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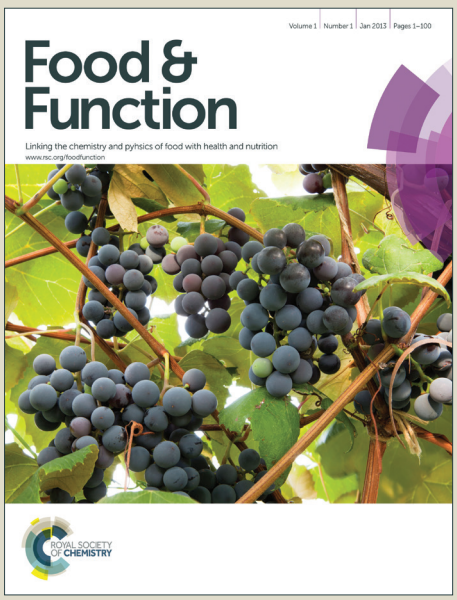

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A methanolic extract of *Ganoderma lucidum* fruiting body inhibits the growth of a gastric cancer cell line and affects cellular autophagy and cell cycle

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Abstract

Ganoderma lucidum is one of the most extensively studied mushrooms as functional food and as chemopreventive agent due to its recognized medicinal properties. Some *G. lucidum* extracts have shown promising antitumor potential. In this study, the bioactive properties of various extracts of *G. lucidum*, from both the fruiting body and the spores, were investigated. The most potent extract identified was the methanolic fruiting body extract, which inhibited the growth of a gastric cancer cell line (AGS) by interfering with cellular autophagy and cell cycle.

Keywords: *Ganoderma lucidum*; methanolic extract; gastric cancer cell line; AGS; growth inhibition; autophagy.

Introduction

The exhaustive search for new ways of treating and preventing cancer has led to the discovery of new drugs based on either natural products or analogs inspired by them.[1] Indeed, although lacking objective biological mechanistic responses, some natural matrices have been investigated as a source of anti-cancer agents and some encouraging findings have been identified.[2,3]

Mushrooms are examples of natural matrices which appear to hold potential health benefits, being recognized as functional foods and as a source of compounds for the development of nutraceuticals or medicines, including compounds with antitumor properties.[4] Medicinal mushrooms are generally well-tolerated with few, if any, side effects. Nevertheless, it is still not completely well understood whether their vast bioactive effects are caused only by a single component or if they are the result of an additive, or even synergistic outcome due to several compounds. It is believed that these benefits are mainly attributed to their richness in polysaccharides (*e.g.*, β-glucans), the primary active immune-enhancing constituents.[5,6]

Ganoderma lucidum extracts and compounds have demonstrated interesting advantages as adjuvants in the prevention and treatment of cancer, possessing anti-proliferative or growth inhibitory properties in various types of human tumor cell lines such as the LNCaP cell line (prostate cancer),[7] sarcoma 180 and Lewis lung carcinoma cell lines (lung cancer),[8] monocytic THP-1 cell line (acute myelogenous leukemia),[9] MCF-7 cell line (breast cancer),[10] and HUC-PC and MCT-11 cell lines (bladder cancer).[11] As a supplement during chemo- or radiotherapy, *G. lucidum* can enhance curative effects and reduce detrimental side–effects associated to this kind of treatments, such as fatigue, immunosuppression, anorexia, hair loss and bone marrow suppression.[12-15]

Taking into consideration the enormous potential of this mushroom and the fact that the underlying molecular mechanisms of the bioactive metabolites are far from being fully understood, the main goal of this work was to further investigate the effect of different extracts of *G. lucidum* (from the fruiting body and spores) on various human tumor cell lines. In addition, the effect of the most potent extract (methanolic extract from the fruiting body) was further studied in the most sensitive cell line, a gastric cancer cell line (AGS), by studying the effect on cellular proliferation, cell cycle profile, programmed cell death and autophagy.

Experimental

Preparation of the *Ganoderma lucidum* extracts

Samples of *Ganoderma lucidum* (Curtis) P. Karst. were collected in Bragança (Northeast Portugal) in July 2011. After taxonomic identification of the sporocarps,[16-18] specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança under the number BRESA-gl01-2011. The fruiting bodies were further separated from spores using a scalpel, and all the samples were lyophilised and powdered (20 mesh). Phenolic (methanolic and ethanolic) and polysaccharidic (boiling water) extracts were prepared from the lyophilised powder following the procedure previously described by us.[19] The phenolic and polysaccharidic extracts from *G. lucidum* fruiting body and spores were chemically characterized in a previous report.[20] The extracts were kept in DMSO and stored at -20 ºC.

