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# *Clitocybe alexandri* extract induces cell cycle arrest and apoptosis in a lung cancer cell line: identification of phenolic acids with cytotoxic potential

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**Abstract**

Mushrooms are a possible rich source of biologically active compounds with potential for drug discovery. The aim of this work was to gain further insight into the citotoxicity mechanism of action of *Clitocybe alexandri* ethanolic extract against a lung cancer cell line (NCI-H460 cells). The effects on cell cycle profile and levels of apoptosis were evaluated by flow cytometry, and the effect on the expression levels of proteins related to cellular apoptosis was also investigated by Western blot. The extract was characterized regarding its phenolic composition by HPLC-DAD, and the identified compounds were studied regarding their growth inhibitory activity, by sulforhodamine B (SRB) assay. The effect of individual or combined compounds on viable cell number was also evaluated using the Trypan blue exclusion assay. It was observed that the *Clitocybe alexandri* extract induced an S-phase cell cycle arrest and increased the percentage of apoptotic cells. In addition, treatment with the $GI_{50}$ concentration (concentration that was able to cause 50% of cell growth inhibition; 24.8 µg/ml) for 48h caused an increase in the levels of wt p53, cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP). The main components identified in this extract were protocatechuic, *p*-hydroxybenzoic and cinnamic acids. Cinnamic acid was found to be the most potent compound regarding cell growth inhibition. Nevertheless, it was verified that the concomitant use of the individual compounds provided the strongest decrease in viable cell number. Overall, we found evidence for alterations in cell cycle and apoptosis, involving p53 and caspase-3. Furthermore, our data suggests that the phenolic acids identified in the extract are at least partially responsible for the cytotoxicity induced by this mushroom extract.

*Keywords: Clitocybe alexandri*; Phenolic acids; Citotoxicity; Cell cycle; Apoptosis

## 1. Introduction

Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008. Cancer related deaths are projected to increase to over 11 million in 2030 (World Health Organization, 2010). Lung cancer is the major cancer killer (being responsible for an estimated 1.4 million deaths in 2008) and a health care problem worldwide with an overall 5-year survival rate of less than 15% (Brescia, 2001; Poleri et al., 2003).

Empirical approaches to discover anticancer drugs and cancer treatments have made limited progress in the past several decades in finding a cure for cancer. The vast structural diversity of natural compounds found in mushrooms (macrofungi) provided unique opportunities for discovering new drugs (Zaidman, Yassin, Mahajana, & Wasser, 2005). Mushrooms have been valued as edible and medicinal resources, and antitumor substances have been identified in many mushrooms species (Zhang, Cui, Cheung, & Wang, 2007). There is a significant interest in the use of mushrooms and/or mushroom extracts as dietary supplements based on the theory that they enhance immune function and promote health (Borchers, Keen, & Gershwin, 2004). Particularly, they can be added to the diet and used orally and they are considered as a safe and useful approach for disease treatment.

Different molecules found in fruiting bodies, mycelia and spores of macrofungi revealed antitumor potential (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007), such as high-molecular-weight compounds (*e.g.* polysaccharides, glycoproteins, proteoglycans and proteins) and low-molecular-weight compounds (*e.g.* quinones, cerebrosides, isoflavones, catechols, amines, triacylglycerols, sesquiterpenes and steroids) (Ferreira, Vaz, Vasconcelos, & Martins, 2010). Among the mentioned compounds, polysaccharides (primary metabolites) are the best studied mushroom-derived

substances with antitumor properties (Wasser, 2002; Zhang, Cui, Cheung, & Wang 2007), while the study of mushrooms secondary metabolites such as phenolic compounds have been overlooked.

Phenolic compounds are aromatic hydroxylated compounds, are commonly found in many food sources and some of them are among the most potent and therapeutically useful bioactive substances. Natural phenolic compounds accumulate as end-products from the shikimate and acetate pathways and can range from relatively simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins) (Bravo, 1998). The main phenolic compounds found in mushrooms are phenolic acids: hydroxybenzoic and hydroxycinnamic acids, which are derived from the non-phenolic molecules benzoic and cinnamic acid, respectively (Ferreira, Barros, & Abreu, 2009).

