Vol. 17(1), pp. 28-36, January, 2023
DOI: 10.5897/JMPR2022.7265
Article Number: FA7703570276
ISSN 1996-0875

http://www.academicjournals.org/JMPRR

Journal of Medicinal Plants Research

Full Length Research Paper

Valuation study on the extracts of *Ilex guayusa* Loes. as an antioxidant and anti-aging raw material

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Received 6 September, 2022; Accepted 31 October, 2022

***Ilex guayusa* Loes. is a medicinal plant used by people of the Ecuadorian Amazon for treating various diseases and for its stimulating properties. In recent years, several studies have shown this plant to possess a high concentration of phenolic compounds and a high antioxidant potential, allowing it to be used as an ingredient for cosmetic formulations. This research confirms the presence of phenolic compounds between 20.18 and 22.44 mg gallic acid equivalents/mL of total phenols and between 7.50 and 3.80 mg hyperoxide equivalents/Ml of total flavonoids, depending on the solvent used (hydroalcoholic or hydroglyceric) to prepare the extract. The evaluation by GC/MS after silylation reveals the presence of 3-hydroxyflavone, techtochrysin, kaempferol and quercetin. The spectrophotometric method of DPPH (2.2-diphenyl-1-picrylhydrazyl) verifies high antioxidant activity in the extracts evaluated and in two cosmetic formulations: Hydrophilic (gel) and lipophilic (cream), with activities similar to ascorbic acid and the natural extract of reference from *Camellia sinensis*. Clinical evaluation of the lipophilic form shows positive results in increasing skin elasticity and firmness in all volunteers. This study verifies the cosmetic potential of *I. guayusa*, as well as the abundant concentration of phenolic compounds and its influence on antioxidant activity, with an industrial use in cosmetics, which could become a source of income for the communities where this plant is grown.**

Key words: *Ilex guayusa*, natural cosmetics, polyphenols, antioxidants, skin elasticity, skin firmness.

INTRODUCTION

Ilex guayusa Loes. is a tree native to Ecuador, that is also found in the Amazon region of Peru, Colombia and Bolivia, at altitudes between 200 and 2,000 m above sea level (Sequeda-Castañeda et al., 2016). The species has

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an average height of 10 m, with trees up to 30 m high, and an average diameter between 50 and 80 cm (Dueñas et al., 2016). *I. guayusa* is a medicinal and ancestral plant used by people of the Ecuadorian Amazon as: Fever reducer, diuretic, pain killers, anti-arthritis, fertility, ritual plant, among others (Dueñas et al., 2016; De la Torre et al., 2008; Villacís-Chiriboga, 2017; Lewis et al., 1991). The most widespread use of the species is that of stimulant (Lewis et al., 1991; Radice and Vidari, 2007), due to its high caffeine concentration, which is usually higher than 3% (Radice and Vidari, 2007; Negrin et al., 2019; Kapp et al., 2016). Generally, the part of the plant used for medicinal purposes are its leaves.

Several studies have been conducted to identify new secondary metabolites in the species, finding terpenes such as ursolic acid, amyrin esters (Chianese et al., 2019) and squalene (Cadena-Carrera et al., 2019); xanthine alkaloids such as theo-bromine and theo-phylline (Pardau et al., 2017); polyphenols (García-Ruiz et al., 2017; Arteaga-Crespo et al., 2020; Chóez-Guaranda et al., 2021; Villacís-Chiriboga et al., 2018) and carotenoids (García-Ruiz et al., 2017). The high and diverse presence of poly-phenolic compounds is noteworthy, among which have been identified chlorogenic acid (García-Ruiz et al., 2017), quercetin (García-Ruiz et al., 2017; Villacís-Chiriboga et al., 2018), kaempferol (Villacís-Chiriboga et al., 2018), caffeoylquinic acid (Villacís-Chiriboga et al., 2018), as the most abundant.