Cell culture of human tumor cell lines

The following cell lines were used in this study: AGS (gastric adenocarcinoma), MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and HCT-15 (colorectal adenocarcinoma). Cells were routinely maintained in RPMI-1640 medium with Ultraglutamine I (Lonza) supplemented with 5% or 10% (depending on the assay to be performed as explained below) heat inactivated fetal bovine serum (FBS, PAA) at 37 °C in a humidified atmosphere containing 5% CO_2. Cell number and viability were assessed with trypan blue exclusion assay.

Primary culture of porcine liver cells

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. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.[21]

Screening for *in vitro* cell growth inhibition

Cell growth inhibition of tumor cell lines and of primary porcine liver cells was studied with the sulforhodamine B (SRB) assay. Tumor cells were plated in 96-well plates

(7.5×10^3 cells/well for the AGS cells, 5×10^3 cells/well for MCF7 and NCI-H460 cells and 1× 10^4 cells/well for HCT-15 cells) and incubated at 37 ºC for 24 h. Porcine liver cells were plated as indicated above in 2.3. Exponentially growing cells were then treated with 5 serial dilutions (1:2) of each extract (ranging from 25 to 400 μg/ml). Following 48 h treatment, cells were fixed with 10% ice cold trichloroacetic acid, washed with water and stained with SRB. After washing with 1% acetic acid, bound SRB was solubilized with 10 mM Tris Base and absorbance measured at 510 nm in a microplate reader (Biotek Instruments Inc. Synergy Mx, USA). For each extract, the corresponding GI_{50} (concentration which inhibited 50% of net cell growth) was determined, as previously described.[22,23] The effect of the vehicle solvent (DMSO) on the growth of each cell line was also analysed by treating cells with the maximum concentration of DMSO used in each assay (0.4%).

AGS cellular treatment with the *G. lucidum* methanolic extract

AGS cells were plated at 1.5×10^5 per well in 6-well plates (in RPMI-1640 medium with Ultraglutamine I supplemented with 10% FBS) and incubated for 24 h. Cells were then treated with complete medium (blank), with *G. lucidum* methanolic fruiting body extract (106 μg/ml) or the equivalent volume of the extract solvent (DMSO).

Following 48 h of treatment, cells were further processed as indicated in the protocols below.

Cell proliferation analysis. Proliferation was analysed with the BrdU incorporation assay. One hour before the 48 h treatment, cells were incubated for 1 h with 10 μM BrdU (Sigma). Cells were then fixed in 4% paraformaldehyde (PFA) in PBS. Cytospins were prepared and incubated in 2M HCl for 20 min. Following incubation with mouse

anti-BrdU (1:10, Dako), cells were further incubated with fluorescein-labeled rabbit anti-mouse antibody (1:100, Dako), as previously described.[24] Slides were mounted in Vectashield Mounting Media with DAPI (Vector Laboratories) and cells were observed in a DM2000 fluorescence microscope (LEICA). A semi-quantitative evaluation of the proliferation levels was obtained by counting a minimum of 500 cells per slide.

Cell cycle profile analysis. Cells were fixed in ice-cold 70% ethanol and kept at 4ºC for at least 12 h. 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. Cellular DNA content was analyzed using a FACS Calibur (BD Biosciences) flow cytometer.[25,26] Analysis of cell cycle profile was carried out using the FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA) after cell debris and aggregates exclusion.

Apoptosis analysis. The levels of apoptosis were analysed by flow cytometry using the Human Annexin V-FITC/PI apoptosis kit (Bender MedSystems, Vienna, Austria), according to the manufacturer's instructions. Flow cytometry was carried out using a FACS Calibur (BD Biosciences) flow cytometer and plotting at least 20,000 events per sample, as previously described.[27] Data was analysed using the FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA).

