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Biochemical Characterization, Antioxidant and Antiproliferative Activities of Different *Ganoderma* Collections

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Short title: Biological Characterization of Different *Ganoderma* Collections

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Abstract

The aim of this study was to conduct a molecular and biochemical characterization and to compare the antioxidant and antiproliferative activities of four *Ganoderma* isolates belonging to *Ganoderma lucidum* (Gl-4, Gl-5) and *Ganoderma resinaceum* (F-1, F-2) species.

The molecular identification was performed by ITS and IGS sequence analyses and the biochemical characterization by enzymatic and proteomic approaches. Antioxidant activity of the ethanolic extracts was compared by three different methods and their flavonoid contents were also analyzed by HPLC. The antiproliferative effect on U937 cells was determined by MTT assay.

The studied mycelia differ both in the enzymatic activities and protein content. The highest content in total phenol and the highest antioxidant activity for DPPH free radical scavenging and chelating activity on Fe^{2+} were observed with Gl-4 isolate of *G. lucidum*. The presence of quercetin, rutin, myricetin and morine as major flavonoids with effective antioxidant activity was detected. The ethanolic extracts from mycelia of *G. lucidum* isolates possess a substantial antiproliferative activity against U937 cells in contrast to *G. resinaceum* in which the antiproliferative effects were insignificant.

This study provides the comparison between *G. lucidum* and *G. resinaceum* mycelial strains showing that *G. resinaceum* could be utilized to obtain several bioactive compounds.

Introduction

Many species from the genus *Ganoderma* P. Karst. (Basidiomycotina, Polyporales, Ganodermataceae), known as the ‘‘mushrooms of immortality’’, include a group of fungi that have long been recognized to promote health and longevity in Asian countries [Boh et al., 2007]. So far, more than 120 species of *Ganoderma* have been reported and the broader definition of Chinese Lingzhi, embraces both *Ganoderma lucidum* (Curtis) P. Karst. and other closely related *Ganoderma* species such as *G. resinaceum* Boud. and *G. sichuanense* J.D. Zhao & X.Q. Zhang [Wang XC et al., 2012]. Following the successful cultivation of *Ganoderma* spp., a range of bioactive compounds have been identified in mushroom fruiting bodies and mycelia to which various pharmacological effects and medicinal properties have been assigned [Boh et al., 2007; Zhou et al., 2007]. Among revealed bioactive chemical constituents there are polysaccharides, triterpenes, sterols, and some proteins such as lectins and LZ-8 [Boh et al., 2007; Ferreira et al., 2010]. It is considered that triterpenoids and polysaccharides are the main active components in *G. lucidum*, however *Ganoderma* spp. contain also phenolic compounds involved in antioxidant and free radical scavenging activity [Kim et al., 2008; Saltarelli et al., 2009], indicating that some other compounds may contribute to their activity. Phenolic compounds have significant biological and pharmacological properties and their bioactivity may be related to their ability to chelate metals, inhibit lipoxygenase, and scavenge free radicals [Duthie et al., 2000]. Flavonoids are a group of polyphenolic compounds with known health-beneficial properties, including free radical scavenging, inhibition of hydrolytic and oxidative enzymes, and anti-inflammatory action. These specific phenolic compounds have been little investigated on *Ganoderma* spp. [Hasnat et al., 2013; Rawat et al., 2013; Saltarelli et al., 2009] and may be involved as key compounds in therapeutical and nutraceutical properties of mushrooms.

Researches in genus *Ganoderma* have been focused only on few species and the most widely used one is *G. lucidum* for which the genome sequence has recently been obtained [Chen et

al., 2012]. *Ganoderma resinaceum* also possesses important medicinal properties of immunomodulation and liver-protection. Some studies were conduct to optimize submerged growth conditions of *G. resinaceum* to enhance the production of bioactive exopolysaccharides by fed-batch culture [Kim et. al., 2006]. More recently, from the fruiting bodies of this fungus, eight new lanostanoids together with six known lanostanoids were isolated; some of these triterpenoids have significant hepatoprotective effects [Peng et al., 2013]. To our knowledge, there are no reports concerning antioxidant activities of ethanolic extracts of *G. resinaceum*.

In this paper the antioxidant activity and the antiproliferative effects of total phenolics isolated from ethanolic extracts of European *G. lucidum* and *G. resinaceum* collections were evaluated. The flavonoids present in the ethanolic extracts from the different isolates were also identified. Moreover, molecular identification by ITS and IGS sequence analyses and biochemical characterization by enzymatic and proteomic approaches of studied collections were conducted.

Results and discussion

Phylogenetic Analyses

The combined ITS-IGS dataset used for phylogentic analysis consisted of 11 taxa and 1491 unambiguously aligned nucleotides (542 and 949 for ITS and IGS, respectively), with 212 parsimony informative and 10 parsimony uninformative characters. BI and MP analyses gave highly congruent topology, thus only the Bayesian phylogram is shown in Fig. 1. Phylogenetic reconstruction clearly included the isolates analysed in this study into 2 separate clades (100% level confidence): Gl-5 and Gl-4 into *G. lucidum* clade and F-1 and F-2 into *G. resinaceum* clade. No nucleotide differences were observed between F-1 and F-2 strains (also considering the Italian *G. resinaceum* isolate Gre4/5133) both in ITS and IGS region. On the contrary, two variable sites were found within the IGS region between Gl-5 and Gl-4 strains.

Genetic diversity of *G. lucidum* and *G. resinaceum* inferred by the phylogenetic tree agrees with the results obtained by Badalyan et al. [2012] and represents the most reliable target to discriminate these two species which form morphologically similar fruiting bodies.

