Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 10 (8): 366-376, 2015
ISSN 1816-4935 / DOI: 10.3923/jm.2015.366.376

Production and Qualitative Analysis of Triterpenoids and Steroids of *Ganoderma* Species Harvested from Cork Oak Forest of North-Eastern Algeria

Melouka Hadda, Chekireb Djamel and Ouartsi Akila
Department of Biochemistry, Faculty of Sciences, University Badji Mokhtar Annaba, BP 12, Annaba, 23000, Algeria

Corresponding Author: Hadda Melouka, Department of Biochemistry, Faculty of Sciences, University Badji Mokhtar Annaba, Algeria

ABSTRACT

Submerged fermentation of higher fungi *Ganoderma* is increasingly, studied for its potential advantages particularly, improving the production biomass and bioactive metabolites. The present study mainly focuses on the qualitative assessment of triterpenoids and steroids in chloroform extracts of harvested mycelia from submerged cultures of three different strains of *Ganoderma* sp. by using a low cost complex medium. For the screening of these bioactive substances we employed a High Performance Liquid Chromatography (HPLC) system based on a gradient of acetonitrile acidified with formic acid, coupled to a Quadrupole Time-of-Flight Mass Spectrometer (QToF-MS) for a tentative determination of Ganoderic Acids (GA) and sterols. The LC-QToF-MS approach was successfully, used in the qualitative analysis of bioactive metabolites. The results show a considerable diversity of produced metabolites in the three samples and indicate the presence of putative target components, such as; GAA, GAH, GA theta, GA beta, GAK, GAE, GAC2, GAR, GAX and ergosterol. The comparison of the compounds according to their retention time and QToF-MS spectra reveals nine shared metabolites produced by the three different strains of *Ganoderma* sp., GAAm1, GAY, GAS, GAT-Q and GAT.

Key words: Secondary metabolites, ganoderic acids, submerged fermentation, *Ganoderma* sp., LC-QToF-MS

INTRODUCTION

Higher fungi represent an immense source of a huge range of structurally diverse compounds with health promoting properties. *Ganoderma* a well-known genus of the Ganodermataceae (Basidiomycetes) (Schmidt, 2006). The species of *Ganoderma* have been currently used for medicinal purposes for thousands years. Over the last decades, the bioactive compounds of these mushrooms were increasingly studied and several pharmacological affects have been revealed. The bioactive properties of *Ganoderma*, such as; antiviral and antibacterial (El-Mekkawy *et al*., 1998; Zjawiony, 2004), anti-androgenic (Liu *et al*., 2010), anti-inflammatory (Ko *et al*., 2008), lowering cholesterol (Berger *et al*., 2004; Hajjaj *et al*., 2005), anti-oxidant (Saltarelli *et al*., 2009), immuno-modulatory (Chen *et al*., 2004; Kuo *et al*., 2006) and anti-tumor activities (Calvino *et al*., 2010; Gao *et al*., 2006; Ma *et al*., 2013; De Silva *et al*., 2012) are mainly due to triterpenoids and polysaccharides and sterols (Boh *et al*., 2007; Petrova *et al*., 2008).

The bio-valuable metabolites have been widely isolated from fruiting bodies and spores (Keypour *et al*., 2010; Seo *et al*., 2009), whilst the field cultivation and the solid state fermentation are laborious, time-consuming and expensive processes. Therefore, submerged fermentation has gained plentiful attention as a promising alternative for producing high biomass yield and bioactive compounds in a compact space, in relatively short time and with lesser chance for contamination (Saltarelli *et al*., 2009; Zhong and Xiao, 2009). Now-a-days, serious efforts are being made to enhance the production of metabolites in submerged fermentation by optimizing the composition of culture media (Fang and Zhong, 2002b; Xu *et al*., 2008; Tang and Zhong, 2002), physicochemical conditions (Fang and Zhong, 2002a; Tang and Zhong, 2003; Zhang *et al*., 2010) and studying biosynthesis pathways and regulation of genes expression (Liang *et al*., 2010; Ren *et al*., 2013; Shi *et al*., 2010).

Moreover, investigators have successfully extracted and characterized GA and sterols from *Ganoderma* species (Hirotani *et al*., 1987; Seo *et al*., 2009; Yuan *et al*., 2007). The use of powerful methods of analysis is crucial to obtain reliable results. Hence, several researches were carried out for developing the analysis techniques of the bioactive components. Liquid Chromatography Combined with Mass Spectrometry (LC-MS) has become the method of choice for screening and characterizing complex mixtures (Liu *et al*., 2011; Wang *et al*., 2006; Yang *et al*., 2009, 2007). However, providing the internal standards for such analysis still being a challenging obstacle to identify all the constituents of complex mixtures (Gao *et al*., 2004; Liu *et al*., 2011).

In this present study, an investigation was carried out on the production of triterpenoids and sterols by *Ganoderma* isolates collected from Kala National Park, El Tarf (Algeria) and grown in submerged fermentation, using a low-cost complex media. The metabolites were assessed with Reversed-Phase High Performance Liquid Chromatography Coupled to Electro-Spray Ionization Quadrupole Time of Flight Mass Spectrometry (RP-HPLC-ESI-QToF-MS).

