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Morphological and molecular barcode analysis of the medicinal tree *Mimusops coriacea* (A.D.C). Miq. collected in Ecuador

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Background

Mimusops coriacea (A.D.C). Miq. is a specie from the Sapotacea Family, originated from Africa. *M. coriacea* plants were introduced to coastal areas in Ecuador and tissues from the tree are regularly used as traditional medicine to treat diseases in humans. Different therapeutically uses of the specie include: analgesic, inflammation and pain purposes to bones and articulation-related diseases. Furthermore, tissues from *M. coriacea* could be used as an anti-oxidant agent. However, limited research has been focused only in few *Mimusops* species including *M. elengi*. Therefore, botanical, chemical, and molecular barcode studies for *M. coriacea* are null. In this study, morphological characterization was performed in different plant tissues including leaves, stem and seeds from fruits. Furthermore, genetic characterization was performed using molecular barcodes for *rbc*L, *mat*k, ITS1 and ITS2 using DNA extracted from leaves.

Methods

Macro-morphological description was performed in fresh plant tissues including leaves, stem and seeds. For micro-morphological evaluation, tissues were embedded in paraffin and transversal dissections were done following incubation with sodium hypochlorite and safranin for coloration and fixated later in glycerinated gelatin. DNA extraction was performed using a modified CTAB protocol from leaf tissues and amplification by PCR was accomplished for the molecular barcodes *rbc*L, *mat*K, ITS1 and ITS2. Sequence and phylogenetic analyses were performed using blastn and MEGA, respectively, with sequences in the GenBank.

Results

For the first time, morphological and genetic characteristics were performed in the *M. coriacea* (A.D.C). Miq. Detailed morphological characteristics were obtained in the different tissues analyzed. Sequence analysis revealed that amplicons were generated using the four barcodes selected. Phylogenetic analysis revealed that the barcodes *rbc*L and *mat*K were not discriminated between species, and different genus

were grouped in one clade of the subfamily Sapotoideae. On the other hand, the ITS1 and ITS2 were discriminative at the level of genus and species of the Subfamily Sapotoideae.

Manuscript Title

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Introduction

Sapotaceae is a botanical Family which contains 58 *genera* of plants with 1271 species. The most important *genera* from this Family includes *Pouteria*, *Palaquium*, *Madhuca*, *Manilkara*, *Sideroxylon*, *Chrysophyllum* and *Mimusops*. *Genera* relevant for geographic distribution and pharmacological applications are *Manilkara*a amd *Mimusops*. In the genus *Mimusops*, a total of 45 species have been described and are distributed in Asia, Africa, Australasia and Oceania. *Mimusops* sp. is a tree reaching a height of 25 meters, with a dense cope and an irregular short trunk and a cracked bark. The tree contains simple leaves which are alternated and clustered with a brilliant color green. The calyx of the flowers contains eight triangular sepals with brown external hairs. This plant species is used in different ethnic groups for medicinal and industrial purposes (Semenya, Potgieter,& Erasmus 2012; Chivandi et al. 2016). For similar species, triterpenoids have been described including phenolics and flavonoids (Chanda et al. 2010; Fayek et al. 2012; Kaneria et al. 2012; Baki et al. 2016). For the genus *Mimusops*, different pharmacological properties have been indicated including antioxidant, hypoglycemic, and antimicrobial activities (Shah et al. 2003; Ali et al. 2008; Baliga et al. 2011;Gami, Pathak, & Parabia2012; Kar et al. 2012; Kiran Kumar et al. 2014; Saradha Gillani et al. 2017). In Ecuador, limited studies have been conducted to species of this genus of the Sapotaceae family; therefore, this report includes morphological and molecular barcode studies of the specie *Mimusops coriacea* (ADC) Miq. including leaves, stems and seeds.

Materials & Methods

Morphological analysis

Plant tissues were recollected in a protected natural vegetative area named “Jardín Botánico” located in the North zone of “Las Orquídeas” area next to the Ave. Francisco de Orellana, in the hills of “Cerro Colorado” of Guayaquil city, Guayas Province, Ecuador (coordinates 02°12′13.6800″S 079°53′50.6400″W). Samples collected corresponded to adult plants of

approximately 30 m of height, with the presence of flowers and fruits. Collected plant samples were cured at the GUAY herbarium of the Faculty of Natural Sciences of Guayaquil University with the accession number 13111.

