Suillus collinitus methanolic extract increases p53 expression and causes cell cycle arrest and apoptosis in a breast cancer cell line

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Abstract

In the present work, methanolic, ethanolic and boiled water extracts of *Suillus collinitus* were chemically characterized and further submitted to evaluation of their bioactive properties (antioxidant potential and cytotoxic activity in tumor cell lines). Phenolic acids and sugars were chromatographically identified and quantified in the methanolic and boiled water extracts, respectively. *S. collinitus* ethanolic extract gave the highest antioxidant activity. Nevertheless, regarding cell growth inhibition, the methanolic extract was the most potent one, particularly in MCF-7 cells (GI_{50} 25.2±0.2μg/ml). Moreover, the GI_{50} concentration induced a G1 cell cycle arrest, with a concomitant decrease in the percentage of cells in the S phase. Furthermore, it caused an increase in the percentage of apoptotic cells, from 6.0±0.2% in untreated cells, to 15.3±2.0% in cells treated with the GI_{50} concentration and to 16.3±2.0% in cells treated with $2\times GI_{50}$ concentration. In addition, 48 h treatment with the GI_{50} concentration caused a strong increase in the levels of p53, p21, and cleaved PARP, together with a decrease in Bcl-2 and XIAP. Results indicate that *Suillus collinitus* may be a promising source of bioactive compounds. Particularly, its methanolic extract appears to have a p53-mediated effect on the normal cell cycle distribution and apoptosis induction in a human breast tumor cell line.

Keywords: Wild mushroom; antioxidant potential; breast cancer; apoptosis induction; p53-mediated effect

1. Introduction

Cancer has become a major public health problem in Europe with an estimated prevalence of about 3%, increasing to 15% at old age. Moreover, cancer related deaths are projected to increase to over 11 million in 2030 (World Health Organization, 2010). Breast cancer is the major health problem in women worldwide, regarding both its incidence and mortality, which is emphasised by the diagnosis of over one million new cases annually (Botha, Bray, Sankila, & Parkin, 2003; Ferlay et al., 2007; Ferlay et al., 2008; Stankov & Stankov, 2011). Indeed, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths (Jemal et al., 2011).

Mushrooms are part of the sexual life cycle of particular fungi with specific metabolic pathways, and therefore may contain a largely unexploited source of powerful new pharmaceutical products with potential antitumor properties. Furthermore, they may have potential as functional foods (Borchers, Keen, & Gershwin, 2004; Ferreira, Vaz, Vasconcelos, & Martins, 2010; Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Zaidman, Yassin, Mahajana, & Wasser, 2005). The most important molecules found in mushrooms with antitumor potential include polysaccharides and phenolic compounds or derivatives (Ferreira et al., 2010; Liu et al., 2009; Vaz, Almeida, Ferreira, Martins, & Vasconcelos, 2012; Wasser, 2002; Zhang, Cui, Cheung, & Wang, 2007; Zhou & Chen, 2011). Furthermore, the antioxidant activity of the latter has been extensively reported, namely as free radical inhibitors (chain breaker), peroxide decomposers, metal inactivators or oxygen scavengers (Ferreira, Barros, & Abreu, 2009; Palacios et al., 2011). Suillin, a derivative of a phenolic acid (protocatechuic acid) belonging to the prenylphenols class isolated from the mushroom *Suillus placidus*, has proven to be a

potent apoptosis inducer in human hepatoma HepG2 cells (Liu et al., 2009). Like *S. placidus*, *Suillus collinitus* is also an edible mycorrhizal mushroom found in European pine forests, belonging to the same genus Suillus in the Suillaceae family. Nevertheless, to the best of our knowledge there is no report of antitumour potential for this mushroom species.

In the present work, methanolic, ethanolic and boiled water extracts of *S. collinitus* were submitted to bioactivity evaluation through in vitro antioxidant potential (mainly free radical scavenging activity and reducing power) and cytotoxic activity in various tumor cell lines: lung, breast, colon and gastric cancer. The extracts were chemically characterized in their phenolic acids and sugars composition by chromatographic techniques. The extract with highest cells growth inhibitory activity (methanolic extract) was chosen to be further investigated regarding its possible mechanism of action in the most susceptible cell line studied (breast cancer), by cell cycle and apoptosis evaluation. Furthermore, being etoposide one of the major standard cytotoxic drugs used in polychemotherapy, its concomitant use with *S. collinitus* methanolic extract was also studied in the same cells.

