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Rhizopogon luteolus and *Ganoderma adspersum* Extracts Inhibit Invasion through the Crosstalk Antioxidant Activity and Apoptosis Induced by pAKT/Rb

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Research Article

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ABSTRACT

Objective: Lung cancer is the primary cause of cancer-related deaths globally, and in NSCLC cancers, kinase protein B (AKT) is associated with pathways such as proliferative, invasion and apoptosis. In this study, the relationship of mushroom extracts with these pathways was investigated. Mushrooms have a long history of use in traditional medicine to treat a variety of illnesses, and scientists have been exploring the potential use of their antioxidant and anticancer properties in modern medicine, a topic to which this paper contributes.

Materials and methods: Mushroom extracts were collected in 2022 and the other phases of the experiment were completed in 2023. We studied the lung cancer cell lines h1299, pc-3 and pc-14. Extracts of *Rhizopogon luteolus* and *Ganoderma adspersum* were applied to these to determine the IC_{50} values. Then, proliferative and invasion effects, apoptosis and antioxidant effects were investigated. Finally, Western blotting was performed to investigate the pathways of these effects.

Results: *Rhizopogon luteolus* and *Ganoderma adspersum* extracts have anti-proliferative and anti-invasive effects on lung cancer cell lines and induce apoptosis, which increases the antioxidant effect. The mushroom extracts work through the p-AKT and Rb pathways.

Conclusions: We anticipate that *Rhizopogon luteolus* and *Ganoderma adspersum* extracts will make effective additions to cancer treatment as agents that work to suppress lung cancer cells *via* the p-Akt and Rb pathways.

Keywords: *Rhizopogon luteolus*; *Ganoderma adspersum*; Lung cancer; Mushroom; Cancer treatment

INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide [1]. Five-year survival is 26% after diagnosis in Non-Small-Cell Lung Cancer (NSCLC), and that drops to just 6% for patients with metastatic-stage NSCLC at the time of diagnosis and who receive cytotoxic treatment [2]. However, the five-year survival of patients with metastatic NSCLC who are eligible for targeted treatment agents or immunotherapies has increased by 15–50% in recent years [3].

Kinase protein B (AKT) protein is associated with many pathways in NSCLC. Phosphorylation of AKT (p-AKT) leads to cell proliferation, migration, increase in invasion capacity and escape from apoptosis [4]. p-AKT also has a role in many cellular pathways; for example, increase in PTEN or decrease in p-AKT may lead to an increase in free radicals [5]. Furthermore, in previous studies on NSCLC cells, it has been reported that increased SOD, GPx and catalase levels induce apoptosis [6]. Mushrooms are organisms commonly found in living ecosystems all over the world. Mushrooms are not only consumed as nutritional foods but also have antimicrobial, antioxidant, anti-proliferative, anti-aging, anticancer, apoptotic and DNA-protective effects [7,8]. These properties are thanks to their bioactive molecules, and accordingly, those molecules are studied in the contents of mushroom composites [9].

Rhizopogon (in the family Rhizopogonaceae, also known as yellow false truffles) is a genus of ectomycorrhizal Basidiomycetes. Its mushrooms usually colonise the roots of coniferous trees such as pine and fir. *Rhizopogon* species play an important role in the recovery of degraded forests [10]. The genus *Ganoderma* was first described in 1881. It is used in traditional Chinese medicine because its species contain compounds with medicinal properties. Studies have reported that the bioactive compounds in *Ganoderma* spp. have antioxidant, antimicrobial, antiproliferative and immunomodulatory effects, as well as degrading environmental pollutants [11].

The main purposes of this study were to investigate the anti-proliferative, antioxidant, apoptotic and invasion inhibition effects of *Rhizopogon luteolus* and *Ganoderma adspersum* extracts and to elucidate the pathways of these effects.

MATERIALS AND METHODS

Mushroom materials

Rhizopogon luteolus Krombh. December and *Ganoderma adspersum* (Schulz) Donk were collected in Mugla, Turkey in November 2022. From those collected, the correct mushrooms were identified and the remainder discarded at the natural products laboratory Fungarium of Mugla Sıtkı Koçman University. Mushroom extracts were collected in 2022 and the other phases of the experiment were completed in 2023.

Extraction process

After *R. luteolus* and *G. adspersum* were dried, they were cut and powdered to form composites. The powdered mushroom (weighing 500 g and 1.9 kg, respectively) was extracted using 15 litres of 100% methanol at room temperature for 15 days. The mixture was filtered and evaporated to obtain methanol extract (68.5 g and 133 g, respectively). Complete dissolution of the methanol extract was achieved with a separatory funnel (wire-cane 2020). This extract was used in all experiments.

