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Isolation and Quantification of Triterpenoid Acids from *Ganoderma applanatum* of Istrian Origin

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Summary

Various *Ganoderma* spp. have been known for their wide range of pharmacologically active compounds. From *Ganoderma applanatum* of Istrian origin, growing on *Populus*, triterpenoid acids were isolated and quantified. An in-house relational information system was built and used as a support for isolation and identification of triterpenoids from different layers of *G. applanatum* fruiting bodies. An optimised extraction procedure was developed, and IR, UV and NMR spectra were used for the identification of ganoderic and ganoderenic acids. The highest fraction of triterpenoid acids was found in the tubes (6.4 mg/g of air-dry weight), followed by the dark context layer, which is the young part of the pileus (2.5 mg/g). The white context layer of the older pileus and the upper surface of the fruiting body contained only about 0.6 mg/g of triterpenoid acids.

Key words: Ganoderma applanatum, triterpenoids, ganoderic acids, ganoderenic acids, extraction, isolation, quantification

Introduction

Although *Ganoderma* spp. have been used for millennia in Chinese and Japanese traditional medicine for the treatment of several types of diseases, systematic research into their pharmacological effects started only about 25 years ago. As *Ganoderma* is scarce in nature, the amount of wild fungus is not sufficient for commercial exploitation. Its cultivation on solid substrates, stationary liquid media or with submersed fermentation has become essential to meet the increasing demands of international markets *(1)*. Successful artificial cultivation was reported on solid substrates, utilising for example sawdust and agricultural wastes as the main media components *(2)*, as well as submerged cultivation in liquid media *(3)*. The quality and content of physiologically active substances vary from strain to strain, and also depend on location, culture conditions *(4)* and the growth stage of the fungus *(5)*.

Diverse groups of chemical compounds with pharmacological activity have been isolated from the mycelium and fruiting body of *Ganoderma*: triterpenoids, polysaccharides, proteins, amino acids, nucleotides, alkaloids, steroids, lactones, fatty acids and enzymes *(6)*. Over 100 triterpenoids were found in *Ganoderma* spp., such as ganoderic (highly oxygenated C_{30} lanostane-type triterpenoids), lucidenic, ganodermic, ganoderenic, ganolucidic and applanoxidic acids, lucidones, ganoderals and ganoderols *(7–13)*. Representative examples are shown in Figs. 1–4.

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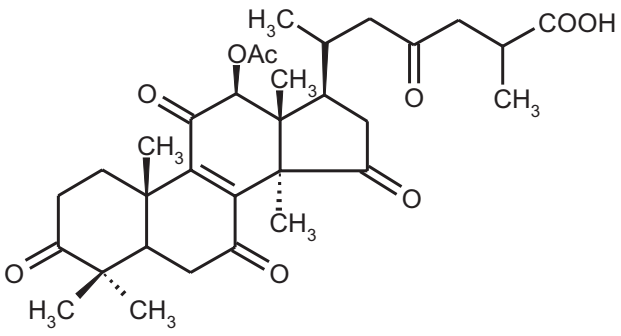

Fig. 1. Ganoderic acid F
12β-acetoxy-3,7,11,15,23-pentaoxo-5α-lanost-8-en-26-oic acid

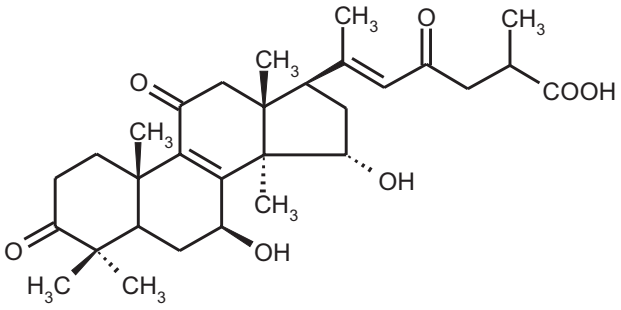

Fig. 2. Ganoderenic acid A
(20E)-7β,15α-dihydroxy-3,11,23-trioxo-5α-lanosta-8,20-dien--26-oic acid

