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Antioxidant Activity and Some Biochemical Properties of *Ganoderma applanatum* (Pers.) Pat. from Iran

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Abstract

Members of *Ganoderma*, belonging to Basidiomycota, such as *Ganoderma applanatum* (Pers.) Pat., have been recognized in traditional and modern medicine and pharmacology for their biochemical properties. In this study, first, the fruit bodies of *G. applanatum* growing on three tree species, *Carpinus betulus* L. (common hornbeam), *Prunus cerasifera* Ehrh. (cherry plum), and *Prunus avium* (L.) L. (sweet cherry), were collected in Neka, a county in Mazandaran Province in Iran. Then, their antioxidant activities were measured by the 1,2-diphenyl-2-pricrylhydrazyl (DPPH) radical scavenging method and the ferric reducing antioxidant power (FRAP) assay method, and their bioactive compounds contents were examined by a spectrophotometer and the HPLC method. The total phenols and flavonoids content and also antioxidant activity measured by the DPPH method in fungi growing on *C. betulus* were higher than the others. The fungi growing on *P. cerasifera* had the highest antioxidant activity examined by the FRAP method and the total polysaccharides content. Fungi growing on *P. avium* had the highest content of total proteins. Also, ursolic acid was not found in the samples, and betulinic acid was only seen in the samples growing on *C. betulus*. Oleanolic acid was not found in fungi growing on *C. betulus* and its amount in the fungi growing on *P. cerasifera* was higher than in samples growing on *P. avium*.

1. Introduction

Some Basidiomycota members have been observed in traditional and modern medicine and pharmacology, because of their medicinal properties and bioactive compounds. Members of the *Ganoderma* genus, such as *G. applanatum* (Pers.) Pat., belong to the family Ganodermataceae, class Basidiomycota. *G. applanatum* doesn't have stipe and its perennial and bracket form fruiting body is hard, grayish brown to red brown, and with a woody- textured and concentric or irregular striations on its upper side. Its underside has white spores soon turns brown by any rubbing or scratching. It broadly distributed and has been reported on the woods and trees of the north and north- western forests of Iran (Asef Shayan, 2016).The history of medicinal uses of this fungus goes back thousands of years in East Asian civilization (Abugry & McElhenney, 2013). Modern studies have also reported useful and medicinal metabolites, such as phenolic and flavonoid compounds in *G. applanatum* (Nagaraj et al., 2014; Vazirian et al., 2014). Other

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investigations revealed that some of its bioactive compound contents are higher than the other species of *Ganoderma* (Abugry & McElhenney, 2013; Rajoriya et al., 2015). It has been confirmed that there are about 150 triterpenoids with antioxidative properties in *Ganoderma* species. Some scientists have reported oleanolic acid, ursolic acid, and betulinic acid from *Ganoderma* species (Dzubak et al., 2006), but without detailed information or experimental data. Due to the uncertainty of the presence of these terpenoids, their analysis was evaluated in this study by the HPLC method.

2. Materials and methods

2.1. Fungal samples and extraction

Fresh mature fruiting bodies of *G. applanatum* were collected from the sweet cherry (*Prunus avium* (L.) L.), cherry plum (*Prunus cerasifera* Ehrh.), and common hornbeam (*Carpinus betulus* L.), in Neka, Mazandaran Province, Iran, in August 2017, and labeled as Ga/Pa, Ga/Pc, and Ga/Cb, respectively, in the figures and Table 2. The scientific name of the species was approved by M.R. Asef from the Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO). The fungi were deposited in the Herbarium of AREEO with voucher specimen numbers of IRAN 18089 F, IRAN 18090 F, and IRAN 18091 F, respectively. The shade and air-dried fungi were ground in a spice grinder and stored in a freezer at -20 °C. For extraction, 5 g of air-dried and ground samples were separately extracted with 100 ml of methanol 75% for 48 hours at room temperature. Samples were boiled in distilled water to extract the polysaccharides.

2.2. Antioxidant assays

DPPH radicals scavenging activity assay of the fungal extract was evaluated by a methanol solution of DPPH (1,2-diphenyl-2-pricrylhydrazyl) to measure free radical scavenging activity according to Blois (1958) and Mohammadifar et al. (2020). Inhibition free radical DPPH in percent (I%) was calculated as follows:

$I\% = (A_{blank} – A_{sample}/ A_{blank}) \times 100$

FRAP (ferric reducing antioxidant power) amounts were evaluated according to the Benzie and Strain method (1996) using the standard curve, and expressed in μM $FeSo_4$/ g dry wt.

