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GC-MS metabolite profiling of the hexane extract and antimicrobial characterization of the Philippine endemic Rubiaceae species *Uncaria cordata* var. *circa*, *Psychotria luzoniensis*, and *Psydrax puberula*

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Recent studies showed that the Rubiaceae family, the 4th largest family among dicotyledonous flowering plants, contains compounds that exhibit antimicrobial properties. This study aims to characterize the antimicrobial activity of the extracts and determine the chemical constituents of the hexane extract from endemic Philippine Rubiaceae species *Uncaria cordata* var. *circa*, *Psychotria luzoniensis*, and *Psydrax puberula*. The crude leaves extract of each plant species were subjected to solvent-solvent extraction to obtain the hexane, chloroform, and butanol extracts. The sub-extracts of each plant were subjected to antimicrobial micro-plate dilution assay. Extracts from the three plants exhibited promising MIC and MBC activities against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923. Characterization of the hexane extracts using GC-MS showed that these plants contained compounds that exhibited biological activities. The results provided in this study have shown that the three endemic plants are new sources of biologically-active secondary metabolites.

Keywords: *Rubiaceae*, *Psychotria*, *Uncaria*, *Psydrax*, MIC, MBC, GC-MS

Introduction

The Rubiaceae family comprises of 659 genera and approximately 13,000 species, making it one of the largest in number among vascular flora [1]. This plant family is known to produce bioactive metabolites with great pharmacological potential, such as iridoids, indole alkaloids, terpenoids, flavonoids, and anthraquinones, giving rise to new nature-oriented drugs with anti-inflammatory, mutagenic, antiviral, analgesic, antibacterial, and antioxidant effects on vascular diseases [2]. Majority of the phytochemical and pharmacological analyses have been done from the genera *Uncaria*, *Psychotria*, *Hedyotis*, *Ophiorrhiza*, and *Morinda* [2]. The Philippine Rubiaceae is currently composed of 80 genera, having one of the largest number of indigenous species, a number of endemic species, and five endemic genera (*Antherostele* Bremek.,

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Greeniopsis Merr., *Sulitia* Merr., *Villaria* Rolfe, and *Kanapia* Arriola & Alejandro) [2]. Three genera from the family of Rubiaceae are the focus of this study, namely *Psydrax, Psychotria*, and *Uncaria* with their respective endemic species namely *Psydrax puberula*, *Psychotria luzoniensis*, and *Uncaria cordata* var. *circa.*

Psydrax is a genus found throughout the Old World tropics from Africa, throughout southern and Southeast Asia, to Australia [3]. This genus is yet to be studied for their anecdotal therapeutic uses, except for *Psydrax amplifolia*, which was used against diarrhea [4]. Literature search revealed a dearth on the phytochemical investigation on the Psydrax species. Cyanogenic glucosides, iridoid dimers, flavonoids, and triterpenoids [5] have been previously identified from this genus [5].

Psychotria is one of the largest genera in the Psychotrieae tribe, and is well-known to be a rich source of the secondary metabolites. Among these compounds, the presence of various types of alkaloids including monoterpene indole, quinoline, isoquinoline, and benzoquinolizidine type have been well-established [6]. Non-alkaloidal compounds such as terpenoids, steroids, phenolics, flavonoids, coumarins, cyclic peptides and aliphatic compounds have also been isolated from various Psychotria species [6]. The presence of 112 Psychotria species in the Philippines had been documented and all of them are endemic in the country [7].

The genus *Uncaria* contains approximately 34 species, which is distributed in tropical regions, such as Southeast Asia, Africa and Southeast America. Mostly, the hooks of this species are applied to treat wounds and ulcers, fever, asthma, rheumatism, hyperpyrexia, hypertension, headaches, gastrointestinal illness, and bacterial and fungal infections [8]. Chemical studies on the different *Uncaria* species have elaborated the presence of alkaloids, terpenoids and their glycosides, flavonoids and their glycosides, coumarins, sterols, quinolic acid, phenolics, and tannins [9].

We herein report the determination of the secondary metabolites present in the hexane extract of *Psydrax puberula*, *Psychotria luzoniensis*, and *Uncaria cordata* var. *circa* with the aid of GC-MS, and to assess the anti-microbial properties of their crude and semi-crude extracts.

METHODOLOGY

The plant materials. Fresh leaves *P. puberula* were collected from Mt. Mingan, Dingalan, Aurora in March 2015. Voucher specimens were deposited at the UST-Herbarium, Research Center for the Natural and Applied Sciences, with Accession #15-528.

