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Original article

Effect of Different Extracts of *Ganoderma lucidum* on Ochratoxin A Formation by *Aspergillus ochraceus*

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Abstract

Background and objectives: Mycotoxins are secondary metabolites produced by fungi contaminated agricultural products and have toxic effect in human and animals. Ochratoxin A is produced by many filamentous fungi species such as *Aspergillus* and *Penicillium*, and is classified as a possible carcinogen and nephrotoxic. *Ganoderma lucidum* is a famous mushroom with antifungal properties and probably preventive effect on toxin production. The aim of this study was to investigate the antitoxigenic properties of Iranian strain of *G. lucidum* as a natural antitoxigenic agent against harmful filamentous fungi in food industry. **Methods:** Different extracts of *G. lucidum* were prepared and the antifungal and antitoxigenic effects were studied on toxigenic *Aspergillus ochraceus*. Ethanolic, hydroalcoholic and aqueous extracts were prepared by cold maceration method and compared with commercial extracts. Ochratoxin A was analyzed by high performance liquid chromatography (HPLC) coupled with fluorescence detector in *A. ochraceus* culture media which was treated with the extracts. **Results:** The results expressed that the alcoholic extracts could inhibit fungal growth, but the aqueous extracts were able to significantly prevent toxin production while the fungus totally grew. **Conclusion:** *Ganoderma lucidum* and its products, may be used as a pharmaceutical and nutraceutical mushroom and could be considered as safe and useful agents for prevention of fungal growth and mycotoxin formation in food and agricultural products.

Keywords: *Aspergillus ochraceus; Ganoderma lucidum*; HPLC; mycotoxins; ochratoxins

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Introduction

Mycotoxins are hazardous secondary metabolites produced by fungi growth on different organic substrates [1]. The role of mycotoxins in food contamination was first recognized in 1960s with several studies on toxic fungal metabolites [2]. More than 400 different mycotoxins have been identified worldwide in food and feed products [3,4]. Global occurrence of mycotoxins is very high (72% of positive samples worldwide) which indicate their crucial role in human health because of their undeniable role in chronic foodborne diseases and the economic loss resulting from over-contamination [5]. Almost all main groups of fungi can produce mycotoxins but genus *Aspergillus* is definitely the most important species [1].

Among different mycotoxins produced by *Aspergillus* spp., ochratoxin is of great importance because of its high prevalence and serious health risks [6,7]. Three forms of ochratoxin have been identified so far (A, B, and C with ochratoxin A as the most prevalent one) [8]. They are produced by many filamentous species. *Aspergillus* (*Aspergillus ochraceus* and

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Aspergillus carbonarius) and *Penicillium* have been classified as possible carcinogens for humans. Also, ochratoxin A nephrotoxic properties have been related to kidney damages [1,9,10]. Several foods such as cereals (maize, wheat, barley, oats and rice), groundnuts [11], meat products, coffee, grape juice, milk, cocoa, spices, dried fruits [12,13], green or black tea [14] and sugarcane [15] are known to be susceptible to ochratoxin contamination.

In the last 20 years, biological methods have been introduced to be efficient and environmentally friendly for detoxification of food products from mycotoxins. Many researchers look for new biological agents such as bacteria, yeasts and fungi as new natural agents for elimination of mycotoxins from food products. The use of non-toxin *Aspergillus* species on agricultural products has gained significant success in reducing mycotoxin contamination. This is because some certain fungi can use their degradation products as a valuable source that helps to the detoxification of mycotoxins [13].

Ganoderma lucidum also known as "Reishi' or "Ling Zhi" is a famous mushroom with significant pharmacological and nutraceutical properties [16-18]. In addition, *G. lucidum* has been reported in recent studies as a novel antifungal agent against some well-known fungal species including *Aspergilus sp*., *Fusarium sp*. and *Penicillium sp*. [19,20]. Because of this antifungal property, we conducted an experiment on *Ganoderma* and experimentally tested whether *G. lucidum* can be used as a natural agent for detoxification of food and agricultural products from mycotoxins such as ochratoxin A.

Material and Methods

Ethical considerations

The authors have considered the ethics in research in the present study.

