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Insights in the knowledge of two Argentine medicinal species of the genus *Hybanthus* (Violaceae)

[Contribución al estudio de dos especies medicinales argentinas del género *Hybanthus* (Violaceae)]

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Abstract

A differential morphoanatomical study was performed in two Argentine species: *Hybanthus parviflorus* and *Hybanthus bigibbosus*. Fresh material fixed in acetoalcoholic formalin and material from herbarium were employed. Paradermal and longitudinal sections of stems and transversal sections of leaves were prepared and stained with aqueous alcoholic safranin. The hydrochloric fluoroglucyn test was performed. Known and novel characters (distribution of vascular bundles of the primary stem and type and relative length of cover hairs and cuticular ornamentation of the adaxial epidermis, respectively) prove to be of value for the recognition of these species.
Furthermore, the intestinal propulsor activity, related to the popular uses of these species was also assessed. Infusions of the whole plant, their roots and the 50% EtOH extracts of the aerial parts of *H. parviflorus* and *H. bigibbosus* were tested by the activated charcoal method. The extracts of *H. parviflorus* proved to have a significant activity which would allow validating its popular use.

Keywords: *Hybanthus parviflorus*, *H. bigibbosus*, morphoanatomy, intestinal propulsion, popular uses
.

Resumen

Se encaró el estudio morfoanatómico diferencial de dos especies argentinas: *Hybanthus parviflorus* e *Hybanthus bigibbosus*. Se trabajó con material fresco fijado en formalina aceto-alcohólica y de herbario. Se realizaron preparados paradermales y cortes longitudinales y transversales de tallos y transversales de hoja empleando safranina alcohólico-acuosa y el test de floroglucina clorhídrica. Caracteres conocidos (distribución de haces vasculares en tallo primario) y novedosos (tipología y longitud relativa de tricomas tectores; ornamentación cuticular de la epidermis adaxial) poseen valor diferencial para el reconocimiento de estas especies.
Asimismo se determinó la actividad de propulsión intestinal de ambas especies, relacionada con su uso popular. Se ensayaron las infusiones de la raíz y de las partes aéreas y el extracto EtOH 50% de las partes aéreas de *H. parviflorus* e *H. bigibbosus*, empleando el método del carbón activado. Los extractos de *H. parviflorus* presentaron una actividad significativa que permitiría validar su uso popular.

Palabras Clave: *Hybanthus parviflorus*, *H. bigibbosus*, morfoanatomia, propulsion intestinal, uso popular.

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INTRODUCTION

The genus *Hybanthus* belongs to the Violaceae plant family which comprises 22 genera and 900 species. Violaceae are generally perennial herbaceous plants (Hoffmann et al., 1992). Sparre (1950) has provided a brief key on the most relevant differences of the three Argentine genera: *Hybanthus*, *Anchietea* and *Viola.*

Sixteen native species belonging to the genus *Hybanthus* grow in Argentina: *H. albus* (A. St.-Hil.) Baill.; *H. atropurpureus* (A. St.-Hil.) Taub.; *H. bicolor* (A. St.-Hil.) Baill.; *H. bigibbosus* (A. St.-Hil.) Hassl.; *H. calceolaria* (L.) Schulze-Menz; *H. circaeoides* (Kunth) Baill.; *H. communis* (A. St. Hil.) Taub.; *H. graminifolius* (Chodat) Schulze-Menz; *H. hasslerianus* (Chodat) Hassl.; *H. hieronymi* (Griseb.) Hassl.; *H. longistylus* Schulze-Menz; *H. paraguariensis* (Chodat) Schulze-Menz; *H. parviflorus* (Mutis ex L.f.) Baill.; *H. serratus* (Phil.) Hassl. *H. velutinus* Schulze-Menz (Zuloaga and Morrone, 1999). *H. ipecacuana* (L.) Baill., Toursrkissian (1980) which is cited in "Medicinal Plants of Argentina". Also *H. leucopogon* Sparre, which is an endemic species (Zuloaga and Morrone, 1999),

Hybanthus parviflorus (Mutis ex L.f.) Baill. is a minor shrub which grows at 0 to 1000 m above sea level. It is a branchy erect plant no more than 50 cm high (Cabrera, 1965). This plant is found in Argentina, Bolivia, Brazil, Chile, Paraguay and Uruguay (Zuloaga and Morrone, 1999).