Macro-morphological description of different organs was performed on fresh specimen tissues with a stereoscope Zeizz LUMAR.V12 (Germany) with a light source MC 1500 and KL 2500 LCF with a power supply Zeiss HBO100, adapted with an ACXION MRc5 camera. The software used was AXION VISION Rel 4.8 (Zeizz, Germany), according to the methodology described (Miranda & Cuéllar 2000). Different characteristics were described in the leaf including shape, edge, apex, base, petiole, venation, consistency, and color. Size was measured in length and width of 100 leaves with a micrometer. For the stems, the characteristics analyzed includes shape, color, external and internal surfaces, and fracture. For fruit characterization, 60 fruits and extracted seeds were analyzed in shape and dimensions, seed coat, and endosperm. For histological analysis, transversals cuts of fresh leaves were performed manually, which were hydrated and clarified with 1% sodium hypochlorite. Tissues were colored with 1% safranin in water, following fixation with glycerinated gelatin according to Gattuso & Gattuso (1999). To analyze anatomical aspects of the leaf epidermis, a longitudinal cut followed with a diaphanization technique was performed. Cleared leaves were obtained with sodium hypochlorite following incubation with 1% safranin in water. Micro-morphological characteristics of cortex were performed to the drug in powder, performing histochemical reactions including: starch determination (Lugol reagent), lignine (1% saphranine in water), and essential oil (5% Sudan III solution in 70% ethanol) (Gattuso & Gattuso 1999). Micromorphology of seeds was performed using dried fragmented material following the procedure described above for leaves and cortex.

DNA extraction and PCR.

Leaves from collected samples were ground using liquid nitrogen in the grinder MM400 (Retsch) and stored at -80C upon DNA extraction. Approximately, 100 mg of leaf was used for DNA extraction using a CTAB protocol with some modifications (Pacheco Coello et al. 2017). PCR was performed using the 2x GoTaq® master mix (Cat. # M7123, Promega) using 0.5 µM of each primer (Table 1). The final volume was 50 µl per reaction. PCR conditions were 95°C to start denaturation; 35 cycles of: 95°C for 30 s, 60°C (for *rbc*L) or 56°C (for *mat*K, ITS1 and

ITS2) for 30 s, 72°C for 90 s, with a final extension of ~~y~~ 72°C for 5 min. Five microliter of PCR reaction was loaded on a 1.5% gel to check for the presence of amplicons. The remaining 45 µl were purified using the Wizard SV Gel and PCR Clean-Up System (Cat. # A9282, Promega) and sequenced commercially (Macrogen, Maryland, USA).

Bio-informatics analysis of sequences

Sequences were trimmed from low quality using FinchTV or Chromas 2.6.5 (Technelysium). Processed sequences were blast (Zhang et al. 2000) in the GenBank using the nucleotide database. Selected sequences with similarities were selected for phylogenetic analysis using MEGA 7.0.26 (Kumar, Stecher, & Tamura 2016). For each barcode, the recommended model from the MEGA7 was used for the phylogenetic analysis after alignment with MUSCLE. For the phylogenetic analysis, around 50 accessions for each barcode of different *genera* from the subfamily Sapotoideae were queried from the GenBank (5th December 2018) and the phylogenetic analysis was also performed independently from selected accession from the blast result.

Results

Morphological evaluation of the leaves:

The macro-morphological evaluation allowed the observation of oblong leaves of coriaceous-waxy texture, short petiole, retuse apex, entire border and obtuse base (Miranda & Cuéllar 2000; Gami, Pathak, &Parabia 2012). The venation is a closed type, which corresponds to a reticular system (the veins branch and anastomose with each other forming a network that facilitates the diffusion of liquids); which is very common in the dicotyledons. In this case, of the penninervia type, the vascular system is one of the most advanced systems that ensures nutrition to all parts of the leaf (Gami, Pathak, &Parabia 2012). Macroscopic details of the leaves are shown (Fig. 1). In respect to the dimensions of the leaves, the average value observed for the length of the leaves was 13.56 ± 1.46 cm with a width of 7.49 ± 0.65 cm.

Morphological evaluation of the crust: The crust presented a rugose cuticle of intense gray color, with an underneath slightly brown outer surface (Fig. 2A) with rough streaks. The internal surface was reddish brown, fibrous and furrowed (Fig. 2B).

156

157 **Morphological evaluation of the seeds:** In the macro-morphological study, the length and
158 width of the green and ripe fruits, the seeds with the husk and the endosperm of the seeds were
159 taken into account (Fig. 3). The fruit is rounded, contains one or two seeds, with dimensions of
160 2.97 ± 0.18 cm long and 3.14 ± 0.25 cm wide when green, reducing their size at maturity to 2.89
161 ± 0.2 cm in length and 2, 97 ± 0.25 cm wide. The seeds with a peel are dark brown with 1.66 ±
162 0.13 cm long by 1.15 ± 0.21 cm wide when the fruit is green and 1.79 ± 0.09 x 1.20 ± 0.09 cm,
163 when the fruit is ripe, with an increase in size when the fruit ripens.