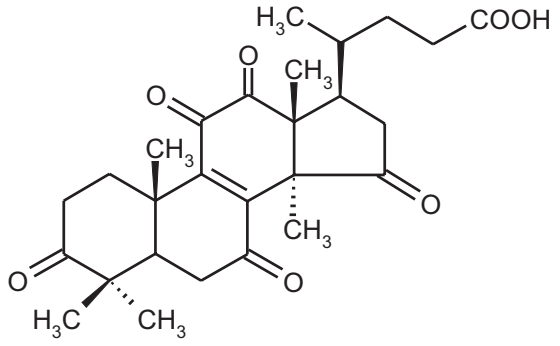

Fig. 3. Lucidenic acid D1
4,4,14α-trimethyl-3,7,11,12,15-pentaoxo-5α-chol-8-en-24-oic acid

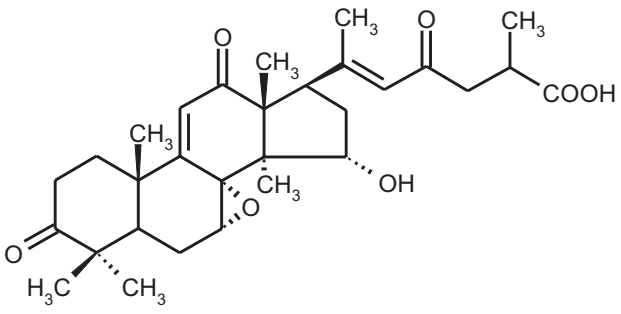

Fig. 4. Applanoxidic acid A
(20E)-15α-hydroxy-7α,8α-epoxy-3,12,23-trioxo-5α-lanosta--9(11),20-dien-26-oic acid

In Asian traditional medicine, particularly in China and Japan, the fruiting body of *Ganoderma*, known as Reishi in Japanese or Ling Zhi in Chinese, has been used as a popular remedy to treat a series of diseases, including hepatitis, nephritis, arthritis, bronchitis, asthma, atherosclerosis, hypertension, diabetes and gastric ulcers *(6)*. Other investigations report on its anti-allergic constituents *(14)*, immunomodulatory action – both immune system suppression or stimulation *(15)*, which may be promising in organ transplants *(16)* and the treatment of HIV infections *(17,18)*, antitumor *(19)* and cardiovascular effects *(20)*, liver protection and detoxification, and effects on the nervous system *(21)*. Triterpenoids have antihepatotoxic *(22)*, antihypertensive *(10)*, hypocholesterolemic *(23)*, and antihistaminic activity *(24)*. Today, whole fruiting bodies, their extracts and active components are used as nutraceuticals, nutriceuticals and pharmaceuticals *(25)*.

Materials and Methods

Information support

In order to accelerate research and to avoid its duplication, all research and developmental phases can be supported by the application of appropriate information tools, ranging from bibliographic and specialised numeric databases to data sources on molecular structures, spectral analyses and molecular modelling *(26)*. As an information support to laboratory research, a relational information system on *Ganoderma* has been developed (Fig. 5) with the main modules on: pharmacological effects, taxonomy, fermentation processes, extraction and isolation of pharmacologically active compounds, chemical compounds – structures and properties, commercial products and formulations based on *Ganoderma*, and bibliographic references.

Plant material

G. applanatum is wide spread in the Northern hemisphere both in temperate and tropical zones. It causes intensive white rot in wood of many deciduous trees such as *Acer, Betula, Castanea, Fagus, Fraxinus, Populus, Quercus* and *Tilia.* Very seldom it is found on the wood of coniferous trees. For our experimental work, about 5 kg of *G. applanatum* fruiting bodies were collected on *Populus* in Slovenian and Croatian parts of Istria in June 1997.