H. parviflorus is a species known by many popular names in the folk medicine of the countries where it grows. In Colombia it is known as "teatina" or "chuchunchullo". Its roots are used in the form of decoction or infusion as a mild laxative, emetic, to cure the amoebic dysentery, the liver hypertrophy and the chronic diarrhoea (García Barriga, 1992). In Chile where it is named "maitencillo", the roots are employed as a substitute of the ipecacuanha as it seems to have the same medicinal properties. Administered as a powder or as infusion of the roots, it is also employed as emetic and purgative. The decoction of the leaves mixed with the other aromatic plants is used to alleviate neuralgia, mainly those of the rheumathic origin (Murillo, 1889; Pizarro, 1966). In Peru it is known as "pachaga pichinya" and it is used as an emetic agent (Rutter, 1990; Soukoup, 1986). In Uruguay it is named "maitencillo", maitecillo" or "maytencillo". The infusion of the aerial parts is employed in cases of rheumatism and neuralgia. At low doses, the roots have emetic and purgative properties whereas at high doses it may cause gastroenteritis and may even cause death (Lombardo, 1979). In Argentina it is known as "violetilla". Its roots are also employed as emetic and laxative (Domínguez, 1903; Marzocca, 1997).

Previous phytochemical studies of this species led us to the isolation and determination of the primary structure of a novel macrocyclic polypeptide, the cyclotide hypa A (Broussalis et al., 2001). In addition, we determined the occurrence of the polyphenols quercetin, quercetin-3-methyl ether, apigenin, luteolin, kaempferol, rutin, caffeic acid and chlorogenic acid and ursolic acid and β-sitosterol (Broussalis et al., 2010).

Hybanthus bigibbosus (A. St-Hil.) Hassl. is a native species to the Argentine flora which also grows in Brazil and Paraguay (Zuloaga and Morrone, 1999). It is a shrub 1-2 m high (Sparre, 1950) that grows in pasturelands located at 0 - 1000 m of altitude in shadow areas either as a wild shrub or as a weed (Biganzoli and Múlgura de Romero, 2004).

In Brazil it is known as "canela-de-veado" and the decoctions of its roots and rhizomes are employed as purgative. The antiallergic properties of this species have also been described (Presibella et al., 2003). The phytochemical analysis of the 20% V/V ethanolic extract of *H. bigibbosus* demonstrated the presence of alkaloids and saponins. The presence of flavonoids, tannins, cardiotonic glycosides or anthraquinones was not investigated in such extracts (Presibella *et al*., 2003).

In 1928, Dominguez indicated in "Contribuciones a la Materia Médica Argentina" ["Contributions to the Argentine Pharmacognosy"]: "Whenever a plant species is studied, it is necessary to precisely determine its provenance, mainly those active plants which their geographical distribution is known or easy to determine. This knowledge will help to identify the best location to collect the plant material for future studies or applications". In order to achieve a proper use of medicinal plants, it is necessary to know in detail the species employed, the preparation and dosage as well as the safety guidelines that must be followed.

In this context, the link between traditional and scientific medicine, through ethnobotanical research, the study of the active principles and the validation of the therapeutic activities of plants will allow the use of natural regional resources to treat the most common ailments.

Finally, it is very important to regain and validate the use of medicinal plants, to broaden the knowledge on the field, to preserve and foster their cultivation so the cultural legacy of populations keeps growing. This will provide benefits for those who have accumulated and preserved for centuries this beneficial knowledge (Mejía and Rengifo, 1995).

Due to the lack of pharmacological research related to the ethnomedical uses of the genus *Hybanthus*, the aims of this work were:

-To establish the botanical characteristics required for the correct species identification.

-To assess the intestinal propulsor activity related to its popular use.

-To make a contribution to the present knowledge about the genus *Hybanthus* by studying the species *H. parviflorus* and *H. bigibbosus*.

PLANT MATERIAL

Collection and botanical identification

Hybanthus parviflorus [*syn*. *Ionidium glutinosum* Ventetat, *Viola parvifolia* Roemer and Shultes (Ballard and Jorgensen, 1997)], family Violaceae was collected, in part, in the intersection of the Nº 12 National Road and the Feliciano stream, La Paz Department, in the Province of Entre Ríos, Argentina in October 1998. The plant was identified by Dr. Juan de Dios Muñoz, keeping a voucher specimen - Muñoz 1514 (ERA) – in the Herbarium of the School of Agricultural Sciences, National University of Entre Ríos, Paraná city, Argentina.