Analysis of Some Enzymes of Primary Metabolism

Investigation of basic metabolism is a fundamental preliminary approach to examine fungal behaviour and to assess the metabolic state of a mycelial colony. Despite its importance, our knowledge is limited and often focused on the study of secondary metabolism of *Ganoderma* spp. as recently reported in the genome sequence project of the model medicinal mushroom, *G. lucidum* [Chen et al., 2012]. The activity of some enzymes of primary metabolism in different collections of *G. lucidum* and *G. resinaceum* is reported in Table 1. No significant differences in the regulatory steps of glycolysis except for phosphofructokinase activity that was lower in *G. lucidum* Gl-5 strain than in other ones were found. However, the activity level of this strain is comparable to that previously revealed in Italian *G. lucidum* strain [Saltarelli et al., 2009]. Furthermore, all these glycolytic enzymes showed a good activity indicating a remarkable efficiency of anaerobic glucose catabolism in this fungus. The activity levels of glucose-6-phosphate dehydrogenase, an enzyme of penthose phosphate pathway which supply both reduced nicotinamide adenine dinucleotide phosphate (NADPH) and pentose phosphates for biosynthetic reactions, were significantly increased in *G. lucidum* Gl-4. Finally, fermentative, glycogen and mannitol metabolisms were investigated evaluating the activity of lactate dehydrogenase, phosphoglucomutase, mannitol dehydrogenase and mannitol-1-phosphate dehydrogenase. The level of lactate dehydrogenase was very low in the studied strains indicating that the fermentative carbohydrate metabolism is limited in these fungi under these growth conditions, as previously reported also for Chinese and Italian *G. lucidum* strains [Saltarelli et al., 2009]. The phosphoglucomutase plays a pivotal role in the synthesis and breakdown of glycogen and its activity was considerable in all *Ganoderma* strains tested even if the *G. resinaceum* F-2 showed the lower level activity than other strains. 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 [Ceccaroli et al., 2007]. In our previous work [Saltarelli et al., 2009] we suggested that in *G. lucidum* other carbohydrates are able to substitute the mannitol as a storage compound (e.g glycogen and/or threhalose) since the mannitol dehydrogenase activity was very low. In this work, the activities of mannitol dehydrogenase and mannitol-1 phosphate dehydrogenase were very low in all tested strains, giving rise to our previous assumption.

Proteomic Analysis

In order to evaluate the protein profile of the different strains of *Ganoderma* spp a 2-DE was performed (Fig. 2). Gels were analysed for number, intensity and size of spots. The comparison of protein patterns of the strains revealed the presence of substantial differences in protein synthesis both qualitative and quantitative except for the two strains of *G. lucidum* Gl-4 and Gl-5 showing a very similar profile of protein expression; therefore we have chosen to show only the gel image relative to Gl-4 (Fig. 2a). Overall, *G. lucidum* appeared to have more complex protein patterns compared to *G. resinaceum* strains (Fig. 2b and c) which also differed among each other. The comparison between *G. lucidum* and *G. resinaceum* F-2, showed at least 33 protein spots exclusively present (white arrows) and at least 12 proteins more abundant (black arrows) in *G. lucidum* compared to *G. resinaceum* F-2. On the other hand, *G. resinaceum* F-2 showed 10 proteins exclusively expressed (white arrows) and 2 up-regulated (black arrows) compared to Gl-4 strain. A marked difference was in the 2D map of the *G. resinaceum* F-1, in which a less number of proteins were revealed, however, we could detect the presence of 7 (white arrows) of the 10 proteins typical of F-2 in addition to 2 up-regulated (black arrows). In the literature few papers report the differential profiles of proteins among strains of *Ganoderma* spp. The analysis of different *Ganoderma* isolates has shown that all strains shared two common proteins of approximate molecular weight of 69 K and 16 K [Rajamannar et al., 2000]. All of therapeutic effects of *Ganoderma* spp are attributed mainly to triterpenoids and polysaccharides, but more recently a new glycoprotein class in *Ganoderma* spp. named proteins FIPs was identified as immunomodulatory

and anticancer [Lin et al., 2010]. Another report suggests that the mycelia of *G. lucidum* have high potential in lowering blood pressure level due to the presence of several antihypertensive proteins [Ansor et al., 2013]. For this reason MS analyses are in progress to identify the proteins differently synthesized among the strains and to test their potential therapeutic effects.

Antioxidant Assay

The antioxidant activities of ethanol extracts of mycelia of different *Ganoderma* strains were evaluated using DPPH free radical scavenging and lipoxygenase assays, and the iron (II) chelating ability. As reported in Fig. 3a all mushroom extracts had radical scavenging activity toward hydroxyl radicals in a dose-dependent manner showing an inhibition ranging from 60 to 85% in all *Ganoderma* strains at concentration over 3 mg/ml. At lower concentration (0.1 to 1 mg/ml) the *G. lucidum* Gl-4 strain was more efficient in DPPH scavenging activity than other strains as demonstrated by its lowest EC_{50} value (0.64±0.04 mg/ml). This value was comparable to that reported for Chinese strain [Saltarelli et al., 2009] whereas EC_{50} values obtained in other analysed strains (1.52±0.16, 1.27±0.32, 1.15±0.6 mg/ml in Gl-5, F-1 and F-2, respectively) were in agreement to that revealed in mycelium of *G. lucidum* from Northeast Portugal [Heleno et al., 2012].

The chelating effect of *Ganoderma* strains is shown in Fig. 3b. Only Gl-4 has a very high chelating ability also at low concentrations and the lowest EC_{50} value (0.25±0.01 mg/ml). However, the EC_{50} values of other *Ganoderma* strains (4.02±0.6, 3.2±0.3, 3.2±0.5 mg/ml in Gl-5, F-1 and F-2, respectively) were similar to that previously reported for Italian and Chinese isolates (2.8 ± 0.03 and 3.36 ± 0.02 mg/ml, respectively) [Saltarelli et al., 2009].

Antioxidants interact non-specifically with lipoxygenase by scavenging radical intermediates and/or reducing the active heme site. All extracts inhibited the oxidation of linoleic acid catalysed by lipoxygenase in a dose dependent manner (Fig. 3c). Also in this case the most efficient was the Gl-4 strain even if its EC_{50} value (1.12±0.34 mg/ml) was not significantly different

from the other tested strains (0.93±0.04, 0.90±0.03, 1.11±0.215 mg/ml in Gl-5, F-1 and F-2, respectively).