164

165 **Anatomical evaluation:**

166 **Leaves:** In the leaf anatomy at the level of a cross section of the central nerve (Fig. 4A) the
167 adaxial surface is convex, slightly wavy and the abaxial face is concave. An enlarged view of the
168 nerve (Fig. 4B) shows a cuticle of waxy texture that covers the entire leaf, and well visible in the
169 macro-morphological study, followed by the epidermis, which is made up of tabular cells, which
170 gives way to the set of cells that form the spongy parenchyma, given the intercellular spaces
171 which are defined. Possible crystals of calcium oxalate are also observed. Bordering the central
172 part of the central nerve, a cord is observed (Fig. 4C) with color red, corresponding to the
173 endodermis, the structure that surrounds the pericycle. In the middle, the conductive tissue
174 formed by the vascular system xylem and phloem is observed (Fig. 4C).
175 An image of the leaf mesophyll (Fig.4D) shows a somewhat thick cuticle on the abaxial surface,
176 followed by the epidermis, a parenchyma palisade with elongated cells that at times become
177 stratified. In the same way, the entire center of the structure occupied by the spongy parenchyma
178 is observed, which borders on the upper epidermis that ends with the cuticle, previously
179 mentioned.

180

181 The diafanization of a portion of the leaf by the adaxial side showed an epidermis with cells of
182 variable shape and size (Fig.4E). However, the abaxial epidermis evidenced many anomocitic
183 type stomata, where the epidermal cells surrounding the pair of occlusive cells are not
184 morphologically different from the rest of the epidermal cells (Fig.4F). A stain with Sudan III
185 reagent at the level of the epidermis, allowed the visualization of bags with essential oils, which
186 took reddish coloration (Fig.4G).

The information referenced in the literature regarding the characteristics of the leaves is scarce; thus, comparisons with respect to two species of the genus was performed.

For *Mimusops elengi* L. (Gami, Pathak & Parabia 2012), authors reported that the leaves are elliptical in shape, little acuminate at the apex, glabrous with an acute base, and petioles 1.3 - 2.5 cm in length,. The dimensions of the leaves range between 6.3 - 10.0 cm long by 3.2 - 5.0 cm wide, while *Mimusops hexandra* Roxb (without *Manilkara hexandra* Roxb), presents oblong leaves, rounded at the apex, glabrous, dark green in the beam and clear on the underside, with a dimension of 2.5 - 11 cm long and 1.0 - 6.0 cm wide (Chanda, Nagani & Parekh 2010). Some species genetically similar to the species under study, present some differences especially in the dimensions of the leaves with respect to those studied, which are superior.
The microscopic analysis of the powder drug showed different fibers and vascular bundles, in this case belonging to the xylematic tissue, classified as scalariform. Figure 5 shows the observed microscopic characteristics.

Bark: The micro-morphological analysis of the powder drug showed different fibers and the vascular system, belonging to the xylematic tissue, responsible for the transport of the crude sap to the photosynthetic centers and the circulation of the highest percentage of water. The xylematic vessels are classified as scalariform (Fig. 5). Related to the cortex, no referenced information was found.

Seeds: The micro-morphological analysis of the seed powder (Fig. 5), allowed the visualization of a section of the episperm (outer layer of the seed or testa) where the presence of cells of the sclerenchyma tissue corresponding to the supporting tissue is observed. These cells have a well-defined compact arrangement and the walls are slightly thick. The sclerides of the macro-sclerosis type and elements of the conductive tissue was observed. Histochemical reactions on the samples, demonstrated a well-defined red-colored oil pocket that could be observed through the reaction with the Sudan III reagent. Starch granules of ovoid shape and blackish color were also observed when using the Lugol reagent.
For the seeds, significant differences were observed between the evaluated parameters of the whole fruits and their seeds at maturity (Gopalkrishnan, & Shimpi 2011); for *M. elengi* seed husk

was light brown to blackish, with measures of 1.7-1.9 cm long and 1.2-1.5 cm wide, which differs from those obtained for the species studied. The endosperm presented dimensions of 1.42 x 1.00 cm when it came from green fruits and 1.43 x 0.91 cm when it came from ripe fruits, decreasing its thickness in this case.

Molecular barcode of *M. coriacea.*

As a complement analysis for characterization and identification of the *M. coriacea* specimen, PCR for the amplification of the *loci rbc*L, *mat*K, ITS1 and ITS2 was performed. Amplicons were detected for all the molecular barcodes and the two samples tested (Fig. 1). Accesion number of the sequences in the GneBank are indicated (Table 2).

After alignment of the barcodes sequences from the GenBank with the *M. coriacea* specimen, the best model for phylogenetic analysis are shown (Table 3). The phylogenetic analysis revealed that for the barcodes *rbc*L and *mat*K, most of the *Mimusops* spp. are clustered together with other *genera* (Fig. S1). On the other hand, the ITS1 and ITS2 sequences revealed several clades for the different genera including the *Mimusops* (Fig. S2).