*Clitocybe alexandri* is an edible saprophytic Basidiomycotina mushroom belonging to the family of Tricholomataceae that can be found wildly in Northeast Portugal. We have recently reported the growth inhibitory activity of methanolic, ethanolic and boiling water extracts from *Clitocybe alexandri*, in human tumour cell lines (NCI-H460, MCF-7, HCT-15 and AGS) (Vaz et al., 2010). The ethanolic extract was the most potent one, particularly in NCI-H460 cells ($GI_{50}$ 24.8 ± 2.3 μg/ml) (Vaz et al., 2010). Herein, we intend to further evaluate the potential of this extract as a possible source of cytotoxic compounds.

## 2. Materials and methods

### *2.1. Standards and reagents*

Ethanol was of analytical grade purity and supplied by Pronalab (Lisbon, Portugal). Acetonitrile 99.9% was of HPLC grade from Lab-Scan (Lisbon, Portugal). Water was

treated in a Milli-Q water purification system (TGI Pure Water Systems, USA). Fetal bovine serum (FBS), L-glutamine, phosphate buffer saline (PBS), trypsin and RNase A were from Gibco Invitrogen Co. (Paisley, UK). RPMI-1640 medium was from Lonza (Basel, Switzerland). Acetic acid, dimethylsulfoxide (DMSO), sulforhodamine B (SRB), trypan blue, propidium iodide (PI) and phenolic standards were from Sigma Chemical Co. (St. Louis, USA). Tricloroacetic acid (TCA) and Tris were sourced from Merck (Darmstadt, Germany). Primary and secondary antibodies were from Santa Cruz Biotechnology Inc. (Heidelberg, Germany).

*2.2. Samples and sample preparation*

Samples of *Clitocybe alexandri* (Gillet) Gillet (edible mushroom) were collected under Q*uercus pyrenaica* Willd, in Bragança (Northeast Portugal), in autumn 2008. Taxonomic identification of sporocarps was made according to Moreno (2005) and online keys (http://www.mycokey.com/), and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. The samples were lyophilised (Ly-8-FM-ULE, Snijders, Holland) and reduced to a fine powder (20 mesh).

The ethanolic extract was prepared from the lyophilised powder (extraction yield 3.5 ± 0.2%) following the procedure previously described by us (Vaz et al., 2010). For the assays, the extract was re-dissolved in DMSO and diluted in media to the final concentrations used: $GI_{50}$ (24.8 μg/ml) or 2 × $GI_{50}$ (49.6 μg/ml). The $GI_{50}$ was previously obtained from the growth inhibitory activity of *C. alexandri* ethanolic extract in NCI-H460 cells (Vaz et al., 2010).

*2.3. Cell line*

NCI-H460 (non-small cell lung cancer) were routinely maintained as adherent cell cultures in RPMI-1640 medium with 5% heat-inactivated FBS, in a humidified incubator at 37 ºC with 5% $CO_2$. All assays were performed with cells in exponential growth, with viabilities over 90% and repeated at least in three independent experiments.

*2.4. Cell cycle distribution analysis and apoptosis detection*

For the analysis of cell cycle phase distribution, NCI-H460 cells were plated at $1.5 \times 10^5$ cells/ml in 6-well plates and left incubating for 24 hours. Cells were then incubated with complete medium only (blank), medium with the solvent DMSO or with *C. alexandri* ethanolic extract at $GI_{50}$ (24.8 μg/ml) and $2 \times GI_{50}$ (49.6 μg/ml) concentrations. The $GI_{50}$ concentration had been previously determined by the SRB assay (Vaz et al., 2010). Cells were harvested following a 48 h incubation with the extract and further processed for either cell cycle analysis or apoptosis detection. For cell cycle analysis, cells were fixed in 70% ethanol for 10 min at room temperature. After centrifugation cells were incubated with PI (5 μg/ml) and RNase A in PBS (100 μg/ml) for 30 min on ice (Vasconcelos et al., 2000; Palmeira et al., 2010). Induced apoptosis was assayed by the Human Annexin V-FITC/PI apoptosis Kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions (Queiroz et al., 2011).