The results obtained using three different testing systems suggest that ethanol extracts of mycelia of tested *Ganoderma* collections had direct and potent antioxidant activity. Among *G. lucidum* strains, the most effective was Gl-4. Moreover, on the basis of the assays performed in this study and the comparison with *G. lucidum,* also *G. resinaceum* mycelium could be utilized as a source of health beneficial natural antioxidants. To the best of our knowledge, few research works have been focused on G. *resinaceum* mainly concerning its endopolysaccharides [Silva et al., 2013] and the identification of two new lanostanoids [Niu et al., 2007]. A paper about antimicrobial, cytotoxic and antioxidant activities of selected Basidiomycetes from Yemen, describes its antibacterial and strong antioxidantive effect in agreement with our data [Al-Fatimi et al., 2005].

Total Phenolic Content

Total phenolic compounds such as flavonoids, phenolic acids, and tannins are considered to be major contributors to the antioxidant capacity of plants. Also mushrooms are found to be rich source of antioxidants [Kim et al., 2008; Saltarelli et al. 2009]. Among four selected *Ganoderma* strains, *G. lucidum* Gl-4 contained the highest amount of total phenolics (39.47±3.23 mg/g) whereas *G. resinaceum* F-2, the lowest (16.25±2.47 mg/g). In Gl-5 and F-1 strains the total phenolic content was 19.72±1.65 and 20.13±1.59 mg/g, respectively. To our knowledge, phenol level in *G. resinaceum* species was never reported before but it was comparable to that reported for Chinese and Italian *G. lucidum* strains (16.50 to 27.9 mg/g) [Saltarelli et al., 2009] and for *G. tsugae* (24.0 to 35.6 mg/g) [Mau et al., 2005]. These phenolic compounds have been little studied in the genus *Ganoderma*. A comparative study on fruiting body, spores and mycelium of *G. lucidum* from Northeast Portugal reveals that fruiting body phenolic extracts provided the highest content in total phenolics (~29 mg/g) whereas in mycelium extract was only 6.03±0.98 mg/g [Heleno et al., 2012]. Different value in total phenolic fraction in mycelium could be due to different culture media utilised for the fungal growth [Heleno et al., 2012; Saltarelli et al., 2009]. In fact optimizing a

culture medium, an increased production of biomass and phenolic compounds in *G. lucidum* was reported [Zárate-Chaves et al., 2013]. Furthermore, the effect of tryptophan on the total phenolic compounds in the mycelial culture of *G. neo-japonicum* was demonstrated [Park and Lee, 2010].

The highest content of total phenols in all tested mycelial samples particularly in Gl-4 strain might account for its elevated antioxidant activity. To elucidate the relationship between phenol content and antioxidant activity, we calculated the correlation with total phenol versus scavenging activity, chelating effect and lipoxygenase activity in all extracts (Supplementary data Table S2). The relationship between antioxidant activity and phenolic content for all tested medicinal *Ganoderma* isolates was a positive and highly significant linear correlation since R^2 ranged from 0.9941 to 0.9966, from 0.7465 to 0.9977 and from 0.7530 to 0.9454 in DPPH assays, in chelating activities and in lipoxygenase inhibition activity, respectively. These results suggest that the phenolic compounds contribute significantly to the antioxidant capacity of the *G. lucidum* mycelia as reported previously [Saltarelli et al., 2009]. Furthermore, these results are in agreement with those reported for *G. lucidum* from Northeast Portugal in which phenolic extracts of fruiting body and mycelia proved to have higher antioxidant potential than their corresponding polysaccharidic extracts highlighting the higher contribution of free phenolic compounds than the ones linked to polysaccharides [Heleno et al., 2012].

Analysis of Flavonoid Compounds

The presence of bioactive compounds was also measured by high performance liquid chromatography (HPLC). Among phenol compounds we focused our attention on flavonoids which are the most common bioactive molecules found in plants as well as in fungi, and are known to possess several important biological activities such as antioxidant, anticancer, antimutagenic, antimicrobial and anti-inflammatory [Hasnat et al., 2013]. The chromatographic profiles of *G. lucidum* Gl-4 and *G. resinaceum* F-2 and F-1 collections are presented in Fig. 4. *G. lucidum* Gl-5, is not reported because showed a very similar profile with Gl-4. Injection of flavonoid standards allows identifying quercetin, morin and myricetin in strains of *G. lucidum*; morin, myricetin and

rutin in *G. resinaceum* F-2. None of above mentioned compounds were detected in *G. resinaceum* F-1.

To our knowledge there are few reports concerning the identification of flavonoids in *Ganoderma* spp.. In a recent work the methanolic extracts of *G. lucidum* mushroom powder samples were subjected to HPTLC analysis and the flavonoid content was separated in six different peaks. These peaks were used for the assessment of antibacterial activity against different selected pathogens [Sakthivigneswari and Dharmaraj, 2013]. Furthermore, the quercetin as the major component in water extrats of *G. lucidum* grown on germinated brown rice has been reported [Hasnat et al., 2013].

Antiproliferative Effects of Mycelial Extracts

Human promonocytic U937 cells were exposed to incremental concentrations (0-2.25 mg/ml) of mycelial extracts of different *Ganoderma* spp. for 2 h. The residual growth of cells was determined by MTT assay after 48h of culture. The results showed that isolates Gl-4 and Gl-5 of *G. lucidum*, effectively inhibited the proliferation of tumor cells in a dose dependent manner (Fig. 5). The growth inhibitory effects were similar for both extracts, reaching over 80% inhibition at a concentration of 0.225 mg/ml. Concerning *G. resinaceum*, the strain F-2 showed a U937 residual growth of about 40% at 0.75 mg/ml, whereas *G. resinaceum* strain F-1, appeared ineffective on the U937 cell viability. The results reported for Gl-4 and Gl-5 mycelial extracts were comparable with that reported for G. *lucidum* fruiting body extracts in which the effective dose inhibiting 50% of U937 cell growth was 63 µg/ml and this effect was associated with an increased cells apoptosis [Müller et al., 2006]. Our results indicate that the ethanol extracts from G. *lucidum* mycelia have also an effective activity against leukemic monocyte lymphoma cells supporting previous data obtained in other human leukemia cell lines (i.e. THP-1, HL-60, NB4) utilizing lipids of spores or methanol extracts of fruiting body of *G. lucidum* [Calviño et al., 2010; Wang JH et al., 2012]. The antiproliferative effect of *G. lucidum* has been attributed to either the polysaccharide fraction, which is responsible for the stimulation of the immune system, or to the triterpenes, which demonstrate

cytotoxic activity against a variety of cancer cells [Suarez-Arroyo et al., 2013]. Our results suggest a possible involvement of other compounds such as total phenolics that are present in the extracts since a cytotoxic effect was also described for several phenolic compounds naturally occurring on different cancer cell lines [Nakajima et al., 2009]. Furthermore, the inefficacy of *G. resinaceum* F-1 on U-937 proliferation could be correlate in particular to the absence of flavonoid fraction as revealed by HPLC analysis (Fig. 4c).