Extraction of triterpenoids

Based on the information from the module of isolation schemes, a modified procedure for the extraction of triterpenoids from *G. applanatum* was developed by the comparative analysis and synthesis of data from 9 procedures, which were designed for extraction of triterpenoids from *G. lucidum* (*5,7,9–11,24,27–29*). In this paper, the following procedure was used for the extraction of triterpenoids from *G. applanatum*:

Air-dried fruiting bodies were cut into four separate layers: tubes (285 g), the pileus dark context layer (725 g), the pileus white context layer (270 g) and the upper surface of the fruiting body (250 g). Separate layers of fruiting bodies were crushed and extracted with 90 % methanol in a shaker in darkness for 7 days at room

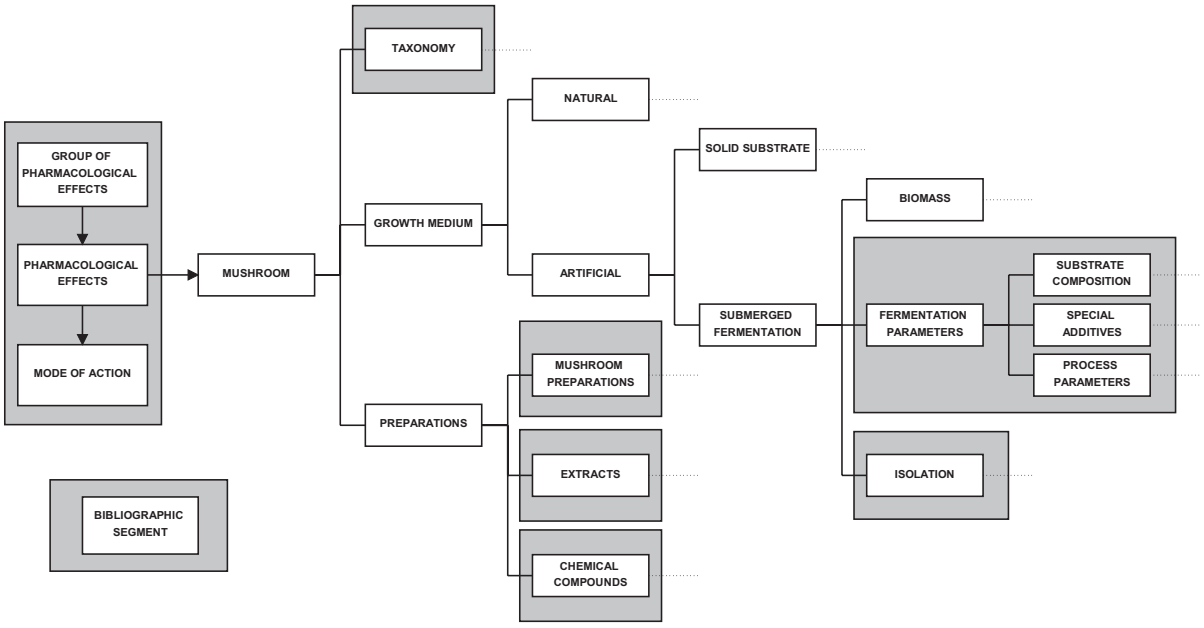

Fig. 5. Main modules (dark boxes) of the relational information system on *Ganoderma*, built in Paradox for Windows with connections to SnapGraphics (isolation schemes), ISISDraw (structural formulae) and CDS/ISIS2.3 (bibliography)

temperature. The homogenate was filtered and the residue was extracted again with the same solvent for 7 days. For each layer the filtrates were combined and the organic solvent was removed under reduced pressure at 40 °C. The mass of crude methanol extracts were: tubes 16.6 g, the pileus dark context layer 25.9 g, the pileus white context layer 5.9 g and the upper surface of the fruiting body 15.4 g. These were processed separately as follows:

