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Full Length Research Paper

Biological and antioxidant activity of *Gunnera tinctoria* (Nalca)

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The endemic Chilean edible plant *Gunnera tinctoria* (Nalca) is highly appreciated in the south of Chile by the small farmers. Nevertheless, no background exists about his secondary metabolites. In the present study, in the leaf from *G. tinctoria* was investigated the content of bioactive compounds like coumaric acid, ascorbic acid and total phenols; the antioxidant capacity was evaluated by 3-ethylbenzothiazoline-6-suslfonic acid (ABTS), oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods reducing sugars were measured. Finally, the biological activity was evaluated against *Cladophialophora* and *Cryptococcus laurentii*. The results suggest that the most abundant constituent in the extract were catechin (1344.97 mg/100 g dry weight) and epicatechin (1429.28 mg/100g dry weight), and was confirmed and quantified by high performance liquid chromatografy (HPLC-PDA); while the ORAC methodology showed a high antioxidant capacity (192000.0±5.91 umol Trolox Eq/100 g dry weight). On the other hand, the extract had a fungicide effect against both microorganism assayed, inhibiting the growth of *Cladophialophora´s* mold- and the yeast *Cryptococcus laurentii*. This is the first report of antioxidant capacity, bioactive compounds and biological activity of *G. tinctoria*, and these findings suggest that an extract prepared from the Nalca leaf may be a promising source of antioxidant and bioactive compounds and as a research object by being an antifungal and therapeutic alternative in development.

Key words: *Gunnera tinctoria*, Nalca, antioxidant capacity, biological activity, nutraceutical.

INTRODUCTION

Numerous studies have established that a high consumption of fruits and vegetables leads to an important intake of bioactive compounds (Torres et al., 2011); which are strongly associated with the reduction in

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the incidence of cardiovascular diseases (Pounis et al., 2013), various types of cancer (Zamora-Ros et al., 2013), type II diabetes (Anhê et al., 2013), immune disorders and neurodegenerative disorders (Marszalek et al., 2017), at the gastrointestinal level (Ozdal et al., 2016), and in general in all diseases in which oxidative stress has an important role (Quiles et al., 2006). Among importance bioactive compounds are p-coumaric acid, which has hepatoprotective (Calil Brondani et al., 2017) and UV-protection qualities (Aguilar-Hernández et al., 2017). Flavanols like catechin and epicatechin are acknowledged for their antibacterial capacity (Hamilton-Miller, 1995), anti-inflammatory activity and cancer preventives for humans (Fujiki et al., 2002), and ascorbic acid which can reduce the malignant potential of melanoma cells (Fischer and Miles, 2017). Also, these secondary metabolites can inhibit the growth of various mold, bacteria and yeast species (Gramza and Korczak, 2005; Almajano et al., 2008), acting as antimicrobials (Han et al., 2007).

Gunneracea is a monogeneric family (Genus *Gunnera L*.), of which 6 subgenera, with approximately 65 species on the planet have been described (Bergman et al., 1992). Its distribution extends to Central and Southern Africa, Madagascar, New Zealand, United Kingdom, Tasmania, Indonesia, Philippines, Hawaii, Mexico, Venezuela, Colombia, Chile and Argentina; having been reported in South America 23 species in Colombia, 12 in Chile, 7 in Ecuador, 6 in Peru, 5 in Argentina and Bolivia, 3 in Venezuela, 2 in Brazil and one in Uruguay, including *Gunnera tinctoria (Mol.) Mirb*., *Gunnera magellanica Lam*., *Gunnera brephogea Linden* and *Gunnera manicata* (Bergman et al., 1992; Wanntorp and Klackenberg, 2006).

G. tinctoria (Molina) Mirb. (*Gunnera chilensis*), commonly known as Nalca, Chilean Rhubarb or Pangue, is a non-timber forest product (NTFPs) (Valdebenito et al., 2003) of recognized importance in southern Chile, especially at smallholder agriculture. Which predominates subsistence exploitation, since it also provides resources and higher incomes to rural people and/or groups that are engaged in the collection and informal sale of this wild plant (Petzold et al., 2006). In South America, this species is distributed from Chile and Argentina, to Colombia and Venezuela; it has also been reported in Peru, Ecuador and Bolivia (EPPO, 2014). It is worth mentioning that in both New Zealand and the UK, *G. tinctoria* seeks to be controlled and eradicated, since it is classified as an invasive species of rapid propagation and difficult management (Hickey and Osborne, 1998, Williams et al., 2005).

In Chile, it is one of the few ornamental plants that has an edible petiole, so it is widely consumed during the spring and summer seasons. Its leaves can be consumed directly as an infusion, or they are used in the native kitchen of the island of Chiloé (Chile) being used to cover other foods during their cooking. It has been described that the maceration of its flowers and leaves constitutes a refreshing infusion. On the other hand, in medicinal terms (at the popular and rural level) it is known as an antifebrile, and applied in hemorrhages, dysentery, diarrhea, mouth, throat, liver and stomach affections (Montes and Wilkomirsky, 1985; Valdebenito et al., 2003). It has also been described that, at the agronomic level, *G. tinctoria* is a very good nitrogen-fixing species, which could be useful for the recovery of soils depleted in this nutrient (Troncoso et al., 2013).

This study is the first work that seeks to determine the nutritional characteristics, antioxidants and effects against microorganisms of *G. tinctoria*. The objective of this research was to characterize this plant, both from the nutritional point of view and the bioactive compounds, determining the antioxidant capacity of the *G. tinctoria* leaf, as well as determining the content of reducing sugars, total polyphenols and related metabolites directly with antioxidant activity, specifically Catechin, Epicatechin and p-coumaric acid, in addition to evaluating its antifungal effect on *Cryptococcus laurentii* and *Cladophialophora*.

MATERIALS AND METHODS

Reagents and equipment

Diphenyl-1-2,2 picrylhydrazyl (DPPH); 2,2'-azobis (2-amidino-propane) (AAPH); fluorescein; 6-hydroxy-2,5,7,8-tetramethyl chromane-2-carboxylic acid (trolox); 2,4,6-tris-(2-pyridyl-s-triazine) (TPTZ); 3,4,5-trihydroxybenzoic acid (gallic acid) and (+)-catechin and (-)-epicatechin standards were obtained from Sigma-Aldrich® (St. Louis, MO, USA). Sodium carbonate and folin carbonate were purchased from Merck (Germany). Methanol and other solvents were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). Ultraviolet-visible measurements were performed on a Multiskan Spectrum spectrophotometer (Thermo Scientific). The decrease in fluorescence intensity measured in the ORAC assay was performed on a Perkin-Elmer LS-55 (U.K.) Spectrofluorimeter. The chromatographic assays were carried out on a Shimadzu® series Prominence® UFLC liquid chromatograph.

Vegetal material

The plant material was collected in the commune of Los Lagos -Los Ríos Region, Chile- (31 m.s.n.m and 12.3°C average temperature) (IPNI, 2012; The plant list, 2013). After being stored in perforated polypropylene bags were lyophilized and taken to the laboratory for the respective analyzes.

Preparation of the sample

For the antioxidant and secondary metabolite assays, 2.0 g of samples dissolved and homogenized with 20 ml of acidified water (1% v/v HCl) were weighed into an Ultra-Turrax (IKA-WERK®). The extract was centrifuged at 5000 rpm for 12 min at room temperature, taking the supernatant and making the appropriate dilutions with type I water. For the biological activity tests 2.5 g of samples dissolved and homogenized were weighed with 25 ml of MeOH-Ac. Formic (99:1). The extract was centrifuged at 5000 rpm for 12 min at room temperature. The solutions were stored at 4°C

until analysis.

Chemical and antioxidant capacity

Proximal analysis

Total ash (Bateman, 1970; AOAC, 1996; method 942.05), moisture (Bateman, 1970; AOAC, 1996; method 930.15), crude protein (AOAC, 1996; method 990.03), ethereal extract (Bateman, 1970; AOAC, 1980; method 832p) and crude fiber (AOAC, 1996; method 978.10) were determined. Carbohydrates, was determined by difference. Each sample was analyzed in triplicate, and all the proximate values were reported in percentage.

Determination of reducing sugars - DNS colorimetric method

The determination of reducing sugars was performed as described by Najmus and Whitney (2011). 500 uL of a 1% solution of 3,5-dinitrosalicylic acid in NaOH is added to 500 uL of the sample to be evaluated. The mixture was heated at 90°C for 5 min and its absorbance determined at 540 nm. The contents were determined by comparing with a standard curve using glucose as standard. The results were expressed as mg of equivalent glucose/100 g dry weight.