MATERIALS AND METHODS

Chemicals: Chloroform R.P. Normapur analysis grade was purchased from Prolabo (Fontenay-sous-Bois, France). Formic acid (analytical reagent grade) was purchased from Merck (Darmstadt, Germany). Acetonitrile LC/MS grade and methanol absolute LC/MS grade were purchased from Biosolve (Valkenswaard, The Netherlands). Pure water was prepared from a Milli-Q SP Regent Water system (Millipore, Bedford, MA, USA).

Fungal isolates: Three *Ganoderma* sp. strains were isolated from *Ganoderma* basidiocarps harvested from different host trees from El Kala National Park, El Tarf (Algeria). Small pieces from each fruiting body were suspended in sterile distilled water, after shaking (2 min), 0.1 mL of suspension was aseptically transferred into Petri dishes containing Potato Dextrose Agar (PDA), or PDA supplemented with Rose Bengal (50 mg L^{-1}). The incorporation of Rose Bengal in the medium was to inhibit the rapidly spreading of fungal colonies (Jarvis, 1973; King *et al*., 1979) to suppress most of bacteria (Ottow, 1972). The plates were incubated at 30°C until fungal growth. Then the three isolates of *Ganoderma* sp. designated G1, G2 and G3 were identified on the basis of their macroscopic and microscopic features.

Maintenance of fungi and media: The stock cultures were maintained on a PDA slants and routinely subcultured every month and the slants were incubated at 30°C for a week and then

stored at 4°C. The submerged cultivation process was performed as described by Xu *et al*. (2008). The seed medium consisted of the following components, glucose 40 g L^{-1}, peptone 4 g L^{-1}, KH_2PO_4 1.5 g L^{-1}, $MgSO_4.7H_2O$ 1.0 g L^{-1} and vitamin B1 0.01 g L^{-1}. The fermentation medium contained, glucose 16 g L^{-1}, peptone 2.93 g L^{-1}, corn flour 20.93 g L^{-1} and soybean powder 6.44 g L^{-1}. Corn and soybean were purchased from a local market then ground to fine powders.

Inoculum and liquid shake cultivation: Actively growing mycelia obtained from a newly prepared agar-plate culture, after it was incubated for 5-7 days at 30°C, around 5-6 of 0.5 mm diameter disks were punched out and then transferred into a 250 mL Erlenmeyer flask with 50 mL of the seed medium. Cultures were incubated at 30°C for 7 days with shaking at 150 rpm. The seed medium in pre-culture flasks was poured carefully, while keeping most of pellets and then it was replaced by 100 mL of the fermentation medium, the culture flasks were maintained at 30°C and 125 rpm for 4 days. All experiments were carried out at least in duplicate to ensure reproducibility.

Sample extraction and preparation: The fermented broths were filtered through layers of sterile gauze. Then the pellets were dried at 50°C to a constant weight after repeated washing with distilled water. The amount of formed mycelia was determined by measuring the dry weight. Previous method reported by many authors (Keypour *et al*., 2010; Wang *et al*., 2006; Yang *et al*., 2007), which was modified and used for extraction of triterpenoids and sterols from the three samples (G1, G2 and G3). Two grams of powdered dried mycelia was extracted with 40 mL of chloroform with shaking for 20 min. The extraction process was repeated twice. The extracts were filtered, combined and evaporated to dryness at room temperature. A small amount of each dry residue was dissolved in 400 μL methanol in an ultrasonic bath for 5 min, after addition of 400 μL of Milli-Q water, the mixture was filtered through a 0.45 μm Millipore filter unit. A 20 μL aliquot of each sample was analyzed by LC-QToF-MS.

LC-QToF-MS conditions: Chromatographic analysis were performed on the agilent 1290 Infinity LC system equipped with a binary pump, an autosampler, a column oven and a Diode-Array Detector (DAD) coupled to an Agilent 6538 QToF mass spectrometer equipped with a dual Electro-spray Ionization (ESI) source. Twenty microliter of each sample was separated on Phenomenex Luna C18(2) 100 Å column (3 μm, 150×4.6 mm i.d.). The mobile phase consisted of 0.2% formic acid in water (A) and acetonitrile (B) using, a linear gradient program of 32% B over the first 15 min, 32-82% B in 15-40 min, 82-100% B in 40-42.50 min and held at 100% B in 42.50-48 min. The flow rate was 0.85 mL min^{-1} and column temperature was set at 20°C. The DAD was monitored at 220, 252 and 280 nm for acquiring chromatograms, the on-line UV spectra were recorded in the range of 190-400 nm.

The ESI MS spectra were acquired in both positive and negative ion modes, the conditions for ESI operation were as follows: Drying gas flow, 12 L min^{-1}; nebulizing pressure, 45 psi; drying gas temperature, 350°C; capillary voltage, 3.8 kV and fragment or voltage, 180 V. Mass spectra were collected at a frequency of 4 GHz and scanned over a mass range of m/z 50-1700 with a scan rate of 1.5 spectra sec^{-1}.

RESULTS AND DISCUSSION

Submerged fermentation of fungi: The growth of *Ganoderma* isolates in the seed medium was important. The fungal isolates have grown vigorously in the production complex medium and in the end of the fermentation process, the turbid broth culture became more and less clear, with quite similar amount of biomass.

