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Phytochemical standardization of hydroalcoholic extracts of ishpingo, *Ocotea quixos* (Lam.) Kosterm

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Investigations have proved the presence of components such as cinamaldehyde, cinamyl acetate and phenolic compounds in *Ocotea quixos,* these components have been attributed to antimicrobial disinfectants, antifungal and healing activities. It is important to point out that, to evaluate any biological activity, it is necessary to have standardized extracts to obtain reliable and reproducible results. The present investigation consist of standardizer hydroalcoholic extracts of ishpingo, *O. quixos,* evaluating the influence of three experimental variables: vegetal material (fresh and dry), solvent (ethanol and cane ethanol) and solvent concentration (90, 70 and 50%) v/v through an experimental factorial design, from which 12 different extracts with 3 repetitions of each were obtained. The extracts were obtained through a percolation process considering the variables of the experimental design. A phytochemical screening showed the presence of polyphenols, tannins, catechins, saponins, quinones, coumarins, lactones and alkaloids in the 36 extracts; the Folin- Ciocalteu method was used in order to quantitatively determine the total phenol concentration and, by statistical analysis ANOVA (Tukey 95%), it was proved that there is a significant difference between the 12 established treatments where the extracts with the highest quantity of polyphenols are AMF50:50 (alcohol, fresh material, concentration 50% v/v) and CMF90:10 (cane alcohol, fresh material, concentration 90% v/v) with 12,538 and 13, 298 mg of gallic acid / mL of extract, respectively; concluding that, this last combination obtain greater quantity of total polyphenols following the established standardized protocol.

Key words: *Ocotea quixos,* ishpingo, total polyphenols, folin ciocalteu, phytochemical screening, gallic acid.

INTRODUCTION

In recent years, ethnobotanical information has been very useful in the search for new active compounds with possible biological activity present in wild and domesticated plants (Santayana and Pellón, 2002). In addition, there is an increase in interest to know and verify the benefits of medicinal plants, which has led to the development of research to describe the cure of diseases (Martínez, 2006; Carrier et al., 2002).

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Ecuador has a wide variety of plants with pharmacological properties, which can be used as raw material for the preparation of extracts, by extraction, isolation and purification of natural substances (De la Torre et al., 2006). Observation allows a better analytical characterization of the compounds of interest (Sharapin, 2000).

Ocotea quixos is a species with very few studies; however, there are some references about the pharmacological potential of this species (Montealegre, 2011; Bruni et al., 2004; Naranjo et al., 1981). Nonetheless, it is known that interest in *O. quixos* began since 1514, as in that year the Amazonas River was discovered in the expedition commanded by Orellana, Pizarro and Spanish soldiers and some plants with therapeutic properties were found within this group *O. quixos*, which contains cinnamaldehyde and other compounds of pharmacological interest (Gottlieb, 1982; Ballabeni et al., 2007).

According to ethnobotanical knowledge, *O. quixos*, can be used to treat gastric and intestinal complaints, flu, colds, vomiting, diarrhea, wound healing and local anesthetic (Fundación Chankuap, 2014; Alain et al., 1994; Campos et al., 2002). Some investigations attribute the therapeutic properties to components such as cinnamaldehyde, cinnamyl acetate, cinnamyl alcohol and phenolic compounds (Montealegre, 2011). *O. quixos* contains five components of pharmacological importance (α-pinene, β-elemene, β-cariophyllene, germacrene-D y δ-cadinene) and the chemical characteristics of *O. quixos* are very different compare to other species of the same family (Takaku et al., 2007; Fréderick et al., 2005).

Recent research has demonstrated the presence of phenolic compounds in extracts of *O. quixos* by a qualitative test (Cárdenas-Tello et al., 2016). Rolli et al. (2014) demonstrated the presence of monoterpenes, sesquiterpenes and polyphenols, to those mentioned above; these has assumed to be the aptitude used to inhibit the microorganisms' growth which could be related to its antibacterial and antifungal activity, demonstrated in the research realized by Noriega and Decarro (2008). These secondary metabolites have been attributed to the ability to inhibit the growth of microorganisms, so that they may be in contact with their antibacterial and antifungal activity demonstrated in the research done by Noriega and Decarro (2008).