Discussion

The identification of plant material used as a phytotherapeutic product is a challenge in natural products. One of the many drawbacks is the management of vulgar or regional plant names, the lack of knowledge of the organ or the part of the plant where the active ingredients are found, and the recognition of the macroscopic and microscopic characteristics of plant drugs. Macro-morphological and micro morphological studies are essential in the control of the quality of plant drugs, as well as significant details to confirm the identity of the plant, and identification of possible adulterants. Therefore, analysis of the morphology and the molecular barcodes is the first step in the characterization of the *Mimusops* spp. for medicinal application. Molecular barcodes are useful to genetically characterized organisms; and different *loci* have been proposed to be universal for land plants (CBOL Plant Working Group 2009). Although, the two proposed *loci* for barcodes are from chloroplast genome and includes the *rbc*L and *mat*K, other *loci* including ITS1 and ITS2 are widely used for medicinal plants (Kim et al. 2016). Furthermore,

the ITS2 region is suggested as a barcode for species identification over *rbc*L and *mat*K (Techen et al 2014; Zhang et al. 2016).

Conclusions

For the first time, the macro and micro-morphological characteristics of the leaves, stems and seeds, of the *M. coriacea* collected in Ecuador were performed. The evaluation of the identity of the species, which is classified taxonomically as *Mimusops* sp., is a novelty of this work, and was confirmed by using molecular barcodes. Most important, the ITS1 and ITS2 indicated more resolution at the species level (*M. coriacea*) than the *rbc*L and *mat*K, confirming published results in medicinal plants. However, further molecular barcode characterization should be performed in *Mimusops* spp. to further validate resolution at the species level as a complement for proper identification using morphological characteristics. Further pharmacognostic analysis will be performed to study medicinal properties of *M. coriacea*.

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Figure 1. Macro morphological details of leaf from *M. coriacea.*

A: retuse apex, B: whole edge, C: obtuse base, D, E and F: closed rib

Figure 2. Macro morphological details of crust from *M. coriacea.*

A: external surface, **B**: internal surface

Figure 3. Macro morphological characters of fruits and seeds from *M. coriacea.*

a) green fruit, b) ripe fruit, c) seeds green fruits with peel, d) seeds ripe fruits with peel, e) endosperm green seeds, f) endosperm mature seeds

Figure 4. Microscopic characteristics of leaf from *M. coriacea.*

Transversal section of the central nerve of the leaf (I): A, central nerve of the leaf; B and C, enlarged view of the central nerve; D, mesophilic; Cu, cuticle; Ep, epidermis; COC, calcium

oxalate crystals; SP, spongy parenchyma; VS, vascular system; En, endodermis; AdE, adaxial epidermis; PP, palisadeparenchyma; AbE, abaxial epidermis.
Diafanized of the leaf (II): E, adaxial epidermis; F and G, abaxial epidermis; EpC, epidermal cells; S, stomata; EO, essential oils.

Figure 5. Powder drug characteristics of *M. coriacea.*
VS: vascular system, F: fibers, S: starch, ST: suberoustissue, SF: septate fibers, COC: calcium oxalate crystal, SC:sclerides cells, MS: macrosclerides, OB: oil bag, SG: starch granules

Figure 6. Gel electrophoresis of amplicons generated for the molecular barcodes with the genomic DNA of *M. coriacea*. A amplification of rbcLA_F/ rbcLA_R. **B** Amplification of matK_3F_KIM f/matK_1R_KIM R. **C** amplification of ITS 5a_F/ITS 4_R (ITS I). **D** amplification of S2f/S3R (ITS II). Numbers from 1 to 3 are replicas of DNA of each species. + is the positive control. – is the negative control. M is the 100 bp DNA Ladder (Cat. # 62101, Promega).

Table 1(on next page)

Primers used for amplification of *rbc*L, *mat*K, ITS1 and ITS2

1 Table 1. Primers used for amplification of *rbc*L, *mat*K, ITS1 and ITS2.

Primer pairs	**Sequence**	**Size (bp)**	***Locus***	**Reference**
rbcLA_F/	ATGTCACCACAAACAGAGACTAAAGC	550	rbcL	Costion et al. 2011
rbcLA_R	GTAAAATCAAGTCCACCRCG			
matK_3F_KIM	CGTACAGTACTTTTGTGTTTACGAG	850	matK	Costion et al., 2011
f/matK_1R_KIMR	ACCCAGTCCATCTGGAAATCTTGGTTC			
ITS 5a F/	CCTTATCATTTAGAGGAAGGAG	700	ITS1	Schultz et al. 2005
ITS 4 R	TCCTCCGCTTATTGATATGC			
S2F/	ATGCGATACTTGGTGTGAAT	400	ITS2	Schultz et al. 2005
S3R	GACGCTTCTCCAGACTACAAT			

2
3

Table 2(on next page)

Samples and sequences submitted in the GenBank from the samples of *M. coriacea* barcoded

Table 2. Samples and sequences submitted in the GenBank from the samples of *M. coriacea* barcoded.

