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ORIGINAL ARTICLE

Production of ganoderic acid by *Ganoderma lucidum* RCKB-2010 and its therapeutic potential

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Abstract The newly isolated basidiomycetous fungus, identified as *Ganoderma lucidum* RCKB-2010 was tested for production of ganoderic acid (GA) under submerged fermentation conditions. Production of GA under liquid static cultivation condition was found to be 2,755.88 mg L^{-1} on the 25th day of incubation, whereas under shaking cultivation conditions the maximum production of GA was observed to be 373.75 mg L^{-1}. ^{1}H NMR analysis revealed clearly that the fungal extracts possessed a lanostane skeleton, confirming the presence of GA. Interestingly, GA was found to have potential to inhibit the proliferation of HeLa cells and U87 human glioma cells in a dose dependent manner. In addition, GA was also found to possess antibacterial activity, exhibiting a minimal inhibitory concentration of 0.25 mg mL^{-1} against standard strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. GA produced in the present study holds potential as a potent anticancer agent.

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Introduction

Ganoderma, a basidiomyceteous white-rot fungus, normally growing on woody plants and wood logs, is rich in novel "mycochemicals", including triterpenes, polysaccharides, steroids and fatty acids, etc. Among them, triterpenes possess diverse and potentially significant pharmacological activities. In China, *Ganoderma* (also known as "Lingzhi") has been reported for the treatment of various ailments (Shiao et al. 1994). It has also been demonstrated to have anti-cancer and anti-microbial/viral activities, including anti-human immunodeficiency virus (HIV) activity (Yoon et al. 1994; El-Mekkaway et al. 1998; Weng et al. 2009). Extracts of Lingzhi have also been reported to mediate the neuronal differentiation and neuro protection of rat PC12 cells (Cheung et al. 2000). The majority of the potential compounds have been extracted from the fruiting body of Lingzhi; among these, ganoderic acid (GA) is the most potent therapeutic biomolecule known. However, production of GA is limited because of various constraints in the production of fruiting body of *Ganoderma* sp. in a shorter time span under laboratory conditions.

Normally, a fruiting body takes 6 months to mature under solid state fermentation (SSF), and it is also difficult to control the quality of the product during cultivation (Fang et al. 2002). Submerged fermentation (SmF) offers an alternative approach for efficient production of fungal metabolites (Xu et al. 2008). SmF provides better growth of fungi and allows the addition of many substrates with a wide variation in their concentrations without worsening the mass transfer conditions in the culture (Fang et al. 2002).

Recently, various research groups have focussed on developing suitable conditions, such as fed batch culture, addition

of different carbon, nitrogen sources and surfactants in SmF, to improve biosynthesis/production of GA (Wagner et al. 2003; Xu et al. 2010). For example, in static liquid culture the addition of Na^+, or nitrogen-limiting conditions increase GA production (Xu et al. 2013; Zhao et al. 2011). Enhanced production of individual GA-Me—an important bioactive triterpene—was obtained using a statistical culture condition optimization approach and response surface methodology (Liu et al. 2012). Furthermore, increased production of GA is also possible by implementing mycelium culture on solid medium (You et al 2012).

Keeping in view the high demand and short supply of high quality fruiting bodies of *Ganoderma* sp. for the extraction of bioactive compounds, the present study focussed on production of GA from the newly isolated fungus, *G. lucidum* RCKB-2010 under liquid static and shaking cultivation conditions. Further, to establish the anticancerous potential of GA, crude GA was evaluated against human cancerous cell lines, and its antibacterial activity was assessed against Gram positive and Gram negative bacteria.

Materials and methods

Isolation of fungus and its maintenance

The growing fruiting body of fungal isolate RCKB-2010 was collected from the deteriorating stem of *Prosopis julifora* (Mesquite), brought to the laboratory and cut into small pieces (3 mm × 3 mm) , then sterilised by treating with 0.1 % Hg_2Cl_2 (w/v) for 1 min followed by washing in 75 % ethanol (Kuhad and Johri 1989). Thereafter, it was washed thoroughly with sterile milli-Q water to maintain aseptic conditions. The fruiting body was then dissected with the help of a sterilised scalpel to obtain the basidiospores, which were inoculated on malt extract agar (MEA) supplemented with augmentin (100 μg mL^{-1}) and incubated at 30 °C in BOD incubator (Hicon, India). Fungal culture was maintained and subcultured regularly on MEA consisted of (g L^{-1}); malt extract 20, $Ca(NO_3)_2{\cdot}4H_2O$ 0.5, $MgSO_4{\cdot}7H_2O$ 0.5, KH_2PO_4 0.5 and agar 20.0 (Dhawan and Kuhad 2002).