Methanol extract was dissolved in water (75 mL) and extracted with chloroform (5 × 100 mL). The combined chloroform fractions were concentrated under reduced pressure at 30 °C to 50 mL volume and extracted with saturated aqueous sodium hydrogen carbonate solution (3 × 70 mL). The combined aqueous layer was acidified to pH = 2–3 with 6 M hydrochloric acid under ice-cooling and extracted with chloroform (3 × 150 mL). The combined chloroform fractions were concentrated under reduced pressure to 100 mL, washed with water, dried with sodium sulphate and evaporated to dryness to yield a mixture of yellow and colourless crystals and yellow and white powder. The yields of acidic fractions were: tubes (1.83 g), pileus dark context layer (1.80 g), pileus white context layer (0.16 g), upper surface of fruiting body (0.16 g).

Purification

Each acidic fraction was chromatographed on a silica gel column (25 × 30 mm).

A mass of 1 g of the tubes acidic fraction was eluted with 300 mL of chloroform (fraction T*-A), 100 mL of chloroform/methanol (99:1 volume ratio) (fraction T-B), 50 mL of chloroform/methanol (98:2) (fraction T-C), 50 mL of chloroform/methanol (96:4) (fraction T-D), 100 mL of chloroform/methanol (9:1) (fraction T-E), 50 mL of chloroform/methanol (6:1) (fraction T-F), 100 mL of chloroform/methanol (2:1) (fraction T-G).

A mass of 1 g of the pileus dark context layer acidic fraction was eluted with 200 mL of chloroform (fraction D**-A), 150 mL of chloroform/methanol (99:1) (fraction D-B), 50 mL of chloroform/methanol (98:2) (fraction D-C), 100 mL of chloroform/methanol (95:5) (fraction D-D), 50 mL of chloroform/methanol (6:1) (fraction D-E), 75 mL of chloroform/methanol (2:1) (fraction D-F).

A mass of 160 mg of the pileus white context layer acidic fraction was eluted with 100 mL of chloroform (fraction W***-A), 300 mL of chloroform/methanol (99:1) (fraction W-B), 200 mL of chloroform/methanol (98:2) (fraction W-C), 150 mL of chloroform/methanol (97:3) (fraction W-D), 200 mL of chloroform/methanol (95:5) (fraction W-E), 300 mL of chloroform/methanol (9:1) (fraction W-F), 100 mL of chloroform/methanol (4:1) (fraction W-G).

A mass of 160 mg of the upper surface of fruiting body acidic fraction was eluted with 200 mL of chloroform (fraction U****-A), 200 mL of chloroform/methanol (99:1) (fraction U-B), 200 mL of chloroform/methanol (98:2) (fraction U-C), 300 mL of chloroform/methanol (97:3) (fraction U-D), 150 mL of chloroform/methanol (96:4) (fraction U-E), 150 mL of chloroform/methanol (95:5) (fraction U-F), 325 mL of chloroform/methanol (9:1) (fraction U-G), 100 mL of chloroform/methanol (6:1) (fraction U-H), 100 mL of chloroform/methanol (2:1) (fraction U-I).

The chloroform/methanol mixture were always done as volume ratio.

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* series T – from tubes
** series D – from the dark context layer
*** series W – from the white context layer
**** series U – of upper surface of fruiting body

Table 1. Examples of patents on *Ganoderma* applications in medicine, food and drinks (data from in-house information system)

