Open Access Library Journal
2018, Volume 5, e4921
ISSN Online: 2333-9721
ISSN Print: 2333-9705

Pharmacognostical, Phytochemical and Antioxidant Evaluations of *Guettarda calyptrata* A. Rich.

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How to cite this paper: Gaitén, Y.I.G., Yaque, J.G., Lizama, R.S., Torres, Z.O., González, A.F., Jiménez, J.L.M. and Monan, M. (2018) Pharmacognostical, Phytochemical and Antioxidant Evaluations of Guettarda calyptrata A. Rich. *Open Access Library Journal*, **5**: e4921.
https://doi.org/10.4236/oalib.1104921

Received: September 18, 2018
Accepted: October 23, 2018
Published: October 26, 2018

Abstract

Guettarda calyptrata A. Rich., originally from Cuba and known as "Contraguao", has traditionally been used to counteract quickly and effectively burns produced by *Comocladia dentate* Jacq (Guao), vegetable species that secretes a highly caustic latex for skin and mucous membranes. A burn is a traumatic injury resulting in local and systemic injury with oxidative changes; in this sense the antioxidants play an important role. In order to offer aspects related to the quality and effectiveness of the plant, its pharmacognostic, phytochemical and antioxidant activity is presented. The morphoanatomical evaluation was carried out, physical-chemical parameters were determined for the crude drug and for the aqueous extract. The chemical profile of the extract was estimated by thin layer chromatography, ultraviolet-visible and quantification of total phenols by Folin-Ciocalteu and total flavonoids by the colorimetric method of aluminum trichloride ($AlCl_3$). Finally, the antioxidant activity was tested by the FRAP and DPPH techniques. Through the pharmacognostic study, the quality specifications of the drug and the extract were established. The methods of analysis used for the chemical profile suggested the presence of flavonoids and phenols in general. The aqueous extract showed antioxidant properties by the two methods evaluated. The study of *G. calyptrata* provided pharmacognostic, phytochemical and effectiveness as an antioxidant evidence, aspects to consider in the possible use of the plant by our Natural and Traditional Medicine.

Subject Areas

Pharmacology, Plant Science

Keywords

Guettarda calyptrata, Pharmacognostic Study, Phytochemical, Antioxidant Activity

1. Introduction

G. calyptrata, which belongs to the botanical family of Rubiacea, is characterized by the production of bioactive metabolites (iridoids, alkaloids, antraquinones, triterpenes, saponins, etc.) with great pharmacological potential. These metabolites can be used as chemotaxonomic markers even for genera and subfamilies [1] [2]. *G. calyptrate* is a shrub or tree endemic to Cuba, common in all the provinces, savannahs and stony, arid and cuabales lands. It reaches a size of up to 6 m in height; presents coriaceous leaves oblong to oval or round-ovate, obtuse at the apex and heart-shaped at the base. **Figure 1** shows the macromorphological characteristics of the leaves. The plant is known by the common names of contraguao, guayabillo, leather of green leaves. Traditionally, it is attributed to the leaves and barks (decoction of 2 to 3 times a day, on the skin), the medicinal property of relieving or curing burns produced by *Comocladia dentata* Jacq., plant that is characterized by secreting highly caustic latex from the skin and mucous membranes [3] [4].

The natural antioxidants present in plants have gained great interest in recent decades, since oxidative stress (an unbalance between oxidants and pro-oxidants) is implicated in a large number of health conditions. It has been demonstrated that burned is a traumatic wound that result in a local and systematic damage with oxidative changes. This kind of lesion increases the xanthine-oxidase enzyme and byproducts of Lipidic peroxidation. It has been demonstrated that substances with antioxidant properties are effective in the treatment of burns, among them, antioxidants from natural fonts [5].

There is not any evidence in scientific literature of studies related with this medicinal plant, for that purpose, the aim of this research work was to evaluate the pharmacognostic, phytochemical and antioxidant activity of *G. calyptrata*to known necessary aspects in the development of its monograph and to support its medicinal use.

Figure 1. Morphological details of *G. calyptrata.*

2. Materials and Methods

2.1. Sample Collection and Processing

The plant was collected in November 2016 in shore area of Cojímar, Habana del Este municipality, Havana, Cuba. Plants were in phonologic state and were harbored an identified at Johannes Bisse herbarium in National Botany Garden where a voucher specimen (HFC-089021) was deposited. Only leaves of the plant were used in this research.