2. Materials and methods

2.1. Standards and reagents

Acetonitrile 99.9% was of HPLC grade from Lab-Scan (Lisbon, Portugal). All the other solvents and chemicals were of analytical grade purity. Ethanol was supplied by Pronalab (Lisbon, Portugal). Sugar standards (L(+)-arabinose, D(−)-fructose, D(+)-mannitol and D(+)-trehalose) were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). 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). Trichloroacetic 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 preparation of the extracts

Samples of *Suillus collinitus* (Fr.) Kuntz (edible mushroom) were collected in Bragança (Northeast Portugal). Taxonomic identification of sporocarps was made according to Coutecuisse & Duhem (2005) 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).

Methanolic, ethanolic and boiled water extracts were prepared from the lyophilised powder following the procedure previously described by us (Vaz et al., 2010). For the antioxidant activity assays, each extract was re-dissolved in the corresponding solvent, while for the tumour cell growth screening assays, the extracts were re-dissolved in DMSO (a stock solution was prepared and then further diluted until the determination of GI_{50} values). Final concentration of DMSO showed no interference with the biological activity tested. The solutions were stored at -20 ºC until further use.

2.3. Chemical characterization of the extracts

Phenolic acids were analysed by high performance liquid chromatography coupled to a photodiode array detector (HPLC-DAD) according to a previous report of the authors (Vaz et al., 2011a).

Sugars were determined, after crude polysaccharides hydrolysis, by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) as previously described by the authors (Heleno et al., 2012), using fructose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco) and a RI detector (Knauer Smartline 2300). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 µm, Knauer) operating at 30 ºC (7971 R Grace oven). The mobile phase was acetonitrile:deionized water, 70:30 (v/v) at a flow rate of 1 ml/min. Sugars identification and quantification was made according to standards and data were analysed using Clarity 2.4 Software (DataApex). The results are expressed in g per kg of dry weight (dw).

2.4. Screening of antioxidant activity

DPPH radical-scavenging activity. This methodology was performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc), according to Vaz et al. (2011b). The reaction mixture in each one of the 96-wells consisted of one of the serial concentrations of the extracts (30 µl) and aqueous methanolic solution (80:20 v/v, 270 µl) containing DPPH radicals (6×10^{-5} $mol.l^{-1}$). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level,

and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard.

Reducing power. The serial concentrations of the extracts (0.5 ml) were mixed with sodium phosphate buffer (200 $mmol.l^{-1}$, pH 6.6, 0.5 ml) and potassium ferricyanide (1 % w/v, 0.5 ml). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 ml) was added. The mixture (0.8 ml) was poured in the 48-wells, as also deionised water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml), and the absorbance was measured at 690 nm in the Microplate Reader described above (Vaz et al., 2011b). The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 690 nm against extract concentration. Trolox was used as standard.

Inhibition of β-carotene bleaching. The antioxidant activity of the extracts was evaluated by the β-carotene linoleate model system. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 ml). Two millilitres of this solution were added to a 100 ml round-bottom flask. After the chloroform was removed at 40 ºC under vacuum, linoleic acid (40 mg), Tween® 80 emulsifier (400 mg), and distilled water (100 ml) were added to the flask and vigorously shaken. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing serial concentrations (0.2 ml) of the extracts. The tubes were shaken and incubated at 50 ºC in a water bath (Vaz et al., 2011b). As soon as the emulsion was added to each tube, the zero time absorbance at 470 nm was measured. Inhibition of β-carotene bleaching was

calculated using the following equation: Absorbance after 2 h of assay/Initial Absorbance) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of antioxidant activity percentage against extract concentration. Trolox was used as standard.

2.5. Screening of growth inhibition activity in human tumour cell lines

The effects of the extracts on the growth of human tumour cell lines was evaluated according to the procedure adopted in the NCI's *in vitro* anticancer drug screening, which uses sulforhodamine B (SRB) assay to assess cell growth inhibition (Skehan et al., 1990). Four human tumour cell lines were used: NCI-H460 (lung cancer), MCF-7 (breast cancer), HCT-15 (colon cancer) and AGS (gastric cancer). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 5% heat-inactivated FBS at 37 ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (5.0×10^3 cells/well for NCI-H460 and MCF-7, 1.0×10^4 cells/well for HCT-15 and 7.5×10^3 cells/well for AGS) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. The DMSO concentrations used (0.2 to 0.8%) have no growth inhibitory effect in these cell lines.

