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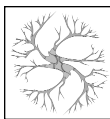
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Ganoderma lucidum inhibits invasiveness of breast cancer cells

Veronika Slivova, BS; Tatiana Valachovicova, BS; Jiahua Jiang, PhD; Daniel Sliva, PhD

ABSTRACT

Ganoderma lucidum (Reishi) is a popular Asian medical mushroom, which has been widely used in traditional Chinese medicine to treat a variety of diseases. Although originally used as a mushroom for longevity, the dried powder of Ganoderma lucidum was recommended as a cancer chemotherapy agent in ancient China. Recent in vitro and animal studies have suggested that Ganoderma lucidum exhibits anticancer activity, mainly through stimulation of the host immune system by polysaccharides or by the cytotoxic effects of triterpenes. We have demonstrated that purified spores or fruiting body of Ganoderma lucidum down-regulated the expression of urokinase plasminogen activator (uPA) and uPA receptor (uPAR), which resulted in the suppression of cell motility in cancer cells. In this study, we investigated how Ganoderma lucidum, in the form of a dietary supplement, can modulate the metastatic behavior of the highly invasive human breast cancer cells MDA-MB-231. Our data demonstrate that Ganoderma lucidum inhibits cell adhesion, cell migration, and cell invasion of highly metastatic breast cancer cells. Furthermore, Ganoderma lucidum suppressed the anchorage-independent growth (colony formation) of MDA-MB-231 cells. Based on these results, Ganoderma lucidum may contribute to reducing invasion and metastasis of breast cancers by inhibiting cancer cell adhesion, cell migration, cell invasion, and growth of cancer cells.

Key words: Ganoderma lucidum, breast cancer, adhesion, migration, invasion, anchorage-independent growth

INTRODUCTION

Breast cancer is the most common malignancy in women in the United States, accounting for about 33 percent of all cancers diagnosed in females.¹ The typical treatment for

breast cancer is surgery and/or adjuvant therapy.² However, breast cancer often progresses from the nonmetastatic and therapy-responsive phenotype to the highly invasive and metastatic phenotype, which is usually resistant to the standard therapeutic procedures. Therefore, breast cancer is the second leading cause of cancer death in the United States, and death usually results from invasion and metastasis.² Cancer metastases and invasion are complex, interrelated processes, consisting of cell adhesion, proteolytic degradation, and cell migration, allowing the spread and colonization of cancer cells in distant sites of the body.³ Furthermore, cancer cells can also metastasize by an additional mechanism (anchorage-independent growth), which does not require integrin-mediated adhesion.⁴ Thus, the inhibition of either cell adhesion, migration, invasion, or anchorage-independent growth could suppress the potential for breast cancer cells to metastasize.

Ganoderma lucidum (Reishi), a medical mushroom, has been used as a home remedy for numerous types of chronic diseases.⁵ Many bioactive components isolated from *Ganoderma lucidum* have been demonstrated to possess antioxidative, antihypertensive, and anticancer effects.⁶⁻⁹ Polysaccharides, mainly with the structure of β -D-glucans, exert anticancer effects against leukemic cells,¹⁰ and some triterpenes also exhibit cytotoxic activity against sarcoma and lung carcinoma cells in vitro.¹¹ On the molecular level, we have recently reported that *Ganoderma lucidum* suppresses constitutively active transcription factors AP-1 and NF- κ B, which results in the down-regulation of urokinase plasminogen activator (uPA) and uPA receptor (uPAR) expression in highly invasive human breast and prostate cancer cells.¹² Furthermore, we have shown that spores or fruiting body of *Ganoderma lucidum* inhibit cell motility in both breast and prostate cancer cells.¹² Although the uPA/uPAR system was originally recognized for its proteolytic activity (breaking down the extracellular matrix proteins), which is necessary for cell invasion, uPA/uPAR is also responsible for cell adhesion and cell migration.¹³

The present study was undertaken to further characterize the effect of *Ganoderma lucidum* on the behavior of highly