RP-HPLC analysis: Obtained chromatograms showed a very different composition among the three extracts, The peaks were severely overlapping, by other means, each peak included several compounds with close retention times (Rt) on the analytical chromatography (Tang *et al*., 2006) (Fig. 1). This usually occurs in separation of complex mixture of natural products with very similar chemical structure, which require a longer elution time to achieve better separation (Gao *et al*., 2004; Liu *et al*., 2011). In such cases, the ESI-MS spectra provide additional information on the chromatographic peaks.

QToF-MS data analysis: The separated ions were detected and reported from accurate-mass scan data using, Agilent Mass Hunter Qualitative Analysis software. The putative assignment of acquired QToF-MS spectra was based on matching between the accurate measured and the theoretical masses using in-house molecular formula database of known triterpenoids and sterols previously isolated from *Ganoderma*. The match score and the mass accuracy (Δm) were calculated for each retrieved chemical formula in different samples. The identification of detected peaks could not be confirmed owing to the unavailability of standard compounds. For more credibility, the tentative assignments were compared by published data (Chen *et al*., 2008; Keypour *et al*., 2010; Yang *et al*., 2007).

Numerous target compounds were identified with very narrow mass tolerance (<10 ppm) and several unknown compounds were detected in the survey scan in the extracts of the *Ganoderma* samples.

In G1 extract, the compound 6 showed an accurate mass of [M+H]+ ion at m/z 530,2877, corresponding to the molecular formula $C_{30}H_{42}O_8$, compound 6 was tentatively assigned to be GA theta. Another compound 10 gave rise to a quasi-molecular ions [M+H]+ at m/z 574,3139, the molecular formula was defined as, $C_{32}H_{46}O_9$, compound 10was tentatively identified to GAK.

The mass spectrum of compound 9 had a molecular ion peak at m/z 517,3165 [M+H]+, the molecular formula was defined as, $C_{30}H_{44}O_7$. However, compound 9 (Rt = 19,343 min) is an isomer of compound 15 detected in extract G3 (Rt = 23,781 min) and compound 22 in G2 (Rt = 26,749 min), the three components were assigned to GAA.

The compounds 8,11 and 16 detected in G2 extract, were plausibly identified to be GAH, GAE and GAC2, respectively (8 at m/z 572,2996 [M+H]+ calcd. $C_{32}H_{44}O_9$, 11 at m/z 512,275 [M+H]+ calcd, $C_{30}H_{40}O_7$ and 16 at m/z 518,3234[M+H]+ calcd. $C_{30}H_{46}O_7$).

Compound 53 appeared in G2 at Rt = 40,859 min, has a molecular formula of $C_{28}H_{44}O$, which is an ergosterol isomer.

The molecular formula of compounds 24 and 46 was determined to be $C_{32}H_{46}O_5$ (m/z 510,3339 [M+H]+) and $C_{30}H_{40}O_7$ (m/z 512,2799 [M+H]+), which were tentatively assigned to be GAT-Q and GAE, respectively.

Unfortunately, the identification based on chemical formula or accurate mass alone is insufficient, even for components expected to be present.