This important presence of flavonoids and other polyphenols in guayusa would suggest the species as a good cosmetic raw material, due to its known antioxidant and anti-inflammatory properties, confirmed in evaluations *in vitro* (Arct and Pytkowska, 2018; Chuarienthong et al., 2010; Hui-zhi, 2010; Benavente-García et al., 1997; Noriega et al., 2015; García-Ruiz et al., 2017; Arteaga-Crespo et al., 2020; Pardau et al., 2017; Villacís-Chiriboga et al., 2018). Vegetable extracts with polyphenols are used frequently in cosmetic anti-aging products (de Lima Cherubin et al., 2020; Panzella, 2020), for this reason, guayusa could to considered as important raw material with industrial potential.

The principal aims of this research is to value extracts from the *I. guayusa*, as active ingredients for the cosmetic industry, due to the presence of its secondary metabolites, as well as its results in *in-vitro* and clinical activity tests. Studies evaluating natural active ingredients for the cosmetic industry are scarce, in the case of *I. guayusa* as it is a plant that is found in a small geographical area of the Amazon even more, this justifies the importance of this research.

MATERIALS AND METHODS

Plant material

Dried and ground leaves of *I. guayusa* were provided by the Chankuap Foundation Resources for the Future, from the Upano Valley, Macas parish, Morona Santiago province, at the following coordinates: Latitude: S 2° 10', Length: W 78° 0', Altitude: 1080 m above sea level, the collection was executed in January 2017. Voucher of the plant was deposited in the herbarium of Salesian Polytechnic University code HUPS-aq-006, the identification was made the botanist Marco Cerna. The collected leaves provided from trees of more than five years were composite and young.

Extracts preparation

Three fluid extracts were prepared from *I. guayusa* leaves (concentration 1 g of plant /mL extract) using different ethanol/water mixtures (80, 50 and 20% and a hydroglyceric fluid extract (concentration 1 g of plant/mL extract) using glycerin and water (1:1, v/v). The technique used was percolation, to reach the concentration; the solvent was evaporated under reduced pressure, on a rotary evaporator, based on the research of Tatiana et al. (2017). The residue was filtered on Whatman grade one filter paper. A fluid extract of *Camellia sinensis* was prepared as a control extract using a ethanol/water solvent at 50%.

Quantification of total phenols and total flavonoids

Folin-Ciocalteu method described by Singleton and Rossi (1965) was used to evaluate the concentration of total polyphenols. Due to the nature of the extracts, the preparation of the solutions was modified in a similar way as described by Noriega et al. (2015); the calibration curve was prepared with a number of gallic acid standards. Total flavonoids were quantified according to Lamaison and Carnat method (1991), with hyperoside as standard (Wolniak et al., 2007). In each case, four replications were conducted for each trial.

Free radical scavenging activity of the extracts and cosmetic products

The free radical scavenging activity was evaluated by the spectrophotometric method of DPPH 2,2-diphenyl-1-picrylhydrazyl, which has been used in medicinal plants rich in polyphenols (Sembiring et al., 2018; Xiang et al., 2017; Nunekpeku et al., 2018). The fluid extracts evaluated (equal to 1 g of drug / 1 mL solution) were prepared using several hydroalcoholic and hydroglyceric solvents. The concentration range of the extracts was from 10 to 500 µg/mL. Ascorbic acid and ethanolic extract of green tea (*C. sinensis*) were used as positive controls. The methodology uses a DPPH solution with a 1×10^{-4} M concentration and various concentrations of the extracts to measure their absorbances at a wavelength of 517 nm, after 30 min of agitation. With the data obtained from absorbance, oxidation inhibition percentages were calculated by means of the following equation.

$$\%\ \text{inhibition } 1 - (AA/AB) \times 100$$

Finally the C_{50} was calculated I in µg/mL and the values are compared, considering that the lower the IC_{50} value the higher its activity. For each determination 4 replications were done.

Polyphenols analysis (GC/MS after silylation)

Poly-phenolic compounds such as flavonoids were separated and analyzed in a gas chromatograph-mass spectrometer, even though these compounds are not volatile. One of the mechanisms used to make these molecules easily evaporate and enter a capillary

Table 1. Composition of the hydrophilic cosmetic formula gel with and without *I. guayusa* extract as an active ingredient.