Apparatus

For optical density detection of fungal suspension, UV/Visible Spectrophotometer (Cecil CE 1021, MA, USA) was used at 530 nm.

In addition, HPLC along with fluorescence detector (FLD) was used for analysis of ochratoxin A (Waters e2695 Technologies, Milford/MA, USA). An auto sampler was used as an injection system of HPLC.

A reverse phase LC column (C_{18}-150 mm×4.6 mm) with ID 3 µm particles was used (Waters, Milford/MA). Immunoaffinity Columns (IACs) were wide-bore (WB) type that was used for mycotoxins with a total volume of 3 mL and purchased from VICAM (Milford, USA).

Chemicals

Potato dextrose broth (PDB) and yeast extract media (Ibresco, Italy) and sabouraud dextrose agar (SDA) media were purchased from Merck (Germany). Ochratoxin A reference standard was purchased from Sigma-Aldrich Chemical Ltd (St Louis, USA). Tween 80 and normal saline containing 0.9% sodium chloride were used for preparing fungal suspensions. Also, commercial standardized aqueous extract of *G. lucidum* was obtained from Biocan Pharma (North York, Canada) with at least 30% polysaccharide content. Other chemicals such as acetonitrile, acetic acid, toluene, methanol, ethanol 99.8%, phosphate buffer saline (PBS) were obtained from Merck (Germany). Pure water was provided from a milli-Q system (Millipore, USA).

All prepared solvents that were used for high performance liquid chromatography (HPLC) analysis were bought as analytical grade from Merck (Germany).

Ganoderma lucidum

The dried fruiting bodies of *G. lucidum* were obtained from Sarin Fam Company (product no. AS-12) originated from Waz forest, Chamestan, Noor city, Mazandaran Province, Iran.

Preparation of the extracts

Cold maceration method was used for preparation of *G. lucidum* extracts. Ten g of dried powdered *G. lucidum* fruiting bodies was weighed. Next, 200 mL of each solvent (sterile distilled water, ethanol 99.8%, ethanol/water 60:40) was poured into a 500 mL screw cap bottle covered by an aluminum foil. The ethanolic and hydroalcoholic extracts were remained at room temperature for two weeks. The two abovementioned extracts were regularly shaken by hand and checked for any possible cross-contamination detected by naked eye. Then, extracts were filtered twice through Whatman filter No. 1.

The aqueous extract was shaken for 24 h at 150 rpm by an orbital shaker and then it was filtered twice by a Whatman paper filter No.1 and was kept at 4 °C for additional tests. Also, the hydroalcoholic and ethanolic extracts were concentrated by rotary evaporator close to

dryness at a controlled temperature (40 °C). Then, the concentrated extract was dissolved in dimethyl sulphoxide (DMSO) to achieve desired concentrations [21].

Preparation of *Aspergillus ochraceus* strain

Toxigenic *A. ochraceus* fungal strain was purchased from Pasteur Institute of Iran (PFCC no. 401-10). The fungus was then sub-cultured for 7 days on SDA medium at 30 °C.

Then, the colonies were covered with sterile solution (100 mL saline normal + 10 µL tween 80) and were probed with a transfer pipette for preparing the suspensions. Next, the mixtures were transferred to a sterile tube and were settled for 3 to 5 min. Afterwards, the supernatant was added to a sterile tube and mixed for 15 s with a vortex [22]. The spectrophotometric method was used for measuring OD of microbial suspension. The OD for the genus *Aspergillus* was adjusted as 0.09-0.13 [22]. The final concentration was 1.2×10^6 spore/mL.

Toxigenic assays

Preparation of concentrations

All extracts were filtered by 0.45 micron syringe filter and were then diluted in order to make 5 desired concentrations (5, 4, 3, 2, 1 mg/mL).

Preparation of test tubes

The tubes were prepared for minimum inhibitory concentration (MIC) assay and ochratoxin A analysis according to Table 1.

As it is shown in Table 1, culture media (PDB) fortified by yeast extract was added to the test tubes and the ethanolic, hydroalcoholic and aqueous extracts with desired concentration, and 1 mL *A. ochraceus* suspension were added to achieve 10 mL final volume for all tubes. Each concentration was inoculated in triplicate into a 15 mL falcon tube. The positive (culture medium with fungal suspension) and negative control (culture media) as were prepared in triplicate. The tubes were incubated with 25-30 °C for two weeks.

