Running title: *Suillus luteus* inhibits proliferation of a colon cancer cell line

Suillus luteus methanolic extract inhibits cell growth and proliferation of a colon cancer cell line

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Abstract

Several edible mushrooms extracts are known to have tumour cell growth inhibitory potential. The objective of this work was to study this potential in extracts of *Suillus luteus* collected from the Northeast of Portugal. Various extracts were prepared and their effect on tumour cell growth was studied with the SRB assay in four human tumour cell lines: MCF-7 (breast), NCI-H460 (non-small cell lung cancer), AGS (gastric) and HCT-15 (colon). The methanolic extract of *S. luteus* was the most potent extract. This extract was slightly more potent in the HCT-15 cells (with mutant p53, GI_{50}=17.8 ± 1.6 μg/mL) than in the other cell lines tested, which suggested that its effect was not p53-dependent. Indeed, in HCT-15 cells, an increase in the levels of p53 was detected but no alterations in some of the proteins transactivated by p53 (e.g. p21 or Bax) were found. The extract caused an increase in the cellular levels of p-H2A.X, which is suggestive of DNA damage. Growth inhibition in these cells was mostly due to inhibition of cell proliferation and an increase in the % of cell in the G1 phase of the cell cycle. An increase in cell death was also found, even though to very low levels. In addition, this extract was not cytotoxic to primary cultures of porcine hepatocytes (GI_{50}>400 μg/mL). Together, these results indicate that the *S. luteus* methanolic extract may be an interesting source of compounds that inhibit the proliferation of tumour cells but further studies should be carried out in order to understand its potential.

Keywords: Suillus luteus; methanolic extract; tumour cell growth; tumour cell proliferation.

1. Introduction

The majority of new drugs have been generated from either natural products (secondary metabolites) or analogs inspired by them. Many drugs such as antibiotics (penicillin, tetracycline and erythromycin), antiparasitics (avermectin), antimalarials (quinine, artemisinin), lipid control agents (lovastatin and analogs), immunosuppressants for organ transplants (cyclosporin, rapamycins) or anticancer drugs (taxol, doxorubicin) have been found or produced in this way (Li & Vederas, 2009).

Mushrooms (macrofungi) contain a vast diversity of biomolecules with nutritional (Kalač, 2009) and/or medicinal properties (Poucheret, Fons, & Rapior, 2006). They have been recognized as functional foods and as a source of compounds for the development of nutraceuticals or medicines, including compounds with antitumour properties (Zaidman, Yassin, Mahajna, & Wasser, 2005; Moradali, Mostafavi, Ghods, & Hedjaroude, 2007).

The most studied compounds are high-molecular-weight ones, such as the β-glucans "Lentinan" from the fruiting bodies of *Lentinus edodes*, "Schizophyllan" from the culture fluid of *Schizophyllum commune*, or the polysaccharopeptides PSP and "Krestin" (PSK) from the cultured mycelium of *Coriolus versicolor* (Ferreira, Vaz, Vasconcelos & Martins, 2010; Luk et al., 2011). Despite their commercialization, limited clinical studies in cancer patients have been conducted. In addition to the mentioned well-known medicinal mushrooms, previous work from our research group showed that other wild unexplored species from Portugal are notable for their promising antitumour potential, such as *Clitocybe alexandri* and *Suillus collinitus*. Indeed, their phenolic extracts (including low molecular weight compounds) showed effects on cell

cycle and induced apoptosis in human lung and breast cancer cell lines, respectively (Vaz et al., 2012a; Vaz et al., 2012b).

Following our research in wild edible species from Northeast Portugal, the present work describes the cytotoxic activity of methanolic, ethanolic and boiled water extracts of *S. luteus* in various human tumor cell lines: lung, breast, colon and gastric cancer. The extract with the highest cell growth inhibitory activity (methanolic extract) was chosen to be further investigated regarding its possible mechanism of action in the most susceptible cell line studied (colon cancer), by evaluation of its effect on the cell cycle profile, cellular proliferation and apoptosis. The chemical characterization of *S. luteus* was previously reported by our research group. This mushroom presented protochatechuic acid (0.47 mg/100 g dry weight), cinnamic acid (0.41 mg/100), α-tocopherol (19.14 µg/100 g), β-tocopherol (15.34 µg/100 g), γ-tocopherol (366.77 µg/100 g), δ-tocopherol (78.51 µg/100 g), mannitol (1.29 g/100 g), trehalose (1.35 g/100 g), polyunsaturated fatty acids (52.75%, with linoleic acid- 52.31%- as the main fatty acid), monounsaturated fatty acids (32.93%, with oleic acid- 31.24%- as the main fatty acid), and saturated fatty acids (14.32%, with palmitic acid- 10.57% as the main fatty acid) (Reis et al., 2011).

2. Materials and Methods

2.1. Sample collection and preparation of the extracts

Samples of *Suillus luteus* (L.: Fries) Gray (edible mushroom) were collected in Bragança (Northeast Portugal), in autumn 2009. 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).