Cellular DNA content (for cell cycle distribution analysis and presence of sub-G1 peak, suggestive of apoptosis induction) and measurement of phosphatidylserine externalization were analyzed using an Epics XL-MCL Coulter flow cytometer plotting at least 20,000 events per sample. Cell cycle distribution and apoptosis data analysis were subsequently performed using the FlowJo 7.2 software (Tree Star, Ashland, USA).

Three to six independent experiments were performed in duplicate and the results were expressed as mean values ± standard deviation (SD). Statistical analysis was performed by the non-parametric Friedman's test followed by Dunn's Post-test using GraphPad Prism 5 software. $P$ values < 0.05 were considered as statistically significant.

*2.5. Protein expression analysis*

For analysis of protein expression, NCI-H460 cells were treated with complete medium (blank), medium with the solvent (DMSO) or with *C. alexandri* ethanolic extract at 24.8 µg/ml, the $GI_{50}$ concentration previously determined by the SRB assay (Vaz et al., 2010), and processed 48 h after incubation. Cells were lysed in Winman's buffer (1% NP-40, 0.1 M Tris-HCl pH 8.0, 0.15 M NaCl and 5 mM EDTA) with EDTA-free protease inhibitor cocktail (Boehringer, Mannheim, Germany). Proteins were quantified using the DC Protein Assay Kit (BioRad, Hercules, CA, USA) and separated in 8% or 12% *tris*-glycine sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were then transferred to a nitro-cellulose membrane (GE Healthcare, Madrid, Spain). The membranes were incubated with the following primary antibodies for poly (ADP-ribose) polymerase (PARP) (1:4000), Actin (1:2000), p53 (1:250) and caspase 3 (1:2000), and further incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) diluted 1:2000 in 5% non-fat dried milk in T-TBS. The signal was detected with the Amersham ECL kit (GE Healthcare). Hyperfilm ECL (GE Healthcare) and Kodak GBX developer and fixer twin pack (Sigma) (Palmeira et al., 2010).

*2.6. Identification of phenolic compounds and evaluation of their cell growth inhibitory activity*

Phenolic acids of *C. alexandri* extract were analysed using high performance liquid chromatography (HPLC) equipment consisting of an integrated system with a Varian 9010 pump, a Varian Pro star diode array detector (DAD) and a Jones Chromatography oven column heater (model 7981). Data were analysed using Star chromatography workstation version 6.41 software (Varian). The chromatographic separation was achieved with an Aqua (Phenomenex, Torrance, CA) reverse phase $C_{18}$ column (3 µm, 150 mm × 4.6 mm i.d.) thermostatted at 30 ºC. The mobile phase and the gradient employed were described previously (Vaz et al., 2011). Injection volume was 20 µl. Detection was carried out in a diode DAD, using 280 nm as the preferred wavelength.

The effects of the identified compounds (protochatequic, *p*-hydroxibenzoic and cinnamic acids) on the growth of NCI-H460 were evaluated according to the procedure adopted in the NCI's *in vitro* anticancer drug screening, which uses SRB assay to assess cell growth inhibition (Skehan et al., 1990). This colorimetric assay estimates cell number indirectly, by staining cellular protein with the protein-binding dye SRB. For such, the cell line was plated at an appropriate density ($5.0 \times 10^3$ cells/well) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various concentrations of the compounds. Following this incubation period, the adherent cells were fixed with 10% trichloroacetic acid, washed with 1% acetic acid and stained with SRB. The bound stain was solubilised with 10 mM Tris and the absorbance was measured at 490 nm in a microplate reader (BIORAD 680 model). The concentration that inhibited growth in 50% ($GI_{50}$) was calculated as described by Monks et al. (1991). The growth inhibitory activity of the compounds was inferred from the SRB assay by comparing the absorbance of the wells containing extract-treated cells with the absorbance of the wells containing untreated cells, 48 h following treatment with the compounds, and subsequently comparing these results with the ones obtained for cells

that had been fixed at time zero (time at which the extracts were added) (Vaz et al., 2010). Doxorubicin was tested in the same manner to be used as a positive control. Three to six independent experiments were performed in duplicate and the results were expressed as mean values ± standard deviation (SD).