Application	Preparation / Compound	Source	Ref.
Medicine			
liver function stimulants	ganodosterone, ganoderic acid R, S, T	*G. lucidum*	*30*
immunostimulant	protein	*G. lucidum* mycelium	*31*
immunosuppressive agent	glycoprotein	*G. lucidum* mycelium	*32*
anti-retro virus drug, agent	protein LZ-8	*Ganoderma* (Mannentake) mycelium	*18*
antiviral agent	polysaccharide, protein polysaccharide	*Ganoderma* fruiting body, artificial culture cell culture medium	*17*
oral liquid for eyesight improvement	extract	*G. lucidum*	*33*
antiteratogenic agent	glycoprotein	*Ganoderma* fruiting body, mycelium culture medium	*34*
immunostimulant antitumor agent	proteoglikan G009	*G. lucidum* IY009	*35*
antitumor agent	β-D-1,3-glucan	*G. applanatum* mycelium	*36*
Drinks			
health drink	fermented liquor	glossy *Ganoderma* S-W119 strain	*37*
health beverage	fermented liquor containing polysaccharides, amino acids and trace elements	*G. sinense*	*38*
health tonic	protein-polysaccharide	*G. lucidum* mycelium	*39*
nutritious drink		glossy *Ganoderma* mycelium	*40*
health tea	organogermanium	glossy *Ganoderma* hyphae	*41*
health tea	extract and finely cut fruiting body	glossy *Ganoderma*	*42*
coffee	extract	*G. lucidum*	*43*
alcoholic beverage		*Ganoderma*	*44*
beer	extract	*G. lucidum*	*45*
beer	aroma (instead of hops)	*G. lucidum*	*46*
wine	fruiting body	*G. lucidum*	*47*
health drink vinegar	fruiting body	*G. lucidum*	*48*
Food			
fermented food	mycelium	*G. lucidum*	*49*
health food		*G. applanatum*	*50*
food and drug	extract	*G. lucidum* and *applanatum*	*51*
food containing dietary fiber	extract powder	*G. lucidum*	*52*
food or beverage for improving saccharide metabolism	extract	*G. lucidum*	*53*
yoghurt	mycelial powder	*G. lucidum*	*54*
healthy candy	fruiting body powder or extract	*G. lucidum*	*55*
jelly	culture medium containing high viscous polysaccharides	*G. lucidum* mycelium	*56*
thickeners, emulsifiers, humeactants, *etc.* in food, chemical pharmaceutical industries	viscous polysaccharides	*G. lucidum* mycelium culture medium	*57*

Identification

UV spectra were recorded with a UV spectrophotometer Shimadzu UV 2401 PC for each 25 mL portion of chromatographic eluates. ^{13}C NMR and IR spectra were recorded for three triterpenoid chromatographic fractions from tubes: 50–75 mL chloroform; 325–350 mL chloroform/methanol (99:1); 500–525 mL chloroform/methanol (9:1), and for two triterpenoid chromatographic fractions from pileus dark context layer: 25–50 mL chloroform; 250-275 mL chloroform/methanol (99:1). IR spectra were recorded with an IR spectrophotometer Perkin-Elmer 1310. NMR spectra were recorded with a NMR spectrometer VXR-300 at 75 MHz in $CDCl_3$ solutions, with TMS as an internal standard, and chemical shifts were recorded in δ values.

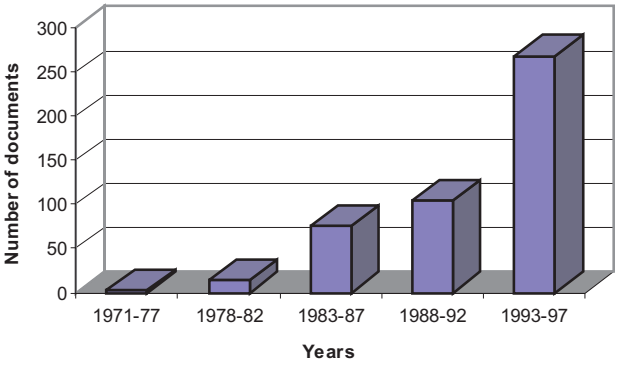
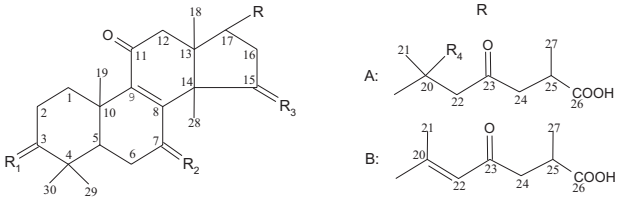


