Optimizing cephaeline and emetine production from Ipecac root and antimicrobial activity through maturity harvesting and processing.

Optimización de la producción de cefaelína y emetina a partir de la raíz de Ipecacuana y actividad antimicrobiana durante procesamiento y cosecha

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Abstract

[Objetive] This study aimed to determine whether there is an optimal harvest time for emetine, cephaeline, and total alkaloids Ipecac (*Carapichea ipecacuanha* [Brot.] L. Andersson) production. Also, the relationship between antibiotic activity and harvest time was tested. is an herb with medicinal properties cultivated in Northern Costa Rica. The root of this plant is valued according to its alkaloid concentration, which underlies its numerous biological activities. **[Methodology]** Two Ipecac fields from northern Costa Rican producers were sampled every ~2 months during a year. Samples were dried (through sun and oven), ground, extracted, and analyzed by HPLC and titration to evaluate the alkaloid concentration. Also, antimicrobial activity was determined using the Kirbi-Bauer test. **[Results]** Our main findings revealed that there is no significant difference between the oven-dried samples and the sun-dried samples. Also, an increase in total alkaloid production is observed in roots until during the first 16 months. Also, there is a variation in alkaloid composition: the cephaeline/emetine ratio increases after 16 months. **[Conclusions]** The highest alkaloid concentration occurs when plants are harvested between 16 and 19 months old.

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Additionally, the antibiotic activity is maximum at 16 months, and the cephaeline/emetine ratio is 2 at the same harvest time.
Keywords: *Carapichea ipecacuanha*, Ipecac, emetine, caphaeline, antibiotic.

Resumen (tittle centered, Times New Roman 11, bold)

[Objetivo] Este estudio tuvo como objetivo determinar si existe un momento óptimo de cosecha para la producción de emetina, cefaelína y alcaloides totales de Ipecacuana (*Carapichea ipecacuanha* [Brot.] L. Andersson). Además, se probó la relación entre la actividad antibiótica y el momento de la cosecha. Es una hierba con propiedades medicinales cultivada en el norte de Costa Rica. La raíz de esta planta se valora según su concentración de alcaloides, que subyace a sus numerosas actividades biológicas. **[Metodología]** Se muestrearon dos campos de Ipecac de productores del norte de Costa Rica cada ~2 meses durante un año. Las muestras se secaron (al sol y en horno), se molieron, se extrajeron y se analizaron mediante HPLC y titulación para evaluar la concentración de alcaloides. Además, se determinó la actividad antimicrobiana utilizando la prueba de Kirbi-Bauer. **[Resultados]** Nuestros principales hallazgos revelaron que no hay una diferencia significativa entre las muestras secadas al horno y las muestras secadas al sol. Además, se observa un aumento en la producción total de alcaloides en las raíces hasta los primeros 16 meses. También hay una variación en la composición de alcaloides: la relación cefaelina/emetina aumenta después de 16 meses. **[Conclusiones]** La concentración más alta de alcaloides ocurre cuando las plantas se cosechan entre los 16 y 19 meses de edad. Además, la actividad antibiótica es máxima a los 16 meses, y la relación cefaelina/emetina es de 2 en el mismo momento de la cosecha.
Palabras clave: *Carapichea ipecacuanha*, Ipecacuana, emetina, cefaelina, antibiótico.

Introduction

Carapichea ipecacuanha (Brot.) L.Andersson (ipecac) is a medicinal herb from South and Central America. The syrup prepared with Ipecac extract is used as an expectorant, amebicide, anti-bronchitis, and vomiting inducer (for poisoning treatment) (Patel & Patel, 2021). Alkaloids are responsible for biological activity. Two major alkaloids: emetine and cephaeline, account for 84% of the total alkaloid content of its roots (Smajlović & Dučić, 2021; Uzor, 2016)). Additionally, emetine and/or cephaeline have been tested as potential treatments for tumors and viruses (against dengue and SARS-CoV-2)(Bleasel & Peterson, 2020b). Although, there is still some ongoing debate regarding the utilization of Ipecac for antiviral purposes (Bleasel & Peterson, 2020b). Other compounds from Ipecac have been associated with bioactivities as well, e.g., some circular peptides have immunomodulatory effects (Rosales-López, Muñoz-Arrieta, & Abdelnour-Esquivel, 2020), and the polyphenols are antioxidants (Ben Hlel et al., 2019).