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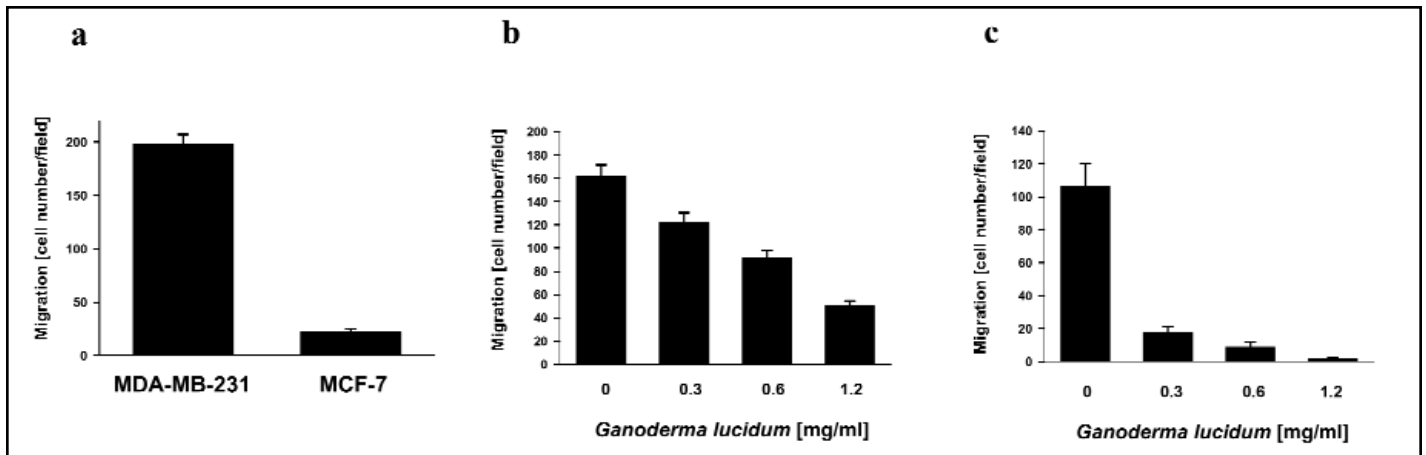


Figure 1. *Ganoderma lucidum* inhibits migration of breast cancer cells. (a) Cell migration of 5×10^4 MDA-MB-231 or MCF-7 cells were determined after 24 hours of incubation in Transwell chambers, as described under Material and Methods. (b) Migration of MDA-MB-231 cells (1.5×10^5) in the presence of *Ganoderma lucidum* (0-1.0 mg/ml) was determined after 6 hours of incubation. (c) Migration of MCF-7 cells (1.5×10^5) in the presence of *Ganoderma lucidum* (0-1.0 mg/ml) was determined after 72 hours of incubation. Data are the means (\pm SD) of triplicate determinations. Similar results were obtained in at least two additional experiments.

invasive breast cancer cells in vitro. Here we demonstrate that the dietary supplement, *Ganoderma lucidum*, inhibited adhesion, migration, and cell invasion of breast cancer cells MDA-MB-231. Furthermore, *Ganoderma lucidum* also inhibited another characteristic of tumorigenic cells, anchorage-independent growth of cancer cells. Thus, *Ganoderma lucidum* suppresses the invasive behavior of breast cancer cells and may have potential therapeutic use to prevent breast cancer metastasis.

MATERIALS AND METHODS

Materials

Ganoderma lucidum (Reishimax™) was purchased from Pharmanex (Provo, UT). According to the manufacturer, this sample contains 13.5 percent polysaccharides and 6 percent triterpenes. *Ganoderma lucidum* was dissolved in boiled water, stored at 4° C, and reheated to 70° C for 10 minutes before every experiment.

Cell culture

Human breast cancer MCF-7 and MDA-MB-231 cells were purchased from ATCC (Manassas, VA) and were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (50 units/ml), streptomycin (50 units/ml), and 10 percent fetal bovine serum (FBS). Media and supplements came from GIBCO BRL (Grand Island, NY). FBS was obtained from Hyclone (Logan, UT).

Cell adhesion assay

Cell adhesion was performed with Cytomatrix Adhesion

Strips coated with human fibronectin or vitronectin (Chemicon International, Temecula, CA), according to the manufacturer's instructions. MDA-MB-231 cells were treated with *Ganoderma lucidum* (0-1.0 mg/ml) for 24 hours, harvested, and counted. Cells (10^5) were applied to the rehydrated fibronectin or vitronectin strips in a 96-well plate and incubated for 1-1/2 hours at 37° C. The cells were stained and washed, and the absorbance was determined with a microplate reader, as previously described.¹⁴ The adhesion of cells treated with the vehicle was established as 100 percent. Data points represent the average (\pm SD) of three individual wells within one representative experiment, repeated at least twice.