*2.7. Viable cell number*

Viabel cell number was determined with the trypan blue dye exclusion test (Renzi, Valtolina, & Foster, 1993) after incubation of NCI-H460 cells ($7.5 \times 10^4$ cells in 12-well plates) with complete medium (blank), medium with the solvent (DMSO), with cinnamic acid (A) at $GI_{50}$ concentration (845.9 μM), protochatequic acid (B) at $GI_{50}$ concentration (1616.9 μM) or with *p*-hydroxibenzoic acid (C) at 3000 μM. Cells were also treated with a combination of the previous treatments: A+B, B+C, A+C and A+B+C. Following a 48 h incubation, viable cell number was determined in a Neubauer chamber. Three independent experiments were performed and the results were expressed as mean values ± standard deviation (SD). Statistical analysis was performed by the non-parametric Friedman's test followed by Dunn's Post-test using GraphPad Prism 5 software. $P$ values $< 0.05$ were considered as statistically significant.

## 3. Results and discussion

*3.1. Effect of Clitocybe alexandri estract on cell cycle profile and levels of apoptosis*

The most active *Clitocybe alexandri* extract- the ethanolic extract- reported in our previous study (Vaz et al., 2010) was chosen to be further investigated regarding its possible mechanism of action. The NCI-H460 cell line was incubated with the $GI_{50}$ (24.8 μg/ml) or $2 \times GI_{50}$ (49.6 μg/ml) concentrations of the mushroom's extract for 48 h

and their effects on the normal cell cycle distribution and induction of apoptosis were studied.

Analysis of the effect of the ethanolic extract on cell cycle was performed by flow cytometry and results show a dose-dependent increase in the percentage of cells in the S-phase of the cell cycle, with a concomitant decrease in the percentage of cells in the G1 and G2/M phases (**Figure 1**). *C. alexandri* extract therefore seems to be an inducer of S-phase cell cycle arrest, after 48 h of treatment.

Additionally, it was investigated whether *C. alexandri* induced apoptosis in the NCI-H460 cell line, by the annexin V-FICT/PI flow cytometry assay. NCI-H460 cells treated with the 2×$GI_{50}$ concentration (49.6 µg/ml) of the ethanolic extract for 48 h presented a statistically significantly increase in the percentage of apoptotic cells (28.6% ± 0.9%), in comparison to the blank cells (6.6% ± 0.3%). Cells treated with the $GI_{50}$ concentration (24.8 µg/ml) of the extract for 48 h had 7.3% ± 2.1% of apoptotic cells, similarly to the blank.

Furthermore, the effect of the mushroom extract on the expression of some proteins involved in the apoptotic process was determined by Western blot. Results show that treatment of NCI-H460 cells with the $GI_{50}$ concentration (24.8 µg/ml) of the extract for 48 h caused an increase in the levels of wt p53, cleaved caspase-3 and cleaved PARP (**Figure 2**).

*3.2. Chemical characterization of the extract and further evaluation of the effect of the identified compounds on cell growth and viable cell number*

The ethanolic extract was analysed by HPLC-DAD, and two phenolic acids and a related compound were identified (**Figure 3**) and quantified: protocatechuic acid (16.4 ±

2.2 mg/kg of dry weight), *p*-hydroxybenzoic acid (8.3 ± 0.4 mg/kg of dry weight) and cinnamic acid (6.4 ± 0.3 mg/kg of dry weight).

The *in vitro* NCI-H460 growth inhibitory activity of the identified compounds was evaluated by SRB assay, after a continuous treatment during 48 h. Cinnamic acid was found to be the most potent compound regarding cell growth inhibition ($GI_{50}$ value 845.9 ± 97.5 μM) (**Figure 4**). Protocatechuic acid revealed a $GI_{50}$ value of 1616.9 ± 75.3 μM, while *p*-hydroxybenzoic acid did not show any activity at the highest tested concentration tested (3000 μM) (**Figure 4**). Despite the much lower $GI_{50}$ value obtained for the positive control Doxorubicin (0.07 ± 0.02 μM), it should be highlighted that phenolic acids are natural compounds and can act synergistically with other compounds present in the mushroom extract. Therefore, the comparisons between the positive control (highly toxic synthetic compound) and the individual compounds found in the extract, and mostly with the whole extract, should be made carefully.