Protein expression analysis. 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 (Roche). Protein content was quantified with the DC Protein Assay kit (BioRad). Protein lysates (20 µg) were electrophoresed on 12% SDS-PAGE and transferred to a nitro-cellulose membrane (GE Healthcare). Membranes were incubated

with the following primary antibodies: rabbit anti-VPS34 (1:1000, Cell Signaling), rabbit Beclin-1 (1:1000, Cell Signaling), rabbit anti-Light Chain 3 B, LC3 (1:1000, Cell Signaling), goat anti-Actin antibody (1:2000, Santa Cruz Biotechnology) and with the corresponding secondary antibody: donkey anti-goat IgG-HRP (1:2000, Santa Cruz Biotechnology) or goat anti-mouse IgG-HRP (1:2000, Santa Cruz Biotechnology). Signal was detected using Amersham™ ECL Western Blotting Detection Reagents (GE Healthcare), the Amersham Hyperfilm ECL (GE Healthcare) and the Kodak GBX developer and fixer (Sigma), as previously described.[28]

Visualization of autophagosomes. Cells were incubated for 1 h with freshly prepared 50 μM monodansylcadaverine (MDC, Biochemika) and fixed in 4% paraformaldehyde (PFA) in PBS. Cytopsins were prepared and mounted in Vectashield Mounting Media with DAPI. Cells were then observed using a fluorescence microscope (Axio Imager.Z1 coupled with ApoTome Imaging System microscope, Zeiss) for the observation of autophagosomes, as previously described.[29]

Statistical analysis

Statistical significance was determined with a two tailed Student's t-test, except for the data presented in Table 2 in which the unpaired Student's t-test was used.* Indicates $p<0.05$.

Results and discussion

Effect of the different G. lucidum extracts on the in vitro growth of human tumor cell lines

It is known that the use of different fractions of *G. lucidum* may have different outcomes in disease treatments.[30] Therefore, in this study, different *G. lucidum* extracts, obtained from the spores or from the fruiting body of this mushroom, were evaluated regarding their effect on the *in vitro* growth of four human tumor cell lines: AGS (gastric adenocarcinoma), MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and HCT-15 (colorectal adenocarcinoma). This was carried out using the sulforhodamine B (SRB) assay which quantifies protein thereby allowing to indirectly assess cell growth[22,31] and to determine the GI_{50} concentration of the extracts (corresponding to the concentration that inhibits 50% of net cell growth).

Results (**Table 1**) showed that polysaccharidic extracts from *G. lucidum* presented no cytotoxic activity since all of them presented GI_{50} higher than 400 μg/ml (for all of the cell lines studied). The mentioned extracts were previously characterized by the authors, being detected, after polysaccharides hydrolysis, the same sugars in the fruiting body and spore extracts: fructose (0.65 and 2.15 g/100 g dry weight, respectively), glucose (0.55 and 0.83 g/100 g), mannitol (7.36 and 8.24 g/100 g) and trehalose (2.76 and 3.27 g/100 g) in the fruiting body extract.[20]

On the other hand, the phenolic extracts (methanolic) from both the fruiting body and the spores showed *in vitro* cell growth inhibitory activity, particularly the one from the fruiting body, which was the most potent extract in all cell lines studied. The mentioned extract contains *p*-hydroxybenzoic acid (0.58 mg/100 g dry weight), *p*-coumaric acid (0.38 mg/100 g) and cinnamic acid (0.28 mg/100 g), as previously reported by the authors.[20] The higher activity revealed by the extract obtained from fruting body might be related to its higher phenolic compounds content in comparison with the extract prepared from spores, that included only *p*-coumaric (0.28 mg/100 g) and cinnamic (0.33 mg/100 g) acids.

Regarding the phenolic extract (methanolic) from the spores, different effects were observed depending on the cell lines analyzed. This extract was more potent in the HCT15 cells followed by NCI-H460 cells, but showed no cytotoxic effect in the other two cell lines studied (MCF-7 and AGS), presenting a GI_{50} concentration higher than the maximum concentration tested (400 μg/ml). This may indicate that the mechanisms of action of this extract are more relevant in the HCT15 and NCI-H460 cell lines than in the MCF-7 and AGS cells, possibly due the different genetic background of those human tumour cell models. However, the GI_{50} concentrations determined for the methanolic extract of the spores were considered high and therefore this extract was no further studied in the present work.

The fact that the phenolic (methanolic) extract showed cytotoxic towards human tumor cells is in agreement with what has been previously published.[32] Furthermore, up to 400 μg/ml, the evaluated extracts did not show cytotoxicity against the primary culture of porcine liver cells, PLP2.

