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Inhibition of *Pseudomonas aeruginosa* by the extracts of indigenous plants from the Cordillera Region, Philippines

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The Cordillera Administrative Region (CAR) in the Philippines has a rich floral biodiversity and its people have been using various indigenous plants for various purposes including treatment of several ailments for a long time. Despite of such a rich bio-resource, very limited biodiversity studies have been done and the scientific basis for ethno-medicinal plants is not well-established. This study therefore aimed to test the antibacterial activity of 13 indigenous plants against *Pseudomonas aeruginosa*, an opportunistic pathogen that has attained serious therapeutic challenge in the fields of medicine and pharmacy. Standard Kirby-Bauer assay was used to evaluate the antibacterial activity of the selected indigenous plant extracts by measuring the zones of inhibition. Four out of the 13 plant extracts screened showed significant zones of inhibition namely: *Nepenthes alata* Blanco. (11.66 mm), *Bauhinia purpurea* L. (14 mm), *Ficus septica* Burm.f. (14.50 mm) and *Melastoma malabathricum* L. (10 mm). Moreover, *N. alata* Blanco obtained the lowest minimum inhibitory concentration (MIC) at 250 μg/mL. This study is one of few studies that were conducted to provide a scientific basis for the antibiotic potential of the indigenous folkloric plants from CAR.

Keywords: Antibacterial activities, Indigenous plant extracts**,** *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic pathogen that has attained serious therapeutic challenge in the world of pharmaceuticals due to its rapid ability to develop resistance to multiple classes of antibiotics. It can be acquired in the communities or even in hospitals[1-3]. It is known to be the causative agent of pneumonia. This bacterium is considered as the leading pathogen among patients with diffused panbronchitis, chronic obstructive pulmonary disease and cystic fibrosis[4-5]. Aside from different lung diseases, *P. aeruginosa* can contribute to serious bloodstream infection which causes high rate of patient mortality and health-care costs[6]. Due to their high surface area, the nutrients easily diffuse to their cellular membranes, allowing them to replicate faster and produce mutants that are resistant to the previous drugs used. This phenomenon is called antibiotic resistance. Misuse of the drugs by the physician or/ and the patient can also lead to antibiotic resistance. Plasmids of the surviving bacteria can be passed to the others by horizontal gene transfer[7]. The treatment of bacterial infections become more impenetrable nowadays and imposes immense medical problems. Consequently, the critical role of *P. aeruginosa* in the fields of medicine and pharmaceuticals has led to numerous efforts towards the development of antimicrobials. For instance, a research was done in India to screen the antimicrobial activity of Indian medicinal plants used in folkloric medicine. The plants were tested against eight gram-negative bacteria and two gram-positive bacteria and four fungi. One of bacteria used was *Pseudomonas aeruginosa* and out of the 50 plants, 17 plants exhibited antibacterial activity against the said bacterium[8]. Another study evaluated the antimicrobial activity of *Bauhinia purpurea* Linn. Leaves against six species of pathogenic and non-pathogenic microorganisms: *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *P. aeruginosa* and *Candida albicans* using the disk diffusion method. Significant inhibitory activity was observed with methanol extracts of plant against the test microorganisms. They also identified the possible bioactive compounds that inhibit microbial growth such as lupeol, stigmasterol, lanosterol, ergosterol, beta-tocopherol, phytol, hexadeconic acids,

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hexadeconic acids methyl esters, octadecadienoic acids and octadecatrienoic acid[9].

Even with the above mentioned contributions, there is still a vast array of folkloric medicinal knowledge yet to be proven. Hence, the principal aim of the study was to screen the ethanolic extracts of indigenous plants obtained from the different areas of the Cordillera region for antibacterial activity against *P. aeruginosa*. With the changing climate and the rapid appearance of emerging diseases, most of which have developed resistance to currently available drugs, the discovery of less expensive antibiotics from natural resources like plants has to cope with the need for them. Moreover, the information gained in this study may add to the scant scientific basis of their ethno-medicinal purposes and may raise awareness especially on the conservation and preservation of flora of Cordillera before they become extinct due to human exploitation.

Methodology

Site of study

The Cordillera Administrative Region (CAR) (Fig. 1) is a landlocked area which is located at the northern part of the Philippines. It is one of three mountain ranges, the CAR being the largest, relative to the other two mountain ranges namely Sierra Madre (the longest) and Caraballo mountain range (the smallest). The Cordillera is geographically and politically composed of six provinces namely Benguet, Ifugao. Mountain Province, Abra, Kalinga and Apayao. Each of these provinces is inhabited by various local communities with characteristically varied and unique culture and languages. With a temperate climate, CAR is bestowed with a diversity of plants which are being harnessed by the local communities to their advantage. The local communities have a close interaction with their environment hence; have developed indigenous knowledge on how they use their natural resources including medicinal plants.

