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Research Article

Pharmacognostic and Phythochemical Studies of *Croton Linearis* Jacq. Leaves.

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ABSTRACT
Leaves from *Croton linearis* Jacq are usually used by Cuban population to treat thefever, but there is almost no information about this plant; that's why in the present research we accomplish a phytochemical and pharmacognostical study of *Croton linearis* Jacq. leaves. Methods: Macro and micromorphological analysis, residual moisture andtotal ashesof the leaveswere performed.An experimental design consisting in the combination of three extractive methods (soxhlet, ultrasound and stirring maceration) and foursolvents (Petroleum ether, ethyl acetate, ethanol and water) was alsocarried out (12 extracts) in order to determine the best extractive combination through the determinationof the total solublesubstances andqualitativechemical composition.To the best extractive combination, its physicochemical (pH, refraction index and relative density)and chemical parameters (fingerprint and thin layer chromatography) were determined. Results: The macro and micromorphological description of the leaves, the values of residual moisture (11.0 ± 1.41 %,) and total ashes (4.54 ± 0.57 %) is informed. The ethanol extracts showed the highest values of total solids for the three extractive methods (0.840 ± 0.071, 0.553 ±0.058 y 0.478 ±0.139), presenting the major diversity of metabolites: alkaloids, triterpenes, carbohydrates, reducing sugars, amino acids, flavonoids and tannins whilethe stirring maceration was the best method. The pH, refractive index and relative density of the extract results of this combination (Ethanol/stirring maceration) is informed as well as it UV-Visible fingerprint. The TLC study showed presenceofa majority substance with UV/Visible absorption at λ 665 ηm. Conclusions: The pharmacognostical and phytochemical parameters determinedwill be useful in order to authenticate and standardize the vegetal drug, while thephysical-chemical and chemical parameters of ethanol extract can be used forits quality control.

keywords: *Croton linearis*, leaves, pharmacognostical parameters, fingerprint

INTRODUCTION

With over 1300 species Croton is one of the most representative genders in the plant kingdom. It is widely distributed throughout tropical and subtropical regions. For Croton species the reported medicinal uses are related to the treatment of cancer, colds, digestive issues, diabetes, hypercolesterolemy, hypertension, inflammation, pain, malaria and ulcers[1,2]. A wide amount of metabolites has beenreported in phytochemical studies carried out about the genus, such as: terpenoids[3], essential oils[4], flavonoids and tanins[5] and alkaloids[6,7]. Alkaloids, phenolic compounds (flavonoids and tanins), saponins, triterpenes and sterols, were qualitatively detected in a phytochemical study carried out in Cuba on 14 *Croton* species[8]. *Croton linearis* Jacq.is widely distributed in the Eastern South shore of Cuba, however bibliographic reports are uncommon. Ethnobotanical information's refer its effectiveness as insecticidal, sedative and as antimicrobial agent[9]. The presence of morphinanedienone type alkaloids is reported in a phytochemical study developed in Jamaica[10,11]. Also, the insecticidal activity of an isolated diterpene from their leaves has been evaluated[12]. Besides these studies, no other scientific papers related to the species were found during the bibliographic revision; remaining its study ofgreat interest. Therefore, this paper intends to accomplish a pharmacognostic and phytochemical study of *Croton linearis* Jacq leaves in order to enrich the existing scientific information about this species, providing valuable data for the standardization of the plant material and its derived extracts.

MATERIALS AND METHODS

Plant Material

Leaves of *C linearis* Jacq were collected from Siboney-Juticí Ecological Reserve, Santiago de Cuba, Cuba in

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September, 2012. A specimen of the plant was identified and authenticated by the taxonomist Felix Acosta and a voucher sample was deposited at the Herbarium of the Eastern Center of Ecosystems and Biodiversity (Code: 21 659). The plant material was dried in the shade at room temperature for 14 days until an invariable weight measure was obtained.

Leaves pharmacognostic parameters

Macroscopic Evaluation:

To determine this parameter a 100 leaves sample from 10 individuals belonging to the species was taken, width and length mean dimensions were established to describe the external morphology (shape), the disposal of the stem, venation, shape of the vertex and base, by margin or edge, consistency and by the color and nature of the upper and lower surfaces.