Fresh leaves of *U. cordata* var. *circa* were collected from Orani, Bataan in August 2015. A voucher specimen was deposited at the UST Herbarium with Accession #12261-62.

Fresh leaves of *P. luzoniensis* were collected from Ilocos Norte in August 2015. A voucher specimen was deposited at the UST Herbarium with Accession #12259-60.

Extraction of the plant materials

Psydrax puberula: Air-dried, ground leaves (2 kg) were percolated with a total volume of 22.4 L of methanol for three consecutive days. The collected methanol extracts were concentrated under reduced pressure to afford the crude extract. A portion of the marc (160 g) was dissolved in 200 mL of distilled water and partitioned with hexane (1100 mL). The collected hexane layer was dried with anhydrous sodium sulfate and concentrated under reduced pressure to afford the hexane extract (Pp-H, 20.0 g).

Uncaria cordata* var. *circa: Air-dried, ground leaves (900 g) were extracted with 13 L of 1:1 MeOH:DCM for three consecutive days. A 45 g

of the crude extract was obtained after concentration under reduced pressure. The crude extract was subjected to the same partitioning procedure using hexane (700 mL) to afford the semi-crude extract (Uc-H, 0.030 g).

Psychotria luzoniensis: Air-dried, ground leaves (175 g) were subjected to exhaustive extraction using 4 L of 1:1 DCM: MeOH. The crude extract was subjected to the same procedure for partitioning using hexane (900 mL) to afford the semi-crude extract (Pl-H, 10 g).

Antimicrobial assay. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the different extracts were determined using micro plate dilution assay. Extracts were diluted with DMSO to a concentration of 1 mg/mL, placed in microwells, then serially diluted (1:2) into eight wells to a final volume of 100 μL for each test organism. Final concentrations of the extracts were 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9 μg/mL. The following bacteria were used for the assay: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 49212 and *Pseudomonas aeruginosa* ATCC 27853. A 100 μL of bacterial suspension (1.5×10^8 CFU/mL) was added to each well and incubated at 37°C for 24 h. The concentration in the last well with no growth as determined by the absence of turbidity after 24 h was reported as the MIC. All wells with no growth were then sub-cultured into nutrient agar (NA) plates to determine the MBC. The lowest concentration of extract which did not show bacterial growth in the NA plates after 24 h was reported as the MBC. All setups were done in triplicate for each of the compounds. DMSO was used as the negative control and Ciprofloxacin as the positive control.

GC-MS analysis. GC-MS technique was used in this study to identify the components present in the extract. The analysis was carried out at the Research Center for Natural Sciences, University of Santo Tomas, Manila. A concentration of 2 mg/mL of the extracts of the three plants was used for GC-MS analysis. For GC-MS, a concentration of about 2 mg/mL of the extracts was analyzed using a Perkin-Elmer gas chromatograph model Clarus 680 coupled to a Perkin Elmer mass spectrophotometer model Clarus SQ8T, equipped with an HP5 column (20 m × 0.32 mm ID; 0.25 μm) programmed from 50°C (5 min) to 300°C at 50°C/min and 5 min hold. The carrier gas was helium (1.0 mL/min); injection was set in the split mode (1/10). Injector temperatures were 250°C. Mass spectral data was acquired in the scan mode in the 33–450 m/z range.

Results and discussion

GC-MS analysis. GC-MS is one of the best techniques to identify the constituents of volatile matter, whether it may be long-chained or branched hydrocarbons, alcohols, esters, and acids [10]. There are twelve identified compounds in hexane extract of *P. puberula*, Pp-H (Table 1), four in *P. luzoniensis*, Pl-H (Table 2), and four in *U. cordata* var. *circa*, Uc-H (Table 3). These components were compared with the NIST 11 MS Search 2.0 library, characterized and identified, along with their retention time and respective biological activity. The compounds identified have ≥98% similarity search. The structures of the compounds are presented in Fig. 1.