MIC assay

MIC testing method was adopted with visual observation which is applied for *A. ochraceus*.

Ochratoxin A analysis

After two weeks, the tubes containing *A. ochraceus* and probably produced ochratoxin A were extracted by IACs and were ready for detection by HPLC-FLD.

The isocratic mobile phase in the experiment contained deionized water (102 mL), acetonitrile (96 mL), and acetic acid (2 mL) (51:48:1) with a flow rate of 1 mL/min and the column was set at room temperature. Ochratoxin A stock standard solution was prepared at a concentration of 200 µg/mL in toluene-acetic acid (99:1) and secondary standard was prepared at a concentration of 10 µg/mL that was confirmed by ultraviolet (UV) spectrophotometry. The intermediate standard used as calibration curve was prepared with a concentration of 1 µg/mL for ochratoxin A in toluene-acetic acid (9:1). Standard calibration curves were prepared in concentrations of 0.25, 0.5, 1, 2, 2.5, 5, 10 and 15 ng/mL for ochratoxin A in methanol-water-acetic acid (30:70:1). Samples were prepared based on instruction of AOAC Official Methods (999.07) [23] with some minor modifications [24]. Ochratoxin A was extracted from the sample (10 mL) by 50 mL acetonitrile/water (60:40).

The mixture was shaken for 30 min (Heidolph, Germany) and filtered through glass microfiber filter (1.5 µm). Next, 6 mL of extract was mixed with 34 mL of deionized water. Then IAC conditioned with 10 mL of PBS before used, after that, 10 mL of diluted extract was passed the IAC. In the following step, the column was washed twice with 0.5 methanol and collected in a vial and collected in a vial. Fifty µL of the contents of the vial were injected into the HPLC device and were determined by reversed-phase liquid chromatography with fluorescence detector at excitation 333 nm and emission 460 nm. The amount of octratoxin A was determined after calculating the area under the curve. The injection of each standard and each sample was repeated three times.

Statistical analysis

The anti-toxigenic properties of the studied extracts tests were presented as mean±SD of three replications. The findings were statistically compared between the control group and the extracted solvents groups by one-way ANOVA. Statistical significance p-level was selected at 5% significance.

Table 1. Preparation of the extract concentrations for *Aspergillus ochraceus* growth and toxin formation

Concentration (mg/mL)	PDB+0.5% yeast extract (mL)	Extract, 50 mg/mL (mL)	Fungal suspension (mL)	Final volume (mL)
1	8.8	0.2	1	10
2	8.6	0.4	1	10
3	8.4	0.6	1	10
4	8.2	0.8	1	10
5	8	1	1	10

Results and Discussion

Growth of *A. ochraceous* was observed for all extracts specially for the aqueous and commercial extracts at all concentrations (1 to 5 mg/mL). Meanwhile, ethanolic and hydroalcoholic extracts at concentrations of 2 and 4 mg/mL were determined regarding MIC_{50}, respectively. Due to the fact that fungal growth was observed in all Falcon tubes, all tubes were analyzed to measure ochratoxin A to observe the prevention of toxin production.

Figure 1 shows the standard chromatogram of ochratoxin A at a concentration of 0.5 ng/mL, the chromatogram of ochratoxin A in a control sample (middle) and the chromatogram of a sample of ochratoxin A from the commercial extract of *G. lucidum* strain at the concentration of 2 ng/mL. The retention time of the standard ochratoxin A chromatogram was approximately 10.2 minutes.

