

PHYTOCHEMICAL SCREENING, FREE RADICAL SCAVENGING, AND ANTI-INFLAMMATORY ACTIVITY OF *CROTON LEPTOSTACHYUS* KUNTH LEAF EXTRACTS**WILMER FERNANDO SÁNCHEZ PERALTA^{1*}, LUIS FERNANDO OSPINA GIRALDO²,
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ABSTRACT

Objective: To study the biological potential of *Croton leptostachyus* Kunth (Mosquero) leaves through phytochemical screening and determination of the free radical scavenging capacity (against 2,2-diphenylpicrylhydrazyl [DPPH•] and 2,2'-azinobis-[3-ethylbenzothiazoline-6-sulfonic] acid [ABTS⁺]) and anti-inflammatory activity through a tetradecanoylphorbol acetate (TPA) induced auricular edema model in ICR mice of the crude ethanolic extract and sub extracts (of n-hexane, ethyl acetate, and ethanol).

Methods: The *C. leptostachyus* leaf extracts were obtained through ethanolic maceration and subsequent partition of the dry ethanolic crude extract with n-hexane and ethyl acetate, the aforementioned samples were tested for phytochemical composition, antiradical activity (DPPH• and ABTS⁺), and anti-inflammatory activity (TPA induced auricular edema model in mice).

Results: The phytochemical plant profile showed a diversity of secondary metabolites such as terpenic compounds (triterpenes and saponins), phenolics (flavonoids, tannins, and phenylpropanoids), alkaloids, and a minor proportion of iridoids and coumarins. Free radical scavenging assays indicated a presumable combined effect of the *C. leptostachyus* leaf metabolites considering the fact that the crude extract was the most active scavenger of DPPH• and ABTS⁺. However, between the subextracts and the ethanolic subextract showed the highest antiradical activity indicating a presumable major contribution of phenolic compounds in this result. The topical anti-inflammatory action of ethyl acetate sub extract showed a possible enhanced effect due to a higher concentration of medium polarity compounds; no anti-inflammatory activity of the subsequent fractions of this sample was observed indicating a probably interaction of the metabolites contained therein.

Conclusion: *C. leptostachyus* leaf extracts showed a potential as free radical scavengers and anti-inflammatory compounds sources.

Keywords: *Croton leptostachyus*, Free radical scavenging activity, Anti-inflammatory activity.

INTRODUCTION

Inflammation plays a key role in the host defense against harmful agents, whether physical, chemical, or biological, allowing the containment and elimination of pathogens, repair of the affected tissue and the preservation of homeostasis of the host [1]. Depending on how long persists the inflammatory response, it can be classified into two phases: Acute or chronic. The acute phase is characterized by five cardinal signs are a pain, redness, fever, tumor, and loss of function. If the inflammation persists for prolonged periods beyond the resolution, this becomes chronic and pathological; many, high cost, and highly disabling chronic diseases as cancer [2], asthma [3], Type 2 diabetes [4], and Alzheimer [5] are close related with inflammatory processes.

Among the signaling pathways implicated in the inflammatory response, free radicals play an important role, since they are very potent chemical mediators involved in the activation of transcription factors such as nuclear factor-kappa B [6,7] that promotes the expression of proteins such as cytokines (interleukin [IL-1 β], tumor necrosis factor [TNF]- α , interferon- γ , IL-6, IL-2, and IL-8), and leukocyte adhesion molecules [8]. Additional to these physiopathological responses, free radicals induce inflammatory processes by direct damage to DNA [9] and cell membrane that triggers and increment in the intracellular Ca⁺⁺ [10], resulting in activation of phospholipase A2 and the production of prostanoids by enzymatic mechanisms [11], as well as isoprostanes by direct lipid peroxidation [12,13].

Non-steroidal anti-inflammatory drugs are the first therapeutic option in the treatment of inflammation. However, their effectiveness, the

gastric damage caused by their non-specific action in the inhibition of cyclooxygenases 1 and 2 (COX-1 and COX-2), and their toxicity mediated by the pro-oxidant action of many of the compounds belonging to this family drug [14,15] have motivated the search for new, safer, and more effective anti-inflammatory compounds; in this context, natural products from plants pose as promising materials for the synthesis of drugs with greater spectrum of bioactivity and safer [16-18].