Identification of the fungal isolate

Fungal genomic DNA was isolated following the method described earlier (Kuhad et al. 2004). The polymerase chain reaction (PCR) was conducted using the universal ITS primers ITS1-5′ TCC GTA GGT GAA CCT GCG G-3′ (forward) and ITS4-5′ TCC TCC GCT TAT TGA TAT GC-3′ (reverse). The *Taq* buffer, dNTP mix, $MgCl_2$ and *Taq* DNA polymerase were obtained from New England Biolabs, UK. The amplification conditions were 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 52–56 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min. The reaction product was electrophoresed on agarose gel to check the amplification. The amplified PCR product was then purified using a Real Biotech Corporation PCR purification kit and sequence was analysed using NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST) to locate the taxonomic position of the fungus. Sequence variations in internally transcribing spacers (ITS) regions were used for taxonomic and phylogenetic studies for identification of the fungus. The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei 1987). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are presented as units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions with less than 95 % site coverage were eliminated. That is, no greater than 5 % alignment gaps, no missing data, and no ambiguous bases were allowed at any position. Evolutionary analyses were conducted using MEGA5 (Tamura et al. 2011).

Production of GA under different cultivation conditions

Liquid static culture

Each 250 mL Erlenmeyer flask contained 50 mL sterile malt extract broth (MEB), comprising: malt extract 20 gL^{-1}, $Ca(NO_3)_2{\cdot}4H_2O$ 0.5, $MgSO_4{\cdot}7H_2O$ 0.5 and KH_2PO_4 0.5, was inoculated with two agar plugs (8 mm diameter each) obtained from the periphery of a 7-day-old fungal culture. The flasks were incubated under static cultivation conditions at 30 °C for 5, 10, 15, 20, 25, and 30 days. The flasks were harvested at regular intervals and analysed for biomass production, change in pH and GA production. All experiments were performed in triplicate unless otherwise stated.

Liquid shake culture

The fungus was grown for 7 days in liquid static culture and the mycelial biomass obtained was crushed in a sterile pestle-mortar and used as inoculum. Each 250 mL Erlenmeyer flask containing 50 mL sterile MEB was inoculated with 4 % (v/v) of crushed mycelium. The flasks were incubated under shaking cultivation conditions at 30°C, 150 rpm for 5, 10, 15, 20, 25 and 30 days. The flasks were harvested at regular intervals and analysed for biomass production, change in pH and GA production.

Analytical procedures

Estimation of fungal biomass

The contents of the flask were filtered through pre-weighed oven dried (W1) Whatman filter paper (Whatman no. 1). The

filter paper with mycelial mass was then dried at 60 °C until constant weight (W2) was achieved (Shrivastava et al. 2011). The difference between W2 and W1 was taken as the dry weight of fungal biomass.

Estimation of GA and intracellular polysaccharide

The GA content in fungal mass was determined using a standard method as described by Tsujikara et al. (1992). The GA from the dried mycelium (100 mg) was extracted in 50 % (v/v) ethanol (3 mL) for 1 week (twice). The mycelium was separated from the sample by centrifugation and the supernatants were dried at 50 °C under vacuum. The mycelial biomass was suspended in water and later extracted with chloroform. The GA extracted in chloroform was further extracted by 5 % (w/v) $NaHCO_3$, and the pH of the $NaHCO_3$ layer was adjusted to less than 3 by adding 2 M HCl. The GA in the $NaHCO_3$ layer was again extracted with chloroform. The chloroform was removed by evaporation at 40 °C and GA was dissolved in absolute ethanol and the absorbance was read at 245 nm in a spectrophotometer. A standard for GA was prepared using a commercial preparation of Ganoderic acid A (Chromadex, Irvine, CA).