Fig. 6. The number of new publications indicates an increasing scientific interest in *Ganoderma* research (processing the bibliographic module by publication year)

Table 2. Data from the ^{13}C NMR spectrum of triterpenoids from *G. applanatum*

Atom	Chemical shift	Functional group
C1	34.0 - 36.2	
C2	33.8 - 34.5	R_1=O
C2	27.0 - 27.6	R_1=OH
C3	215,2 - 217,9	R_1=O
C3	77,2 - 77,9	R_1=OH
C4	45.9 - 47.5	R_1=O
C4	37.8 - 39.4	R_1=OH
C5	48.1 - 50.7	
C6	35.0 - 38.2	R_1=O
C6	27.0 - 29.0	R_1=OH
C7	197.1 - 200.3	R_2=O; R_3=O
C7	204.5 - 207.3	R_2=O; R_3=OH
C7	66.3	R_2=OH; R_3=O
C7	68.7 - 68.9	R_2=OH; R_3=OH
C8	149.5 - 150.3	R_2=O
C8	156.2 - 159.2	R_2=OH
C9	148.2 - 153.8	R_2=O
C9	139.9 - 141.2	R_2=OH
C10	37.8 - 39.4	
C11	197.1 - 201.2	
C12	48.1 - 52.7	
C13	45.3 - 49.0	
C14	58.6 - 59.7	R_3=O
C14	51.8 - 53.4	R_3=OH
C15	204.5 - 207.3	R_3=O; R_2=O
C15	215.2 - 218.0	R_3=O; R_2=OH
C15	72.5 - 73.2	R_3=OH
C16	36.8 - 39.4	R_3=O
C16	33.8 - 38.2	R_3=OH
C17	50.4 - 52.7	ganoderenic acid
C17	45.3 - 49.6	ganoderic acid
C18	16.8 - 19.5	
C19	17.1 - 19.9	
C20	152.8 - 157.6	ganoderenic acid
C20	31.7 - 35.5	ganoderic acid R_4=H
C20	72.5 - 73.2	ganoderic acid R_4=OH
C21	20.3 - 21.4	ganoderenic acid
C21	18.1 - 19.9	ganoderic acid R_4=H
C21	27.0 - 27.6	ganoderic acid R_4=OH
C22	124.1 - 124.7	ganoderenic acid
C22	48.0 - 49.6	ganoderic acid R_4=H
C22	51.8 - 53.4	ganoderic acid R_4=OH
C23	197.2 - 200.3	ganoderenic acid
C23	204.5 - 207.3	ganoderic acid R_4=H
C23	210.3 - 210.5	ganoderic acid R_4=OH
C24	46.5 - 48.5	
C25	33.8 - 35.5	
C26	178.8 - 180.7	
C27	16.9 - 17.4	
C28	24.6 - 25.0	R_3=O
C28	19.3 - 21.4	R_3=OH
C29	27.0 - 29.0	
C30	29.0 - 21.1	R_1=O
C30	16.9 - 17.4	R_1=OH



A: ganoderic acids, B: ganoderenic acids

Results and Discussion

Processing the in-house information system of Ganoderma spp.

Synthesis of data from the bibliographic module enabled the recognition of trends in the field of *Ganoderma* research. The number of new publications indicates an increasing scientific interest in *Ganoderma* research (Fig. 6). An analysis by location of authors suggests a strong influence of Asian research institutions (Japan 35 %, China 18 %, S. Korea 9 %, Taiwan 8 %, India 3 %, USA 4 %, others 23 %). Processing the module on commercial products shows that several *Ganoderma* preparations and products have been patented (Table 1).

Identification of triterpenoids

UV spectra of the chromatographic fractions (elution chromatograms are shown in Fig. 7) showed an absorption at 240–255 nm, which is characteristic for the α, β-unsaturated carbonyl group, and is in accordance with the literature data: ganoderenic acids exhibit maximal absorption at 240–250 nm, and ganoderic acids at 250–256 nm *(7,11,28,58)*.