Different concentrations of standard ochratoxin A solution were injected into the HPLC to prepare a calibration curve according to the described contents (15-0.25 ng/mL). The correlation coefficient and its linearity indicate the accuracy of the analysis according to the following equation:

$$y = 18323x - 1341.7,\ R^2 = 0.9998$$

The preventive effect of ochratoxin A production by different extracts of *G. lucidum* is shown in Figure 2. The percentage of reduction in toxin production for ethanolic, hydroalcoholic, commercial and aqueous extracts was 13-34%, 14-28%, 46-56% and 36-43%, respectively. In presence of the commercial strain extract the fungi totally grew but the toxin formation was significantly prevented in comparison with the control tube. Also, among other extracts, the aqueous one could reduce the toxin production rather than ethanolic and hydroalcoholic extract. The toxin formation of both alcoholic extracts in comparison with control was not statistically significant difference. All tested extracts were compared with the control that was the growth media of the fungi.

Ganoderma lucidum has shown antifungal effect in various articles that have been reviewed, but so far, no published article studied the inhibitory properties of *G. lucidum* for the production of ochratoxin A by the fungal species *A. ochraceous* in the presence of *Ganoderma* extracts. The extract may have different compositions depending on the type of extraction solvent. This difference was investigated according to the solvent used to inhibit the production of ochratoxin A.

Based on the results, it was observed that standard commercial aqueous extract showed a more significant effect in preventing the production of ochratoxin A than other *G. lucidum* extracts. The commercial powder is standardized based on its polysaccharide content. The polysaccharides may play an important role in preventive effect of toxin formation and could suppress the ochratoxin A production by *A. ochraceus*. Also, in comparison with the aqueous extracts from *G. lucidum*, it was observed that the effect of reducing toxin production was more significant than alcoholic and hydroalcoholic extracts.

Very few research has been done on the effect of *G. lucidum* extracts on the growth of *A. ochraceous*, but the results of a same research suggest that *G. lucidum* extracts can be harmful for growth of this fungus. For example, Heleno et al. [20] in the study of *G. lucidum* extracts obtained a MIC value of 0.75 mg/mL. Or in a study by Stojkovic et al. [25], the MIC values obtained for the methanolic extract of *G. lucidum* against *A. ochraceous* for fungal species collected from Serbia and China were 0.15 and 0.10 mg/mL, respectively. The results of comparing the present study with above-mentioned studies show a significant difference between the MIC values obtained for ethanolic, hydroalcoholic and aqueous extracts. This difference in results can be attributed to several factors. The first factor can be the fungal species

used, so that according to the study of Stojkovic et al. [25], extracts from different origins get different MIC results. Another reason can be attributed to the method used for the experiment; *G. lucidum* extracts in the two above-mentioned studies were obtained by powdering and drying the *G. lucidum* fungus, while in the present study, the extracts were not dried and prepared from concentrated extracts and also aqueous extract was used directly after filtering. Regarding the fact that the standard commercial aqueous extract in the form of lyophilized powder was more suitable in preventing the production of toxins, it can be attributed to its more effective compounds, that prevent the production of toxins.