Content of secondary metabolites with antioxidant properties

Total phenols

The determination of phenols was done by the colorimetric method of Folin-Ciocalteu designed by Singleton and Rossi (1965). 50 µL of sample were added to 125 µL of Folin's reagent and 400 µL of 7.1% (w/v) sodium carbonate, adjusting with distilled water to 1000 µL, the spectrophotometric reading was performed at 760 nm and compared with Standard curve using gallic acid as standard. The results were expressed as mg gallic acid equivalent: GAE/100 g dry weight.

Determination of ascorbic acid

The determination of ascorbic acid was performed through HPLC, following the method described by Kelebek et al. (2009). The aqueous extract was filtered with cellulose acrodiscs of pore size 0.45 µm. The samples were analyzed on a Shimadzu® liquid chromatograph model LC-20AD, equipped with a SIL-20A/HT auto injector, a CBM-20A communication module and a PDA detector SPD-M20A; a Merk® (250 mm long and 4 mm internal diameter) LiChrospher® 100 C-18 (5 µm, 250 mm × 4.6 mm) column. As a mobile phase 0.1% formic acid in purified water was used, by isocratic elution system, at a temperature of 35°C and flow of 0.8 mL/min. Detection was performed at 245 nm. The identification and quantification was done with calibration curves elaborated with different concentrations of ascorbic acid.

Determination of (+)-catechin and (-)-epicatechin

Confirmation and quantification of (+)-catechin and (-)-epicatechin were analyzed by direct injection of the samples, previously filtered through a 0.45 µm pore-size nylon filter, in a liquid chromatography (HPLC-DAD) using a Shimadzu LC-20AD/T HPLC equipped with a SPD-6AUV detector (Kyoto, Japan) and a Pinacle (II) C-18 column (5 µm) 250 × 4.6 mm (Restek©, Bellefonte, USA) with an autoinjector and a photodiode array detector (PDA). (+)-catechin and (-)-epicatechin were adopted as the standard of identification and quantification at 280 nm. The mobile phase was methanol (A) acidified water (0.1% formic acid) (B), with gradient elution of 0.01 min 60% A was used; 5-12 min 80% A; 13-14 min 60% A. Flow rate of mobile phase was 1.0 mL/min (Oliveiro et al., 2009).

Phenolic acids

The p-coumaric phenolic acid was determined by direct injection of the samples, previously filtered through a 0.45 µm pore-size nylon filter, in a HPLC-DAD using a Shimadzu LC-20AD/T HPLC equipped with a SPD-6AUV detector (Kyoto, Japan) and a Pinacle (II) C-18 column (5 µm) 250 × 4.6 mm (Restek©, Bellefonte, USA) with an autoinjector and a photodiode array detector (PDA). P-coumaric acid was adopted as the standard for its identification at 320 nm. The mobile phase was a mixture of acetonitrile, acidified water (phosphoric acid at pH = 2.5) (40:60) v/v, supplied at a rate of 0.8 mL/min (Kelebek et al., 2009).

Assessment of antioxidant capacity

Ferric reducing/antioxidant power assay (FRAP)

This method evaluates the reducing potential of a sample based on its ability to reduce ferric iron (Fe^{+3}) present in a complex with 2,4,6-tris (2-pyridyl-s-triazine) (TPTZ) to the ferrous form (Fe^{+2}). The absorbance was determined at 593 nm. In brief, 50 µL of sample were added to 900 µL of pH 3.4 acetate buffer, TPTZ, $FeCl_3$, in a 10:1:1 ratio; after 30 min of reaction the absorbance was determined, this value was compared to the reference curve constructed with ascorbic acid as the standard, and the results were expressed as AEAC (Ascorbic Acid Equivalent Antioxidant Capacity: mg ascorbic acid per 100 g dry weight) (Benzie and Strain, 1996).

DPPH assay

Radical scavenging activity against the stable radical DPPH was measured using the methods proposed by Brand-Williams et al. (1995), with certain modifications. The method is based on the reaction of 10 µL of sample with 990 µL of DPPH solution for 30 min at room temperature. The absorbance decrease, associated with a reduction in the DPPH concentration, was measured at 517 nm. The results were expressed in µmol trolox equivalents per 100 g dry weight.

ABTS•+ assay

The method of Re et al. (1999) was used with some modifications. 10 µl of sample was added to 990 µl of a solution of ABTS•+. The ability of the samples to trap the cationic radical ABTS•+ was evaluated by means of the decrease in absorbance read after 30 min of reaction at a wavelength of 732 nm. The absorbance value was compared with the reference curve constructed with Trolox as the primary standard, and the results were expressed as TEAC values (µmol Trolox per 100 g dry weight).

Oxygen radical absorbance capacity (ORAC) assay

This methodology is used to evaluate the ability of a sample to trap peroxyl radicals (ROO•). The method described by Prior et al. (2005) and Romero et al. (2010) was used. 30 µl of the sample was

Table 1. Physico-chemical composition of *Gunnera tinctoria*.

Proximate composition variable (%)	Nalca leaf
Humidity	90.42 ± 0.31
Ashes	7.23 ± 0.23
Protein	19.95 ± 0.48
Ethereal Extract	1.15 ± 0.1
Crude fiber	12.40 ± 0.2
Carbohydrates***	59.27

*The results of each analysis are presented as the mean ± standard deviation (n=3). ** Results expressed on dry weight. *** Determination of Carbohydrate by difference.

added to 21 µl of 1×10^{-2} M fluorescein in PBS (75 mM); 2,899 µl PBS (75 mM); and 50 µL of 0.6 M AAPH in PBS (75 mM). The temperature was controlled at 37°C and the pH was maintained at 7.4. The readings were made at an excitation λ 493 nm and excitation slit 10 nm; and an emission λ 515 nm with an emission slit 15 nm, with 1% attenuator and no attenuator plate. The protective effect of the antioxidant is calculated using the differences in areas under the decay curve of fluorescein between the blank (reaction in the absence of the sample) and the sample. It was compared against the curve of the primary Trolox® standard. The results were expressed as TEAC, µmol equivalents of Trolox per 100 g dry weight according to Equation (1).

$$ORAC = \frac{AUC - AUC^{\circ}}{AUC_{Trolox} - AUC^{\circ}} f[Trolox] \quad (1)$$

Where AUC is the area under the curve of the sample, AUC° area under the curve for the control, AUC_{Trolox} area under the curve for the Trolox, f is the dilution factor of the extracts.

Biological activity

Fungal strains

Fungal strains of *C. laurentii* and *Cladophialophora* were used for the determination of antifungal activity. The microorganisms used were isolated from a cheese ripening room, belonging to small producers in the region of Los Ríos. Sampling, culturing, isolation and identification were done at the Clinical Microbiology Institute of the Universidad Austral de Chile.

Evaluation of antifungal activity

Suspension preparation

From each strain a suspension was prepared, which consisted of an inoculum of the microorganisms, resuspended in sterile physiological serum. It was homogenized to a turbidity equal to that presented in a 0.5 Mc Farland tube. This comparison was made visually.

Susceptibility testing

The susceptibilities of the isolated microorganisms were determined by the modified Kirby-Bauer disc diffusion method with Muller Hinton agar plates (Bauer et al., 1996). All the media used in the present investigation were obtained from Oxoid, Hampshire, UK.

Agar well diffusion method

Antifungal activity of *G. tinctoria* was determined by agar well diffusion method (Bauer et al., 1996). To apply this technique, 6 wells were made in the agar (6 mm diameter). Afterwards, the plate was inoculated, where the fungal suspension (0.5 Mc Farland) was absorbed with a sterile cotton swab and then smeared on the agar in three different directions at a 90° angle. In parallel, 5 dilutions were prepared from the pure extract, of which in each well were deposited 25 ul of extract. The final well contained 25 ul of solvent (70% alcohol), which corresponded to the negative control. Later, the plates were incubated at 25 and 32°C (mold and yeast respectively) for 20 days. The experiments were carried out in triplicates.

Determination of fungal activity

Antifungal activity was assessed by observing and determining the presence and/or absence of inhibition halos formed around the well containing the pure and diluted extracts. The determination of the fungistatic and/or fungicidal effect was done visually considering the time of inhibition of growth in Mueller Hinton agar. These measurements were performed after seven and 20 days.