IR spectra of fractions showed the presence of hydroxyl groups (3450–3500 cm^{-1}), carbonyl groups (1720 and 1660 cm^{-1}), and α,β-unsaturated carbonyl group (1700 cm^{-1}).

For the identification of triterpenoids from the ^{13}C NMR spectrum (Table 2), chemical shifts were compared with literature data *(7,11,28,58)*. ^{13}C NMR spectra showed the presence of unconjugated carbonyl groups (δ = 204.5 – 210.3 and δ = 215.2 – 217.9), conjugated carbonyl groups (δ = 197.1 – 200.3), a carboxyl group (δ = 178.8 – 180.7), double bonds (δ = 139.9 – 159.2, tertiary carbons, and δ = 124.1 – 124.7, a secondary carbon) and secondary carbons (δ = 66.3 – 68.9, δ = 72.5 – 73.2 and δ = 77.2 – 77.9) bearing a hydroxyl group, which are characteristic for ganoderic and ganoderenic acids. The presence of lucidenic acids is possible, because they have similar chemical shifts as ganoderic acids.

Information on triterpenoids from *G. applanatum* is scarce. Protiva and coworkers *(59)* isolated four triterpene compounds from the light petroleum extract of *G. applanatum* found in the Czech Republic, but none of them was a triterpenoid acid. Since there are no research data available on triterpenoid acids from European *Ganoderma* spp., the comparison of results is only possible with the Japanese *(58)* and Indonesian (*12*) samples of *G. applanatum.*

As in the case of *G. applanatum* from Asia, the Istrian fungus contained ganoderic and ganoderenic acids. The yield of acidic fractions was very similar. However, the ^{13}C NMR spectra did not show any presence of an epoxy group, characteristic for applanoxidic acids

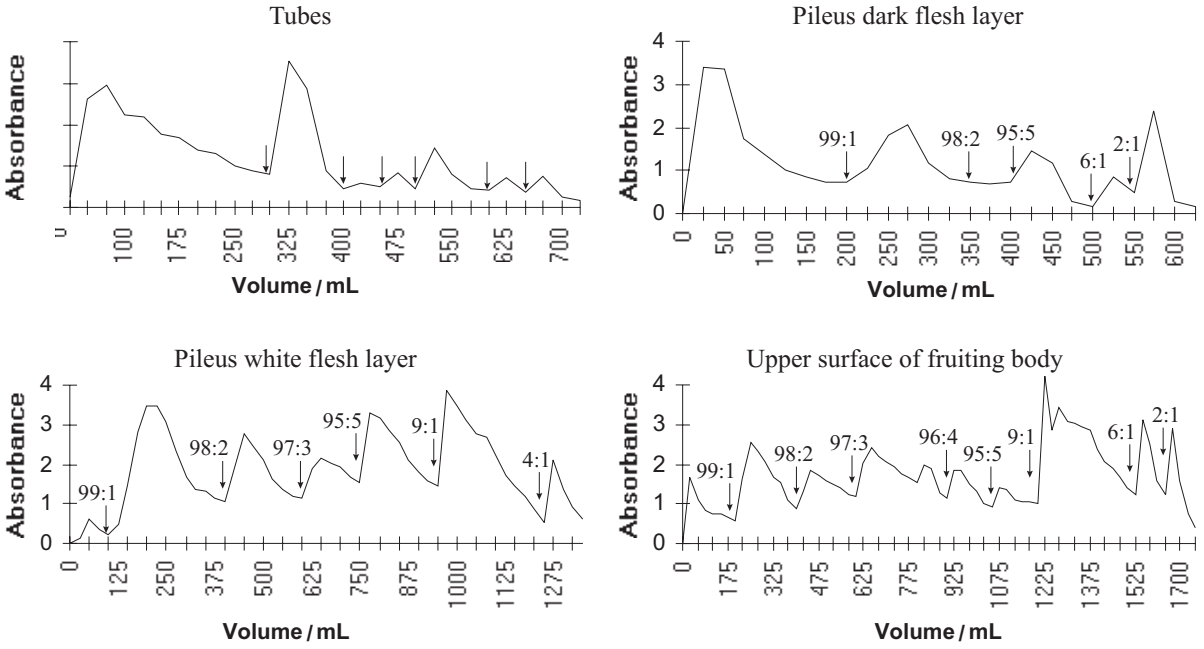


Fig. 7. Elution chromatograms of acidic fractions with the UV absorbance within the range 240-255 nm (tubes and pileus dark flesh layer fractions are diluted 30:1)