The leaves were dried at 40˚C in an oven model AISET model YLD-6000 (China) using 100 g of each sample per replica, determining the loss of weight and the time of dried (every 12 hours) according to NRSP 309, 1992; Miranda and Cuéllar, 2000 [6] [7].

2.2. Pharmacognostic Analysis

2.2.1. Macromorphology

Macromorphological characters of the 100 leaves like leaf shape, size, color, texture, margin type, apex, base and petiole size, flower color and length etc were observed. Measurements were carried out using line ruler and a Stereoscopic microscope NTB-2B with camera model HDCE-50B (China) [7].

2.2.2. Microscopic Analysis

Microscopic analysis was carried out on the powdered sample using a light microscope NOVEL (China) with 10× microscope objective lens, and coupled to HDCE-50B digital camera (China) and Scope Image Dynamic Pro software. Ground powder was cleared for some minutes in sodium hypochlorite solution. It was washed in water and then coloured with saffranin at 1% and stained in glycerinated gelatin according to Gattuso M and Gattuso S, 1999; and Miranda and Cuéllar, 2000 [7] [8].

2.2.3. Physicochemical Analysis

Physicochemical analyses were carried out on the powdered sample following standard methods. Moisture content, alcohol extractive values at 30%, 50% and 80%), water extractive value and total ash, water soluble ash and acid insoluble ash value were tested for using a MUFFLE FURNACE SX2-12TP (China) [6] [8] [9] [10].

2.2.4. Extract Preparation

The extracts were prepared with the ground material (20 g × 100 mL of water) getting the physico-chemical parameters like organoleptic properties (odor and color), pH, refraction index, relative density and total solids [7] [11].

2.2.5. Phytochemical Profile of the Extract

1) TCL

TLCP (thin-layer chromatography plate) on silica gel with fluorescent indicator 254 nm on aluminum cards (layer thickness 0.2 mm) (10 × 20 cm) using n-butanol: acetic acid: water (BAW 65:25:10) as developing agent (v/v/v), con-

centrated sulfuric acid plus heat, $FeCl_3$ and $AlCl_3$ were the chromogenic agents. The TLCP were examined under ultraviolet (254 nm and 365 nm) and ordinary light. Vanillin at 1%, Rutin (R) and quercetin (Q) (Merck) were used as standard.

2) UV spectroscopy

The extract was analyzed on a UV-Visible spectrometer Anlytikjena Specord-200 plus model (Germany). The scan range was 200 to 700 nm.

3) Total phenolic and total flavonoid content

Total phenols was calculated by the Folin-Ciocalteu method [12], using as reference the gallic acid (Sigma-Aldrich) at concentrations of 10, 20, 30, 40 and 50 mg/mL. On the other hands, the content of total flavonoids was carried out by the colorimetric method according to Pourmorad *et al.*, 2006 [13], using aluminum trichloride and quercetin (Sigma-Aldrich) as reference substance at the concentrations of 10, 15, 25, 50 and 100 µg/mL. In each case, calibration lineal curve was constructed with absorbance readied in a spectrophotometer Rayleigh UV-1601 (China) at 715 nm *vs.* concentration of reference compound, which was then obtained respective concentration of total content of phenol or flavonoids and SD in the studied extract (mg/mL).

2.3 Antioxidant Activity of the Extracts

2.3.1. Ferric Reducing Antioxidant Power [FRAP] Assay

The Ferric Reducing Antioxidant Power (FRAP) assay was measured as described previously by Benzie and Strain (1996) [14], which determine the ability of the sample to reduce iron ferric (Fe^{3+}) to ferrous (Fe^{2+}). The determinations were carried out in a spectrophotometer Rayleigh UV-1601 (China) at 593 nm. The extract was tested at 20, 30 and 40 µg/mL concentrations. The results were expressed as µmol equivalent of Vitamin C (purity 99%, Sigma-Aldrich), according to the standard curve of ascorbic acid (20, 50, 100, 400 and 800 µmol/L).

2.3.2. Free Radical Scavenging Activity

The reduction of 2,2-diphenyl-1-picrylhydracil (DPPH; Sigma-Aldrich) radical in 2,2-diphenyl-1-pryryl hydrazine was used for the antioxidant action of compounds containing -OH groups that decolorize said reagent according to Brand-Williams *et al.*, 1995 [15]. The extract was tested at 25, 37, 5 and 50 µg/mL concentrations. The absorbance was read at 517 nm in a spectrophotometer Rayleigh UV-1601 (China) and the percentage inhibition of DPPH (% DPPH) staining was calculated by the following formula: % inhibition of the DPPH = (Abs control − Abs sample/Abs control) × 100. Experiment was carried out in triplicate and results were expressed as mean and SD.