The most potent extract against tumor cell lines, the methanolic extract from the *G. lucidum* fruiting body, was chosen to be further studied regarding its effect in cellular proliferation, cell cycle profile and cell death. For this, AGS cells were analyzed following treatment with 106 μg/ml of phenolic extract (methanolic) of the fruiting body, hereafter referred to as "*G. lucidum* methanolic fruiting body extract".

Effect of the *G. lucidum* methanolic fruiting body extract in AGS cellular proliferation and cell cycle profile

Prompted by the effect found on cell growth, it was intended to investigate if that effect was due to alterations in cellular proliferation. Therefore, the effect of *G. lucidum* extract on AGS proliferation was analyzed by determining the percentage of BrdU-

incorporating cells 48 h following treatment (**Figure 1**). Results showed a decrease on the proliferation levels of AGS cells (from 36% in blank and from 35% in DMSO, respectively) to approximately 19%, following treatment with the *G. lucidum* extract **(Figure 1**).

In addition, possible alterations in the cycle profile were investigated by flow cytometry following PI labeling of the cells. Results (**Figure 2**) showed that the *G. lucidum* extract caused a statistically significant increase in the percentage of AGS cells in the G1-phase of the cell cycle, together with a decrease in the percentage of cells in the G2-phase of the cell cycle. Moreover a decrease in S-phase was observed, although this was not considered statistically significant.

Other authors had previously reported a G1 cell cycle arrest in breast cancer (MCF-7) cells treated with *G. lucidum* extracts.[33]

Effect of the *G. lucidum* methanolic fruiting body extract in programmed cell death

The fact that no alteration in the sub-G1 peak was previously observed in the cell cycle analysis of AGS cells following treatment with the extract suggested that its mechanism of action did not involve apoptosis. Nevertheless, other studies have indicated that some *G. lucidum* extracts (such as unboiled aqueous extract and a methanol-extracted column-chromatography semipurified fraction) induced apoptosis.[34] Likewise, an ethanolic fraction of *G.lucidum* was shown to induce apoptosis in AGS cells, not only via the intrinsic mitochondrial pathway but also through the death receptor-mediated extrinsic apoptotic pathway.[35]

Therefore, it was further confirmed if the methanolic extract from the fruiting body of *G. lucidum* induced programmed cell death, by analyzing the levels of apoptosis by flow cytometry following Annexin V/PI labeling, which is an apoptosis-specific assay.

Results (**Table 2**) showed that no alterations in the levels of apoptosis following treatment with this extract. This is possibly due to the concentrations and time points tested in the present study. Indeed, in the previously mentioned study of Calvino and collaborators, concentrations tested were far superior and the time points were inferior to the ones tested in the present study.[34]

Effect of the *G. lucidum* methanolic fruiting body extract in autophagy

It has been suggested that *G. lucidum* associates with autophagy.[36-38] In fact, treatment with a triterpene extract from *G. lucidum* suppressed proliferation of HT-29 colon cancer cells and inhibited growth of the respective xenograft tumor model. This effect was shown to be due to the induction of autophagy, with the extract inducing the formation of autophagic vacuoles and upregulating the expression of autophagy-associated proteins, such as Beclin-1 and LC-3, both in HT-29 colon cancer cells as well as in the xenograft tumors.[36] Recently, *G. lucidum* was also shown to induce autophagy in a breast cancer cell line, promoting cell death.[37]

Therefore, in this study, in order to confirm if the extract was interfering with cellular autophagy, the expression levels of some autophagic proteins were analysed. Results (**Figure 3**) showed no alterations in the levels of VSP-34 and Beclin-1. However, a clear increase in the autophagy marker LC3-II, was evident in AGS cells treated with the extract. As expected, there were no alterations in the Bcl-2 (antiapoptotic) protein levels when cells treated with the extract were compared with control (DMSO treated) cells.

This interference with cellular autophagy was further confirmed by an increase in the monodansylcadaverine (MDC) labeling of autophagosomes, observed in AGS cells following treatment with the extract (**Figure 4**).

Conclusion

In summary, the *G. lucidum* methanolic fruiting body extract inhibits the growth of a human gastric cancer cell line (AGS), by interfering with cellular autophagy and cell cycle. Further studies of the cellular and molecular mechanisms involved will be pursued in future work.

Competing interests

The authors declare no competing financial interest.