Enriched phenolic (methanolic and ethanolic) and polysaccharidic (boiled water) extracts were prepared from the lyophilised powder following the procedure previously described by us (Vaz et al., 2010). Briefly, to obtain the methanolic extract, the lyophilized sample (~2 g) was extracted twice with methanol (50 mL) mixture at -20 °C for 6 h. The extract was sonicated for 15 min, centrifuged at 4000 × *g* for 10 min, filtered through Whatman nº 4 paper and concentrated under reduced pressure (rotary evaporator Büchi R-210). To obtain the polysaccharidic (boiling water) extract, the lyophilized sample (~1.5 g) was extracted three times with water at boiling temperature (50 mL) for 2 h and subsequently filtered through Whatman No. 4 paper. The combined extracts were lyophilized, and then 95% ethanol (10 mL) was added and polysaccharides were precipitated overnight at 4 ºC. The precipitated polysaccharides were collected after centrifugation (Centorion K24OR- 2003 refrigerated centrifuge) at 3100 × *g* for 40 min followed by filtration, and then were lyophilized, resulting in a crude polysaccharidic sample. The ethanolic supernatant was concentrated under reduced pressure, giving the ethanolic extract.

For the subsequent assays, the extracts were re-dissolved in DMSO and the solutions were stored at -20 ºC until further use.

2.2. Cell Culture

2.2.1. Human tumour cell lines. The cell lines used were: NCI-H460 (lung cancer), AGS (gastric cancer), MCF-7 (breast cancer) and HCT-15 (colon cancer). Cells were plated in RPMI 1640 medium supplemented with 5% heat-inactivated FBS (Lonza). All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2.2. Primary cells of porcine liver. A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL penicillin, 100 µg/mL streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 ºC with a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. The cells were cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (Abreu et al., 2011).

2.3. Analysis of cell growth

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 (Skehan et al., 1990). 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, and 1.0×10^4 cells/well for PLP2) 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 presented 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 510 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 according to Monks et al. (1991) and as previously described by the authors (Vaz et al., 2010).

2.4. Cell cycle analysis

For the analysis of cell cycle phase distribution, HCT-15 cells were plated at 2×10^5 cells/well in 6-well plates and incubated for 24 h. Cells were then incubated with complete medium (blank), with *S. luteus* methanolic extract at the GI_{50} and $2\times GI_{50}$ concentrations or with DMSO (control). Cells were harvested following a 24 and 48 h of incubation period with the extract, further fixed in 70% ethanol and kept at 4ºC until analysis. Prior to analysis, cells were incubated with Propidium Iodide (5 μg/mL) and RNase A in PBS (100 μg/ml) for 30 min on ice (Vaz et al., 2012a).

Cellular DNA content was analyzed at the Advanced Flow Cytometry Unit (IBMC/INEB) using a FACS Calibur (BD) flow cytometer. Cell cycle profile was subsequently analysed using the FlowJo 7.2 software (Queiroz et al., 2010).

2.5. Programmed cell death (TUNEL) and proliferation (BrdU) asssay

For the TUNEL and BrdU assay, cells were plated, respectively at 7.5×10^4 per well in 12-well plates, and 2×10^5 per well in 6-well plates, and incubated for 24 h. Cells were then further incubated with complete medium (blank), with *S. luteus* methanolic extract at the GI_{50} and $2\times GI_{50}$ concentrations or with DMSO (control).

For the TUNEL assay, cells were harvested following a 24 and 48 h incubation with the extract, further fixed in 4% PFA and kept in PBS at 4 ºC until analysis. Cytospins were prepared and cells were permeabilized in ice cold 0.1% Triton X-100 in 0.1% sodium citrate for 2 min. Programmed cell death was assessed using the "in situ cell death

detection kit" (Roche, Basel Switzerland). Briefly, cells were incubated with the TUNEL reaction mix (enzyme diluted 1:20) for 1 h, as previously described (Palmeira et al., 2010).

For the BrdU assay, 23 h following incubation with the extract, cells were given a pulse of BrdU (10 μM, Sigma) for 1 h and were then washed and fixed in 4% paraformaldehyde in phosphate buffer saline. Cytospins were prepared and incubated in 2M HCl for 20 min. They were then incubated with mouse anti-BrdU (1:10, Dako) and further incubated with fluorescein-labeled rabbit anti-mouse antibody (1:100, Dako), as previously described (Palmeira et al., 2010).

For the analysis of slides from both the TUNEL and the BrdU assay, slides were mounted in Vectashield Mounting Media with DAPI (Vector Laboratories). Cells were observed in a DM2000 microscope (LEICA) and a semi-quantitative evaluation of the levels of programmed cell death or proliferation was performed by counting a minimum of 500 cells per slide.