from Indonesian *G. applanatum*, which is evidence that these compounds are absent in Istrian *G. applanatum.*

Quantification of triterpenoid acids in different layers of the fruiting body showed an uneven distribution (Fig. 8). The highest amount of triterpenoid acids was found in the tubes (6.4 mg/g of air-dry weight). The pileus dark context layer, which is the younger part of a cap, contained 2.5 mg/g, while the pileus white context layer and the upper surface of the fruiting body contained only 0.6 mg/g of triterpenoid acids.

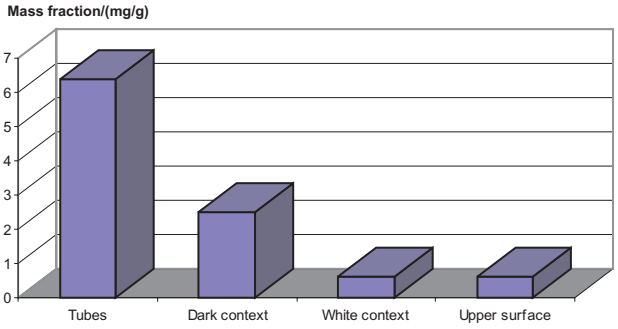


Fig. 8. Differences in triterpenoid acids content in different parts of *G. applanatum* fruiting bodies (mg/g of air-dry weight)

Conclusions

Ganoderma spp. have been known for their numerous pharmacological effects. As a systematic research into *Ganoderma* active compounds has been focussed primarily on *G. lucidum* from Asian habitats, there is little known about other species from other geographical regions. G*. applanatum* is spread in temperate and tropical zones in the Northern hemisphere, and can be found also in Slovenian and Croatian parts of Istria.

Isolation and quantification of triterpenoid acids from Istrian *G. applanatum* showed the presence of ganoderic and ganoderenic acids. The overall yield of acidic fractions from the fruiting body was similar to the reported quantities from Asian *G. applanatum*. Further quantification of triterpenoid acids in different layers of the fruiting body showed the highest concentration in the tubes, followed by the pileus dark context layer, while the older parts of the fruiting body contained triterpenoid acids in much lower quantity.

According to the bibliographic search in Chemical Abstracts and Biosis databases, this is the first reported identification of triterpenoid acids in *G. applanatum* from European habitats and their quantification in different layers of the fruiting body.

Acknowledgements

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Izolacija i kvantitivno određivanje triterpenskih kiselina iz *Ganoderma applanatum* istarskog podrijetla

Sažetak

Razne vrste *Ganoderma* spp. imaju raznolike farmakološki aktivne spojeve. Iz gljive *G. applanatum* istarskog podrijetla, koja raste na drvetu *Populus*, izolirane su i kvantitativno određene triterpenske kiseline. Vlastitim postupkom provedena je izolacija i identifikacija triterpenoida iz različitih dijelova gljive *G. applanatum.* Provedena je optimalna ekstrakcija a za identifikaciju ganoderne i ganoderenske kiseline primijenjeni su IR, UV i NMR spektri. Najveća količina triterpenskih kiselina utvrđena je u stapkama (6,4 mg na zraku sušene gljive), a nešto manje u tamnom sloju ovojnice koji je dio izbojka pileusa (2,5 mg/g). Bijeli sloj starijeg pileusa i gornja površina gljive sadržavali su samo 0,6 mg/g triterpenskih kiselina.