Journal of Medicinal Plants Research Vol. 5(18), pp. 4579-4583, 16 September, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Anatomical and molecular identification of "guaco" *Mikania glomerata* and *Mikania laevigata* (Asteraceae), two important medicinal species from Brazil

Carolina Lopes Bastos[1]*, Camila Souza da Mata[1], Vitor Hugo Maia[2], Rafael Augusto Xavier Borges[1], Luciana Ozório Franco[1], Paulo Cavalcanti Gomes Ferreira[2] and Neusa Tamaio[1]

[1]Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Diretoria de Pesquisa Científica, Rua Pacheco Leão 915, Rio de Janeiro, RJ 22460-030, Brazil.
[2]Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941-590, Brazil.

Accepted 22 June, 2011

Mikania glomerata **Sprengel and** ***Mikania laevigata*** **Schultz Bip. ex Baker are native Brazilian lianas, popularly known as** ***guaco*** **and mainly used as bronchodilators. Based on morphological and anatomical leaf features, preliminary data indicate substantial similarity between the two species, requiring further studies to increase the accuracy of their diagnosis. However, it is still unclear which of the two species is commercially sold. Therefore, in the anatomical analysis, three individuals of each species were treated according to the usual methodology for stem anatomy. Molecular analysis was performed on four specimens of** ***M. glomerata*** **and six of** ***M. laevigata*****, using three plastid loci (*matK, rbcL* and *trnH-psbA*) and the ITS2 locus, which have been proposed as plant barcodes. Our results, including anatomical descriptions and statistically derived quantitative data, show that the anatomical structural standard is very similar between the species. Molecular data corroborate the morphological data in pointing to the total similarity between the two species observed in the loci used. Based on these results, we conclude that** ***M. laevigata*** **and** ***M. glomerata*** **could be unified in terms of nomenclature.**

Key words: Asteraceae, liana, *matK*, *rbcL*, *trnH-psbA,* ITS2, plant molecular biology, stem anatomy.

INTRODUCTION

Leaves and young stems of several species of the genera *Mikania* Willd. (Eupatorieae, Asteraceae), commonly called *guaco* (Pio Corrêa, 1931) are used by the Brazilian population because of their known medicinal effects. Officially, according to Santos (2005), *Mikania glomerata* Sprengel was part of the Brazilian Pharmacopeia of 1929, and *Mikania laevigata* Schultz Bip. ex Baker was only recently included in the 2005 version of that publication (Gasparetto et al., 2010). Both species are native lianas from Brazil, and their leaves and stems are popularly used in "natura" for the treatment of influenza and common cold symptoms, based on their bronchodilation and expectorant properties (Castro et al., 2005; Neves and Sá, 1991; Oliveira et al., 1986a, b). Such properties are commonly associated with the presence of Coumarin (1,2-benzopirona) in the extracts (Do Amaral et al., 2003), but other metabolics probably contribute to the pharmacologic effect (Gasparetto et al., 2010). *M. laevigata* and *M. glomerata* are interchangeably used due to their morphological similarity (Gasparetto et al., 2010). Morphologically difficult to distinguish, these two species present flowers with similar corolla, cypselae and pappus. According to Barroso (1958) and Ritter and Miotto (2005), flower morphology differs in quantitative and inconsistent characteristics [corolla tube size (ca. 1 mm) and pappus size (ca. 2 mm)]; both studies suggested that only the shape of basal leaves could be used to segregate these taxa.

Microscopic analysis may be used as a tool to assist in discriminating the species. Studies reporting the micromorphology of fruits and the anatomy of leaves, reproductive axis, roots and stems have already been

*Corresponding author. E-mail: carol_l_b@ig.com.br Tel: (55) (21) 3204-2094.

Table 1. Studied species with number of collector, collection and accession numbers.