2.6. Protein expression analysis

Cells were plated at 2×10^5 per well in 6-well plates and incubated for 24 h. They were then treated with complete medium (blank), the solvent of the extract (DMSO) or with the GI_{50} or $2\times GI_{50}$ concentration of the methanolic extract of *S. luteus* and processed 48 h later. Briefly, 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). Proteins were quantified with the DC Protein Assay kit (BioRad), separated in 12% (resolving) and 5% (Stacking) tris-glycine sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to a nitro-cellulose membrane (GE Healthcare). Membranes were incubated with the following primary antibodies for p53

(1:200, Calbiochem), p73 (1:500), Bax (1:500, Santa Cruz Biotechnology), p21 (1:250, Thermo scientific), p-H2A.X (1:200), Actin (1:2000, Santa Cruz Biotechnology), and then incubated with the respective secondary antibodies- horseradish peroxidase (HRP) conjugated (1:2000, Santa Cruz Biotechnology). The signal was detected with the Amersham ECL kit (GE Healthcare), Hyperfilm ECL (GE Healthcare) and Kodak GBX developer and fixer twin pack (Sigma), as previously described (Vasconcelos et al., 2000). The intensity of the bands obtained in each film was further analyzed using the software Quantity One – 1D Analysis (Bio-Rad), as previously described (Palmeira et al., 2010).

2.7. Statistical analysis

Results were expressed as mean values ± standard deviation (SD) of at least 3 independent experiments, performed in duplicate. Statistical significance was tested with a two tailed paired Student's t-Test using the DMSO as a negative control. * Indicates $p<0.05$.

3. Results and Discussion

3.1. The methanolic extract of S. luteus presents cell growth inhibitory activity in various human tumour cell lines but not in primary porcine liver cells

The enriched phenolic (methanolic and ethanolic) and polissacharidic (boiled water) extracts of *S. luteus* were screened for tumour cell growth inhibitory potential in four human tumour cells lines (NCI-H460, MCF-7, HCT-15 and AGS). The determined values of GI_{50} (concentrations that caused 50% cell growth inhibition) are expressed in **Table 1**. The methanolic extract was the most potent extract in all the cell lines tested,

since the other extracts did not reach the GI_{50} at the concentrations tested (up to 400 µg/ml). Léon et al. (2008) had previously shown that suillumide, a ceramide isolated from the ethanolic extract of *S. luteus*, presented cytotoxic activity against a human melanoma cell line (with IC_{50} values of approximately 10 µM following a 72 h treatment). Nonetheless, in our study the ethanolic extract did not present significant cytotoxic activity (in the studied cell lines and up to the concentrations tested).

S. luteus methanolic extract had previously been reported to present cytotoxic activity against a murine cancer cell line (Tomasi, 2004). Nevertheless, to our knowledge, this is the first study that shows a cytotoxic activity in human tumour cell lines.

The cell line which was most sensitive to the effect of this extract was the HCT-15 cell line (GI_{50} = 17.75±1.6 µg/mL). Therefore, this cell line was selected to further study the effect of this extract. Interestingly, this extract was not cytotoxic to primary cultures of porcine hepatocytes (GI_{50} > 400 µg/mL, **Table 1**).

3.2.The methanolic extract of S. luteus reduces the proliferation of HCT-15 cells and causes a cell cycle arrest in G1

The HCT-15 cells were treated with the GI_{50} (17.75 µg/mL) or twice the GI_{50} concentration (35.5 µg/mL) of the methanolic extract of *S. luteus*. The effect on cellular proliferation was analysed upon 24 h incubation and on cell cycle profile upon 24 h and 48 h incubation. Results from the cell cycle profile analysis show that this extract caused an increase in the % of cells in the G1 phase of the cell cycle, which was more evident (and statistically significant) with twice the GI_{50} concentration, mostly 24 h following treatment (**Figure 1A**) . In addition, treatment with the extract caused a statistically significant decrease in the % cells in the S and G2/M phases of the cell cycle, when cells were treated with twice the GI_{50} concentration for 24 h (**Figure 1A**).

A decrease in the % cells in the S phase was also obtained when cells were treated with the GI_{50} for 24 h and a statistically significant decrease in G2/M when cells were treated with twice the GI_{50} concentration for 48h (**Figure 1B**).

In agreement with this cell cycle arrest in G1, treatment of HCT-15 cells with the extract caused a concentration dependent decrease in cellular proliferation, which was very strong and statistically significant when cells were treated with twice the GI_{50} concentration (**Figure 2**).

3.3.The methanolic extract of S. luteus does not interfere with programmed cell death of HCT-15 cells

The effect of the extract on programmed cell death of HCT-15 cells was investigated with the TUNEL assay. Treatment during 24h with the GI50 concentration or during 24h or 48h with twice the GI_{50} concentration of the extract increased cell death, even though to very low levels (**Table 2**).