Ingredients	Product with *I. guayusa* extract	Product without *I. guayusa* extract
	Amount (g)	**Amount (g)**
Deionized water	70	79
Ethyl alcohol 70º	20	20
Carbomer	0.5	0.5
Triethanolamine	0.5	0.5
Ilex guayusa leaf extract	9	-

Source: Authors 2022

Table 2. Composition of lipophilic cosmetic formula (cream) with and without *I. guayusa* extract as an active ingredient.

Ingredient	**Product with *I. guayusa* extract**	**Product without *I. guayusa* extract**
	Amount (g)	**Amount (g)**
Isopropyl palmitate	7.5	7.5
Plukenetia volubilis seed oil	4.0	-
Ceteareth-20 · Cetearyl alcohol	5.0	5.0
Triethanolamine	4.0	4.0
Ilex guayusa leaf extract	5.0	-
Phenova (phenoxyethanol, methylparaben, ethylparaben, propylparaben, butylparaben, isobutylparaben)	0.7	0.7
Deionized water	73.8	82.8

Source: Authors 2022

column is the chemical derivatization reaction known as silylation, in which the -OH groups are replaced by the -O-Si-$(CH_3)_3$ group (Proestos and Komaitis, 2013). Previously, poly-phenols were subjected to an acidic hydrolysis process in order to separate them from their sugar, using a 6 N HCl solution with a process described in a previous investigation (Proestos et al., 2006), where polyphenol extract is prepared prior to silylation. Subsequently, the extract is derived by reacting with the reagent composed of BSA + TMCS Sigma Aldrich code 33019-U; finally, the substance obtained was filtered and injected into the GC/MS system.

Analysis conditions were as follows: The equipment used was composed of a Varian gas chromatograph, model 4000 and a Varian Saturn 2100 mass spectrometer. A VF5 column was used (5% -phenyl-95% dimethylpolysiloxane) (internal diameter of 30 m × 0.25 mm, 0.25 µm film thickness). The carrier gas was helium with a constant flow of 1.9 mL/min and a split ratio of 1/25. The analysis started at 75°C and was carried on until it reached 135°C at a speed of 10°C/min; the temperature was then raised to 270°C at a rate of 3°C/min, remaining at that temperature for 10 min. The ionization energy of the mass spectrometer was 70 eV and the mass range was 40 to 650 m/z. For the identification of the compounds, molecular mass values were compared with flavonoids after silylation.

Cosmetic formulations

For evaluating the antioxidant and anti-aging clinical properties, two formulas of hydrophilic nature (gel) and lipophilic (cream) nature were prepared, similarly to what was done by Noriega et al. (2015). In both formulas, insertion properties of compounds were previously observed in order to set the ideal percentage of *I. guayusa* extract that is necessary in the achievement of a stable formula and with good organoleptic and application characteristics. The formulas are detailed in Tables 1 and 2.

Free radical scavenging activity of the hydrophilic and lipophilic formulations

Free radical scavenging activity was determined in the hydrophilic (gel) and lipophilic (cream) cosmetic formulations in the same way as detailed for the extracts above, using ascorbic acid and the fluid extract of green tea as positive controls. Absolute ethanol was used as solvent at a concentration range from 10 to 500 ug/mL.

Clinical evaluation of cream efficacy

The cream with hydroglyceric extract of *I. guayusa* at 5% was clinical evaluated by a panel of 10 women between 30 and 45 years old. The conditions of the volunteers were previously analyzed. Table 3 shows the criteria of admission and exclusion. This study compiled ethical considerations of the clinical studies applied in Ecuador. Consent was signed before giving them the cream. Each volunteer received two samples of 125 g of product (creams), one without active ingredient (NAP) and the other with active ingredient (AP), for their application in a part of the cheek. The left side of the cheek was destined for the cream NAP and the right side for AP. The application frequency was once a day, at night. Measurements of firmness and elasticity of the skin were done in two different

Table 3. Inclusion and exclusion criteria for selecting the participants.