Following this incubation period, the adherent cells were fixed with 10% trichloroacetic acid (final concentration), stained with SRB and excess dye washed with 1% acetic acid. The bound SRB was solubilised with 10 mM Tris and the absorbance was measured at 490 nm in a microplate reader (Multi-mode microplate synergy™ MX, Biotek) and analysed with the Gen5™ software (Biotek). The concentration that inhibited growth in 50% (GI_{50}) was calculated as described by Monks et al. (1991). The growth inhibitory activity of the extracts 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 extracts, and subsequently comparing these results with the ones obtained for cells that had been fixed at time zero (time at which extracts were added).

2.6. Cell cycle distribution analysis and apoptosis detection

For the analysis of cell cycle phase distribution, MCF-7 cells were plated at 1.5×10^5 cells/ml in 6-well plates and left incubating for 24 hours. Cells were then incubated with complete medium (blank), or with *S. collinitus* methanolic extract at the GI_{50} and $2\times GI_{50}$ concentrations. The DMSO concentrations used have no effect on the cell cycle profile. Cells were harvested following a 48 h incubation period with the extract and further processed for either cell cycle analysis or apoptosis detection. For cell cycle analysis, cells were fixed in 70% ethanol and kept at 4 ºC until analysis. Prior to analysis, cells were incubated with PI (5 μg/ml) and RNase A in PBS (100 μg/ml) for 30 min on ice (Vaz et al., 2012). Apoptosis was assayed with the Human Annexin V-FITC/PI apoptosis Kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions, as previously described by us (Queiroz et al., 2010).

Cellular DNA content (for cell cycle distribution analysis and presence of sub-G1 peak, which is suggestive of apoptosis induction) and measurement of phosphatidylserine externalization (for apoptosis detection) 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 (Queiroz et al., 2010).

2.7. Protein expression analysis

For analysis of protein expression, MCF-7 cells were treated with complete medium (blank), or with *S. collinitus* methanolic extract at the GI_{50} and $2\times GI_{50}$ concentration, and processed 48 h after incubation. DMSO controls were also tested. 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 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), p53 (1:200), XIAP (1:1000), Bcl2 (1:100), p21 (1:200) and Actin (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) using the Hyperfilm ECL (GE Healthcare) and the Kodak GBX developer and fixer twin pack (Sigma) (Vaz et al., 2012).

2.8. Cell growth inhibition following treatment of cells concomitantly with etoposide and S. collinitus methanolic extract

The effects of the concomitant use of etoposide and *S. collinitus* methanolic extract on the growth of MCF-7 cells was evaluated according to the procedure previously described in section 2.5. Etoposide was used at 1 µM (approximately the GI_{50} concentration in this cell line, previously determined), and the extract was used at the previously determined GI_{50} concentration.

2.9. Statistical analysis

For each assay 3 to 6 independent experiments were performed in triplicate (antioxidant activity assays) or duplicate (antitumour activity assays), and the results were expressed as mean values ± standard deviation (SD) or standard error (SEM). One-way analysis of variance (ANOVA) followed by Tukey's HSD Test was performed with the SPSS v.16.0 software. Differences in *p* values bellow 0.05 were considered statistically significant.

3. Results and discussion

3.1. Chemical characterization and screening of bioactive properties

The methanolic and boiled water (after hydrolysis of crude polysaccharides with trifluoroacetic acid) extracts were analyzed by HPLC-DAD and HPLC-RI, respectively and the results are given in **Table 1**. Two phenolic acids, *p*-hydroxybenzoic and protocatechuic acids, and one related compound, cinnamic acid, were found. Regarding sugars composition, mannitol, arabinose and trehalose were identified and quantified in *S. collinitus* (**Figure 1**).

Three different assays were carried out for the *in vitro* evaluation of the antioxidant properties of the methanolic, ethanolic and boiled water extracts: scavenging activity on DPPH radicals, inhibition of lipid peroxidation in a β-carotene-linoleate system and reducing power. The methanolic extract revealed the lowest antioxidant activity (highest EC_{50} values; extract concentration that achieves 50% of antioxidant activity or 0.5 of reducing power) in all the assays (**Table 2**). The antioxidant properties of ethanolic and boiled water extracts were similar (without significant statistical differences), except for reducing power which was higher in the ethanolic extract (1.3 mg/ml, **Table 2**). The observed antioxidant potential might be related to the phenolic acids found (**Table 1**) and their reported chemoprotective effects against oxidative stress-mediated disorders

mainly from their free radical scavenging and metal chelating properties (Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005).