In few studies, *O. quixos* have use essential oils or extracts of different parts of the plant, to evaluate its therapeutic properties (Mosquera and Veloz, 2011; Noriega and Samaniego, 2006; Cazorla, 2013; Julkunen-Tiitto, 1985). Nevertheless, to evaluate any biological activity, it is important to count with phytochemically standardized extracts because this allows in the reduction of variables used in the investigation of the different biological activities and facilitating the reproducibility of the results (Miño, 2007). Also, it helps to secure a natural product which has been proven effective. The standardization of an extract generates specifications of percentage of groups' phytochemicals (Sharapin, 2000). This is why the present research seeks to obtain hydroalcoholic extracts of *O. quixos* phytochemically characterized taking as a reference the total polyphenols, to give scientific value to the traditional use of this species.

The extract will act as a chemical marker from which future research can be carried out reducing the variability of the results and improving its reproducibility, since it has a raw material with proven effectiveness. In addition, a protocol will be provided to obtain the extract, in which the optimal parameters are established in drying, grinding, percolation, concentration and clarification processes.

MATERIALS AND METHODS

Experimental design

From the experimental design, the research was based on a totally randomized factorial design model for three experimental variables: "type of vegetal material" (dry and fresh), "type of solvent" (potable ethanol and cane ethanol) and "percentage alcoholic solvent" (50, 70 and 90%) v/v.

Obtaining and identifying vegetal material

The vegetal material was collected in the Province of Morona Santiago, Canton Macas, in August and September, 2016 during the winter. A total of 6 kg vegetal material was obtained; once in the laboratory, it was washed, identified and stored in hermetic containers, keeping it in refrigeration at 5°C for later use. The identification was taken by comparison with vegetal species of *O. quixos* from Salesian Polytechnic University herbarium. To prepare the extracts, the leaves of *O. quixos* were used.

Hydroalcoholic extracts

The process of obtaining hydroalcoholic extracts was carried out by percolation, following the methodology of the United States Pharmacopeia 30. The percolation process consists of: milling and wetting of the plant material, percolation for 48 h, concentration on rotates, clarification by filtration and centrifugation and maintenance of the extract on 4°C, to control it stability.

Quality control of fluid extracts

The organoleptic characteristics of the state, color, smell and taste of the 36 extracts were determined by means of a sensorial analysis; physical-chemical tests carried out are: pH, refractive index, density and total solids. The microbiological analysis consists in determining the presence of total aerobes, total coliforms and molds and yeasts in culture in 3M Petrifilm plates. All these tests were carried out by triplicate for each extract.

Phytochemical screening

The phytochemical groups present in the hydroalcoholic extracts

were determined by means of qualitative tests; allow to describe the presence or not of these. The metabolites analyzed were: polyphenols and tannins (5% ferric chloride test), saponins (foam test), catechins, coumarins and lactones (Baljet assay), quinones (Borntrager assay) and alkaloids (Dragendorff, Wagner and Mayer tests) (Miranda and Cuellar, 2001).

Quantification of total polyphenols

The quantification of the total polyphenols of the extract was performed by the Folin-Ciocalteu method, in a UV-VIS spectrophotometer at 765 nm wavelength, from a calibration curve with gallic acid.

The method of Folin Ciocalteu consist of 3950 uL of distilled water, 250 uL of the reagent Folin Ciocalteu and 750 uL of carbonate of sodium (20% v/v) for 2 h, with spectrophotometer which obtained the content of total polyphenols.

