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Published at http://www.ijate.net http://dergipark.gov.tr/ijsm **Research Article**

DNA barcoding and phytochemical profiling of wild plant "Lal lat tan" from Imugan, Sta Fe, Nueva Vizcaya, Philippines

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Abstract: The Philippines hosts a wide-ranging diversity of plant species with pharmacological potential. Species identification on the basis of DNA sequences has been done for some time in plants and used to detect variations among the sequences specifying genetic divergence as a result of molecular evolution during the course of time. In this study, molecular approach as well as phylogentic analysis were conducted to identify the wild plant "Lal lat tan" collected from Imugan, Sta Fe, Nueva Vizcaya. In addition, thin layer chromatography was conducted to determine the bioactive compounds present in the wild plant. For molecular approach, the genomic DNA was extracted from the young leaves using CTAB and amplified using the nrDNA ITS marker. The PCR amplified product was sequenced and subjected for search query analysis using BLAST and was identified as *Dendrocnide meyeniana*. Using chloroform+methanol as solvent system, the result revealed the presence of saponins, phenols, tannins, flavonoids, anthrones, anthraquinones, terpenes and steroids which are considered as active medicinal phytochemical constituents. Lal lat tan is a wild plant and was known for having stinger leaf that lead to severe itchiness and swelling of eyes and face when touched.

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1. INTRODUCTION

Ever since, in search for rescue to treat disease, people looked for drugs in nature. The beginnings of the medicinal plants' use were instinctive, as is the case with animals. In view of the fact that during that time there was not sufficient information either concerning the reasons for the illnesses or concerning which plant and how it could be utilized as a cure, everything was based on experience [1].

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Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug compounds or components because of the unmatched availability of chemical diversity [2]. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs [3]. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases [4]. Medicinal plants are important source of producing valuable bioactive compounds which is great importance for the health of individuals and communities.

The medicinal values of the plants are due to the chemical substances that produce a definite physiological action on human body and are called phytochemicals [5]. After various observations and experimentations, medicinal plants were identified as a source of important medicine, therefore, treatment through these medicinal plants, began in the early stages of human civilization. Several phytochemical surveys have been published, including the random sampling approach which involved some plant accessions collected from all parts of the world. Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients. They protect plants from disease and damage and contribute to the plant's color, aroma and flavor [6]. Various method of extraction for the plant is commonly used method since the extract was rich in chemical compounds and also the bioactivity of plant extracts depends on the water concentration used in the extraction process [7].

DNA barcoding technique is a new tool for taxon recognition and classification of biological organisms based on sequences of a fragment of mitochondrial gene, cytochrome c oxidase I (COI) [8]. DNA barcoding is a diagnostic technique which based on sequence variation at a small fragment of the mitochondrial c oxidase I (COI) [9] that provides an inexpensive and simple tool for identifying novel species [10] and also for describing cryptic species which are difficult to detect phenotypically [8, 11].

2. MATERIALS and METHODS

2.1 Collection and processing of sample

The plant material was collected at Imugan Falls, Santa Fe, Nueva Vizcaya, Philippines (N 16° 11" 38.76" E 121° 6" 36.72" with elevation of 1,685 meters above sea level. Matured leaves of Lal lat tan were collected using clean knife and was placed in a large plastic bag. Sample was cleaned and the external moisture was wiped out using tissue paper. The sample was dried under full sunlight condition and kept for phytochemical analysis. Some fresh leaves were kept in a ziplock and refrigerated before DNA extraction.

2.2 Genomic DNA Extraction, PCR and Sequencing

About 2-3 g of fresh plant material homogenized using mortar and pestle with liquid nitrogen. Powdered sample was transferred in new tubes and 400µl Lysis Buffer PA1 was added and mixed using vortex. Sample was added by 10µl RNase and then mixed and then incubated at 65°C for 10 min. The lysate was loaded onto a new 2ml tube with the ISOLATE II (Bioline) filter and centrifuged in 11,000xg in two minutes. After the collection of the clear flow-through, the ISOLATE II filter was decanted and discarded. The clear supernatant was transferred carefully without disturbing the pellet to a new tube. A 450µl binding buffer PB was added and mixed using vortex. The ISOLATE II Plant DNA (Bioline) spin column was placed into a new 2ml tubes and the sample and was centrifuged at 11,000xg for one minute and the flow-through was discarded. The silica membrane was washed and dried by adding 400µl wash buffer PAW1, centrifuged at 11,000xg for one minute and the flow-through was discarded. And then washed again by adding 700µl wash buffer PAW2, centrifuged at 11,000 x g for one minute and the flow-through was discarded. And then another 200µl wash buffer PAW2 was added, centrifuged at 11,000xg for two minutes to remove wash buffer and to dry silica membrane completely. The ISOLATE II Plant DNA spin column was placed into a new tube added by 50µl preheated elution buffer PG (65°) onto center of silica membrane. It was incubated at 65°C for 5 minutes and then the step was repeated with another 50µl elution buffer PG and eluted into same tube. A 2µl stock DNA mix with 1µl loading dye was loaded into 1% agarose gel containing 1µl of gel into red (GelRed™ Nucleic acid, Biotium) and run in gel electrophoresis system (Endruro Gel XL).

