Molecular Confirmation on the Synonymy of Phaeanthusebracteolatusand P. Ophthalmicusincluding Biological Activities of Its Phytochemical Constituent

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Abstract **- The genus Phaeanthus Hook.f & Thomson of the family Annonaceae is a medicinal plant essentially characterized by inner petals that are longer than outer petals, numerous carpels and stamens, and monocarpous fruits. Previous studies have recognized Phaeanthus ebracteolatus as a synonym of P. ophthalmicus based on the morphological examination of limited herbarium specimens. In order to determine the validity of the finding, this study aims to verify the synonymy of P. ebracteolatusand P. ophthalmicus using combined matK and rbcL dataset, as well as computing its sequence divergence; and, to further explore the phytochemical and biochemical components of the plant.Collected plant samples were subjected to morphological characterization followed by molecular analysis through DNA extraction, amplification, purification, sequencing, sequence analysis and data analysis. The crude leaf extracts were subjected to phytochemical screening by thin layer chromatography and three colorimetric assays such as α-glucosidase inhibition, anti-tyrosinase and anti-Staphylococcus. This study confirms that the two Phaeanthus species are conspecific using combined matK and rbcL dataset which is strongly supported and computed sequence divergence which includes 5 bp (0.81%) and 3 bp (0.41%) in matK and rbcL regions, respectively. Overlapping morphological characters such as axillary inflorescence, valvate inner and outer petals, truncate stamens, club-shaped carpels, and globose monocarps also support the finding. The crude leaf extract yields positive to different antioxidant constituents and demonstrated a high potency in α-glucosidase inhibition. The study validated the synonymy of P. ebracteolatus and P. ophthalmicus using sequences and morphology, with P. ophthalmicus being acknowledged as its correct name. Furthermore, the plant extracts proved to be efficient as an α-glucosidase inhibitor.**

Keywords: **α-glucosidase, matK, Phaeanthusophthalmicus, phytochemical constituents, rbcL**

I. INTRODUCTION

The family Annonaceae, known as the "custard apple family", is characterized by alternate, exstipulate leaves with mostly trimerous flowers [21]. Its plants are also known to possess numerous truncate-free stamens and free carpels. Annonaceae is one of the major angiosperms which, according to Chatrou et al. [3], is comprised of 135 genera and 2500 species. About 50 genera and 950 species are confined in Asia and Australia [10]. In terms of usage and purpose, the family Annonaceaeare generally recognized for their significance in diverse fields.

Economically, the family is of appreciable importance as a source of edible fruits such as the pawpaw or Asiminatriloba and the custard apple or Annonareticulata [10]. Even oils from the seeds of Annonaceous plants are used to manufacture edible oils and soaps. Some of their woods are also being employed for alcohol production. The family is famously distinguished because of its odorous flowers; thus, species of Annonaceae have been synthesized to procure various fragrances [14]. The Canangaodorata or commonly known as the ylang-ylang is one example.

One of the 135 genera of Annonaceae is the genus PhaeanthusHook.f & Thomson. It includes 36 species; however, only 4 are currently accepted, while the rest are still unresolved [11]. Majority of the species remain ambiguous for data sources are not sufficient or available to conclude whether they are accepted or possible synonyms. In the Philippines, the species itself can be sometimes mistaken as either UvariaRoxb. ex G. Don or Goniothalamus(Blume) J.D. Hook. & Thomson due to the similar appearance of their fruits and flowers, respectively. One of the common species of Phaeanthusin the country isPhaeanthusebracteolatusor locally known as kalimatas [11]. In fact, it is acknowledged as a remedy for eye diseases such as sore eyes [10]. In the recent study of Turner &Veldkamp [20], it has been suggested that P. ebracteolatusshould be correctly referred to as P. ophthalmicus. However, limited morphological features were presented in their study. In terms of phytochemical

constituents, Lebeouf [8] has stated that the species from family Annonaceae is rich in alkaloids, which indicates that Phaeanthus can have probable use in various biological and medical fields.

Although P. ebracteolatus is recognized to cure eye infections, no other studies have shown its capacity to be of great use to other types of illnesses. With this, queries regarding its potential importance in other areas of medicine have been raised.