Inclusion	**People who:** Are women whose age is from 35-50 years, mean between aging II and III. The benefits they receive from applying cosmetic formula are higher than the risks. Sign appropriate informed consent to ensure the confidentiality of the volunteer and the privacy of her identity
Exclusion	**Women who:** Have an occupation or perform activities involving the frequent use of chemicals. Are pregnant or breastfeeding. Have solar exposure between 3 and 6 hours a day. Experience skin reaction after solar exposure. Do not use sunscreen frequently. Smoke regularly. Present all medical conditions mentioned. Use night creams frequently or consistently. Use medications frequently or consistently.

Source: Authors 2022

Table 4. Total phenolics and total flavonoids of various extracts from *I. guayusa*.

Fluid extract	Total phenolics as mg of gallic acid equivalents/mL	Total flavonoids as mg of hyperoside equivalents/mL
I. guayusa (20% ethanol solvent)	21.46 ± 0.35	5.42 ± 0.24
I. guayusa (50% ethanol solvent)	22.44 ± 0.63	7.50 ± 0.37
I. guayusa (80% ethanol solvent)	20.18 ± 0.45	3.80 ± 0.21
I. guayusa (water: glycerin 50:50)	21.75 ± 0.78	7.40 ± 0.65
C. sinensis (50% ethanol solvent)	50.28 ± 0.17	4.92 ± 0.35

Source: Authors 2022

times: Time 1 within 30 days and time 2 after 60 days, compared to the initial measurement time (To). Two AP groups with active substance and NAP without *I. guayusa* extract active are established in the assay. Applications of both products were done on the same volunteer on each of their cheeks. Elasticity and firmness were determined using a Cutometer Dual MPA 580, brand MICROCAYA, the units for measurement was millimetres (Leyva-Gómez et al., 2017). This study was executed in the science life laboratories in the Salesian Polytechnic University, Quito Ecuador, between March and May 2017.

Statistics

To assess the results of the elasticity and firmness measurements, Kruskal-Wallis, non-parametric test was used to verify statistical differences between treatments in relation to control for a significance level (α=0.05). Mann-Whitney's U-test was used to establish the differences between the means of the two treatments, 30 and 60 days. The program used was the free software "R version 3.6.3 (2020-02-29). Relative standard deviations and statistical significance (student´s test; $p < 0.05$), one way ANOVA and LSD post hoc Fisher´s honest significant difference test, were used, when appropriate, for all data collected in total phenolics, total flavonoids and IC_{50} DPPH. All computations were made using the statistical software STATISTICA 6.0.

RESULTS

Quantification of total phenols and total flavonoids

The results of these measurements are described in Table 4, in mg/mL concentrations of the average and its standard deviation, expressed in mg gallic acid equivalents/mL for total phenols, and in mg hyperoside equivalents/mL for total flavonoids, for each analysis was made 4 repetitions, Fluid extract of *I. guayusa* prepared with ethanol 50%, showed the highest concentration of total phenolics and flavonoids; this indicates that the extract contains a very good amount of secondary metabolites.

Free radical scavenging activity

The activity was valued in ethanolic extracts, hydroglyceric extracts, hydrophilic cosmetic formulation (gel) and in a lipophilic formulation (cream). Ascorbic acid and an ethanolic extract of *Camellia sinensis* were used as a reference of activity. The values (50% oxidation inhibition DPPH) IC_{50} DPPH in µg/mL are the average and its standard deviation, for 4 repetitions (Table 5).

Lower values of IC_{50} mean higher free radical scavenging activity. In this proof *I. guayusa* extract prepared with ethanol 50% shows a less value; this result is very similar to citric acid, which denotes a high activity.

Free radical scavenging activity for the ascorbic acid with IC_{50} DPPH 18.69 ug /mL ± 0.03 compared to the extract prepared with 50% ethanol (IC_{50} 23.85 ± 0.02 ug /mL) showed little difference between the extract and the

Table 5. IC_{50} DPPH values for various extracts and cosmetic products.

Fluid extract or cosmetic products	IC $_{50}$ (DPPH) µg/mL
I. guayusa (20% ethanol solvent)	57.25 ± 0.24
I. guayusa (50% ethanol solvent)	23.85 ± 0.02
I. guayusa (80% ethanol solvent)	25.05 ± 0.02
I. guayusa (water: glycerin 50:50)	34.88 ± 0.13
C. sinensis (50% ethanol solvent)	32.54 ± 0.14
Hydrophilic formulation (gel), with 9% of the fluid extract of *I- guayusa* (50% ethanol solvent)	209.34± 2.72
Lipophilic formulation (cream), with 5% of the fluid extract of *I. guayusa* (hydroglyceric extract)	55.78 ± 0.68
Ascorbic acid	18.69 ± 0.03

Source: Authors 2022

Table 6. Characterization phenolic compounds with a silylation derivatization reaction.