2.4.The activity of the methanolic extract of S. luteus does not depend on p53

The HCT-15 cell line has heterozygous mutations in the p53 tumour suppressor gene (O'Connor et al., 1997). Even though it would be possible that the remaining wild-type allele could remain functional, this cell line has been documented, by a reporter assay monitoring p53-dependent transactivation in cells, to lack functional p53 (Falck et al., 2001). Therefore, the fact that the methanolic extract of *S. luteus* was more potent in this cell line than in the other studied cell lines indicated that the effect of this extract was, at least in this cell line, not p53 dependent. Indeed, even though HCT-15 cells treated with the extract presented an increase in p53 expression (**Figure 3A**), there was no increase in some of the proteins transactivated by wild-type p53, such p21 or Bax

(**Figure 3B and 3C**). In fact, the levels of Bax, a proapoptotic protein that has the ability to form oligomers on the outer mitochondrial membrane causing membrane permeabilization and apoptosis (Lamb & Hardwick, 2013), were surprisingly decreased. The reason for this decrease in the levels of Bax is not understood, since equal levels were expected as mutant p53 in known to be unable to activate Bax transcription (Campomenosi et al., 2001). Nevertheless, this decrease in Bax might explain the low levels of cell death that were detected upon treatment of cells with the mushroom extract.

The p53 family has several isoforms and many of the functions of the p53 tumour suppressor protein may be shared by other members of this family, such as the p73 protein (Müller et al., 2006). Therefore, the effect of the extract on the levels on p73 protein was studied. No difference in p73 protein levels was found, indicating that p73 is not involved in the effect of this extract (**Figure 3D**).

Finally, the levels of p-H2A.X were determined and found to be increased upon treatment of the cells with twice the GI_{50} concentration of the extract (**Figure 3E**). This increase is suggestive of DNA damage and could justify the increase in the p53 levels previously found.

In conclusion, it may be interesting to further study this extract, considering that many tumours present mutant p53 tumour suppressor gene and that this extract is capable of decreasing cellular proliferation in the absence of functional p53, an event most likely triggered by an increase in DNA damage. Nonetheless, further studies regarding the cellular effects resulting from the increase in mutant p53 levels need to be carried out, since some mutant p53 have gain of function and may lead to a decrease in apoptosis, decrease in growth arrest, decrease in senescence and increase in chemoresistance

(Strano et al., 2007). Therefore, an increase in mutant p53 may have deleterious cellular effects.

The mechanisms or pathways leading to the observed decrease in cellular proliferation may be complex and need to be further studied. We found no evidence of p73 being involved in this pathway, at least in the HCT-15 cell line (which presents mutant p53), but other p53 family proteins (DeYoung and Ellisen, 2007) may be involved. In addition, many studies have demonstrated that Bcl-2 and Bax also have an effect on cellular proliferation. For example, bax has been shown to reverse the proliferation retarding activities of Bcl-2 (Borner, 1996 and O'Reilly, 1996). Therefore, the decrease in Bax levels that were found following treatment with the mushroom extract may be related to the observed decrease in cellular proliferation. Further studies need to be carried out in order to evaluate this hypothesis. It would be interesting to identify the compound or compounds responsible for the biological activity here described. Nevertheless, in a previous work we identified and quantified protocatechuic and cinnamic acids in *S. luteus* methanolic extract, and these compounds could possibly be related not only to the antioxidant activity reported in that study (Reis et al., 2011), but also to the capacity to decrease cellular proliferation in the herein tested colon cancer cell line.

Acknowledgements

The authors are grateful to Fundação para a Ciência e a Tecnologia (FCT, Portugal) and COMPETE/QREN/EU for financial support to this work (research project PTDC/AGR-ALI/110062/2009) and to CIMO (strategic project PEst-OE/AGR/UI0690/2011). They also thank to University of Porto and Santander Totta for financial support. J.A Vaz thanks to FCT, POPH-QREN and FSE for her grant (BD/43653/2008). G.M. Almeida is

supported by FCT and the European Social Fund. IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT.

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Table 1. Growth inhibitory activity of different extracts of *S. luteus* on human tumour cell lines and on primary cells of porcine liver.

	Tumour cell lines				Primary cells
	NCI-H460	**HCT-15**	**MCF-7**	**AGS**	**PLP2**
Boiled water	> 400	> 275[#]	> 400	> 400	> 400
Methanolic	30.33±1.1	17.75±1.6	32.25±5.7	30.30±3.1	> 400
Ethanolic	> 400	> 400	> 400	> 400	> 400