The present study aims to: (1) confirm the synonymy between P. ebracteolatus and P. ophthalmicus through molecular and additional morphological analysis, (2) compare and compute sequence divergence using matK and rbcL sequences of the two species and (3) further expound the phytochemical constituents of P. ebracteolatus and assess its biological properties by subjecting to different bioassays.

II. MATERIALS AND METHODS

Taxon sampling. Leaf samples and voucher specimen of Phaeanthusebracteolatus, which was labeled as P. ebracteolatus15-623B, were collected in Mount Balabag, Barangay Tig-baboy, in the province of Antique. Leaves were placed in a ziplock, containing silica gel for molecular analysis; and, fruiting and flowering branches served as voucher specimen. Field photographs (Fig. 1.), and field notes were taken. About 20 kilograms of leaves were also collected for phytochemical analysis and bioassay.

Fig 1. Field photo-documents of P. ebracteolatus15-623B. (A) open flower, (B) flower buds and young fruit, (C) infruitescences.

Morphological analysis.Morphological characters of P. ebracteolatus15-623B were carefully examined. Its vegetative parts such as leaves, petioles and branches were thoroughly described as well as the reproductive parts namely flowers, fruits, and seeds. Cross-section of seeds were observed while the flowers were dissected longitudinally to better examine their parts. The plant was, then, compared to protologues of P. ophthalmicus [17] in order to establish similar and distinct characteristics.

DNA extraction, amplification, purification, and sequencing. Total genomic DNA was extracted from silica gel-dried leaf samples following the protocols of the DNeasy Plant Mini Kit (Qiagen, Germany). Universal primer pairs for two chloroplast regions namely matK and rbcLwere amplified using KapaTaq PCR Kit (Kapa, USA) in Biometra® T-Gradient Thermal Cycler. PCR conditions were set at 90s at 97˚C for the initial denaturation, followed by 35 cycles of 30s 95˚C for secondary denaturation, 20s with a temperature of 50˚Cfor the primer extension, finishing with a temperature of 72˚C for 10 min for the DNA to be incubated and to fill up incomplete strands.

Agarose gel electrophoresis was utilized to confirm the presence of amplicons. The generated DNA fragments were purified using the QIA-quick PCR Purification Kit (Qiagen, Germany). Purified DNA fragments were sent to Macrogen Inc. Seoul, South Korea for bi-directional sequencing.

Phytochemical extraction. The process for extraction of Morschheuser [12] was used. Four hundred grams of air-dried leaf samples were extracted with 1:1 technical-grade methanol and dichloromethane (DCM-MeOH) and was concentrated in vacuo at 45°C using a rotary evaporator (Buchi R-200 and Eyela N-1200A). The concentrated crude leaf extract was labeled (Px) and acid-base extraction was done in order to obtain the alkaloids. Pxwas extracted three times with ethyl acetate and the pH was adjusted to 5 using ammonium hydroxide (NH_4 OH). This was filtered and was concentrated in order to acquire the first crude alkaloid extract (PxA). The ethyl acetate layer from this extraction was, then, labeled as the non-alkaloidal extract (PxE). Px was also increased to a pH of 9 (base) usingNaCl and this was labeled as the second crude alkaloid extract (PxB). The fractions with the concentrated crude leaf extracts were, then, subjected to phytochemical screening and bioassay experimentations.

Thin layer chromatography. The method made by Davies [5] was used. Seven thin layer plates were prepared and marked. Each plate was divided into four parts to accommodate the four different extracts namely Px, PxA, PxB and PxE. These extracts were carefully placed in the plates using a capillary tube to avoid contamination. The 7 plates were then air dried and placed in a glass jar lined with filter paper containing the solvent system. After which, the plates were removed, air dried and subjected to seven constituent tests. The plates were consequently sprayed with antimony (III) chloride, potassium ferricyanide-ferric chloride, Kedde reagent, Borntrager reagent, Van Urk-Salkowski, and vanillin-sulfuric acid. The plates sprayed with antimony (III) chloride and Borntrager reagents were viewed under UV light at 365 nm.α-glucosidase colorimetric assay.