Microscopic Evaluation

Five adult leaves from 4 individuals were chosen for the foliating anatomy study, which were hydrated with distilled water and glycerin (1:1) because of their membranous texture. Afterwards, transverse cuts were made to the whole foliating gill, besides from transversal and lengthwise cuts to the medium vein; these were performed manually using a paraffin support. The transversal cuts were died with safranin and differentiated with alcohol 70 %, in order to be subsequently washed with distilled water, according to the methodology described by Peña and Saralegui[13]. The number of cellular stratums, the thickness of the cuticle compared to the epidermis, the thickening or not of the anticlinal and periclinal cellular walls and the shape of the anticlinal walls, were the analyzed characters regarding adaxial epidermis. On the other hand, for the abaxial epidermis, the shape of the cells observed in the paradermal surface, the kinds and number of stomas on both surfaces of the leaves and the nature of the mesophyll were considered. Taking into account the criteria of Theobald and collaborators[14], the type of trichome found in the abaxial and adaxial surfaces was determined. Also, the presence of wax was assessed by analyzing the way it is arranged and using the terminology of Barthlott[15]. Observations were made using a NOVEL N-220M bright field microscope 400X and a NOVEL NSZ-606 stereo-microscope 40X, both from china.

Residual Moisture

The presence of essential oils has been reported for several species of the *Croton* genus, therefore this pharmacognostic parameter was determined by the toluene azeotropic method on the shadow-dried drug, as it is described in the compendium for Quality Control of Medicinal Plants[16]. Equation 1 was used to calculate this parameter. The experiment was developed by triplicated.

$$Equation\ 1 \quad H = \frac{(V_2 - V_1)}{M} \times 100$$

Where:

V_2: Final volumeof water (mL).

V_1: Initial Volume of water (mL).

M: Mass of the sample (10 g)

Total Ashes

Total Ashes were determined according to the Quality Control of Medicinal Plants Compendium[16]. A sample of 2g of dried plant material was placed in a crucibleand incinerated in a ML.-12 muffle (Germany) for 2 hours. Results were reported based on the percentage of ashes in anhydrous base, using equation 2. The mean of the 3 replicates is informed.

$$Equation\ 2 \qquad C_t = \frac{(M_2 - M_1) \times 100}{M} \times \frac{100}{100 - \mathrm{H}}$$

Where:

C_t: totalashes in anhydrous base

M: Mass of the sample (2 g).

M_1: Mass of the empty crucible (g).

M_2: Mass of the crucible containing ashes (g).

H: % Residual Moisture.

Soluble Substances

A 4x3 experimental design was developed to determine this parameter using 4 solvents (petroleum ether, ethyl acetate, ethanol and water) and 3 different extraction methods (Soxhlet, ultrasonic, and stirring maceration). In all cases 5g of dried leaves and 100ml of solvent were employed. The percentage of extracted substances (equation 3) was considered as quantitative criterion while the diversity of metabolites that tested positive when faced to the chemical essays described by Komathi and Rajalakshmi was considered as qualitative one[17]. All experiments were carried out by duplicated. The extraction methods were developed under the following conditions:

- Hot Continuous Extraction (Soxhlet): during 4 hours after the first Soxhlet reflux.
- Ultrasonic extraction: At 40 kHz during 4 hours controlling temperature at 40 ^{0}C in an ultrasonic bath SB-3200DTD (China).
- Stirring maceration: during 24 hours in a Shaker JPSelecta 3000974 (Spain).

$$Equation\ 3 \qquad Ss = \frac{Pr - P}{V} 100$$

Where:

Ss: Soluble Substances

P: Mass of the empty capsule (g)

Pr: Mass of capsule containing theresidue(g)

V: Volume of the essay portion (5 mL)

Considering these criteria the best combination of solvent/extraction method is chosen to prepare an extract for posterior standardization.

Pharmacognostic parameters of the selected extract

pH

This variable was determined using a digital pH-meter (Hanna instruments, Spain) with a combined glassy and calomel electrode with temperature control as it is established in Health Ministery National Standards 312/91. Three measurements were fulfilled and the mean was reported.

Refraction index

The determination of the refraction index was accomplished with a German ABBE refractometer as established in the Health Ministery National Standards 312/91[18]. Three measurements were fulfilled and the mean was reported.