Barcode	Accesion
*rbc*L	2198607
*mat*K	2199742
ITS1	MK577640
ITS2	MK577643

Table 3(on next page)

Best model to describe the substitution pattern using Mega7

Table 3. Best model to describe the substitution pattern using Mega7.

Barcode	Best model
*rbc*L	JC
*mat*K	T92
ITS1	T92+G
ITS2	T92+G

KG: Kimura 2-parameter; +G: Gamma distribution; T92: Tamura 3-parameter; GTR: General Time Reversible. K2: Kimura 2-parameter. JC: Jukes-Cantor.

Figure 1(on next page)

Macro morphological details of leaf from *M. coriacea*

A: retuse apex, B: whole edge, C: obtuse base, D, E and F: closed rib

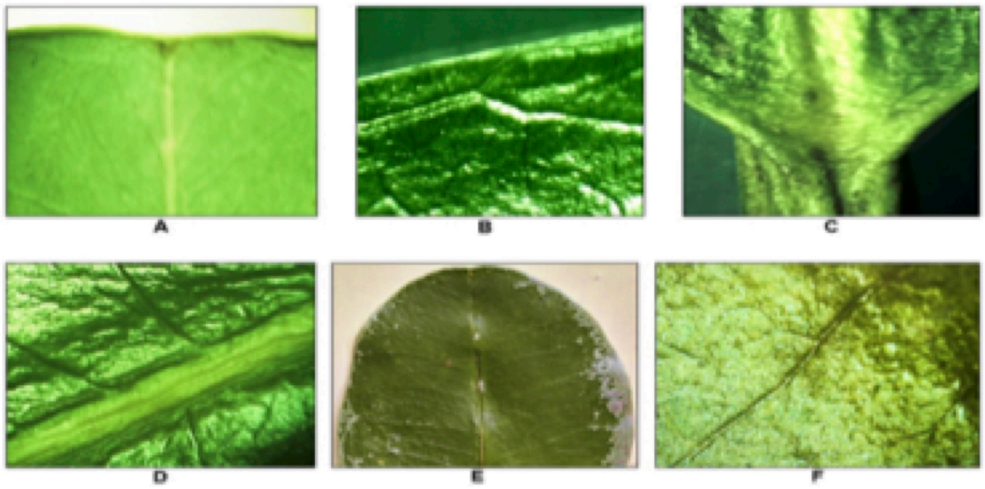

A
B
C
D
E
F

Figure 2(on next page)

Macro morphological details of crust from *M. coriacea*

A: external surface, **B**: internal surface

A B

Figure 3(on next page)

Macro morphological characters of fruits and seeds from *M. coriacea*

a) green fruit, b) ripe fruit, c) seeds green fruits with peel, d) seeds ripe fruits with peel, e) endosperm green seeds, f) endosperm mature seeds

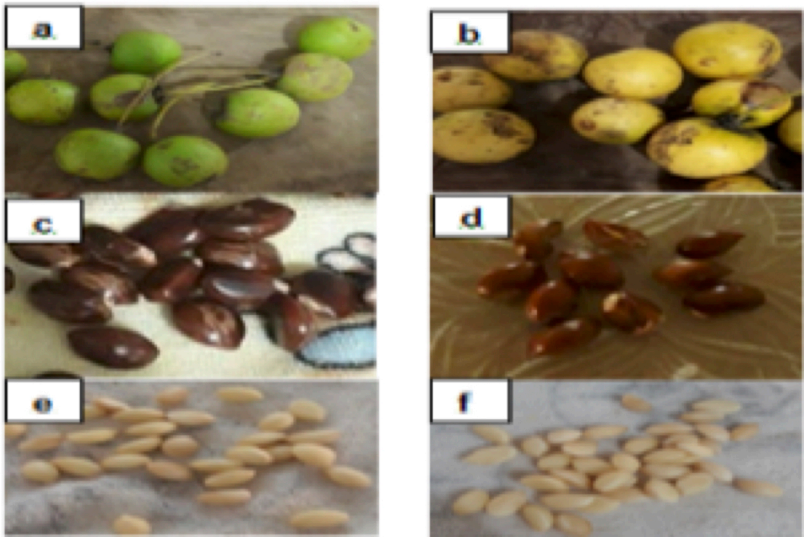

a
b
c
d
e
f

Figure 4

Microscopic characteristics of leaf from *M. coriacea*

Transversal section of the central nerve of the leaf (I): A: central nerve of the leaf, B and C: enlarged view of the central nerve, D: mesophilic, Cu: cuticle, Ep: epidermis, COC: calcium oxalate crystals, SP: spongy parenchyma, VS: vascular system, En: endodermis, AdE: adaxial epidermis, PP:palisadeparenchyma, AbE: abaxial epidermis.