Analytical grade reagents used both in α-glucosidase and tyrosinase inhibition assays were bought from Sigma-Aldrich, except the α-glucosidase enzyme which is from Wako Fine Chemicals. The α-glucosidase inhibition was determined with slight modification of the method reported by Senthilkumar and Sudha [16]. Test samples Px, PxA, PxB and PxE were initially prepared by dissolving each sample with dimethylsulfoxide (DMSO) followed by the dispensation of 140 µl phosphate buffer with a pH of 6.8 on each well to accommodate the extracts with varying concentrations of 16.67 µg/ml, 13.33 µg/ml, 10ug/ml, 6.67 µg/ml, 3.33 µg/ml and 0 µg/ml.

Tyrosinase colorimetric assay.Slight modifications from the methods of Johns et al. [14] were applied. The process of tyrosinase inhibition is the same with α-glucosidase inhibition but with few exceptions. L-3, 4-dihydroxyphenylalanine (L-DOPA) was used as the substrate, tyrosinase was used as the enzyme and kojic acid was used as the positive control. The gathered absorbance for each concentration in both tyrosinase and α-glucosidase assays were calculated to determine their percent inhibition which will, then, lead to their IC50 values. Supplementary 3 and 4 provides the graph for percent inhibition of both colorimetric assays.

Staphylococcus aureus inhibition. For the antimicrobial assay, bacterial suspension was prepared by mixing 90 µl of Mueller Hinton Broth (MHB) and 10 µl of 1.5 x 108 CFU/ml bacterial solution of Staphylococcus aureus, which was compared to 0.5 McFarland standard until turbid. Afterwards, bacterial suspension together with the plant extracts dissolved in pure DMSO was diluted to get the final concentrations of 1000 µg/ml, 500 µg/ml, 250 µg/ml,125µg/ml, 62.5 µg/ml, 31.3 µg/ml, 15.6 µg/ml, 7.8 µg/ml, 3.9 µg/ml and 1.95 µg/ml on each well. Minimal inhibitory concentration was determined by observing the plate after 24 hours of incubation. This assay was performed in triplicates with a positive (Streptomycin) and negative control (DCM), with minor modifications [6]. RESULTS AND DISCUSSION Sequence variation in matK and rbcL.The aligned data matrix in matK and rbcL contained a total of 613 base pairs (bp) and 477 bp, respectively. The sequences in both matK and rbcL regions for P. ophthalmicus and P. ebracteolatus were obtained from GenBank to serve as the basis for alignment and comparison. Computed sequence divergence included 0.81% (5 bp) in matK and 0.41% (2 bp) in rbcL, without insertion-deletions.

Phylogenetic positions of the genus Phaeanthus. A parsimony analysis of combined datasets of matK and rbcL resulted in 1000 equally parsimonious trees (CI = 0.7433, and RI = 0.9547). The strict concensus tree (Fig. 1) shows the conspecific relationship of P. ebracteolatusand P. ophthalmicus. This molecular confirmation, combined with the computed sequence divergence, supported the study of Turner and Veldkamp [20] which suggested the synonymy of the said Phaeanthusspecies. Thus, P. ebracteolatusshould be, at present, referred to as P. ophthalmicus. Phylogenetic analysis. Fig. 2 shows that the genus Phaeanthusforms a strongly supported (BS=97%) clade; and that P. ebracteolatus 15-623B has grouped with P. ophthalmicus.