Relative density

Relative density was accomplished using a 25 mL picnometer as established in the Health Ministery National Standards 312/91[18]. Three measurements were fulfilled and the mean was reported.

UV-Visible Fingerprint

In order to be able to determine absorbance peaks in UV Visible region from 200 to 900ηm, a volume of extract is dissolved in methanol. Measurements were made in a double bundle spectrophotometer RAY LEIGH UV-2601 (China).

Thin Layer Chromatography (TLC)

The selected extract was chemically evaluated bythin layer chromatography on silica gel Merck platescontaining an UV-fluorescent indicator (Germany). Four solvent systems were considered: hexane/chloroform/methanol (15:12:5), hexane/chloroform (3:7), Hexane /methanol (3:7), chloroform/methanol (7:3). An UV lamp WD.9403E (China) was used at λ 254 and 363 ηm to detect the bands. Retention times were calculated.

When a good separation was obtained, preparative plates were prepared in order to remove the bands and dissolved in methanol.Further UV-Visible spectrums (λ200-900 ηm) were measured in the double bundle spectrophotometer RAY LEIGH UV-2601 (China) previously mentioned.

Statistical Analysis

To select the optimal solvent/extraction method combination, an ANOVA analysis was performed using the software Statgraphics Plus Version 5.1 for Windows, professional edition. Differences that exceeded a 0.05 value of α were considered as significant for the Low Significant Differences Turkey-Kramer test.

RESULTS AND DISCUSSION:

Leaves Pharmacognostic Parameters

Macroscopic Evaluation

Leaves of the species are alternate, linear, forming terminal fascicles, pinnate, acuminate in the apex, obtuse in the base, with whole-limbo edges and membranous consistency. Dark-green upper surface leaf is less colored and pubescent than the lower surface which happens to be light green. In generally this description coincides with the "Bush Medicine of the Bahamas" and with other related bibliographies. The study of the dimension of the leaves indicates values of length and width of 8.148 ± 1.07 cm and 3.731± 0.49 mm respectively. These results also match the ones reported en literature for this species, which inform ranges of length values from 3.5 to 10 cm and width from 2 to 6 mm[19]. This variability in sizes of the leaves could be related to the development stage of the plant and environmental changes.

Microscopic Evaluation

The foliating surface is surrounded by hairs that abounds in the adaxial side, and is formed by starred trichomes with 3-16 unicellular hairs, that have an acuminated vertex and are arranged pelted in adaxial and sitting in abaxial. A thick cuticle appears in the adaxial side with more than one third of the epidermis thickness. The epidermis consists of a single polygonal cells cellular stratum, showing thickening of the straight periclinal cellular walls and straight and concave anticlinal cellular walls in the farthest surface of the central nerve (Figure 1).

The abaxial epidermis has only one polygonal cells cellular stratum with anomocytic stomas from two to three per field (100X). A great number of round glandular cells appear exceling the epidermis occasionally associated to the insertion of trichomes (Figure 1). They are arranged sort of regularly all along the surface except over the medium nerve. A palisade parenchyma stratum is found in the mesophyll and beneath it a spongy parenchyma with one or two associated stratums (Figure 1). Wax was observed all along the leaf upper surface, like consequence to the xerophyte nature of the vegetable formation where it was collected (Coastal Xeromorphic Thicket).

Residual Moisture

The moisture content of shadow-dried leaves was 11.0 ± 1.41 %, this value is withinlimits of 8 and 14% established by British Pharmacopeia[20]. According to this it is possible to dictate that the shadow-drying process is effective in reducing the amount of water in the species' leaves, which offers stability guaranties for the vegetable drug. Regardless the drying process; leaves kept its characteristic aromatic scent, also confirming the suitability of this method for species that contain essential oils.

Total ashes

Calculated values of total ashes for the three replicas resulted in 4.54 ± 0.57 % which is within the admissible range for vegetable species that can reach values exceeding 11 %. This parameter is associated to the presence of inorganic material in the leaves. In a commercial process where collecting volumes are significantly high, this indicator is very important, because it can reveal the presence of dirt or dusty material; however for small collected amounts, values of total ashes are more related to the amount of inorganic microelements that the plant storages for its metabolic development.

Soluble substances: Influence of the solvent.