Cell culture

Human lung cancer cell lines H1299, PC3 and PC14 were provided by Dr. Hakan Akca (University of Pamukkale University, Denizli, Turkey). H1299, PC3 and PC14 cells were incubated in RPMI-1640 medium with 10% Foetal Bovine Serum (FBS) in a humid environment containing 5% CO_2 at 37 °C.

Cell viability analysis

H1299, PC3 and PC14 cell lines were seeded in 96-well plates at 5 × 10^3 cells. Then, the mushroom (*Rhizopogon luteolus* and *Ganoderma adspersum*) extracts were applied to these cell lines at different concentrations. The extracts were prepared as 1 mg/ml master stock and cytotoxic activity was determined by using the CellTiter Glo 2.0 Kit (Promega) *via* luminescence-based measurements at 0, 24, 48 and 72 hours. Regression analysis was performed to calculate IC_{50} values for the extracts on the different cell lines.

Invasion assay

H1299, PC3 and PC14 cell lines were seeded into invasion chambers with serum-free medium at 1 × 10^5 cells. The outer part of each invasion chamber was incubated with serum in medium. The cell lines were incubated with mushroom extracts (IC_{50} value) in medium for 24 hours. After 24 hours, the membrane was fixed with methanol and stained with 1% borax, 1% toluidine blue. Finally, the cells were counted under a microscope according to the manufacturer's protocols.

TUNEL assay

H1299, PC3 and PC14 cell lines were seeded in flasks at 5 × 10^5 cells so we could perform terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labelling (TUNEL). To do so, the cell lines were treated with the mushroom extracts at the IC_{50} values determined in the cytotoxicity experiment. Then, samples were collected at 0, 24, 48 and 72 hours. The TUNEL test (Invitrogen) was performed on those samples, and apoptotic cells were counted under a microscope according to the manufacturer's protocols.

Evaluation of SOD, CAT and GPx enzyme activities

The cell lines were treated according to the IC_{50} values determined for the mushrooms extracts. After 24 hours' incubation, the cells were washed in PBS and trypsinised. Then, we examined the effects of the mushroom extracts on Superoxide Dismutase (SOD), CAT and GPx enzyme activity. The SOD activity measurement method was based on the principle whereby xanthine reacts with xanthine oxidase to generate superoxide radicals, which react with tetrazolium salt to form a red formazan dye; the SOD activity is measured by the degree of inhibition of this reaction. The catalase assay, meanwhile, was based on the reaction of the enzyme with methanol in the presence of the optimal concentration of hydrogen peroxide; a Catalase Assay Kit (Cayman) was used to perform this measurement. Lastly, GPx was measured based on the principle that it catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidised glutathione is immediately converted to the reduced form with concomitant oxidation of NADPH to NADP+. The decrease in absorbance of NADPH was measured at 430 nm using a GPx Assay Kit (Cayman), and that corresponded to the GPx activity.

Western blotting assay

H1299, PC3 and PC14 cell lines were seeded in flasks at 5 × 10^5 cells. Then, mushroom extracts were applied at the IC_{50} values determined for 24 hours. After incubation, the cell lines were treated with RIPA buffer and were collected with a cell scraper on an ice block. Protein was obtained from the collected cell lysates. Protein samples of 100 µg were run on 2–15% polyacrylamide gel (Pierce) and transferred to PVDF membrane (PharmaciaBiotech Amsterdam). AKT, p-AKT (Ser473), p53, MAPK and GAPDH (Santa Cruz) antibodies were used at a dilution ratio of 1:1000. After labelling with the second antibodies containing peroxidase conjugate, the samples were finally labelled with SuperSignal West Pico chemiluminescence substrate

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and then visualised using hyperfilm ECL.

Statistical analysis

We used GraphPad 8.0 and SPSS 17.0 to perform a statistical analysis of the results obtained from the cell viability, invasion, TUNEL and antioxidant ELISA assays. One-way ANOVA, Tukey's post hoc test and a paired T-test were used. The statistical significance level for all comparisons was set at $p<0.05$.