There are several studies suggesting that flavonoids exhibit biological activities, including antioxidants, antiallergenic, antiviral, anti-inflammatory and anticancer. For this reason, different methods of extraction were tested to obtain a large amount of polyphenols and flavonoids in several fungi including also *Ganoderma* spp. [Abugri and McElhenney, 2013]. More recently, detectable quantities of flavonoids found in the methanol soluble fraction of the *G. lucidum* extract inhibited the neuraminidase activity of influenza virus H5N2 [Shamaki et al., 2013].

Concluding Remarks

Significant differences in the enzymatic activities of primary metabolism and in the protein patterns have been highlighted in studied collections of *Ganoderma* spp.. Furthermore, using three different testing systems, the ethanolic extracts from tested *Ganoderma* spp. show high antioxidant activity. Among *G. lucidum* strains the Gl-4 is the most effective and this elevate antioxidant activity is positively correlated by the highest content of total phenols in this strain. Moreover, the ethanolic extracts from G. *lucidum* (Gl-4 and Gl-5) have also an effective antiproliferative activity against leukemic monocyte lymphoma cells. To our knowledge there are no available reports concerning the comparison of biochemical characteristics, antioxidant and antiproliferative activities between *G. lucidum* and *G. resinaceum* mycelial strains. The results reported herein show that *G. resinaceum* could also be utilized to obtain several bioactive compounds even if its extract appears less effective on leukemic cell viability than *G. lucidum*. The obtained results could be of

important relevance from the viewpoint of antitumor actions of bioactive compounds from different *Ganoderma* collections.

Experimental Procedures

Fungal Cultures

Genetically identified *Ganoderma* collections were used in current study (Supplemetary data Table S1). Mycelial cultures of *G. lucidum* were isolated from immature fruiting bodies found in Armenia (strains Gl-4 and Gl-5) and cultures of *G. resinaceum* were isolated from fruiting bodies collected in France (strains F-1 and F-2). Mycelia are preserved in the Culture Collection of the Laboratory of Fungal Biology and Biotechnology (FBBL), Yerevan State University [Badalyan et al., 2012], whereas the fruiting bodies were conserved as exsiccates in the mycological herbarium of the Mycological Centre of the Bologna University (CMI-Unibo). 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 week old to maintain their viability and bioactivity. Isolates were grown in a liquid medium containing: 20 g/l of glucose, 2.0 g/l of polypeptone, 2.0 g/l of yeast extract, 5.0 g/l of KH_2PO_4, 2.5 g/l of $MgSO_4$ and 10 g/l of maltose, pH 5.7 in 250 ml Erlenmeyer flasks, each containing 100 ml of medium, and kept in a growth chamber at 30°C with agitation (250 rpm/min). After 20 days of growth the mycelia were recovered from the medium, washed with distilled water, dried at 60^oC and used for all experiments reported below.

Molecular Characterization and Phylogenetic Reconstruction

Molecular characterization of studied collections and other conspecific isolates of *G. lucidum* and *G. resinaceum* was carried out by sequencing the ITS1-5.8S-ITS2 and a portion of the IGS rDNA regions. Genomic DNAs were isolated from 100 mg of fresh mycelium scraped from 1-wk old cultures growth on cellophane-covered Potato dextrose agar (20 g/l). DNA extractions were carried out using Nucleospin Plant II kit (Macherey-Nagel) following to the manufacturer's

protocol for fungi. ITS and IGS regions were amplified using the primer pairs ITS1f-ITS4 [Gardes and Bruns, 1993] and CNL12-5SA [Henrion et al., 1992], respectively. PCRs were performed on 50 µl volume reactions using a T gradient Thermal Cycler (Biometra). Reaction mix contained 50 ng of total DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM for each dNTP, 400 nM for each primer and 1.5 U of "Dream Taq" DNA polymerase (Thermo Scientific). The cycling parameters were as follows: 3 min of initial denaturation at 95 °C; 30 cycles of 94 °C for 30 s, 55 °C (ITS) or 58 °C (IGS) for 30 s and 72 °C for 1 min (ITS) or 1.5 min (IGS); and a final extension step of 72 °C for 10 min. Amplified ITS fragments were separated in a 1% agarose gel and visualized by staining with ethidium bromide. PCR products were purified using the "PCR clean up" kit (Macherey-Nagel) and sequenced in both directions with primers mentioned above.

Sequences were edited using Chromas v2.3 (Technelysium) and deposited in GenBank database with the accession numbers (Supplementary data Table S1). ITS and IGS sequences generated in this study were analysed together with those obtained by other Authors [Wang XC et al., 2012] for *G. lucidum* (European and Chinese lineages) and *G. resinaceum* vouchers. Chinese *G. lucidum*, reclassified as *Ganoderma sichuanense* by Wang XC et al. [2012], was specified as outgroup. Alignments were performed by CLUSTAL W and gaps and ambiguous sites were excluded prior to phylogenetic analyses. Bayesian inference (BI) and Maximum Parsimony (MP) were used to confirm phylogenetic affiliation of isolates used for biochemical analysis. BI analysis was performed in MrBayes 3.2 [Ronquist et al., 2012] using a partitioned likelihood approach, where ITS and IGS regions were treated as independent partitions. The best-fit model of nucleotide substitution was determined using the jModelTest 2.1.4 [Darriba et al., 2012]. K80+I and TrNef+I were identified as the best models for ITS and IGS partitions, respectively. Two independent runs, with 4 chains each, were conducted for 5 million generations, sampling every 100 generations. A 50% majority-rule consensus tree was generated after the exclusion of the first 250 trees. Equally weighted MP analysis was conducted using a heuristic search in PAUP 4.0b10 [Swofford, 2002]

with 1000 random sequences additions. Branch support was assessed by bootstrap analysis of 1000 replicates.