In the accomplished experiment four solvents were used: petroleum ether, ethyl acetate, ethanol and water, for each of the considered extraction methods (Soxhlet, Ultrasound and Stirring Maceration). Standard deviationsand means of each experiment are reported, as a result of the two carried out replicas (Table 1). For the Soxhlet extraction method, ethyl acetate and petroleum ether had a similar behavior according to Tuckeys' LSD test, showing the lowest absolute values. On the other hand, ethanolshows the better results, been statistically differentto water solvent. This behavior indicates that the semipolar properties of ethanol turn up suitable for the extraction of total metabolites of *Croton linearis* Jacq leaves. For the Stirring Maceration method, ethyl acetate and petroleum ether again showed similar behavior and statistical differences regarding water and ethanol, showing the lowest values of soluble substances. This proves that the nonpolar properties of petroleum ether and the low polar properties of ethyl acetate are not effective in the extraction of high percent of soluble substances. Between water and ethanol no significant differences were observed according to Tukey's LSD test. However ethanol shows a high value of

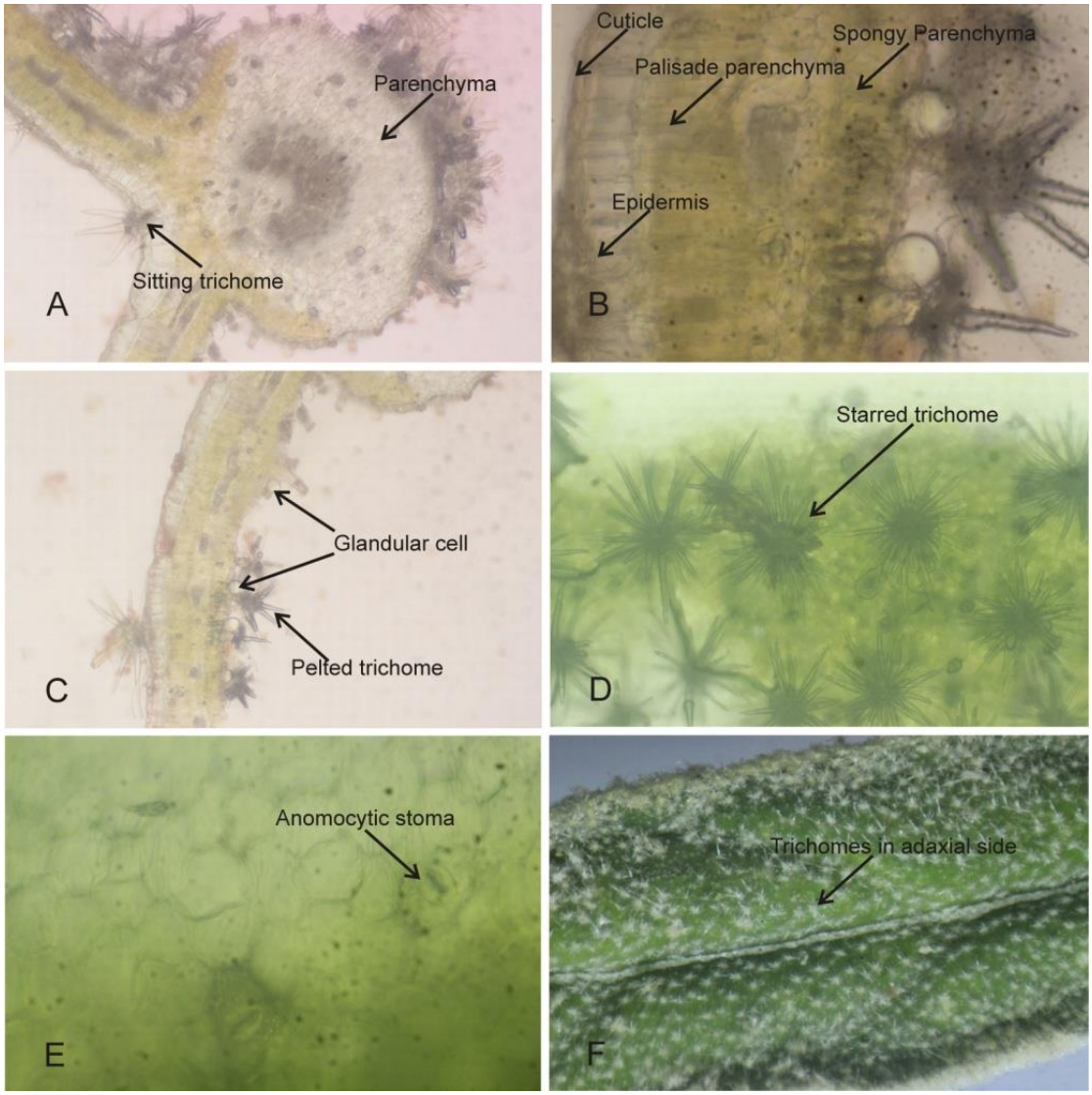

Figure 1: Transverse section of mesophyll (A, B, and C), longitudinal section (D and E) and adaxial side (F) of *Croton linearis* Jacq. leaves.

extracted substances. Finally, for ultrasound extraction ethyl acetate, petroleum ether and water showed the same statistical behavior, but different regarding ethanol, which again shows the highest percent. These results illustrate that petroleum ether and ethyl acetate are not suitable for the developed extraction methods. On the other hand, while for an extraction method (Stirring Maceration) ethanol and water behaved statistically similar, it is clear that ethanol reports the highest values of soluble substances, which combined to a higher microbiological stability, points out that the most quantitatively effective solvent for extraction of total metabolites from leaves of *Croton linearis* Jacq is indeed ethanol.

Soluble substances: Influence of the extraction method