Electrophoresis was carried out at 100 V for 30 minutes. The gel was viewed in gel documentation system (Enduro™ GDS). The genomic DNA was diluted 1:100 using sterilized distilled water. To identify the identity of the sample, the chloroplast gene region was amplified using the *nr*DNA ITS (Table 1) using PCR machine (2720 Thermal Cycler). One (1) µl of diluted DNA was mixed with PCR components and the PCR profile was set as follows: 35 cycles with an initial denaturation at 94°C for 5 minutes, final denaturation at 94°C also for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 45 seconds, final extension for 10 minutes at 72°C and hold at 10°C. Final mixtures of PCR components and DNA were transferred to PCR tubes and were subjected to PCR reaction. The PCR product was sent to Apical Scientific Sequencing in Malaysia for PCR purification and sequencing procedure. The sequences were queried on BLAST (basic local alignment search tool) to check the percent similarity in Genbank.

Table 1. Primers use to amplify nrDNA ITS, *rbc*L and *mat* K

Primer pair	Sequence
ITS3F	5'-GTAAGTGCCGAATTCGAAATAACTGAATGGGA- 3'
ITS4R	5'-AGGGCTGTGGGATCCTCATTCAGGTCTATCACCTC-3'

The chromatogram result was evaluated using BioEdit software [12]. Sample sequence was queried using nucleotide BLAST (basic local alignment search tool) [13]. Default search parameters on the standard nucleotide BLAST (blastn) web interface were used. Other related species sequences were downloaded and were used for evolutionary relationship and phylogenetic analysis.

2.2. Phytochemical Analysis

Dried sample was homogenize using blender. 3.0 g of dried powdered plant material was defatted with 10 mL of hexane and heated over a water bath for 5 minutes. The solvent was decanted and discarded. The residue was extracted with 10 mL of a mixture of methanol and water, CH3OH:H2O (1:1) and heated over a water bath for 5 to 10 minutes. The solution was filtered and the filtrate was labeled and stored. The plant residue was then discarded. Thin layer plate (Merck Millipore) was cut into size of 1X2 inch. The plates were marked 1cm in both ends. About 0.1 mL of solution were applied as spots on the each plates respectively; the chromatogram were developed with different solvent systems suggested in Table 2.

Table 2. Solvent system used for Thin Layer Chromatography.

Absorbent	Solvent System
Silica gel G	Chloroform- methanol (5:1) n- Butanol- acetic acid- water (4:1:5) upper phase Ethyl acetate- pyridine- water (5:1:4)

The chromatogram was air dried and visualized with the following spray reagents shown in Table 3. Positive tests are likewise indicated.

Table 3. List of plant constituents, their visualizing agents and indication of a positive test

Constituents Tested	**Spray Reagents**	**Observable Result for a Positive Test**
Saponins	Antimony (III) chloride	Intense yellow to orange visible zone appear on spraying
Phenols, Tannins, Flavonoids	Potassium ferricyanide-ferric chloride	Blue spots
Alkaloids	Dragendorff's reagent	Brown- orange visible spots immediately on spraying colors aren't stable
Cardenolides	3,5 Dinitrobenzoic acid; Kedde reagent	Blue to red- violet colored zones
Coumarins, Anthraquinones, anthrones, Phenols	Methanolic potassium hydroxide (Borntrager reagent)	Anthraquinones give orange coloration Anthrones give yellow (UV365 nm) zones Coumarins react to form blue (UV365 nm) colored zone
Anthraquinones	Magnesium acetate	Orange- violet color
Indoles	Van Urk- Salkowski Test	Blue- violet spots
Terpenes, Steroids, Phenols	Vanillin- sulfuric acid	Red-violet or purple spots.