The effects of the *S. collinitus* extracts on the growth of four human tumour cell lines (NCI-H460, MCF-7, HCT-15 and AGS), represented as the concentrations that caused 50% of cell growth inhibition (GI_{50}), are summarized in **Table 3**. The methanolic extract was the most potent in all tested cell lines, presenting GI_{50} values that ranged from 25.2 to 103.2 µg/ml for the MCF-7 and HCT-15 cells, respectively. Its activity could be related to the phenolic compounds found in the extract who have already demonstrated capacity to decrease viable cells number, mostly when they are concomitantly used (Vaz et al., 2012).

The boiled water extract did not show any effect on the cell lines at the tested concentrations (up to 400 µg/ml), as it can be observed, for example, in the dose-response effect of the three extracts on MCF-7 cell line (**Figure 2**).

MCF-7 cell line was the most susceptible (presenting the lowest GI_{50}) to *S. collinitus* methanolic extracts. Therefore, this most active extract was chosen to be further investigated regarding its possible mechanism of action in this cell line.

3.2. Effects on cell cycle profile and apoptosis induction

The MCF-7 cell line was incubated with the GI_{50} (25.2 µg/ml) or twice the GI_{50} (50.4 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 methanolic extract on cell cycle was performed by flow cytometry and results show that both the GI_{50} concentration and $2\times GI_{50}$ concentration induced a G1 cell cycle arrest, with a concomitant decrease in the percentage of cells in the S phase (**Figure 3**).

Additionally, it was investigated whether *S. collinitus* induced apoptosis in the MCF-7 cell line, using the annexin V-FICT/PI flow cytometry assay. MCF-7 cells treated with the methanolic extract for 48 h presented an increase in the percentage of apoptotic cells, from 6.0±0.2% in untreated cells, to 15.3±2.0% in cells treated with the GI_{50} concentration and to 16.3±2.0 % in cells treated with $2\times GI_{50}$ concentration (**Table 4**).

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 MCF-7 cells with the GI_{50} concentration of the extract caused a strong increase in the levels of p53. This effect was even stronger when cells were treated with $2\times GI_{50}$ concentration, suggesting that the effect is concentration-dependent. Accordingly, the levels of p21 whose expression is regulated by p53 and related to cell cycle arrest were also increased in a concentration dependent manner with the GI_{50} and with the $2\times$ GI_{50} concentrations. Finally, treatment of the cells with the extract caused a decrease in the levels of XIAP and Bcl-2 and a concentration dependent increase in the levels of cleaved PARP, which are consistent with an apoptotic process of cell death (**Figure 4**).

Etoposide is one of the major standard cytotoxic drugs used for different cancers and frequently used in polychemotherapy. Therefore, the effect of treating MCF-7 cells concomitantly with *S. collinitus* (methanolic extract) and etoposide was also studied, by verifying the % of cell growth upon a 48 h incubation with the previously determined approximate GI_{50} concentration of etoposide (1 μM) and with the GI_{50} (25.2 μg/ml) of the extract. The combined use of the methanolic extract and etoposide caused a greater decrease in the % of cell growth, when compared to either of them used individually, indicating the potential benefit of this combination (**Figure 5**).

Overall, the *S. collinitus* ethanolic extract gave highest scavenging properties and reducing power, while the methanolic extract was the most potent inhibiting the growth of tumour cell lines (NCI-H460, MCF-7, HCT-15 and AGS).

Natural antioxidants may have different means for exerting their action, including interactions with intracellular signal transduction pathways or inducing the expression of antioxidant and detoxification enzymes. In this context, it is becoming now more evident that the pro-oxidant effect, rather than the antioxidant, is the key event at the basis of antiproliferative effect exerted by natural antioxidants on the cells (Chen et al., 2008; Ullah et al., 2011; Weisburg et al, 2010; Zhang et al., 2010). Nevertheless, within the present study it is not clear if antioxidant or pro-oxidant effects are related to antiproliferative activity. Based on the *in vitro* assays performed, only antioxidant effects were found: mainly scavenging activity and reducing power. Apparently, there is no correlation between both activities (*in vitro* antioxidant activity and antiproliferative activity using human cells), because ethanolic extract revealed the highest antioxidant potential, while methanolic extract gave the highest antiproliferative activity.

MCF-7 cells were the most susceptible of the cells studied, and the methanolic extract appears to have a p53-mediated effect on the normal cell cycle distribution and apoptosis induction in these cells. *Suillus collinitus* is a promising source of bioactive compounds, however further studies will be necessary such as analysis of its effect in non-tumour cells and the isolation and identification of the compounds present in the methanolic extract which may be responsible for the obtained results. Nonetheless, it is possible that the activity here described may be caused by the combination of several compounds and not by any individually isolated compound.