Specimens were also collected in Cerro Azul, Experimental Station of the National Institute of Agricultural Technology (INTA), J. Urdampilleta, L. N. Alem Department, in the Province of Misiones, Argentina. This vegetal material was identified by Dr. Aníbal Amat and a voucher specimen is kept in the Herbarium of the Pharmacy Department of the School of Exact, Chemical and Natural Sciences (MNEF 3980).

Hybanthus bigibbosus (A. St-Hil.) Hassl. (Violaceae) was collected in Posadas, in the Province of Misiones. The plant material was identified by Dr. Anibal Amat. A voucher specimen is kept in the Herbarium of the Pharmacy Department of the School of Exact, Chemical and Natural Sciences (MNEF 3984).

A. MORPHOANATOMICAL STUDY OF *H. parviflorus* AND *H. bigibbosus* (*Violaceae*)

MATERIALS AND METHODS

Methodology (D´Ambrogio de Argüeso, 1986)

Fresh material fixed in acetoalcoholic formalin (AAF) and material obtained from a herbarium were employed, the latter was restored by incubation in a humid chamber and subsequent fixation with AAF.

Paradermal sections were prepared for the observation of the epidermis. This procedure was performed by scraping. Longitudinal and transversal sections of the primary and secondary stems and transversal sections of the leaves were also cut employing a manual microtome. In paradermal studies, epidermal casts were prepared with acrylic resins. Macerates with Jeffrey's mixture (10% chromic acid-10% nitric acid 1:1) were done to study the histological elements isolated.

Aqueous alcoholic safranin was used as stain. The hydrochloric fluoroglucyn test was employed for the detection of lignified tissues and isolated elements. Micrographs were taken with a Pentarex ™ camera coupled to a Nikon ™ Labophot-2 microscope and employing a 100D Kodak™ film

Some micrographs were also taken with a Nikon Coolpix 4300 digital camera coupled to the microscope.

RESULTS

Differentiation between *Hybanthus parviflorus* and *Hybanthus bigibbosus* is based on marked morphological characteristics related to habitat, foliar and fruits morphology.

Despite the presence of common characters in the two species that account for the known affinities within the genus, there are quali- and quantitative differences that allow distinction from each other.

Hybanthus parviflorus is an herb of small size, rarely a sufrutex, not exceeding 30 cm in height

Figure 1: ***Hybanthus parviflorus***

The primary stem, in transversal section (Figure 2), presents a furrowed strong cuticle, with uniseriated, wartly ornated 4-7-celled cover hairs (Figure 3 and 4) with a furrowed cuticle in the base up to 450 µm long; a subepidermic angular collenchyma arranged in 1-2 layers; a chlorenchyma with isodiametric cells; endodermis on starchy sheat conspicuous; schlerenchymatic caps presenting an oblong or lenticular outline with big stone cells (75-175 µm long in major axis) in pericyclic position and associated to parenchymatic cells with 5-85 µm long prismatic crystals. An almost continuous ring of collateral bundles, in which the phloem is well developed and the xylem (as observed in macerated preparations) is composed by pointed vessels (ca. 250 µm long and ca. 175 µm diameter) and abundant end-rounded, clearly intrusive developed fibers (more than 500 µm long). The parenchymatic pith, totally or partially reabsorbed may contain crystals. Occasional early secondary thickening may be observed: in this case the cork is 3-4 layered, the cells have thin walls with big lonely crystals like that described for the primary structure.