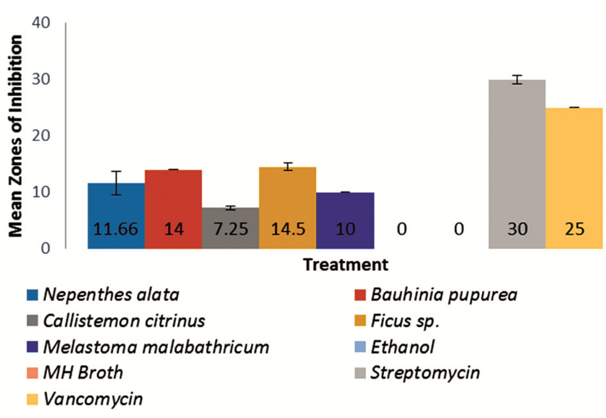

Fig. 1 — Mean of the inhibition zones of the different plant extracts against

The indigenous plants

Table 1 shows the thirteen ethanolic plants, their locality and the plant parts used as medicinal purposes based on ethnobotanical knowledge. The plants were selected based on availability and ethnobotanical use categories relating to treatment of various bacteria-related ailments. The plants were collected previously in another project, were extracted using 95% ethanol, the solvent was retrieved using a rotary evaporator and the extract was completely dried using a shaking water bath. To prevent degradation of the bio-active compound, the plant extracts were deposited in a -40^{0}C freezer until ready to use. The extraction process followed the standard methods.

The plant extracts subjected to antibacterial test were dissolved in Mueller-Hinton broth to a final concentration of 4mg/mL[2]. This concentration was used in the antibacterial test against *P. aeruginosa.*

Biomonitor organism

The test organism used is the clinical isolates of pathogenic gram-negative *P.aeruginosa,* with accession number ATCC27853, was obtained from the bacterial culture collection of the Natural Sciences Research Institute of University of the Philippines Diliman. These bacterial strains can survive at a wide range of temperature, from 4°C up to 42°C but grows best at 37°C. They proliferate best with aeration but are also capable of surviving anaerobically[10]. Fresh inoculants of *P.aeruginosa* were prepared for testing the antibacterial potential of all the plant ethanolic extracts.

Screening of antibacterial activities through disk diffusion assay

Standard disk diffusion assay was used to evaluate the antibacterial activity of the selected indigenous plant extracts by measuring the zones of inhibition. Briefly, 20 µL of each extract (4 mg/mL) was loaded onto sterile disks (6 mm diameter) and then placed on each Mueller-Hinton (MH) plates spread with overnight culture of 100 µL *P. aeruginosa* in MH broth. The plates were incubated for 24 hours at 37°C. The antibiotics used as positive controls were streptomycin and vancomycin and the negative controls are ethanol and MH broth. A visible clear

Table 1 — The 13 indigenous plants from the Cordillera region and their locality, that were tested for antibacterial activities against P. aeruginosa

Species	Local/ Common name	Family	Parts used	Locality	Herbarium Code
Nepenthes alata Blanco.	*kakallong*	Nepenthaceae	pitcher	Mt. Polis, Mt. Province	DOST-006
Bauhinia purpurea Linn.	butterfly tree	Fabaceae	leaves	Palali, Sablan	DOST-129
Callistemon citrinus Stapf.	bottlebrush	Myrtaceae	leaves	Baguio, Benguet	DOST-UPB_12
Ficus septica Burm.f.	*tibig*	Moraceae	stem	Mt. Polis, Mt. Province	DOST-074
Mangifera indica Linn.	manga	Anacardiaceae	leaves	Poblacion, Atok	DOST-143
Melastoma malabathricum Linn.	*bakgi*	Melastomataceae	leaves	Poblacion, Atok	DOST-012
Elephantopus mollis Kunth.	*malatabako*	Asteraceae	leaves	Tuba, Benguet	DOST-103
Dicranopteris linearis (Bum.f.) Underw.	*ro-ot*	Gleicheniaceae	leaves	Banayakew, Atok	DOST-014
Verbena bonariensis Linn.	purple top	Verbenaceae	leaves	Halsema Road, Atok, Benguet	DOST-039
Drynaria cordata Linn.	*agayap di nuang*	Polypodiaceae	stem	Poblacion, Atok	DOST-013
Ficus minahassae Miq.	*sabfog*	Moraceae	leaves	Bayabas, Sablan, Benguet	DOST-145
Ageratum conyzoides Linn.	*puriket or burburtak*	Asteraceae	leaves	Poblacion, Atok	DOST-018
Coffea robusta Linn.	*kape*	Rubiaceae	leaves	Banayakew, Atok	DOST-110