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Table 1. Phenolic acids, derivatives and sugars (after crude polysaccharides hydrolysis) in the *Suillus collinitus* extracts.

Methanolic extract*	Protocatechuic acid	5.2 ± 0.2
	p-Hydroxybenzoic acid	14.1 ± 1.2
	Cinnamic acid	1.3 ± 0.2
Boiled water extract	Arabinose	30.3 ± 5.5
	Mannitol	32.2 ± 4.1
	Trehalose	11.0 ± 0.2

Results are expressed in mg/kg (phenolic acids) or g/kg (sugars) in dry weight basis, and show means ± SD of 3 independent experiments performed in triplicate.*Vaz et al., 2011.

Table 2. Antioxidant activity of *Suillus collinitus* extracts.

Extracts	DPPH scavenging activity	β-carotene bleaching inhibition	Reducing power
Methanolic*	14.1 ± 1.2^{a}	1.2 ± 0.1^{a}	3.0 ± 0.0^{a}
Ethanolic	7.3 ± 0.7^{b}	1.2 ± 0.1^{a}	1.3 ± 0.0^{c}
Boiled water	7.9 ± 0.5^{b}	1.2 ± 0.0^{a}	2.1 ± 0.0^{b}

Results are expressed as EC_{50} (concentration of extract in mg/ml that cause 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay), and show means ± SD of 3 independent experiments performed in triplicate. In each column, different letters mean significant differences between results ($p<0.05$).* Heleno et al., 2010.

Table 3. Growth inhibitory activity of *Suillus collinitus* extracts on human tumour cell lines.

Extracts	NCI-H460 (lung cancer)	MCF-7 (breast cancer)	HCT-15 (colon cancer)	AGS (gastric cancer)
Methanolic	62.5 ± 6.3^{b}	25.2 ± 0.16^{b}	103.2 ± 9.9^{b}	79.2 ± 15.5^{b}
Ethanolic	253.7 ± 2.3^{a}	101.8 ± 8.9^{a}	139.4 ± 34.1^{a}	170.7 ± 35^{a}
Boiled water	> 400	> 400	> 400	> 400

Results are expressed as GI_{50} (concentration of extract in µg/ml that cause 50% of cell growth inhibition), and show means ± SD of 3-6 independent experiments performed in duplicate. In each column, different letters mean significant differences between results ($p<0.05$).

Table 4. Percentage of apoptotic MCF-7 cells induced by the methanolic extract of *Suillus collinitus*.

	Blank	GI_{50}	$2\times GI_{50}$
Apoptotic cells (%)	6.0 ± 0.2^{b}	15.3 ± 2.0^{a}	16.3 ± 2.0^{a}

Results are the mean ± SD of at least 3 independent experiments, performed in duplicate. In the row, different letters mean significant differences between results ($p<0.05$).

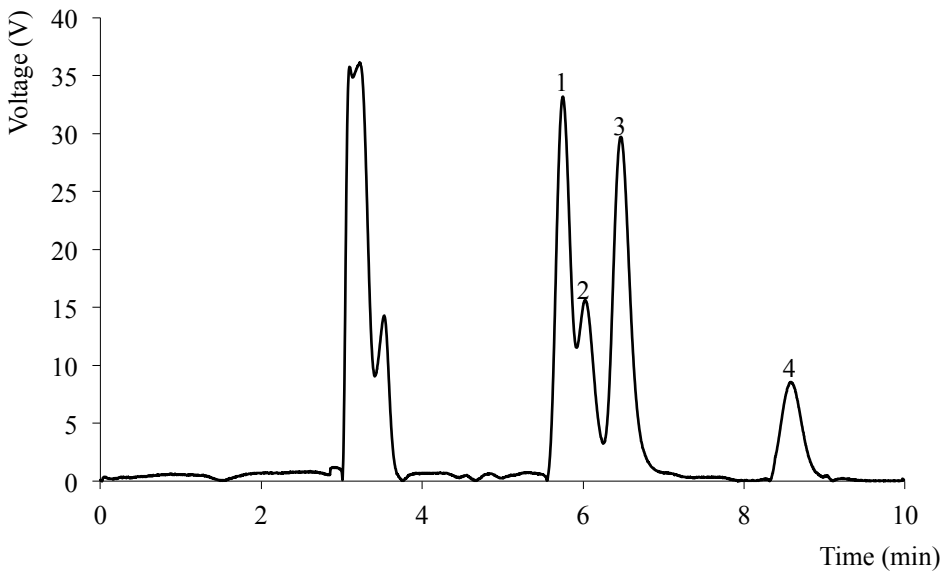


Figure 1. Sugars profile of *Suillus collinitus* obtained by HPLC-RI. 1. Arabinose; 2-Fructose (IS); 3- Mannitol; 4- Trehalose.