3. RESULTS and DISCUSSION

3.1 Molecular identification and phylogeny

Lal lat tan is a small tree, growing to a height of 3 to 5 meters. Leaves have numerous, conspicuous stinging hairs. The hairs have a large bulbous base from which projects a long tapering tube that ends in a curved tip that breaks off easily. Contact with the leaves causes breaking of the tips of the hairs (trichomes) is released that causes immediate and intense skin irritation. The stings are painful and may cause the formation of blisters, immediate and possibly progressing to confluence. DNA from the plant sample was successfully extracted and was subjected in agarose gel to check for DNA quality. The gene markers were used in amplification of the genomic DNA. About 450 base pair fragments of ITS was amplified based on its expected size after PCR amplification. After sequencing the representative sequences of the plant sample were queried against GenBank nucleotide database using BLAST to determine the sequence similarity to the known plant sequence with maximum percent identity. BLAST analysis showed that the plant species collected was identified as *Dendrocnide meyeniana* (KM58432) with 99% identity.

Table 4. Identities of the specimens after BLAST analysis.

Primer used	GenBank Accession Number	Match Accession Name	Maximum % Identity
ITS	*Dendrocnide meyeniana*	KM58432	99%

The sequences were used to construct phylogenetic tree using Mega6 [14] and Clustal W [15], with a bootstrap value of 1000 replicates, respectively. The evolutionary history was inferred based on Neighbour-Joining method [16] and the evolutionary distances were computed using the Maximum Composite Likelihood Method (Figure 1).

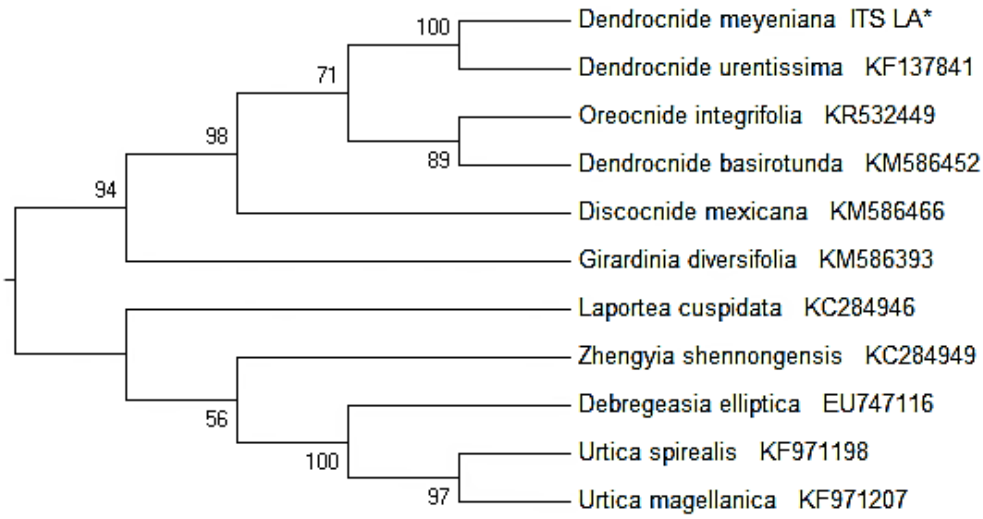

Figure 1. The molecular phylogenetic tree of the Lal lat tan inferred from nrDNA ITS gene marker

The molecular phylogeny tree involved 11 nucleotide sequences that was divided into two major branches and all are sister related taxa up to their genera. The plant sample are in the first branch. All the eleven species are angiosperms, under the order *Rosales* and a family *Urticaceae* and have the characteristics of being armed with stinging hairs.

The nettle family (Urticaceae) is a family of flowering plants. This includes about 2600 species, grouped into 54 to 79 genera according to the database of Royal Botanic Gardens, Kew [17]. The largest genera are *Pilea, Elatostema, Urtica* and *Cecropia.* Urticaceae can be herbs, subshrubs, or shrubs, rarely trees, very rarely climbing, stems often fibrous, sometimes succulent and armed with stinging hairs [18]. The stinging trichomes of *Urtica* consist of a stinging cell with surrounding pedestal cells. When a touch with the human skins, the toxin in the stinging trichomes is released to human and gives pain, wheal, or stinging sensation, and the sensation can be lasted for several hours [19][20]. It was also noted that other than used for animal defence, the function of the stinging trichomes is also regarded as secretion of metabolites [21].

Three genera of *Dendrocnide* including the *Oreocnide integrifolia* in the first branch formed into clade that are all known to be trees. *D. Mexicana* and *G. diversifolia* are herbs so that they separated from the four tree species which formed another subclade. The second group are shrubs and were clustered closely to the first branch. The longer branches in the horizontal dimensions represents the larger amount of evolutionary lineages changed.