2.4. Statistical Analysis

Results are presented as mean ± SD. Statistical analyses were performed by Student's t-test. The values of $p < 0.05$ were considered significant. Duncan test was used utilizing the Statgraphics® Plus, version 5.0 program. The mean effective

concentration (IC_{50}) was determined with the help of the Graphprism 5.0 statistical program.

3. Results and Discussion

Botanical Characterization of *G. calyptrata*

1) Macromorphological evaluation of the leaves

The macromorphological ealuation represented in **Figure 2**, allow the observation of decussate leaves on the stem, with aovade-eliptic shape, coreaceus texture, penninervous, short petiole (0.9 ± 0.31), cordade base, obtuse-oblongate apex and ondulate margine. Trichomes on the abxial and adaxial surfaces. Leaf length 6.79 cm (SD = 1.05) and width of 4.21 cm (SD = 0.70). Macromorpholic characteristis are according to literature data for this spice as discussed by Bisse in 1988 [3].

2) Micromorphological evaluation of the powder drug (leaves)

Helical xylematic vessel tissue according to Gattuso M and Gattuso S, 1999 [3], with unicellular trichomes, which were identified under macroscopic analysis. Stomata and epidermal cells were observed with lightly grossed walls, and variable size and shape (**Figure 3**).

3) Physicochemical parameters of the leaf powder

Table 1 summarizes the results of determination of physicochemical para meters of powered drug. Moisture content is according with the range accepted for medicinal plants. Water soluble extractive is acceptable while alcohol soluble extractives are higher than the aqueous extractive indicating that chemical compounds have a half-polarity, increasing the values with the ethanol concentration. All ashes values are beneath the permitted values, demonstrating that the drug was clean and with a low content of metals [6] [7] [9].

4) Physicochemical Parameters of the extract

Table 2 summarizes the results of physicochemical parameters. The pH of the extract was noted to be 4.01 ± 04 (lightly acid), total solids value is low but is in correspondence with the results obtained in water soluble extractive, indicating that their chemical components are less polar or middle polar. The extraction with water was done according to popular use in the Cuban traditional medicine.

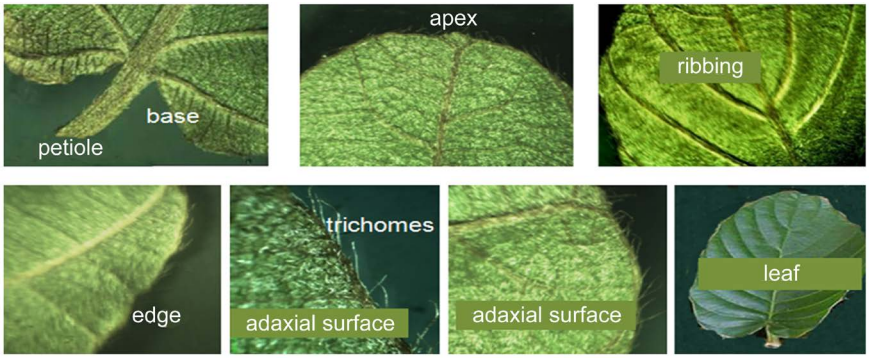

Figure 2. Macromorphological characteristics of the leaves of *G. calyptrata.*

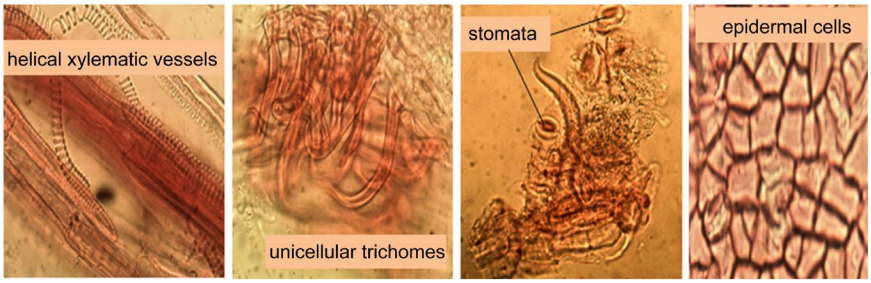

Figure 3. Powder microscopic characteristics of *G. calyptrata.*

Table 1. Physicochemical parameters of the leaf powder.

