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Antibiotic Properties of the Endophytic *Streptomyces* spp. Isolated from the Leaves of Myanmar Medicinal Plants

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Abstract

Three medicinal plants of Myanmar are selected in the study of endophytic microorganisms and are taxonomically classified and identified to be Sa-ba-lin (*Cymbopogon citratus* Stapf.), Shazaung-tinga-neah (*Euphorbia splendens* Bojer. ex Hooker) and Ma-shaw (*Sauropus grandifolius* Pax. and Hoffm.). The screening of endophytic microorganisms is performed according to the ISP method (International *Streptomyces* Projects 1993). The morphological and physicochemical properties of isolated strains are studied and identified to be the Genus *Streptomyces*. The test of apparent antimicrobial activity of isolated *Streptomyces* is done on 18 strains of pathogenic bacteria. It is found that the isolated endophytic *Streptomyces* showed the significant antibacterial activity on most of the test organisms.

Keywords : *Streptomyces, Endophytic microorganisms,* Antibiotics

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Introduction

Biologists with very different objectives are much concerned in the aerial surface of plants and the microorganisms which exists there. It is very interesting to know these microhabitat better, how they live and adapt in such an environment very different from soil where most living organisms spend their lives. In a plant, the leaves constitute the major visible surface and are open to infection by airborne or dispersed microorganisms. The leaf surface tissues are not only a potential microenvironment for microorganisms but also interfaces of exchange mechanisms with the external environment. Some pathways existing for these purposes may also serve as channels of entry for microorganisms (Cutter, 1976). The isolation and characterization of microflora of the leaves of higher plants has been reviewed by Preece and Dickinson (1971). These phylloplane or endophytic microflora in natural habitat has not yet been well studied compared to those of soil although they play a significant role in protection and infection of some important agricultural crops. In the course of screening antimicrobial metabolic products from phylloplane microorganism, an antibacterial antibiotic AP-001 has been detected in the culture broth of *Streptomyces* isolation of *Streptomyces species*. fermentation parameter, isolation and purification of novel antibiotic AP-001 and some physicochemically properties as well as biological activity have been reported. In the present study, some endophytic microorganism which show potent antibiotic activity were screened from 3 medicinal plants, namely *Cymbopogon citratus* Stapf (Saba-lin). *Euphorbia. splendens* Bojer. ex Hooker (Shazaung- tinga-neah) and *Sauropus grandifolius* Pax & Hoffm (Ma-shaw). The fermentation parameter and apparent test of antibacterial activity on 18 strains of pathogenic test organisms, systematics of isolated endophytic microorganisms some physicochemical as well as biological significance were investigated.

Materials and Methods

Collection of Medicinal Plants and Plants Systematic Study

The Myanmar medicinal plants used in screening of endophytic microorganisms were collected from Myit-kyi-na and Yangon University Campus. The study of plant systematics was carried out in the herbarium, Department of Botany, Y. U., with the help of literatures of Hooker (1885) and Hundley (1987).

Isolation and Identification of Endophytic Microorganisms

Three strains of endophytic microorganisms were screened from the leaf segments of *Cymbopogon citratus* Stapf. (Gramineae), *Euphorbia splendens* Bojer. (Euphorbiaceae) and *Sauropus grandifolius* Pax. & Hoffm (Euphorbiaceae) by the methods of ISP as well as Nyunt Phay *et al.* (1996). The pure- culture isolates were designated as AP-101, AP-102 and AP-103 respectively. Taxonomic characterization of isolated strains was carried out according to the methods shown in the Bergey's Manual of Determinative Bacteriology (8th edition, Buchanan and Gibbon, 1974).