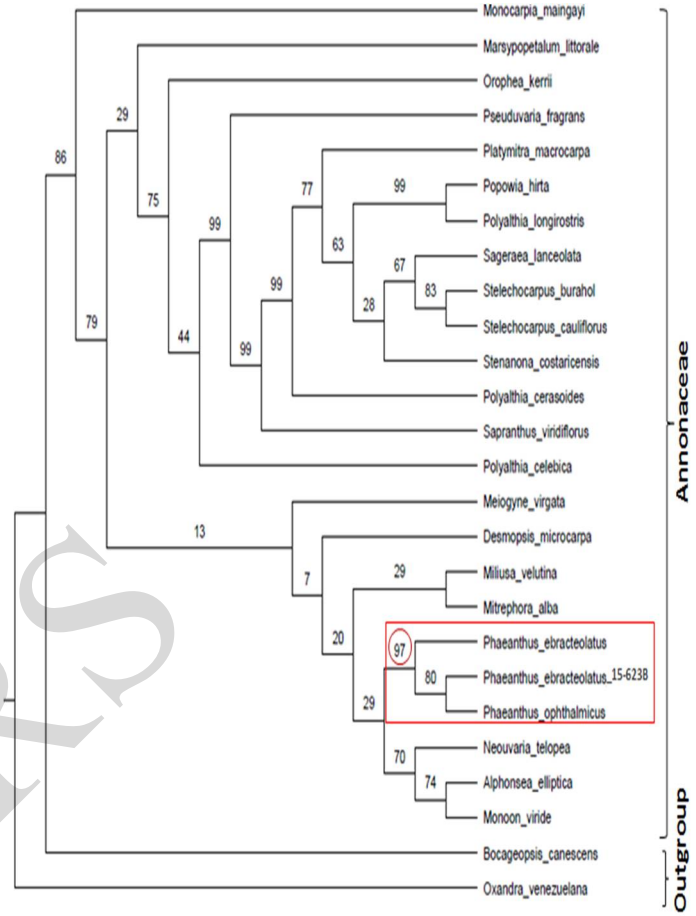

Fig 2. Strict consensus tree derived based from combined analysis of matK and rbcL data set. Numbers above the nodes corresponds to bootstrap values >50%. Bracket line shows outgroup taxa while the rest of the species is from the family Annonaceae.

Morphology of P. ebracteolatus and P. ophthalmicus.

The P. ebracteolatus15-623B and P. ophthalmicusare shown to have overlapping characters. The characters of the two Phaeanthusspecies are highlighted in Table 1 showing similarities (yellow) and differences (green). These characteristics were based from the protologue found in Garden Bulletin [19] and from a published journal by Mols and Kebler entitled Revision of the Genus Phaeanthus (Annonaceae)[17]

Table I: Characteristics Of P. Ebracteolatusand P. Ophthalmicus

Characters	*P. ebracteolatus*15-623B	*P. ophthalmicus*
Habit	- small tree	- small tree
Branches	- terete - brown when dried - lozenge-shaped striations on bark	- terete - brown to ash-grey when dried - bark with lozenge-shaped striations - glabrous to pilose to strigose
Petioles	- brown to black - longitudinal - strigose or piloseindument (few) - about 4 mm long	- black - longitudinal (slightly) grooved or flattened - glabrescent, pilose to strigose - 3-11 mm long
Leaves	- entire, lanceolate - papyraceous to pergamentaceous - shiny appearance - brownish to grayish when dried - base: obtuse - apex: attenuate to acuminate - midrib: sunken above, prominent below, strigose or piloseindumenta - secondary veins: anastomosing - 11-20 x 2.5-3 cm	- glabrous or midrib short to long pilose or strigose above - midrib and lateral veins pilose to strigose below - veins 9-14 pairs - anastomosing - 7.4-25.3 x 2.6-10.1 cm
Infloresences	- axillary - peduncle: brown or black when dried, glabrescent - pedicel: brown to black when dried, slender, about 8.5 cm, pilose - cyme	- reduced to one axis - 1-4 flowered - peduncle: 1-13 mm long, pilose to strigose or glabrescent - pedicel: 1.2-4.1 cm long, sparsely strigose
Sepals	- not seen	- 0.5-1.75 x 0.75-1.75 mm - apprisedpilose to strigose outside - glabrous inside
Outer Petals	- not seen	- 3 petals - valvate - sepal-like - much smaller than inner ones
Inner Petals	- yellow to cream - one set of petals only - 3 petals - valvate - triangular - base broad: strigose to piloseindument - 190 mm	- inner petals: - yellow to cream - 3 petals - valvate - triangular - base and apex pilose to strigose - 4-6 veins - visible
Torus	- hemispherical	- hemispherical
Stamens	- truncate - filament short - edges wavy - flat-topped	- 30-100 - truncate - filament short - connective prolongation flat - oblique - edges wavy
Carpels	- cylindrical - appressed rusty or ochre strigose - no style - club-shaped stigma	- margins and top cobwebbed - glabrescent - style short or absent - stigma ellipsoid to club-shaped
Fruit	- many - globose - black when dry - apiculate - raphe visible when dry - stipe: rounded - green-yellow-orange-dark red to purple - about 1 cm long	- many (up to 60) - monocarps: globose than ellipsoid, stipe pilose to strigose - black when dry - apiculate - raphe visible when dry - stipe rounded to angular, sometimes grooved - green-yellow-orange (young), dark red to purple (mature
Seeds	- 1 seeded - globose - papyraceous seed coat	- 1 or 2 - globose - seed coat papyraceous