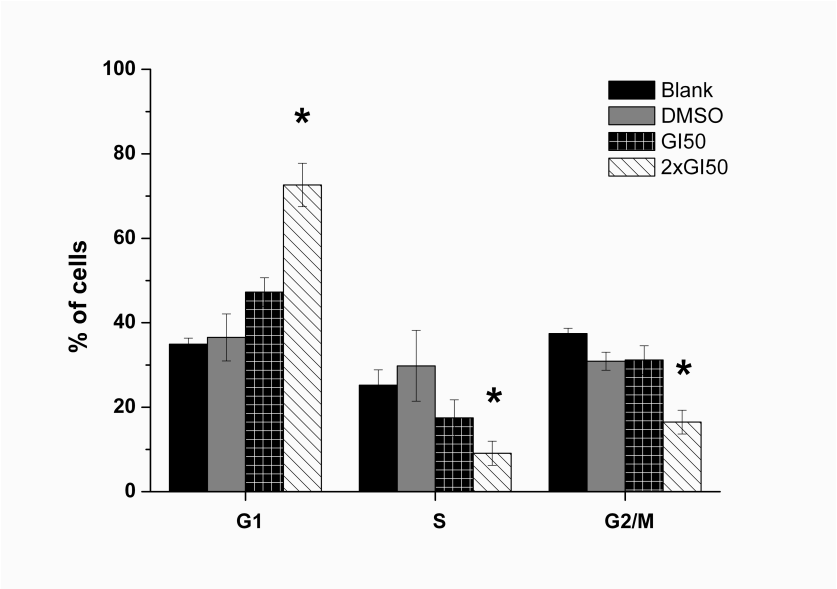
Results are expressed as GI_{50} (concentration of extract in µg/mL that causes 50% of cell growth inhibition), and show means ± SD of 3 independent experiments performed in duplicate. [#]The GI_{50} concentration could not be determined but was always superior to 275 µg/mL.

Table 2. Percentage of HCT-15 cells undergoing programmed cell death, following treatment with the methanolic extract of *S. luteus.*

	Time	
	24h	**48h**
Blank	0.23±0.27	0.39±0.15
DMSO	0.25±0.03	0.34±0.23
GI_{50} concentration	0.76±0.15*	0.66±0.18
2×GI_{50} concentration	1.64±0.45*	1.66±0.31*

Results were determined by TUNEL assay and are the mean ± SD of at least 3 independent experiments, performed in duplicate. Statistical significance was tested by two tailed paired Student's t-Test using DMSO as a negative control. * Indicates $p<0.05$. Doxorubicin was used as a positive control. The percentage of cells undergoing programmed cell death following 24h of treatment with doxorubicin was 1.95 ± 0.61% and following 48h of treatment was 17.73 ± 3.41%.

A.



B.

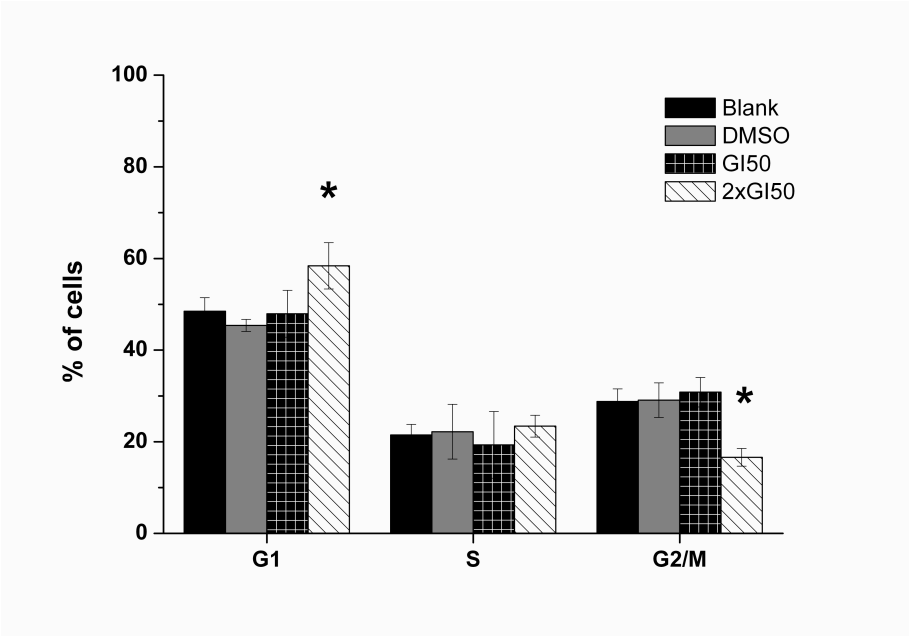


Figure 1. Cell cycle analysis of HCT-15 cells treated with the methanolic extract of *S. luteus*. Cell were treated for 24h (**A**) or 48h (**B**) with the GI_{50} (17.75 µg/mL) or twice the GI_{50} concentrations (35.5 µg/mL) of the extract. Untreated cells (blank) and cells treated with DMSO (concentration corresponding to $2xGI_{50}$ concentration) were used as controls. Results are the means ± SD of 3 independent experiments performed in

duplicate. Statistical significance was tested by two tailed paired Student's t-Test using DMSO as a negative control. * Indicates $p<0.05$.