2.3. Total phenolic content (TPC) assay

The total phenolic constituent in a methanol extract was performed employing the methods in Wang et al. (2005). Briefly, 0.5 ml of extract solution (diluted 1:10%), 5 ml FCR (diluted 1:10 %), and 4 ml of Na_2CO_3 (1 M) were mixed, and the mixture was allowed to stand in an ultrasonic bath at 45 °C for 15 minutes. The absorbance was measured at 765 nm. Total phenolic content was expressed as gallic acid equivalent in mg per gram of dry weight (mgGA.g^{-1} dry wt) of *G. applanatum* sample using the calibration curve.

2.4. Total flavonoid content (TFC) assay

Total flavonoid content was measured by the Chang et al. (2002) method. 0.5 ml of methanol extract (previously diluted 1:10) was mixed with 105 ml of methanol (80%), 0.1 ml of aluminum chloride (0.1%), 0.1 ml of sodium acetate (0.1%), and 2.8 ml of distilled water. After shaking, absorbance was determined at 765 nm. The calibration curve was prepared using quercetin as the standard. Flavonoid contents were expressed as mg quercetin equiv per gram (mgQE.g^{-1}) of sample dry weight using the calibration curve.

2.5. Total sugar content (TSC) assay

The total sugar content was measured according to the Dubois et al. (1956) method. 1 g of fungal material was mixed with 10 mL of distilled water and boiled for 1 minute. Water extract was filtered with Whatman filter paper. An aliquot of 1 ml of the test samples or glucose standards was mixed with 1 ml of 5% phenol in a test tube followed by the addition of 5 ml of 97% sulfuric acid and then was shaken quickly. The mixture was allowed to rest for 20 minutes at room temperature and its absorbance was read at 470 nm. The samples absorptions were compared to the calibration curve of different concentrations of glucose.

2.6. Total protein content (TP) assay

The total protein content was determined by the Bradford (1976) method. Bovine serum albumin

was used as the standard. To prepare the standard curve, different concentrations of it (0.2- 1 $mg.ml^{-1}$) were mixed with distilled water to bring the final volume to 60 µL. 0.5 g of fungi powder was ground thoroughly to a fine powder in liquid nitrogen. 10 ml of tempered Tris buffer was added to the sample and boiled for 3- 4 minutes, and let stand at room temperature for 15 minutes. Then it was centrifuged for 5 minutes at 14000× g at 4 °C. The residue was discarded with the collection of supernatant. The centrifugation was repeated several times until the prepared liquid was clear.

The sample solution and acetone (99%) were mixed in a 1:3 ratio and allowed to stand for 3- 4 hours to form protein sediment. After evaporating the acetone, 10 ml of Tris (1 M, pH= 8) was added to the protein and its absorbance was read at 595 nm. The results were prepared using the standard curve.

2.7. Quantitative analysis of triterpenoids using HPLC method

A Waters liquid chromatography apparatus consisting of a Separations module (Waters 2695; USA) and a Dual Absorbance Detector (waters 996; USA) was used for the HPLC analysis. The details of the experiment were performed according to previous studies (Mohammadifar et al., 2020).

2.8. Statistical analysis

Using IBM SPSS Statistics 21, a Kruskal Wallis nonparametric test type (Chi- Square statistics), at 5% level of significance, was performed to compare the mean distribution of variables. Pearson's correlation coefficient was used to represent the correlation between antioxidant activity and bioactive compounds contents. All experiments were replicated three times to obtain mean values.

3. Results and Discussion

3.1. Antioxidant assay

The antioxidant activity was measured using the DPPH method to evaluate free radicals scavenging shown as IC_{50}. The IC_{50} value of *G. applanatum* grown on *Carpinus betulus* was found to be at about 0.4 $mg.ml^{-1}$ and the lowest one. Therefore this sample had the highest antioxidant activity for the ability of scavenging DPPH radicals (Figure 1). Also, statistical tests showed that the mean distribution of the IC_{50} variable in the studied samples was different and the analysis was significant at Chi-square 7.261, at level 0.027.