Statistical analysis

A statistical descriptive analysis of the absorbance values of each of the 36 extracts of *O. quixos* was carried out, using SPSS 24 software (Statistical Product and Service Solutions) as well as an ANOVA test, to determine significant differences in treatments as a function of the experimental variables: types of solvents, type of vegetal material and solvent concentration followed by a Tukey HSD test among the 12 treatments, to determine the presence of subgroups between them.

RESULTS AND DISCUSSION

Experimental design

The experimental design based on the three variables was an image design in a 2x2x3 type factorial design, obtaining 12 treatments with three replicates of each unit, with a total of 36 experimental units. The variables considered in the experimental design are based on previous research related to the percolation extraction process.

In the case of the type of solvent, two options are presented: cane ethanol and potable ethanol. According to Cárdenas et al. (2016), the solvents that best extract the secondary metabolites are cane ethanol and potable ethanol, when compared with other solvents such as water and hexane.

Another factor to consider in the solvent extraction process is the characteristic of the plant material (dry and fresh). It is recommended to use dry plant material to prevent processes of microbial contamination (Naveda, 2010). However, the content of polyphenols is usually affected during the drying processes and for this reason, it is necessary to establish a temperature that does not denature the secondary metabolites of interest (Gil, 2012).

Quality control of vegetal material

The percentages of foreign matter, humidity, total ash, insoluble ash in hydrochloric acid and water-soluble ash are presented in the Table 1, compared with the World Health Organization (WHO) limit values for phytochemicals, as well as the values of microorganism test by culture in plate and maximum permissible values in vegetal drugs.

As shown in Table 1, the values obtained in the evaluation of plant material do not exceed the limits established by the American Pharmacopoeia 33. The values reported in the microbiological control show absence of microbial contamination. For this reason, there is a good hygiene of plant material.

In the percentage of foreign material, the value was 0.24%, below the limit and this refers to the presence of material not belonging to the study species (sand, stones, parts, of other plants, etc.). The total ash helps to determine adulterations in plants, as well as the presence of sandy materials from the harvest (Montesdeoca, 2010).

Obtaining fluid extracts

A total of 36 fluid extracts were obtained, corresponding to the 36 treatments of the experimental design which consider the proposed variables; type of vegetal material (fresh and dry) and type of solvent (potable ethanol and cane ethanol); solvent percentage (50, 70, 90%) v/v.

Each extract was identified by the following coding: 1) Type of solvent: “A” or “C” for potable ethanol or cane ethanol; type of vegetal material: “MS” for dry material or “MF” for fresh material; 3) repetition number: 1, 2 or 3 and 4) solvent percentage: 90:10, 70:30 or 50:50 according to solvent: water ratio.

Quality control of fluid extract

The average of physicochemical characteristics is pH 6.605±0.5429. This value is beneficial because in therapeutic products of external use, it is recommended that the product has a pH similar to the pH of the skin (Ferraro et al., 2016). The refractive index of 1.35 ± 0.007 °Brix is close to that of water (1.33°Brix), the highest value in the extracts indicates the presence of compounds extract by the solvent (Urquizo, 2010), the density is of 0.95 ± 0.03 g/cm3, in this case the value obtained is intermediate between the density of the ethanol (0.789 g/cm3) and the density of the water (1 g/cm3), which is logical because the extracts are constituted by one part of ethanol and other part of water. And total solids of 1.9870 ± 0.7661 g.

In the microbiological control, the total absence of the evaluated microorganisms (total aerobes, total coliforms and molds and yeasts) was evidenced.

Phytochemical screening

Phytochemical screening was performed to qualitatively

Table 1. Quality control of vegetal material.

Parameter	Reported mean value (%)	Limit value (%)
Foreign matter	0.2375	10
Humidity	48.606	-
Total ash	1.9197	12
Water soluble ash	0.4755	7
Insoluble ash in hydrochloric acid	0.1108	5
Test	**UFC / g (Reported)**	**UFC / g (Límit)**
Total aerobes	1	100
Total coliforms	0	100
Molds and yeasts	2	100

Note: The limit value for the parameter of quality control for vegetal material and UFC limit were established by World Health Organization (WHO), 1998 and UPS 33, respectively.