RESULTS

The proximal physico-chemical composition of the *G. tinctoria* leaf is shown in Table 1, and Table 2 shows that the Nalca leaf has a significant amount of metabolites, which have been quantified as total gallic acid equivalent polyphenols. Also, reducing sugars and bioactive compounds such as ascorbic acid, epicatechin, catechin and p-coumaric acid were determined. The antioxidant and reducing activity of nalca leaf extracts was characterized by different assays (ABTS, FRAP, DPPH, ORAC). The results of these analyzes can be seen in Table 3.

Due to the high antioxidant potential and the abundant concentration of phenolic compounds, biological activity tests were carried out in which the pure extract exerted a fungistatic and fungicidal effect on yeast *C. laurentii*

Table 2. Bioactive compounds and reducing sugars quantified in *G. tinctoria* leaf.

Compound	Nalca leaf
Total phenols (mg gallic ac. eq/100 g DW)	10684.20±128.75*****
p-coumaric acid (mg/100 g DW)	48.69
Catechin (mg/100 g DW)	1344.97
Epicatechin (mg/100 g DW)	1429.28
Ascorbic acid (mg/100 g DW)	46.79
DNS (mg glucose eq/100 g DW)	7353.39 ± 0.36*****

* The results of each analysis are presented as the mean ± standard deviation (n=3). ** Results expressed in 100 g DW.

Table 3. Antioxidant activity in *G. tinctoria* leaf.

Methodology	Nalca leaf
FRAP (mg ascorbic acid/100 g DW)	11194.50±303.42
ABTS (µmol Trolox Eq/100 g DW)	120094.22±5950.44
DPPH (µmol Trolox Eq/100 g DW)	83266.76±3944.77
ORAC-H (µmol Trolox Eq/100 g DW)	192006.07±5911.13

* The results of each analysis are presented as the mean ± standard deviation (n=3). ** Results expressed in 100 g DW.

Table 4. Biological activity. Presence or absence of inhibition halos when applying *G. tinctoria* leaf extract against *Cryptococcus laurentii*.

Species/extract	Day 7			Day 20		
	S.E	F.T	F.G	S.E	F.T	F.G
Cryptococcus laurentii						
Well 1		√			√	√
Well 2		√			√	√
Well 3		-			-	-
Well 4		-			-	-
Well 5		-			-	-
Well 6 (N.C)		-			-	-

*Well 1: Pure extract. Well 2: 1:10 dilution. Well 3: 1:100 dilution. Well 4: 1:1000 dilution. Well 5: 1:10000 dilution. Well 6: negative control. ** (√): Presence of halo (inhibitory effect obtained by the extracts)); (-): Absence of halo; N.C: Negative control; SE: No inhibitory effect. FT: Fungistatic effect. FG: Fungicidal effect.

(Table 4 and Figure 1) and *Cladophialophora* mold (Table 5 and Figure 2).

DISCUSSION

Physico-chemical characterization

According to Bello (2000) in food or plant species the water content and its location within the matrix are factors that influence specific characteristics such as appearance, texture and color. The percentage of humidity for leaf of *G. tinctoria* is of 90.42% being a value of water content similar to those reported for kiwi (83.98%) (Morillas-Ruiz and Delgado-Alarcón, 2012), orange (85.70%) Berlitz and Grosh (1999), mango (82.10%) (Moreiras et al., 2006), and slightly higher than those determined for avocado (72.07%) and cherry (75.84%) (Morilla-Ruiz and Delgado-Alarcón, 2012). On the other hand, the total ash, corresponding to the mineral elements of a plant structure, presented a percentage of 7.23%; which is slightly higher than that found in apricot (3,0%), peach (4.0%), cherry (4.7%) (Lazos, 1991), orange (5.02%) and lemon (5.17%) (Ibrahim et al., 2011).

Proteins are macronutrients whose main function in the body is to form and regenerate tissues. In the leaves of

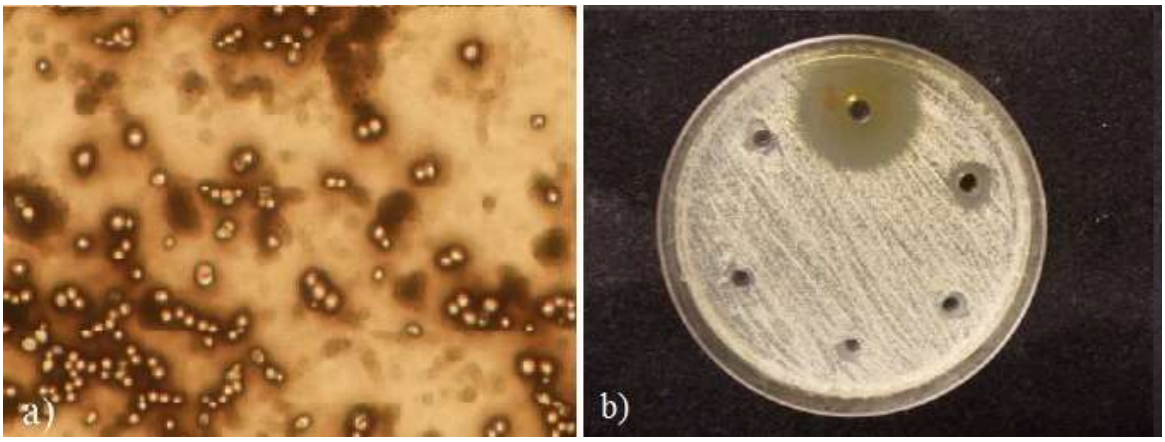

Figure 1. *Cryptococcus laurentii*. (a) Microscopic examination (fresh examination, chinese ink), and (b) Effect exerted by leaf extract.

Table 5. Biological activity, presence or absence of inhibition halos when applying *G. tinctoria* leaf extract against *Cladophialophora*.

Species/extract	Day 7			Day 20		
	S.E	F.T	F.G	S.E	F.T	F.G
Cladophialophora						
Well 1		√			√	√
Well 2		-			-	-
Well 3		-			-	-
Well 4		-			-	-
Well 5		-			-	-
Well (N.C)		-			-	-

*Well 1: Pure extract. Well 2: 1:10 dilution. Well 3: 1:100 dilution. Well 4: 1:1000 dilution. Well 5: 1:10000 dilution. Well 6: negative control. ** (√): Presence of halo (inhibitory effect obtained by the extracts)); (-): Absence of halo; N.C: Negative control; SE: No inhibitory effect. FT: Fungistatic effect. FG: Fungicidal effect.

G. tinctoria a value of 19.95 ± 0.48% was found which is much higher than that reported for fruit pulps such as cherry (4.23-5.91%) (Mahmood et al., 2013), orange (6.40%) and lemon (7.04%) (Ibrahim et al., 2011). The difference between the values obtained for leaf and pulp is based on the fact that the role of proteins in living organisms is mainly structural. The ethereal extract or lipid fraction recorded a percentage of 1.15% which is considerably lower than those of cherry (6.39%), kiwi (4.36), plum (3.49%), the order of orange (0.33%) and papaya (0.16%) (Morillas-Ruiz and Delgado-Alarcón, 2012).

For crude fiber, a percentage of 12.40% was determined, which is similar to those reported for cherry (9.50%) (Lazos, 1991), and avocado (9.11%) (Morillas-Ruiz and Delgado-Alarcón, 2012); and considerably higher than orange (3.30%), kiwi (5.71%) and plum (3.0%) (Morillas-Ruiz and Delgado-Alarcón, 2012). Since raw fiber is the non-digestible portion of food and consists of cellulose, hemicellulose and lignin, this finding gives the *G. tinctoria* leaf a high dietary value.

Although the leaves of one plant species are not comparable with the pulp of other matrices (because they are different structural zones); in the case of *G. tinctoria* it is important to make such comparisons (in addition to highlighting its high percentage of raw fiber, high protein content and low ethereal fraction) since in the peasant culture of southern Chile the leaf is consumed directly as a salad and/or as infusions or beverages.