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Table 1. GI_{50} concentrations of various extracts from the fruiting body or spores of *G. lucidum* in four human tumor cell lines.

		GI_{50} (µg/ml)			
		NCI-H460	**HCT-15**	**MCF-7**	**AGS**
Fruiting body	Phenolic extract (Methanolic)	107.5 ± 5.3	103.4 ± 13.2	112.6 ± 6.7	93.3 ± 9.1
	Phenolic extract (Ethanolic)	> 400	> 400	> 400	> 400
	Polysaccharidic (Boiling water)	> 400	> 400	> 400	> 400
Spores	Phenolic extract (Methanolic)	386.9 ± 11.15	280.8 ± 11.17	> 400	> 400
	Phenolic extract (Ethanolic)	> 400	> 400	> 400	> 400
	Polysaccharidic (Boiling water)	> 400	> 400	> 400	> 400

Results are the mean ± SE of 3 independent experiments. Values >400 indicate that the GI_{50} concentration was not found when testing extracts up to 400 µg/ml (maximum concentration tested).

Table 2. Levels of apoptosis of AGS cells following treatment with *G. lucidum* methanolic fruiting body extract.

	Apoptotic cells (%)
Blank	7.9 ± 1.1
DMSO	5.9 ± 1.1
Extract	9.5 ± 3.2

Results are the mean ± SE of 3 independent experiments.

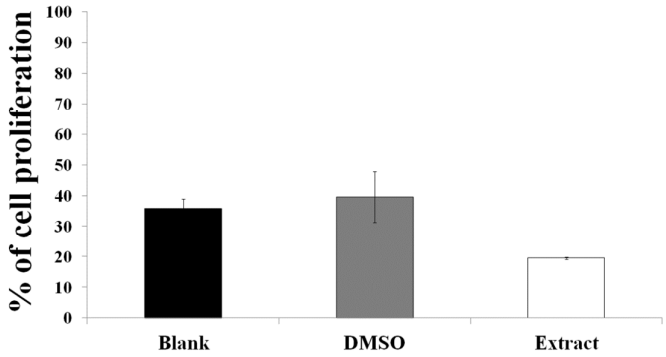

Figure 1 – Effect of *G. lucidum* methanolic fruiting body extract in AGS cellular proliferation. Cells were treated for 48 h with complete medium (Blank), *G. lucidum* extract or with corresponding vehicle (DMSO, control). Results are the mean ± SE of three independent experiments.

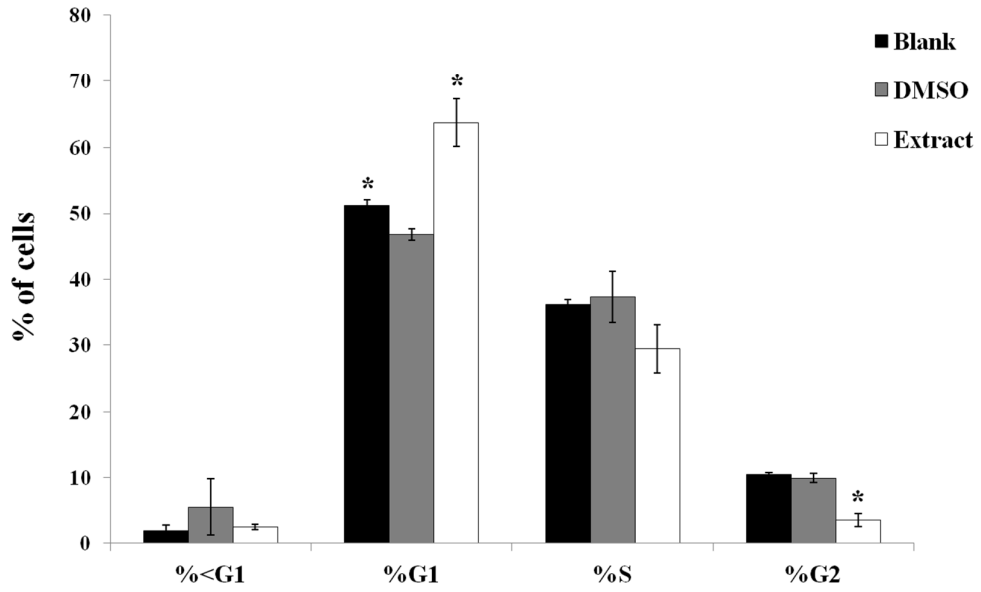

Figure 2. Effect of *G. lucidum* methanolic fruiting body extract in AGS cell cycle profile. Cells were treated for 48 h with medium (Blank), *G. lucidum* extract or with corresponding vehicle (DMSO, control). Results are the mean ± SE of 3 independent experiments. *Indicates $p \leq 0.05$ between treatment with the extract and with the DMSO (control).