Phenolic compounds	Molecular ion of TMS derivaties	Charesteristic fragments
3-Hidroxyflavone	310	295, 280, 237, 181, 152, 77
Techtochrysin	340	325, 310, 295, 268, 239
Kaempferol	574	559, 501, 282
Quercetin	647	575, 487, 297, 137

Source: Authors 2022

antioxidant molecule, which shows a high activity. This is best confirmed when comparing the IC_{50} values with the natural extract of *C. sinensis*, in this case several extracts have lower values, which implies a higher activity.

Phenolics compounds characterization

The silylated phenolic compounds were identified in the mass spectrum. Extract of *I. guayusa* (50% ethanol solvent) was used in this experiment to obtain the major concentration of phenolics compounds. Quercetin and kaempferol flavonoids were identied, which are described in several papers as the most representatives in *I. guayusa*. In addition, molecular ions matching 3-hydroxyflavine and techtochrysin were found. Table 6 shows the results obtained from the mass spectra.

Cosmetic efficacy

The results allow to observe significant differences between those treatments with and without the extract of *I. guayusa*, either when comparing the data obtained individually by each volunteer (Figures 1 and 2) or when comparing treatments (Figures 3 and 4).

The values were the results of measuring elasticity and firmess in 10 volunters, and at two different times (30 daysand 60 days) and in the presence of the *I. guayusa* extract (AP) or withouth *I. guayusa* extract (NAP). For each measure, 4 repetitions were executed.This difference in the values of the individual data towards a lower measurement value with respect to the initial measurement and treatments without *I. guayusa* shows cosmetic efficacy on the elasticity and firmness parameters of the skin.

Regarding the treatment that differentiates measurement times, it is clear that increased application time positively affects values that determine improved firmness and elasticity.

DISCUSSION

Both qualitative and quantitative determinations confirm the information described in the literature, mentioning a significant number of polyphenols present in *I. guayusa* (Pardau et al., 2017; Villacís-Chiriboga et al., 2018; Wise and Santander, 2018; Radice et al., 2016; Manzano et al., 2018). concentrations similar to an investigation conducted using alcoholic solvents and ultrasound in the extraction was found; the maximum values reported by the study are around 30 mg gallic acid equivalents/mL (Arteaga-Crespo et al., 2020), while in this study 22 mg gallic acid equivalents/mL was reported. The extract obtained using 50% ethanol solvent provided the best extraction values; for this reason, it was the one used in the elaboration of the hydrophilic formula (gel). Similarly, that hydroglyceric extract contains significant concentrations of both total phenolic compounds and flavonoids was determined; subsequently this was used