Species	Collector number	Collection number	*matK*	GenBank accession number		
				rbcL	*trnH-psbA*	ITS2
M. laevigata	A.C.Rabonato 2	RB 490677	JF826298	JF826311	JN052778	JN083864
M. laevigata	A.C.Rabonato 3	RB 490679	JF826299	JF826310	JN052779	JN083855
M. laevigata	A.C.Rabonato 4	RB 490680	JF826300	JF826305	JN052780	JN083863
M. laevigata	A.C.Rabonato 5	RB 490681	JF826301	JF826313	JN052781	JN083860
M. laevigata	A.C.Rabonato 8	RB 490685	JF826302	JF826304	JN052782	JN083862
M. laevigata	A.C.Rabonato 9	RB 490686	JF826303	JF826306	JN052783	JN083857
M. glomerata	A.C.Rabonato 10	RB 490673	JF826297	JF826309	JN052774	JN083856
M. glomerata	A.C.Rabonato 11	RB 490674	JF826294	JF826312	JN052775	JN083859
M. glomerata	A.C.Rabonato 12	RB 490672	JF826295	JF826308	JN052776	JN083858
M. glomerata	A.C.Rabonato 13	RB 490675	JF826296	JF826307	JN052777	JN083861

carried out (Ceolin et al., 2006; Ritter and Miotto, 2006; Santos, 2005; Neves and Sá, 1991; Oliveira et al., 1986a, b). In spite of the obvious morphological similarity of these species, no comparative stem anatomy, performed in a way that yields conclusive results, has been done.

DNA barcoding, which is the use of short, highly variable and universally amplified DNA sequences, has also been employed as a tool for discriminating species. It has been successfully applied in many animal groups (Smith et al., 2006; Herbert et al., 2003, 2004). The mitochondrial cytochrome c oxidase I [cox1 or CO1] gene is variable enough to allow discrimination among closely related species, and yet it possesses highly conserved regions that can be easily sequenced with standard protocols. In plants, however, slower substitution rates (Mower et al., 2007) and rapidly changing gene content and structure (Adams and Palmer, 2003) have led to the search for alternative barcoding regions outside the mitochondrial genome.

Therefore, selecting a similar general barcode for plants has proven to be more challenging. After evaluating the performance of seven leading candidate plastid DNA regions (atpF–atpH spacer, matK gene, rbcL gene, rpoB gene, rpoC1 gene, psbK–psbI spacer, and trnH–psbA spacer), the consortium for the barcode of life (CBOL) plant working group recommended the two-locus combination of rbcL + matK as the plant barcode (CBOL Plant Working Group, 2009). This dual-locus combination was considered the best option to meet the requirements of universality, sequence quality, discrimination and costs. The authors pointed out that species discrimination was successful in 72% of cases in the sample set examined.

However, further resolution be could achieved by using supplementary loci as non-coding plastid regions and/or internal transcribed spacers of nuclear ribosomal DNA, when direct sequencing of this locus is possible. Among non-coding plastid regions, the trnH-psbA spacer remains the leading candidate as a source of additional data (CBOL Plant Working Group, 2009).

The purpose of this study is to investigate characteristics that can be used to distinguish *M. glomerata* from *M. laevigata,* using an anatomical approach corroborated by a molecular study with recently proposed plant plastid barcoding loci.

MATERIALS AND METHODS

Plant material

Four specimens of *M. glomerata* and six of *M. laevigata* were used for all analysis. They were collected from the experimental field of the Research Center for Chemistry, Biology and Agriculture (CPQBA, State University of Campinas, São Paulo, Brazil) (Table 1). The vouchers of each specimen were deposited in the herbarium of the Botanical Garden of Rio de Janeiro (RB).

Anatomical procedures

Stem samples with ca. 0.5 cm were selected and fixed in FAA (Formaldehyde, acetic acid and ethyl alcohol 70%, 18:1:1). After suitable trimming, they were stored in 70% ethanol. The mature portion of the stem was processed according to the usual methods for Hydroxyethylmethacrylate (Gerrits and Smid, 1983) in order to obtain transversal sections between 3 and 7 µm in thickness using a rotary microtome. The sections were stained with 0.05% toluidine blue (O'Brien and McCully, 1981). The dissociation of the xylem cellular elements was performed according to Franklin (1945). Descriptions and measurements of cellular elements followed the recommendations of the IAWA Committee (1989). Measurements were made using Image Pro Plus 4.5 software.

Statistical analyses

The following species variables were quantified: vessel diameter, vessel length, fiber diameter, fiber length and fiber wall thickness. For each variable, 25 measurements from three individuals for each species were made. Statistical descriptive analysis and variance analysis (Kruskal-Wallis, ANOVA) were carried out using Statistica for windows software, version 7.0.