Intracellular polysaccharide (IPS) from the dried mycelium (100 mg) was extracted with a solution of 1 M NaOH at 60 °C for 1 h (Berovic et al. 2003). The polysaccharide extracted was then quantified using the phenol-sulphuric acid method described by Dubois et al. (1956).

Nuclear magnetic resonance

The chloroform extract of mycelia mass containing GA was chromatographed on silica gel and separated before subjecting to ^1H nuclear magnetic resonance (NMR) (Hirotani et al. 1986). ^1H NMR of GA was recorded on a JEOL JNMECX 400P FT NMR system available at the University Science Instrumentation Facility, University of Delhi, North Campus, New Delhi, India, using tetramethylsilane (TMS) as an internal standard. The chemical shift values were recorded on δ scale.

Cell proliferation assay

A cell proliferation assay was carried out using the Millipore Colorimetric kit for cell survival and proliferation (Liu et al. 2009). The viability of cells was evaluated by MTT [3-(4, 5-dimethylthiazo l-2-yl)-2, 5-diphenyltetrazolium bromide] reduction method. The cells (1×10^5 cells mL^{-1}) were plated in a 96-well tissue culture plate and treated with different concentrations of GA (5–50 μg mL^{-1} for HeLa and 1.25–2,500 μg mL^{-1} for U87 human glioma cells) for 4 h. The cells were incubated for another 24 h for cell proliferation. The cells were then stained with MTT for 4 h and then 0.1 mL isopropanol and 0.04 *N* HCl was added. Optical density at 570 nm was monitored to determine cell viability. Effects of GA on inhibition of cell growth were calculated; cells treated with DMSO at the same concentrations were used as a control.

Antibacterial activity

Screening for GA antibacterial activity was carried out by the Broth Microdilution minimum inhibitory concentration (MIC) method with standard bacterial strains as described earlier (Kidwai et al. 2005). The antibacterial test by broth microdilution method was carried out in sterile microdilution plates with 96 flat-bottomed wells. The different bacterial cultures (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*) were suspended in saline solution with a turbidity equivalent to a McFarland 0.5 turbidity standard. The inoculum was suitably diluted and a volume of 100 μL was dispensed into separate wells, which contained 100 μL GA (ranging from 1,000–2, 500 μg mL^{-1}). In each plate, one well was kept as positive control (broth + inoculum) and another as negative control (broth only) and ampicillin was taken as antibiotic standard. The plates were incubated at 37 °C overnight. The turbidity was measured by comparing the growth of the bacterium in the wells with the positive control. The lowest concentration at which 50 % of the growth of bacterium was inhibited in comparison to the positive control was identified as the MIC.

Statistical analysis

All experiments under liquid static and shaking culture conditions were carried out in triplicate and the average of three observations is reported (SD<10 %) unless otherwise stated. Differences in various means during the cell cytotoxicity assay were tested by one way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Statistical analyses were performed using Sigma Stats 3.0 (Systat Software, San Jose, CA) for Windows.

Results and discussion

Identification of new fungal isolate

The fruiting body of the new fungal isolate RCKB-2010 appeared leathery with hard-crust shiny surface and when brought into culture, grew as thick white mycelium and attained full growth on MEA plate after 6th day. The ITS rDNA partial gene sequence when compared with database, the fungal isolate was found to exhibit maximum homology with the ITS sequence of the fungus belonging to genus *Ganoderma* and species *lucidum* (GenBank accession nos. HM130706, FJ463925 and FJ463920) (Fig.1 a, b and c). The