Fermentation

The culture of isolated endophytic strains was grown at 30° C for 5 days on nutrient agar was inoculated into 500 ml conical flasks containing 100 ml the seed medium. It was composed of soluble starch 3%, polyepeptone 0.5%, glucose 0.2%, yeast extract 0.3 %, ammonium sulphate 0.1% and calcium carbonate 0.3%. The pH was adjusted to 7.0 before autoclaving. The flasks were incubated at 30° C for 3 days on a shaker at 130 revolution per minutes. After the incubation, fermentation

broth was centrifuged at 3000 rpm for 20 minutes. The supernatant of the fermented broth was collected and used as the main source of antibiotic on pathogenic test organisms by agar disc diffusion technique.

Paper Disc Diffusion Technique

Paper disc diffusion technique reported by Bauer (1966) was applied in the study of antibiotic sensitivity test. The Toyo filter paper discs (8 mm in diameter, Type 26 Advantage) were sterilized by autoclaving followed by one hour drying in 60° C and then impregnated with supernatant fermented broth in which sources of antibiotic metabolite were provided. Approximately 70ul / disc of fermented broth was needed to soak a filter paper disc and allowed to dry at the room temperature.

The test organisms were cultured by streaking on the surface of nutrient agar and them the filter paper discs soaked with fermented broth were placed on the agar surface. With the help of a flamed forceps tips the discs were generally pressed down to ensure the contact. In the centre was a control disc incorporated with sterilized basal medium and the culture plates were incubated at 37°C overnight. After the incubation period, the diameter of observed clear zones around each disc was measured and recorded. The complete inhibition of growth as determined by unaided eye was assumed sensitive. The mean values of clear zones of sensitivity were recorded from triplicate experiments.

Study on Cultural and Physicochemical Characteristic of Isolated Endophytic Strains

Some cultural as well as physicochemical characteristic of isolated strains was studied by culturing on different agar media according to the method shown in Bergey's manual of determinative bacteriology 8^{th} ed., 1974.

The strains preserved in the Bacteriology Research Division, Department of Medical Research were used as test organisms in the present study and shown in Table 2.

Estimation of Minimum Inhibitory Concentration (MIC)

The MIC of secondary metabolites produced by isolated AP-102 from *Euphorbia splendens* was estimated by serial dilution method (Linton, 1983) combining with the agar disc diffusion techniques Bauer (1966)

Results and Discussion

The collected plant samples for the present study are classified as following according to Hooker (1885) and Hundley (1987).

Family - Gramineae

Botanical name - *Cymbopogon citratus* Stapf.

Vernacular Name - Sabalin

Perennial, highly aromatic plant, forming dense turf, laminae linear, flat, 3-10 mm broad; inflorescence consisting of 6-15 mm long, spicate branches breaking into segments with fruits, usually arranged two on common peduncles in axils of apical leaves, flowering very rarely. South and Southeast Asia where it is cultivated to obtain aromatic oil.

Family- Euphorbiaceae

Botanical name- *Euphorbia splendens* Bojer.

Vernacular name- Shazaung-tinga-neah

The Shazaung-tinga-neah is monoecious shrub. Stems woody, cylindrical, covered with stipular spines. Leaves simple opposite, superposed, subsessile, acute, laminae oblong-lanceolate, stipulate,

stipules spiny, unicostate, reticulate venation. Inflorescence large number of cyathia arranged in peduncled axillary cymes. Flower pedicellate, unisexual, enclosed within involucre of bracts forming cupular structure. Perianth absent, naked flowers. Androecium single stalked stamen representing male flower, bracteate, anthers 2-celled dehiscing longitudinally. Gynoecium a single stalked, tricarpellary pistil represent female flower, it remain surrounded by male flowers (stalked stamens), ovary superior, three-chambered, single ovule in each loculus, axile placentation, three styles, three bifid sigmas. Fruit a capsule.

Family - Euphorbiaceae

Botanical name - *Sauropus grandifolius* Pax . and Hoffm.