Table 2. Phytochemical screening.

Metabolites	Treatments											
	AMF			AMS			CMF			CMS		
	90:10	70:30	50:50	90:10	70:30	50:50	90:10	70:30	50:50	90:10	70:30	50:50
Polyphenols	**	**	***	*	*	*	**	*	*	***	**	***
Tannins	**	*	*	**	**	*	*	**	**	**	*	**
Catechins	***	***	**	**	**	***	**	***	***	**	***	**
Saponins	**	***	***	*	**	**	***	***	***	**	**	**
Quinones	***	***	***	***	***	***	***	***	***	***	***	***
Coumarins	**	**	**	*	**	**	**	**	**	*	*	*
Alkaloids Alcaloides												
(Dragendorff)	***	**	**	**	***	**	***	**	**	**	***	**
(Wagner)	***	**	**	***	**	*	**	**	***	*	**	***
Mayer	***	***	***	**	***	**	***	**	***	*	*	***

*****,** High presence; **, present; *low presence; -, absent.

identify the presence of certain secondary metabolites in each extract. The results are shown in Table 2.

These agree with those obtained by Cárdenas-Tello et al. (2016), which conclude the qualitative analysis. Cane ethanol extracts completely the metabolites of interest with exception of coumarins.

Quantification of phenolic compounds

The gallic acid calibration curve was obtained with the absorbance values at 765 nm as shown in Table 3. The calibration curve with gallic acid as a reference standard in the quantification of phenols serves as a reference for calculating the concentrations of total phenols present in each extract. The absorbance values reported in the Table 3 allowed producing a curve of calibration absorbance vs. concentration (Figure 1).

The analysis of variance of ANOVA of the experimental design allows in determining the significant difference

The results obtained in the phytochemical screening show that all extracts contain polyphenols, tannins, catechins, saponins, quinones, coumarins and alkaloids. between the media of gallic acid per mL of extract; type of plant material, type of solvent and concentration of solvent. Based on the interactions between the experimental design variables, analysis of variance shows that there is no significant difference between the interactions of two factors (type of plant material-type of solvent, type of plant material - concentration, type of solvent - concentration), while the interaction between the three variables shows a significant difference (Sig = 0.00).

By means of the ANOVA test for the 12 treatments, it was found that there is a significant difference (Sig = 0.010) among the 12 treatments, a result concordant with the significance obtained from the analysis of the interaction of the three variables. Tukey's post-hoc test allows the identification of three subgroups of treatments where: the treatment with the lowest concentration of gallic acid corresponds to the AMS50:50 treatment, a

Table 3. Gallic acid concentrations and their respective absorbance.

Standard	Concentration of gallic acid (mg/mL)	Absorbance at 765 nm
Blanco	0	0
C1	0.001	0.007
C2	0.002	0.112
C3	0.02	0.4064
C4	0.05	1.0572