Several commercial formulas contain Ipecac syrup, which have been commercialized in America, India, and Europe since 1762 (Garcia, de Oliveira, Moreira, & Barros, 2005). Both syrup and alkaloids were utilized by the pharmaceutical industry, and it became a major drug during the 1940s in the USA and Europe (Ocampo-Sanchez, 2004). Syrups are prepared

from the dried roots of the Ipecac plant. Plants are produced in three main regions: from Nicaragua to Northern Colombia (from Southern Central America to Northern South America), the Southwestern Brazilian Amazon, and the rainforest near the Brazilian Atlantic Coast (Garcia et al., 2005). 20% and 35% of the global market is provided by Nicaragua and Costa Rica, respectively (Ocampo, 2007). Ipecac root production is a crucial economic activity in the Northern Costa Rican region and Southern Nicaragua. Also, Ipecac roots have been introduced for culturing in India and some regions of Asia, resulting in poor-quality products (Ocampo-Sanchez, 2004).

The final sale price of Ipecac roots depends on the concentration of emetine, cephaeline, and total alkaloids (used as quality parameters). Nonetheless, there is not a systematic study of the relationship between the maturity of the plants and the harvest time to optimize the selling price of the final product. Previously, Alvez-García et al. (Garcia et al., 2005) found some dependence of alkaloid content on the morphologic characteristics of the root. Also, there are some variations in alkaloid occurrence rates, e.g., the cephaeline/emetine ratio has been found to be 60-70% emetine by Ocampo (Ocampo, 2007) but 47% emetine by Rosales-Lopez et al. (Rosales-López et al., 2020). Some of the morphological characteristics could be related to plant maturity and the general production of metabolites.

Our hypothesis is that a specific harvest can be found with optimal alkaloid concentration. This information is relevant for the agricultural production of Ipecac, for a better understanding of the metabolite production, and, eventually, for future evaluation of the potential related species. This study aims to determine the optimal maturity and harvest time for alkaloid production in dry Ipecac roots.

Methodology

Sample collecting and processing

Plants were harvested from two production fields in La Guaria, San Carlos (Alajuela Province, Costa Rica). A total of six samples for each field were taken, 45 days apart from each other approximately, starting one year after sowing. A random sampling of *Carapichea ipecacuana´*s roots from each field was conducted. The roots were collected, cleaned, and divided into two parts for two different drying procedures: oven-dried (40°C, four days) and sun-dried. The sun drying procedure is the method utilized by the Ipecac producers, and it consists of allowing the sample to dry at sun hours until it gets brittle. After that, samples were ground to 1 mm in a Wiley cutting mill grinder (from Thomas Scientific, NJ). Finally, grounded samples were stored under refrigeration at 4°C and used for further analysis.

Total alkaloid determination

Total alkaloids were determined using a previously reported volumetric method (Pharmacopeia, 2014). For this purpose, 3.75 g of dried and powdered Ipecac root samples were added to a flask with 50 mL of ethyl ether and mixed in an orbital shaker at 400 rpm for 5 min. Then, 2.5 mL 6N ammonium hydroxide was added, and the mixture was shaken at the same speed for an additional hour. 2.5 mL of water was added after the agitation, and the flasks were mixed by hand. Then, the flasks were let to settle down for a few minutes. Subsequently, the mixture was filtered through cotton, collecting the organic layer and dumping the aqueous phase. The flask and the cotton were washed with 2x15 mL ethyl ether, and all the ether extractions were combined. The ethyl ether was evaporated in a warm water bath (using a hood to extract vapors) until it dried up. The residue was redissolved into 2.5 mL warm ethanol, let cool down and mixed with 7.5 mL of a standard solution of sulfuric acid 0.1 N. The alkaloids were back-titrated using 0.1 N sodium hydroxide and a mixture of indicators (1 mg/mL methyl red and 0.5 mg/mL methylene blue, using ethanol as solvent).