Figure 2: ***H. Parviflorus*. Transversal section (TS) of the stem. Scale: 1 mm**

Figure 3: *H. parviflorus*. TS of the stem showing stone cells and cover hairs. Scale: 100 µm

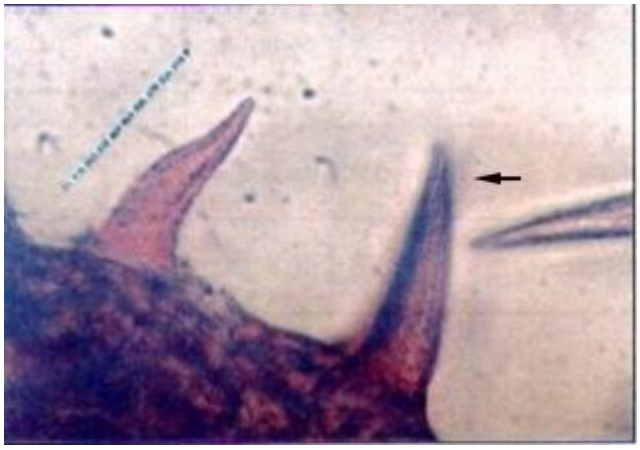

Figure 4: *H. parviflorus*. Unicelular cover hairs. Scale: 100 µm.

In cross sections the petiole is covered by 4-5-celled cover hairs (which are 375 µm long and have a furrowed base and a warty body). In superficial view both the adaxial (Figure 5) and abaxial epidermis have epidermal cells with a sinuate outline, presenting a characteristic cuticular slightly pointed ornamentation and unicellular conic, warty hairs up to 125 µm long, with very thick walls, more frequent on veins and margins. The lamina is hypostomatic, with paracytic stomata (45-50 µm long in main axis, outstanding and associated to a broad subestomatic chamber. The midrib is presented as a collenchymatic prominence in the hypofile, and –as viewed in cross sections-is surrounded by a parenchymatic sheath with big quadrangular crystals similar to those of the stem. The mesophyll has a dorsiventral structure with 3-4-layered palisade chlorenchyma and an abaxial spongy parenchyma with notorious intercellular spaces associated to the subestomatic chambers.

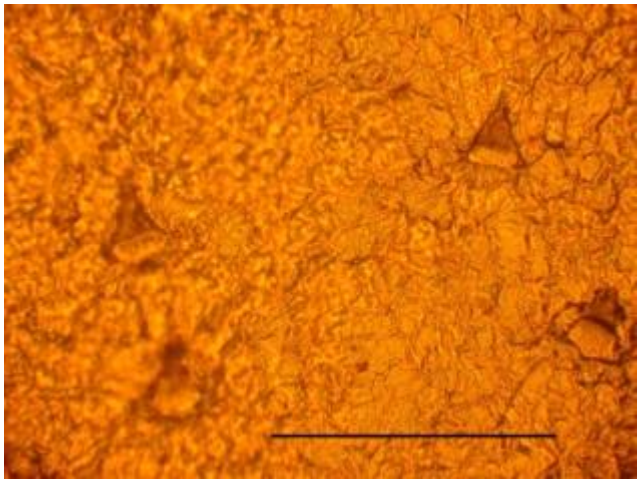

Figure 5: *H. parviflorus*. Adaxial epidermis. Scale: 100 µm.

Hybanthus bigibbosus is a subshrub sufrutex in habitat, reaching ca. 1 m in height (Figure 6).

Figure 6: *H. bigibbosus*

In transverse section, the primary stem (Figure 7) has a similar structure to the former species, but its hairs, also warty, are much longer, the vascular bundles are distinctive and separated between them and are linked al the xylem level by lignified fibers. Schlerenchymatic caps are associated to the phloem, and its elements (as demonstrated by maceration techniques) are stone cells. Stem secondary (Figure 8) growth develops much xylem with pointed vessels with relation to phloem.

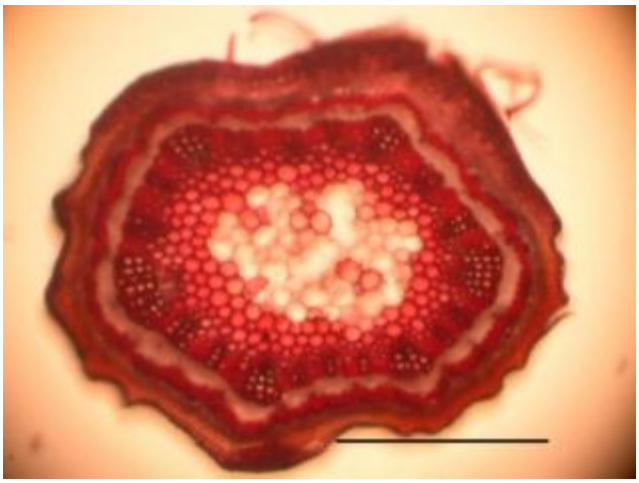

Figure 7: *H. bigibbosus*. Transversal section (TS) of he primary stem. Scale: 1 mm