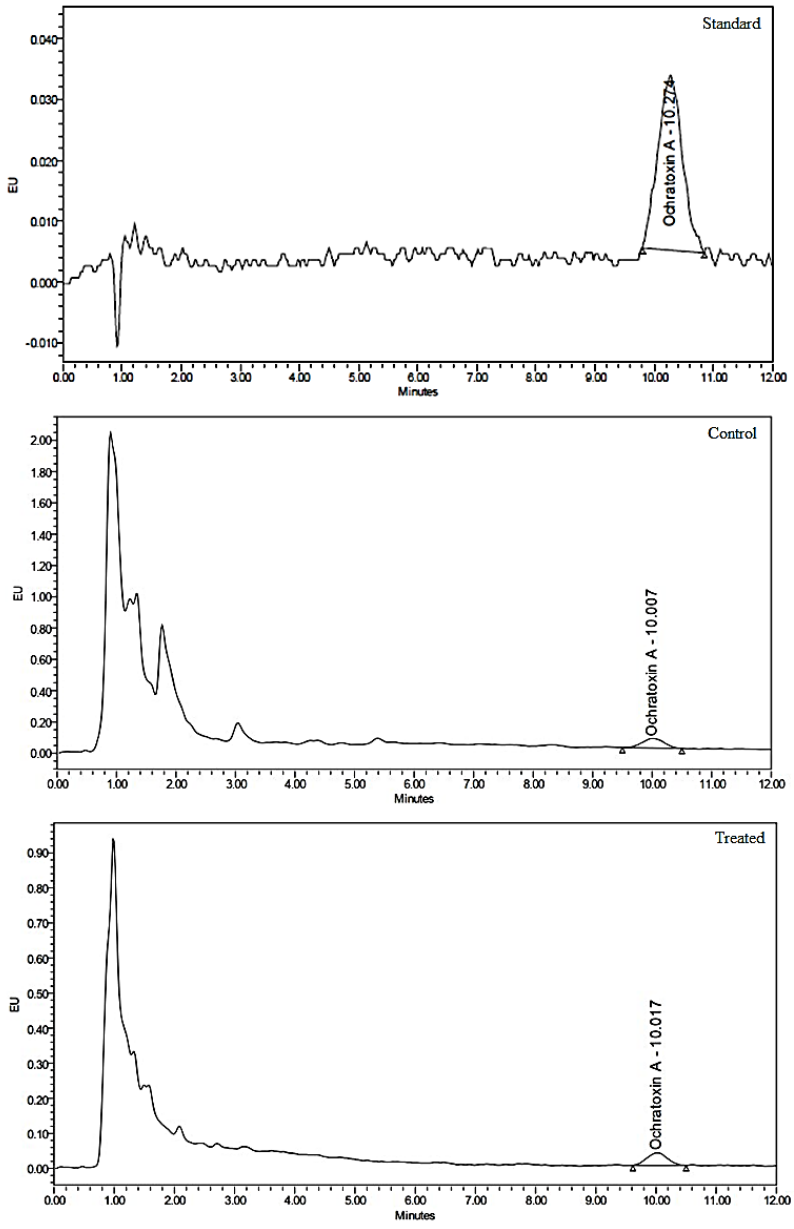

Figure 1. HPLC of chromatograms of ochratoxin A standard (0.5 ng/mL), control, treated with commercial extract of *Ganoderma lucidum*