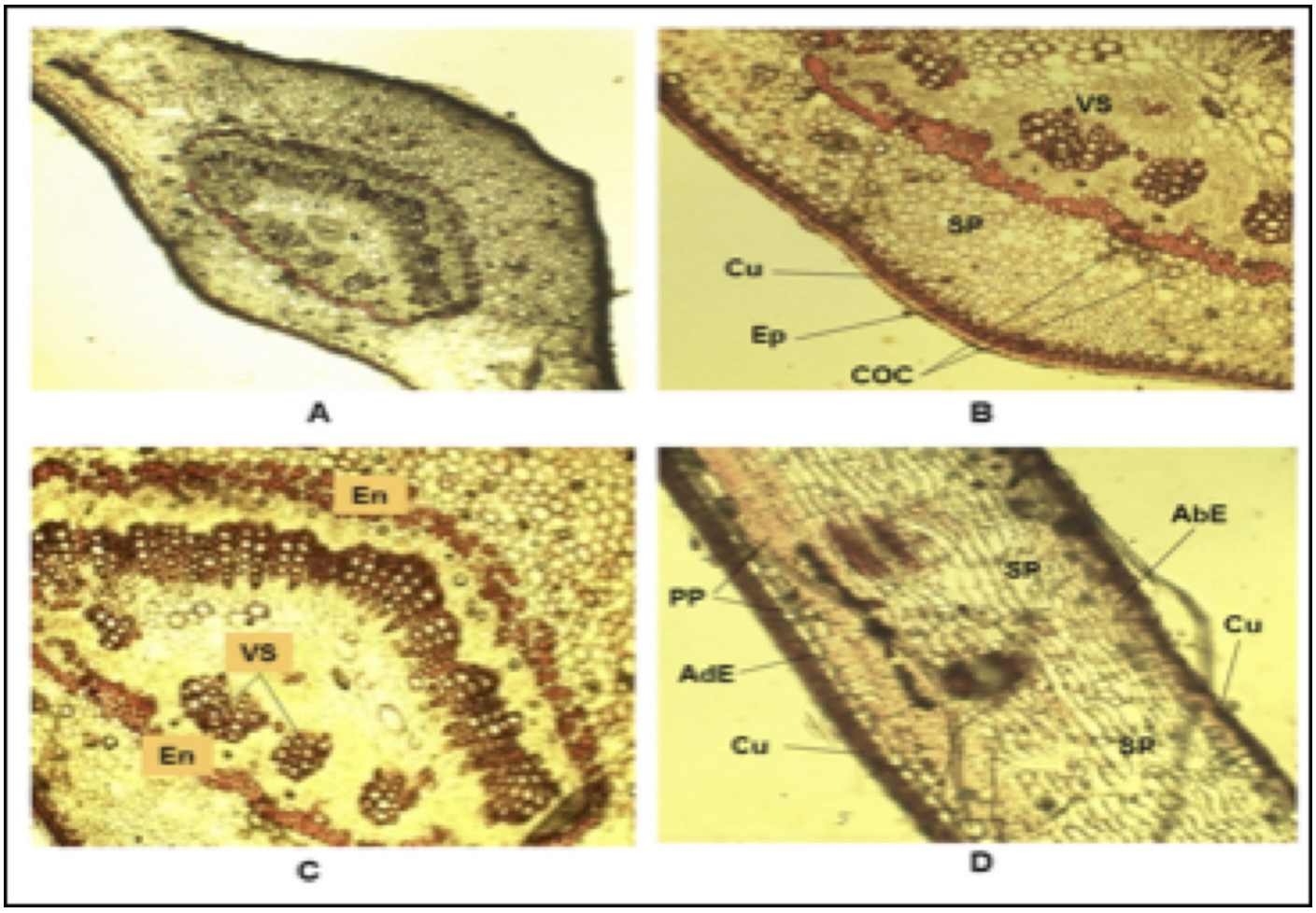


Figure 5(on next page)

Microscopic characteristics of leaf from *M. coriacea*

Diafanized of the leaf (II): E: adaxial epidermis, F and G: abaxial epidermis EpC: epidermal cells, S: stomata, EO: essential oils

