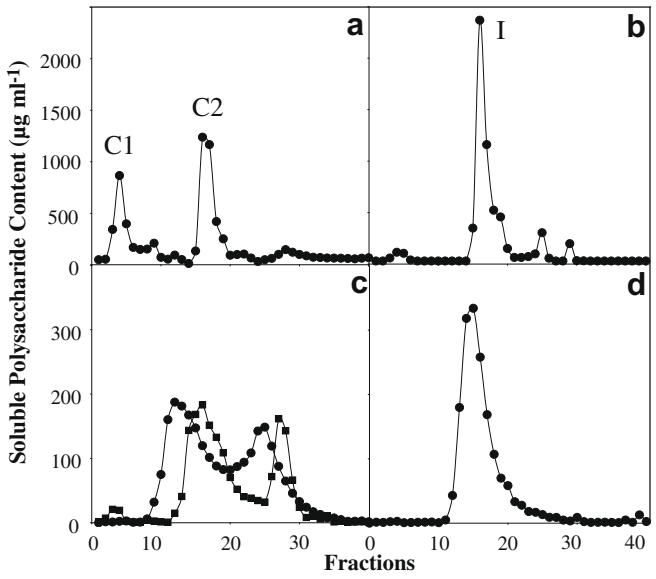


**Fig. 4.** Chromatographic profiles of the soluble polysaccharide content. Elution chromatograms of ion-exchange chromatography (DEAE) obtained loading 5 mg of polysaccharides from Chinese (a) and Italian (b) *G. lucidum* isolates. Elution chromatograms of gel filtration obtained loading 2 mg of each DEAE-polysaccharide peaks: (c) C1 peak (■–■), C2 peak (●–●) from Chinese isolate and (d) I peak from Italian isolate.

the structure and antitumor activity of complex polysaccharides, it has been reported that high molecular weight glucans appear to be more effective than low molecular weight species (Mizuno, 1999). The potential antioxidant activity of polysaccharide fractions from Chinese (PC) and Italian (PI) *Ganoderma* isolates was evaluated in different *in vitro* tests such as DPPH assay, ferrous ion chelation and lipoxygenase inhibition. In our experimental conditions, no antioxidative effects were shown by PC and PI fractions (data not shown). Our results, in agreement with the data reported by Sun, He, and Xie (2004), suggest that the polysaccharide fractions are not involved in free radical scavenging. In the literature it has been reported that polysaccharides extracted from *G. tsugae* have a free radical scavenging ability (Tseng, Yang, & Mau, 2008), but the antioxidant mechanism of polysaccharides is still not fully understood. Tsiapali et al. (2001) speculate that the abstraction of anomeric hydrogen from monosaccharides accounts for their free radical scavenging ability. Polysaccharides enhance antioxidant activity more than monosaccharides because polysaccharides abstract the anomeric hydrogen from one of the internal monosaccharide units more easily than monosaccharides. In this study the PC and PI fractions were not chemically characterised and we can not exclude the possibility that the manner in which the polysaccharides were extracted and solubilised affected their antioxidant ability.

### 3.6. Chelating effect on ferrous ion

The chelating effect of Chinese and Italian *Ganoderma* isolates was evaluated in L fraction, containing mainly low molecular weight compounds. As shown in Fig. 5a, the Italian (LI) and Chinese (LC) fractions showed a chelating activity on ferrous ion which varied according to extract concentrations. At 6.0 mg $ml^{-1}$, the chelating effect for both strains was about 80%, whereas at 2.4 mg $ml^{-1}$ it was around 50% and comparable with the effect reported by Mau et al. (2002). The chelating abilities of LI and LC fractions on ferrous ion were good as shown by their low $EC_{50}$ values (2.8 ± 0.03 and 3.36 ± 0.02 mg $ml^{-1}$ for Italian and Chinese isolates, respectively). This indicates that the chelating activity of Italian and Chinese extracts in metal ion may play an important role in their antioxidant

activity. Iron is essential for life because it is required for several important metabolic processes such as oxygen transport, respiration and enzymatic activity. However, iron is an extremely reactive metal and will catalyse oxidative changes in lipids, proteins and other cellular components. Oxidative damage is induced by hydroxyl radicals generated by the Fenton reaction. As shown in Fig. 5a, the extracts showed chelating effects on ferrous ions, suggesting that they could sequestrate Fe ions or minimise the concentration of metal in the Fenton reaction. Consequently, these extracts could act as liposome, deoxyribose or protein-protectors. Since ferrous ions are the most effective pro-oxidants in the food system, higher chelating effects of extracts from mycelia would be beneficial.