Compound **6** was found in the hexane extract of *P. puberula* and *U. cordata* var. *circa*. Interestingly, the abundant compounds identified in the GC-MS are the steroids β-sitosterol (13.78%) and betulin (10.57%) for *P. puberula*, (3β,22E)-Ergosta-5,22-dien-3-ol (12.98%) for *P. luzoniensis*, and (3β,22E)-Ergosta-5,22-dien-3-ol, acetate (10.05%) for *U. cordata* var. *circa*. Of the 20 identified compounds by GC-MS, 10 were found to have biological activities (Fig. 1). Diverse significant biological activities were noted which include anti-inflammatory (compounds **3, 4, 5, 7, 10, 11**), anti-microbial (**3**), anti-tumor (**7, 11, 15**),

Table 1. Chemical constituents identified in the hexane sub-extract of *Psydrax puberula* (PpH)

RT (min)	Peak Area %	Compound	Biological Uses
8.1	0.37	14α, 18α-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl),(5β)-Pregnane-3,20-β-diol (**1**)	
8.5	1.13	Digitoxin (**2**)	Remedy for heart diseases, suppress cancer growth [11]
8.9	3.45	Ethyl isoallocholate (**3**)	Antimicrobial, diuretic, anti-inflammatory, antiasthma [12]
9	2.34	Methyl palmitate (**4**)	Anti-inflammatory [13]
9.17	1.78	Palmitic acid (**5**)	Anti-inflammatory [14], antioxidant, hypocholesterolemic, nematicidal, pesticidal, hemolytic, antiandrogenic, hemolytic, 5-alpha reductase inhibitor [15]
9.45	1.26	2-[2-(2-(2-pentylcyclopropyl) methylcyclopropyl)methyl] cyclopropyl Cyclopropanebutanoate (**6**)	
9.65	2.01	Oleic acid (**7**)	Antitumor, anti-inflammatory [16]
9.75	2.21	Methyl-6-cis,9-cis,11-trans-octadecatrienoate (**8**)	
10.45	1.03	β-monoolein (**9**)	
10.66	13.78	β-sitosterol (**10**)	Immunomodulator/Anti-inflammatory [17]
11.46	10.67	Betulin (**11**)	Anti-cancer, anti-tumor, Anti HIV; Anti carcinomic; Anti feedant; Antiflu; Anti inflammatory, Antitumor; Antiviral; Aphidifuge, Cytotoxic; Hypolipemic; Prostaglandin-Synthesis-Inhibitor, Topoisomerase-Inhibitor [17]
12.28	2.34	(2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-propanoic acid (**12**)	protein tyrosine phosphatase inhibitor [18]

Table 2. Chemical constituents identified in the hexane sub-extract of *Psychotria luzoniensis* (PlH)

RT (min)	Peak Area %	Compound name	Bioactivity/Uses
1.11	0.89	2-butyne-1,4-diol (**13**)	
8.99	2.46	Z,Z-3,15-octadecadien-1-ol acetate (**14**)	
9.39	1.25	(Z)- methyl ester 9-octadecenoic acid (**15**)	Flavour, fungicide, pesticide, antioxidant, anti carcinogen, peroxisome proliferator [19]
9.44	12.98	(3β,22E)-Ergosta-5,22-dien-3-ol (**16**)	

Table 3. Chemical constituents identified in the hexane sub-extract of *Uncaria cordata* var. *circa* (UcH)

RT (min)	Peak Area %	Compound name	Bioactivity/Uses
8.9	2.34	(11E)-10,13,13-Trimethyl-11-tetradecen-1-yl acetate (**17**)	
9.04	1.76	2-[2-(2-(2-pentylcyclopropyl) methylcyclopropyl)methyl] cyclopropyl Cyclopropanebutanoate (**6**)	
9.37	1.27	9-octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (**18**)	
9.42	10.05	(3β,22E)-Ergosta-5,22-dien-3-ol, acetate (**19**)	antitumor, cytotoxic, rheumatoid arthritis and immune promoter [20]