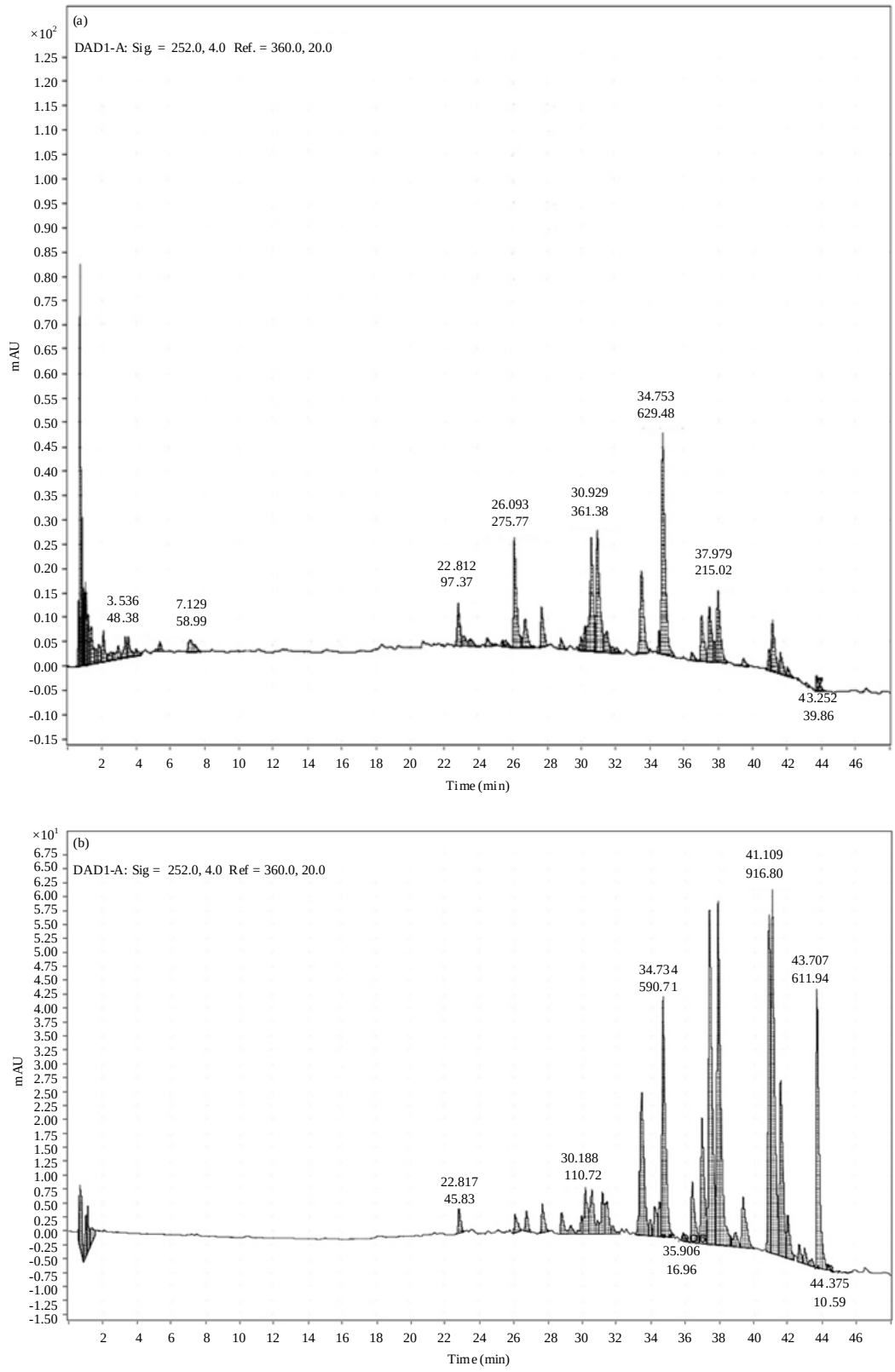

Fig. 1(a-c): Continue

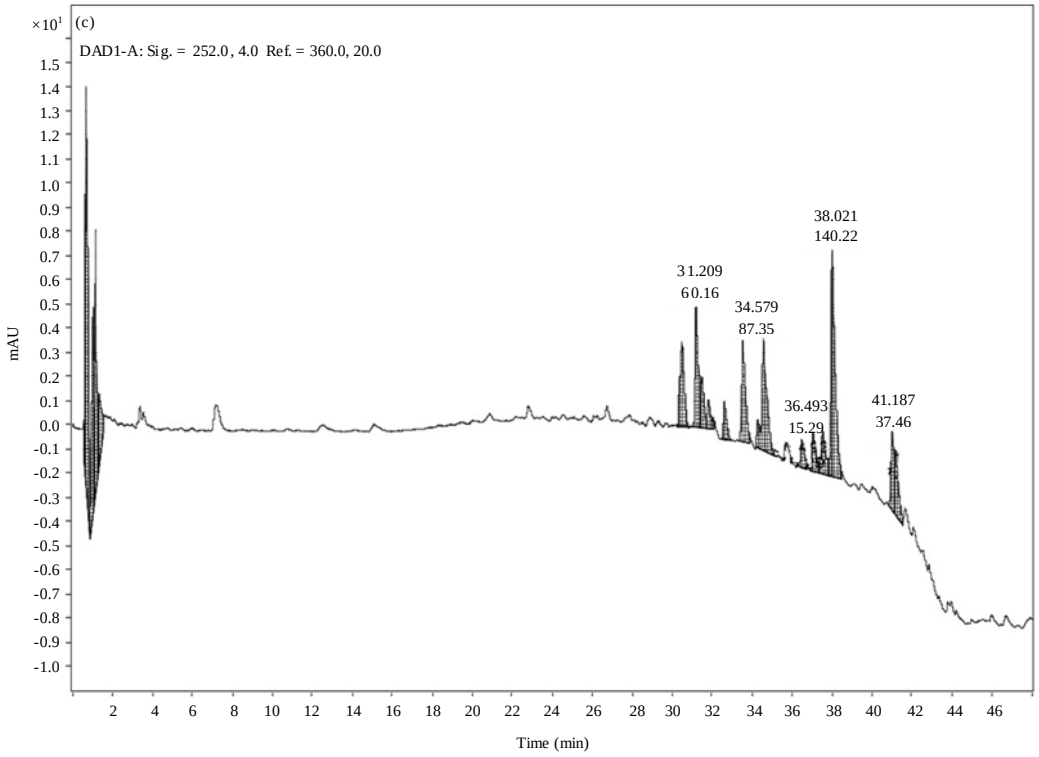

Fig. 1(a-c): LC-DAD-QToF-MS chromatograms at 252 nm of the three extracts (a) G1, (b) G2 and (c) G3, eluted with water with 0.2% of formic acid (A) and acetonitrile (B). The gradient and ESI parameters are described in materials and methods

The MS data matching and the manual retention time alignment allowed the comparison of same metabolites across the different samples (Table 1). Overall, nine common peaks were observed among the three *Ganoderma* samples.

The detected ion peaks in the three *Ganoderma* extracts at Rt = 18.8 min corresponding to the same metabolite compound 7 have a molecular formula of $C_{30}H_{42}O_7$, which was assigned as, GAAm1.