Secondary metabolites

The phenolic compounds constitute a group of secondary metabolites that present antioxidant capacity with multiple biological benefits for the human being. In the present work the concentration determined for polyphenols was 10684.20 ± 128.75 mg gallic acid/100 g DW; Similar to that reported for Curuba (10584.7 mg gallic acid/100 g DW), and higher than blackberry (1864.4 mg gallic

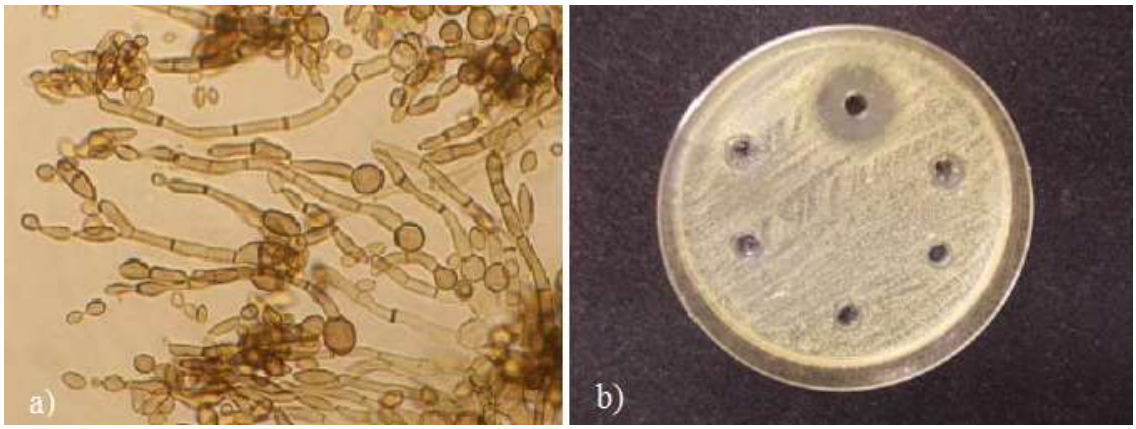

Figure 2. *Cladophialophora. (*a) Microscopic examination (fresh examination, Chinese ink), and (b) effect exerted by leaf extract.

acid/100 g DW), strawberry (1638.4 mg gallic acid/100 g DW), mortiño (1237.2 mg acid (Gallon acid/100 g DW), red apple (426.7 mg gallic acid/100 g DW), grape (290.6 mg gallic acid/100 g DW), cauliflower (440.3 mg gallic acid/100 g DW), kiwi (199.3 mg gallic acid/100 g DW) and carrot (160.0 mg gallic acid/100 g DW) (Zapata et al, 2014).

Compared to leaves of *Swietenia macrophylla*, *Swietenia humilis* and *Swietenia mahagoni*, the total polyphenol content of Nalca is vastly higher (10684.20 mg gallic acid/100 g DW versus 290.5; 250.91, and 146.4 mg gallic acid/100 g DW respectively) (Preciado et al., 2016). It also exceeds the concentrations reported in *Vaccinium meridionale Swart* (Green tea: 85.6 mg gallic acid/100 g DW and Black tea: 189.8 mg gallic acid/100 g DW) (Zapata-Vahos et al., 2015); and, to those of *Ilex paraguariensis* (commonly known as yerba mate) and *Ilex laurina*, which present a minor concentration (10.78 mg gallic acid/100 g DW and 23.70 mg gallic acid/100 g DW, respectively) (Perez et al., 2014).

Usually the leaf is one of the structural parts of plant species with higher content of bioactive compounds, since the synthesis of them is recognized as a necessity by supporting filter functions and protection against ultra violet light (Morales et al., 2017), support mechanical and intervention in the ecological interactions between the plant, its environment and predators, among others (Zobel et al., 1999, Chaves and Escudero, 1999, Zoratti et al., 2014, Ramakrishna and Ravishankar, 2011).

p-coumaric acid (pCA) is present in a wide variety of edible plants, fruits and vegetables (Clifford, 2000). It has been reported to have antioxidant, antimicrobial, anti-inflammatory properties, and it has also been suggested that its participation reduces the risk of stomach cancer (Ferguson et al., 2005) through the reduction of carcinogenic nitrosamines (Kikugawa et al., 1983). It was determined by HPLC-UV, obtaining a value of 38.41 mg/L. Similar to extracts of yerba mate (*Ilex laurina*): 47.32 mg/L; and that of *Ilex paraguariensis* 24.44 mg/L (Pérez et al., 2014). In terms of dry basis (48.69 mg p-coumaric acid/100 g DW) the results of *G. tinctoria* are much higher than those reported for leaves and ginger root: *Boesenbergia pulchella var attenuata*, *Boesenbergia armeniaca* (each with 4.0 mg/100 g DW) (Jing et al., 2010); and greater than Turmeric (*Curcuma longa*) (5.96 mg /100 g DW) (Wojdylo et al., 2007).

The metabolites catechin and epicatechin due to their antioxidant activity are reported as inhibitors and/or retarders of various types of cancer-skin, breasts, prostate and lung (Yang et al., 2002); and for having shown the capacity of arrest the cell cycle and induce apoptosis in cancerous cells (Gupta et al., 2010; Chena et al., 2010). The calculated values for these bioactive compounds were 1060.98 and 1127.49 mg epicatechin/L, respectively (Figure 3). In terms of dry basis, concentrations of 1344.97 mg catechin/100 g DW and 1429.28 mg epicatechin/100 g was determined. These results are higher than those reported for black tea from Mortiño leaves (*V. meridionale Swartz*): 437-972 mg of catechin/100 g DW and 470-1014 mg epicatechin/100 g DW (Zapata-Vahos et al., 2015). On the other hand, compared to the quantified for *Camellia sinensis* (green tea), the leaf extract of *G. tinctoria* showed higher values of both catechin (1060.98 mg/L v/s 270 mg/L) and epicatechin (1127.49 mg/L v/s 738mg/L) (Moore et al., 2009).

Antioxidant capacity

One of the most reliable methodologies for measuring antioxidant capacity is DPPH; since it quantifies only antioxidant compounds, and also presents a high selectivity due to its high molecular impediment, so that the antioxidants that neutralize this radical must be very efficient (Prior et al., 2005; Osman et al., 2006).

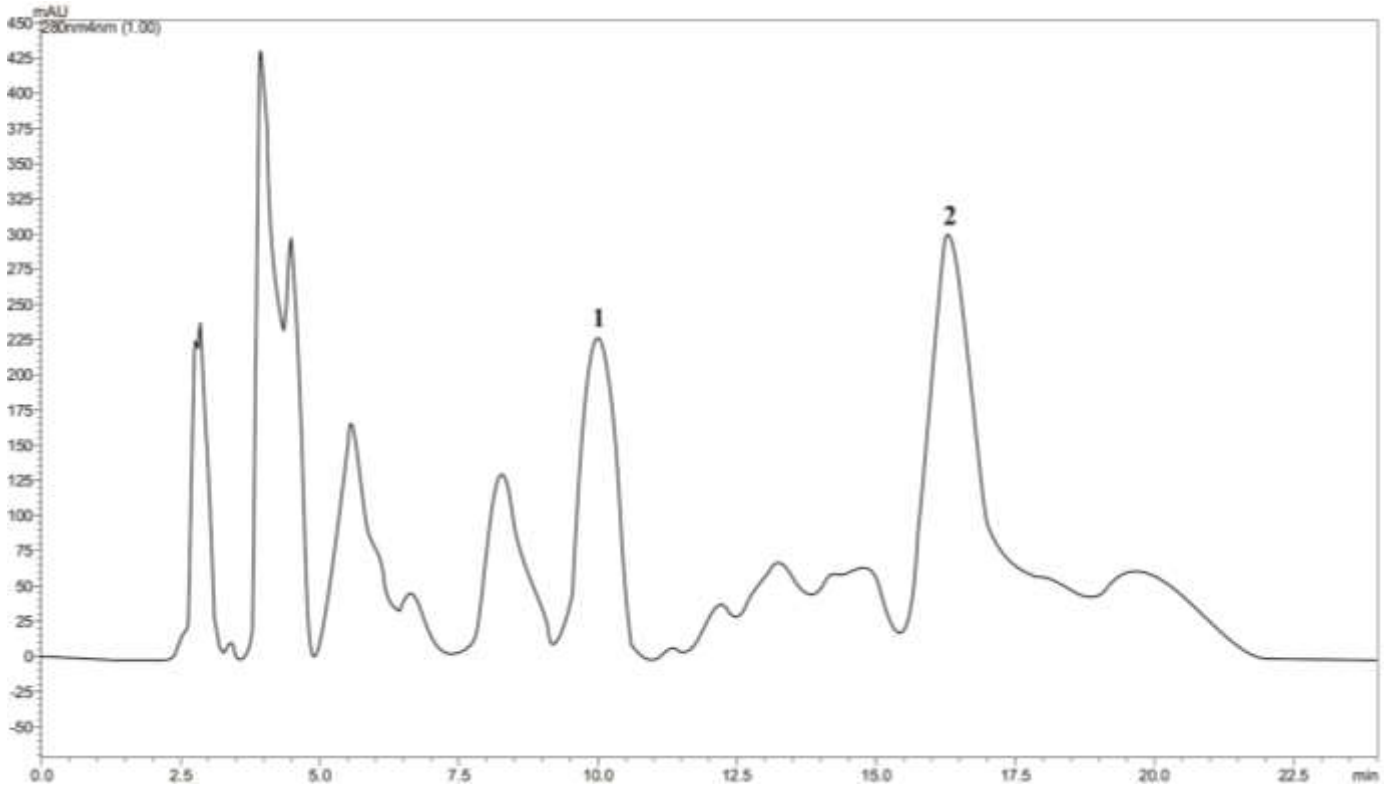

Figure 3. HPLC-PDA chromatograms at 280 nm of (1) catechin, (2) epicatechin from Nalca leaf collected in the commune of Los Lagos, XIV region of Chile.