Enzyme Assay

Mycelial samples were homogenized 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 different enzymes as previously described [Ceccaroli et al., 2007; Saltarelli et al., 2009]. One unit (U) of enzyme is defined as the amount of enzyme which catalyzes the formation of 1 µmol of product per min at 37 °C. The values obtained were the means of four independent determinations. The protein content was spectrophotometrically determined according to Bradford's method [1976]. Bovine serum albumin was used as standard reference.

Protein Analysis

Dried mycelia were ground with liquid nitrogen and homogenized in 7 M urea, 2 M thiourea, 4% 3-CHAPS, 30 mM Tris base using the Sample Grinding Kit (Amersham Biosciences). After centrifugation at 14,000 rpm, protein concentration in the supernatant was determined by the Bradford assay [1976]. Eighty µg 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 to 3500 V during the first 3 h, later stabilized at 5000 V for 20 h. After isoelectric focusing, IPG strips were equilibrated by soaking in 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 (18cm x 20cm x 1.5 mm) at 40 mA/gel constant current, until the dye front reached at bottom gel. Analytical gels were stained with silver nitrate and gel images were acquired by Fluor-S MAX multi-imaging system (BioRad) and the data were analysed, including spots detection,

quantification and normalization, using ImageMaster 2D Platinum version 5.0 software (GE Healthcare).

Extraction of Phenolic Compounds from Mycelia

The mycelia (1-3 g) were dried at 60 °C for 12 h and crashed in a mortar with liquid nitrogen. The samples were treated as reported in Saltarelli et al. [2009]. Briefly, the mycelia were extracted with mixture of 80% ethanol/water (80:20, v/v) at 4 °C under stirring. The fungal extracts were subsequently centrifuged at 1,500 rpm for 15 min and the residues were then extracted with two additional portions of the ethanol/water mixture. All supernatants were combined, evaporated in stove at 60 °C to dryness and stored at 4 °C. The extracts were re-dissolved in ethanol/water (80:20, v/v) for the antioxidant activity assays and further chemically characterizations.

Quantification of Total Phenolics

Total polyphenol content was determined using Folin-Ciocalteau method described by Singleton et al. [1999]. The total volume of the reaction mixture was minimised to 1 ml. The extract solutions (10 µl) were mixed with Folin-Ciocalteu reagent (50 µl) and deionised water (90 µl). Three minutes later 300 µl of 20% (w/v) sodium carbonate was added, and the mixture was brought up to 1 ml with distilled water. The tubes were vortex mixed for 15 s and allowed to stand for 30 min at room temperature for colour development. Absorbance was then measured at 725 nm (UVIKON spectrophotometer). 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 ($R^2 = 0.9973$).

HPLC Analysis of Flavonoids

A liquid chromatographic system from Beckman (Beckman, Berkely, CA, USA) was used. The HPLC apparatus consisted of a Programmable solvent module n. 126, a Model 210A sample injection valve, a 20µl injection loop and a 168 diode-array detector. The system was interfaced with a personal computer utilizing System Gold Karat software for control and data collection. A 5

µm Supelcosil LC-318 analytical column (25 cm x 0.46 mm I.D.) (Supelco, Bellafonte, PA, USA), protected with a guard column (2 cm x 0.21 mm I.D.) was used at the same experimental conditions described by others [Menghinello et al., 1999]. The mobile phase used for the separation consisted of 0.1% (v/v) TFA (Buffer A), and 0.1% TFA in acetonitrile (Buffer B).The following gradient of Buffer B was used: 10-50% in 35 min, 50-100% in 1 min and 5 min at 100%. The initial conditions were restored in 7 min. The flow rate was 1.2 ml/min. Detection was performed at 254 nm and 340 nm and the diode-array detector was set from 190 nm to 400 nm.

Evaluation of Antioxidant Activity

The antioxidant activity of the phenolic extracts was evaluated using three different *in vitro* assays previously described [Saltarelli et al., 2009]: DPPH radical-scavenging activity, chelating activity on Fe^{2+} and lipoxygenase assay. Briefly, the DPPH assay was conducted as follows: a 100 µM $DPPH^{\bullet}$ ethanol solution was prepared and 0.85 ml were added to 0.15 ml of sample (0.1–4.5 mg/ml) diluted in 50 mM Tris-HCl, pH 7.4. The absorbance decrease at 517 nm was recorded after 10 min at room temperature. For determination of Fe^{2+}chelating activity, 200 µl of extract (0.03–8.0 mg/ml) 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. For lipoxygenase assay, 5-lipoxygenase (0.18 µg/ml) was added to the reaction mixture (1 ml) pre-equilibrated at 20 °C for 20 min containing 10 mM linoleic acid, the sample or the same quantity of Tris/HCl, pH 7.0 as blank, and 50 mM sodium phosphate, pH 6.8. The formation of hydroperoxides from linoleic acid was then observed spectrophotometrically at 235 nm at 20 °C.

Evaluation of Cytotoxic Activity

Human promonocytic U937 cells, used for the cytotoxicity test, were cultured in suspension in RPMI 1640 medium supplemented with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), 1.2 mM glutamine and 10% fetal bovine serum. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. For experiments, exponentially growing cells were washed and the growth medium replaced with a saline containing 8.182 g/l NaCl, 0.372 g/l KCl,

0.336 g/l $NaHCO_3$ and 0.9 g/l glucose prewarmed at 37 °C. The cells were resuspended at a number of 4×10^5 cells/treatment condition and incubated with different extracts at a concentration ranging from 0.0375 to 2.25 mg/ml for 2 h. After the treatments, cells were washed with PBS buffer and complete cultural medium added. The effect of different extracts on the viability of U937 cells was evaluated after 48 h postchallenge and determined by the MTT assay. One hundred microliters of MTT solution (5 mg/ml in PBS) were added to each cell suspensions and the cells were further incubated for 30 min at 37 °C. Then the cells were centrifuged at 1,200 rpm for 3 min, the supernatants were removed and the pellets dissolved in 1 ml of dimethyl sulfoxide. Formation of blue formazan was measured spectrophotometrically at 570 nm. Data were expressed as percent of viability compared with control.