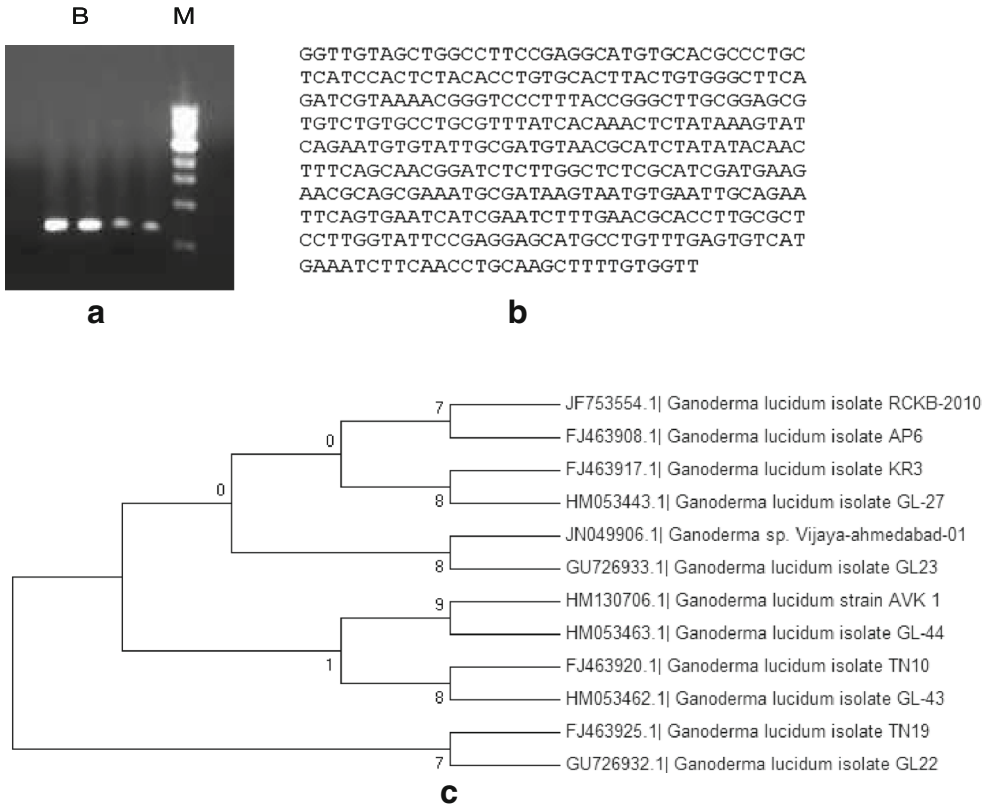

Fig. 1 **a** Internal spacer sequence (ITS) rDNA amplicon. **b** Sequence of the ITS region. **c** Dendrogram showing evolutionary relationship of *Ganoderma lucidum* RCKB-2010 with other *Ganoderma* sp.

sequence of new fungal isolate was submitted to the GenBank with the accession number *JF753554* (*Ganoderma lucidum* RCKB-2010).

Profile of fungal growth, pH and GA production

A time-course production of GA by *G. lucidum* RCKB-2010 was carried out for 30 days, under both stationary and shaking cultivation conditions. The fungus synthesized more GA under static cultivation compared to shaking cultivation conditions. The GA level under static cultivation conditions increased from day 5 (13.38 mg L^{-1}) to day 15 of incubation (1,803 mg L^{-1}), decreased slightly on day 20 (1,585 mg L^{-1}) and increased again on day 25 (2,755.88 mg L^{-1}) (Table 1). On the contrary, under shaking cultivation conditions, GA production was observed to increase up to day 30 (373.75 mg L^{-1}) except for a slight fluctuation on day 20 (115.14 mg L^{-1}) (Table 1). The biomass production and change in pH profile during both the cultivation conditions are shown in Fig. 2a,b. Under static cultivation, fungal biomass of *G. lucidum* RCKB-2010 increased regularly along with an increase in incubation period up to day 25 (9.4 mg mL^{-1}) and started decreasing thereafter. Similarly, *G. lucidum* RCKB-2010 when grown in shaking culture produced maximum biomass on day 30 (7.01 mg mL^{-1}) except for a slight decrease on the 10th day (Fig. 2a). Irrespective of the cultivation conditions, other than slight fluctuation in GA production on day 20, there was a positive correlation between production of GA and fungal growth. However, statistical analysis of the correlation between Biomass and GA revealed only a weak

Table 1 Ganoderic acid (GA) and intracellular polysaccharide (IPS) production under different cultivation conditions. Values are presented as mean of three replicates ± SD