In this investigation the antioxidant capacity of *G. tinctoria* was 83266.76 um Trolox/100 g DW. On the other hand, the antioxidant activities described for *S. macrophylla*, *S. mahagoni* and *S. humilis* (Preciado et al., 2016) are largely overcome by the leaves of Nalca (83266.76 µmol Trolox/100 g DW versus 16190; 10710 and 8900 µmol Trolox/100 g DW respectively). As well as it is larger and in the order of the extracts made from green tea leaf and black tea of mortiño (49158 and 111815 µmol Trolox/100 g DW, respectively) (Zapata-Vahos et al., 2015).

Considering the characteristics and nature of the bioactive compounds it is necessary to combine and execute other techniques that measure the antioxidant capacity of these compounds. In this way, the ABTS methodology has been used extensively to determine it. This test is based on the quantification of the discoloration of the ABTS•+ radical, due to the interaction with hydrogen or electron donor species (Moon and Shibamoto, 2009). According to Lu et al. (2010) the ABTS methodology is a fast and stable technique, which is applied mainly to antioxidants of an aqueous nature. Results obtained from ABTS for Nalca leaf (*G. tinctoria*) indicate a concentration of 120094.22 µmol Trolox/100 g DW. Values higher than those obtained by Kopjar et al. (2015), who in Croatia reported for infusions of yellow, green and black tea leaves concentrations of 40733, 32815 and 27032 µmol Trolox/100 g DW respectively; at 1810 µmol Trolox/100 g DW reported for leaf of *Punica granatum L*. (Pomegranate) (Elfalleh et al., 2012).

In the comparison of the results of the antioxidant activity obtained by both methods, it can be observed that the values for DPPH are lower than those obtained in ABTS. According to Heim et al. (2002), the OH groups at the 3'-, 4'-, 5'- positions of the B ring of flavonoids increase the antioxidant activity of the compound (unlike phenolic compounds with a single group Hydroxyl). And, from the point of view of free radicals, these cause different reactivity with phenolic compounds depending on their structure (Singleton and Rossi, 1965). According to this the ABTS radical has low selectivity in reactions with hydrogen donor atoms (it reacts with any hydroxylated aromatic compound independent of its real antioxidant potential) (Campos and Lissi, 1997). Unlike DPPH, it does not react with flavonoids lacking OH groups in a B ring, nor does it with aromatic acids containing only one OH group (Roginsky and Lissi, 2005). In addition, the results of the study clearly suggest that the antioxidant compounds present in *G. tinctoria* leaves are highly hydrophilic and therefore more sensitive to the ABTS technique.

Through the FRAP assay, the presence of metabolites

with the capacity to stabilize free radicals through the electron transfer mechanism (SET) is expressed. The leaf extract showed an antioxidant capacity of 11194.50 mg ascorbic acid/100 g DW; higher by 42.06% than that reported for Mortiño (*V. meridionale*) green tea leaf (7880 mg Ascorbic acid/100 g DW) (Zapata-Vahos et al., 2015). And, of the order of black tea leaf of Mortiño (15780 mg ascorbic acid/100 g DW) (Zapata-Vahos et al., 2015).

The antioxidant efficacy determined by this assay depends on the redox potentials of the compounds present in the extract, so that, from the study results, it is feasible to indicate that the high reducing capacity of polyphenols is related to the degree of hydroxylation and conjugation of the polyphenol compounds (Pulido et al., 2000).

According to Prior et al. (2005), both the FRAP and Folin-Ciocalteu methods to quantify total phenols are capable of detecting any reducing substance, presenting interference with, among others, free reducing sugars and organic acids such as ascorbic acid. In order to compare this information, the concentration of reducing sugars was quantified by means of the colorimetric technique with DNS (3,5 dinitroxalicylic acid), which was 7353.39 mg equivalent glucose/100 g DW; and by HPLC technique a Vitamin C value of 46.79 mg ascorbic acid/100 g DW.

Since the total phenol test showed results much higher than those reported by other studies, and that on the contrary FRAP was of the order of the mentioned vegetal species; it is possible that the compounds involved were more sensitive to basic pH. Therefore, taking into account that both methodologies may be responding positively to reducing sugars or other components that are not involved in the antioxidant activity, the yields were verified by techniques such as ABST, DPPH and ORAC, which quantify only antioxidant metabolites.

The ORAC methodology measures the ability of the polyphenols to trap the peroxyl radicals generated *in situ* through a hydrogen atom transfer (HAT) proton transfer mechanism; and is the most commonly used technique to measure the total antioxidant potential of foods and nutritional supplements (Wu et al., 2004). It provides a controllable source of peroxyl radicals and can be adapted to detect both hydrophilic and hydrophobic antioxidants by handling the source of the radical and the solvent (Prior et al., 2003; Ou et al., 2005). The antioxidant activity measured by the ORAC method for the Nalca leaf yielded a value of 192006.07 µmol Trolox/100 g DW. These values suggest that the *G. tinctoria* leaf has a high content of hydrophilic antioxidants with the ability to neutralize radicals ROO• peroxides. These are superior to those reported for *S. mahagoni* (125520 µmol Trolox/100 g DW) (Preciado et al., 2016); green tea and black of Mortiño (*V. meridionale*) (77880 and 149682 µmol Trolox / 100 g DW) (Zapata-Vahos et al., 2015); yerba mate (*Ilex laurina*) (58880 µmol Trolox / 100 g DW), *Ilex paraguariensis* (35990 µmol Trolox/100 g DW) (Pérez et al., 2014); *Camellia euphlebia*, *Camellia microcarpa* and *Camellia nitidissima* (85640, 59630 and 52490 µmol Trolox/100 g DW respectively (Song et al., 2011); freeze-dried leaves of Boldo (*Peumus boldus Molina*) (46160 µmol Trolox/100 g DW) (Soto et al. , 2014), acai (43795.7 µmol Trolox/100 g DW), guayava (11988.2 µmol Trolox/100 g DW), cauliflower (10617.7 µmol Trolox/100 g DW), and kiwi (4651.1 µmol Trolox/100 g DW) (Zapata et al., 2014).\

The metabolite content and the antioxidant activity obtained through the ORAC method (together with those quantified by the other methodologies described) are relevant evidence for the study; since they are above those described for other species of recognized pharmaceutical capacity. The antioxidant capacity of *G. tinctoria* leaf measured through ORAC is 147 and 28% higher than the leaf of green tea and black mortiño, a plant species which has shown the ability to reduce the cell viability of SW480 (colon adenocarcinoma cell line) (Zapata-Vahos et al., 2015). A 300% superior to the infusions of *Paemus boldus*, endemic tree of Chile recognized for behaving as an antioxidant, chemo-preventive, digestive stimulant, diuretic, relaxing and in the treatment of disorders of liver and gallbladder (Soto et al., 2014; Simirgiotis and Schmeda-Hirschmann, 2010, Fernandez et al., 2009), and, 226 and 433% higher than extracts prepared from leaves of species of the *Ilex family* (which are widely consumed as tea-like beverage in Argentina, Uruguay, southern Brazil, Bolivia, Chilean Patagonia), and which have been reported to be capable of inhibiting the growth of metastatic-derived cells (SW620) and of primary human colon adenocarcinoma cells SW480, HT-29 and CaCo-2 (Perez et al., 2014; González de Mejia et al., 2010).

