Nova Scientia
Revista de Investigación de la Universidad De La Salle Bajío

Nº 21, Vol. 10 (2), 2018. **ISSN 2007 – 0705**, pp.: 247 - 257
doi.org/10.21640/ns.v10i21.1538

Detección de polisacáridos en extractos de *Ganoderma lucidum*
Detection of polysaccharides in *Ganoderma lucidum* extracts

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Palabras clave: M.A.R.S.III.; cromatografía; método de Fehling; extractos; ácido acetilsalicílico
Keywords: M.A.R.S.III.; chromatography; Fehling´s method; extracts; acetylsalicylic acid

Recepción: 12-06-2018 / Aceptación 15-08-2018

Resumen

Introducción: *Ganoderma lucidum* es uno de los hongos medicinales mas conocidos a nivel mundia; sin embargo, esta especie de hongo de origen mexicano presenta notables diferencias en comparación con su similar siendo aun poco estudiado por lo que es importante determinar sus caracteristicas una vez que se ha cultivado y se han obtenido sus extractos en forma estandarizada.
Método: Los polisacáridos de los extractos de *Ganoderma lucidum* cepa CP-145 se midieron por el analizador de resonancia magnética cuántica MARSIII de Bruce Copen (MARSIII), técnica propuesta para estimar valores de compuestos en el área agrícola, estos resultados se compararon con dos técnicas estandarizadas: el método Fehling de reductores de azúcar y Cromatografía de Gases acoplada a Espectrometría de masas (CG-EM), los extractos a medir se obtuvieron del cultivo tradicional de basidiocarpos (control) y del extracto de basidiocarpos adicionados con ácido acetilsalicílico (AAS)
Resultados: Los resultados para el analizador magnético cuántico MARSIII fueron 4.967 ± 1.016 y 8.110 ± 1.416% en extracto control y extracto adicionado respectivamente, por el método de Fehling 8.784 ± 2.019% y 41.326 ± 1.430% en extracto control y extracto adicionado respectivamente, para CG-EM 7.050 ± 1.527 y 18.456 ± 2.937 % en extracto control y extracto adicionado respectivamente.
Discusión y Conclusión: Se encontraron diferencias significativas para las técnicas de Fehling y para GC-EM para ambos extractos, pero no así en la detección del analizador MARSIII, lo que indica que no es un método adecuado para detectar variaciones en las concentraciones de polisacáridos en este tipo de extractos.

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Abstract

Introduction: *Ganoderma lucidum* is one of the most worldwide known medicinal mushrooms. However, this species of mexican mushroom has notable differences compared to its similar one since it has not been studied enough so it is important to determine its characteristics once it has been cultivated and its extracts have obtained in a standardized form.

Method: The polysaccharides of the extracts of *Ganoderma lucidum* strain CP-145 were measured by the quantum magnetic resonance analyzer MARSIII of Bruce Copen (MARSIII), a proposed technique to estimate values of compounds in the agricultural area, these results were compared with two standardized techniques: Fehling method of sugar reducers and Gas Chromatography - Mass spectrometry (GC-MS), the extracts to be measured were obtained from the traditional culture of basidiocarps (control) and extract of basidiocarps added with acetylsalicylic acid (ASA).

Results: The results for the MARSIII quantum magnetic analyzer were 4.967 ± 1.016 and 8.110 ± 1.416% in control extract and extract added respectively, by the Fehling´s method 8.784 ± 2.019% and 41.326 ± 1.430% in control extract and extract added respectively, for CG- MS 7.050 ± 1.527 and 18.456 ± 2.937% in control extract and extract added respectively.

Discussion and Conclusion: Significant differences were found on the Fehling´s techniques and on the GC-MS for both extracts, but not in the MARSIII analyzer detection, which indicates that it is not an adequate method to detect variations in the concentrations of polysaccharides in this type of extracts.