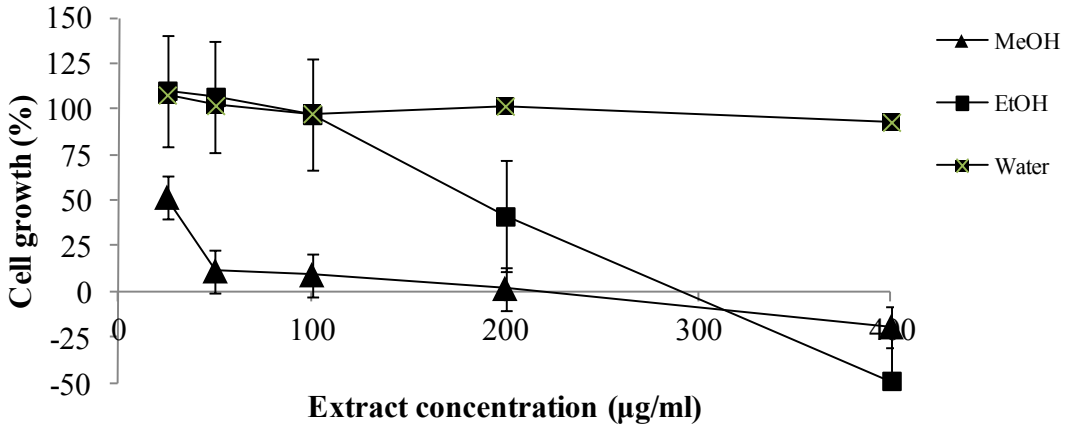


Figure 2. Percentage of cell growth of the methanolic (MeOH), ethanolic (EtOH) and boiled water extracts of *Suillus collinitus* against MCF-7 cells. Results are the mean ± SD of at least three independent experiments, performed in duplicate.

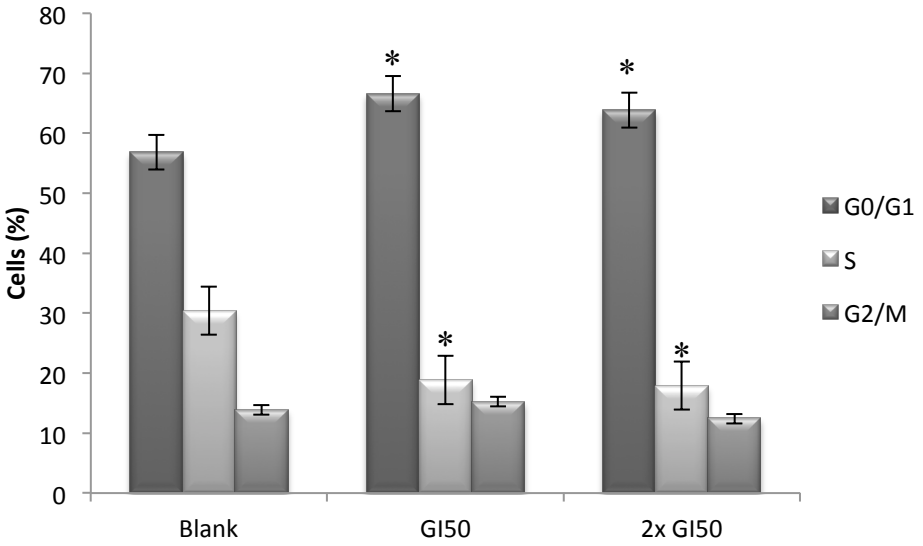
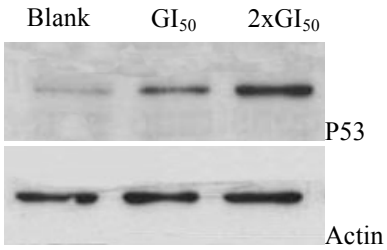
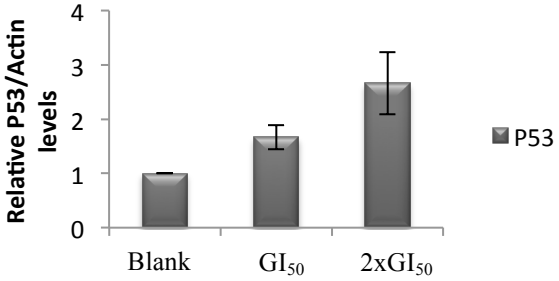
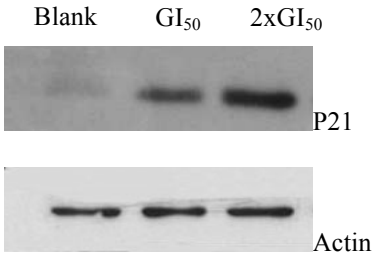


