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GANODERMA LUCIDUM EXTRACT INHIBITS PROLIFERATION OF SW 480 HUMAN COLORECTAL CANCER CELLS

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Aim: ***Ganoderma lucidum*** **is a commonly used Chinese herb and an important ingredient in traditional Chinese medicine herbal formulations for immune dysfunction related illnesses. The effects of this medicinal mushroom on human colorectal cancer cells have not yet been evaluated. In this study, we investigated the effects of *Ganoderma lucidum* extract using SW 480 human colorectal cancer cell line.** ***Materials and Methods:*** **Two different fractions of *Ganoderma lucidum* extract, i.e., a fraction containing mainly polysaccharides (GLE-1), and a triterpenoid fraction without polysaccharides (GLE-2) were analyzed. Their antiproliferative activity was evaluated by cell proliferation assay and ^{3}H-thymidine incorporation assay. Scavenging effects of DPPH radical were assessed using ESR-spectroscopy.** ***Results:*** **Our data showed that both GLE-1 and GLE-2 significantly inhibited the proliferation of SW 480 cells. The inhibitory effect of GLE-2 was much stronger than that of GLE-1. GLE-1 inhibited DNA synthesis in the cells and reduced the formation of DPPH radicals.** ***Conclusion:*** ***Ganoderma lucidum*** **extract inhibits proliferation of human colorectal cancer cells and possesses antioxidant properties.**

Key Words: Ganoderma lucidum, **colorectal cancer, SW 480, proliferation, reactive oxygen species, DPPH radicals.**

Colorectal cancer is the second leading cause of cancer related death in the United States, and the second most prevalent cancer worldwide [1]. Half of the patients diagnosed with colorectal cancer eventually die from the disease; and only less than ten percent of patients with the metastatic cancer survive more than five years after diagnosis [1]. Although in its early stages the cancer can be cured by surgical resection alone, very often surgery is combined with adjuvant radio- and chemotherapy. Patients treated for advanced stage colorectal cancer with adjuvant chemotherapy show a 40% reduced risk of relapse [2]. Recently, controlled clinical trials support a multimodal, multidisciplinary approach to treating both early stage and advanced colorectal cancers [3, 4].

While advances continue to be made in developing effective treatment strategies for colorectal cancer patients, chemotherapies are still limited by severe side effects and dose-limiting toxicity. The drug-related adverse events not only worsen patients' quality of life, but can also lead to their refusal to continue the potentially curative chemotherapy [5]. Patients with cancer often resort to complementary and alternative medical means to treat the side effects of chemotherapy [6].

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Abbreviations used: DMSO – dimethyl sulfoxide; DPPH – 2,2-Diphenyl-1-picrylhydrazyl; ESR – electron spin resonance; GLE – *Ganoderma lucidum* extract; ROS – reactive oxygen species.

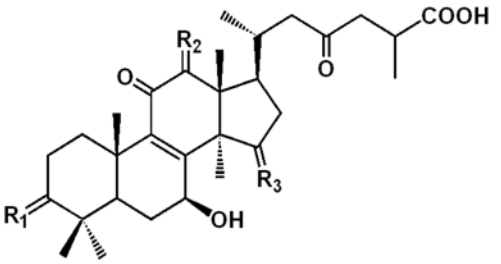
Ganoderma lucidum (or "Lingzhi" in Chinese) has long been recognized by Chinese medical professionals as a valuable herbal medicine in treating a number of different illnesses [7]. Shizhen Li, a highly influential doctor in Chinese medicine from the Ming Dynasty (1368–1644 A.D.), recorded the effectiveness of Lingzhi in his famous book, *Ban Chao Gang Moo* ("Comprehensive Pharmacopoeia"), and stated that taking Lingzhi over the long-term would build a strong, healthy body and assure longevity. *Ganoderma lucidum* is often an important ingredient in traditional Chinese medicine herbal formulations for immune dysfunction related illnesses [7, 8].

Currently, there are several *Ganoderma lucidum* herbal products available. SunRecome is a commonly used *Ganoderma lucidum* product manufactured by Shanghai Green Valley Pharmaceuticals in China. Previous studies showed that this herbal product could attenuate chemotherapy-induced nausea and vomiting in clinical trials [9] and animal studies [10]. Although human observational data in Chinese literature suggest that *Ganoderma lucidum* possesses an anticancer property [9], and the active anti tumor constituents are thought to be on the polysaccharides [11], the effects of this medicinal mushroom on human colorectal cancer cells has not been evaluated. In the present study, we investigated the antiproliferative effects of two fractions of SunRecome, i.e., a fraction containing mainly polysaccharides, and a triterpenoid fraction without polysaccharides, using SW 480 human

colorectal cancer cell line. Potential antioxidant activity of *Ganoderma lucidum* was also evaluated.