zone is indicative of antibacterial activity. The zones of inhibition were measured and compared to the positive and negative controls. The experiment was performed in triplicate[11]. The extracts that yielded positive results were tested to determine their minimum inhibitory concentrations (MICs) through broth dilution method[12].

Statistical Analysis

Descriptive analysis was used in the study. The mean and standard deviation of the values obtained were calculated using GraphPad Prism 5 software.

Results

It has been established for long that plants provide a significant contribution to the development and discovery of novel antibacterial drugs. Hence, plant extracts have been studied *in vivo* and *in vitro* for years against several multi-resistant bacteria such as *P. aeruginosa*[13-14]. At present, various articles were published about the medical importance of indigenous plants from Africa, India, China and other Asian countries[15-22]. These studies demonstrated the usefulness and potential of plants as promising sources of antibiotics. In the Philippines, however, especially in the Cordillera Administrative Region, few studies have been published[23-28]. These studies form the dearth of literature in the CAR, a lot of plants with folkloric medicinal uses are yet to be explored.

In addition to the previous studies, the current investigation revealed that four out of 13 plants tested were found to have antibacterial activity against *P. Aeruginosa* because of the observed clear zones of inhibition. These plants are *N. alata* (Nepenthaceae), *B. purpurea* (Fabaceae), *F. septica* (Moraceae) and *M. malabathricum* (Melastomataceae). Table 2 presents the averages of the three trials of 13 plant extracts and MIC of the extracts that demonstrated positive results. The mean and standard deviation mean (SDM) in the inhibition zones is presented in Fig. 1. It was observed that four extracts were generally partially active (10-13 mm) and active (14-19 mm) based on a standard[29]. In all extracts with a concentration of 4 mg/mL, *F.septica* demonstrated the highest antibacterial activity with an average diameter of 14.50±0.50 mm whereas its MIC is 1000 µg/mL.

Maximum zones of inhibitions were observed in the positive controls, 30±1.30 mm in streptomycin and 25±0.00 mm in vancomycin, indicating that the said bacterium was susceptible in the antibiotics used and the negative controls facilitated its growth. After *F. septica,* the second maximum zone of inhibition is *B. purpurea* with an inhibition zone of 14±0.00 mm. The last two plant extracts, *N. alata* and *M. malabathricum* only showed partial antibacterial

Table 2 — Summary of the antibacterial screening of 13 plant extracts against Pseudomonas aeruginosa and minimum inhibitory concentrations (MICs) of plant extracts with antibacterial properties

Species	Plant parts used	Zone of Inhibition (mm)						(MIC) µg/mL
		T1	T2	T3	Mean	SD	SEM	
***Nepenthes alata* Blanco**	pitcher	12	10	13	11.66 (+)	1.528	0.8819	250
***Bauhinia purpurea* Linn.**	leaves	14	-	14	14 (++)	0	0	4000
***Callistemon citrinus* Stapf.**	leaves	7.5	-	7	7.25	0.3536	0.25	-
***Ficus septica* Burm.f.**	stem	-	15	14	14.50 (++)	0.7071	0.5	1000
***Mangifera indica* Linn.**	leaves	-	-	-	-	0	0	-
***Melastoma malabathricum* Linn.**	leaves	10	10	10	10 (+)	0	0	-
***Elephantopus mollis* Kunth.**	leaves	-	-	-	-			-
***Dicranopteris linearis* (Bum.f.) Underw.**	leaves	-	-	-	-			-
***Verbena bonariensis* Linn.**	leaves	-	-	-	-			-
***Drynaria cordata* Linn.**	stem	-	-	-	-			-
***Ficus minahassae* Miq.**	leaves	-	-	-	-			-
***Ageratum conyzoides* Linn.**	leaves	-	-	-	-			-
***Coffea robusta* Linn.**	leaves	-	-	-	-			-
				Controls Used				
Streptomycin (10 µg/ mL)		30	30	30	30 (+++)			
Vancomycin (10 µg/ mL)		25	25	25	25 (+++)			
Ethanol		-	-	-	-			
Muller Hinton Broth		-	-	-	-			

activity against *P. aeruginosa* with 11.66±0.88 mm and10±0.00 mm respectively.