Cell migration assay

MCF-7 and MDA-MB-231 cells were harvested and incubated with *Ganoderma lucidum*, as indicated in the text. Chemokinesis was assessed in Transwell chambers (6.5 mm diameter polycarbonate filters; 8 μ m pore size) in DMEM containing 10 percent FBS, as previously described.¹⁵ After fixing and staining, we determined the number of migrating cells microscopically by enumeration at 40x magnification from at least four random fields. Data points represent the average (\pm SD) of four individual filters within one representative experiment, repeated at least twice.

Cell invasion assay

MDA-MB-231 cells were harvested and treated with *Ganoderma lucidum* (0-1.0 mg/ml). After 72 hours of incubation, invasion was assessed in Transwell chambers (6.5 mm diameter polycarbonate filters; 8 μ m pore size) coated with

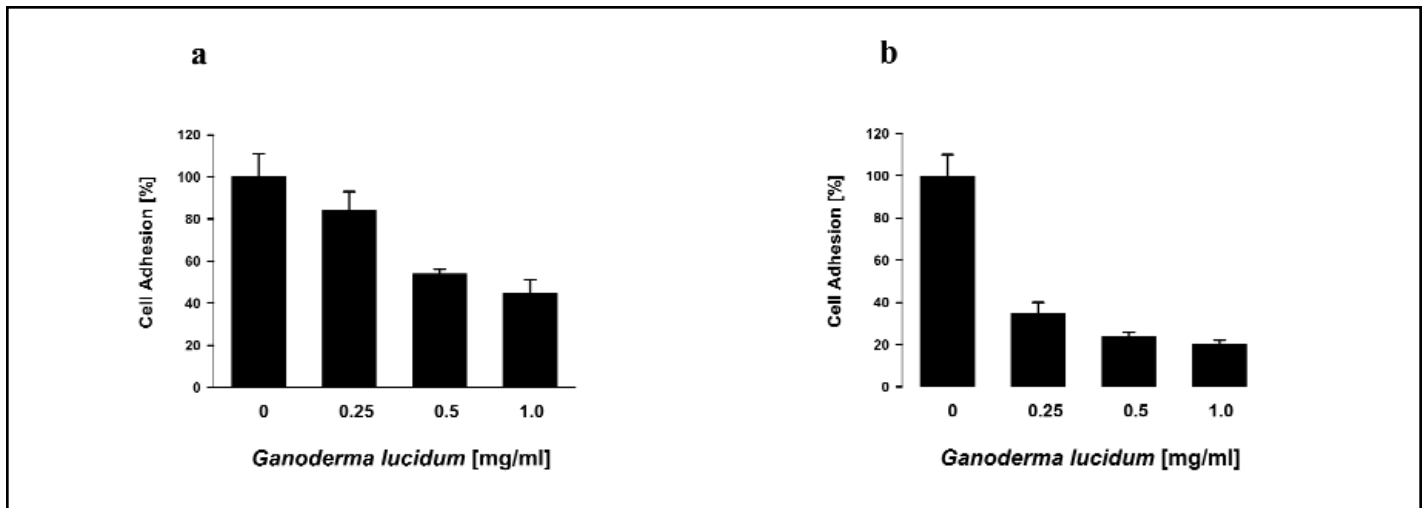


Figure 2. *Ganoderma lucidum* suppresses adhesion of highly invasive breast cancer cells to extracellular matrix proteins. (a) Adhesion to fibronectin. MDA-MB-231 cells were incubated with *Ganoderma lucidum* (0-1.0 mg/ml) for 24 hours and harvested, and adhesion to fibronectin was assessed after an additional 1-1/2 hours of incubation, as described under Materials and Methods. The proportion of adherent cells was counted as a percentage of the control. Data points represent the average (\pm SD) of three parallel wells within one representative experiment repeated at least twice. (b) Adhesion to vitronectin. MDA-MB-231 cells were incubated with *Ganoderma lucidum* (0-1.0 mg/ml) for 24 hours and harvested, and adhesion to vitronectin was assessed after an additional 1-1/2 hours of incubation, as described above. Data points represent the average (\pm SD) of three parallel wells within one representative experiment, repeated at least twice.