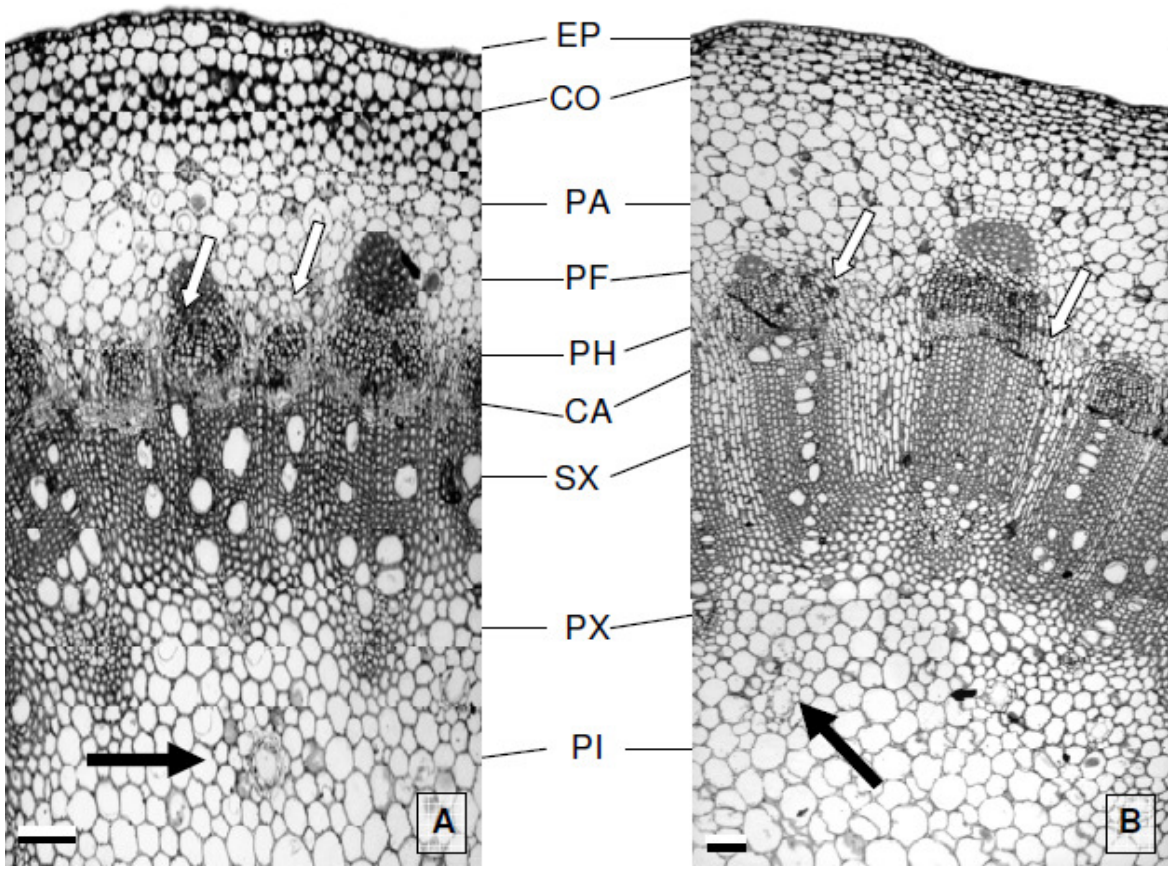

Figure 1. Transverse sections of mature stem showing similar structural patterns. A: *Mikania glomerata*. B: *Mikania laevigata*. Epidermis (EP), collenchyma (CO), parenchyma (PA), perivascular fibers (PF), phloem (PH), primary xylem (PX), secondary xylem (SX), secretory ducts (arrows), cambium (CA), pith (PI). Bar = 100 µm.