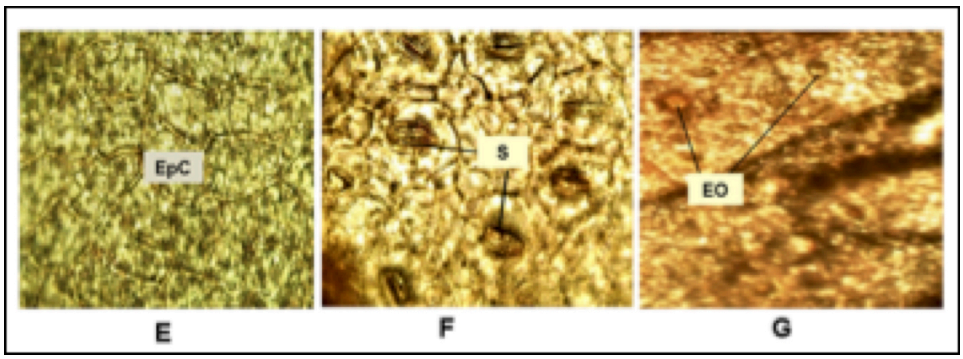

EpC
S
EO
E
F
G

Figure 6

Powder drug characteristics of *M. coriacea*

VS: vascular system, F: fibers, S: starch, ST: suberous tissue,SF: septate fibers, COC: calcium oxalate crystal, SC:sclerides cells, MS: macrosclerides, OB: oilbag, SG: starch granules