Evaluation of extraction conditions

The best extraction conditions were evaluated. 0.1 g of dry and ground samples were mixed with 0.5 mL 6N ammonia. Then each sample in a separate experiment was extracted with 1 to 5 extraction steps. Each extraction step consisting of 2 mL ethyl ether and 10 min in an ultrasonic bath. After each extraction, the mixture was centrifuged for 10 min at 840 x *g*. Then, the other fractions were mixed in a 10 mL volumetric flask and filled with ethyl ether. Finally, 1 mL was transferred to a vial. The solvent was evaporated and redissolved into 1 mL methanol for further HPLC analysis. Three repetitions were performed.

Emetine and cephaeline quantification using HPLC

Main alkaloids were quantified using the procedure reported by Han *et al*. (Han, Wang, Feng, & Jia, 2013) with minor modifications. The methanolic extracts were analyzed in a UHPLC chromatograph Dionex UltiMate3000 (from Thermo Scientific, MA, USA) equipped with an Acclaim™ 120 C18 column, a column oven (kept at 40°C) and a diode array detector. A mobile phase of the components: 0.08% trifluoroacetic acid (TFA, aqueous) and acetonitrile was utilized, following the gradient described in Table 1. The injection volume was 5 µL. Emetine and cephaeline were analyzed at 285 nm.

Table 1.

Solvent gradient utilized for HPLC separation of ipecac alkaloids

Run time (min)	**Flux (mL/min)**	**Solvent components (%)**	
		TFA 0.08%	**Acetonitrile**
0.00	0.6	100	0
0.50	0.6	90	10

1.25	0.6	80	20
2.50	0.6	50	50
3.75	0.6	30	70
4.00	0.3	20	80
4.50	0.3	100	0
6.00	0.3	100	0
6.50	0.6	100	0
7.00	0.6	100	0

Antibiotic activity of Ipecac extracts

The antibiotic activity of Ipecac extracts was evaluated against the ESKAPE pathogen group: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 9027, and *Enterococcus faecium* ATCC 6056.

Modified Kirbi-Bauer for agar well diffusion method was utilized (Balouiri, Sadiki, & Ibnsouda, 2016; Biemer, 1973). For this purpose, 100 µL of *E. coli* suspension (0.5 McFarlan scale) in sterile saline solution (0.85% NaCl) was plated into 90 x 15mm Muller-Hinton agar plates (25mL of agar). Then, four 7 mm wells were perforated into each agar plate using sterile Pasteur pipettes (upside down), and the wells were filled with 50 µL of 60 mg/mL extract solution (in methanol). The other two wells were filled with methanol as negative control and penicillin/streptomycin (1000 µg/mL each) as positive control. Plates were incubated at 37°C during 18 h. The percentage of relative inhibition (%RI) was calculated according to equation (1).

$$\%RI = \frac{DIZ_{sample} - DIZ_{negative\ control}}{DIZ_{positive\ control}} x100 \qquad (1)$$

where DIZ stands for "diameter of inhibition zone".

Statistical analysis

Extraction optimization was evaluated using Minitab®, with α=0.05. Repeated ANOVA measurements for total alkaloids were performed using R-studio. Assumptions of no extreme outliers and normality by Shapiro-Wilk test were verified by utilizing parametric statistics. The pair-wise comparison was performed using the Bonferroni method to adjust p-value. α=0.05. Three repetitions were used for drying conditions and total alkaloids. Standard deviations were calculated for emetine and cephaeline concentrations from six repetitions from two fields.