Introduction

Ganoderma lucidum is the most popular medicinal mushroom also known as Reshi in Japan. It is one of the most studied fungi due to the great variety of bioactive compounds that have been reported in the mycelium, the mature fruiting body contains more than 400 different bioactive compounds, mainly triterpenoids, polysaccharides, nucleotides, sterols, steroids, fatty acids, proteins/peptides and trace elements that have been reported to have various medicinal effects (Cör *et al*., 2017, 129). Within these compounds we can find more than 150 antioxidants and phytonutrients, responsible for the most important immunomodulatory, anticarcinogenic and anti-inflammatory properties, hypoglycemic, hypolipid and hepatoprotective. Among the most active components are the polysaccharides that help strengthen the immune system, intensifying the activity of T cells, applied in the treatment of a variety of diseases, including cancer (Pai-Feng *et*

al., 2012, 1). The importance of polysaccharides lies in the fact that they can provide many benefits related to their intervention in biological metabolisms in humans, immunostimulant activity, inhibition of tumor growth, as well as anti-inflammatory action, which are generally related to their structure, molecular weight and degree of branching. Some studies indicate that the polysaccharides of *Ganoderma lucidum* could promote the release of serum insulin, regulate the activity of enzymes with participation in glucose metabolism and the decrease in plasma glucose (Teng *et al*., 2012, 173).

There is a wide variety of polymeric compounds that form the structure of cells, the mycelium and the fruitful body of micro and macromycetes, mainly glucans that is a monosaccharide (glucose) and exopolysaccharides, however their major composition are various monosaccharides chains of sizes. Determine the exact structure of fungal glucans many analytical methods have been applied such as chemolysis methods that include methylation analysis, oxidation methods (periodate, IV lead acetate), Smith degradation, NMR spectroscopy is the non-destructive method of analysis more effective and updated structural (Synytsya *et al*., 2015, 720; Ruthes *et al*., 2015, 754).

It is important to determine the chemical composition that provides the fungal diversity to optimize the use of their properties, to have simple methods that generate a correlation with other results and make analysis, simpler, faster and less expensive, that is why it has techniques already standardized the new ones offered by the analytical area.

The aim of this study was to investigate the chemical composition of polysaccharides in standardized extract of the fungus *Ganoderma lucidum* when it is stimulated to increase the production of its functional and medicinal compounds by the addition of acetylsalicylic acid (ASA) under controlled conditions, comparing three techniques.

Method

Chemicals and reagents

Acetylsalicylic acid ≥ 99.0%, dimethyl sulfoxide (DMSO) for HPLC, ≥ 99.7% were purchased from Sigma-Aldrich, U.S.A., all other chemicals used were of analytical purity and grade HPLC.

Obtaining basidiocarps

The biological material used was *Ganoderma lucidum* CP-145, belonging to the Centre for Genetic Resources of Edible, Functional and Medicinal Mushrooms, *Campus* Puebla, Mexico of the Colegio de Posgraduados, cultivated in solid sterile substrates based on oak sawdust, two types of basidiocarps were obtained: those added on their substrate with ASA, (10 mM) and control basidiocarps cultured in the traditional way.

Obtaining hydroalcoholic extracts

Obtaining two types of hydroalcoholic extracts: An extract from added basidiocarps and another from basidiocarps from control fungi both extracts were carried out with water-alcohol (80:20) by 24 h, hydroalcoholic extracts were obtained according to patented procedure, each extract was taken at a concentration of 1 g/mL with rotary evaporator at 19°C (Meneses *et al*. 2016, 5).

Determination of polysaccharides by quantum magnetic resonance analyzer MARSIII of Bruce Copen

The sample is placed directly in the sample holder of the equipment obtaining a reading at 10 seconds, without the use of solvents, reagents or other treatment.

The analizer has a database where the compounds of interest are selected to be monitored in this case polysaccharides, generating a table of data comparing the results of the sample against the references of the software, the answer is given in abundance (http://copen.com.mx/nueva/mars3-analisis.php; https://www.copen.us).