For petroleum ether the ultrasound method showed the best results, however, it does not show statistically significant differences when faced to stirring maceration method. For ethyl acetate, statistically significant differences are observed among each of the methods, resulting to be the most effective extraction with Soxhlet. For ethanol, Soxhlet method again showed the highest value of soluble substances; however it is not statistically different to extraction by stirring maceration according to Tuckey's LSD test. Finally for water extraction by stirring maceration yielded the highest amounts without exhibiting statistical differences when faced to Soxhlet extraction. In spite of not being regularity, integral analyses about the influence of the different evaluated extraction methods shows that stirring maceration method is the one with the best extracting results. This observation is ensured when we is considered the simplicity of the method, requirement of complex equipment and energy waist. All together preserves the integrity of the original metabolites, having no possibility to be degraded by physical agents.

Qualitative evaluation. Chemical composition of the extracts.

The qualitative chemical composition was determined for all 12 extracts that were obtained from the 4x3 experiment. Results show that petroleum ether extracts are negative for the majority of the essays (Table 2), which indicates that

Table1: Influence of the solvent and extraction method in the extraction of metabolites (percentage of soluble substances) of *Croton linearis* Jacq leaves.

Solvent	Extraction method		
	Extraction by Soxhlet	Stirring maceration	Ultrasonic extraction
Ethanol	0,840 (± 0,071)[a; I]	0,553 (± 0,058)[a; I, II]	0,478 (± 0,139)[a; II]
Water	0,404 (± 0,054)[b; I,II]	0,499 (± 0,100)[a; I]	0,201 (± 0,024)[b; II]
Ethyl acetate	0,122 (± 0,003)[c; I]	0,038 (± 0,011)[b; III]	0,091 (± 0,001)[b; II]
Petroleum Ether	0,011 (± 0,007)[c; II]	0,021 (± 0,004)[b; I,II]	0,028 (± 0,001)[b; I]

Different letters within the same column indicate significant differences (LSD Tukey test, α= 0.05), Different Romans numbers within the same row indicate significant differences (LSD Tukey test, α= 0.05)

Table 2: Qualitative chemical composition of the extracts obtained from the dried leaves of *Croton linearis* Jacq.