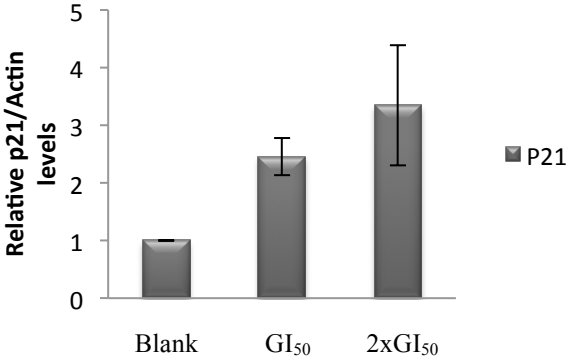
Figure 3. Treatment of MCF-7 cells with the methanolic extract of *Suillus collinitus* increases the % cells in G1 and decreases de % cells in the S phase of the cell cycle.

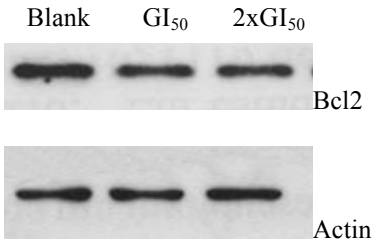
Cell cycle analysis of MCF-7 cells treated for 48 h with the methanolic extract of *Suillus collinitus* at GI_{50} (25.2 µg/ml) or twice the GI_{50} (50.4 µg/ml) concentrations. Untreated cells were used as control (blank). Results are the means ± SEM of 3-6 independent experiments performed in duplicate. *Statistically significant different values when compared to blank ($p<0.05$).