In comparison, the plant sample *Dendrocnide meyeniana* are trees, its bark are smooth and glabrous almost throughout to the end of branchlets [22] and grows to a height of 3 to 5 meters.

3.2 Phytochemical Analysis

Chloroform and methanol extract was used as the extracting solvent. The result of the phytochemical analysis from various fraction and the RF value of the different constituents were shown in Table 5. The phytochemical screening with the extract of Lal lat tan leaves revealed the presence of secondary metabolites such as saponins, phenols, tannins, flavonoids, anthrones, anthraquinones, terpenes and steroids which are considered as active medicinal phytochemical constituents.

The intense yellow zone visible upon submerging of thin-layer plates to the Antimony (III) Chloride showed the existence of saponins in the plant material. Using Potassium ferricyanide- ferric chloride spray reagents, a blue spot were seen in the thin layer plates which indicated the presence of phenols, tannins and flavonoids. The yellow and orange zone observed as the positive result in methanolic Potassium hydroxide (Borntrager reagent) inditates the presence of anthrones, anthraquinones and phenols while negative for the presence of coumarins. And lastly, the blue-violet spots appeared in Vanillin-sulfuric acid spray reagents specifies the presence of terpenes and steroids.

Table 5. Phytochemical profiling of various fractions of Lal lat tan

Constituents Tested	Extracting Solvent (Chloroform+ Methanol)	RF Value
Saponins	+	1.402cm
Phenols	+	1.572cm
Tannins	+	1.374cm
Flavonoids	+	1.264cm
Alkaloids	-	1.582cm
Cardenolides	-	1.364cm
Coumarins	+	1.641cm
Anthraquinones	+	1.469cm
Anthrones	+	1.145cm
Phenols	+	1.000cm
Terpenes	+	1.815cm
Steroids	+	1.575cm
Anthraquinones	-	1.263cm
Indoles	-	1.544cm

+: present, - : absent

4. DISCUSSION

The typical bioactive compounds in plants are produced as secondary metabolites. Thus, a definition of bioactive compounds in plants is: secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals [23]. The phytochemical constituents detected are known to have medical importance and to be biologically active compounds that are responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer [24, 25]. For example, several reports are available on flavonoid groups which exhibited high potential biological activities such as antioxidant, anti-inflammatory, antimicrobial, anti-angionic, anticancer and anti-allergic reactions [26, 27, 28, 29]. Tannins and their derivatives are phenolic compounds considered to be primary antioxidants or free radical scavengers [30] and according to research, are known to have antibacterial, antitumor and antiviral activities.

Phenolic compounds could be a natural source of antioxidants because phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits and vegetables [31]. According to Enrico *et al.* [32] the essential oils, unlike antibiotics, are composed of many molecules so that bacteria cannot resist in mutant. Preventively and curatively, they are especially known for their potent antibacterial, antiviral, anti-inflammatory, anti-fungal, anti-parasitic, antipyretic, expectorant, and mucolytic effects. A large body of literature has demonstrated that the naturally occurring anthraquinones possess a broad spectrum of bioactivities, such as cathartic, anticancer, anti-inflammatory, antimicrobial, diuretic, vasorelaxing, and phytoestrogen activities, suggesting their possible clinical application in many diseases [33]. According to Mazza *et al.* [34], Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are characterized by their structure containing a triterpene or steroid aglycone and one or more sugar chains and also has a biological activity such as anticancer, and anticholesterol activity.

Steroids has therapeutic value as anti-inflammatory agents, anabolic (growth-stimulating) agents, and oral contraceptives. Renewed interest in plant antioxidants has emerged during the recent years, probably due to the appearance of undesirable side effects of certain commercial antioxidant. In medicinal plants world, there are a huge number of different types of bioactive compounds with antioxidant activity that play a significant role in terminating the generation of free radical chain reactions [35]. This research work that focused on phytochemical analysis of the phytochemical constituents of Lal lat tan. The phytochemical analysis of the plants are important and have commercial interest in both research institutes and pharmaceuticals.

5. CONCLUSION

The molecular approached revealed that ITS gene marker possess the high discriminatory power and best among the gene markers used in obtaining the the resolution of sequence of Lal lat tan. After obtaining the sequences on GenBank using nucleotide BLAST, it was identified that the plant sample is *Dendrocnide meyeniana* with 99% identity. The phylogenetic tree showed that the branches are composed of sequences that are sister related taxa and possess matching characteristics. Different secondary metabolites that are essential and known to have medical importance and to be biologically active compounds that are responsible for different activities such as antioxidant, antimicrobial, antifungal, and anticancer were determined to be present in plant material as well as the absences of other phytochemicals in the plant.

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