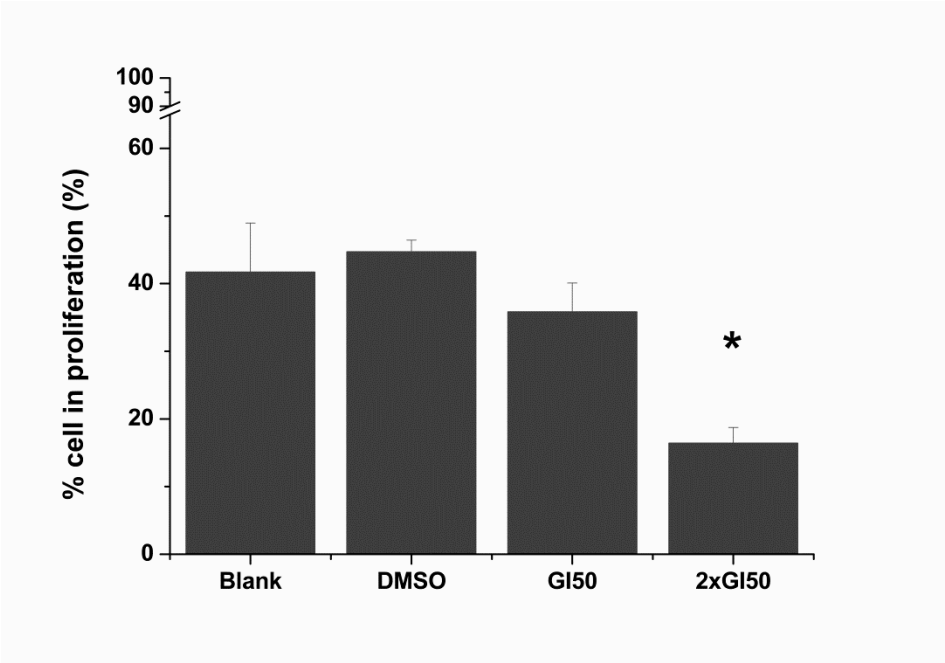
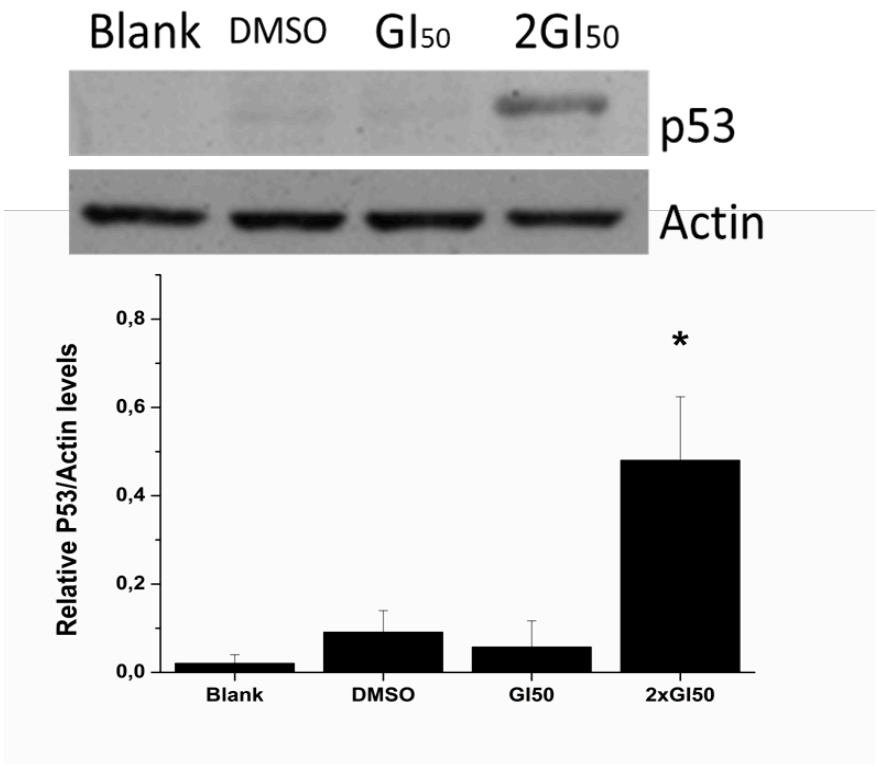
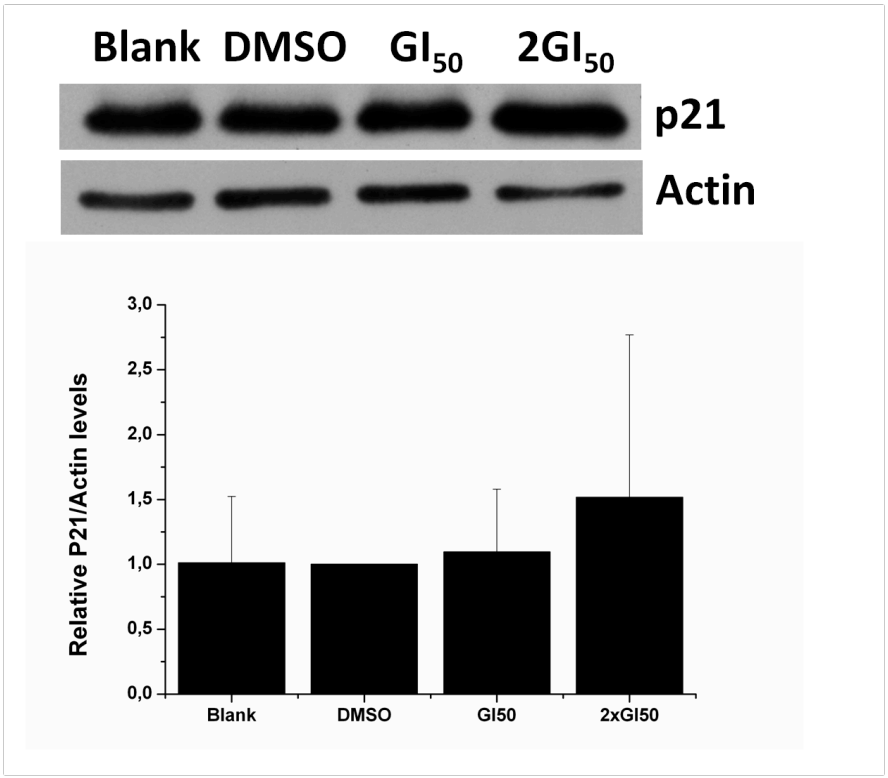