### 3.7. Lipoxygenase inhibitory activity

Lipoxygenase constitutes a family of non-heme enzymes containing dioxygenase group that are widely distributed in plants and animals. In cells, these enzymes play a key role in the biosynthesis of a variety of bio-regulatory compounds and are involved in the metabolism of arachidonic acid. Conversion of this fatty acid via the lipoxygenase pathway is associated with a production of ROS. These reactive forms of oxygen and other arachidonic acid metabolites may play an important role in different diseases (Nie & Honn, 2002). Antioxidants interact non-specifically with lipoxygenase by scavenging radical intermediates and/or reducing the active heme site (Cao, Sofie, & Prior, 1996).

The effect of LI and LC fractions on lipoxygenase activity is shown in Fig. 5b. Both extracts significantly inhibited the oxidation of linoleic acid catalysed by lipoxygenase in a dose dependent manner. At 0.4 and 1.2 mg $ml^{-1}$ both extracts inhibited about 40% and 70% of lipoxygenase activity *in vitro*, respectively. In particular, the extracts showed an inhibition activity with an $IC_{50}$ value of 0.58 ± 0.04 and 0.47 ± 0.06 for Italian and Chinese isolates, respectively. These $IC_{50}$ values are not significantly different ($P > 0.05$) and suggest that both fractions are effective in preventing lipid oxidation. So far, in the literature no information is available concerning the lipoxygenase inhibition assay in *Ganoderma* extracts, but this test was performed with a peptide isolated from fermented *G. lucidum*. This peptide showed a very high lipoxygenase inhibitory activity corresponding to about 90% at 0.3 mg ml mg $ml^{-1}$ (Sun et al., 2004). Since a key step for lipoxygenase activation is the binding of a non-heme-iron at the active site, the inhibitory effect of the LI and LC fractions might be due to their iron-chelating activity.

### 3.8. Radical scavenging activity

The scavenging activity profiles of the LC and LI fractions are shown in Fig. 6a. The radical scavenging activity increased with the concentrations of LC and LI extracts; however, the LI was more efficient at lower concentrations than LC. The LI scavenging effect on DPPH· at concentrations ranging from 0.04 to 0.5 mg $ml^{-1}$ was twice as high as LC inhibition. In particular, there was an increase in the scavenging effect of LI up to a 0.5–0.6 mg $ml^{-1}$ concentration (60%), beyond which there was no significant increase even up 1.5 mg $ml^{-1}$. On the contrary, LC exhibited a progressive increase in the scavenging effect up to 1.2–1.4 mg $ml^{-1}$ (about 65%). The DPPH· scavenging effect of the LI fraction was about 60% at a concentration of 0.5 mg $ml^{-1}$, which is higher than LC (about 36%) and *Ganoderma* methanolic extracts reported in literature. In fact, Mau et al. (2002) report that the DPPH· scavenging ef-

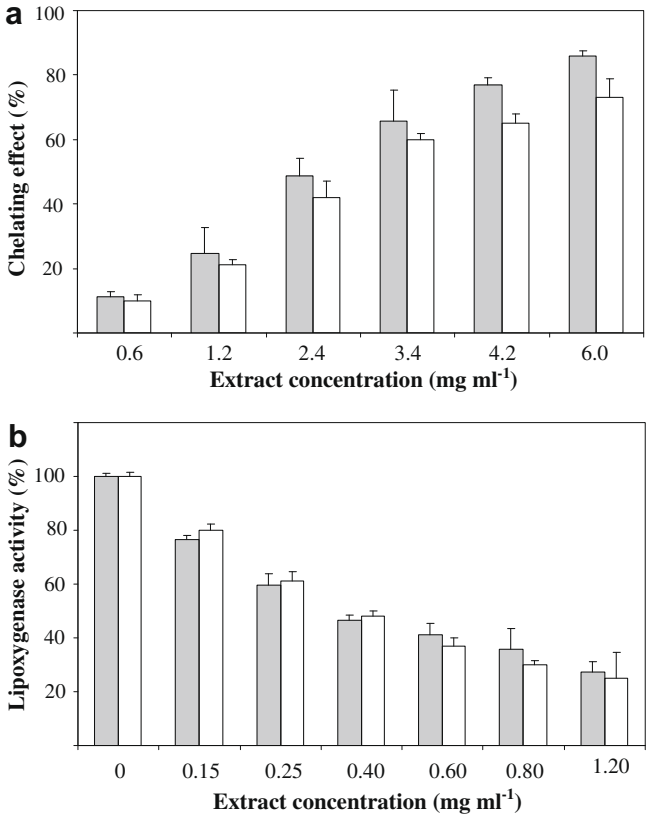


**Fig. 5.** (a) Chelating ability of *G. lucidum* extracts on ferrous ions. (b) Effect of *G. lucidum* extracts on lipoxygenase activity *in vitro*. (■) LI fraction from Italian *G. lucidum* (□) LC fraction from Chinese *G. lucidum*. Each column represents the mean ± standard deviation ($n = 3$).