100 μ l of Matrigel™ (BD Biosciences, Bedford, MA) diluted 1:4 with DMEM. The cells, which invaded through Matrigel, were stained with hematoxylin, and their number was determined microscopically by enumeration at 40x magnification from at least four random fields. Data points represent the average (\pm SD) of three individual filters within one representative experiment, repeated at least twice for verification.

Anchorage-independent growth

MDA-MB-231 cells (2.5×10^5) were harvested and seeded in 6-well plates coated with one percent agarose. Anchorage-independent growth was assessed after incubation for 10 to 14 days in the culture media with or without *Ganoderma lucidum* (0-1.0 mg/ml). The culture media were replaced every four days. Plates were stained with 0.005 percent Crystal Violet, and the colonies were counted manually with a microscope and photographed.

RESULTS

Ganoderma lucidum inhibits migration of breast cancer cells

We have previously shown that the highly invasive human breast cancer cells, MDA-MB-231, migrate spontaneously compared to the less invasive human breast cancer cells, MCF-7, after two hours of incubation.¹⁵ Although MCF-7 cells are less invasive cells, they still should have some

migratory potential. Therefore, the incubation period for the cell migration assay was extended to 24 hours. As shown in Figure 1a, both highly invasive MDA-MB-231 cells and less invasive MCF-7 cells exhibited spontaneous migratory activity, although the MCF-7 cells demonstrated only 10 percent of the activity of MDA-MB-231 cells. We have recently demonstrated that spores or fruiting bodies of *Ganoderma lucidum*, with unidentified chemical content, inhibit the motility of highly invasive breast and prostate cancer cells with varying potency.¹⁶ In the present study, we used the dietary supplement *Ganoderma lucidum*. *Ganoderma lucidum* contains both polysaccharides and triterpenes, which showed activity against different cancer cell lines.^{10,11} As expected, *Ganoderma lucidum* suppressed migration of MDA-MB-231 cells in a dose-response manner (Figure 1b). Furthermore, *Ganoderma lucidum* also inhibited migration of the poorly invasive breast cancer cells MCF-7 (Figure 1c). However, the inhibitory effect of *Ganoderma lucidum* on MCF-7 cells was observable after 72 hours of incubation, whereas *Ganoderma lucidum* suppressed cell migration of MDA-MB-231 cells after six hours. Because *Ganoderma lucidum* inhibits cell proliferation of both MDA-MB-231 and MCF-7 cells in a dose- and time-dependent manner (data not shown), it is possible that the inhibition of migration of MCF-7 cells is the result of the suppression of viability of MCF-7 after extended incubation. However, the inhibition of migration of MDA-MB-231 cells is

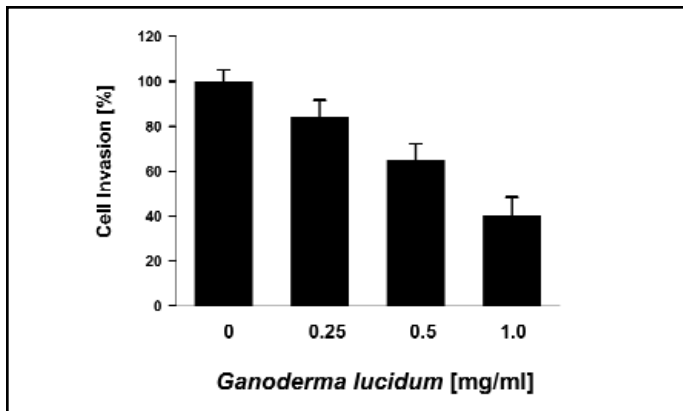


Figure 3. *Ganoderma lucidum* inhibits cell invasion of MDA-MB-231. MDA-MB-231 cells were harvested and treated with *Ganoderma lucidum* (0-1.0 mg/ml). Invasion through Matrigel was assessed after 72 hours of incubation, as described under Materials and Methods. Data are the means (\pm SD) of triplicate determinations. Similar results were obtained in at least two additional experiments.

independent of inhibition of cell proliferation, because the six hours of incubation does not affect the viability of MDA-MB-231 cells (data not shown).