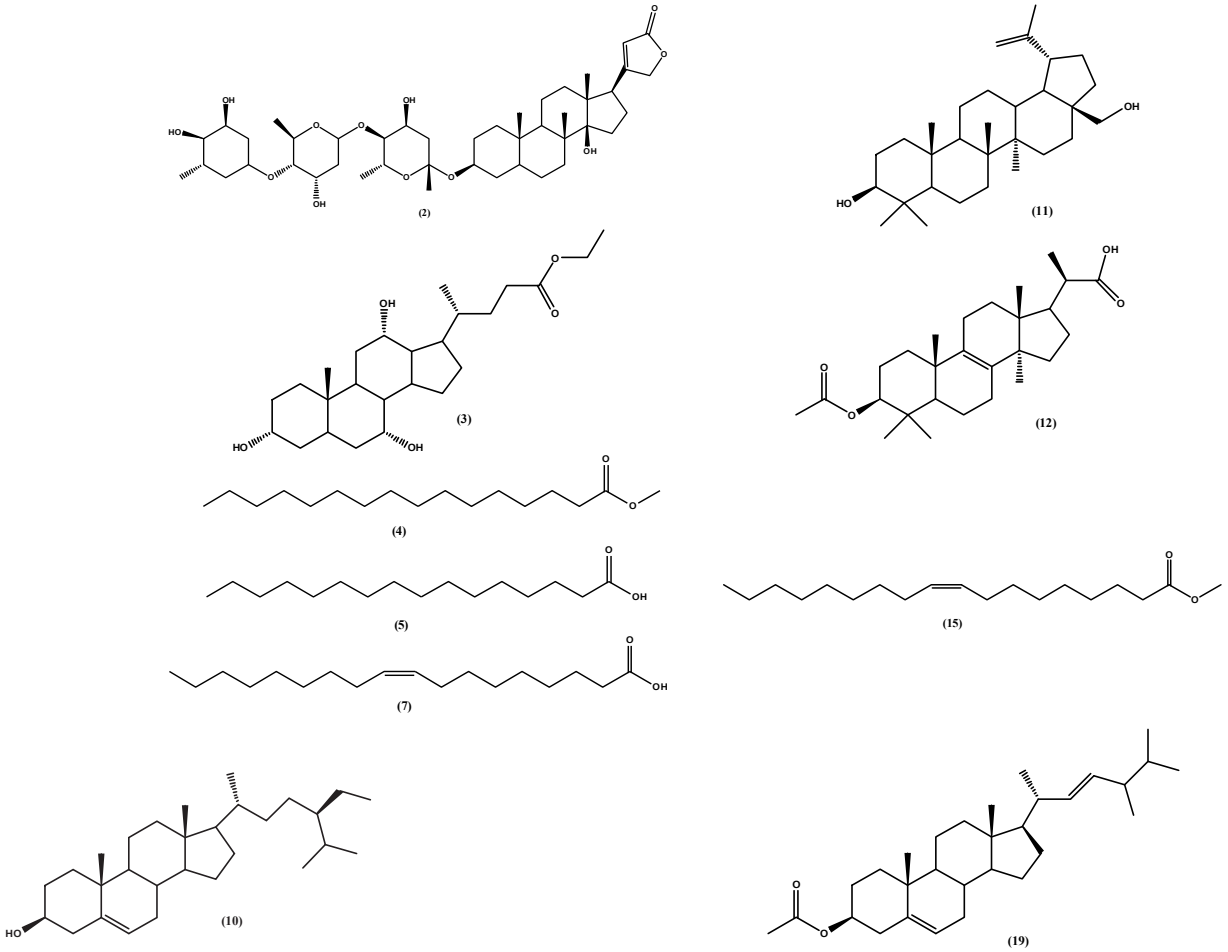


Figure 1. Chemical structures of bioactive compounds identified by GC-MS.

cytotoxicity (**2, 11, 15, 19**), anti-oxidant (**5, 15**), and enzyme inhibitor (**5, 11, 12**). The bioactive compounds are sterols (**2, 3, 10, 19**), terpenes (**11, 12**) or long-chain carboxylic acids or esters (**4, 5, 7, 15**).

The other compounds identified in the GC-MS include long-chain hydrocarbon esters (**6**, **8, 9, 14, 17, 18**), steroid (**16**), alkyne alcohol (**13**), and an alkaloid (**1**). (Fig. 2).

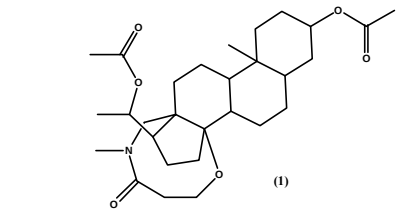
Antimicrobial assay. The antimicrobial activity utilizing the MIC and MBC values of the different extracts were determined using the micro-plate dilution technique. Table 4 shows the results of the extracts and using DMSO as negative control and Ciprofloxacin as the positive control. DMSO showed no activity (>500 μg/mL) while ciprofloxacin exhibited ≤3.91 μg/mL activity.