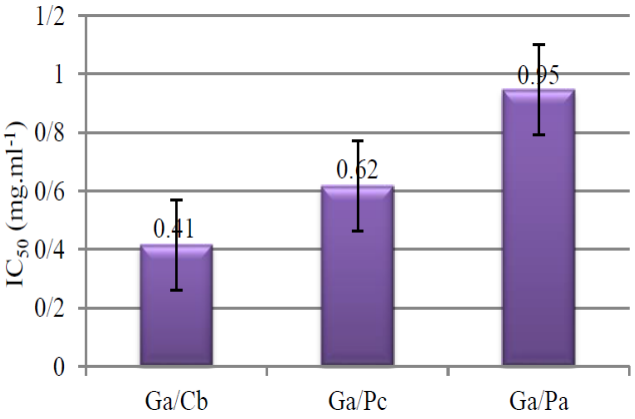

Figure 1. DPPH radicals scavenging activity (IC50) of *Ganoderma applanatum* growing on *Carpinus betulus*, *Prunus cerasifera* and, *Prunus avium* (shown as Ga/Cb, Ga/Pc and Ga/Pa, respectively, in the figure)

According to the FRAP analysis, *G. applanatum* growing on *Prunus cerasifera* showed the highest FRAP amount of up to about 131 µM $FeSo_4.g^{-1}$ dry wt (Figure 2). Also, statistical tests showed that the mean distribution of the FRAP variable in the studied samples was different, and the analysis was significant at Chi-square 7.200, at level 0.027.

Because different reactions occur between different antioxidants and oxidants, two different methods of measuring antioxidants were used in this experiment. Also, substances that have antioxidant properties are not limited to phenolic compounds, and other compounds also have this property. For this reason, the FRAP method was used in addition to the previous method. This method is based on the ability of the sample to reduce Fe^{3+} to Fe^{2+} and chelating Fe^{2+}. Ferrous ion reacts with ferrozin and forms a violet color ferrozin-Fe^{2+} complex, but chelating compounds prevent ferrozin-Fe^{2+} complex formation and decrease its violet color.

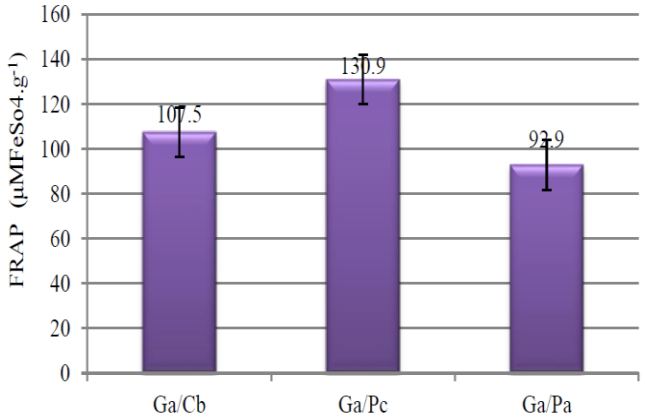

Figure 2. Ferric reducing antioxidant power (FRAP) of *Ganoderma applanatum* growing on *Carpinus betulus*, *Prunus cerasifera*, and *Prunus avium* (shown as Ga/Cb, Ga/Pc and Ga/Pa, respectively, in the figure)

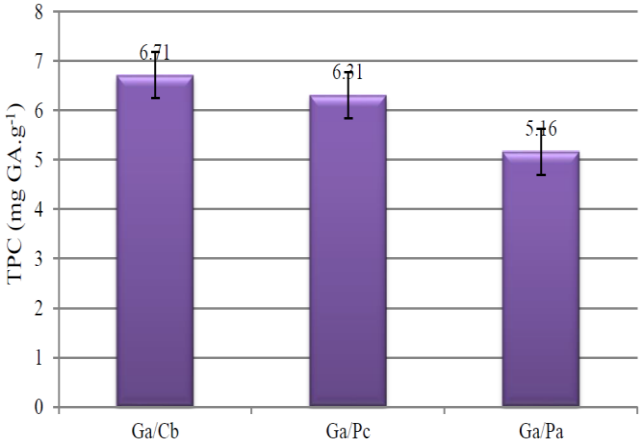

Figure 3. Total phenolic content (TPC) of *Ganoderma applanatum* growing on *Carpinus betulus*, *Prunus cerasifera*, and *Prunus avium* (shown as Ga/Cb, Ga/Pc, and Ga/Pa, respectively, in the figure)

3.2. Total phenolic content (TPC) assay

We confirmed the highest TPC for *G. applanatum* growing on *C. betulus*, while the *G. applanatum* growing on *P. avium* had the lowest amount of TPC (Figure 3). Also, statistical tests showed that the mean distribution of the TPC variable in studied samples was different, and the analysis was significant at Chi-square 7.200, at level 0.027.