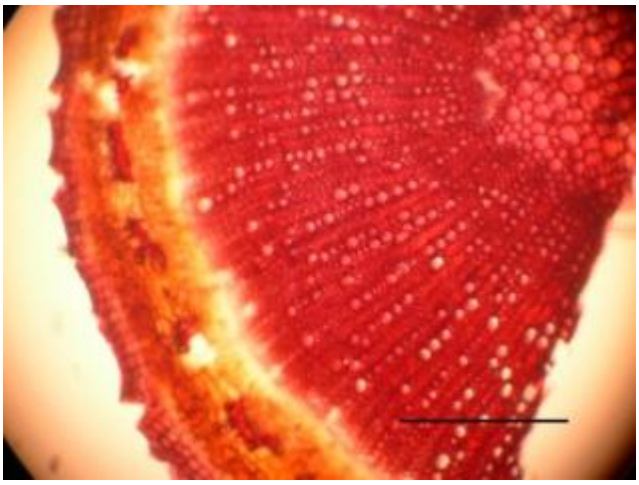

Figure 8: *H. bigibbosus*. TS of the secondary stem and details. Scale: 500 µm

The pith persists even in thick stems, as well as periphloematic schlenrenchymatic caps. A cork with clearly radial disposition of its elements is developed with ulterior fissure formation. Solitary big quadrangular crystals are observed inside the phloem, as well as in inner peridermis layers.

The leaf also has a simple dorsiventral structure, with a thick cuticle on the adaxial surface. The midrib exhibits collenchymatic projections on both surfaces and vascular bundles are surrounded by parenchymatic cells with big solitary crystals which may also be present in other parts of the mesophyll. The lamina is hypostomatic, with paracytic stomata. On both surfaces, but with prevalence on adaxial one, scattered unicellular, thick-walled ornated-base trichomes (up 400 µm long) (Figure 9) are present, more conspicuous on the ribs. An important character, that differentiates the two species, is the cuticle ornamentation of adaxial epidermic cells, which contain marked striae in a nearly radial distribution in relation to each cell (Figure 10). On the other hand, a fine pointed ornamentation is present in *H. parviflorus*.

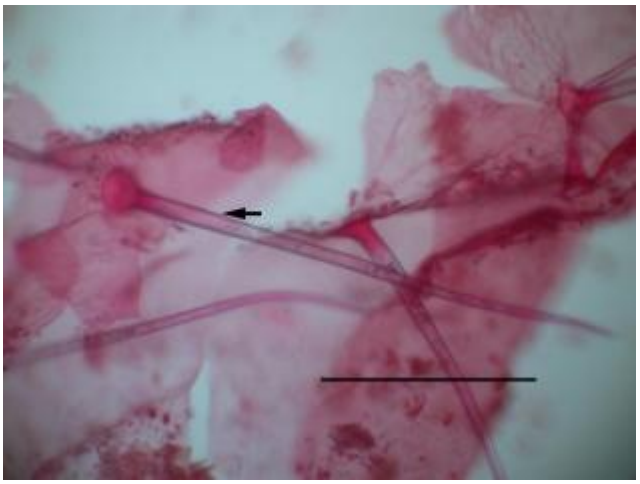

Figure 9: *H. bigibbosus*. Leaf trichomes in powdered material. Scale: 100 µm.

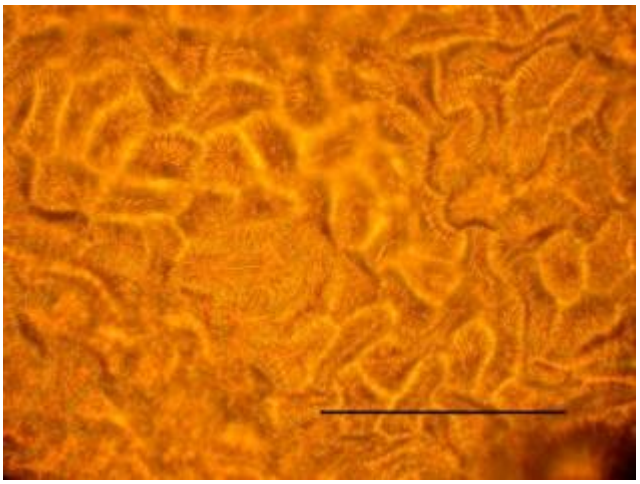

Figure 10: *H. Bigibbosus*, cuticle ornamentation of adaxial epidermis cells, markedly striated. Scale: 100 µm.