Analysis and results

Evaluation of the sun-drying vs. the oven-drying procedure

Local Ipecac producers utilize a sun-dry method for root drying. Root tissue is relatively easy to dry. The traditional method is inexpensive, requires no technological equipment or trained personnel, and is logistically efficient (Belwal et al., 2022). However, an unsuccessful drying step will result in lower alkaloid yield and higher transportation costs. Also, long drying times can lead to a decline in the alkaloid concentration because other organisms can contaminate and degrade it (Kamel, Thabet, & Algadi, 2013)

A comparison between a regular oven-drying process and the traditional sun-drying protocol has been studied -results are shown in Fig. 1. Repeated ANOVA analysis for the average of both fields (Fig. 1 (c)) showed no significant difference between the oven-dried samples and the sun-dried samples. Also, 16-month-old plants did not show any significant difference. 16-month plants are relevant because they yield the highest titer of total alkaloids. However, some of the samples from the individual fields (Fig. 1(a) and 1(b)) had shown significance during some of the months tested. For example, some oven-dried samples (17.5, 19, and 20.5 months) from field #2 contain around 0.1-0.5 more total alkaloids (%mass) than sun-dried. This difference can be explained because older plants developed stronger structural tissue and made the more mature plants harder to dry. Then, the residual moisture lowers the alkaloid content (Ekeoma, Boldrin, Loades, Bengough, & Soil, 2021).

Effect of the plant maturity on total alkaloid production

Fig. 1(c) shows the average concentration of alkaloid production in the combined samples of both fields included in this study. Clearly, the best results are obtained when plants are between 16 and 19 months old, with its maximum at the 16^{th} month. Previously, Yonjan (Yonjan, 1988) found *C. ipecacuanha* increases its alkaloid concentration during the warmer months in the Indian summer, and the maximum increment shows at the beginning of the summer. The increment in the total alkaloid concentration is claimed to be related to the post-reproductive phases. Samples from the 16^{th}-old Ipecac roots included in this study were taken during December, the beginning of the dry season (December to March). After the 16^{th} month, the alkaloid concentration starts decreasing. In the Northern zone, average temperatures are 21-30 C (IMN, 2023). Variations during dry and rainy (April to November) seasons are small ±3 C and probably are not a significant influence over alkaloid production. Precipitation may have some influence over alkaloid concentration, although it was not considered a factor in this study.

Other variables such as explant hardening and lower altitude are reported to increase the total alkaloid concentration (Chatterjee, Bidyawati Yonjan, & Nandi., 1986).