The plant extracts that demonstrated zones of inhibitions of 11±0.00 mm and above, were further tested to determine the minimum inhibitory concentration (MIC) of the plant extracts. The lowest concentration of a drug that prevents the growth of an organism is called MIC which is done using serial dilution test tube preparations of MH broths and inoculated by the *P. Aeruginosa*[30]. Results from this study revealed that the lowest concentration that can inhibit the bacterial growth was observed in *N. alata* Blanco with only 250 µg/mL. Currently, no similar studies provided information about the MIC of *N. alata* Blanco. On the other hand, *B. purpurea* Linn. Showed an MIC of 4000 µg/mL whereas as low as 50 µg/mL exhibited bacterial inhibition was previously demonstrated[31]. Lastly, the MIC of the *F. septica* was 1000 µg/mL, this is relatively higher as compared to another study[32].

Discussion

Pseudomonas aeruginosa is a gram-negative bacterium. The composition of its outer membrane is structural lipopolysaccharides, making the cell impermeable to lipophilic solutes. Furthermore, it also has a characteristic inherent multiple efflux pumps that prevent the intracellular accumulation of antibacterial agent[33]. These factors contribute to its rapid resistance against antibacterial drugs.

As stated earlier, *Ficus septica* Burm.f. demonstrated the strongest antibacterial activity. *Ficus* species has β-sitosterol. β -sitosterol, and phytosterol that has a similar structure with cholesterol, is known, especially in India for its anti-cancer and anti-inflammatory activities and many more. This could be due to its ability to reduce the expression of chemotactic cytokine genes as exemplified by the inhibition of the *P. aeruginosa*-dependent activation of Protein Kinase C isoform alpha which in turns down regulated the expression chemokines IL-8, GRO-α GRO-β, which play a pivotal role in the recruitment of neutrophils in cystic fibrosis inflamed lungs[34].

Bauhinia purpurea Linn. exhibited the second strongest antibacterial activity. It has lupeol, stigmasterol, lanosterol, ergosterol, beta-tocopherol, phytol, hexadeconic acids, hexadeconic acids methyl esters, octadecadienoic acid and octadecatrienoic acid as potential bioactive compounds[35]. These phytochemicals serve as antioxidants by eliminating the effects of free radicals; and inhibit nucleic acid synthesis cytoplasmic membrane function, energy metabolism, attachment of biofilm formation and alter the membrane permeability, leading to the attenuation of the pathogen[36-37].

The antibacterial activity of *N. alata* pitcher extracts exhibited in this study concurred with the results presented by Buch and his colleagues in 2012[38]. The authors further demonstrated that the pitcher fluid contained antimicrobial naphthoquinones, plumbagin and 7-methyl-juglone, and defensive proteins such as the thaumatin-like protein. Naphthoquinones, for instance, reduce bacterial biofilm formation up to 50%, making it better than vancomycin as biofilm forms a barrier against the antibiotic that avoids its action. They target the initial process of bacterial attachment[39]. Similar results was reported by Sunilson (2008)[40]. Results of this study showed the antibacterial and wound healing activities of *M. malabathricum* Linn. in the form of an ointment in two types of wound model in rats. The said plant possesses antinociceptive, anti-inflammatory, and antipyretic activities. Its bioactive compounds can block or reduce pain resulting from direct nociceptor stimulation, modulate inflammatory mediators' action and, eventually cross the blood–brain barrier then control the action of centrally synthesized prostaglandins[41-43].

Conclusion

Preliminary results of this investigation proved that these indigenous folkloric plants indeed have medicinal purposes. Their bioactive compounds can be used as an alternative to the commercially available antibiotics since they can significantly control the growth of the multi-resistant *P. aeruginosa*. The current study indicates that unexplored diversity of indigenous plants in Cordillera has potentials in the field of pharmaceuticals and medicine, thus need to be further preserved and conserved. Thirteen plants tested is just a small representative of the highly diverse and unexplored indigenous plants of Cordillera region. Hence, it is recommended that more plants in the region will be studied to discover useful plant species that have novel antibacterial compounds to attenuate bacterial pathogenicity. Future studies can also focus on the data base development of potential plants for antibacterial activities as well as elucidation of the structures of the said phytochemicals to harness their full potential. Public education and awareness must also be disseminated in order for the local communities and government agencies to be aware of the importance of these folkloric plants and should help to preserve and conserve them.

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