Antifungal activity

According to Gramza and Korczak (2005) polyphenols can inhibit the growth of species of the genus *Clostridium* and *Helicobacter pylori*. It has also been shown that certain extracts of antioxidant tea inhibitors inhibit some food-borne pathogenic microorganisms such as *Bacillus cereus* (Almajano et al., 2008; Si et al., 2006), *Staphylococcus aureus* (Kim et al., 2004; Si et al., 2006; Taguri et al., 2004), *Vibrio cholerae* (Taguri et al., 2004), *Listeria monocytogenes* (Kim et al., 2004; Kim and Fung, 2004; Si et al., 2006), *Escherichia coli* (Cho et al., 2007; Kim and Fung, 2004; Si et al., 2006), *Salmonella typhimurium* (Si et al., 2006), *Salmonella enteritidis* (Kim and Fung, 2004), *Micrococcus luteus* y *Pseudomona aeruginosa* (Almajano et al., 2008); and that in particular green tea catechins may decrease the resistance of *S. aureus* to antibiotics such as oxacillin (Stapleton et al., 2004).

It is possible to attribute this antagonistic effect to the high content of metabolites reported in the Nalca leaf and its synergistic effect, which is directly related to its

antioxidant and biological activity. It is precisely the complex mixture of compounds (usually of low molecular weight) that are part of the leaves of the plants (glucosides, saponins, tannins, alkaloids, polyphenols, flavonols, flavonoids, catechins, epicagallocatechin, organic acids and volatile components, terpenes, among others) (Tajkarimi et al., 2010) that contribute to the antimicrobial activity.

The high levels of catechin and epicatechin obtained from leaf of *C. sinensis* have shown the ability to inhibit the *in vitro* growth of *Candida albicans* (Anand and Rai, 2017; Ning et al., 2015), the most common human fungal pathogen (Pfaller and Diekema, 2007), associated with superficial infections of the skin, hair, oral or vaginal tract and with life-threatening systemic infections (Luo et al., 2013; Paramythiotou et al., 2014). Also, *in vitro* antibacterial activity was reported by Riso et al. (2002) against *E. coli*, *Pseudomonas aeruginosa* and *S. aureus*.

According to Avello et al. (2012), the possible antifungal mechanisms may be due to the volatility and low molecular weight of most of the compounds mentioned, since they can cross resistance structures of the fungal species. Thus, both cytoplasmic membrane damage, interference in folate metabolism and reduction in ergosterol content, promote the formation of pores through which ions and molecules (mainly K^+ and H^+) diffuse, which ultimately compromise viability of the membrane and that according to the concentrations of the extract applied would affect the production of ATP. Also, due to the mixing of a large number of components present, they are considered low risk in the development of resistance by the pathogens, since each of the components has its own biological activity, and it is difficult to correlate the action of a single compound or classes of compounds, because the fungitoxic potential is the result of the synergistic action of the constituents (Burt, 2004; Yahyzadeh et al., 2008).

The *in vitro* antifungal activity of *G. tinctoria* leaf extract was evaluated qualitatively by the presence of inhibition halos. Figure 2 shows the inhibition halos against two fungi, *C*. *laurentii* (yeast) and *Cladophialophora* (mold).

In this research and supported by the studies conducted by Neri et al. (2006) and Amiri et al. (2008) the fungistatic effect was considered when a reactivation of mycelial growth was observed after seven days of incubation, and effect fungicide when there was no mycelial growth of the pathogen after the period of incubation and observation that corresponded to the period of 20 days. According to Gajanan et al. (2011), the fungicidal effect in the laboratory refers to the agent that decreases the colonies by 99.9% from a pure subculture inoculum. In this way, the pure extract of leaf, produced in laboratory conditions have inhibitory effects (fungicidal effect) on both microorganisms; since the effect remained unchanged until the observation made on day 20. In parallel, there were negative controls (Well 6), which after their incubation showed an abundant growth.

These results are promising because in cheeses conserved (even in refrigeration) species of the genus *Cryptococcus* predominate; which interferes in the maturation of this product by metabolizing the lactic acid, raising the pH, favoring the growth of proteolytic bacteria and generating fructifications, undesirable levaduriform flavors and an unpleasant texture for the appearance of the product (Pereira-Dias et al., 2000; Orberá, 2004). On the other hand, in the food industry, *Cladophialophora* has been isolated in non-integral maize flour, tomatoes, carrots, apples, strawberries and pears (Badali et al., 2008; Romão-Dumaresq et al., 2016). In addition, taking into account the content of metabolites and the antioxidant capacity presented by *G. tinctoria*, it is possible to project its biological activity on various microorganisms and to present itself as an alternative to the antagonistic action exerted by extracts from native and endemic Chilean trees such as Laurel (*Laurelia sempervirens*), Boldo (*Peumus boldus*) and Tepa (*Laureliopsis philippiana*) against pathogenic strains of plant species such as *Rhizoctonia solani*, *Pythium irregulare*, *Ceratocystis pilífera*, *Phragmidium violaceum* and *Fusarium oxysporum* (Bittner et al., 2009).

Conclusions

In this research, the antioxidant and antifungal potential of *G. tinctoria* was reported, highlighting its added value compared with other plants, fruits and vegetables that are of mass consumption. The results of this research suggest that the *G. tinctoria* leaf is a rich source of polyphenols and secondary metabolites, which intervene in the high antioxidant capacity quantified by this study. According to this it is possible to consider it as (1) a functional food and rich source of antioxidants and bioactive compounds, and (2) as an antifungal and/or therapeutic alternative.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Optimization of enzyme assisted extraction of polysaccharides from *Poria cocos*

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Water-soluble polysaccharide was isolated from *Poria cocus* by enzyme-assisted method using orthogonal methodology. By single factor test and orthogonal group, the extraction conditions of water-soluble polysaccharide were investigated, such as liquid-solid ratio, temperature, time and pH. The optimum conditions for single factor were as follows Enzyme concentration (%), Extraction temperature oC, Extraction time/h, Extraction pH. The result revealed that 2% complex enzyme, remained most important factor of polysaccharide extraction, followed by temperature and pH. The liquid-solid ratio was 1:50, temperature was 40°C, time was 3.0 h, and pH was 5. The highest extraction rate of crude polysaccharide remained 4.14%. Results indicated that the complex-enzyme assisted remained best technique for extracting polysaccharide from *P. cocus*. It proved to be as highly effective as well as energy and time saving extraction techniques.

Key words: *Poria cocus* enzyme assisted extraction, polysaccharides, alpha amylase, cellulase, Taka-diastase.

INTRODUCTION

Enzyme-assisted extraction (EAE) has been demonstrated to be effective in improving the yield of the model component (Li et al., 2006). Mushrooms have long been used as oriental medicine, as well as dietary supplements and in natural cosmetics (Kaneno et al., 2004). *Poria cocus* is a fungus that grows on the roots of pine trees, and used as most important traditional medicines in China and other Asian countries; moreover, it consists of culinary and medicinal properties such as anti-inflammatory, antitumor, complement activating, and immune stimulating activities (Kanayama et al., 1983; Lee and Jeon, 2003; Yasukawa et al., 1998; Yu and Tseng, 1996). *P. cocus* mainly contain polysaccharide (Jia et al., 2016; Wu et al., 2016). The major chemical constituents of *P. cocus* are polysaccharides, triterpenoids, ergosterol and proteins (Tai et al., 1992, 1995a, b; Wang and Wan, 1998; Yang and Bao, 2005).

Polysaccharide is a vital bioactive substance with some

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physiological functions, such as immune, regulating cell growth and senescence (Jin and Xu, 2002). It can be widely used in medicine, health products and functional foods. Many mushrooms revealed a great range of the healthy properties of polysaccharides, being the most significant modulation of the immune system. Bioactive carbohydrates, including β-glucans, are generally examined as biological response modifiers (BRM) (Wasser, 2002). *P. cocus* useful in health products it can be used as raw material to produce various kinds of health products, such as a *P. cocus* polysaccharide capsule, tablet, electuary and so on. Hot water extraction of several mushrooms used in traditional Chinese medicine believed to be effective in the treatment of different diseases including many forms of cancer.

The enzymatic extraction process is manipulated by several factors, including enzyme concentration, temperature, pH, activators and/or inhibitors (Dam, 2004). While an enzyme is a protein-based catalyst, its rate of reaction depends on its concentration and has an optimum temperature at which shows maximum. Stirring speed also facilitates contact and the reaction between the substrate and the catalyst. An optimization of reaction time is important to ensure maximum extraction without degrading product quality. In order to get the pharmacological composition of mushroom presently, the extraction methods of mushroom polysaccharides (MP), generally include hot water extraction, ultrasonic extraction, enzyme extraction, alkaline extraction, acid extraction and microwave extraction (Jiang et al., 2008; Ghosh et al., 2005; Huang and Ning, 2010; Yu et al., 2009). These methods are regularly associated with higher temperature, extended extraction time and excessive energy consumption and lower yield. Whereas the enzyme assisted extraction with lower temperature, less time, less power consumption and high extraction yield is arising technology in the food industry.