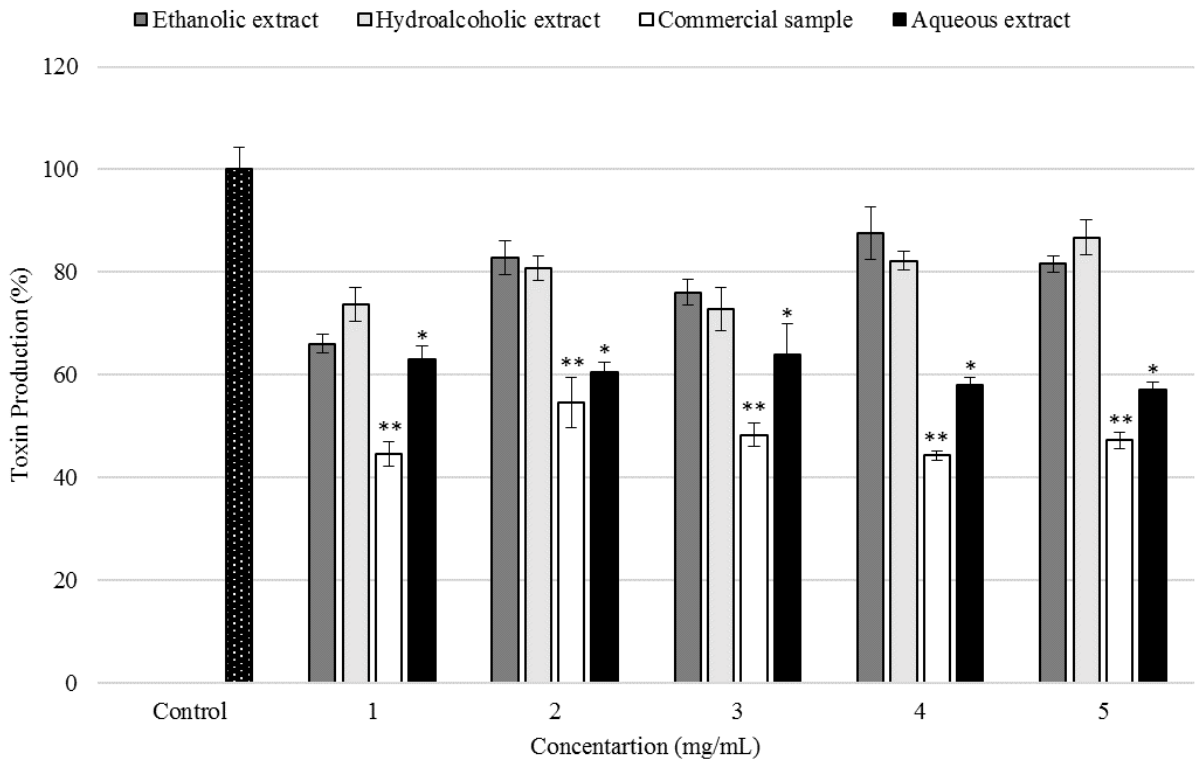

Figure 2. Ochratoxin A production in the presence of different extracts of *Ganoderma lucidum* in concentrations of 1 to 5 mg/mL ($p<0.05$)

Based on the findings of this study, it can be concluded that alcoholic extracts like ethanolic extract contain compounds that can prevent the growth of fungi, but these compounds have no effect on biochemical pathways of toxin formation and cannot have a positive effect on reducing the production of ochratoxin A by toxic species of *A. ochraceous*. Conversely, compounds derived from aqueous extracts are not able to inhibit the growth of the fungus, but can interfere with the mechanism of toxin production by the fungus *A. ochraceous* and prevent the formation of ochratoxin A [26]. Therefore, a direct relationship between inhibition of fungal growth and production of toxins by the studied *Ganoderma* extracts cannot be expected.

Another reason for more promising results of aqueous extracts of *G. lucidum* against the production of ochratoxin A in comparison with alcoholic and hydroalcoholic extracts is the type of solvent used for extraction. It is well known that polysaccharides are one of the most important bioactive compounds found in *G. lucidum* [17]. Polysaccharides are long chains of carbohydrate molecules, with significant inhibitory activity against ochratoxin A production [27]. On the other hand, water has a strong polarity that makes it a proper candidate for efficient extraction of bioactive compounds in *G. lucidum*. Therefore, water is well suited for the extraction, purification and isolation of polysaccharides which makes the aqueous extract a good medium for inhibition of ochratoxin A production by harmful fungi such as *Aspergillus* species compared to ethanolic extracts [28].

Also, the effect of hydroalcoholic extract was better than alcoholic extract in preventing the production of toxins, although no significant difference was observed between the results. Due to the fact that hydroalcoholic extracts have a higher amount of alcoholic phase than the aqueous phase (60 to 40), it can be assumed that a small amount of water-soluble compounds entered this extract. In our study, there was no significant difference between the used concentrations (1-5 mg/mL) but may be in the higher or lower concentrations a rational relationship could be found between the studied concentrations and toxin formation.

Conclusion

Ganoderma lucidum as a fungus with medicinal and therapeutic applications has long been studied for its therapeutic and pharmacological effects and its antifungal and antibacterial effects have been proven and published in various articles. The fact that this fungus with aqueous extracts can be effective in preventing the production of mycotoxins such as ochratoxin A, which is one of the most important contaminants in food products, can be a good strategy to use *Ganoderma* derivatives to prevent fungal contamination or toxin formation in food products.

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Author contributions

Shahram Shoeibi and Marzieh Vahdani were involved in study concept and design; Shahram Shoeibi and Anousheh Sharifan performed analysis and interpretation of data and statistical analysis; Marzieh Vahdani participated in drafting the manuscript; Shahram Shoeibi provided critical revision of the manuscript for important intellectual content.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

CLSI: Clinical Laboratory Standards Institute; DMSO: dimethyl sulphoxide; FLD: fluorescence detector; HPLC: high performance liquid chromatography; IACs: immunoaffinity columns; LC: liquid chromatography; MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration; MIC_{50}: minimum inhibitory concentration required for inhibiting the growth of 50% of organisms; OTA: ochratoxin A; PFCC: pathogenic fungi culture collection; PDB: potato dextrose broth; PBS: phosphate buffer saline; RT: retention time; SDA: Saboraud dextrose agar; UV: ultraviolet