The effect of the individual and combined treatment with the identified compounds was also tested, by verifying the number of viable cells upon a 48 h incubation with the $GI_{50}$ concentrations for cinnamic (845.9 μM) and protochatequic (1616.9 μM) acids and 3000 μM of *p*-hydroxibenzoic acid (the maximum concentration tested in the SRB, since the $GI_{50}$ was not obtained even with this high concentration). Results were compared with those obtained with DMSO control and blank treatment (cells incubated with complete medium). Treatment with the $GI_{50}$ concentration of cinnamic (845.9 μM) and protochatequic (1616.9 μM) acids caused a statistically significantly reduction in the number of viable cells to ≈ 50 %, as expected to occur with the $GI_{50}$ concentration of any compound (**Figure 5**). However, *p*-hydroxibenzoic acid did not show any significantly reduction in the viable cell number, as expected from the results previously obtained with SRB assay. Nevertheless, it was verified that the concomitant use of the

three compounds provided the strongest decrease in the viable cell number, highlighting a concomitant effect of those compounds (**Figure 5**).

Overall, the results reported here are related to the cytotoxicity of *Clitocybe alexandri* in a non-small lung cancer cell line. We found evidence for alterations in cell cycle and apoptosis, involving p53 and caspase-3. Other studies using extracts of plants like *Euchresta formosana* and *Angelica sinensis* (Cheng, et al., 2011; Hsu, et al., 2007) also reported similar effects to the ones observed for *Clitocybe alexandri* extract.

Finally, our data suggests that the phenolic acids identified in the ethanolic extract are at least partially responsible for the cytotoxicity induced by this mushroom extract and that they exert a concomitant effect. Further studies will include analysis of effect in non-tumour cells and the identification of other compounds in the extract.

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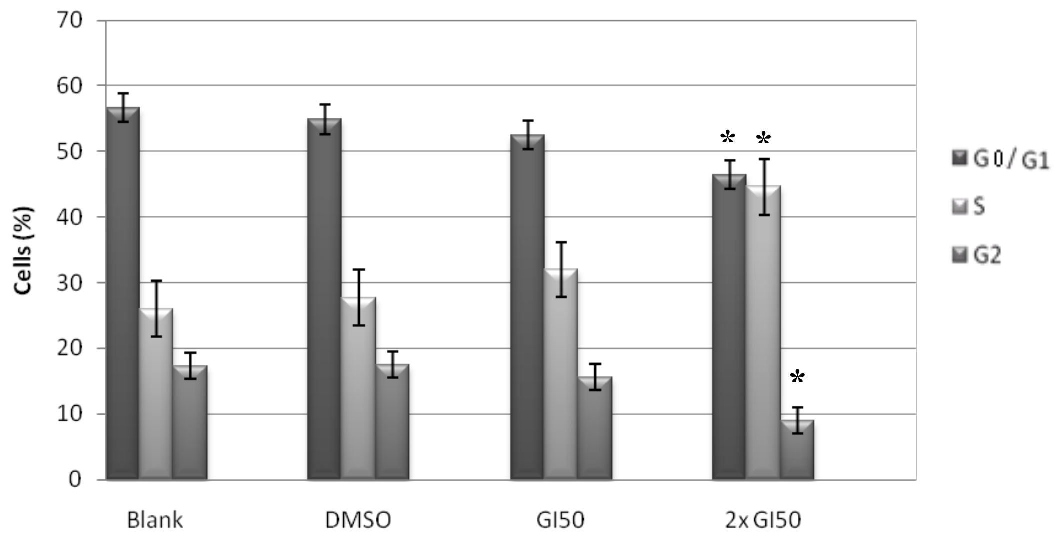


**Figure 1**. Cell cycle analysis of NCI-H460 cells treated for 48 h with the ethanolic extract of *Clitocybe alexandri* at $GI_{50}$ (24.8 μg/ml) or $2\times GI_{50}$ (49.6 μg/ml) concentrations. Untreated cells and the solvent (DMSO) treated cells were used as controls. Results are the mean ± SD of three to six independent experiments performed in duplicate. *Values statistically significantly ($P < 0.05$) different when compared to blank or DMSO.