Statistical Analysis

For each sample assayed three or more replicates were made. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Kruskal-Wallis test for multiple comparison analysis or by the non-parametric test Mann–Whitney U. *p*-values less than 0.05 were considered significant.

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Figure Legends

Fig. 1. Bayesian tree constructed from ITS and IGS rDNA regions (1491 sites). Topologies from equally weighted parsimony and Bayesian inference were congruent at all supported nodes. Thickened branches are supported both by parsimony bootstrap values of >70% and by Bayesian posterior probabilities of >95%. Taxa are labelled by their voucher identifier or isolate code (see Supplementary data Table S1). The tree was edited using TreeGraph 2.0.54-364 beta (http://treegraph.bioinfweb.info/).

Fig. 2. 2-DE of 80 µg of total proteins from different *Ganoderma* strains. (a) Gl-4; (b) F-2; (c) F-1. White arrows mark the proteins exclusively expressed in that isolate, black arrows mark the proteins whose expression is higher than the other strain. IEF was carried out using IPG 3-10 NL followed by second dimension SDS-PAGE (9-16% w/v acrylamide). Gels were stained with silver nitrate.

Fig. 3. Antioxidant capacity of *Ganoderma* spp. mycelial extracts. (a) Scavenging effect on the DPPH test and (b) chelating ability on ferrous ions; Δ Gl-4; ○ Gl-5; ● F-1; ▲ F-2. (c) Effect on lipoxygenase activity *in vitro*. Each value represents the mean ± S.D. of three independent measurements.

Fig 4. HPLC analysis of flavonoid profile. In the figure are represented the chromatograms of HPLC analyses of different strains: (a) GL-4; (b) F-2; (c) F-1. The identified flavonoids are: myricetin (1); morin (2); quercetin (3); rutin (4).

Fig. 5. The effects of mycelial ethanolic extracts of different *Ganoderma* collections on cell proliferation by MTT assay. U937 cells were treated with different extracts at concentration range from 0.0375 to 2.25 mg/ml for 2 h. Viable cells yield were detected after 48 h. The cells untreated

with extracts were used as a control and the cells treated with ethanol alone were reported as 0+et in the graph. MTT assays were performed as described. Results shown represent the mean ± S.D. for at least three separate experiments. * Significant differences at $p < 0.05$

Table 1. Activity of some enzymes in tested *Ganoderma* collections.

Enzyme	Gl-4 (A)	Gl-5 (B)	F-1 (C)	F-2 (D)
Hexokinase	0.977±0.268	0.594±0.123	0.819±0.119	0.76±0.066
Phosphofructokinase	0.179±0.009	0.086±0.055*	0.107±0.044	0.172±0.049
Pyruvate kinase	2.606±0.813	2.655±0.268	2.99±0.689	2.452±0.308
Glucose-6-phosohate dehydrogenase	0.428±0.088*	0.165±0.0175	0.104±0.023	0.11±0.008
Lactate dehydrogenase	0.033±0.009	0.0189±0.001	-	0.0171±0.003
Phosphoglucomutase	2.241±0.241	2.515±0.272	1.401±0.322	1.296±0.060*
Mannitol dehydrogenase	0.0260±0.001	0.0194±0.002	0.0266±0.002*	0.0214±0.002
Mannitol-1-phosphate dehydrogenase	0.0433±0.004*	0.0163±0.001	0.0259±0.017	0.0176±0.001

The values of enzymatic activities are expressed as U mg^{-1} of total proteins. Each value represents the mean of four independent measurements.
Kruskal-Wallis test
Dunn's post test p values are expressed as * ($p < 0.05$),
Significant differences between groups A,B,C,D (Dunn's test)
Phosphofructokinase *B vs A
Glucose-6-phosohate dehydrogenase *A vs C, D
Phosphoglucomutase * D vs B
Mannitol dehydrogenase * C vs B
Mannitol-1-phosphate dehydrogenase * A vs B, D