The genus *Croton* (Euphorbiaceae), comprises about 1300 species of trees, shrubs, and rarely herbs [19]. *Croton* species are characterized by different types of leaves, colored exudate, clear or reddish, often little or apparently absent [20] usually with glands at the base of the blade and/or the petiole, female flowers with reduced or absent petals, ovary usually tricarpeal, with one egg per locule [21]. *Croton* specimens inhabit a very wide range of habitats and can be found in all types of vegetation although most of them can be found in dry or open vegetation [22]. Previous works have studied the antioxidant [23,24] and inflammatory [25-28] properties of species from this genus.

Croton has a wide range of compounds of biomedical importance such as alkaloids, terpenes, and phenols [23,29]. These species are rich in terpenoids (mainly diterpenes) within which highlights the cembranoid, clerodane, neoclerodane, isopimarane, kaurene, sec-kaurene, labdanum, phorbol, and trachiloban types [19]. The major groups of alkaloids reported in *Croton* are the glutarimide [30], proaporphine [31], dihydroproaporphine, aporphine [32], and tetrahydroprotoberberine [33] types. Among the group of phenolic compounds flavonols, flavones [34,35], and phenylpropanoids [36] has been found.

Croton leptostachyus Kunth (Mosquero) is used in the Colombian ethnobotany with food, craft, veterinary, medicinal, ornamental, and fodder purposes [37]. However, this panorama, there are few experimental studies supporting this knowledge; to date, only one report focused on ethnobotany, phytochemical, and antimicrobial activity of this plant can be found [37]. Given the chemotaxonomic background of this genus, traditional uses, and limited scientific knowledge of the species *C. leptostachyus*, the objective of the present study was to establish a phytochemical characterization of its leaves and to evaluate the antiradical capacity *in vitro* by scavenging of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS^{•+}) radicals, and anti-inflammatory activity in a model of TPA-induced ear edema in mice, considering the importance of compounds such as alkaloids, terpenes, and phenols have in preventing free radicals mediated pro-oxidant effects [38-40], and therefore, provide knowledge to set the value of this species as a promising source of bioactive principles of therapeutic interest.

METHODS

Vegetal sample

C. leptostachyus Kunth leaves were collected in the environs of Ibagué, Tolima (Colombia), at the Coordinates 19° 34.4'N and 75°05' 35.07" W. A voucher was deposited in the National Herbarium of Colombia (Code 572765).

Reagents

DPPH, ABTS, (±)-6-Hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox), and potassium per sulfate were purchased from Sigma-Aldrich. Solvents and other reagents used in the chromatographic procedures were acquired from Merck and were analytical grade.

Preparation of extracts

C. leptostachyus leaves were air dried, mill crushed, and macerated with ethanol (1:10 powder/solvent) until exhaustion of the sample. After obtaining the ethanolic extract (MOSQEE), it was dried under reduced pressure (35°C). A portion of the crude extract was destined to phytochemical screening and the remainder was separated by solid-liquid extraction with successive washes, thus, obtaining the n-hexane (HEXMOSQSE), ethyl acetate (ACETMOSQSE), and ethanol (EMOSQSE) sub extracts. The samples were dried under the same conditions of the crude extract, packaged, labeled, and stored under refrigeration (5°C) for further assays.

Phytochemical screening

A phytochemical screening of the ethanolic extract and sub extracts of *C. leptostachyus* leaves was carried out in order to establish a preliminary phytochemical profile of the species. The developed assays (precipitation and TLC) focused on the presence of saponins (Rosenthaler), polyphenols (Folin-Ciocalteu, TLC), tannins (FeCl₃, Gelatin-Salt), flavonoids (Shinoda), phenylpropanoids (Arnow), terpenes and steroids (Lieberman-Burchard; Salkowski, TLC), iridoids (ethanolic vanillin), alkaloids (Dragendorff, Mayer, Erlich, Tanred, Valser, TLC), quinones (Bornträger), and coumarins (TLC) [41].

DPPH• assay

The DPPH scavenging assay was performed as previously described [42], with some modifications. Briefly, 0.5 mL of each of the diluted solutions of the extracts was mixed with an equal volume of a methanolic solution of DPPH 0.02%. The mixture was kept in darkness for 30 minutes and then the absorbance of each mixture was measured at 517 nm against a blank constituted by a mixture of equal volumes of methanol and ethanol (solvents of the extracts and the radical, respectively). The control consisted of a 1:1 mixture of ethanol and methanol solution of DPPH•. A curve of trolox (0.1-1 µg/mL) was used as a standard to compare the antioxidant activity of the extracts. Values of antiradical activity at different concentrations were estimated by the following equation:

$$\% \text{DPPH scavenging} = \left(\frac{A_C - A_M}{A_C} \right) \times 100$$

where: A_C: Control absorbance, A_M: Reaction mixture absorbance

ABTS^{•+} assay

The method described by Kuskoski [43] was followed: The radical was prepared by a mixture of ABTS (7 mM) and potassium per sulfate (2, 45 mM final concentration). This mixture was incubated for 16 hrs at room temperature and then was diluted with ethanol to obtain an absorbance of 0.7±0.02 at 734 nm. After adjusting the radical, the assay was started by mixing 3.43 mL of the adjusted ABTS^{•+} solution with 70 µL of the extracts at different concentrations (0.2-200 µg/mL final concentration); the absorbance was measured after 6 minutes of reaction. Trolox (0.1-1 µg/mL) was used as standard. The percentage of radical scavenging was calculated as follows:

$$\% \text{ABTS scavenging} = \left(\frac{A_{\text{ABTS}} - A_{6\text{minutes}}}{A_{\text{ABTS}}} \right) \times 100$$

where: A_{ABTS}: ABTS^{•+} absorbance before mixing, A_{6minutes}: Mixture absorbance after 6 minutes

Inhibition of TPA-induced ear edema

For this assay, ICR mice (28-40 g weight) supplied by the Department of Pharmacy experimental bioterium of the National University of Colombia. The animals were held under the following conditions: Photoperiod (12 hrs light/12 hrs dark), temperature (21±2°C), and controlled humidity, the supply of filtered air and food and water *ad libitum*.

The ear edema was induced by administration of 13-ethyl-12-O-tetradecanoylphorbol (TPA, 100 µg/ear, 10 µL per side of the ear) as described by De Young [44] with modifications proposed by Payá [45]. Treatments (extracts and sub extracts) and the positive control were applied at a dose of 1 mg/ear to each corresponding experimental groups. After applying the treatment to each animal of a group, a solution of TPA (2.5 µg) dissolved in acetone (20 µL) was applied topically, both inside and outside of the right ear of each mouse (10 µL per side) in order to induce inflammation.

Four hours after TPA application, mice were sacrificed by cervical dislocation and discs (7 mm diameter) of the right ear (treated) and left (non-treated) were obtained with a puncher. The discs were weighed to determine the edema generated as a variation (delta) of the weight (W_t - W_{nt}). The percentage of inhibitory activity of auricular edema (%AEIA) was calculated by the following equation:

$$\% \text{AEIA} = \left(\frac{(W_t - W_{nt})_{\text{Control}} - (W_t - W_{nt})_{\text{Treatment}}}{(W_t - W_{nt})_{\text{Control}}} \right) \times 100$$

where: W_t: Weight of treated ear disc, W_{nt}: Weight of non-treated ear disc

Statistical analysis

Data were expressed as a mean ± standard deviation. Treatments were compared using ANOVA followed by a Bonferroni's test using the statistical package STATGRAPHICS Centurion at 95% of significance (*p<0.05).

RESULTS

Phytochemical screening

The phytochemical composition of the leaves of *C. leptostachyus* is shown in Table 1.

The phytochemical screening revealed the presence of saponins, phenolic compounds, triterpenes, and alkaloids in the *C. leptostachyus* leaves.

Table 1: Phytochemical composition of *C. leptostachyus* crude extract and sub extracts

Secondary metabolite	Sample			
	MOSQEE	EMOSQSE	ACETMOSQSE	HEXMOSQSE
Saponins	+++	ND	++	+
Polyphenols	++	+	++	ND
Tannins	++	+	++	ND
Flavonoids	++	++	+	ND
Phenylpropanoids	+	+	+	ND
Triterpenes and steroids	+++	+	++	+++
Iridoids	++	+	+	ND
Alkaloids	+++	+	++	+++
Quinones	ND	ND	ND	ND
Coumarins	+	ND	+	ND

Conventions: +: Low, ++: Medium content, +++: High content, ND: Not detected under the test conditions, *C. leptostachyus*: *Croton leptostachyus*

In vitro free radical scavenging

The results of the DPPH• and ABTS• scavenging activity of the extracts from *C. leptostachyus* leaves are shown in Table 2.

The crude ethanolic extract and sub extracts from *C. leptostachyus* leaves have antiradical activity.

Inhibition of TPA-induced ear edema in mice

Fig. 1 shows the results of the anti-inflammatory activity of the extracts from *C. leptostachyus* leaves.