In general, these features are in line with the present knowledge about the family *Violaceae* and particularly the genus *Hybanthus* (Melcalfe and Chalk, 1957), thus demonstrating the common pattern that features the family. Nevertheless, some characters analyzed in this work, i.e. the distribution of vascular bundles in the primary stem already described for the family (Meltcalfe and Chalk, 1957, 1988; Watson and

Dallwitz, 2005) and novel features such as the type and relative length of cover hairs and the cuticular ornamentation of the adaxial epidermis, are useful characteristics to be used in the species differentiation according to the following diacritic criteria:

A- Primary stem with vascular bundles in a continuous ring, pith generally reabsorbed and uniseriate, warty cover hairs up to 450 µm long; leaf adaxial epidermis with fine pointed cuticle ornamentation and with conic unicellular hairs up to 375 µm long
Hybanthus parviflorus

AA- Primary stem with vascular bundles separated by lignified fiber elements, pith generally not hollow and uniseriate cover hairs longer than 450 µm, warty body. Leaf epidermis with striae radially orientated (in relation to each cell) and conic cover unicellular hairs longer than 400 µm.
Hybanthus bigibbosus

DISCUSSION

The lack of literature data led us to undertake the morphoanatomical study of *H. parviflorus* and *H. bigibbosus*. Even though most of the anatomical features of these species are in agreement with those previously reported for the Violaceae family, in this work differential characteristics were found which may be valuable for the differential recognition of these two species. These differential features allow the characterization of the material studied from chemical and biological activity standpoints.

On the other hand, and due to the fact that these plant species can be transformed into drugs with potentially interesting uses, this study can contribute to the basis of future monographies and become an important tool in the process of quality control.

B. INTESTINAL POPULSOR ACTIVITY: *H. parviflorus* AND *H. bigibbosus*

MATERIALS AND METHODS

Drugs

Atropine sulfate and carbachol were purchased from Sigma Chemical Co., St. Louis, MO., USA**.**

Animals

Female Swiss mice weighing 25-30 g were used following international principles and local regulations concerning the care and use of laboratory animals (ANMAT, 1996). Animals had free access to a standard commercial diet and water *ad libitum* and were kept in rooms maintained at 22°C ± 1°C with a 12 h light/dark cycle.

Extracts and fractions studied

The intestinal propulsor activity was assessed employing the following extracts:
- Decoction of the roots (aqueous extract) of *H. parviflorus*
- Decoction of the aerial parts (aqueous extract) of *H. parviflorus*
- 50% V/V EtOH extract of the aerial parts

Decoction of the roots

Five g of dried and ground roots were placed in a conical flask with a top and 100 ml of distilled water were added. The top was then slightly tightened and the mixture was softly boiled for 20 min. The mixture was then cooled to 40-45° C and filtered through filter paper. Plant residues were pressed and distilled water was run through to make a 100 ml final volume (Farmacopea Nacional Argentina VI Ed., 1978). The decotion obtained by this procedure was taken to dryness in a rotary evaporator at a temperature < 40° C. Dried material was then solubilized in MilliQ water and lyophilized. After this procedure, 0.9 g and 1.0 g of the decoction of *H. parviflorus* and *H. bigibbosus* were obtained respectively.

Decoction of the aerial parts

The methodology described above was carried out to obtain the decoction of the aerial parts employing 10.0 g of dried and ground material. 3.6 g and 3.8 g of the decoction of *H. parviflorus* and *H. bigibbosus* were obtained respectively.

50 % V/V EtOH extract

Dried and ground aerial parts (30.9 g *H. parviflorus* and 30.2 g *H. biggibosus*) were extracted by maceration with CH_2Cl_2 (300 ml, 1 h under continuous shaking). This procedure was repeated 7 times. Extracts were discarded.