It is therefore necessary to evaluate on the extraction method of polysaccharides; the comparative analysis about extraction method of *P. cocus* polysaccharides has seldom been studied.

Orthographic graph is a statistic method to make the complex work simple. In this investigation, (A) complex enzyme amount (B) extraction temperature (C) extraction time and (D) extraction pH were selected as four independent variables, combined by three levels which formed the orthographic graph (Table 1). Furthermore, all three Taka-diastase, alpha amylase and cellulose enzyme were separately investigated; also, four independent variables (A) enzyme amount (B) extraction temperature (C) extraction time and (D) extraction pH were selected (Tables 2, 3 and 4). This diagram was used to optimize the amount of extract and polysaccharides yield. The aim of this work is to examine the performance of a combination of cellulose, alpha amylase, Taka-diastase, and complex-enzyme-hydrolysis-assist) extraction from *P. cocus*. After a set of prospective tests, four parameters (enzyme ratio, extraction temperature, extraction time and extraction pH) were optimised in order to improve the yields of polysaccharides extraction.

MATERIALS AND METHODS

The samples of *P. cocus* and enzymes were obtained from Wuhan, Hubei Province, China. Other analytical grade reagents were obtained from the Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

Isolation of polysaccharides from *Poria cocos* using complex enzyme

The *P. cocus* were cut into pieces, ground by electric mill, and sieved through screens 60-80 mesh (LD-Y500A, Ding Shuai Hardware Products Co., Ltd. Zhejiang, China). Ten grams of the powder were mixed in 200 mL distilled water respectively, of 9 bottles in which the experiments were carried out. The amounts of complex enzyme were also added corresponding to Table 2. The bottles were labelled and pH for extraction were adjusted for all samples shown in Table 2. The bottles were placed in a water bath at the temperature of 40, 50 and 60°C at extraction time of 3 h, 2 h and 1.5 h, respectively.

The sample was filtered and concentrated to about one-fifth of the original volume in a rotary evaporator (Model RE-2000A Yarong Bio-instrument Shanghai, China) at 40°C. One part of the solution was dried into solid and powdered to calculate the amount of extract. Ethanol was added into the remaining part and kept at room temperature for 24 h. The precipitate was collected after centrifugation (4,000 r/min, 15 min), and then dried to obtain the desired crude polysaccharides (Thulasi et al., 2008). Furthermore, the same methods as mentioned above were applied for the Taka-diastase, alpha amylase and cellulose enzyme and their amount of enzyme and pH were computed in Tables 3, 4 and 5 respectively.

Determination of amount of extract and polysaccharides yield

Glucose was accurately weighted 0.0200 g and dried in 105°C prior to dissolving in 250 mL and the standard solution was diluted to different concentrations making up the final concentration with pure water. Following that, 1.0 mL phenol and 5 mL sulfuric acid were added in 1.0 mL of glucose solution for each concentration, mixed by vortex and reacting samples for 10 min in boiling water bath and cooled at room temperature. The absorbance of each tube was measured at 490 nm with an ultraviolet-visible spectrophotometer (1.0 mL); distilled water was used as blank; standard curve was obtained with x-coordinate for concentration of glucose and y-coordinate for absorption value, and finally, based on the equation of glucose standard curve, the glucose contents of the samples were determined. Considering the dilution ratio, the amount of extract and polysaccharides yield was calculated by the following equation.

$$\text{Amount of extract (\%)} = \frac{\text{Weight of extraction (g)}}{\text{Weight of } \textit{Poria} \text{ powder (g)}} \times 100$$

$$\text{Polysaccharides yield (\%)} = \frac{\text{Polysaccharides yield (g)}}{\text{Weight of } \textit{Poria} \text{ powder (g)}} \times 100$$

Table 1. L9 (3^4) Orthogonal table of complex enzyme method

Level	Complex enzyme amount (%) [A]	Extraction Temperature (°C) [B]	Extraction Time (h) [C]	Extraction pH [D]
1	1.5	40	3	5
2	2	50	2	6
3	2.5	60	1	7

Table 2. L9 (3^4) Orthogonal table of Taka Distances enzyme method.

Level	Complex enzyme amount (%) [A]	Extraction Temperature (°C) [B]	Extraction Time (h) [C]	Extraction pH [D]
1	0.835(g)	40	3	5
2	0.01112(g)	50	2	6
3	0.03896(g)	60	1	7

Table 3. L9 (3^4) Orthogonal table of Alfa amylase enzyme method.

Level	Complex enzyme amount (%) [A]	Extraction Temperature (°C) [B]	Extraction Time (h) [C]	Extraction pH [D]
1	0.835(g)	40	3	5
2	0.01112(g)	50	2	6
3	0.03896(g)	60	1	7

Table 4. L9 (3^4) Orthogonal table of Cellulose enzyme method.

Level	Complex enzyme amount (%) [A]	Extraction Temperature (°C) [B]	Extraction Time (h) [C]	Extraction pH [D]
1	0.835(g)	40	3	5
2	0.01112(g)	50	2	6
3	0.03896(g)	60	1	7

RESULTS

Complex enzyme extraction method

Results of amount of extract and polysaccharides yield with complex enzyme are shown in Table 5. On the basis of single factor experiments, four factors and three levels of L9 (3^4) orthogonal test were selected. Conditions of pH and temperature were modified in each experiment to match the optimum conditions of the measured enzyme. The experiments were carried out for complex enzyme 1.5%, extraction temperature at 40°C, extraction time of 3 h and extraction pH at 5. After this condition, the amount of extract was 4.14% and polysaccharides yield was 5.3%. Furthermore, the results of 9 samples demonstrated in Table 5 and Sample 4 shows the highest amount of extract with Sample 1 showing the higher polysaccharides yield, that is, 7.6%. In order to get the consequence of different independent variables and their levels on polysaccharides yield and amount of extract, the K factor along with R factor were calculated are presented in Table 5.

Cellulose enzyme extraction method

The results of the polysaccharides yield and the amount of extract with cellulose enzyme are shown in Table 6. On the basis of single factor experiments, four factors and three levels of L9 (3^4) orthogonal test were selected. The factor levels are revealed in Table 2. The result showed that highest amount of extract was 8.4%, polysaccharides yield of 2.60% was observed at 50°C with the cellulose enzyme amount of 0.835. Furthermore, the results of 8 samples showed that sample 4 show lowest amount of extract 5.6% and sample 3 show lowest polysaccharides yield 1.76%. In addition, to get the result of different independent variables and their levels on polysaccharides yield and amount of extract, the K factor and R factor were calculated and presented in Table 6. Whereas, K is the standard value of related results of every independent variable. The factors which have an

Table 5. L9 (3^4) Orthogonal experiment results of complex enzyme extraction method (Cellulose, Amylase and Taka-diastase).

Level	Complex enzyme amount (g)	Extraction temperature	Extraction time	Extraction pH	Polysaccharide yield	Amount of extract
1	A1	B1	C1	D1	4.14	5.3
2	A1	B2	C2	D2	2.28	4.5
3	A1	B3	C3	D3	3.88	5.6
4	A2	B1	C2	D3	2.85	7.6
5	A2	B2	C3	D1	2.51	5.9
6	A2	B3	C1	D2	2.2	7.5
7	A3	B1	C3	D2	1.34	6.1
8	A3	B2	C1	D3	3.16	5.9
9	A3	B3	C2	D1	2.71	4.8
						Remarks
K1	5.1	2.77	2.77	5		
K2	7.0	2.85	2.65	5.5	A>D>C>B	
K3	5.6	2.93	2.93	6.0		
R	1.9	0.16	0.28	1.0		

R refers to the result of extreme analysis.

impact on the polysaccharides yield in appropriate order were extraction complex enzyme amount >pH>extraction time > extraction temperature.

Alpha amylase enzyme extraction method

The results for the polysaccharides yield and amount of extract with alpha amylase are shown in Table 7. On the basis of single factor experiments, four factors and three levels of L9 (3^4) orthogonal test were selected (Table 3). The highest amount of the extract 7.6% was in Sample 2, moreover the higher polysaccharides yield 4.24% was found in sample 9 and extract were 6.5%. In order to get the effect of different independent variables and their levels on polysaccharides yield and amount of extract, the K factor along with an R factor were calculated as found in Table 7. The factors which have an impact on the polysaccharides yield in appropriate order were extraction enzyme amount >extraction temperature > extraction time > pH.