DISCUSSION

In the study of natural products, the phytochemical analysis is a key tool to investigate the chemical composition and establish the quality of a plant species [46] and define its potential as a source of bioactive molecules. The phytochemical profile of crude extract of *C. leptostachyus* leaves shows the presence of saponins, phenolic compounds (tannins, flavonoids, and phenylpropanoids), triterpenes, alkaloids, iridoids, and coumarins. It was observed a distribution pattern associated with the polarity of the solvents used in the preparation of the sub extracts. According to above-mentioned, saponins were abundant in the crude extract (MOSQEE), but since they are terpenic/steroidal species, they distributed only in samples of low (HEXMOSQSE) and medium polarity (ACETMOSQSE), predominantly in the second one indicating a probably greater proportion of saponins in these two samples.

The phenolic content is moderate, and thus, phytophenols present in *C. leptostachyus* leaves were distributed in EMOSQSE and ACETMOSQSE. The same as the case of the saponins, the fraction of medium polarity had the higher phenolic content, which may be associated with increased presence of non-glycosylated phenols in the leaves of the plant, with a higher proportion of tannins as compared to flavonoids and phenylpropanoids. The previous studies in other species of *Croton* have demonstrated the importance of these compounds in biological activities such as cardioprotection mediated by its antioxidant action in *C. celtidifolius* [38] or antiviral activity in *C. lechleri* [47]. Considering the fact that most reported of the property for the phytophenolic compounds is free radical scavenging, this result would infer a potential antiradical effect of *C. leptostachyus*.

It was found an abundance of terpenic compounds, which are rich in the resin of species of this genus. Some compounds of this type, isolated from other species of *Croton*, have shown anticancer [48] and antiproliferative [49] effects. These metabolites distributed in all sub extracts being less abundant as the polarity of the solvents increases.

There was a high alkaloid content and according to their structural properties, the order of distribution in these samples is inversely proportional to the polarity of the solvent.

Iridoids present in the plant material are moderated and distributed equally in EMOSQSE and ACETMOSQSE sub extracts. Coumarins were found only in ACETMOSQSE.

Table 2: Free radical scavenging activity of *C. leptostachyus* leaves extracts and sub extracts

Sample	IC ₅₀ (µg/mL)	
	ABTS•	DPPH•
MOSQEE	11.6±0.3	53.5±1.1
HEXMOSQSE	50.72±0.98	2451.29±46.3
ACETMOSQSE	16.53±0.58	491.41±32.2
EMOSQSE	14.05±0.32	740.89±44.8
TROLOX	3.15×10 ⁻⁶	3.98×10 ⁻⁶

Data expressed as the average of three determinations±standard deviation. With a significance level of 95% (*p≤0.05), differences were observed between treatments, DPPH•: 2,2-diphenylpicrylhydrazyl, ABTS•: 2,2'-azinobis-[3-ethylbenzothiazoline-6-sulfonic] acid, *C. leptostachyus*: *Croton leptostachyus*

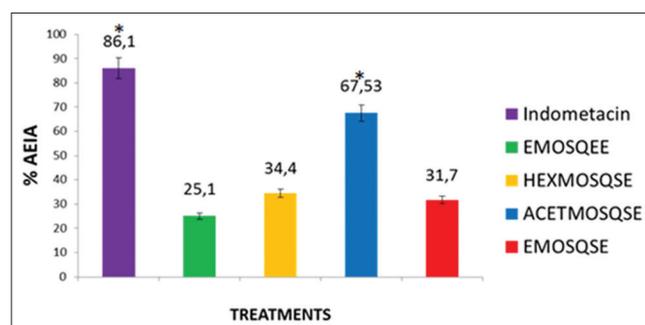


Fig. 1: Inhibitory activity of tetradecanoylphorbol acetate-induced ear edema. Increased activity was observed in *C. leptostachyus* leaf sub extract ACETMOSQSE as compared to other extracts, which showed no statistically difference at a 95% significance level (*p≤0.05)

A previous reports have demonstrated antioxidant activity of different *Croton* species such as *C. lechleri* [50] and *C. celtidifolius* [26]. The authors conclude that results obtained are justified by the presence of phenolic compounds. In some cases, more than 90% of the dry weight of the sap of these species is constituted by flavonoid type phenolic compounds such as proanthocyanidins, catechin, epicatechin, galocatechin, and epigallocatechin [51]. As previously noted in the results of phytochemical screening, some of these compounds were detected in *C. leptostachyus* leaves, which along with the antecedents of other species of this genus supports the idea of considering its antioxidant potential.