MATERIALS AND METHODS

Ganoderma lucidum extract (GLE) and test solution preparation. SunRecome, or *Ganoderma lucidum* extract (GLE), from one lot, was obtained from Shanghai Green Valley Pharmaceuticals, China. The extract was analyzed by Shanghai Allsuccess Commodity Inspection Co., who reported that the extract contained 1.89% terpenoids and 15.8% polysaccharides using spectrophotometric methods. Fig. 1 shows the chemical structure of terpenoids in *Ganoderma lucidum*. The extract was also analyzed by Applied Consumer Services (Hialeah Gardens, USA) to confirm that it was free of contamination such as microorganisms, pesticide residues, and toxic elements.



Compounds	R1	R2	R3
Ganoderic acid A	O	H_2	H, OH
Ganoderic acid B	OH, H	H_2	O
Ganoderic acid C1	O	H_2	O
Ganoderic acid C2	OH, H	H_2	H, OH
Ganoderic acid G	OH, H	OH, H	O
Ganoderic acid γ*	O	H_2	H, OH

*Structure of side chain:

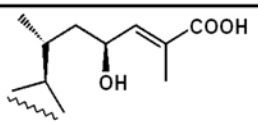


Fig. 1. General structure of terpenoids in *Ganoderma lucidum*

Two different solutions were used to prepare GLE-1 (a fraction containing mainly polysaccharides) and GLE-2 (a fraction containing triterpenoids without polysaccharides). To prepare GLE-1, the extract powder was dissolved in 5% ethanol-water solution and placed in a 4 °C refrigerator over night. The following day the sealed sample was heated in a water bath at 80 °C for 30 min. When the sample temperature cool down to room temperature, the solution was filtered with a Millex 0.2 μm nylon membrane syringe filter (Millipore Co., Bedford, USA). To prepare GLE-2, the extract powder was dissolved in DMSO in room temperature, and then filtered with a Millex 0.2 μm nylon syringe filter. To ensure that the cell growth was not affected by the DMSO, we studied the effect of DMSO alone on cell proliferation as a vehicle.

Cell culture. Human colorectal SW480 cancer cell line (ATCC, Manassas, USA) was maintained in Leibovitz's L-15 medium with 10% L-glutamine (Gibco, USA), supplemented with 10% fetal bovine serum and penicillin-streptomycin (50 units/ml, Invitrogen, USA), in a humidified incubator (5% CO^2 in air at 37 °C) with medium change every 2–3 days. When the cells reach 70–80% confluency, they were trypsinized, harvested, and seeded into a new tissue culture dish (100 mm in diameter).

Cell proliferation assay. On selected days after removal of incubation medium, SW480 cell monolayer was washed twice with phosphate buffered saline (PBS). To examine the antiproliferation effect of the extract, the cells were seeded in a 24-well plate at about 10,000 cells/well with regular medium and allowed to adhere for 24 h. After adhesion of the cells, the culture medium was changed prior to the addition of the test herbal extracts. The cells were incubated with testing material at various concentrations for 72 h. Control cultures were incubated in culture medium alone. At the end of treatments, the cells were detached using trypsin and counted using a Coulter Counter (Counlter Electronics, Hialeah, USA) [12, 13]. All assays were performed at least three times. The inhibition of SW480 cell proliferation was calculated as follows: Cell proliferation (%) = 100 x (each cell number in experimental well/total cell number in the control well).

^{3}H-thymidine incorporation assay. SW 480 cells were seeded in a 24-well plate and allowed to adhere for 24 h. The cells were incubated with GLE-1 at various concentrations in 300 μl media containing 1 λ/ml ^{3}H-thymidine (specific activity: 1.48 TBq /mmol 40.0 Ci/mmol) in each well for 72 h. Control cultures were incubated in the medium with ^{3}H-thymidine only. After washing with PBS and 10% TCA (trichloroacetic acid), 0.5 ml NaOH (0.2 M) was added to each well and agitated for 5 min. Finally, the cell lysates were transferred into vials and 5 ml of 30% liquid scintillation was added and mixed in the vials. The radioactivity was measured using Liquid Scintillation Analyzer (Tri-Carb1500, Packard) [14].