In this project, the antioxidant activity had a positive, strong, and significant correlation with the total phenolic content. Other authors such as Saltarelli et al. (2009), Modi et al. (2014), and Acharya et al. (2015), also documented that total phenolic and flavonoid compounds are the main contributors to the antioxidant activity of the *Ganoderma* genus. Their antioxidant activity is due to their ability to inhibit lipoxygenases, free radical scavenging activity, reducing activity, and indirect effects arising from the chelation of pro-oxidant metal ions, that prevent free radical formation. The reducing power of medicinal mushrooms might be due to their hydrogen-donating ability. Accordingly, they might contain a high amount of reductone, which reacts with free radicals to stabilize them and terminate their chain reactions (Shon et al., 2003; Nagaraj et al., 2014; Modi et al., 2014).

3.3. Total flavonoid content (TFC) assay

The highest contetnt of total flavonoids was obtained for the *G. applanatum* on the *C. betulus*, and the lowest one was for *G. applanatum* on *P. avium* (Figure 4).

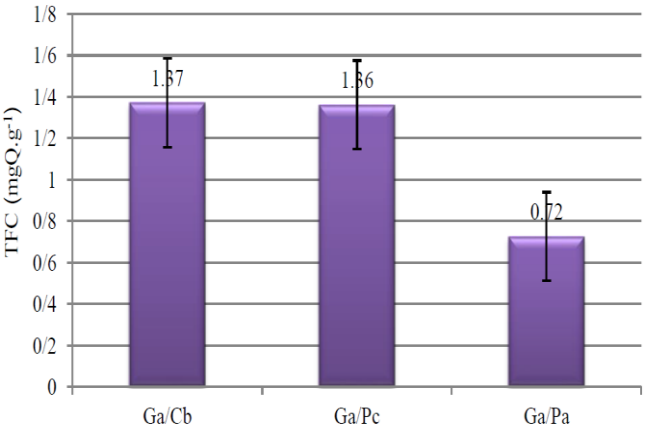

Figure 4. Total flavonoid content (TFC) of *Ganoderma applanatum* growing on *Carpinus betulus*, *Prunus cerasifera*, and *Prunus avium* (shown as Ga/Cb, Ga/Pc, and Ga/Pa, respectively, in the figure)

In this study, there was a strong relationship between total flavonoid compounds content and antioxidant activity. This suggests that flavonoids are important factors of antioxidant properties. Flavonoids are strong inhibitors of peroxide and hydrogen radicals. In this project, *G. applanatum* growing on *Carpinus betulus* had the highest

amount of total phenolic and total flavonoid compounds which suggests that flavonoid compounds are the major group of phenolic compounds in the studied samples. This positive relation has also been shown by Pourmorad et al. (2006) and Modi et al. (2014).

3.4. Total sugar content (TSC) assay

G. applanatum growing on *P. cerasifera* had the highest total sugar content (Figure 5). Also, statistical tests showed that the mean distribution of the TSC variable in the studied samples was different, and the analysis was significant at Chi-square 7.200, at level 0.027.

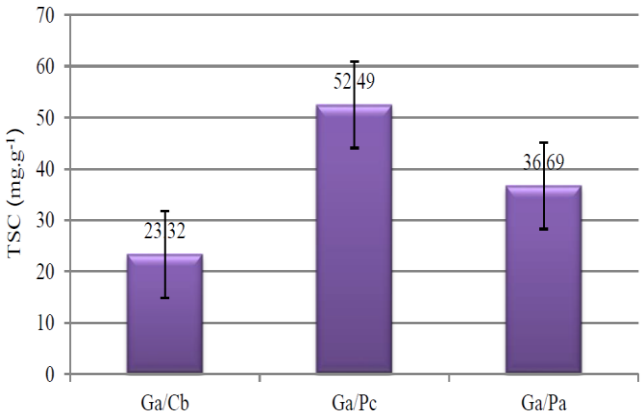

Figure 5. Total sugar content (TSC) of *Ganoderma applanatum* growing on *Carpinus betulus*, *Prunus cerasifera*, and *Prunus avium* (shown as Ga/Cb, Ga/Pc, and Ga/Pa, respectively, in the figure)