***Ganoderma lucidum* inhibits adhesion of highly invasive breast cancer cells**

We have previously demonstrated that *Ganoderma lucidum* inhibits expression of uPA/uPAR as well as secretion of uPA from MDA-MB-231 cells.¹² Furthermore, cell adhesion and migration are mediated through the interaction of uPA/uPAR with integrin receptors that are ligated to the extracellular matrix proteins, such as fibronectin and vitronectin.¹³ Breast cancer cells MDA-MB-231 express integrin receptor for fibronectin $\alpha_3\beta_1$ ¹⁷ and vitronectin $\alpha_v\beta_3$ ¹⁸ Therefore, we hypothesized that *Ganoderma lucidum* will also inhibit cell adhesion. MDA-MB-231 cells were treated with *Ganoderma lucidum* (0-1.0 mg/ml) for 24 hours and harvested, and adhesion to fibronectin was determined. As seen in Figure 2a, *Ganoderma lucidum* inhibited adhesion of MDA-MB-231 cells in a dose-response manner. Further experiments confirmed that *Ganoderma lucidum* also inhibited adhesion of MDA-MB-231 cells to vitronectin (Figure 2b). Taken together, these two results show that *Ganoderma lucidum* suppressed the formation of the fibronectin- $\alpha_3\beta_1$ -uPA/uPAR and vitronectin- $\alpha_v\beta_3$ -uPA-uPAR complexes, resulting in the inhibition of cell adhesion.

***Ganoderma lucidum* inhibits invasion of MDA-MB-231 cells**

In addition to its role in cell adhesion and migration, uPA/uPAR is also crucial for the invasion of cancer cells

through the activation of proteolytic activity that is responsible for the degradation of the components of the extracellular matrix.¹³ Therefore, we were interested in whether *Ganoderma lucidum* also inhibits invasion of highly invasive breast cancer cells. MDA-MB-231 cells were seeded on the Matrigel-coated Transwell filters in the presence of *Ganoderma lucidum* (0-1.0 mg/ml), and the number of invaded cells was determined. As seen in Figure 3, *Ganoderma lucidum* markedly inhibited invasion of MDA-MB-231 cells through Matrigel, confirming its potency to suppress the proteolytic activity of highly invasive cancer cells.

***Ganoderma lucidum* suppresses colony formation of MDA-MB-231 cells.**

Colony formation is one of the typical characteristics of the metastatic potential of cancer cells in vitro and strongly correlates with tumorigenesis in vivo.⁴ To determine whether *Ganoderma lucidum* inhibits colony formation in highly invasive breast cancer cells, we assessed the anchorage-independent growth of MDA-MB-231 cells. We found that MDA-MB-231 cells formed colonies after 14 days of incubation (Figure 4a), and treatment with increased concentrations of *Ganoderma lucidum* resulted in the inhibition of colony formation (Figures 4b-4d). Therefore, *Ganoderma lucidum* inhibits growth of breast cancer cells independently of the inhibition of cell adhesion.

DISCUSSION

Ganoderma lucidum is a popular mushroom that has been widely used in traditional Chinese medicine in many Asian countries. Although the anticancer effects of purified or semipurified compounds from *Ganoderma lucidum* have been described in cell culture and animal studies, the effects of unfractionated mushrooms or their parts remain elusive. The popularity of dietary supplements as alternative therapies for the treatment of cancer has been increasing recently.¹⁹ Since *Ganoderma lucidum* is currently available in the form of dietary supplements, the clarification of its potential anticancer activity would scientifically justify its use by cancer patients. In the present study, we have compared the effects of *Ganoderma lucidum* on the invasive behavior of breast cancer cells. Here we show that *Ganoderma lucidum* inhibits cell migration, cell adhesion, and cell invasion, as well as colony formation of human breast cancer cells. *Ganoderma lucidum* suppressed cell migration of poorly invasive MCF-7 as well as highly invasive MDA-MB-231 cells. However, the mechanism for the inhibition of cell migration in these two types of cancer cells is probably different because of the difference in