Vernacular name - Ma-shaw

Monoecious perennial shrubs, 1.5-20 m high. Stems woody, cylindrical, glabrous. Leaves alternate, simple, two ranked; laminae ovate-lanceolate, the bases acute, the margins entire, the tips acute to acuminate, unicostate, glaucous, stipules lanceolate. Inflorescences axillary 1 to 3 flowered cymes; staminate flowers in the lower axils; pistillate in the upper ones. Flowers unisexual, greenish yellow, 3-merous; sepals 6, basally connate, valvate; petals absent; staminate flowers : (3) stamens, monadelphous, the filaments nearly absent, the anthers dithecous, dorsifixed, extrorse, longitudinal dehiscent; pistillate flowers: 3-carpelled, the stigma 3-fid, the ovary discoid, superior. Fruits berries, globoid. Flowers in October.

Identification of Isolated Endophytic Microorganisms

Three strains of endophytic microorganisms were isolated from the leaf segments of three Myanmar Medicinal plants Sa-ba-lin, Shazaung-tinga-neah, and Ma-shaw. These were designated as AP-101, AP-102 and AP -103 respectively. The isolated strains show the septate mycelium and Gram-positive. The aerial mycelium at maturity forms chains of three to

many spores. Glucose is utilized for growth by all species tested. Temperature optimum 25-35°C; some species show no growth at 37°C. Optimal pH range for growth lies between 6.5 - 8.0.

The results of cultural characteristic (Table 1) as well as some physicochemically tests and fermentation of carbohydrate tests (Table 4) indicated that these endophytic microorganisms are in the genus *Streptomyces*. Particularly, the one that is screened from the leaves of Shazaung-tinga-neah is identified to be *Streptomyces galtieri*.

Table 1. Cultural characteristics of strains AP-101, AP -102 & AP-103

Medium	Growth
Sucrose nitrate agar	Moderate
Glucose-asperagine agar	Poor
International Streptomyces Project	Abundant
Tyrosine agar	Moderate
Nutrient agar	Moderate
Oatmeal agar	Moderate
Starch agar	Moderate
Glycerol-nitrate	Moderate

Table 2. Antibacterial Properties of the Secondary Metabolites Screened from Three Medicinal Plants

No.	Bacterial strains	Sources	Zone of Inhibition (mm)		
			AP-101	AP-102	AP-103
1.	*Escherichia coli*	ATCC 25922	18	12	12
2.	*Escherichia coli*	LT./ 536-2	22	13	11
3.	*Escherichia coli*	EAEC N 10/83	15	12-	
4.	*Escherichia coli*	EPEC 0125 / WT 493	-	12	13
5.	*Escherichia coli*	WT -87	15	13	10
6.	*Plesiomonas shigelloides*	Id 23/ WT 8	16	15	16
7.	*Proteus morganni*	ID-1	16	10	-
8.	*Pseudomonas pyocyanea*	Biken, Japan	19	19	-
9.	*Salmonella typhi*	Id 74 /DTW	-	12	12
10.	*Salmonella typhi*	SEP 69	-	-	10
11.	*Shigella boydii*	Id 22/N 1367	-	20	17
12.	*Shigella dysenteriae*	Id 58/ 2802	17	15	17
13.	*Shigella dysenteriae*	SD 4/ ID 25	-	10	10
14.	*Shigella flexneri*	Id 4.6 N 186/6	26	24	27
15.	*Shigella flexneri*	Id 57/1132	16	10	-
16.	*Shigella sonnei*	Id 56/ N 4812	12	16	14
17.	*Vibrio cholerae*	A-532	27	25	27

AP-101 = Secondary metabolites produced by *Streptomyces* screened from *Cymbopogon citratus*

AP-102 = Secondary metabolites produced by *Streptomyces* screened from *Euphorbia splendens*

AP-103 = Secondary metabolites produced by *Streptomyces* screened from *Sauropus grandifolius*

Table 3. Antimicrobial Activity of AP-102

Microorganisms	MIC mg / ml	Microorganisms	MIC mg / ml
Escherichia coli (5 strains)	1.64	*Shigella boydii*	0.41
Plesiomonas shigelloides	0.82	*Shigella dysenteriae*	0.82
Proteus morganni	<100	*Shigella flexneri*	0.21
Pseudomonas pyocyanea	0.41	*Shigella sonnei*	0.82
Salmonella typhi (2 strains)	<100	*Vibrio cholerae*	0.21