Fig. 1. Saltarelli *et al.*

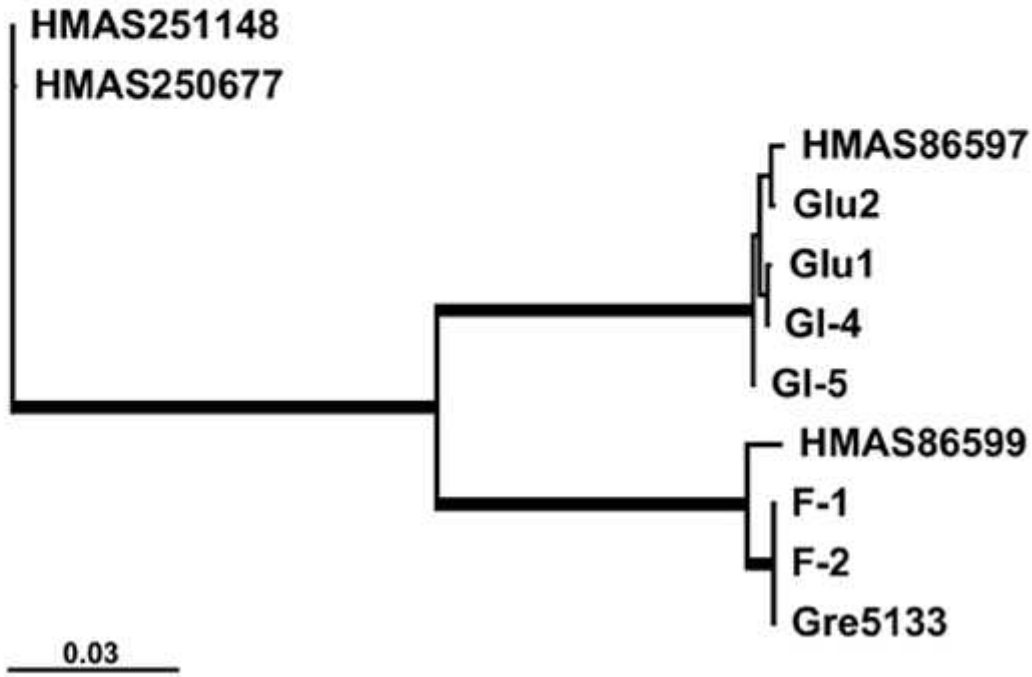
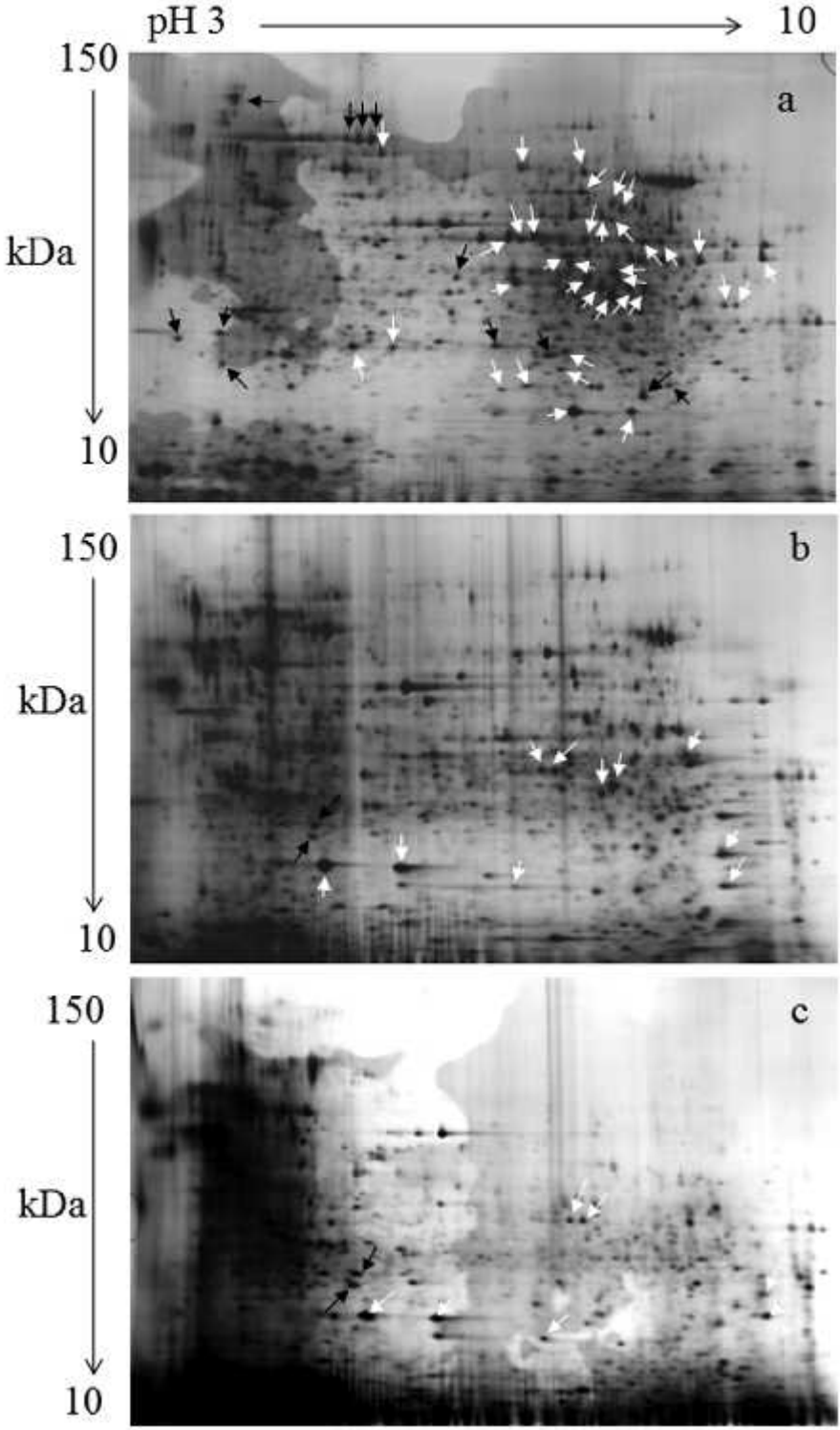