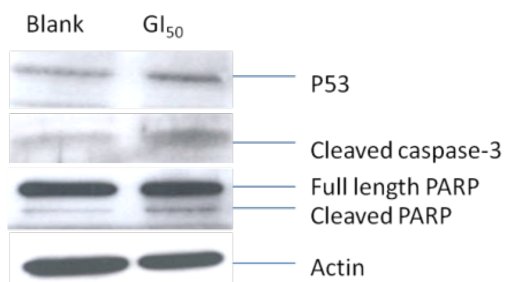


**Figure 2.** The ethanolic extract of *Clitocybe alexandri* alters the expression of some apoptotic proteins. NCI-H460 cells were treated for 48 h with complete medium (blank) or with the extract at $GI_{50}$ concentration (24.8 µg/ml). Actin was used as a loading control. Results are representative of three independent experiments.

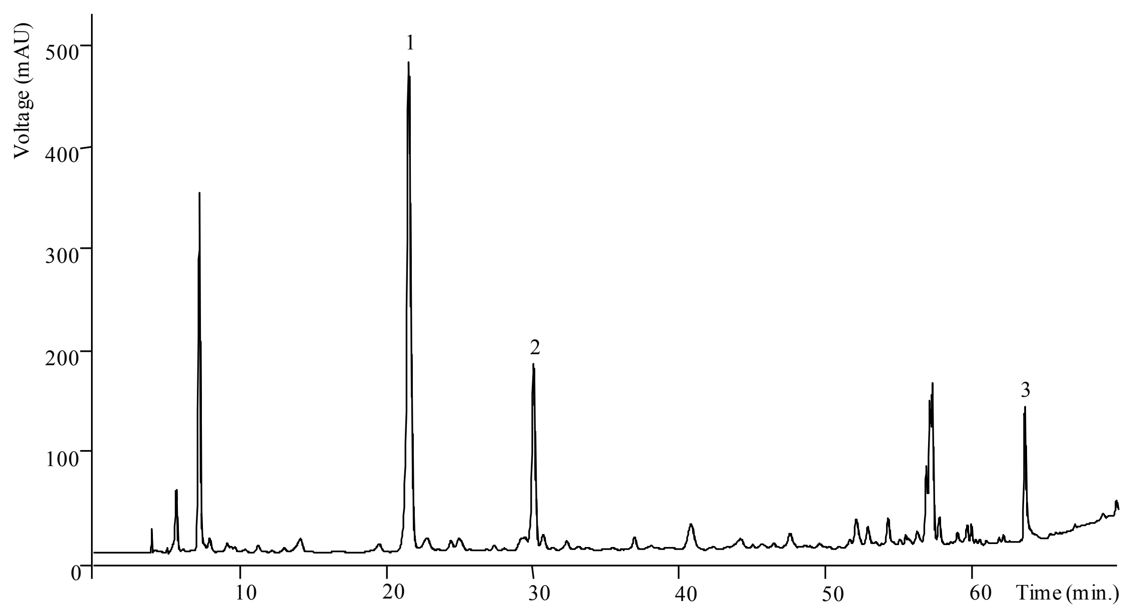


**Figure 3.** HPLC chromatogram recorded at 280 nm of the *Clitocybe alexandri* extract. Only peaks corresponding to phenolic compounds or related compounds are indicated: (1) protocatechuic acid, (2) *p*-hydroxybenzoic acid and (3) cinnamic acid.