Table 4. Physiological Characteristics of isolated Strains AP-101, AP-102 & AP-103

Temperature range for growth (°C)	14 - 40
Optimum temperature (°C)	25 - 30
Lysine iron agar	Positive
Sulfide indole motility	Negative
Triple sugar iron agar	Positive
Urea	Positive

	AP-101	AP-102	AP-103
Utilization of sugars			
D-arabinose	-	-	-
L-arginine	-	-	-
D-cellobiose	-	-	-
Cellulose	-	-	-
D-fructose	+	+	+
D-galactose	+	-	+
D-glucose	+	+	+
Glycerol	+	-	-
Glycine	-	-	-
Lactose	-	-	-
Maltose	-	-	-
D-mannitol	+	-	-
D-mannose	-	-	-
Raffinose	-	-	-
Rhamnose	-	-	-
Soluble starch	+	+	+
D-sorbitol	-	-	-
Sucrose	+	+	+
D-xylose	-	-	-

+ : Positive reaction, - : Negative reaction

Antibacterial Properties of the Secondary Metabolites Screened from Three Medicinal Plants

The metabolites secreted from the *Streptomyces* (AP-101) of Saba-lin inhibited 13 out of 18 test bacteria and the zone size ranges from 12 to 27 mm. It was not active only on one strain of E. coli, two strains of *Salmonella typhi*, one strain of *Shigella boydii* and one strain of *Shigella*

dysenteriae. The metabolite in the supernatant of the fermented broth by *Steptomyces* (AP-102) of Shazaung-tinga-neah showed wide inhibition on 17 out of 18 test bacteria. The inhibitory zones ranges from 10 to 25 mm. It showed no activity on only one strain of *Salmonella typhi*. Similarly, the secondary metabolite from AP-103 of Ma-shaw provided the antibacterial activity on 13 out of 18 test organisms with the 10 to 27 mm ranges of inhibitory zone. It was not active on one strain each of *E.coli, Proteus morganii, Pseudomonas pyocyanea* and two starins of *Shigella flexneri* (Figure 1 and Table 2).

All the test organisms in the present study were directly isolated from the patients suffering from diarrhoea, dysentery and wound sepsis. All the secondary metabolites produced by isolated Streptomyces variably showed the antibiotic activity on different strain on which the potency could be judged by the sizes of clear zones.

Antimicrobial Activity of AP-102

In the present work, the antibacterial activity of AP-102 was examined by serial dilution method using N.S. media and which was incubated at 37°C for 24 - 48 hours. Antimicrobial activity is expressed as the minimum concentration which inhibits growth of the microorganisms (MIC). As shown in Table 3, the prominant MIC of secondary metabolite from AP-102 on pathogenic bacteria *Shigella flexneri* and *Vibrio cholerae* was estimated to be 0.21 mg/ml.

Chater (1981) reported that *Streptomyces* could produce a large number of secondary metabolites, which had important application in medicine and agriculture. Moreover, many of these secondary metabolites had been used as antibacterial, antitumor and antifungal agents and also in agricultural aspect as growth promotors, agents for plant protection, antiparasitic agents and herbicides (Champness and Chater, 1994)

According to the results of present study, it was concluded that all the isolated endophytic microorganisms were morphologically classified to be the genus *Streptomyces*. The one screened from the Shazaung-tinga-neah, was identified as ***Streptomyces galtieri*** by physicochemical properties. Despite of the lack of structural evaluation, the secondary metabolites produced by isolated strains via fermentations had shown significant antibacterial activity on most of pathogenic test organisms. These are highly probable to be new antibiotics of wide spectrum. Further investigations concerning the extraction, purification and structural evaluation of these secondary metabolite will be performed in near future.

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