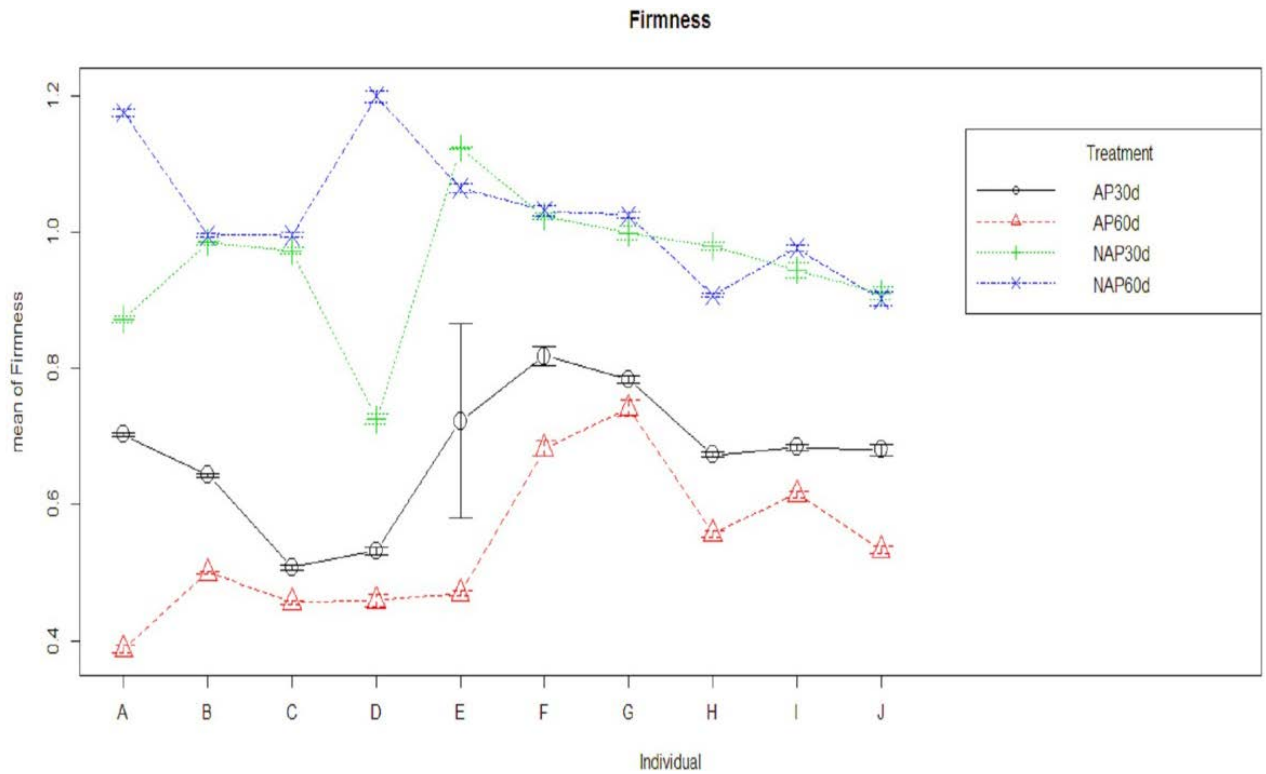

Figure 1. Data of the firmness of the skin in each volunteer. AP with active ingredient, NAP without active ingredient, thirty days and sixty days after starting treatment. Means + SD. Lower values show better firmness.
Source: Authors 2022