DNA extraction, amplification and sequencing

Young leaves dried in silica were disrupted in individual lysing tubes with a bead-mill (Mixer Mill 301 Confort, Retsch). Total genomic DNA was isolated using modified Doyle and Doyle (1987). The two proposed plastid barcoding loci for plants, *matK* and *rbcL* (CBOL Plant Working Group, 2009), were evaluated using available sets of primers (Cuénoud et al., 2002; Fay et al., 1997) respectively. The non-coding *trnH-psbA* spacer was also tested using primers described by Kress et al. (2005). Internal transcribed spacer 2 (ITS2) was amplified according to Chen et al. (2010). PCR reactions contained 1 µl of 30 to 40x diluted DNA template, 1x reaction buffer, 1.5 mM $MgCl_2$, 10 mM dNTPs, 10 pmol of each primer and 2.5 units of Taq DNA polymerase in a final volume of 25 µl. The following PCR profiles and modifications were used:

1) *matK* (modified RBG Kew protocol): 94°C, 5 min; [40 cycles: 94°C, 30 s; 46°C, 40 s; 72°C, 1 min]; 72°C, 7 min.
2) *rbcL*: 94°C, 5 min; [30 cycles: 94°C, 1 min; 50°C, 30 s; 72°C, 1 min]; 72°C, 7 min.
3) *trnH-psbA*: 94°C, 5 min; [35 cycles: 94°C, 30 s; 48°C, 30 s, 72°C, 30 s]; 72°C, 5 min.
4) ITS2: 94°C, 5 min; [40 cycles: 94°C, 30 s; 56°C, 30 s; 72°C, 45 s]; 72°C, 10 min.

The same primers were used for amplifications and sequencing. PCR products were purified and sequenced at Macrogen Inc., Seoul, South Korea, using ABI3730XL. Sequences were edited and aligned with Geneious Pro, version 4.8.2.

RESULTS

As shown in Figures 1a and b, the stem structure of both *M. glomerata* and *M. laevigata* is very similar; therefore, a single description will suffice for both. The epidermis is comprised of a single layer of cells, mostly rectangular, and is covered by a thin cuticle. The vascular bundles are collateral type. The cortex is comprised of several collenchyma layers, between 5 and 7; parenchyma cells, from 6 to 7 layers; and secretory ducts. The fibers of pericyclic origin form a fragmented ring. The secondary phloem is comprised of sieve-tube elements, companion cells and axial parenchyma. The secondary xylem is comprised of elements of solitary and multiple vessels, 2 to 4, with simple perforation plate, wide rays comprised of up to 12 cells, scanty axial parenchyma and fibers of medium thickness. Secretory ducts are observed in the peripheral pith. Significant quantitative difference ($p<0.05$) was recorded for vessel diameter and fiber wall

Table 2. Means values and standard deviation of stem anatomical features analyzed in *Mikania glomerata* and *Mikania laevigata*. Distinct letters indicate significant differences at P<0.05 level among the species.

Stem anatomical features	*M. glomerata*	*M. laevigata*
Vessel diameter	64 ± 26 a	55 ± 25 b
Vessel length	372 ± 102 a	359 ± 72 a
Fiber diameter	20 ± 3.47 a	20 ± 3.72 a
Fiber length	544 ± 185 a	538 ± 121 a
Fiber wall thickness	4 ± 1 a	3 ± 1 b

thickness (Table 2).

In molecular analysis, average sequence lengths were 877, 701, 480 and 228 bp for *matK*, *rbcL*, *trnH-psbA* and ITS2, respectively (Table 1 for GenBank acession numbers). The four loci exhibited exactly the same sequences in all individuals tested, showing no variation within or between the two *Mikania* species

DISCUSSION

Anatomically, the morphological similarity among the two species was confirmed, and no qualitative attribute was identified in the stem that could separate the species. The quantitative data, obtained by measuring the xylem cells, represent the first approach of this type carried out for *M. glomerata* and *M. laevigata*. The comparison of statistical averages showed that the xylem elements of the two species are very similar. Comparing the P values, we observed that stem anatomy did not show statistically significant differences between the species, except for vessel diameter and fiber wall thickness, thus making it difficult to differentiate the species.

Considering that (1) qualitative characters are similar between these two species and (2) the literature points to similarities in both flowers and leaves, we can conclude that it is impossible to separate the two species using only two quantitative characters: vessel diameter and fiber wall thickness. In the present work, the material from both species was collected in the same region, having the same climatic conditions, thus avoiding the influence of environmental conditions. Some previous papers show that the vessel diameter is a character that varies depending on the environment where the plant grows, with narrower vessels being found in xeric environments (Baas et al., 1983), while larger vessels are found in the regions of mesic habitats (Baas et al., 1983; Barajas-Morales, 1985). Some authors associate fiber wall thickness with humidity (Alves and Angyalossy, 2002; Fahn et al., 1986). These authors observed a tendency toward thicker fibers in drier environments. Considering that *guaco* has a large amplitude of occurrence, it would be necessary to analyze samples from various regions to verify if ecological trends can be established based on stem anatomical features and if fiber walls and diameter of vessels have good taxonomic value in the genus. The high degree of anatomical and morphological similarity between the two species prompted us to test the recently proposed barcoding loci, *matK* and *rbcL,* and supplementary markers, *trnH-psbA* and ITS2 (CBOL Plant Working Group, 2009), as a species discriminating tool for the group.