Figure 2. Cellular proliferation of HCT-15 cells treated with the methanolic extract of *S. luteus*. Cells were treated for 24h with the GI_{50} (17.75 µg/mL) or twice the GI_{50} (35.5 µg/mL) concentrations of the extract. Untreated cells (Blank) and cells treated with DMSO (concentration corresponding to $2\times GI_{50}$) were used as controls. Results are the mean± SD of 3 independent experiments performed in duplicate. Statistical significance was tested by two tailed paired Student's t-Test using DMSO as a negative control. * Indicates $p<0.05$.

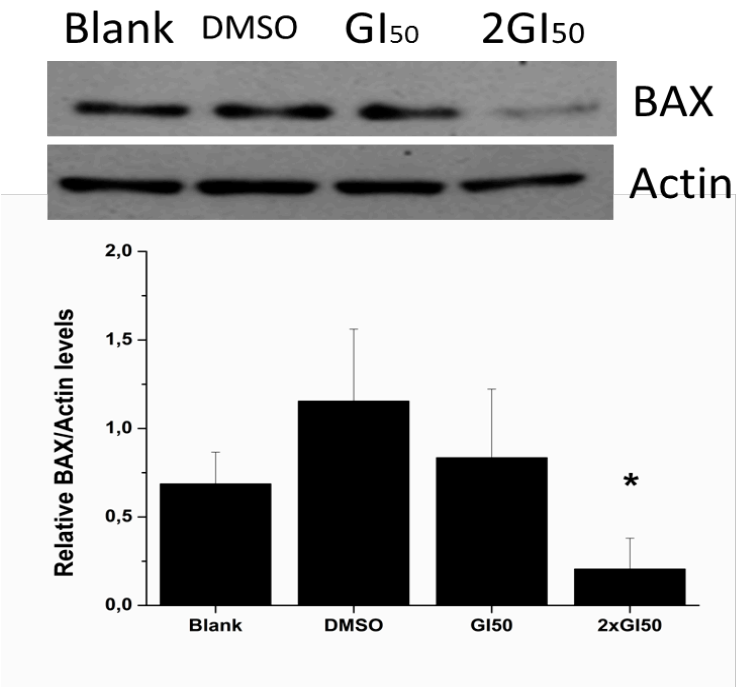
A.


Blank
DMSO
GI50
2GI50
p53
Actin
Relative P53/Actin levels
0,0
0,2
0,4
0,6
0,8
*
Blank
DMSO
GI50
2xGI50