Fig. 2. Saltarelli *et al.*



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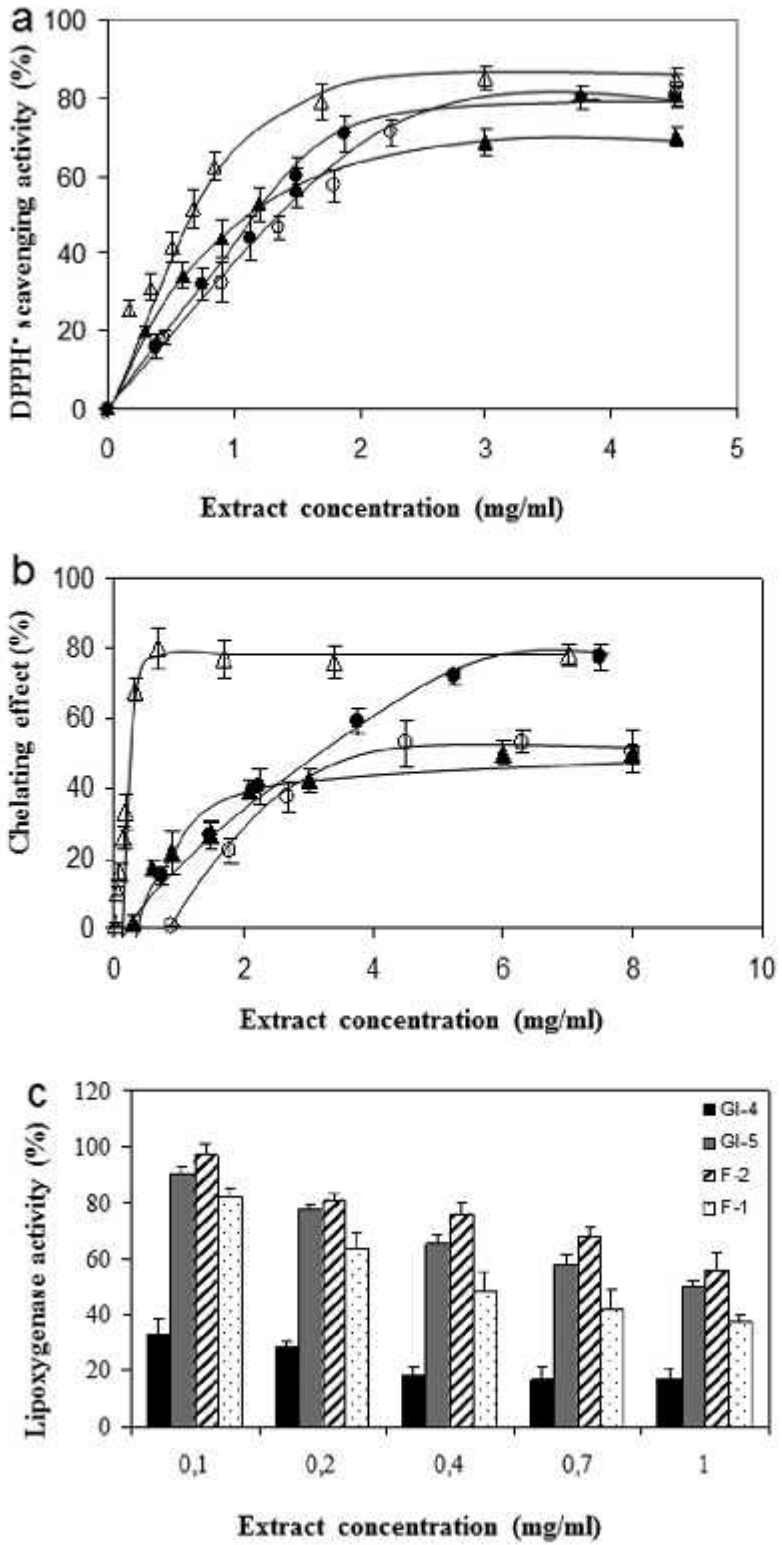

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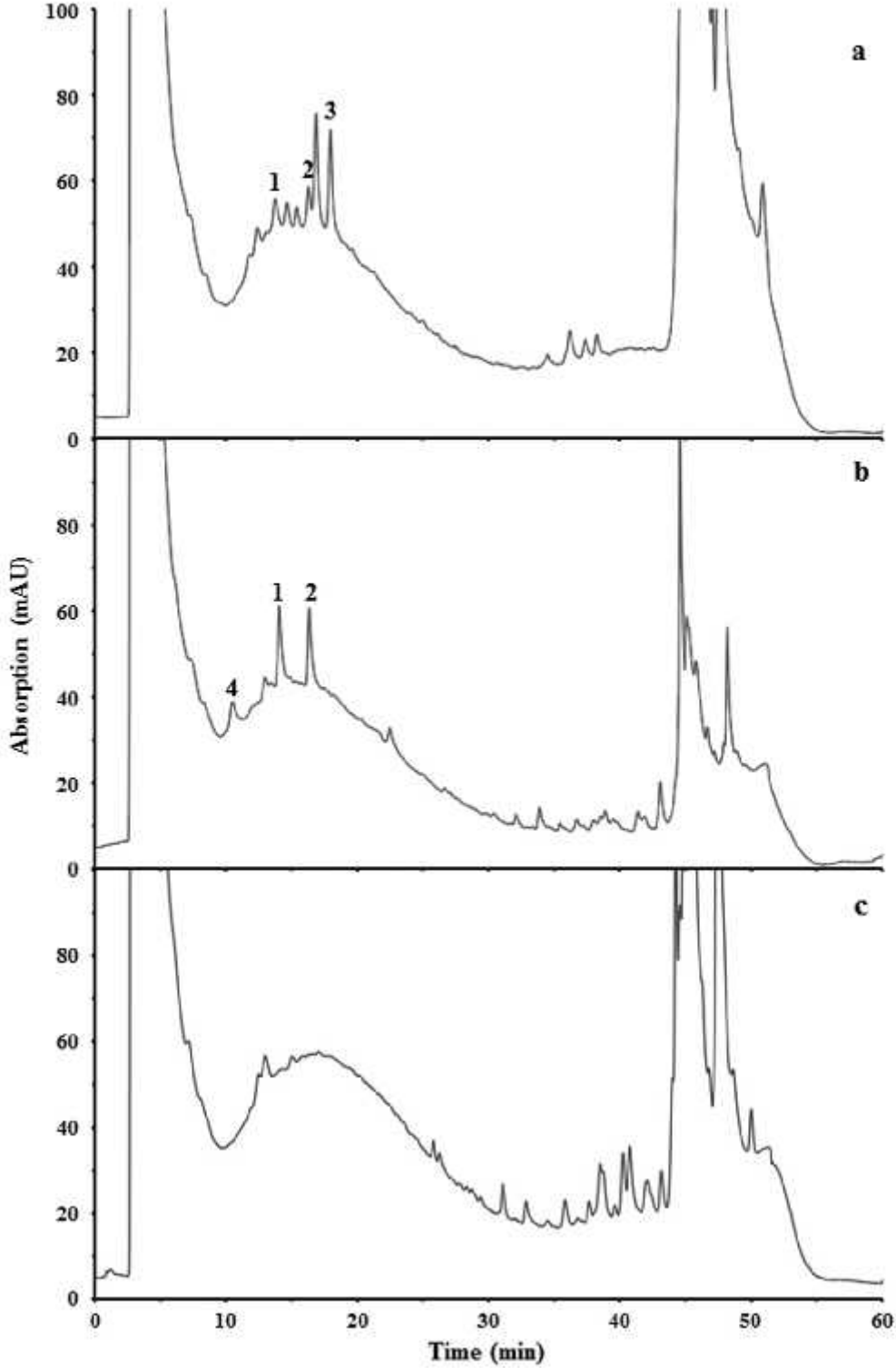

Fig. 5. Saltarelli *et al.*