Taka-diastase enzyme extraction method

The results of the polysaccharides yield and the amount of extract with Taka-diastase are shown in Table 8. On the basis of single factor experiments, four factors and three levels of L9 (3^4) orthogonal test was selected. The factor levels were revealed in Table 4.The experiments were carried out, for example, condition of extraction Sample 1 was a complex enzyme amount of 1.5%, extraction temperature at 40°C extraction time of 3 h and extraction pH at 5. The result shows that the highest amounts was found in Sample 3 with extract of (7.4%), however, the high yield of polysaccharides (4.07%) was found in Sample 8. In order to get the significance of different independent variables and their levels on polysaccharides yield and amount of extract, the K and R factors were calculated as found in Table 8. The factors which have an impact on the polysaccharides yield in appropriate order were extraction temperature > extraction enzyme amount < extraction time > pH.

DISCUSSION

The use of medicinal mushroom extracts for the medicinal use is recognized and familiar in China, Japan, Korea, Russia and now increasingly in the USA (Mizuno et al., 1995). A number of methods have been developed to extract anti-cancer polysaccharides from mushroom fruit-bodies, mycelium and liquid media (Mizuno, 1999). In this study, extraction condition of total polysaccharides for effective yield enzyme amount, extraction temperature, extraction time and extraction pH is investigated. According to results of the orthogonal trial, these four methods were evaluated. Recently, several methods of extracting polysaccharides from mushrooms are reported, such as microwave assisted extraction, water extraction, ultrasonic assisted extraction and some enzymatic assisted methods (Boroushaki et al., 2010; Dkhil et al., 2011; Radheed et al., 2003). The literature reported that the function of enzyme is to degrade cell wall constituents and discharge intracellular contents. Generally, a plant cell wall comprises cellulose, hemicelluloses and pectin, while flesh is main content for pectin and proteins.

Cellulases and pectinases can therefore be used for

Table 6. L9 (3^4) Orthogonal experiment results of Cellulose enzyme extraction method.

Sample No.	Cellulose enzyme amount (g)	Extraction temperature	Extraction time	Extraction pH	Polysaccharide yield	Amount of extract
1	A1	B1	C1	D1	2.16	8.4
2	A1	B2	C2	D2	2.60	7.6
3	A1	B3	C3	D3	1.76	8.2
4	A2	B1	C2	D3	1.24	5.6
5	A2	B2	C3	D1	1.92	6.8
6	A2	B3	C1	D2	2.09	6.9
7	A3	B1	C3	D2	2.08	6.4
8	A3	B2	C1	D3	2.09	6.5
9	A3	B3	C2	D1	2.08	8.1
						Remarks
K1	7.76	2.17	1.82	4.84		
K2	6.53	1.75	2.20	4.74	A> B> C>D	
K3	7.20	1.96	1.85	4.91		
R	1.23	0.42	0.38	0.17		

R refers to the result of extreme analysis.

Table 7. L9 (3^4) Orthogonal experiment results of Alfa Amylase enzyme extraction method.

Sample No	Alfa Amylase enzyme amount (g)	Extraction temperature	Extraction time	Extraction pH	Polysaccharide yield	Amount of extract
1	A1	B1	C1	D1	3.04	6.5
2	A1	B2	C2	D2	1.59	7.6
3	A1	B3	C3	D3	2.2	7.3
4	A2	B1	C2	D3	3.24	6.7
5	A2	B2	C3	D1	3.1	6.4
6	A2	B3	C1	D2	2.65	7.1
7	A3	B1	C3	D2	4.24	6.6
8	A3	B2	C1	D3	2.04	7.4
9	A3	B3	C2	D1	1.48	6.5
						Remarks
K1	7.13	2.27	3.56	4.84		
K2	6.73	2.99	2.24	4.74	C> B>A>D	
K3	6.83	2.64	2.11	4.91		
R	0.40	0.72	1.45	0.17		

R refers to the result of extreme analysis.

degradation of cell structure in the extraction process (Fernandes, 2010). It is observed from the results, that the extraction yield was decreased with the increasing of temperature from 40 to 50°C whereas there was no significant change found amongst all samples. According to Chunhua et al. (2014), enzyme assistant extraction method in polysaccharide extraction is an innovative research in recent years and has advantages over the conventional hot water extraction. Furthermore, complex enzyme hydrolysis method can be performed under low bath temperature (47°C) and the yield of the polysaccharide significantly improved to microwave-assist extraction and hot water extraction method (Chunhua et al., 2014). Enzyme assisted extraction is an advanced and efficient extraction method (Jiang et al., 2010).

Moreover, the study was carried out by the single enzyme assist extraction method, the results of the cellulose, alpha amylase and Taka-diastase enzymes

Table 8. L9 (3^4) Orthogonal experiment results of Taka-diastaseenzyme extraction method.

Sample No	Taka Distance enzyme amount (g)	Extraction temperature	Extraction time	Extraction pH	Polysaccharide yield	Amount of extract
1	A1	B1	C1	D1	1.37	6.4
2	A1	B2	C2	D2	2.53	7.1
3	A1	B3	C3	D3	1.82	7.4
4	A2	B1	C2	D3	3.93	6.1
5	A2	B2	C3	D1	3.63	6.0
6	A2	B3	C1	D2	3.19	5.8
7	A3	B1	C3	D2	3.31	5.8
8	A3	B2	C1	D3	4.07	7.0
9	A3	B3	C2	D1	3.27	5.7
					Remarks	
K1	6.96	1.90	2.87	4.87	B>A>C>D	
K2	5.96	3.58	3.26	4.88		
K3	6.16	3.55	2.76	4.84		
R	1.0	1.68	0.50	0.04		

R refers to the result of extreme analysis.

experiment as were shown in Tables 6, 7 and 8. However, in present study, the highest extraction yield amongst the three enzymes was 4.24% in the optimal conditions (50°C, 120 min,) with alpha amylase. In complex enzyme assay, extraction method and pH are the main factors which manipulated the effective extraction both for amount of extract and polysaccharides yield. pH can affect enzyme activity because every enzyme possesses their optimal pH, while the change of pH affects the structure of enzyme and enzymatic activity (Yin et al., 2011). Further study of Yin et al. (2011) explained the pH can affect enzyme activity as different enzymes have their own optimal pH. Study further revealed that extraction time remained a significant factor to affect the polysaccharides yield; similar findings are reported by Chunhua et al. (2014) in the extraction of polysaccharide from ganoderma lucidum.

The study further demonstrated that yield of polysaccharide increased with increase in temperature and amount of the extract enhanced with different enzymes. The correlated result was reported that due to a higher temperature, the polysaccharides matter leaved out from the mushroom particles into the solution (Li et al., 2006). Long extraction time observed plays a positive role in the production of polysaccharides (Ros et al., 2004). Multi enzymatic hydrolysis helps to split linkage and assists in releasing the polysaccharide, increasing the rate of extraction. Furthermore, the enzyme-hydrolysis-assist extraction showed its advantages in extraction conditions and great specificity for application.

In the meantime, enzyme hydrolysis assists extraction demonstrating its advantages in consideration for extraction conditions and extremely specific application. As it seldom damages the molecular three-dimensional structures, it consequently maintains the bioactivity of mushroom polysaccharide. Furthermore, it also helps to progress the purity of polysaccharide. This result will be consistent with that previous experiment in which the polysaccharide was extracted from the fruit body of mushroom *C. maxima* (Yang et al., 2011). Multi enzymatic hydrolysis, help to break down the linkage and assist in discharging the polysaccharide and raise the extraction rate as reported by Chen et al. (2013).

Conclusions

Extraction pH is the most important factor which influenced the extraction efficiency both for amount of extract and polysaccharides yield. Enzyme-hydrolysis-assist extraction remained effective. It can hydrolyze the cellulose, pectin and break down the cell walls. This study reports an effective extraction method for poly-saccharide, the best optimal conditions were determined: complex enzyme amount of 2%, extraction temperature at 40°C, extraction time is 3 h and extraction pH is 5. While the best optimal conditions for the amount of extract were as follow: complex enzyme amount of 2%, extraction temperature at 60°C, extraction time 1 hr and extraction pH is 6. Furthermore best optimal conditions with alpha amylase assist extraction as follows for enzyme amount of 0.03896%, extraction temperature at 50°C, extraction time of 2 h, and extraction pH is 6. Further studies on the complex enzyme hydrolysis assist extraction and single enzyme necessitates is suggested for finding out the best extraction condition.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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