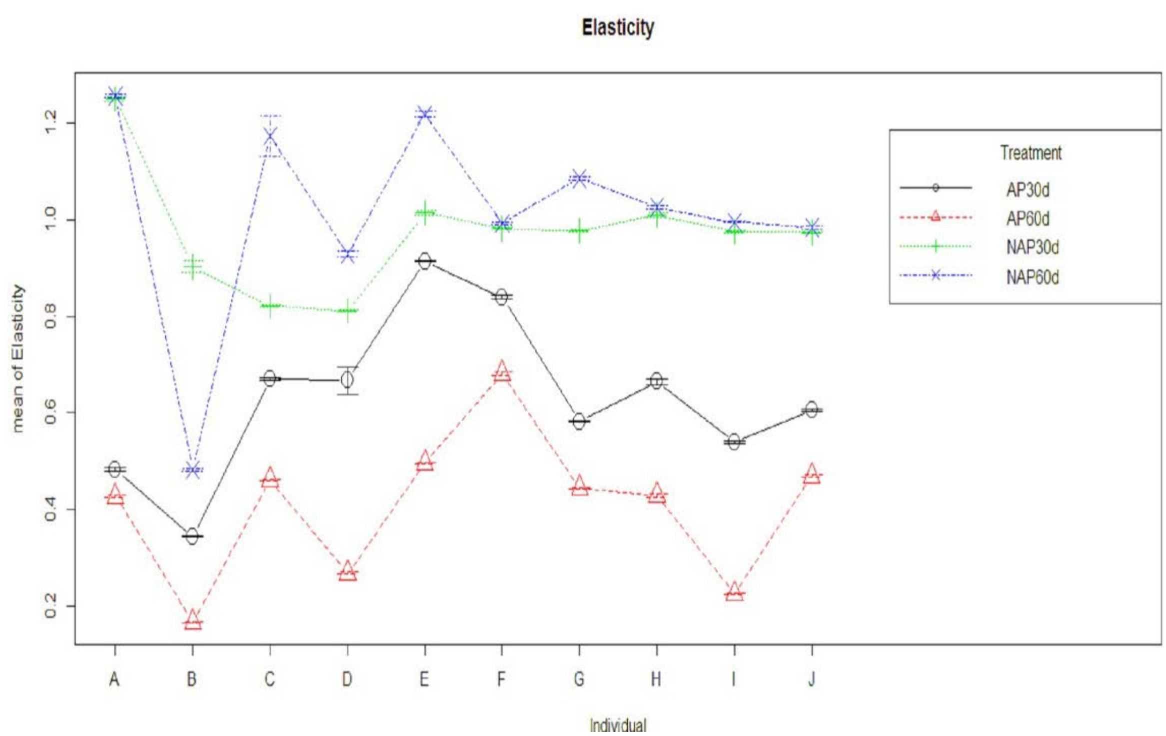

Figure 2. Data of the elasticity of the skin in each volunteer. AP with active ingredient, NAP without active ingredient, thirty days, sixty days after staring treatment. Means + SD.
Source: Authors 2022

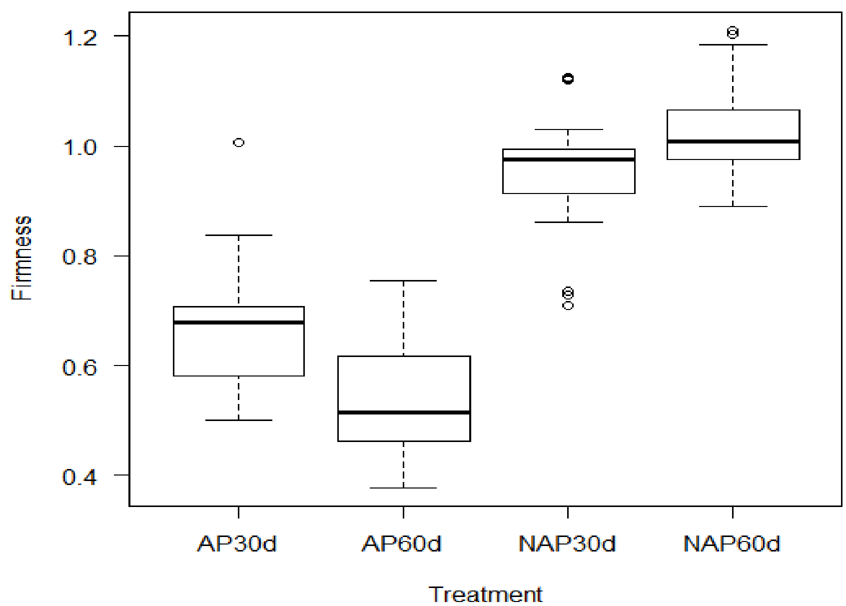

Figure 3. Data of variation (explain how variation is shown) of the skin firmness in 10 volunteers for each treatment. AP with active ingredient, NAP without active ingredient, thirty days and sixty days after starting treatment.
Source: Authors 2022