Measurement of free radical scavenging ability of GLE-1 in a cell-free system. Antioxidant activity of *Ganoderma lucidum* was evaluated on the basis of its scavenging effect of DPPH radical using ESR spectroscopy [15]. Conventional ESR spectra were obtained with a Varian E-109 X-band spectrometer. ESR signals were recorded with 10 mW for DPPH, and 100 kHz field modulations of 2G. All measurements were performed at room temperature.

The DPPH stable radical scavenging activity of GLE-1 was estimated by ESR according to the method of Yamaguchi et al. [16]. The reaction mixture contained 50 μl test sample and 50 μl DPPH solution (0.5 mM). The proportion of ethanol/water in the reaction mixture was 5/95. ESR signaling was recorded at room temperature. Time dependence of the scavenging effect was determined by adding GLE-1 to the reaction mixture at 5 mg/ml for 2–15 min after mixing sample. Scavenging effect was determined by comparison with a control group. The dose dependency of the scavenging effect was also determined after GLE-1 addetion to the reaction mixture for 15 min.

GLE-2 was diluted in DMSO. Since DMSO possesses antioxidant activity, we were unable to evalu-

ate the free radical scavenging ability of GLE-2 in the cell-free system.

Statistical analysis. Data are expressed as mean ± standard error (S.E.). Statistical analysis was performed using ANOVA in combination with Student's *t*-test. Differences were considered significant if $P < 0.05$.

RESULTS

Inhibitory effect of GLE-1 on SW 480 cells. The inhibitory activity of GLE-1 on the proliferation of SW 480 human colorectal cancer cells at 72 h is shown in Fig. 2, *a*. GLE-1 decreased cell proliferation significantly in a concentration related manner. Compared to control group (100%), GLE-1 reduced cell proliferation by 3.8 ± 4.5% at 0.05 mg/ml, 8.9 ± 5.2% at 0.25 mg/ml ($P < 0.05$), and 10.2 ± 5.0% at 1 mg/ml ($P < 0.05$).

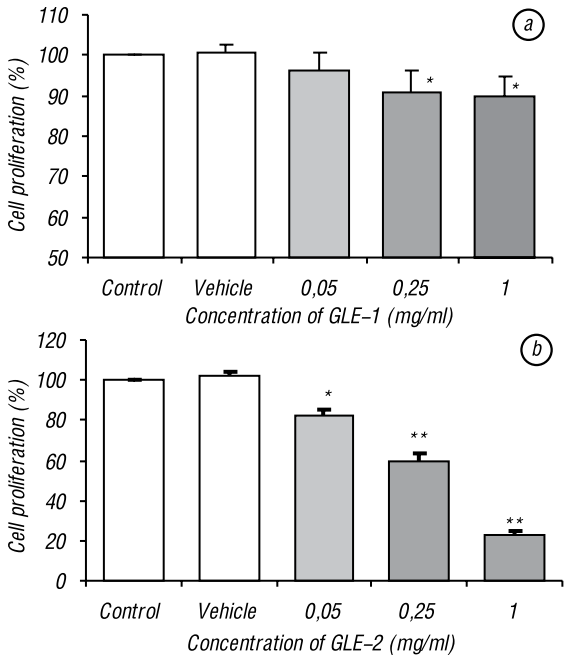


Fig. 2. Antiproliferative effects of *Ganoderma lucidum* extract on SW 480 colorectal cancer cells after 72 h treatment. *(a)* Effects of GLE-1. *(b)* Effects of GLE-2. * $P < 0.05$; ** $P < 0.01$.

Inhibitory effect of GLE-2 on SW 480 cells. The inhibitory activity of GLE-2 on the proliferation of SW 480 cells at 72 h is shown in Fig. 2, b. GLE-2 decreased the cell proliferation significantly in a concentration dependent manner. Compared to the control group, GLE-2 reduced cell proliferation by 18.1 ± 3.1% at 0.05 mg/ml ($P < 0.05$), 40.2 ± 3.7% at 0.25 mg/ml ($P < 0.01$), and 76.7 ± 1.8% at 1 mg/ml ($P < 0.01$).