RESULTS

Rhizopogon luteolus and *Ganoderma adspersum* suppressed cell proliferation and invasion

To determine the anti-invasive and anti-proliferative effects of mushroom extracts on the H1299, PC3 and PC14 cell lines, we performed a cytotoxicity assay with the CellTiter Glo 2.0 kit and an invasion assay with a Boyden invasion chamber. Our results showed that the mushroom extracts inhibited the cell proliferation of the H1299, PC3 and PC14 cell lines in a time- and dose-dependent manner. The half maximal Inhibitory Concentration (IC_{50}) doses of the mushroom extracts for 24 hours in the H1299, PC3 and PC14 lung cells were found to be 25.04, 11.73 and 16.54 for *R. luteolus* and 2.97, 1.53 and 1.01 for *G. adspersum*, respectively. *G. adspersum* had a stronger cytotoxic effect than *R. luteolus* on the H1299, PC3 and PC14 cell lines according to our results (Figure 1). Our next goal was to carry out an invasion experiment to investigate whether *G. adspersum* and *R. luteolus* extracts blocked invasion. The cell lines were treated with mushroom extracts at the IC_{50} values in the invasion chamber for 24 hours. After incubation, we compared each of the control groups with the study groups. We analysed the results by counting the whole area four times. Our results showed that *R. luteolus* had a stronger anti-invasive effect than *G. adspersum* on the H1299, PC3 and PC14 cell lines (Figure 2).

Figure 1. The cytotoxic effects of *R. luteolus* and *G. adspersum* in H1299, PC3 and PC14 NSCLC. IC_{50} values was calculated by using data in this graphs.

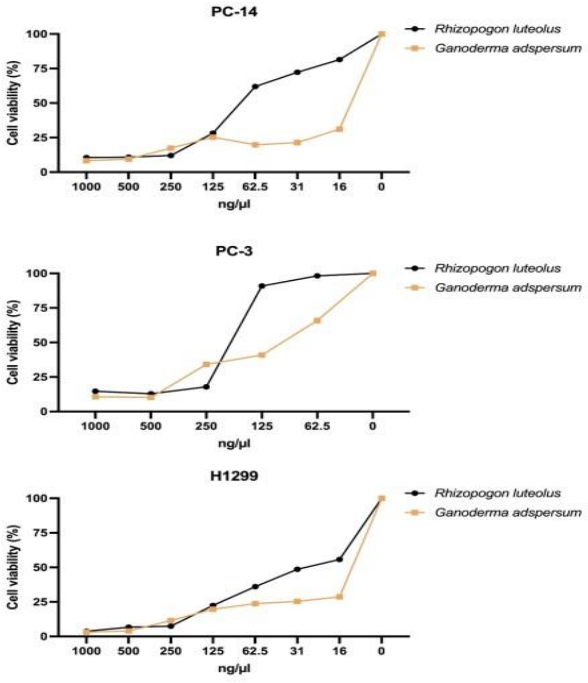

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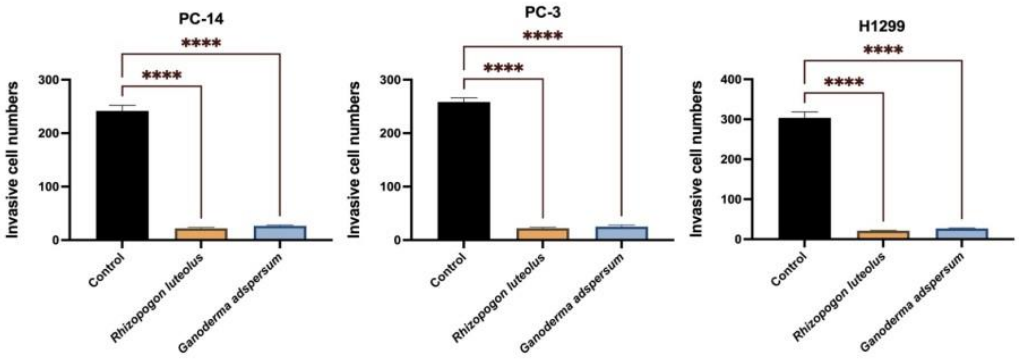
Figure 2. The effects of *R. luteolus* and *G. adspersum* about on cell invasion in H1299, PC3 and PC14 NSCLC.