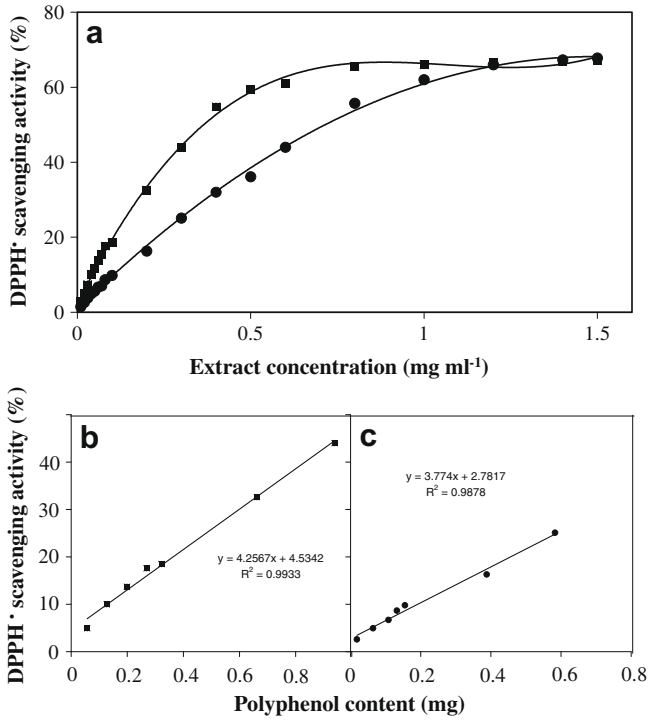


**Fig. 6.** Antioxidant capacity of *G. lucidum* extracts. (a) Scavenging effect on the DPPH· test of LC (●–●) and LI (■–■) fractions from Chinese and Italian isolates, respectively. The data represent the percentage of inhibition induced by increasing concentrations of LC and LI extracts. (b) (c) Correlation between total polyphenol content and DPPH· scavenging activity of LI (a) and LC (c) fractions. Each value represents the mean of three independent measurements and varied from the mean by no more than 5%.

fect of fruiting body methanol extracts of *G. lucidum*, and *G. tusgae* was about 45% at a concentration of 0.5 mg $ml^{-1}$. The results obtained are confirmed by the $EC_{50}$ values achieved by interpolation from linear regression analysis. The $EC_{50}$ value in scavenging ability on DPPH· radicals of LI was significantly lower than those obtained for LC. In fact, the LI fraction showed maximum scavenging ability with an $EC_{50}$ value of 0.23 ± 0.05 compared to an $EC_{50}$ value of 0.69 ± 0.02 for LC fraction ($P < 0.005$).

Among the compounds that exhibit antioxidant properties we evaluated the polyphenol content in both extracts. The total polyphenol content is higher in LI than in LC corresponding to 27.9 and 16.5 mg $g^{-1}$ extract, respectively. The polyphenol content of the LI fraction is comparable to that reported for the methanolic extract from *Antrodia camphorata* submerged culture (38.0 ± 0.7 mg $g^{-1}$ extract) (Song & Yen, 2002). To elucidate the relationship between the polyphenol content and free radical scavenging activity, we calculated the correlation with total polyphenol and scavenging effect in both extracts. As shown in Fig. 6b and c, the scavenging effect correlates directly with the different polyphenol content and there is a linear relationship with $r^2 = 0.9933$ and $r^2 = 0.9878$ in LI (Fig. 6b) and LC (Fig. 6c) extracts, respectively. This result suggests that the antioxidant power observed may be due to the presence of polyphenols. Our data are in agreement with those reported for *A. camphorata* (Song & Yen, 2002) and *Inonotus obliquus* (Nakajima, Sato, & Konishi, 2007) in which the antioxidant abilities of extracts were correlated with their total polyphenol content based on the evaluation of different antioxidant test systems. It was reported that the antioxidant activity of phenolics was mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and single oxygen quencher (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). Hence we could not exclude that some other components contribute in part to the antioxidant properties of these mushrooms.

## 4. Conclusions

In conclusion, our analysis showed significant differences in the enzymatic activities, protein patterns and soluble polysaccharide content in the Italian and Chinese isolates grown in submerged culture. Furthermore, the ethanolic extracts containing mainly low molecular weight compounds from both *G. lucidum* showed good antioxidant activities. In particular, the scavenging activity on the DPPH· radical was higher in the Italian *G. lucidum* isolate than in the Chinese isolate. As discussed above, the latter property could be largely dependent on phenol compounds.

The results reported herein demonstrate that the Italian *G. lucidum*, though phylogenetically distinct from the thoroughly studied Asian *G. lucidum sensu latu* strains, could be used to obtain several bioactive components. In order to investigate the antioxidant mechanism of some potential antioxidant molecules, the fractionation and the identification of the ethanolic extract containing the low molecular weight compounds are in progress.

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