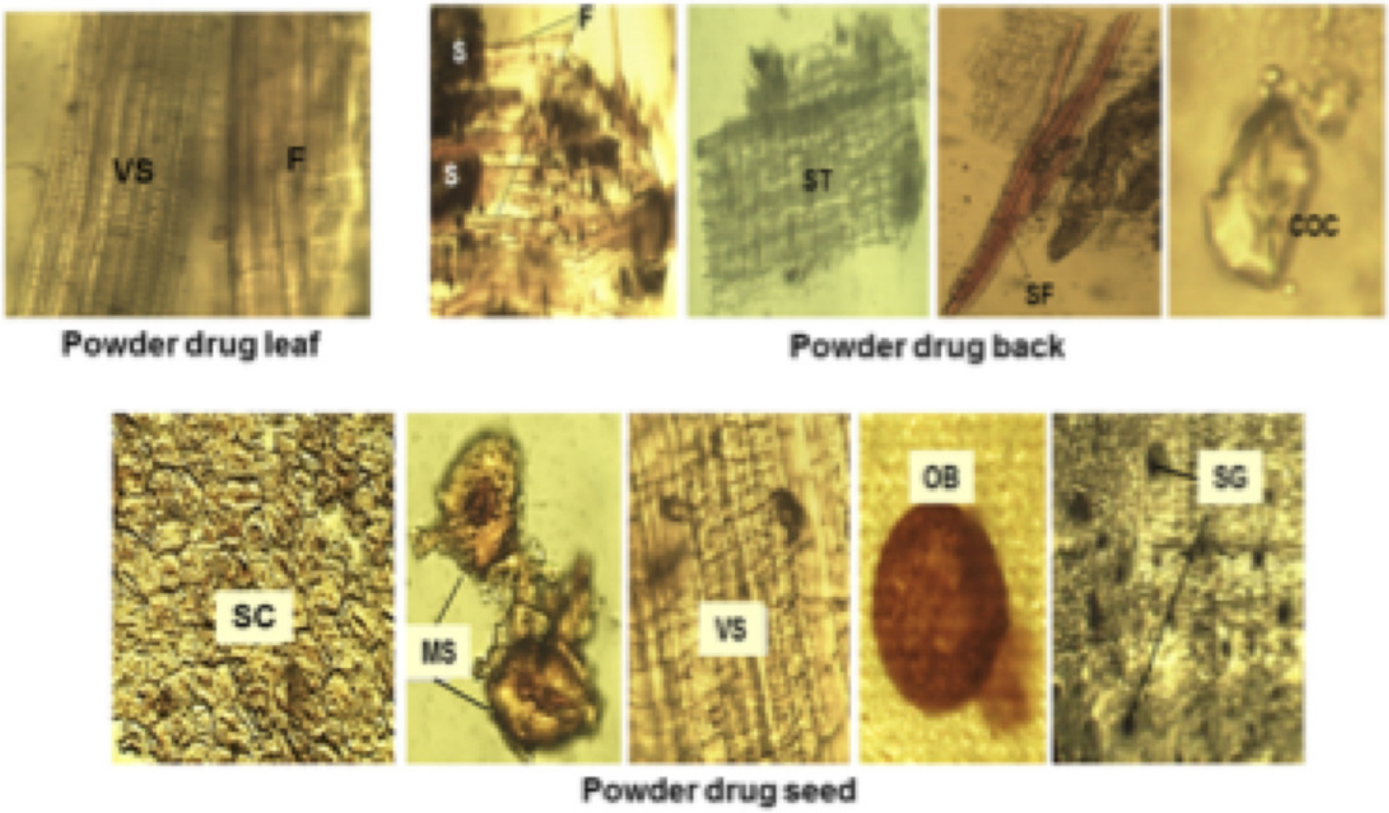
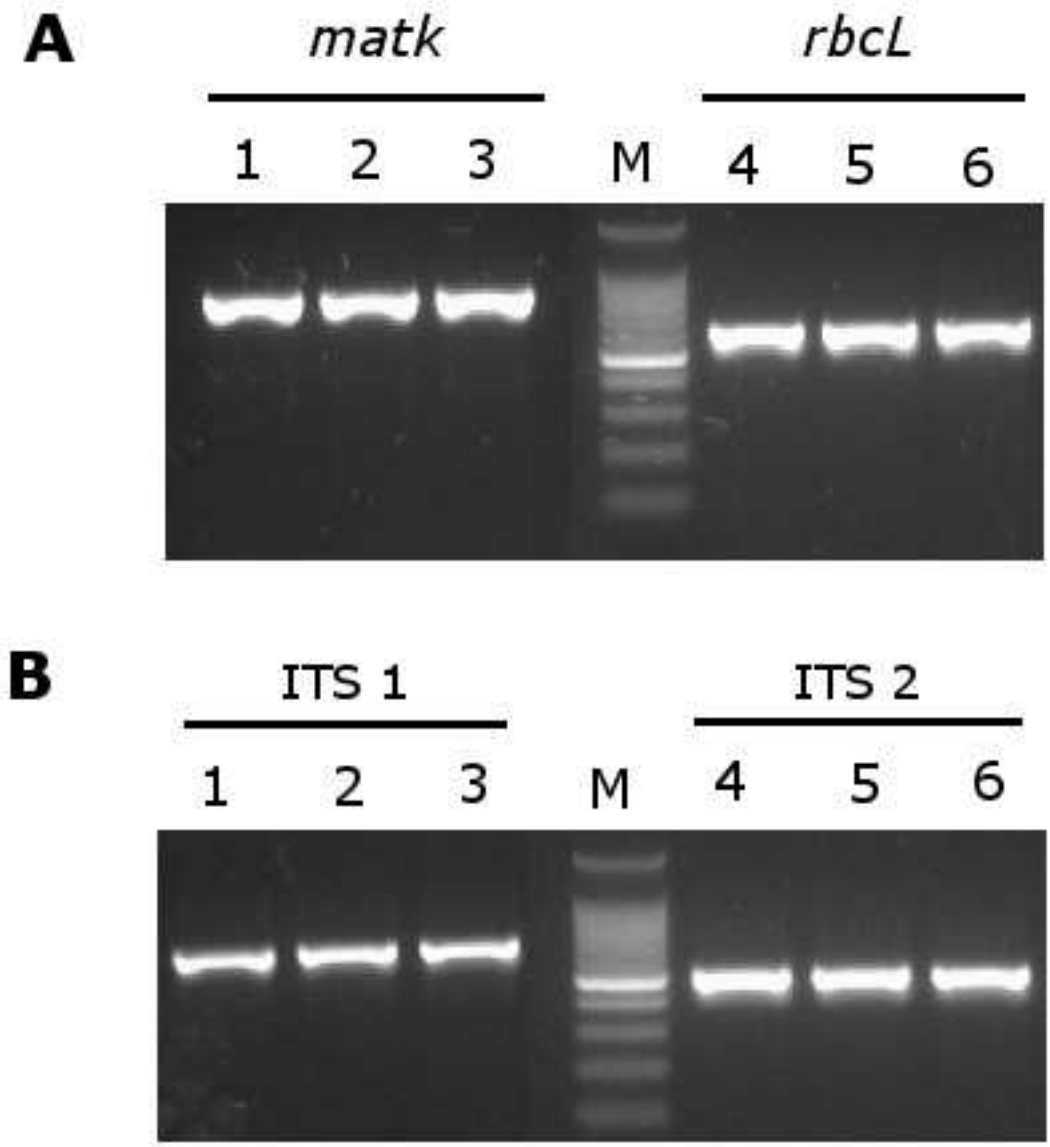


Figure 7 (on next page)

Gel electrophoresis of amplicons generated for the molecular barcodes with the genomic DNA of *M. coriacea*

A amplification of rbcLA_F/ rbcLA_R. **B** Amplification of matK_3F_KIM f/matK_1R_KIM R. **C** amplification of ITS 5a_F/ITS 4_R (ITS I). **D** amplification of S2f/S3R (ITS II). Numbers from 1 to 3 are replicas of DNA of each species. + is the positive control. – is the negative control. M is the 100 bp DNA Ladder (Cat. # 62101, Promega).


A
matk
rbcL
1
2
3
M
4
5
6
B
ITS 1
ITS 2
1
2
3
M
4
5
6