Rhizopogon luteolus and *Ganoderma adspersum* induced apoptosis by triggering antioxidant activity in NSCLC

After demonstrating that mushroom extracts affected cell proliferation and invasion, we next examined the relationship with cell apoptosis. We applied a TUNEL test to determine whether the IC_{50} values obtained in the cell viability assay were due to apoptosis or necrosis of the cells. According to the data we obtained, *G. adspersum* and *R. luteolus* extracts had an apoptotic effect on lung cancer cell lines ($p<0.05$) (Figure 3). The antioxidant effects of mushrooms are known, and we hypothesized that those triggered apoptosis in cancer cells. To investigate our results in this direction, we examined the antioxidant effects. To that end, we incubated the cells for 24 hours according to the IC_{50} values for the mushroom extracts. Next, the cells were washed twice with PBS and then lysed. We used these lysates to determine the antioxidant enzyme activity, which according to our results, the mushroom extracts increased ($p<0.05$) (Figure 4). Western blotting was used to investigate the cellular pathways for the anti-proliferative and invasive effects of mushroom extracts, as well as their antioxidant and apoptotic effects. We collected the cells that we had incubated for 24 hours according to the IC_{50} values, and we applied those to lung cancer cell lines with RIPA buffer. The Western blotting results were analysed in the ImageJ 5.0 program. As shown in Figure 5, there was a statistically significant increase in p-AKT, p53 Rb and p-MAPK proteins.

Figure 3. *R. luteolus* and *G. adspersum* induce apoptosis in H1299, PC3 and PC14 NSCLC.

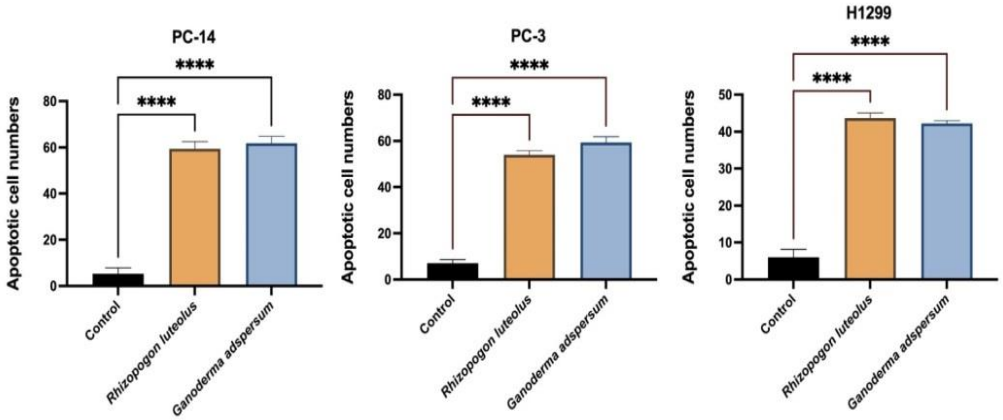


Figure 4. The effects of *R. luteolus* and *G. adspersum* in cellular anti-oxidant activiy at H1299, PC3 and PC14 NSCLC. A) SOD, CAT, GPx leves were measured in PC14 treated with *R. luteolus* and *G. adspersum*. B) SOD, CAT, GPx leves were measured in PC3 treated with *R. luteolus* and *G. adspersum*. C) SOD, CAT, GPx leves were measured in H1299 treated with *R. luteolus* and *G. adspersum*

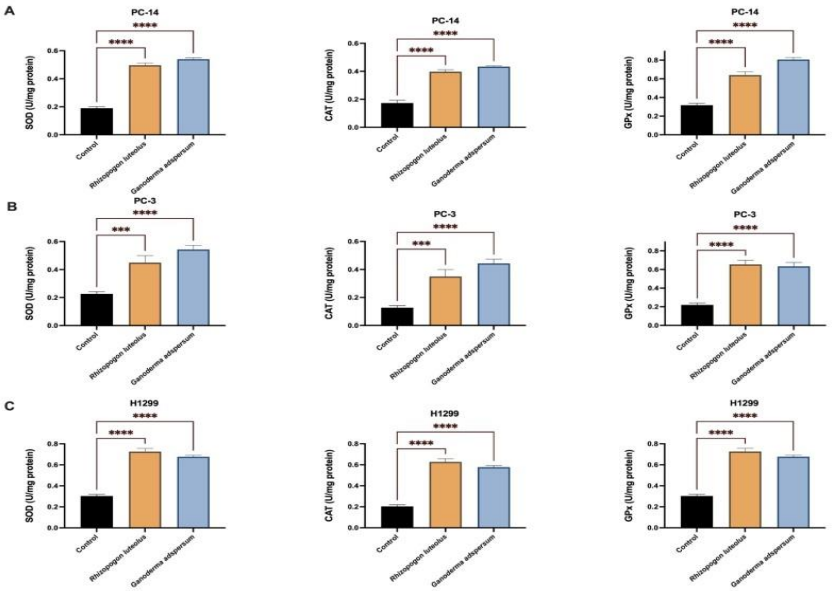
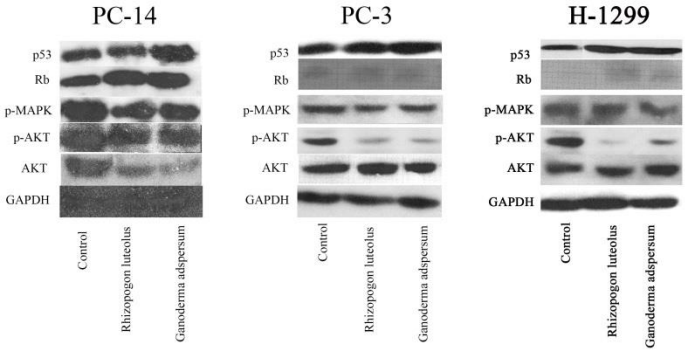