Determination of polysaccharides by the Fehling´s method

This technique was used to determine the content of polysaccharides in the form of glucose, polymers of glucopyranosides and polyalcohols present in the extracts of *Ganoderma lucidum*. In this test the presence of aldehydes but not ketones are detected by reduction of the deep blue solution of copper (II) to a red precipitate of insoluble copper oxide. Two solutions are required: Fehling's "A" uses 7 g of $CuSO_4.5H_2O$ dissolved in distilled water containing 2 drops of dilute sulfuric acid. Fehling's "B" uses 35 g of potassium tartrate and 12 g of NaOH in 100 mL of distilled water.

The mixture was made with 15 mL of solution "A" with 15 mL of solution "B", 2 ml of this mixture added to an empty test tube and 3 drops of the compound to be tested added to the tube. It was placed in a water-bath at 60°C. A positive test is indicated by a green suspension and a red precipitate. The test was so sensitive enough that even 1 mg of glucose produced the characteristic red colour of the compound (Ávila *et al*., 2012, 131; Laine *et al*., 2002, 608).

Extraction with DMSO for polysaccharides and polyalcohols by GC-MS

Derivatization was performed to obtain them as ester by methylating the polysaccharides, 2 mL of alcohol-alkalinized with sodium hydroxide (10:2) were added to 5 mL of each control extract and to 5 mL with the addition of ASA separately, 0.5 mL of DMSO were added, the sample was maintained 30 min in an ultrasonic bath at room temperature, subsequently it was heated for 1 h at 90°C, cooled, 25 mL of distilled water were added, the aqueous phase was extracted with 10 mL of dichloromethane, the filtrate was centrifuged at 1400 rpm, filtered with a bed of dry anhydrous sodium sulfate (Synytsya and Novák, 2013, 3; Kao *et al*., 2012, 2).

Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) and its respective multiple comparison of Tukey the level of significance was set at $p<0.05$ using the statistical software OriginPro Version 8.0

Results

Determination of polysaccharides by quantum magnetic resonance analyzer MARSIII of Bruce Copen

The results were obtained by the MARSIII analyzer were with the selection of four types of sugars in the database, the variations of their standard deviations are large and there is no significant difference to 95% confidence between the control extract and the added extract. This technique only generates comparison results between the two samples without giving a value of the total content of the sugars present in each sample (Figure 1).

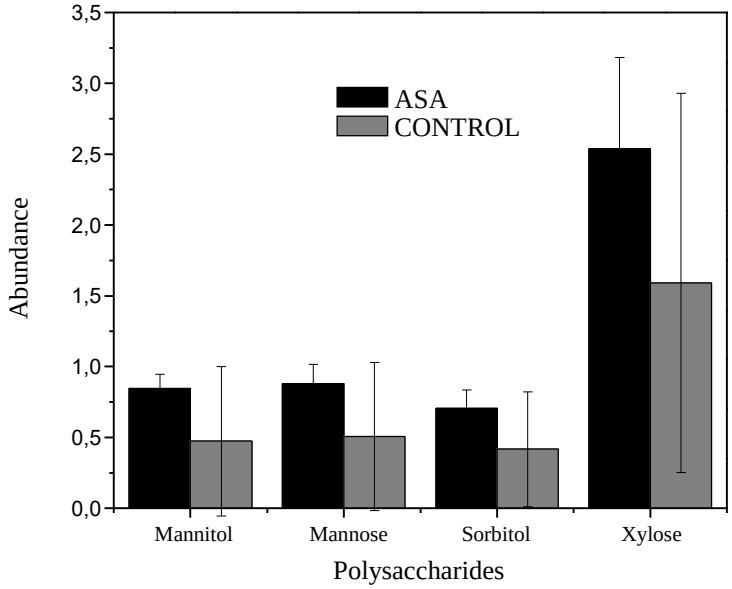

Figure 1. The comparison of the obtained results is presented using control extract and extract added by the method of quantum magnetic resonance analyzer MARSIII.