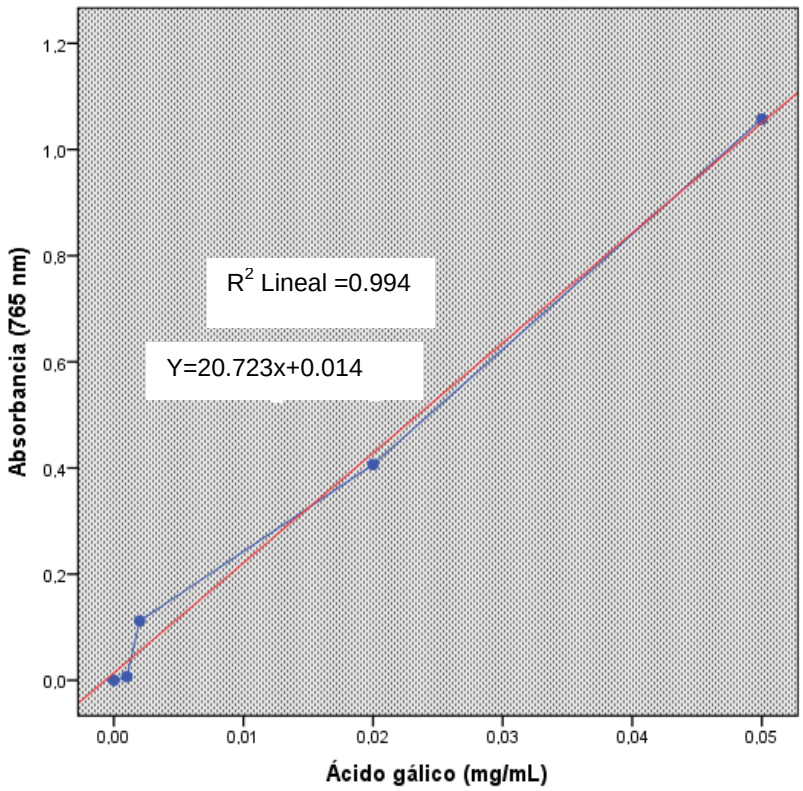

Figure 1. The gallic acid calibration curve (Absorbance vs concentration).

subgroup of 10 treatments is identified that share both subgroups (1 to 2) and were determined as the best treatments at AMF50:50 and CMF90:10, although they share means of subgroup 2, their greatest potentiality is distinguished as optimal treatments to obtain high concentrations of gallic acid (Table 4).

As it can be evidenced in the histogram (Figure 2), on the concentration media of mg. of gallic acid per treatment, the extracts codified as CMF90:10 present a higher concentration of total phenols, which is in agreement with the research carried out by Cárdenas et al. (2016) which conclude that, ethanol from cane was the most efficient in extracting the secondary metabolites of interest in *O. quixos* and *Piper carpunya*, as it improves the penetrability of lysigenic structures of plant organs and extracts the metabolites of interest.

It is important to mention that the second treatment with the highest amount of total phenols is AMF50:50, which is an indication that the concentration could be influenced when extracting the phenols from the leaves. As Caldas (2012) mentions, the use of high concentrations of the solvent can extract compounds that inhibit the extraction of the metabolite of interest; whereas too low concentrations fail to extract the metabolites. This is verified by the results of this research where the treatments AMS50:50 and CMF50:50 had the lowest concentrations of total phenols.

It should be noted that, the alcoholic strength of drinking ethanol is 96°GL while that of cane ethanol is 58°GL and when combined with water, the alcohol content decreased. For the case of plant material there was no significant difference, that is, fresh or dry material

Table 4. Tuckey post – hoc test (95%).

Treatments	mg gallic acid / mL extract		
	Number	Subset	
		1	2
AMS50:50	3	6.239444	-
CMF50:50	3	8.008815	8.008815
CMS90:10	3	8.938539	8.938539
CMS70:30	3	9.511171	9.511171
AMF70:30	3	9.670415	9.670415
CMF70:30	3	10.365295	10.365295
AMS90:10	3	11.129341	11.129341
AMF90:10	3	11.545947	11.545947
AMS70:30	3	11.803310	11.803310
CMS50:50	3	11.968988	11.968988
AMF50:50	3	-	12.538403
CMF90:10	3	-	13.297624
Sig.	-	0.065	0.113

HSD Tukey is the means for each groups in which homogeneous subsets are visualized. It´s base on the averages observed. The error term is the quadratic mean (Error) = 4.066. (a) Uses the simple size of the harmonic mean = 3.000, (b) α = 0.05.