Figure 5. *R. luteolus* and *G. adspersum* supress invasion/proliferation *via* p53, Rb, pAKT/ AKT and pMAPK axis. These protein levels were detected with Western Blot analysis.



DISCUSSION

Mushrooms have been used in traditional medicine for centuries to treat various diseases. In research on modern medicine, researchers have been focused on investigating mushrooms for their anticancer and antioxidant properties. Among more than 11,000 mushrooms found in nature, 2000 of them are non-toxic, but only 300 of these mushrooms have medicinal properties [12].

In this study, we conducted a series of experiments to investigate whether *G. adspersum* and *R. luteolus* extracts blocked invasion through a mix of apoptotic, proliferative and cellular antioxidant activities in NSCLC cells (H1299, PC3 and PC14). In previous research, Tel-Çayan et al. obtained fumaric acid, gallic acid and protocatechuic acid in the phenolic profile when they

analysed *R. luteolus* extracts, and fumaric acid, caffeic acid and 2,4-dihyroxybenzoic acid in *G. adspersum* [13]. In the fatty acid profiles of these two mushroom species, linoleic and oleic acid were obtained, and galactomannan 1 and 2 were isolated as polysaccharides in a study conducted with the same extracts in 2020 [14]. Kaur et al., reported a significant increase in SOD, GPx and CAT enzymes with fumaric acid treatment in cadmium-induced liver damage in their study [15]. Here, we obtained results in good agreement with those previously published, and we propose that fumaric acid, which was highly represented in the phenolic profile, caused the antioxidant effects.

The antioxidant activities of *R. luteolus* and *G. adspersum* mushrooms are known, and the increase in antioxidant substances indicates the suppression of oncogenic genes [11]. Tosun et al., reported in their study with *R. luteolus* that the increase in ROS caused an increase in intrinsic apoptosis [16]. Mahajna et al., meanwhile, reported that *Ganoderma lucidum* blocked cell proliferation *via* the PI3K/Akt/NF-kB pathway [17]. It was also reported to block the G_1 for the S-phase transition through the CDK- and CDK6 pathways. In the Western blot analysis, the amounts of the p53 and Rb proteins increased, while the amounts of p-AKT and p-MAPK decreased. Akça et al., reported that the decrease in p-AKT and increase in SOD, GPx and CAT were mediated by PTEN [5], while another study with linoleic acid reported anti-proliferative and apoptotic effects to occur *via* the PI3K/Akt and ERK pathways [18]. Our results showed that the extracts were effective in decreasing the p-AKT level and increasing the Rb level. Kimura et al., reported that oleic and linoleic acid blocked tumour growth, invasion and metastasis in lung cancer cell lines [19]. In his study with galactomannan, a polysaccharide obtained from *Punica granatum*, Manu reported that it has a cytotoxic effect in many cancer types, and he found that the effectiveness of chemotherapy was improved when this polysaccharide was introduced.

CONCLUSION

According to our research, *R. luteolus* and *G. adspersum* exhibit potent anticancer and antioxidant effects on the human lung cancer cell lines H1299, PC3 and PC14, and those effects occur through the activation of apoptosis, triggered by the accumulation of p53 and Rb. We propose that the two mushroom species may include active compounds with potential as therapeutic agents that can be added into combined treatment strategies for human lung cancer.

ACKNOWLEDGMENT

Not applicable.

FUNDING

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AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHORS' CONTRIBUTIONS

AH and MME designed the study and confirm the authenticity of all the raw data. DA, KER, TO and ÇGT performed the experiments. DA and KER were major contributors to the writing of the manuscript. All authors have read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

PATIENT CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTEREST

All authors declare that they have no competing interest.

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