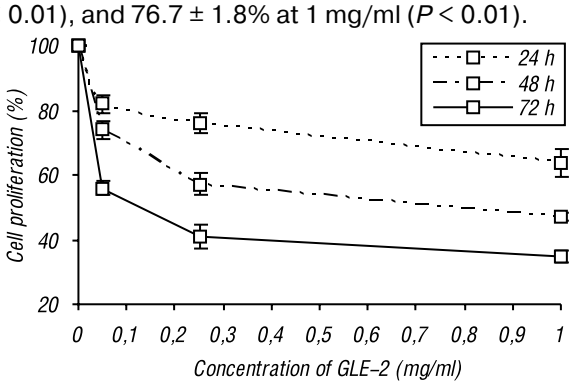


Fig. 3. Time- and concentration-dependent inhibitory effects of *Ganoderma lucidum* extract on SW 480 colorectal cancer cells. GLE-2 was used in this experiment. * $P < 0.05$; ** $P < 0.01$.

Fig. 3 shows the time and concentration related inhibitory effects of GLE-2 on the proliferation of SW 480 cells.

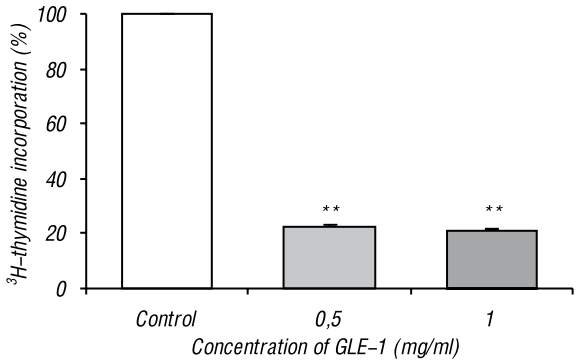


Fig. 4. Effects of *Ganoderma lucidum* extract on DNA synthesis. ^{3}H-thymidine incorporation in SW 480 colorectal cancer cells was measured after 72 h of treatment. ** $P < 0.01$

Effects of GLE-1 on DNA synthesis by measuring ^{3}H-thymidine incorporation. As shown in Fig. 4, c, treatment with GLE-1 for 72 h at 0.5 and 1 mg/ml suppressed cellular incorporation of 3H-thymidine significantly by 70.0 ± 0.6% and 73.0 ± 1.5% ompared with the control (both $P < 0.01$), respectively.

Free radical scavenging ability of GLE-1 in cell-free system. The radical scavenging activities of GLE-1 in a cell free system are shown in Fig. 5. Addition of GLE-1 to the DPPH system at a concentration of 5mg/ml remarkably reduced the free radical ESR signal after 2 min. After 15 min, the ESR signal of DPPH was totally eliminated. This time-related effect is shown in Fig. 5, *a*. The concentration-related scavenging effect of GLE-1 for 15 min is shown in Fig 5, *b*.

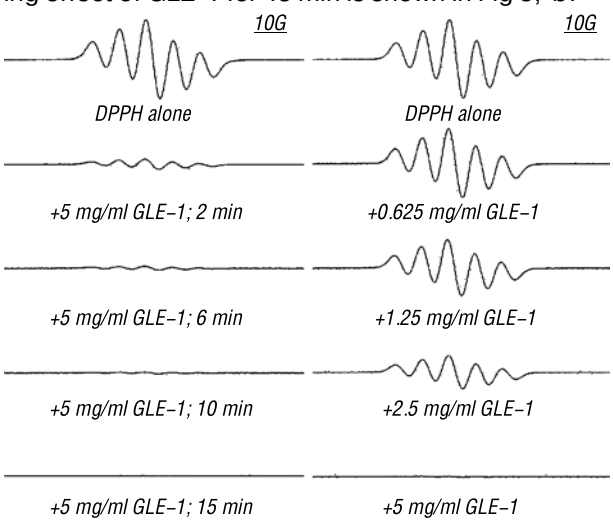


Fig. 5. Effects of *Ganoderma lucidum* extract on the ESR spectra of the DPPH radical in ethanol/water (20/80) solution. ESR time points *(b)* spectra of the DPPH radical with GLE-1 are shown for different, and at different concentrations (*a)*

DISCUSSION

Ganoderma lucidum is a commonly used herbal medicine in many Oriental countries. The anticancer effects of polysaccharides extracted from another species of genus *Ganoderma* were initially reported

in 1971 [17]. Recently, some researchers observed that triterpenoids in *Ganoderma lucidum* also are active antitumor constituents [18, 19].