3.5. Total protein content (TP) assay

G. applanatum growing on *P. avium* showed the highest total protein content of up to 19.16 $mg.g^{-1}$ dry wt (Figure 6). Also, there was a strong relationship between total protein and antioxidant activity, and this has been documented by other authors such as Sa-ard and Sarnthima (2015) and Sarnthima et al. (2017). The Pearson correlation coefficient represented the correlation between antioxidant properties and biochemical compounds (Table 1).

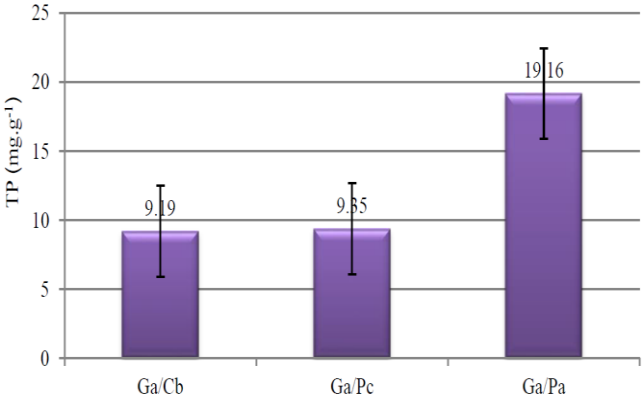

Figure 6. Total protein content (TP) of *Ganoderma applanatum* growing on *Carpinus betulus*, *Prunus cerasifera*, and *Prunus avium* (shown as Ga/Cb, Ga/Pc, and Ga/Pa, respectively, in the figure)

Table 1. correlation between variables

	TPC	TFC	IC_{50}	FRAP	TS	TP
TPC	1	.895**	-.993**	.925**	.378	-.710**
TFC	.895**	1	-.870**	.836**	.349	-.940**
IC_{50}	-.993**	-.870**	1	-.921**	-.355	.665*
FRAP	.925**	.836**	-.921**	1	.678*	-.625*
TS	.378	.349	-.355	.678*	1	-.208
TP	-.710**	-.940**	.665*	-.625*	-.208	1

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

3.6. Quantitative analysis of triterpenoids using HPLC method

According to the HPLC method results showing the existence of triterpenoids, *G. applanatum* fungi growing on *C. betulus* included betulinic acid, and the fungi on *P. cerasifera* and *P. avium* showed oleanolic acid (Table 2, Figures 7, 8, and 9; Mohammadifar et al., 2020).

Table 2- Comparison between triterpenoid amounts of *Ganoderma applanatum* growing on *Carpinus betulus*, *Prunus cerasifera*, and *Prunus avium* (shown as Ga/Cb, Ga/Pc, and Ga/Pa, respectively, in the table) measured by the HPLC method.

	betulinic acid ($mg.g^{-1}$)	oleanolic acid ($mg.g^{-1}$)	ursolic acid ($mg.g^{-1}$)
Ga/Cb	1.546035	-	-
Ga/Pc	-	1.178139	-
Ga/Pa	-	0.968121	-