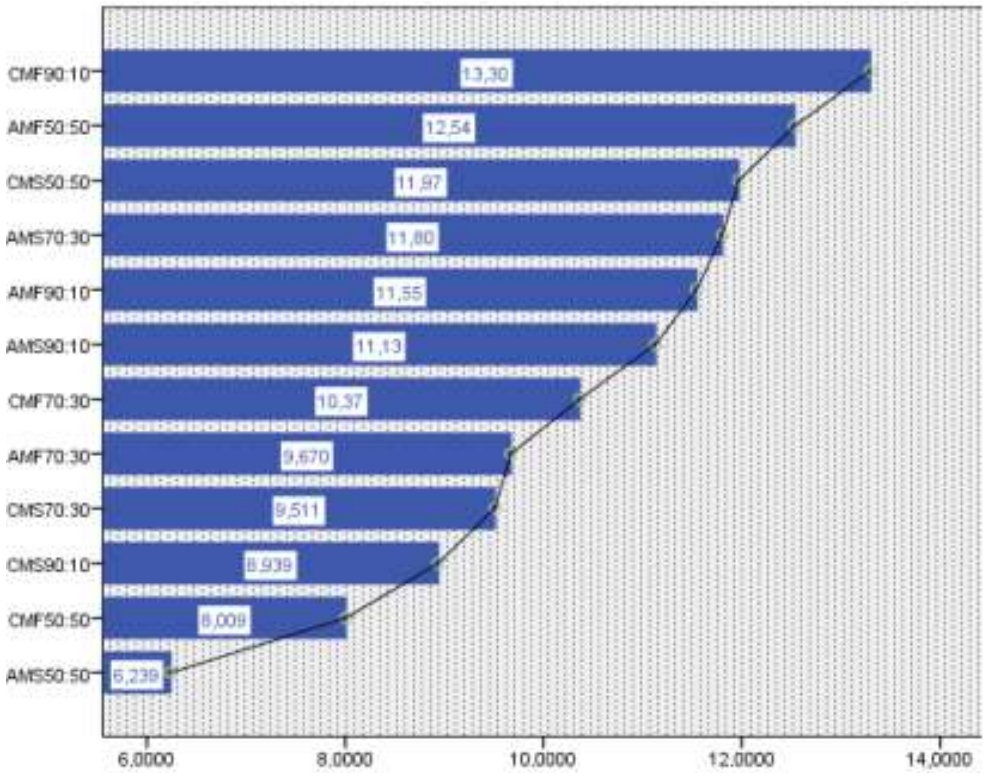

Figure 2. Histogram of averages of mg gallic acid/mL extract with the different treatments.

can be used and the final content of total phenols will not vary significantly as evidenced in the ANOVA statistical tests. In this case, it is advisable to use dry material to facilitate grinding processes and avoid microbial contamination (Pérez, 2009).

Although, one of the great advantages of *O. quixos*

extracts is the high content of phenols and as explained above, phenols have proven antimicrobial and antifungal activity (Noriega and Decarro, 2008). In addition, ethanol has been used as solvent, which is considered a growth inhibitory agent of microorganisms (Sánchez and Sáenz, 2005).

Finally, as evidenced by the statistical test of Tukey, there is no significant difference when analyzing each of the variables independently, but rather at the moment in which the variables interact, significant changes in the concentration of total phenols can be reported.

Conclusions

The 36 hydroalcoholic extracts had significant amounts of total polyphenols. However, in the statistical analysis of the results, it was possible to conclude that the interaction between the variables considered in the experimental design (solvent type, plant material status and solvent concentration) influences the final result of total polyphenols.

The best treatment was CMF90:10, with a total of 1,330 mg gallic acid/mL extract; while the treatment with the lowest concentration of total polyphenols was AMS50:50, in which 6,239 mg gallic acid/mL extract was obtained. It is important to mention that there was no significant difference when the variables were analyzed independently, whereas for the interactions variables, it can be concluded by means of the statistical analyses which confirm that there are significant differences in the content of total polyphenols.

The content of total polyphenols in the extracts was the parameter that allowed standardizing each one of the extracts. Thus, the reproducibility of *O. quixos* hydroalcoholic extracts is guaranteed, which can be used in future investigations.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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