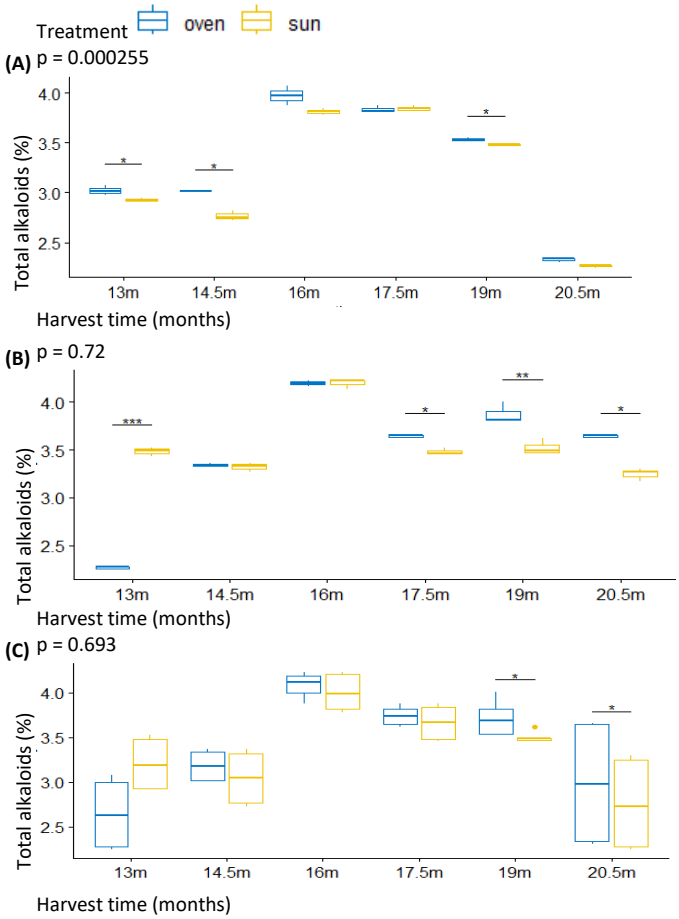

Figure 1. *Boxplot graph of the effect of drying treatment on final alkaloid concentration. (a) field #1, (b) field #2, (c) both fields combined.*

*Note: Boxes represent t-test for sun drying and oven drying pair-wise comparison (Two-way ANOVA). The p-value on top of each graph represents the pairwise comparison between treatment groups. Significance codes for pair-wised boxes are: 0.001 ´**´, and 0.01´*´. The p-adjust utilized is Bonferroni.*

Cephaeline and emetine production

Fig. 2(A) shows the emetine and cephaeline concentrations of Ipecac roots during maturity. Results are consistent with total alkaloid productions, showing its maximum cephaeline concentration between 16 and 19 months old (3.2-3.7%) and emetine's maximum titer between 14.5 and 19 months (1.4-1.7%). Also, cephaeline/emetine (C/E) ratio is around 2 in plants ranging from 13 to 17.5 months old plants, and then it increases to approximately 3 at 20.5 months (Fig. 2(B)). According to US pharmacopeia, oral formulations containing Ipecac extracts should not exceed 2.5 C/E ratio (Bleasel & Peterson, 2020a). Emetine's highest titer is reached earlier (around 6 months), while cephaeline's top concentration is

reached at 19 months. C/E ratio's trend is increasing at 19 months, although concentration of both alkaloids is decreasing. All this data suggests the same conclusion: optimal harvest time is around 16 months.

Cephaeline and emetine are monoterpenoid-isoquinoline alkaloids. The biosynthetic pathway comprises the transformation of loganin → deacetylisoipicoside → protoemetine → cephaeline → emetine (Jha, Sahu, Sen, Jha, & Mahato, 1991). Many of the present alkaloids only differ in the O-methylation over some of the phenolic residues regulated by O-methyltransferases (OMT). The conversion of cephaeline in emetine is catalyzed by ipeOMT1 (Nomura & Kutchan, 2010). Then, it is reasonable to think that C/E ratio is related to a change in the expression of ipeOMT1 at different growth stages, although additional experimentation is needed to agree to take that hypothesis.

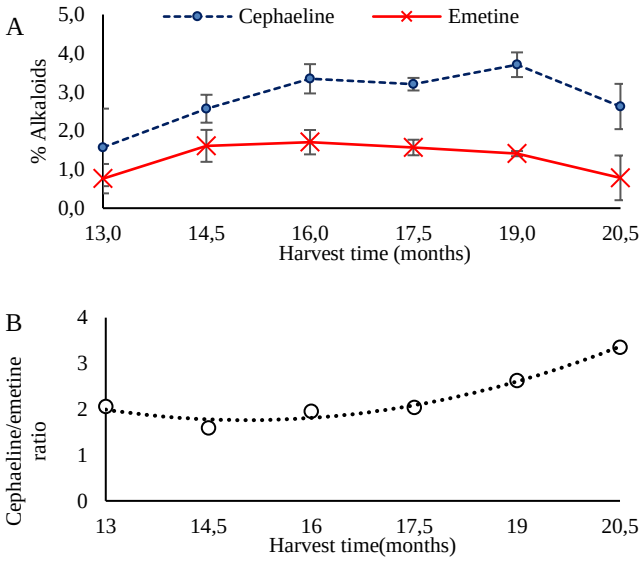

Figure 2. *Main alkaloids in C. ipecacuanha roots. (A) Cephaeline and emetine concentration in dried roots vs. maturity time. Error bars represent standard deviation. (B) Cephaeline/emetine ratio during maturity. The dotted line represents the ratio's trend.*