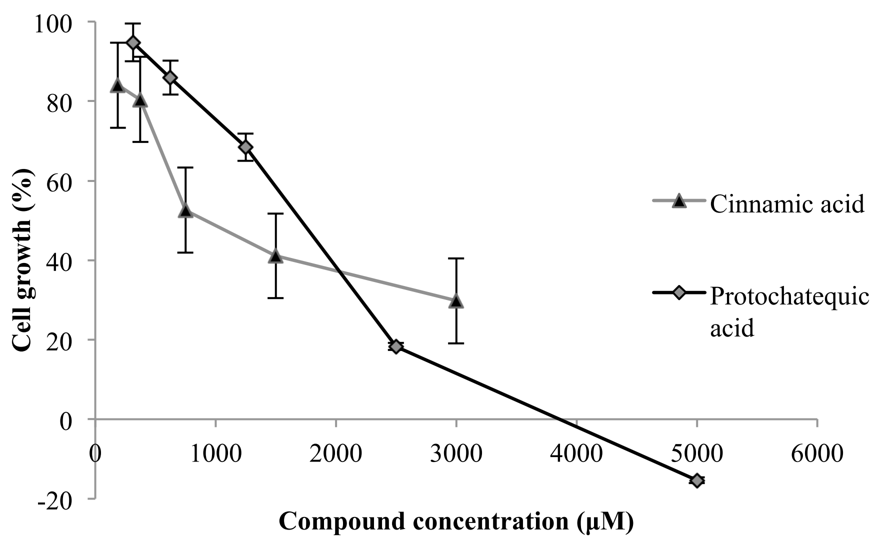


**Figure 4.** Percentage of cell growth inhibition induced in the NCI-H460 cells by the main compounds identified in the ethanolic extract of *Clitocybe alexandri*. Results are express as a % of the control. $GI_{50}$ value for the positive control Doxorubicin: 0.07 ± 0.02 µM. Results are the mean ± SD of three to six independent experiments, performed in duplicate.

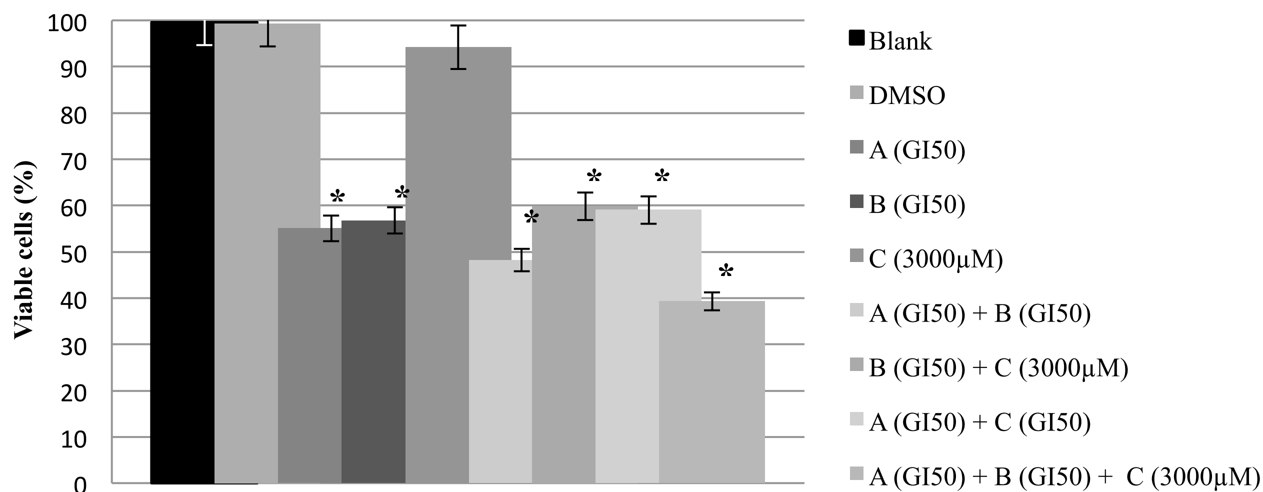


**Figure 5**. Viable cell number after treatment of NCI-H460 cells with the compounds identified in the ethanolic extract of *Clitocybe alexandri*, for 48 h: A- cinnamic acid (at $GI_{50}$ concentration; 845.9 µM), B- protochatequic acid (at $GI_{50}$ concentration; 1616.9 µM), C- *p*-hydroxibenzoic acid (at 3000 µM). Untreated cells and the solvent DMSO were used as controls. Results are the mean ± SD of three independent experiments performed in duplicate. *Values statistically significantly ($P < 0.05$) different when compared to blank or DMSO.