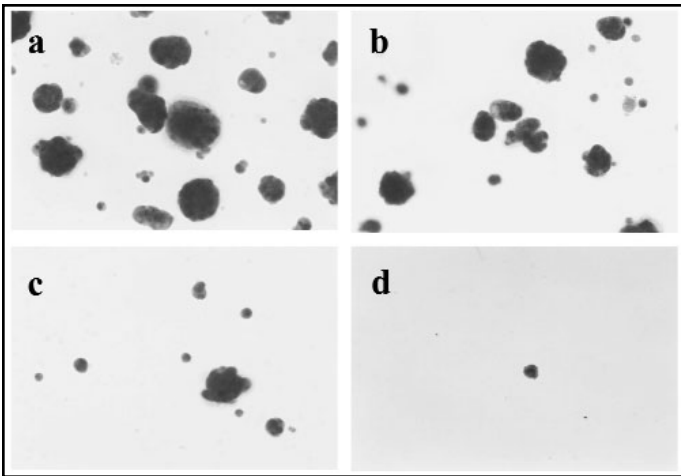


Figure 4. *Ganoderma lucidum* suppresses colony formation of MDA-MB-231 cells. MDA-MB-231 cells were harvested and seeded in 6-well plates coated with agarose. Anchorage-independent growth was assessed as described under Materials and Methods. (a) 0 mg/ml, (b) 0.25 mg/ml, (c) 0.5 mg/ml, and (d) 1.0 mg/ml *Ganoderma lucidum*.

time needed for incubation before inhibition occurs. The inhibition of migration of poorly migrating MCF-7 cells after three days of incubation is caused by the inhibition of cell proliferation, whereas the migration of MDA-MB-231 cells is inhibited after six hours of incubation (an incubation time that does not affect proliferation of MDA-M-231 cells). Since MCF-7 cells are poorly invasive, the number of migrating cells is low for the determination of the inhibitory effect of *Ganoderma lucidum* on cell migration. After an extended period of time, the decrease in migrating cells is caused by cell death. This observation is in agreement with a recent study demonstrating the induction of apoptosis of MCF-7 cells by *Ganoderma lucidum* extract.²⁰ In addition, the inhibition of migration of highly invasive MDA-MB-231 cells is a result of the inhibition of constitutively active NF- κ B and secretion of uPA, which are not active in poorly invasive MCF-7 cells.²¹ Finally, we have previously shown that *Ganoderma lucidum* inhibits NF- κ B and expression of uPA and uPAR and secretion of uPA.¹² Therefore, we hypothesized that the anticancer effects in MDA-MB-231 cells by *Ganoderma lucidum* were caused by the inhibition of the uPA/uPAR system. As expected, *Ganoderma lucidum* inhibited cell adhesion to the extracellular matrix (ECM) proteins fibronectin and vitronectin, which resulted from the suppression of formation of the complexes consisting of ECM proteins with integrin receptors and uPA/uPAR (ECM-IR-uPA/uPAR). Our data agree with recent observations demonstrating that disruption of the ECM-IR-uPA/uPAR complex resulted in the inhibition of cell adhesion and cell migration

of cancer cells.^{14,22} Furthermore, *Ganoderma lucidum* also suppressed invasion of MDA-MB-231 cells through Matrigel, suggesting its inhibitory effect on the proteolytic activity of secreted uPA. Alternatively, inhibition of uPA can result in the suppression of the proteolytic activity of matrix metalloproteinases (MMPs).¹³ In addition, *Ganoderma lucidum* can down-regulate the expression of MMP-1, -3, and -9 through the inhibition of NF- κ B.^{23,24}

In the present study, we also show that *Ganoderma lucidum* inhibits the anchorage-independent growth (colony formation) of breast cancer cells. Because anchorage-independent growth correlates with the capacity of cancer cells to metastasize and colonize in the human body,⁴ *Ganoderma lucidum* could have preventive effects against secondary metastases, which are responsible for the high mortality of breast cancer. In summary, *Ganoderma lucidum* demonstrated a strong inhibitory effect against the invasive behavior of highly metastatic breast cancer cells in vitro. We used *Ganoderma lucidum* in the form of a dietary supplement, without other extraction or purification steps, which could possibly increase the biological effects of *Ganoderma lucidum*. Furthermore, this application is in accord with the practice of herbal medicine, in which the use of the whole product could reduce the toxicity of its purified components, and the interaction between different biologically active components can increase the therapeutic activity of the whole product.²⁵

Although polysaccharides and lipids extracted from *Ganoderma lucidum* demonstrated inhibitory effects on tumor growth in animal studies,⁵ the effects of digestion and metabolic degradation on its anticancer activity remains to be investigated. Thus, animal experiments are in progress to demonstrate the anticancer effect of *Ganoderma lucidum* in vivo and to scientifically justify the use of the dietary supplement *Ganoderma lucidum* (Reishi) for the prevention and treatment of invasive cancers.

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