After the maceration with CH_2Cl_2, the vegetal residue was air-dried overnight at room temperature. The following day, the material was extracted by maceration with 50% V/V EtOH (400 ml, 1 h). This procedure was repeated 3 times. Hydroalcoholic extracts were pooled and taken to dryness in a rotary evaporator. At the end of the procedure, 5.61 g and 5.74 g of extracts of *H. parviflorus* and *H. bigibbosus* were obtained respectively.

Effect on small intestinal transit in mice.

The effect of *H. parviflorus* on small intestinal transit in unanaesthetized mice was tested using the charcoal method (Wendel et al., 2008).

Mice were divided into 5 groups of 10 animals each. The first group served as control and was orally administered with the vehicle (0.5% CMC). The second and third groups received the reference drugs atropine and carbachol by i.p. route at doses of 10 and 6 mg/kg respectively. The remaining two groups received, the ethanolic extract or the decoctions of roots and aerial parts of *H. parviflorus* at doses up to 1 g/kg body weight p.o. using a gavage and the last group was administered with the decoctions of roots and aerial parts of *H. bigibbosus* p.o. Simultaneously with the i.p. administrations and 30 min after the oral administration, all the animals were given 0.1ml/10g of an aqueous suspension of 10% charcoal and 2% CMC using a gavage and 30 min later, mice were killed by cervical dislocation. The stomach and intestines were excised from the gastroesophageal junction to the ileocaecal junction and laid on white filter paper for inspection and measurement of distances traversed by the charcoal. The distance travelled by the charcoal meal from the pylorus was measured, and expressed as the percentage of the total length of the small intestine from the gastropyloric junction to the iliocaecal junction (intestinal transit).

Statistical analysis was carried out using unpaired Student`s *t* test. A level of significance of $P < 0.05$ was regarded as statistically significant.

RESULTS

(Figure 11) Atropine (10 mg/kg) reduced significantly ($P< 0.05$) the propulsion of charcoal meal through the gastrointestinal tract. On the other hand, carbachol (6 mg/kg) produced a significant increase ($P<0.01$) in the charcoal propulsion. In a similar manner the ethanol extract and the two decoctions of *H. parviflorus* significantly increased ($P<0.05$) the charcoal transit. Decoctions and the ethanolic extract of *H. bigibbosus* showed no significant differences when compared to the control group.

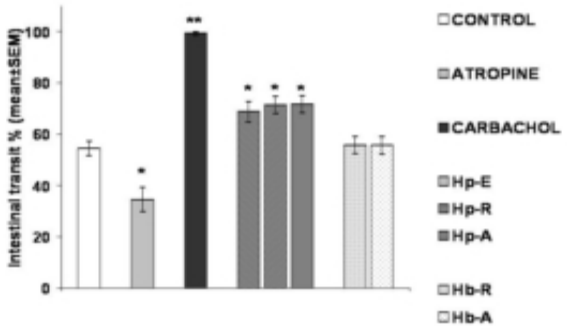

Figure 11. Effect of *H. parviflorus* (Hp) and *H. bigibbosus* (Hb) on the small intestinal transit in mice. E: Ethanol, R: roots; A: aerial. Each row correspond to mean ± SEM. *P < 0.05; **P << 0.01 vs control

The extracts of *H. bigibbosus* did not display significative activity on the intestinal propulsor activity of mice.

DISCUSSION

The present study suggests that the extracts of *H. parviflorus* induced an excitatory effect in the gastrointestinal tract, evidenced by a significant increase in the propulsion of charcoal meal. This effect is mediated, at least in part, through the cholinergic system. The precise mechanism involved in this process is difficult to elucidate due to the complexity of the physiological control of intestinal functions, where intrinsic and extrinsic nerves, autacoids and hormones are involved (Bennet, 1992).

CONCLUSIONS

In this work, the differential morphological features of *H. parviflorus* and *H. biggibosus* are reported for the first time. These plant species possesses potentially interesting uses, therefore, this study can contribute to the basis for future monographies and become an important tool in the processes of quality control.

The roots of *H. parviflorus* are employed in the folk medicine as mild purgative. This work demonstrated that the decoctions of the roots and the aerial part, and the ethanolic extract of this species produced a significant increase in the intestinal propulsor activity, thus validating its uses in the traditional medicine. Further studies comparing the propulsor activity of this species with synthetic prokinetic drugs currently employed would be of great interest.

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