At Rt = 31,1 min, compound 33 gave rise to the following ion peaks [M+H]+ at m/z 454.3447 (G2), m/z 454.3454 (G1) and m/z 454.3445 (G3), the molecular formula was established to be $C_{30}H_{46}O_3$ which was tentatively identified as GAY. The compound 42 observed at Rt = 35.6 min in the three *Ganoderma* extracts is an isomer of compound 33 and it was also assigned to be GAY.

The component 39 is produced by the three *Ganoderma* samples at slightly different retention times (G1 and G3 at Rt = 33.604 min, G2 at Rt = 34.055 min), this compound was assigned to be GAT-Q.

Similarly, another common component 48 between the three *Ganoderma* samples (Rt = 38.0 min) with the chemical formula of $C_{36}H_{52}O_8$ was tentatively identified as GAT.

From the above results, the production of metabolites differed significantly from a strain to another, which is almost explained by the genetic differences between these fungi isolates, considering that they had the same cultivation conditions (Wang *et al*., 2006). The screening revealed the presence of similar components in the analyzed extracts.

Table 1: Interpretation of acquired QToF-MS data of the three analyzed extracts

Cpd	Rt	Extract	Best possible formula	Calculated mass (Da)	Measured mass (Da)	Δm (ppm)	Assigned molecule
1	0.668	G2	$C_{30}H_{48}O_3$	456.3603	456.3590	2.80	Unknown
	0.752	G3			456.3601	0.45	
2	0.764	G3	$C_{28}H_{46}O_4$	446.3396	446.3417	-4.77	Unknown
	0.817	G1			446.3445	-10.87	
3	1.088	G1	$C_{28}H_{44}O_4$	444.3240	444.3249	-1.93	Unknown
4	1.164	G2	$C_{30}H_{42}O_8$	530.288	530.286	-3.70	Ganoderic acid theta
	1.228	G3			530.2867	-2.54	
5	6.111	G1	$C_{28}H_{44}O$	396.3392	396.3371	5.28	Unknown
6	7.653	G1	$C_{30}H_{42}O_8$	530.288	530.2877	-0.65	Ganoderic acid theta
7	18.869	G2	$C_{30}H_{42}O_7$	514.2931	514.2923	1.55	Ganoderic acid Am1
	18.897	G3			514.2921	1.88	
	18.927	G1			514.2931	0.02	
8	18.882	G2	$C_{32}H_{44}O_9$	572.2985	572.2996	1.88	Ganoderic acid H
9	19.343	G1	$C_{30}H_{44}O_7$	516.3087	516.309	0.55	Ganoderic acid A
10	20.584	G1	$C_{32}H_{46}O_9$	574.3142	574.3139	-0.49	Ganodericacid K
11	21.563	G2	$C_{30}H_{40}O_7$	512.2774	512.275	-4.63	Ganoderic acid E
12	21.595	G2	$C_{30}H_{40}O_7$	512.2774	512.2744	5.86	Unknown
13	22.854	G3	$C_{30}H_{44}O_4$	468.324	468.3238	-0.38	Ganoderic acid DM
	23.228	G2		468.324	468.3249	1.94	
14	23.467	G3	$C_{28}H_{46}O$	398.3549	398.3551	-0.54	Unknown
	23.588	G1			398.3512	-6.37	
15	23.781	G3	$C_{30}H_{44}O_7$	516.3087	516.3102	2.97	Ganoderic acid A
16	23.836	G2	$C_{30}H_{46}O_7$	518.3244	518.3234	-2.00	Ganoderic acid C2
17	24.522	G1	$C_{30}H_{44}O_5$	484.3189	484.3199	2.01	Ganoderic acid beta
	24.522	G2			484.3204	3.15	
18	26.144	G1	$C_{28}H_{46}O_4$	446.3396	446.3338	13.08	Unknown
	26.713	G3		446.3396	446.3421	-5.52	
	26.889	G2			446.3459	-14.13	
19	26.156	G1	$C_{31}H_{46}O_3$	466.3447	466.3386	12.98	Unknown
	26.159	G3			466.3490	-9.14	
20	26.159	G3	$C_{28}H_{44}O_4$	444.3240	444.3258	-4.02	Unknown
	26.425	G2			444.3223	3.72	
21	26.725	G2	$C_{30}H_{44}O_4$	468.324	468.3254	2.96	Ganoderic acid DM
	26.746	G1			468.3255	3.26	
22	26.749	G2	$C_{30}H_{44}O_7$	516.3087	516.3073	-2.64	Ganoderic acid A
23	26.766	G1	$C_{31}H_{46}O_3$	466.3447	466.3484	-7.87	Unknown
24	26.787	G3	$C_{32}H_{46}O_5$	510.3345	510.3339	-1.22	ganoderic acid T-Q
25	28.167	G3	$C_{32}H_{44}O_9$	572.2985	572.2977	-1.46	Ganoderic acid H
	28.2	G1			572.2972	-2.23	
27	28.588	G1	$C_{28}H_{46}O$	398.3549	398.3504	9.39	Unknown
28	30.197	G3	$C_{30}H_{42}O_7$	514.2931	514.2915	3.10	Unknown
29	30.276	G3	$C_{30}H_{48}O_3$	456.3603	456.3593	2.23	Unknown
	30.305	G2			456.3605	-0.54	
30	30.554	G2	$C_{34}H_{54}O_9$	606.3768	606.3796	-4.69	Unknown
	30.59	G1			606.3802	-5.54	
31	30.655	G1	$C_{32}H_{46}O_5$	510.3345	510.3356	2.10	Ganoderic acid T-Q
32	30.658	G1	$C_{30}H_{48}O_5$	488.3502	488.3528	-5.40	Unknown
	30.661	G3			488.3521	-3.82	
	30.667	G2			488.3550	-9.88	
33	31.112	G2	$C_{30}H_{46}O_3$	454.3447	454.3447	-0.04	Ganoderic acid Y
	31.122	G1			454.3454	1.48	
	31.129	G3			454.3445	-0.35	
34	31.272	G3	$C_{34}H_{54}O_9$	606.3768	606.3690	12.94	Unknown
35	31.321	G1	$C_{30}H_{46}O_3$	454.3447	454.3449	0.49	Ganoderic acid Y
36	33.497	G1	$C_{30}H_{44}O_3$	452.329	452.3303	2.80	Ganoderic acid S
	34.359	G3			452.3294	0.86	
37	33.563	G2	$C_{34}H_{54}O_9$	606.3768	606.3774	-1.00	Unknown
	33.647	G3			606.3701	11.13	
38	33.575	G2	$C_{28}H_{44}O_3$	428.3290	428.3283	1.60	Unknown
	33.576	G1			428.3323	-7.71	
	33.591	G3			428.3318	-6.55	