Metabolites	Results											
	Petroleum Ether			Ethyl acetate			Ethanol			Water		
	S	*M*	*U*	*S*	*M*	*U*	*S*	*M*	*U*	*S*	*M*	*U*
Alkaloids	-	-	-	-	-	-	+++	+++	+++	±	±	±
Triterpenes& steroids	+	-	-	+	+	±	+	+	+	n/r	n/r	n/r
Coumarins	+	-	-	-	-	-	±	±	±	n/r	n/r	n/r
Resinas	n/r	n/r	n/r	n/r	n/r	n/r	-	-	-	n/r	n/r	n/r
Essential oils	-	-	-	-	-	-	-	±	-	n/r	n/r	n/r
Phenols & tannins	-	-	-	+	+	+	+	+	+	+	+	+
Free amino acids	-	-	-	-	-	-	+	+	+	n/r	n/r	n/r
Cardiacglycosides	-	-	-	-	-	-	-	-	-	-	-	-
Flavonoids	-	-	-	±	±	±	++	++	++	+	+	+
Carbohydrates	n/r	n/r	n/r	+	+	+	+	+	+	+	+	+
Quinones	-	-	-	-	-	-	+	+	±	n/r	n/r	n/r
Reducing sugars	-	-	-	±	±	±	+	+	+	+	+	-
Saponins	n/r	n/r	n/r	n/r	n/r	n/r	-	-	-	+	+	+

S: Extraction by Soxhlet M: Stirring maceration U: Ultrasonic extraction
+++intensepositive ++ evident positive + slight positive ± uncertain- negative**n/r**not done

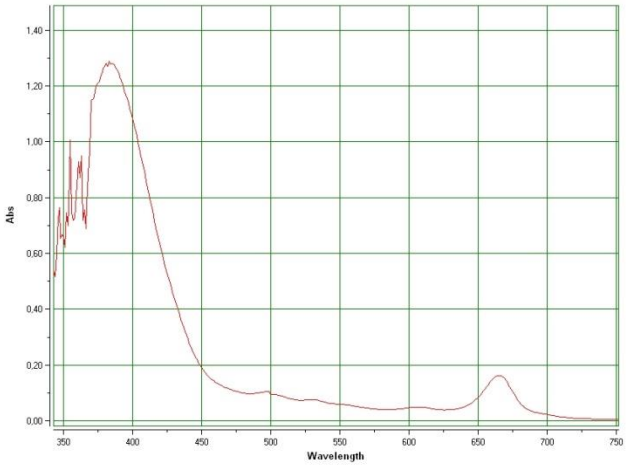

Figure 2A : Near-UV/Visible spectrum of the extract of Croton linearisJacq.leaves: (20 µL the extract in 3mL Methanol)

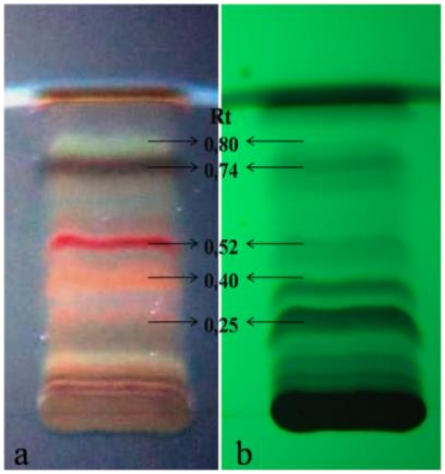

Figure 2B: The thin layer chromatography analysis carried out on the extract of *Croton linearis*

the high nonpolar properties of this solvents does not propitiate extraction of a wide variety of metabolites, resulting positive only for triterpenes and steroids essay in one of the extraction methods. On the other hand in the ethyl acetate extracts a higher number of positive essays was observed, indicating that the polarity of this solvent accomplishes the extraction of other metabolite groups that could not be extracted with petroleum ether, like tanines and flavonoids, as well as the presence of carbohydrates and reductive sugars. Nevertheless those qualitative results do not have a quantitative relevance in the total soluble substance test as was discussed before. The detection of carbohydrates and reductive sugars in ethyl acetate extracts should be related to glycones commonly found in triterpenes and steroids and flavonoids as has been previously reported for other *Croton* species[22]. Ethanolic extracts turned out as the highest chemical qualitative variety, extracting the wider diversity of metabolites such as alkaloids, triterpenes, phenolic compounds, carbohydrates and others (Table 2). This abundance and

Table 3. Pharmacognostic Parameters determinate to extract of *Croton linearis* Jacq leaves.

Pharmacognostic Parameters	Results (n=3)
pH	4,8733 ± 0,0208
Refraction index	1,3829 ± 0,0006
Relative density	0,9616 ± 0,0014

Table 4. Retention times and absorption maximums (UV-visible) of the bands obtained in thin layer chromatography TLC study on the extracts of *Croton linearis* Jacq leaves.