Content of reducing sugars

The sugar reducers Fehling´s method is adequate because it takes advantage of the reduction properties of the sugars present in the extracts with a value of 8.784 ± 2.019% in the control basidiocarps extract and 41.326 ± 1.430% in the extract of basidiocarps added, increasing the concentration of these 4.7 times more, there is a significant difference between the control extract and the added extract to 95% de reliability. The result obtained from saccharides is expected by the presence of the ASA (Meneses *et al*., 2016, 12).

Determination of polysaccharides and polyalcohols

Ganoderma lucidum is a mushroom with substantial number of active biological compounds present in the form of polysaccharides when analyzed by GC-MS. The extraction with DMSO is recommended for the detection of polysaccharides wich can be seen in Figure 2, the compound with the highest concentration is β-glucopyranoside methyl 41.53 y 36.44 abundance respectively for the added extract and the control extract, along with this we find other polysaccharides and polyalcohols as the expected composition characteristic of this fungus.

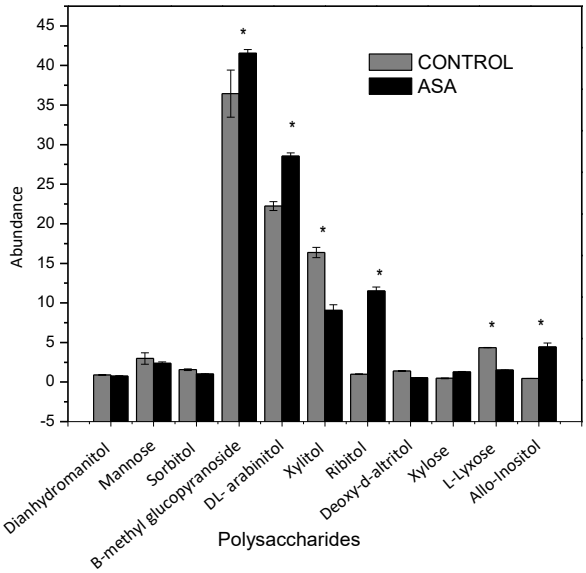

Figure 2. Sugars determined by GC-MS, * indicates $p<0.05$ compared to control and extract added with ASA.

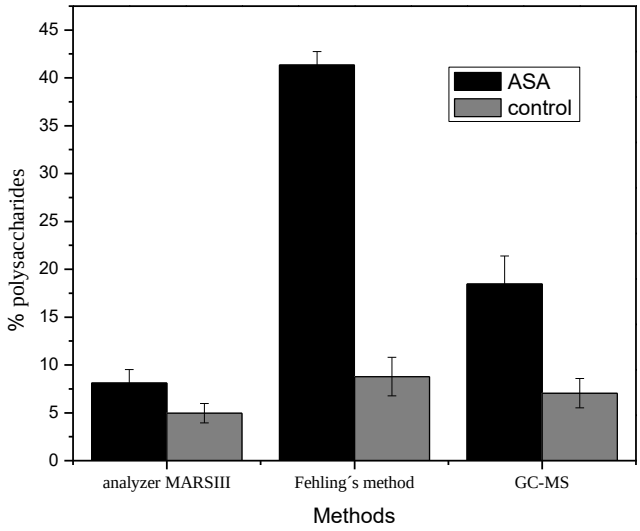

Figure 3. Comparative of the total polysaccharides found by each method.

The quantum magnetic resonance analyzer MARSIII by Bruce Copen for the characterization of agricultural area materials such as plants and fungi was evaluated through the determination of polysaccharides in extracts of *Ganoderma lucidum*, even though it has characteristics that make it an attractive method since it does not require reagents or sample handling

(http://copen.com.mx/nueva/mars3-analisis.php; https://www.copen.us), it was not possible to detect a significant diference between the control extracts and those added with ASA.