Table 1: Continue

Cpd	Rt	Extract	Best possible formula	Calculated mass (Da)	Measured mass (Da)	Δm (ppm)	Assigned molecule
39	33.604	G1	$C_{32}H_{46}O_5$	510.3345	510.3359	2.71	Ganoderic acid T-Q
	33.604	G3			510.3356	2.20	
	34.055	G2			510.3366	4.04	
40	34.832	G1	$C_{33}H_{46}O_8$	570.3193	570.3142	9.02	Unknown
	35.695	G3			570.3203	-1.81	
41	34.875	G2	$C_{28}H_{46}O$	398.3549	398.3547	0.47	Unknown
42	35.595	G2	$C_{30}H_{46}O_3$	454.3447	454.3452	1.04	Ganoderic acid Y
	35.635	G1			454.3449	0.38	
	35.654	G3			454.3446	-0.31	
43	35.939	G2	$C_{36}H_{52}O_8$	612.3662	612.3657	-0.83	Ganoderic acid T
	36.028	G1			612.3662	0.01	
44	36.528	G1	$C_{30}H_{44}O_3$	452.329	452.3308	4.07	Ganoderic acid S
	36.558	G3			452.33	2.32	
45	37.076	G1	$C_{32}H_{48}O_5$	512.3502	512.351	1.54	Ganoderic acid X
	37.123	G3			512.3515	2.61	
46	37.476	G3	$C_{30}H_{40}O_7$	512.2774	512.2799	4.84	Ganoderic acid E
47	38.009	G2	$C_{30}H_{46}O_4$	470.3396	470.3432	-7.63	Unknown
	38.044	G1			470.3428	-6.73	
	38.093	G3			470.3420	-5.15	
48	38.028	G2	$C_{36}H_{52}O_8$	612.3662	612.367	1.37	Ganoderic acid T
	38.029	G3			612.3668	1.04	
	38.052	G1			612.3655	-1.21	
50	38.507	G2	$C_{30}H_{50}O_3$	458.3760	458.3786	-5.57	Unknown
	38.576	G1			458.3784	-5.22	
51	39.038	G2	$C_{34}H_{54}O_9$	606.3768	606.3725	7.17	Unknown
52	41.113	G3	$C_{28}H_{44}O_3$	428.3290	428.3288	0.56	Unknown
53	40.859	G2	$C_{28}H_{44}O$	396.3392	396.3384	2.01	Unknown
54	40.963	G2	$C_{34}H_{50}O_6$	554.3607	554.3622	2.68	Ganoderic acid R
55	41.979	G2	$C_{28}H_{44}O$	396.3392	396.3418	-6.56	Unknown
56	42.561	G3	$C_{30}H_{50}O_3$	458.3760	458.3777	-3.75	Unknown

CONCLUSION

Production of bioactive metabolites by submerged fermentation of higher fungi has gained popularity over the past years and many papers about optimization of the fermentation process for more efficient production have been published. Besides, relevant researches have been made in isolation and structural elucidation of active constituents.

This study reports for the first time the production of triterpenoids and sterols by Algerian *Ganoderma* sp. strains. The used culture medium was suitable for mycelial growth and many compounds were produced in the submerged fermentation process. In the attempt to screen triterpenoids and sterols by using a LC-QToF-MS method, many targeted compounds were successfully assigned. Conclusive identification of targeted compounds was constrained by the unavailability of authentic standards. Besides unknown compounds that could not be identified through database searching or the literature. The obtained results highlight a varied production of metabolites, however, few compounds are commonly produced by the three strains.

These findings show the need that future studies in screening approaches of bioactive metabolites require complementary analytical techniques to improve accuracy and confidence in compound identification.

REFERENCES

Berger, A., D. Rein, E. Kratky, I. Monnard and H. Hajjaj *et al*., 2004. Cholesterol-lowering properties of *Ganoderma lucidum in vitro*, *ex vivo* and in hamsters and minipigs. Lipids Health Dis., Vol. 3.