Day	Liquid static cultivation		Liquid shaking cultivation	
	GA (mg L^{-1})	IPS (mg L^{-1})	GA (mg L^{-1})	IPS (mg L^{-1})
5	13.38±0.87	237.82±12.78	66.22±4.32	667.53±32.23
10	80.65±6.70	238.32±20.56	131.85±8.79	2,034.63±89.78
15	1,803.53±33.89	367.68±9.78	209.16±13.82	604.67±23.28
20	1,485.29±35.15	255.19±18.76	115.14±9.57	3,225.86±112.38
25	2,755.88±56.78	314.41±22.56	297.79±18.74	2,193.68±68.78
30	1,205.44±90.34	271.38±13.24	373.75±12.22	2,039.08±48.73

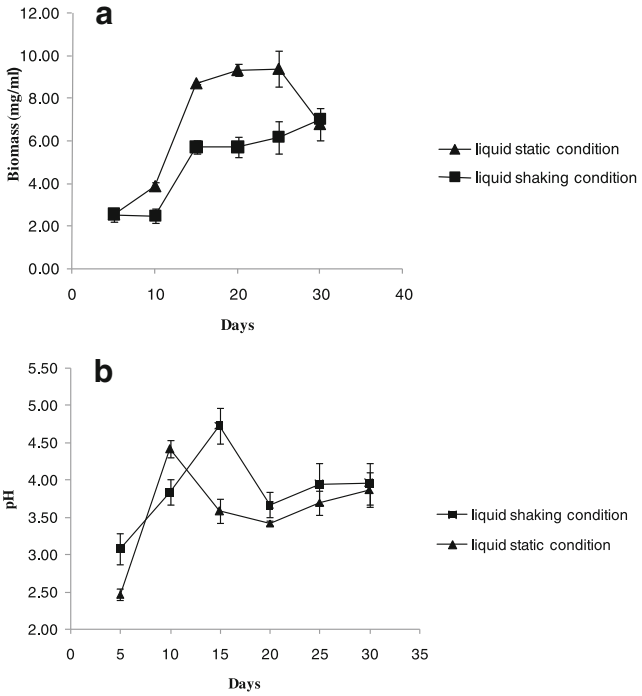

Fig. 2 **a** Biomass, and **b** pH profile during submerged fermentation of the fungus *Ganoderma lucidum* RCKB-2010. Δ Liquid static cultivation, □ liquid shaking cultivation

correlation coefficient under both static (R^2=0.4831) and shaking (R^2=0.774) culture conditions. Similar observations about GA and biomass production has been reported by Zhang and Zhong (2010) and Xu and Zhong (2012).

The dry cell mass of *G. lucidum* RCKB-2010 obtained in the present study was observed to be slightly less (2–10 g L^{-1}) than that found in various other reports under similar conditions (10–17 g L^{-1}) (Fang and Zhong 2002a, b). This could be due to limited initial feeding of carbon and nitrogen source (malt extract and micronutrients) in the present experiment instead of providing higher levels of carbon and nitrogen sources (peptone, yeast extract: 2.5–10 and glucose: 20–65 g L^{-1}) (Fang and Zhong 2002a). In addition to that, fungal cells are also known to grow under a certain pH range and metabolite formation is also known to be affected by medium pH (Fang and Zhong 2002b). Higher GA production with an increase in incubation period could also be explained by a change in medium pH causing variable cell membrane function, cell morphology and structure, solubility of salts, ionic states of substrates and nutrient uptake (Fang and Zhong 2002b). Similarly, other than a slight fluctation, we also observed a change in the initial pH of the fermentation medium from 5.4 to 3.0 (Fig. 2b).