Band	Rt	λ_{max} (nm)
1	0,25	250
2	0,40	253
3	0,52	225, 251, 414, 665
4	0,74	225, 260, 432
5	0,80	239, 260, 434

diversity of metabolites concurs with the semipolar characteristics of this solvent, and matches at the same time with the accomplished high yielding of soluble substances. Finally, in the aqueous extracts carbohydrates, phenolic compounds, reductive sugars, saponines and other high polarity compounds are mainly extracted (Table 2). Regarding the extraction methods, these do not have a great influence in the chemical quality of the extracts (Table 2). In some cases slight differences are observed: the presence of triterpenes, steroids and cumarines in petroleum ether extracts only for Soxhlet method, indicating that higher temperatures straight forwardness the extraction of these compounds in the solvent. Also, in the aqueous extract by ultrasonic extraction there is no evidence of reductive sugars while they are present in Soxhlet and stirring maceration methods. All those observations confirm that the most proper solvent for the extraction of metabolites is ethanol, because the remaining solvents: petroleum ether, ethyl acetate and water manage to extract only specific groups of metabolites according to their miscibility in themselves. A general analysis derived from quantitative (percent of soluble substances) and qualitative (chemical composition), shows that combining the stirring maceration and ethanol is the most promising choice, therefore this combination was used to prepare and characterize *Croton linearis* Jacq leaves extract.

Pharmacognostic Parameters of the extract.

Determination of pH, refraction index and relative density

Obtained values of these parameters for the extract are disposed in Table 3. As this table illustrates, the extract has an acid pH which coincides with the presence of numerous phenolic nature compounds, as it was described in results of the qualitative chemical analysis. The efficiency of the process is once again evident in the values of refraction index and relative density, which are quite high compared to those reported for the pure ethanol solvent.

UV-Visible Fingerprint

An important aspect to consider when evaluating a plant extract is its fingerprint behavior, because it illustrates its characteristics in the matter of chemical composition working as pattern comparison. The measurement of this parameter results in the presence of multiple absorption maximums in the UV-visible region causing a distortion of the spectrum; nevertheless in the near and visible UV region it is possible to identify two maximums (λ= 383 and 665 ηm) characteristic of the extract, in a way that they can be used as its own fingerprint (Figure 3). Multiple maximums in the UV region may be related to the presence of an important number of phenolic compounds, whose presence was previously described in the qualitative chemical results and frequently informed in other species of the gender[23,24]. In a recent study, our research group reported the presence of the flavonoid 5-hidroxy-3,7,4´-trimethoxyflavone[25]. This light yellow colored compound, or some of its structural analogies might be responsible for the maximums observed in the near and visible UV region of the extract; confirming once again the validity of this technique when establishing a chemical marker of each vegetable extract.

TLC fingerprint Study

The thin layer chromatography analysis carried out on the extract proved that any of the five employed solvent systems could be used to separate a wide range of secondary metabolites, nevertheless; with hexane/chloroform (3:7) better results were obtained. Using this system it was possible to observe bands of well-defined intensity and coloring, mostly when λ=254 nm is used as revealer, allowing to characterize some of them by their retention time (Figure 3). Table 4 shows λ_{max} of each interest bands, showing differences among them in their UV-visible spectra. The multiple peaks in the region from 225 to 260 matches with the UV-Visible fingerprint behavior previously described, demonstrating the influence that those compounds gives to the *Croton linearis* leaves extracts. In addition, the red band with Rt=0.52 presented a λ_{max}=665 *nm* in the visible region of the spectrum, is similar to the λ_{max} observed for the total extract (Figure 2). All those results suggest us that the compounds separated by TLC are present in high concentrations. The presence of multiple bands of diverse intensity and coloring stands out the complexity of the evaluated chemical extract, however the obtained resolution for all 5 of the bands characterized in its retention times can be used as a chemical marker of the analyzed vegetable extract.

CONCLUSIONS

The present study gives an account on pharmacognostic and phytochemical screening of *Croton linearis* Jacq leaves which may useful in order to authenticate, standardize and avoid any adulteration. On the other hand, the UV/Vis fingerprint and TLC studies as well as the determination of physic-chemicals parameters will help to establish the quality control parameters for the ethanol crude extract.

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