Boh, B., M. Berovic, J. Zhang and L. Zhi-Bin, 2007. *Ganoderma lucidum* and its pharmaceutically active compounds. Biotechnol. Annu. Rev., 13: 265-301.

Calvino, E., J.L. Manjon, P. Sancho, M.C. Tejedor, A. Herraez and J.C. Diez, 2010. *Ganoderma lucidum* induced apoptosis in NB4 human leukemia cells: Involvement of Akt and Erk. J. Ethnopharmacol., 128: 71-78.

Chen, H.S., Y.F. Tsai, S. Lin, C.C. Lin, K.H. Khoo, C.H. Lin and C.H. Wong, 2004. Studies on the immuno-modulating and anti-tumor activities of *Ganoderma lucidum* (Reishi) polysaccharides. Bioorganic Med. Chem., 12: 5595-5601.

Chen, Y., Y. Yan, M.Y. Xie, S.P. Nie, W. Liu, X.F. Gong and Y.X. Wang, 2008. Development of a chromatographic fingerprint for the chloroform extracts of *Ganoderma lucidum* by HPLC and LC-MS. J. Pharm. Biomed. Anal., 47: 469-477.

De Silva, D.D., S. Rapior, F. Fons, A.H. Bahkali and K.D. Hyde, 2012. Medicinal mushrooms in supportive cancer therapies: An approach to anti-cancer effects and putative mechanisms of action. Fungal Diversity, 55: 1-35.

El-Mekkawy, S., M.R. Meselhy, N. Nakamura, Y. Tezuka and M. Hattori *et al*., 1998. Anti-HIV-1 and anti-HIV-1-protease substances from *Ganoderma lucidum*. Phytochemistry, 49: 1651-1657.

Fang, Q.H. and J.J. Zhong, 2002a. Effect of initial pH on production of ganoderic acid and polysaccharide by submerged fermentation of *Ganoderma lucidum*. Process Biochem., 37: 769-774.

Fang, Q.H. and J.J. Zhong, 2002b. Submerged fermentation of higher fungus *Ganoderma lucidum* for production of valuable bioactive metabolites-ganoderic acid and polysaccharide. Biochem. Eng. J., 10: 61-65.

Gao, J.J., N. Nakamura, B.S. Min, A. Hirakawa, F. Zuo and M. Hattori, 2004. Quantitative determination of bitter principles in specimens of *Ganoderma lucidum* using high-performance liquid chromatography and its application to the evaluation of Ganoderma products. Chem. Pharm. Bull., 52: 688-695.

Gao, J.J., A. Hirakawa, B.S. Min, N. Nakamura and M. Hattori, 2006. *In vivo* antitumor effects of bitter principles from the antlered form of fruiting bodies of *Ganoderma lucidum*. J. Nat. Med., 60: 42-48.

Hajjaj, H., C. Mace, M. Roberts, P. Niederberger and L.B. Fay, 2005. Effect of 26-oxygenosterols from *Ganoderma lucidum* and their activity as cholesterol synthesis inhibitors. Applied Environ. Microbiol., 71: 3653-3658.

Hirotani, M., I. Asaka, C. Ino, T. Furuya and M. Shiro, 1987. Ganoderic acid derivatives and ergosta-4,7,22-triene-3,6-dione from *Ganoderma lucidum*. Phytochemistry, 26: 2797-2803.

Jarvis, B., 1973. Comparison of an improved rose Bengal-chlortetracycline agar with other media for the selective isolation and enumeration of moulds and yeasts in foods. J. Applied Bacteriol., 36: 723-727.

Keypour, S., H. Rafati, H. Riahi, F. Mirzajani and M.F. Moradali, 2010. Qualitative analysis of ganoderic acids in *Ganoderma lucidum* from Iran and China by RP-HPLC and electrospray ionisation-mass spectrometry (ESI-MS). Food Chem., 119: 1704-1708.

King, D.A.Jr., A.D. Hocking and J.I. Pitt, 1979. Dichloran-rose bengal medium for enumeration and isolation of molds from foods. Applied Environ. Microbiol., 37: 959-964.

Ko, H.H., C.F. Hung, J.P. Wang and C.N. Lin, 2008. Antiinflammatory triterpenoids and steroids from *Ganoderma lucidum* and *G. tsugae*. Phytochemistry, 69: 234-239.

Kuo, M.C., C.Y. Weng, C.L. Ha and M.J. Wu, 2006. *Ganoderma lucidum* mycelia enhance innate immunity by activating NF-κB. J. Ethnopharmacol., 103: 217-222.

Liang, C.X., Y.B. Li, J.W. Xu, J.L. Wang and X.L. Miao *et al*., 2010. Enhanced biosynthetic gene expressions and production of ganoderic acids in static liquid culture of *Ganoderma lucidum* under phenobarbital induction. Applied Microbiol. Biotechnol., 86: 1367-1374.

Liu, J., K. Shimizu and R. Kondo, 2010. The effects of ganoderma alcohols isolated from *Ganoderma lucidum* on the androgen receptor binding and the growth of LNCaP cells. Fitoterapia, 81: 1067-1072.