A similar trend of GA production by *Ganoderma lucidum* was reported earlier by Fang and Zhong (2002a). The overall profiles of fungal cell growth and GA production under both cultivation conditions have shown that, under liquid shaking conditions, higher shaking speeds perhaps favoured IPS production greatly over that of GA (Table 1). The suppressed production of GA under shaking conditions may be due to decreases in the adsorption of the secreted polysaccharides on the cell wall providing stimulus for further polysaccharide synthesis (Wagner et al. 2003). Alternatively, under static cultivation conditions, restriction of oxygen and nutrient diffusion due to the presence of a thick layer of mycelium or an increased transcription of its biosynthetic genes (*hmgr*, *sqs*, and *ls*) could explain the stimulation of GA production (Wagner et al. 2003; Xu et al. 2010). However, sporulation is also known to be another reason for the accumulation of GA in the static liquid culture of *Ganoderma* sp. (Zhang and Zhong 2010). Moreover, in the present study, use of complex medium (MEB) could also have resulted in higher GA production by eliminating the negative effect of catabolic repression under submerged culture of *G. lucidum* (Fang and Zhong 2002a; Jia et al. 2009; Xu et al. 2008). The decrease observed in GA production in late middle phase could be due to a decrease in the transcription of genes involved in the biosynthesis of GA (Xu et al. 2010).

Confirmation of GA

The GA produced was analysed by ^{1}H NMR. The ^{1}H NMR spectrum of crude extract from *G. lucidum* RCKB-2010 clearly suggested that the GA possessed a lanostane skeleton (Fig. 3). The presence of GA extracted from fungal mycelium was confirmed by comparing with a standard peak of GA reported earlier (Hirotani et al. 1986) (Table 2). Moreover, lanosterol with its lanostane ring skeleton is reported to be an important precursor of GAs (Shiao 1992).

Evaluation of therapeutic potential of GA

Antibacterial activity

The antibacterial activity of GA from *G. lucidum* RCKB-2010 was evaluated against Gram positive and Gram negative bacteria (Table 3). Among the bacteria used in this

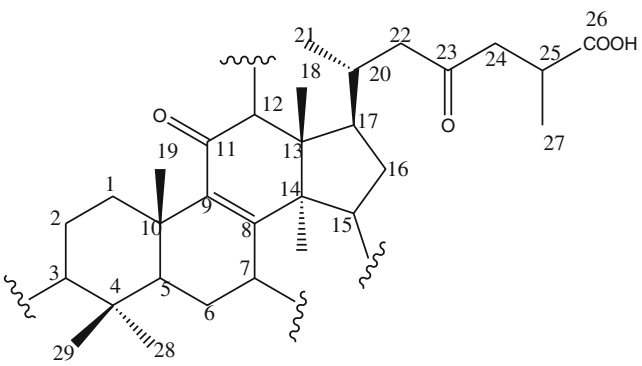

Fig. 3 Chemical structure of ganoderic acid (GA)

Table 2 NMR spectrum data of GA in $CDCl_3$ (δ in ppm)

Position	δ_H (J, Hz)
1	1.4-1.5 (2H, overlapping m)
2	1.8-1.9 (2H, overlapping m)
3	4.1 (1H, overlapping m)
4	–
5	1.6-1.7 (1H,m)
6	1.6-1.7 (2H, m)
7	3.9 (1H, m)
8	–
9	–
10	–
11	2.1–2.2 (2H, m)
12	1.9–2.0 (2H, m)
13	–
14	–
15	4.9–5.0 (m)
16	2–2.1 (2H, m)
17	1.75 (1H, overlapping)
18	0.68 (3H, S)
19	0.96 (3H, S)
20	1.5 (1H, overlapping)
21	0.90 (3H, S)
22	3.25 (2H, overlapping, m)
23	2.3–2.5 (2H, overlapping, m)
24	2.2–2.3 (2H, m)
25	–
26	–
27	–

study, *S. aureus* and *S. epidermidis* were found to be inhibited maximally in the presence of GA and its MIC was 0.25 mg mL^{-1}, which shows its higher efficiency compared to earlier reports, i.e. 0.75 mg mL^{-1} against *Micrococcus luteus* (Yoon et al. 1994). However, the present study revealed that the antibacterial activity of GA was more potent against Gram positive bacteria than Gram negative bacteria. Similar antimicrobial properties of methyl australate extracted from *Ganoderma austral* have been reported (Smania et al. 2007).