Thin Layer Chromatography.

TLC plates revealed that P. ebracteolatus is rich, predominantly, in phenols. Other constituents found are tannins, flavonoids, coumarins, anthraquinones, and anthrones (Table

Table II: Constituents found in P. ebracteolatus

Spray Reagent	Constituents Tested	Px	PxA	PxB	PxE
Vanillin-sulfuric acid	Higher alcohols, phenols, steroids, essential oils	-	-	-	-
Potassium ferricyanide-ferric chloride	Phenols, tannins, flavonoids	+	+	+	+
Antimony(III) chloride	Flavonoids, steroids	-	-	-	-
Van Urk-Salkowski Test	Indoles	-	-	-	-
Magnesium acetate	Anthraquinones	-	-	-	-
Menthanolic potassium hydroxide (Borntrager reagent)	Coumarines, anthraquinones, anthrones, phenols	+	+	+	+
3,5 Dinitrobenzoic acid: Kedde reagent	Cardenolides	-	-	-	-

It has also exhibited that in the Potassium ferricyanide-ferric chloride test and in the Borntrager test, all four fractions of the crude plant extract have tested positive for its comprised constituents (Table 2). The plant extracts were found to be rich in Phenols and other antioxidant constituents (Table 3). The presence of such compounds could signify their antimutagenic importance.

Table III: Results of α-glucosidase and tyrosinase inhibition

Fractions	Anti-tyrosinase (IC_{50} values)	Anti-glucosidase (IC_{50} values)
Px	11.6057	0.19721
PxA	20.9695	0.10411
PxB	8.29228	0.66907
PxE	30.9884	0.18993
Positive Control	1.58721 (*Kojic Acid*)	0.32545 (*Carbose*)

Biological Assays. IC50 values were determined for anti-tyrosinase and anti-glucosidase. The IC50 value is a measures as the effectiveness of a compound in inhibiting certain functions. The positive control serves as a basis of the efficacy of inhibition of the plant extract. Values that are indicator of inhibition are those that are lower than the positive control. Fraction yielded higher values for anti-tyrosinase while samples, with the exception of PxB, presented lower that standard (Table 3) making the plants a viable source for the procurement of an anti-diabetic drug. The result of anti-staphylococcus, on the other hand, was determined using minimum inhibitory concentration (MIC). MIC is the lowest concentration of the antimicrobial that will inhibit bacterial growth. All plant extract had a low inhibitory effect on S. Aureus compared to the positive control (Table 4).

Table IV: Results of S. aureus inhibition

Plant Extracts (Fractions)	Minimal Inhibition Concentration (MIC)
Px	125 µg/ml
PxA	250 µg/ml
PxB	125 µg/ml
PxE	125 µg/ml
Positive Control (Streptomycin)	3.91 µg/ml

III. CONCLUSION AND RECOMMENDATIONS.

The study provided sufficient evidence based on sequences and morphology to support the synonymy of the P. ebracteolatusand P. ophthalmicus, with the latter being established as the current name of the species. Through thin layer chromatography, antioxidant components and phenolic compounds are revealed; while in the colorimetric assay, the plant extracts proved to be efficient as an α-glucosidase inhibitor. Further development can be employed in order to produce a viable drug for diabetes due to the inhibiting factor of P. opthalmicus in the anti-glucosidase colorimetric assay. Moreover, the researchers also recommend further studies to maximize the medicinal potential of the plant.

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