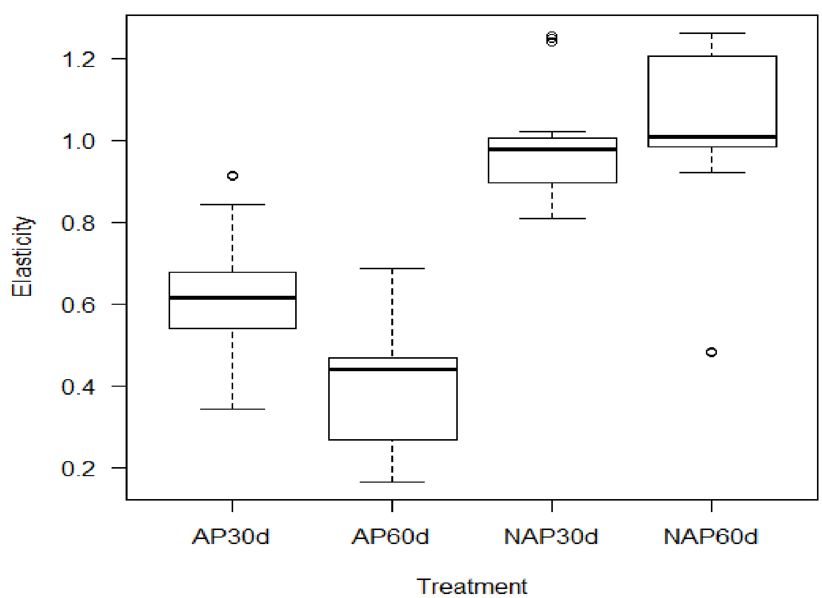

Figure 4. Data of variation of the skin elasticity in 10 volunteers for each treatment. AP with active ingredient, NAP without active ingredient, thirty days and sixty days after starting treatment.
Source: Authors 2022

in the elaboration of the lipophilic cosmetic formula (cream).

From a qualitative point of view, the presence of quercetin and kaempferol flavonoids described in the scientific literature (García-Ruiz et al., 2017), is confirmed, as well as the presence of 3-hydroxyflavone and techtochrysin. Several studies mention the presence of chlorogenic acid and caffeoyl (García-Ruiz et al., 2017; Villacís-Chiriboga et al., 2018); however, silylation products of these compounds have molecular ions that exceed the mass spectrometer resolution of 650 m/z, which were not possible to detect here.

The assessment of antioxidant activity by the DPPH method reveals a very high potential of the species, which remains noticeable in cosmetic formulations, whether hydrophilic (gel) or lipophilic (cream). The extract prepared with 50% ethanol solvent is the one with the best activity, which coincides with the highest presence of phenolic compounds; thus, verifying an activity superior to green tea (C. sinensis), which is already a validated raw material in antioxidant and antiaging cosmetic products (Koch et al., 2019; Gianeti et al., 2013; Kaur and Saraf, 2011). Regarding the lipophilic formulation, the antioxidant capacity is very interesting and is surely enhanced by the presence of *Plukenetia volubilis* oil, which is rich in polyunsaturated fatty acids and with high antioxidant capacity (Cisneros et al., 2014; Wang et al., 2018; Mosquera et al., 2012).

Clinical efficacy studies of the lipophilic formulation confirm good performance to improve the elasticity and firmness characteristics of the skin in all volunteers who used the product for 60 days; for this reason, this species is proposed as a good cosmetic raw material option.

Statistics analysis (Figures 4 and 5) show the minor values for the individual mediation (elasticity and firmness), in all the volunteers; this means that independently in all of them there was a positive result, whose response was inherent in each volunteer.

Results about the efficacy in the treatments, independent of volunteers, shown in the Figure 3 and 4 that the treatment AP60d (with active ingredient and sixty days of apply) is the best to improve elasticity and firmness in the skin.

A relashionship betwen *in-vitro* studies (antioxidant activity) and clinical studies (elasticity and firmness) is not possible, because the nature of the assays is different, but it is known that antioxidant compounds have a possitive effect for maintaining the good state of the skin.

Conclusion

Secondary metabolites such as phenolic compounds have gained importance in recent years for their various beneficial properties for human health and well-being. *I. guayusa* is an Amazonian species that grows in Ecuador, whose composition of this type of molecules has been recognized in recent years. This study shows a plant that can be considered as promisor in quantity and quality of polyphenolic compounds, many of them as the quercetin and kaempherol, with an interesting biological activity for the pharmacy and cosmetics. The research carried out in the Laboratories of Life Sciences of the Salesian Polytechnic University confirm the beneficial properties of the species, not only evaluating the antioxidant activity and polyphenolic chemical composition in various extracts, but also analyzing its use in cosmetic formulas. The studies *in vitro* (antioxidant gel) and in clinical, elasticity and firmness (antiaging cream), confirm the good proprieties of *I. guayusa*. The results are encouraging and propose *I. guayusa* as raw material for the design and creation of cosmetic products.

CONFLICT OF INTERESTS

The authors have not declared any conflict interests.

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