When the determinations were made with the methods of sugar reduction and GC-MS that are already standardized methods, significant differences were obtained between both extracts as shown in Figure 3, the differences that these last two methods show in their detections suggest Interferences due to the chemical composition of the extracts. In the Fehling´s method the presence of calcium is relevant to generate results that are not affected by this element, being a non-specific technique that yields a total value of reducing sugars and in the chromatograph method the manipulation of samples to make the reaction of methylation generates decrease in the recovery of each of its compounds, although its greatest advantage is to generate very specific results by identifying compounds that can be made through the comparison of the NIST library in the operating software.

Discussion and Conclusion

The increase of the presence of a compound in the added extract shows the effect of the addition of the ASA that stimulates the production of more functional molecules with respect to the control extract, the polysaccharides found are compounds of significant importance and although they have already been reported, their detection is relevant since these compounds are presented in large quantity as a characteristic, in this work a higher concentration of β-methyl glucopyranoside is detected followed by DL-arabinitol and ribitol for the added extract. However, xylitol and L-lyxose are present with greater presence in the control extract, all these compounds with antioxidant activity (Ruthes *et al*., 2015, 756; He *et al*., 2006, 708-709).

The main composition of the extract is the polysaccharides that stand out: dianhydromannitol, mannose, sorbitol, β-methyl glucopyranose, DL-arabinitol, ribitol, deoxy-d-altritol, Allo-inositol, as can be seen in Figure 2 where the results obtained are compared in the control extract and the extract added with ASA. Detection of these compounds are common in both extracts: D-arabinitol, ribitol, mannitol, β-D-glucopyranoside, methyl β-D-ribopyranoside, which are alcohols and sugars of five carbon atoms. These polysaccharides with demonstrated activity in the stimulation of the immune system also have potential in the treatment of human diseases (Synytsya and Novák, 2013, 805; He *et al*., 2006, 708-709). The carbohydrate content of edible fungi varies with species and ranges between 35 and 70% DW. It is believed that edible fungi

contain a high level of oligosaccharides and only a low level of total soluble sugars (Rathee *et al*., 2012, 460). Unlike works where the addition of SA and ASA in plants allows to obtaining of crops that develop greater vigor and tolerance to various environmental factors to face the problem due to climate change (Soliman *et al*., 2018,7), there are few works where these compounds are added to edible and medicinal mushroom, research has been carried out to reduce its browning, such as the case of *Agaricus bisporus*, where treatment with SA at 250 μM can be used as a useful technology to alleviate post-harvest browning of the fungus carphophore by maintaining the integrity of the membrane due to the improvement of the activity of the antioxidant system and also the accumulation of phenols (Magdziak *et al*., 2016, 771; Dokhanieh and Aghdamb, 2016,147), another research is aimed at increasing the content of polysaccharides and triterpenes with the addition of SA to *Ganoderma lucidum* where 12.41 ± 0.32 and 12.62 ± 0.31 mg / g of control polysaccharides and sample additioned with SA were obtained respectively (Ye *et al*., 2018,7), the combined induction increased polysaccharide 9.02% of polysaccharides being lower concentrations than those detected in this study, this research sought to modulate the properties of *Ganoderma lucidum* with ASA, in a previous quantification of the extract 0.78% of carbohydrates and glucose were obtained using the ion chromatography and colorimetric method (Meneses *et al*., 2016, 18) which demonstrated that it is necessary to have more specific techniques for the characterization of these extracts.

There were differences between the polysaccharide profiles of the control extract and the added extract, these differences were originated by the addition of ASA (10 mM) to the substrate, since all the other variables remained constant during the culture of basidiocarps, species, strains, substrate and environmental conditions (Meneses *et al*., 2016, 15).

The results obtained indicate that it is necessary to continue with the search for new tools that facilitate the analysis of the extract of *Ganoderma lucidum* to maximize the use of the properties of this medicinal fungus.

Agradecimientos

To Consejo Nacional de Ciencia y Tecnología (CONACYT) for the scholarship grant to the first author (CVU: 348734).

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