Parameters (%)	Results ± SD	Limit value for herb (Commission, 2015)	Lou-Zhicen (1980)
Moisture content	8.00 ± 0.0	10.0	8.0 - 14.0
Water soluble extractive	10.57 ± 0.14	-	-
Alcohol soluble extractive at 30%	15.47 ± 0.07	-	-
Alcohol soluble extractive at 50%	19.29 ± 0.13	-	-
Alcohol soluble extractive at 80%	20.55 ± 0.08	-	-
Total ash content	3.26 ± 0.11	15.0	5.0
Water soluble ash	1.41 ± 0.16	8.0	-
Acid insoluble ash	1.81 ± 0.10	1.5	2.0

Table 2. Physicochemical parameters of aqueous extract of *G. calyptrata.*

Parameters	Results ± SD
pH	4.01 ± 0.04
Total Solids (%)	1.61 ± 0.05
Refraction index	1.3297 ± 0.0001
Relativedensity (g/mL)	0.9811 ± 0.0006

Refraction index and Relative density are characteristic of this spice.

5) Phytochemical Profile of the extract

The chemical components were separated in some way taking into account their polarities, suggesting the presence of phenolic compounds and among them, rutin flavonoid, on behalf of the Rf and the chromogenic agents used. The results are showed in **Figure 4**.

6) UV-Visible spectroscopy

Figure 5 shows the UV-Visible spectroscopy analysis of the extracts showed absorption bands between 270 and 285 nm and another one between 325 and 330 nm. This behavior could be related with the presence of phenolic compounds into the extracts, especially flavonoids, which exhibit two bands in the ultraviolet-visible region: Band I at 300 - 400 nm and band II at 200 - 285 nm according to Abad-Garcia, 2009 and Martínez *et al.*, 2012 [16] [17].

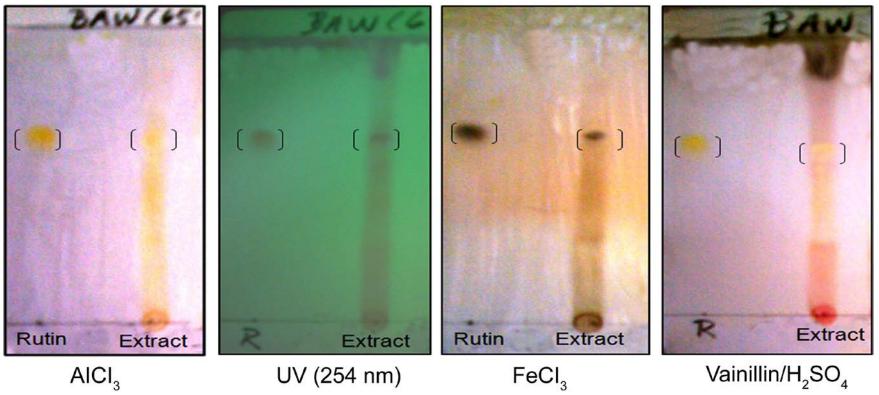

Figure 4. TLC profile of *G. calyptrata* extract.

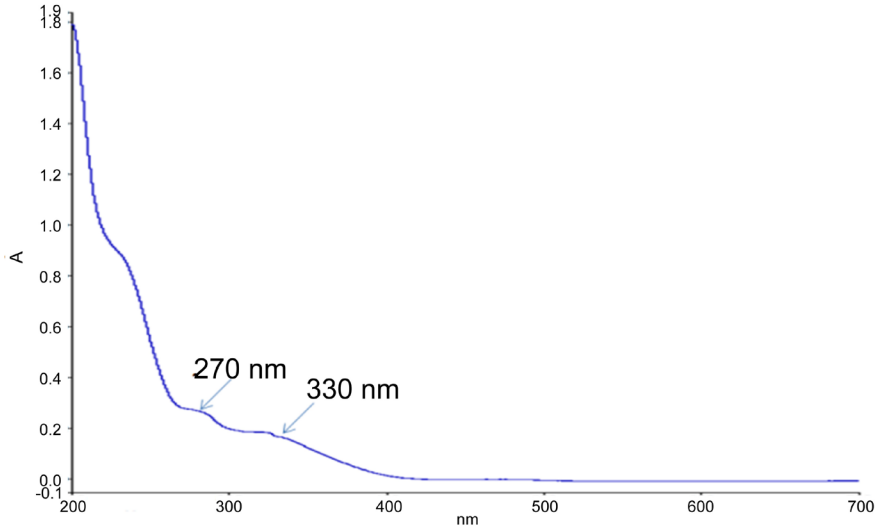

Figure 5. UV-Visible spectrum of aqueous extract of *G. calyptrata.*

7) Phenol and flavonoid contents

Table 3 demonstrates that both results showed low concentration, suggesting that the increments of the value are possible with the use of hydroethanolic solvent and heating the extract.