Comprehensive studies revealed that adding more plastid markers provided clear benefits for resolving species (Fazekas et al., 2008; Newmaster and Ragupathy, 2009). In spite of paralogy issues, ITS2 has been reported as the best marker for discriminating species in Asteraceae (Gao et al., 2010). In our study, we aimed to use the molecular-based approach to corroborate morphological and anatomical findings, and we found that all four *M. glomerata* and six *M. laevigata* individuals exhibited exactly the same sequences for each locus. It is well known that Asteraceae have been recently radiated (Holmes, 1995) which could explain the lack of variation in the sequences reported here, as well as in morphological and anatomical characters.

In conclusion, the observed similarities might suggest that both species could be treated as one. Notwithstanding the results of the present study, delimitation could be better resolved with population genetics and reproductive biology studies, which have, thus far, not been carried out for these species.

ACKNOWLEDGMENTS

We thank Dr. Glyn M. Figueira for providing information about the species and Dr. Roberto L. Esteves for confirming the identification of the material. V.H.M is indebted to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for graduate fellowships. P.C.G.F receives support from CNPq research grants. We are also thankful to Natura for financial support.

REFERENCES

Adams KL, Palmer JD (2003). Evolution of mitochondrial gene content: Gene loss and transfer to the nucleus. Mol. Phyl. Evol., 29: 380-395.

Alves ES, Angyalossy-Alfonso V (2002). Ecological trends in the wood anatomy of some Brazilian species. 2. Axial parenchyma, rays and fibres. IAWA J., 23: 391-418.