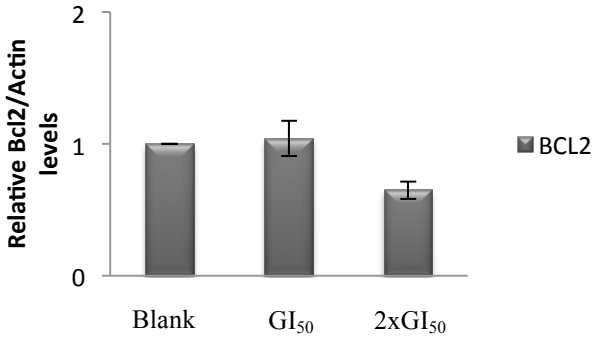

Blank
GI50
2xGI50
P53
Actin

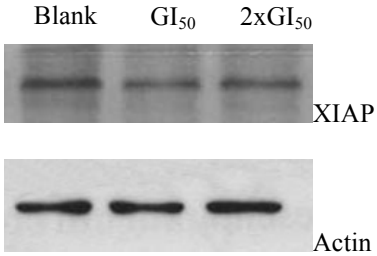

Relative P53/Actin levels
4
3
2
1
0
Blank
GI50
2xGI50
P53

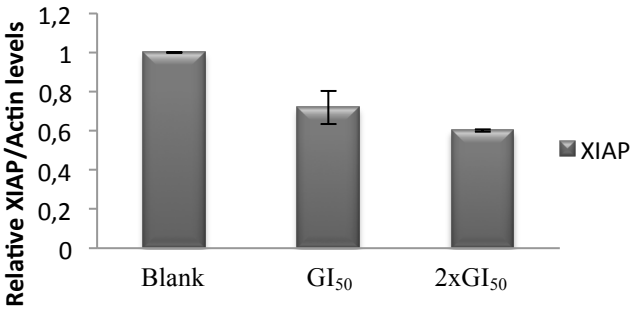

Blank
GI50
2xGI50
P21
Actin

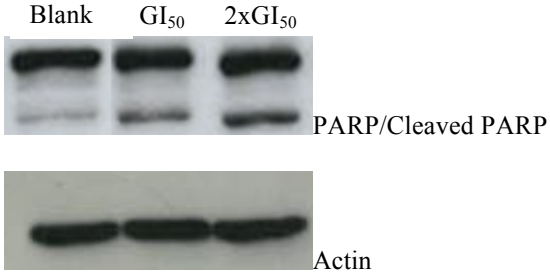

Relative p21/Actin levels
5
4
3
2
1
0
Blank
GI50
2xGI50
P21


Blank
GI50
2xGI50
Bcl2
Actin


Relative Bcl2/Actin levels
2
1
0
Blank
GI50
2xGI50
BCL2


Blank
GI50
2xGI50
XIAP
Actin


Relative XIAP/Actin levels
1,2
1
0,8
0,6
0,4
0,2
0
Blank
GI50
2xGI50
XIAP


Blank
GI50
2xGI50
PARP/Cleaved PARP
Actin

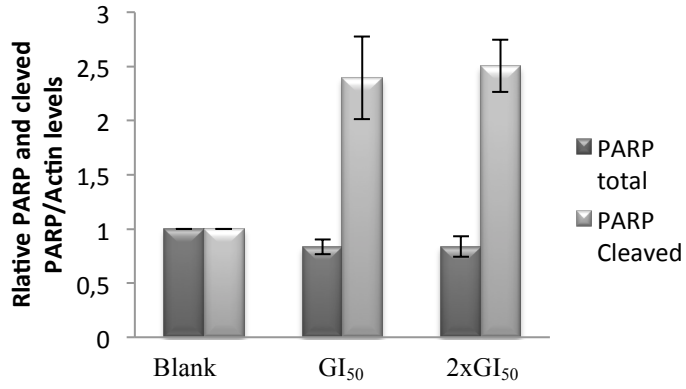

Rlative PARP and cleved PARP/Actin levels
3
2,5
2
1,5
1
0,5
0
Blank
GI50
2xGI50
PARP total
PARP Cleaved

Figure 4. Treatment of MCF-7 cells with the methanolic extract of *Suillus collinitus* increases p53 levels and PARP cleavage. MCF-7 cells were treated for 48 h with complete medium (blank) or with the extract at GI_{50} concentration (25.2 μg/ml) or $2\times GI_{50}$ concentration (50.4 μg/ml). Actin was used as a loading control. DMSO controls had no effect on the expression of the studied proteins. Results are representative of 3 independent experiments.

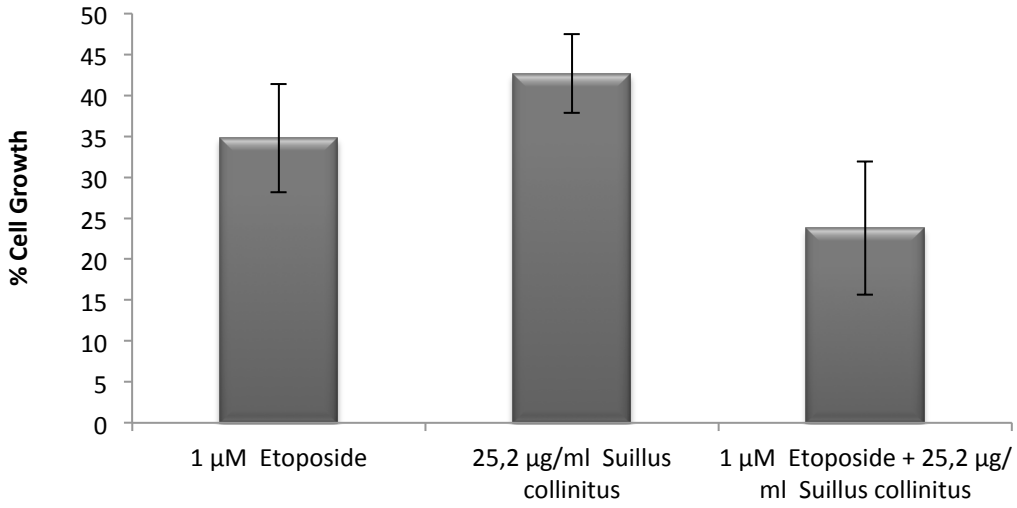


Figure 5. Treatment of MCF-7 cells concomitantly with etoposide and *Suillus collinitus* (methanolic extract) has a greater cell growth inhibitory effect than treatment with either of them alone. MCF-7 cells were treated with 1 µM of etoposide, 25.2 µg/ml of the methanolic extract of *S. collinitus* or with both together. Cell growth inhibitory activity was confirmed with the SRB assay. Appropriate DMSO controls had no effect on cell growth. Values are the mean of 4 independent experiments ± S.E.