Liu, Y., Y. Liu, F. Qiu and X. Di, 2011. Sensitive and selective liquid chromatography-tandem mass spectrometry method for the determination of five ganoderic acids in *Ganoderma lucidum* and its related species. J. Pharm. Biomed. Anal., 54: 717-721.

Ma, Q.Y., Y. Luo, S.Z. Huang, Z.K. Guo, H.F. Dai and Y.X. Zhao, 2013. Lanostane triterpenoids with cytotoxic activities from the fruiting bodies of *Ganoderma hainanense*. J. Asian Nat. Prod. Res., 15: 1214-1219.

Ottow, J.C.G., 1972. Rose Bengal as a selective aid in the isolation of fungi and actinomycetes from natural sources. Mycologia, 64: 304-315.

Petrova, R.D., A.Z. Reznick, S.P. Wasser, C.M. Denchev, E. Nevo and J. Mahajna, 2008. Fungal metabolites modulating NF-κB activity: An approach to cancer therapy and chemoprevention (Review). Oncol. Rep., 19: 299-308.

Ren, A., X. Ouyang, L. Shi, A.L. Jiang and D.S. Mu *et al*., 2013. Molecular characterization and expression analysis of GlHMGS, a gene encoding hydroxymethylglutaryl-CoA synthase from *Ganoderma lucidum* (Ling-zhi) in ganoderic acid biosynthesis pathway. World J. Microbiol. Biotechnol., 29: 523-531.

Saltarelli, R., P. Ceccaroli, M. Iotti, A. Zambonelli and M. Buffalini *et al*., 2009. Biochemical characterisation and antioxidant activity of mycelium of *Ganoderma lucidum* from Central Italy. Food Chem., 116: 143-151.

Schmidt, O., 2006. Biology. In: Wood and Tree Fungi, Schmidt, O. (Ed.). Springer, Berlin, Heidelberg, ISBN: 978-3-540-32138-5, pp: 3-52.

Seo, H.W., T.M. Hung, M. Na, H.J. Jung and J.C. Kim *et al*., 2009. Steroids and triterpenes from the fruit bodies of *Ganoderma lucidum* and their anti-complement activity. Arch. Pharm. Res., 32: 1573-1579.

Shi, L., A. Ren, D. Mu and M. Zhao, 2010. Current progress in the study on biosynthesis and regulation of ganoderic acids. Applied Microbiol. Biotechnol., 88: 1243-1251.

Tang, Y.J. and J.J. Zhong, 2002. Fed-batch fermentation of *Ganoderma lucidum* for hyperproduction of polysaccharide and ganoderic acid. Enzym. Microb. Technol., 31: 20-28.

Tang, Y.J. and J.J. Zhong, 2003. Role of oxygen supply in submerged fermentation of *Ganoderma lucidum* for production of *Ganoderma* polysaccharide and ganoderic acid. Enzyme Microb. Technol., 32: 478-484.

Tang, W., T. Gu and J.J. Zhong, 2006. Separation of targeted ganoderic acids from *Ganoderma lucidum* by reversed phase liquid chromatography with ultraviolet and mass spectrometry detections. Biochem. Eng. J., 32: 205-210.

Wang, X.M., M. Yang, S.H. Guan, R.X. Liu, J.M. Xia, K.S. Bi and D.A. Guo, 2006. Quantitative determination of six major triterpenoids in *Ganoderma lucidum* and related species by high performance liquid chromatography. J. Pharm. Biomed Anal., 41: 838-844.

Xu, P., Z.Y. Ding, Z. Qian, C.X. Zhao and K.C. Zhang, 2008. Improved production of mycelial biomass and ganoderic acid by submerged culture of *Ganoderma lucidum* SB97 using complex media. Enzyme Microb. Technol., 42: 325-331.

Yang, M., X. Wang, S. Guan, J. Xia, J. Sun, H. Guo and D.A. Guo, 2007. Analysis of triterpenoids in *Ganoderma lucidum* using liquid chromatography coupled with electrospray ionization mass spectrometry. J. Am. Soc. Mass Spectrometry, 18: 927-939.

Yang, M., J. Sun, Z. Lu, G. Chen and S. Guan *et al*., 2009. Phytochemical analysis of traditional Chinese medicine using liquid chromatography coupled with mass spectrometry. J. Chromatogr. A, 1216: 2045-2062.

Yuan, J.P., J.H. Wang and X. Liu, 2007. Distribution of free and esterified ergosterols in the medicinal fungus *Ganoderma lucidum*. Applied Microbiol. Biotechnol., 77: 159-165.

Zhang, W.X., Y.J. Tang and J.J. Zhong, 2010. Impact of oxygen level in gaseous phase on gene transcription and ganoderic acid biosynthesis in liquid static cultures of *Ganoderma lucidum*. Bioprocess Biosyst. Eng., 33: 683-690.

Zhong, J.J. and J.H. Xiao, 2009. Secondary Metabolites from Higher Fungi: Discovery, Bioactivity and Bioproduction. In: Biotechnology in China I, Zhong, J.J., F.W. Bai and W. Zhang (Eds.). Springer, Berlin, ISBN: 978-3-540-88414-9, pp: 79-150.

Zjawiony, J.K., 2004. Biologically active compounds from aphyllophorales (Polypore) fungi. J. Nat. Prod., 67: 300-310.