Baas P, Werker E, Fahn A (1983). Some ecological trends in vessel characters. IAWA Bull., 4: 141-159.
Barajas-Morales J (1985). Wood structural differences between trees of two tropicals forests in México. IAWA Bull., 6: 355-364.
Barroso GM (1958). Mikaniae Brazil. Arch. Bot. Gardener. Rio de Janeiro, 16: 239-330.
Castro EM, Pinto JEBP, Melo HC, Soares AM, Alvarenga AA, Lima Júnior EC (2005). Anatomical and physiological aspects of guaco plants under different photo periods guaco. Hortic. Bras., 23: 846-850.
CBOL Plant Working Group (2009). A DNA Barcode for land plants. P. Natl. Acad. Sci., 106: 12794-12797.
Ceolin ACG, Bratti C, Vieira SCH, Scalon SPQ (2006). Structural organization of the stem of *Mikania cordifolia*, *Mikania laevigata* and *Mikania glomerata*. Hortic. Bras., 24: 2637-2640.
Chen S, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y, Ma X, Gao T, Pang X, Luo K, Li Y, Li X, Jia X, Lin Y, Leon C (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS ONE, 5: e8613.
Cuénoud P, Savolainen V, Chatrou LW, Powell M, Grayer RJ, Chase MW (2002). Molecular phylogenetics of Caryophyllales based on nuclear 18S rDNA and plastid rbcL, atpB, and matK DNA sequences. Am. J. Bot., 89: 132-144.
Do Amaral RR, Arcenio NF, Carvalho ES, Teixeira LA, De AGL, Sharapin N, Testa B, Gnerre C, Rocha L (2003). Evaluation of IMAO and antibacterial extracts of *Mikania glomerata* Sprengel. Rev. Bras. Farmacogn., 13: 24-27.
Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull., 19: 11-15.
Fahn A, Werker E, Baas P (1986). Wood anatomy and identification of trees and shrubs from Israel and adjacent regions. The Israel Academy of Sciences and Humanities, Jerusalem.
Fay MF, Swensen SM, Chase MW (1997). Taxonomic affinities of *Medusagyne oppositifolia* (Medusagynaceae). Kew Bull., 52: 111-120.
Fazekas AJ, Burgess KS, Kesanakurti PR, Graham SW, Newmaster SG, Husband BC, Percy DM, Hajibabaei M, Barrett SCH (2008). Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. PLoS ONE 3: e2802.
Franklin GL (1945). Preparation of thin sections of synthetic resins and wood-resin composites, and a new macerating method for wood. Nature, 155: 51.
Gao T, Yao H, Song J, Zhu Y, Liu C, Chen S (2010). Evaluating the feasibility of using candidate DNA barcodes in discriminating species of the large Asteraceae family. BMC Evol. Biol., 10: 1-7.
Gasparetto JC, Campos FR, Budel JM, Pontarolo R (2010). *Mikania glomerata Spreng*. and *M. laevigata* Sch. Bip. Ex Baker, Asteraceae: agronomic ,genetic, morphological and anatomical, chemical, pharmacological, toxicological studies and use on phytotherapy programs in Brazil. Rev. Bras. Farmacogn., 20: 627-640.
Gerrits PO, Smid L (1983). A new, less toxic polymerization system for the embedding of soft tissues in glycol methacrylate and subsequent preparing of serial sections. J. Microsc., 132: 81-85.
Herbert PDN, Cywinska A, Ball SL, De WJR (2003). Biological identifications through DNA barcodes. Proc. Royal Soc. B Biol. Sci., 270: 313-321.
Herbert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004). Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly Astraptes fulgerator. PNAS, 101: 14812-14817.
Holmes WC (1995). A review preparatory to an infrageneric classification of *Mikania* (tribe: Eupatoriae). In: Hind DJN, Jeffrey C, Pope GV (eds), The Royal Botanic Gardens, Kew. Adv. Compos. Syst., 1: 239-254.
IAWA Committee (1989). IAWA list of microscopic features for hardwood identification. IAWA Bull., 10: 219-332.
Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005). Use of DNA Barcodes to identify flowering plants. PNAS, 102: 8369-8374.
Mower J, Touzet P, Gummow J, Delph L, Palmer J (2007). Extensive variation in synonymous substitution rates in mitochondrial genes of seed plants. BMC Evol. Biol., 7: 135.
Neves LJ, Sá MFA (1991). Contribution to the study of medicinal plants *Mikania glomerata* Sprengel. Rev. Bras. Farmcogn., 72: 42-47.
Newmaster SG, Ragupathy S (2009). Testing plant barcoding in a species complex of pantropical *Acacia* (*Mimosoideae, Fabaceae*). Mol. Ecol. Res., 9: 172-180.
O'Brien TP, McCully ME (1981). The study of plant structure principles and select methods. Melbourne: Termarcarphi Pty, P. 45.
Oliveira F, Akisue G, Akisue MK, Jorge LIF (1986a). Morphodiagnosis of *Mikania laevigata* Schultz Bip. ex. Baker - Guaco-do-mato from the woods - axis study. Rev. Bras. Farmacogn., 6: 45-57.
Oliveira F, Akisue G, Akisue MK, Jorge LIF (1986b). Morphodiagnosis of leaves and reproductive aerial parts of *Mikania laevigata Schultz* Bip. ex Baker. Rev. Bras. Farmacogn., 6: 20-34.
Pio Corrêa CM (1931). Dictionary of useful plants from Brazil and exotic crops. Natl Press Rio De Janeiro, Brazil, 2: 707.
Ritter MR, Miotto STS (2005). Taxonomy *of Mikania* Willd. (Asteraceae) in Rio Grande do Sul, Brazil. Hoehnea 32: 309-359.
Ritter MR, Miotto STS (2006). Micromorphology from the surface of the fruit species of *Mikania* Willd. (Asteraceae) species from the State of Rio Grande do Sul, Brazil. Acta Bot. Bras., 20: 241-247.
Santos SC (2005). Chromatographic Characterization of extracts of medicinal guaco: *Mikania laevigata* Schultz Bip. Ex Baker and *M. Glomerata* Sprengel and the action of *M. laevigata* in allergic pulmonaryinflammation. Master dissertation , University of Vale do Itajai, São Paulo, Brazil.
Smith MA, Woodley NE, Janzen DH, Hallwachs W, Hebert PDN (2006). DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: *Tachinidae*). PNAS, 103: 3657-3662.