8) Antioxidant activity of the extract

a) Ferric reducing antioxidant power (FRAP) assay

FRAP assay showed in **Table 4**, based on the reduction of ferric tripyridyltriazine complex to its ferrous colored form according to Afsar *et al.*, 2018 [18]. The results were expressed as μM equivalent of ascorbic acid, the standard used for the analysis. The results displayed in table show the ability of the extract to reduce Fe^{3+} to Fe^{2+}. Was visualized an intense blue color complex. The best result was obtained at the concentration of 40 μg/mL.

b) Free radical scavenging activity

The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accept an electron or hydrogen radical to become a stable diamagnetic molecule. The

Table 3. Phenol and flavonoid content of aqueous extracts of *G. calyptrata.*

	Results
Compounds (mg/mL)	$\bar{x} \pm SD$
Phenol contents	2.83 ± 0.04
Flavonoid contents	1.14 ± 0.005

Table 4. Antioxidant activity of the extract in the FRAP assay.

Concentration (µg/mL)	(µM equivalents of ascorbic acid) ± SD
20	21.24 ± 1.75^{a}
30	44.04 ± 1.76^{b}
40	162.76 ± 6.16^{c}

Values are expressed as mean ± Standard deviation (n = 3); means with superscript with different letters (a - c) are significantly ($p < 0.05$). Data analyzed by using one way ANOVA followed by Duncan test.

reduction capability of DPPH radicals was determine by the decrease in its absorbance at 517 nm induced by antioxidants [18] [19].

Qualitatively was observable a change in the color of the solution from purple to yellow in ll concentrations evaluated, indicating that the higher inhibition percentage of DPPH radical was at the higher concentration evaluated (50 µg/mL). **Table 5** shows the results with DPPH radical scavenging activity and IC_{50}.

4. Conclusions

From the study, important diagnostic characters that might be useful in determining authenticity and identifying adulteration of the crude drug are observed. These are found in the in the abundant long unicellular unbranched trichomes, helical xylematic vessels and epidermal cells with lightly grossed walls. The micromorphological results of powdered drug of *G. calyptrata* have not been reported previously, this is an important contribution to know this spice.

An aqueous extract was elaborated using decoction method taking into account the traditional use giving by Cuban population. Physicochemical parameters to establish its quality were determined.

Phytochemical study allowed detects phenolic compounds and particularly rutin according the conditions tested. The presence of phenolic compounds in this spice has a great coincidence with those results reported for Guettarda gender, where were found flavonoid glycosides like quercetin-3-O-B-D-galactopiranoside y quercetin-3-O-B-D-glucopiranoside [20]. The results exhibit the first evidences of the preliminar chemical composition in *G. calyptrata.*

Natural antioxidants play an important role in front of oxidative stress, possessing antimutagenic, anticarcinogenic, and antiinflammatory and neuroprotective effects discussed by Hsu *et al.*, 2012; Afsar *et al.*, 2018 [18] [21]. These properties could be closely related to phenolic compounds that contain in its

Table 5. Antioxidant activity of the extract in the DPPH assay.

Concentration (µg/mL)	DPPH radical scavenging activity ± SD
25	40.09 ± 0.22[a]
37.5	42.25 ± 0.34[b]
50	52.92 ± 0.11[c]
IC_{50}	38.41

Values are expressed as mean ± Standard deviation (n = 3); means with superscript with different letters (a - c) are significantly ($p < 0.05$). Data analyzed by using one way ANOVA followed by Duncan test.

chemical structures, a variable number of hydroxy groups that react with free radicals according to Csepregi *et al.*, 2016; Sepahpour *et al.*, 2018 [22] [23].

According with those results, the presence of phenolic compounds and particularly rutin was detected and recognized by its antioxidant activity. Last evidence suggests that at least part of the antioxidant effect founded for the aqueous extract of *G. calyptrate* could be associated with this component.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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