According to the values obtained in the two vitro antiradical activity tests, increased capacity of the reference standard (IC₅₀ of 3.15×10⁻⁶ and 3.98×10⁻⁶ µg/mL for ABTS• and DPPH•, respectively) was observed. However, the crude extract showed a higher antiradical activity than the sub extracts, with IC₅₀ values of 6.11 µg/mL for ABTS•

and 53.5 µg/mL for DPPH•. Concerning to the sub extracts antiradical activity, MOSQSE was the most active followed by HEXMOSQSE and ACETMOSQSE, respectively, and although phytochemical screening showed a greater presence of phenols in ACETMOSQSE the highest free radical scavenging was observed in EMOSQSE. It may be due to the hydrophilic environment, in which the assays were developed, favoring the interaction of polar compounds with the radical species used.

This trend may indicate a combined effect in the composition of the plant suggesting that the whole *C. leptostachyus* leaves constituents exerts their antiradical action more effectively. Significantly, the medium polar compounds, among which may be aglycone flavonoids and some terpenic compounds, also offer considerable potential contribution to the sample, considering the mode of action the underlying in the methods (proton donation in DPPH• and electron transference in ABTS•).

According to the results of the anti-inflammatory assay, the crude extract and sub extracts of leaves from *C. leptostachyus* possess anti-inflammatory potential, although the response pattern differs from the observed in the antiradical activity, where the crude extract showed a higher scavenging of DPPH• and ABTS•, this result could be justified considering the modes of action exerted on the biological reagent and the complexity of cellular responses in the affected tissue. Statistical analysis (ANOVA, *p<0.05) revealed no difference in the activity of both crude extract and the hexanic and ethanolic subextracts.

The relationship between the phenolic content, antiradical/antioxidant, and the anti-inflammatory activity of plant species has been evaluated by several authors [52-54]. ACETMOSQSE showed the highest auricular edema inhibitory effect (67.5±11%) despite being lower than that of indomethacin (86.1±8%) is significant and appears to be associated with a higher concentration of medium polarity compounds; if the chemical antecedents of the genus are considered, as well as the phytochemical screening results, it seems likely to assume that tannin type compounds and, in a minor proportion non-glycosylated flavonoids and phenylpropanoids, as well as a considerable ratio of terpenes, saponins, and even coumarins contained in this sample could be involved in this response. All of this would indicate a diverse range of modes of action in which the polar compounds exert greater antioxidant effect, and the grouping of medium polar compounds exerts anti-inflammatory effect.

Considering the screening nature of this test, and that values >40% inhibition define the potential of an extract or sub extract as a promising anti-inflammatory, column fractionation with silica gel 60 was developed to define the secondary metabolites responsible for the anti-inflammatory activity of ACETMOSQSE using as eluents n-hexane-ethyl acetate (9:1), ethyl acetate-methanol (7:3), and methanol-water (7:3). The obtained fractions identified as F1, F2, and F3 were tested following the same conditions of the initial test, showing null anti-inflammatory activity (data not shown); reinforcing the idea of combined action of these metabolites in ACETMOSQSE and suggesting its potential application as a topical anti-inflammatory.

The previous research has demonstrated the antioxidant and anti-inflammatory activity of other species (*C. celtidifolius*) [25,26] in different *in vivo* inflammation models and gives support to this work with the idea of dual biological effects (antiradical and anti-inflammatory) of *C. leptostachyus* supported by the chemotaxonomic antecedents of this genus.

CONCLUSIONS

The free radical scavenging and anti-inflammatory activities of the crude ethanolic extract and subextracts of *C. leptostachyus* were demonstrated. The ethanolic extract was the most effective concerning the antiradical capacity, but the activity reported by the ethanol sub extract (EMOSQSE) suggests a high contribution of the polar compounds, among which is included phenolic types. In contrast, the anti-inflammatory response

revealed by the ethyl acetate sub extract (ACETMOSQSE) associated with their phytochemical composition, probably is due to a significant contribution of some medium polarity terpene, saponin, and phenolic-type compounds. Further fractionation of the sample and the consequent loss of activity of the fractions obtained can be indicative of an interaction exerted by the secondary metabolites of this sub extract. It is important to evaluate other mechanisms of antioxidant and anti-inflammatory activity in these samples for both *in vitro* and *in vivo* in order to obtain a better knowledge of the biological potential of *C. leptostachyus* in the treatment of conditions associated with oxidative stress and inflammation.

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