Antibiotic activity of Ipecac extracts

Alkaloids are known for being antimicrobial as well (T. T. Cushnie, B. Cushnie, & A. J. Lamb, 2014b). Then, the antimicrobial properties of the Ipecac extracts and how they change with the harvest time was explored. Fig. 3 summarizes antibiogram results for the antimicrobial activity *of C. ipecacuanha* against six bacterial strains. All the extracts showed antimicrobial properties, being at least more than half penicillin/streptomycin activity (54-94%). Interestingly, the antimicrobial activity does not decay in the presence of Gram-positive bacteria. Moreover, the activity of the extracts against *E. faecium* is almost as vast

as the positive control (penicillin-streptomycin), which is remarkable since this bacterium is one of the most problematic intra-hospital microbes due to its high capacity to generate resistance to antibiotics (Miller, Munita, & Arias, 2014).

Antibiotic activity is probably related to alkaloid concentration (T. T. Cushnie, B. Cushnie, & A. Lamb, 2014a). Then, the extracts from plants harvested at 16 months old are the most active, as we expected. This is because of the high alkaloid concentration (Cushnie et al., 2014a).

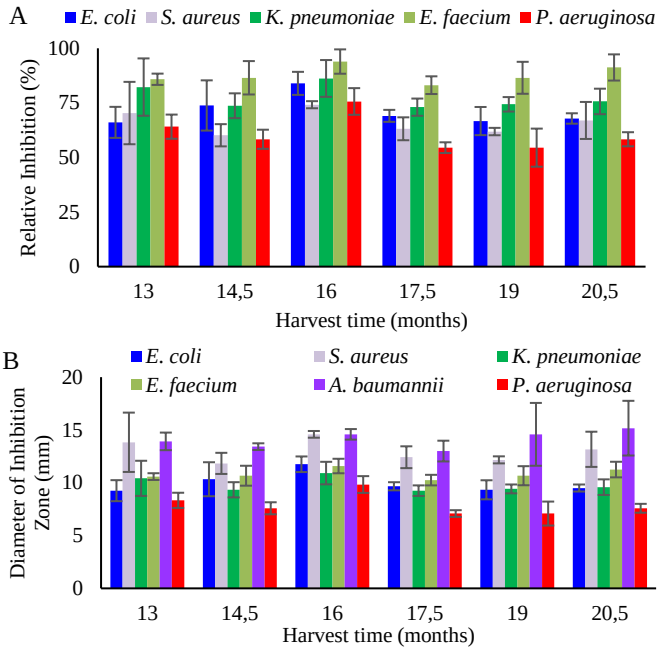

Figure 3. *Antibiotic activity of Ipecac extracts vs. harvest time. Error bars represent the standard deviation. (A) Percent of relative inhibition using penicillin-streptomycin 1000 µg/mL as positive control. A. baumannii was excluded because it was not inhibited by the positive control (B) Diameter of inhibition zones in antibiograms.*

Conclusions

The concentration of emetine, cephaeline, and total alkaloids in *C. ipecacuanha* depends on the harvest time. 16-month-old plants are considered the best because of the high alkaloid concentration, emetine, cephaeline, and low C/E ratio. Antibiotic activity is present and is high, even reaching similar activity than traditional antibiotics, for some samples. The antibiotic activity is probably related to the alkaloid concentration.

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Conflict of Interest

-The authors declare no competing interests.

Author contribution statement

All the authors declare that the final version of this paper was read and approved.

The total contribution percentage for the formal analysis, visualization conceptualization, preparation and correction of this paper was as follows: MAV-R., 40 %., R.S-L. 10%, J.A.R-R 10%, G.R-R 15%, V.Á-V 15%, P.J-B 10%,

Data availability statement

The data supporting the results of this study will be made available by the corresponding author, **V.A-V.** upon reasonable request.

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