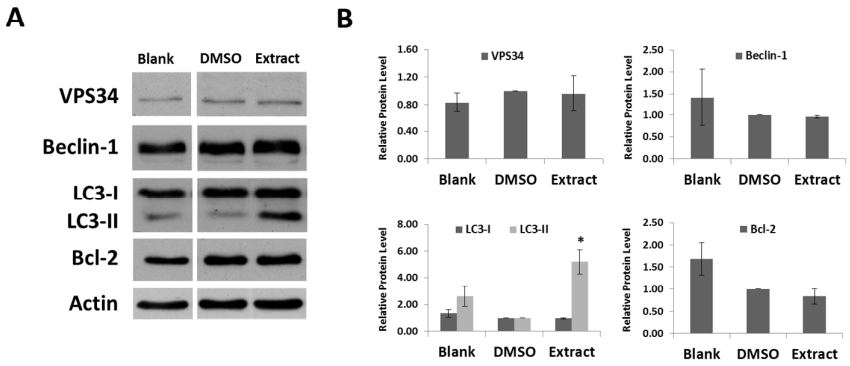

Figure 3. Expression of autophagy- and apoptosis-related proteins in AGS cells following 48 h treatment with *G. lucidum* methanolic fruiting body extract. A) Western Blot images representative of at least 3 independent experiments. Actin was used as loading control. B) Densitometry analysis of the Western blots. Results are the mean ± SE of three independent experiments and are expressed after normalization of the values obtained for each protein with the values obtained for actin and further expressed in relation to control cells. * Indicates $p<0.05$ between treatment with the extract and with the DMSO (control).

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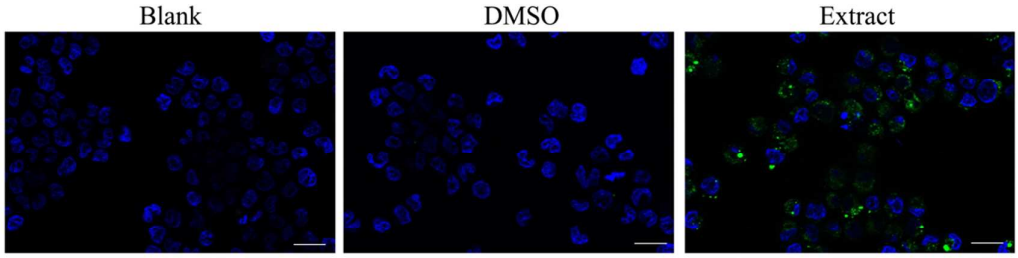

Figure 4. Analysis of the effect of *G. lucidum* methanolic fruiting body extract in the presence of autophagosomes. Fluorescence microscopy images after MDC incorporation (green) are representative of 2 independent experiments. Cell nuclei are stained with DAPI (blue). Bar corresponds to 20 μm.

TOC graphic

A methanolic extract of *Ganoderma lucidum* fruiting body inhibits the growth of a gastric cancer cell line and affects cellular autophagy and cell cycle

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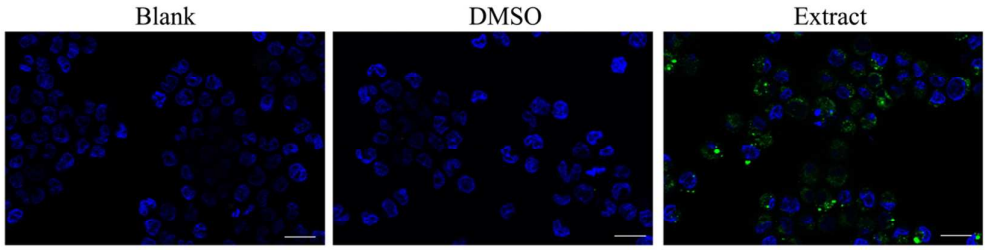

G. lucidum methanolic extract inhibited the growth of a gastric cancer cell line (AGS) by interfering with cellular autophagy and cell cycle.