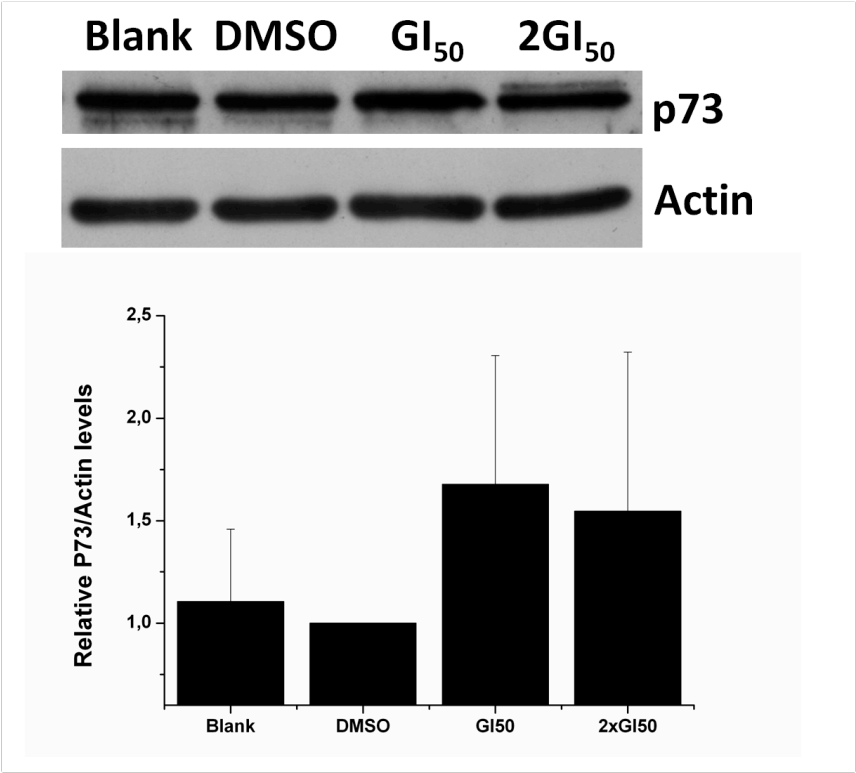
B.


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

C.


Blank
DMSO
GI50
2GI50
BAX
Actin
Relative BAX/Actin levels
2,0
1,5
1,0
0,5
0,0
*
Blank
DMSO
GI50
2xGI50

D.


Blank
DMSO
GI50
2GI50
p73
Actin
Relative P73/Actin levels
2,5
2,0
1,5
1,0
Blank
DMSO
GI50
2xGI50

E.

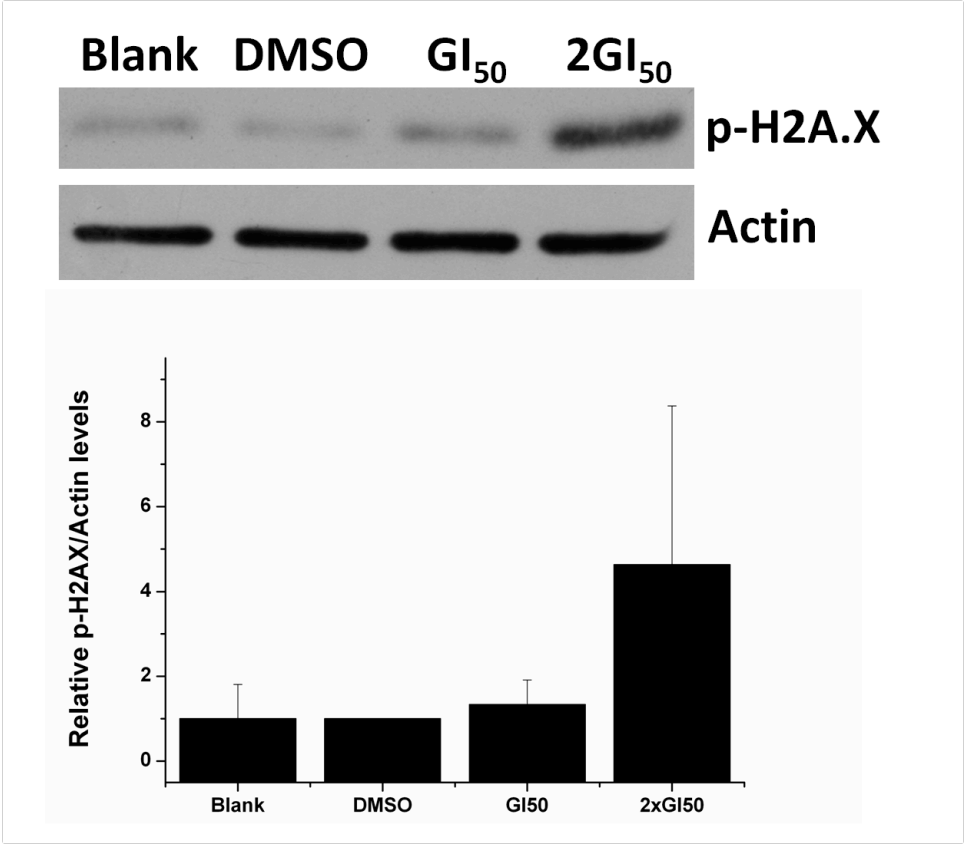


Figure 3. Protein expression of HCT-15 cells treated with the methanolic extract of *S. luteus*. The levels of p53 (**A**), p21 (**B**), Bax (**C**), p73 (**D**) and p-H2A.X (**E**) of cells treated for 48 h with the GI_{50} (17.75 µg/mL) or twice the GI_{50} (35.5 µg/mL) concentrations of the extract are shown. Untreated cells (Blank) and cells treated with DMSO (concentration corresponding to $2xGI_{50}$) were used as controls. Actin was used as the loading control. Results are the mean ± SD of 3 independent experiments. Statistical significance was tested by two tailed paired Student's t-Test using DMSO as a negative control. * Indicates $p<0.05$.