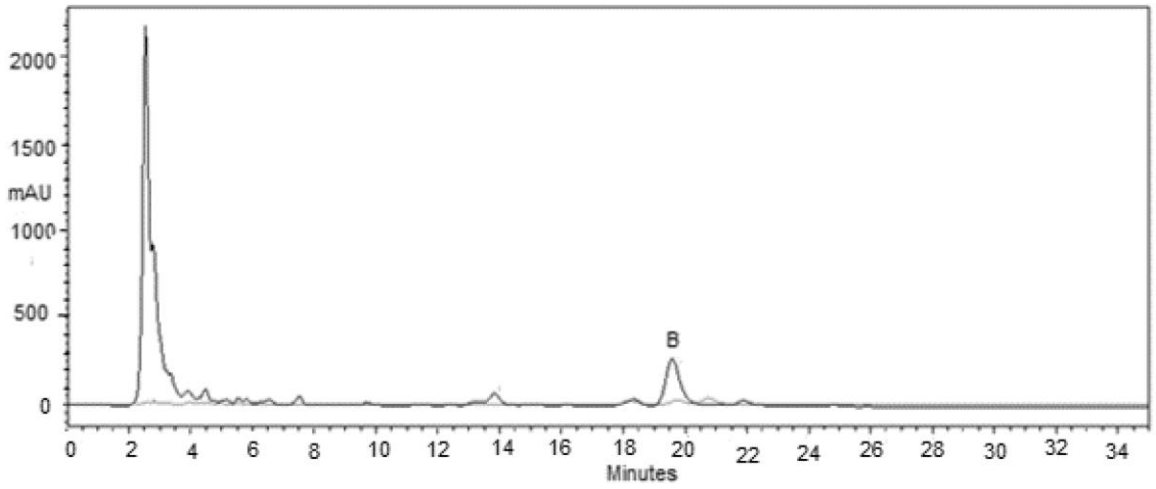

Figure 7. HPLC chromatogram of triterpenoids (ursolic acid, oleanolic acid, and betulinic acid: B) of *Ganoderma applanatum* growing on *Carpinus betulus* (Mohammadifar et al., 2020)

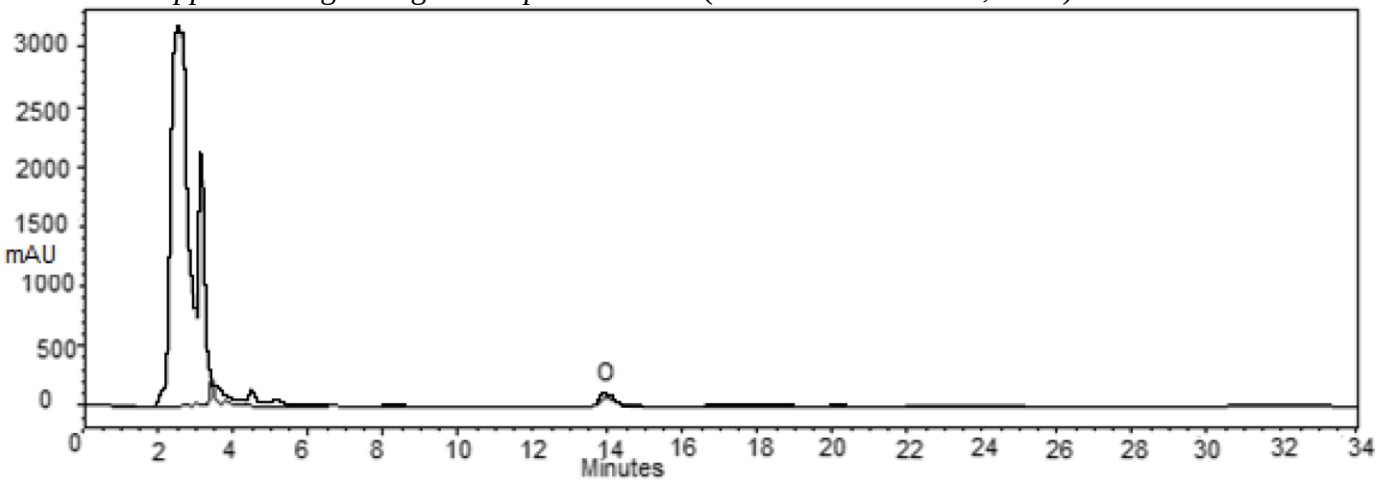

Figure 8. HPLC chromatogram of triterpenoids (ursolic acid, oleanolic acid: O, and betulinic acid) of *Ganoderma applanatum* growing on *Prunus cerasifera*