In this study, two different solutions were used in the preparation of GLE-1 and GLE-2. Although both of the extracts showed inhibitory effects on the proliferation of SW 480 human colorectal cancer cells, the percentage of inhibition was different, with much stronger inhibition using GLE-2. GLE-1 was prepared with 5% ethanol-water solution, which presumably dissolved mostly polysaccharides and a small percentage of terpenoids, while GLE-2 was prepared with DMSO, which presumably dissolved mostly terpenoids without any polysaccharides [11]. Thus, it seems that terpenoids in the GLE exert significant antiproliferative effects on the colorectal cancer cells in our experiments. In order to explore the possible mechanisms of *Ganoderma lucidum*'s anticancer activity, we evaluated the effect of the extract on the incorporation of ^{3}H-thymidine in SW480 cells by using ^{3}H-thymidine labeling assay. Our data suggest that the extract directly inhibits the synthesis of DNA in the cells [14].

Many herbal medicines possess antioxidant properties [20]. Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species (ROS). Some ROS, such as superoxide and hydrogen peroxide, are normally produced in cells as by-products of biochemical reactions or as signaling molecules. When ROS-generating reactions are activated excessively, pathological quantities of ROS are released to create an imbalance between antioxidants and ROS, resulting in cellular damage. Oxidative stress was linked with the pathogenesis of many human diseases including cancer, aging, and atherosclerosis [21]. Herbal antioxidants may protect against these diseases by contributing to the total antioxidant defense system of the human body [20, 22]. We observed the antioxidant effect of *Ganoderma lucidum* extract in our ESR study. The relationship between the observed antioxidant activity and the antiproliferation effect of SW 480 cells remain to be elucidated. In future investigations, we will also use a panel of cell lines to provide us with the opportunity to evaluate whether the effects of a medicinal herb can be generalized across the spectrum of human cancers.

In addition to its possible anticancer effect, *Ganoderma lucidum* may also help in decrease chemotherapy-induced side effects. We have demonstrated that the extract of *Ganoderma lucidum* attenuated cisplatin-induced nausea and vomiting in a rat model [10]. Cancer patients are known to use herbal products more often than the general population, and thus, well-investigated herbal therapies can offer patients with a practical alternative [6]. Moreover, if synergistic effects between herbal medicines and chemotherapy agents can be identified, reduction of chemotherapy dose in combination with herbs can further decrease dose-related drug toxicity. The identification of nontoxic anticancer herbal medicines remains an essential step in advancing the treatment of cancer. Data obtained from our studies has the potential to advance treatment regimens, and improve the quality of life of patients suffering from colorectal cancer.

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ТОРМОЖЕНИЕ ПРОЛИФЕРАЦИИ КЛЕТОК КОЛОРЕКТАЛЬНОГО РАКА ЛИНИИ SW 480 ЭКСТРАКТОМ *GANODERMA LUCIDUM*

Цель: **для лечения заболеваний, связанных с нарушениями функционирования иммунной системы, в традиционной китайской медицине часто используют препараты на основе *Ganoderma lucidum*. В данной работе было изучено влияние экстракта *Ganoderma lucidum* на клетки колоректального рака человека линии SW 480.** ***Материалы и методы:*** **изучали две фракции экстракта *Ganoderma lucidum:* фракцию GLE-1, содержащую полисахариды, и тритерпеноидную фракцию GLE-2. Их рост-ингибирующую активность определяли с помощью анализа клеточной пролиферации и по включению ^{3}H-тимидина. Для оценки антиоксидантной активности использовали ECP-спектроскопию.** ***Результаты:*** **обе фракции *Ganoderma lucidum* подавляли пролиферацию клеток SW 480. Рост-ингибирующий эффект GLE-2 был более выраженным, чем фракции GLE-1. Фракция GLE-1 подавляла синтез ДНК в клетках линии SW 480 и понижала уровень формирования DPPH радикалов.** ***Выводы:*** **экстракт *Ganoderma lucidum* подавляет пролиферацию клеток колоректального рака человека и обладает антиоксидантными свойствами.**

***Ключевые слова: Ganoderma lucidum*, колоректальный рак, SW 480, пролиферация, активные радикалы кислорода, DPPH радикалы.**