Table 3 Minimal inhibitory concentrations (MICs) of GA obtained from *Ganoderma lucidum* RCKB-2010 against Gram negative and Gram positive bacteria

Organism	MIC
Escherichia coli	1 mg mL^{-1}
Pseudomonas aeruginosa	1 mg mL^{-1}
Staphylococcus aureus	0.25 mg mL^{-1}
Staphylococcus epidermidis	0.25 mg mL^{-1}

Cell cytotoxicity assay

The effect of GA from *G. lucidum* RCKB-2010 on the growth of HeLa cell line and U87 human glioma cell line was observed. Proliferation of HeLa and U87 glioma cancer cells was found to be inhibited in a dose-dependent manner. GA from *G. lucidum* RCKB-2010 at 50 μg mL^{-1} and 2,500 μg mL^{-1} caused nearly 90 and 95 % inhibition, respectively, of HeLa and U87 glioma cells growth after 24 h incubation (Fig. 4a, b). Cytotoxicity against tumour cells, as observed in the present study, might be due to cell cycle arrest at G2/M phase and induction of apoptosis (Yue et al. 2008). Moreover, it has also been reported earlier that eIF5A—a protein important in translation initiation—might contribute to the growth inhibition of HeLa cells induced by GA (Tzivion et al. 2006). However, the effect of individual GA could not be studied because different GAs have different biological activities and it has proved difficult to separate and purify them due to their close physical and chemical properties (Zhang and Zhong 2010).

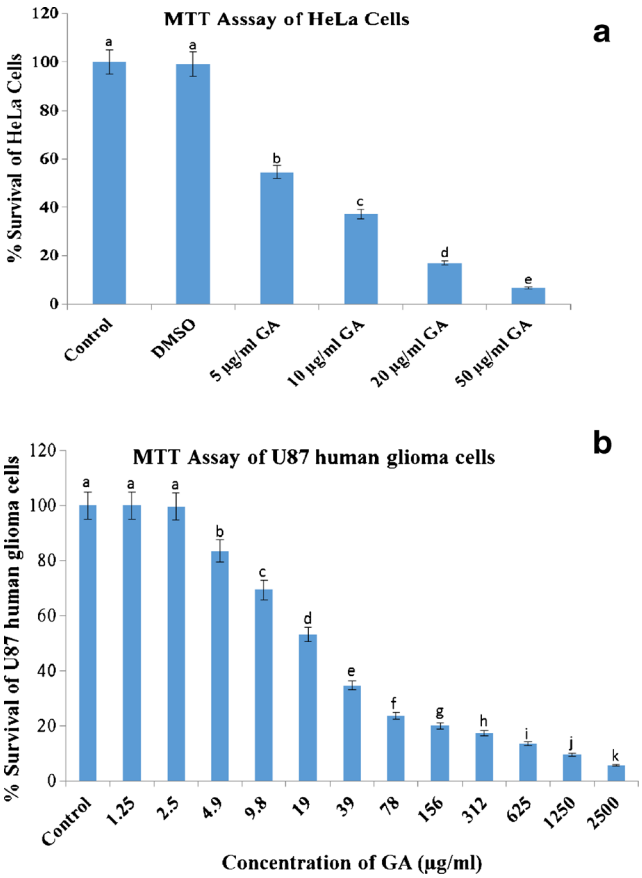

Fig. 4 Effect of GA on the growth survival of (**a**) HeLa cells (**b**) U87 Glioma cells. **a** Means bearing different superscripts (*a–e*) denote significant difference ($P<0.05$) with respect to control and same superscript denotes non-significant differences, analysed by ANOVA followed by Dunnett's post hoc test. **b** Means bearing different superscripts (*a–k*) differ significantly ($P<0.05$) and same superscript denotes non-significant difference, analysed by ANOVA followed by Dunnett's post hoc test

Conclusions

In summary, *G. lucidum* RCKB-2010 used in this study produced several fold higher amounts of GA when cultured in liquid static cultures than in shake cultures. The GA in crude mycelial extract exhibited antibacterial properties against Gram positive and Gram negative bacteria, indicating its possible usage as a broad spectrum antibiotic compound. Another property of GA to inhibit the proliferation of HeLa and U87 human glioma cells in a dose-dependent manner opens up the possibility of using it as a potent anticancer drug.

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