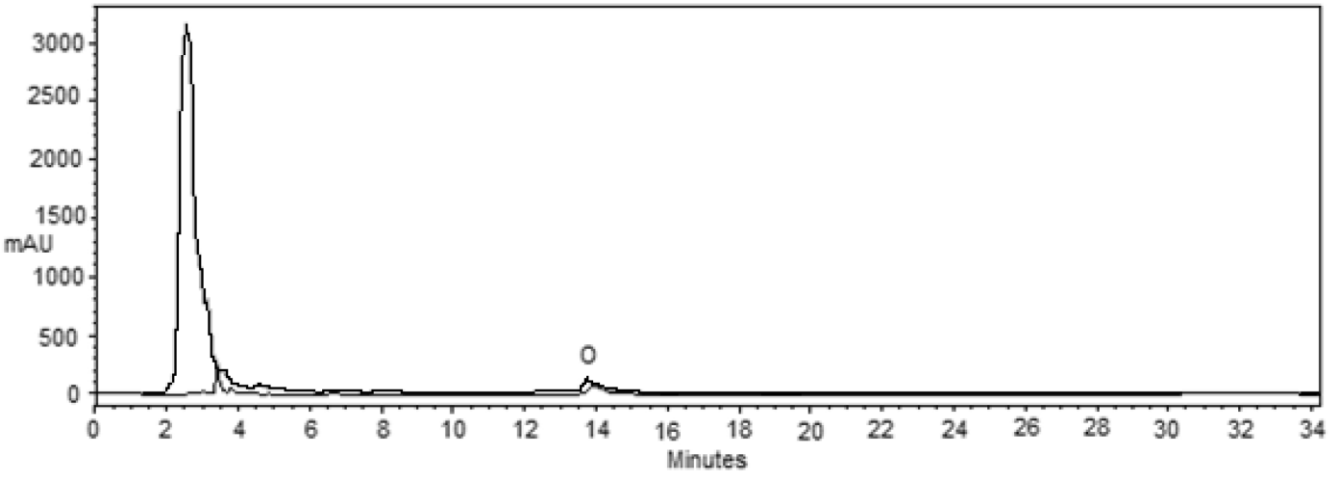

Figure 9. HPLC chromatogram of triterpenoids (ursolic acid, oleanolic acid: O, and betulinic acid) of *Ganoderma applanatum* growing on *Prunus avium*

The HPLC method has been used to measure terpenoids of *Ganoderma* since 1987. Since then, the effect of factors, such as habitat and other environmental conditions of the growth medium on the production of various terpenoids of *Ganoderma* species has been investigated (Lin & Shiao, 1987; Ha et al., 2015).

The analyzes performed in this study show that *G. applanatum* native to Iran, has valuable metabolites for therapeutic applications, and in some cases, the amount of beneficial compounds of this fungus is even higher than samples grown in other countries (Table 3). For this reason, it is recommended that the cultivation of this medicinal mushroom be widespread in Iran.

Table 3- Comparative values of antioxidant activity and biochemical compound values for extracts of *Ganoderma applanatum* in Iran (the present study) and other countries (Nagarj et al., 2014; Rajoriya et al., 2015).

	DPPH ($mg.mL^{-1}$)	FRAP ($\mu MFeSo4.g^{-1}$)	TPC ($mgGA.g^{-1}$)	TFC ($mgQ.g^{-1}$)	TSC ($mg.g^{-1}$)	TP ($mg.g^{-1}$)
the present study (Iran)	0.41	107.5	6.71	1.37	23.32	9.19
	0.61	130.9	6.31	1.36	52.49	9.35
	0.95	92.96	5.16	0.72	36.69	19.16
other countries	6	nd*	11.6	0.4	nd	nd

*- has not yet been measured.

4. Conclusions

There was a significant difference in the amount of total phenolic, flavonoid, polysaccharide, and protein compounds in samples growing on different hosts in this project. The results probably indicate that the host trees are able to influence the chemical properties of fungi. Previous studies have focused on the effect of other environmental factors, such as growth substrate, temperature, altitude, and climate on *Ganoderma* species properties, especially *G. lucidum* (Gąsecka et al., 2016; Obodai et al., 2017). For example, according to the study of Poomsing et al. (2013), the wood of *Dimocarpus*

longan had the best effect on the production of polysaccharides in *G. lucidum*.

The free radicals scavenging ability (shown as IC_{50}) of the methanolic extract and concentrations of phenolics and flavonoids of *G. applanatum* growing on *Carpinus betulus* were higher than other samples, but antioxidant activity measured by the FRAP analysis and the amount of polysaccharides of *G. applanatum* growing on *Prunus cerasifera*, and protein concentration of samples growing on *P. avium* were higher than the others.

Oleanolic acid, ursolic acid, and betulinic acid are terpenoids that researchers have noted in the fungi of the genus *Ganoderma* (Dzubak et al., 2006; Kozai et al., 1987). Unfortunately, their information was neither exact nor sufficient. Due to the uncertainty of the presence of these terpenoids in *Ganoderma* fungi, their analysis was evaluated in this study.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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