Results indicate that the extracts of *P. puberula* showed antimicrobial activity against all the organisms. Over-all, *P. puberula* extracts displayed an inclination of antimicrobial activity towards the Gram-positive bacteria (*S. aureus*). The extracts of *U. cordata* var. *circa* also showed promising activity (MIC) against the three

Table 4. MIC and MBC of the crude and sub-extracts

Microorganism	Extracts*	MIC µg/mL	MBC µg/mL
***Escherichia coli* ATCC 25922**	Pp-CR	125	250
	Pp-H	250	250
	Pp-B	125	250
	Pp-C	250	250
	Uc-H	31.25	125
	Uc-C	31.25	125
	Uc-B	31.25	>500
	Pl-H	>500	>500
	Pl-C	>500	>500
	Pl-B	62.5	125
***Pseudomonas aeruginosa* ATCC 27853**	Pp-CR	62.5	125
	Pp-H	125	125
	Pp-B	125	125
	Pp-C	125	125
	Uc-H	62.50	125
	Uc-C	62.50	125
	Uc-B	62.50	>500
	Pl-H	>500	>500
	Pl-C	>500	>500
	Pl-B	62.50	125
***Staphylococcus aureus* ATCC 25923**	Pp-CR	31.25	125
	PpH	31.25	62.5
	PpB	31.25	250
	PpC	62.50	250
	Uc-H	31.25	125
	Uc-C	31.25	62.50
	Uc-B	31.25	>500
	Pl-H	>500	>500
	Pl-C	>500	>500
	Pl-B	62.5	125

*Pp – *Psydrax puberula*, Pl – *Psychotria luzoniensis*, Uc – *Uncaria cordata* var. *circa*, H – hexane, C – Choloform, B – *n*-butanol, CR – Crude extract



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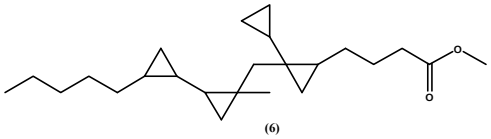

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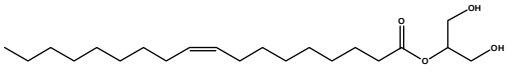

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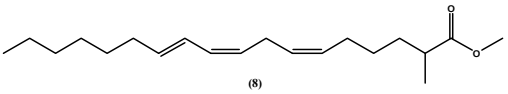

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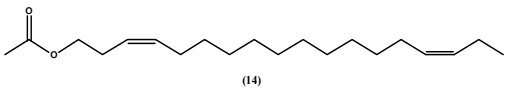

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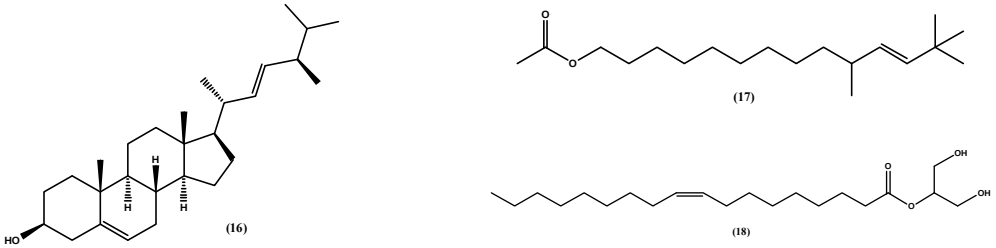

Figure 2. Other compounds identified from the GC-MS

bacteria. MBC values of *U. cordata* var. *circa* showed, however, that the butanol extract did not exhibit good activity (>500 μg/mL) as compared with the hexane and chloroform extracts. The observed antimicrobial activity of *U. cordata* var. *circa* is consistent with the other species of the genus *Uncaria* [2]. Analysis of the antimicrobial activity of the *P. luzoniensis* extracts may indicate that the compounds responsible are polar in nature. This was in accordance with the observed MIC and MBC values of both the hexane and chloroform extracts (>500 μg/mL) versus the butanol extract. Based on the identified metabolites by GC-MS, only ethyl isoallocholate (**3**) have a reported antimicrobial activity.

The antimicrobial results obtained from the three Philippine endemic *Rubiaceae* species warrant phytochemical investigation into the compounds responsible for the activity.

Conclusion

The *Rubiaceae* family continuously proves itself to be rich in secondary metabolites with great pharmacological activity. In this study, 20 components were characterized from the hexane extracts of the endemic *Rubiaceae P. puberula*, *P. luzoniensis*, and *U. cordata* var. *circa*. It has also been proven that these three plants have antimicrobial properties effective against both gram-positive and gram-negative bacteria. Through GC-MS analysis, it was also discovered that all three plants may contain compounds with interesting activities and could be a new source of biologically-active materials.

Acknowledgment

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