ISOLATION OF ENDOPHYTIC BACTERIA FROM *GARCINIA MANGOSTANA* L. AND STUDY ON ITS ANTIBACTERIAL ACTIVITY

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Abstract

Endophytes are ubiquitous organisms that live within the host plants without causing any apparent symptoms of disease. In the present study the endophytic microorganisms were isolated from plants *Garcinia mangostana* L. and 12 endophytic bacteria were successfully screened from different parts of leaves, bark and fruit. Characterization of endophytic bacteria was performed based on the morphology, biochemical characteristic and the antimicrobial properties. Screening of potential antibacterial metabolites was done by using paper disc diffusion method and bioautography. The antibacterial activity was tested against eight test organisms. Cell morphology and colony characters of isolated bacteria were investigated and three bacteria which show antimicrobial activity (B-2, B-7 and B-9) were selected and applied in the fermentation was media using nutrient broth for two days. After separate fermentation, the crude compounds were extracted by using ethyl acetate and butanol and partially purified by TLC. The results of TLC showed the presence of Quercetine, Tannin and Xanthone in each extract of B-2, B-7 and B-9.

Keywords- Endophytic bacteria, antimicrobial activity, biochemical characterization of endophytes.

Introduction

Garcinia belongs to the family Clusiaceae, which is native to Asia, Australia, Southern Africa, and Polynesia, and that produces edible fruits. This plant is known as "queen of the tropical fruit" in Malaysia and its scientific name is *Garcinia mangostana* L. (Mangosteen), which is famous for its sweet, creamy, and fragrant edible flesh (Phongpaichit *et al*., 2006). Different parts of *Garciniamangostana* L., such as the hull, bark, and leaf, have been used as traditional medicine to treat various diseases for hundreds of years. Applications of the mangosteen include leaves infusion to the circumcision wound to prevent infection and root decoction for the regulation of female menstruation (Moongkarndi *et al*., 2004; Nakatani *et al*., 2002). Studies based on *Garcinia* species has been greatly reviewed its biological potential activity, but the analysis based on the endophytic fungi that coexist within the fruit tree is very limited (Phongpaichit *et al*., 2007; Phongpaichit *et al*., 2006).

Endophyte simply means the location of an organism, with "endo" means "inside" and "phyte" means "plants". Therefore, endophyte refers to organisms that live within plants (Wilson, 1995). Bacteria are the most common organisms associated with the term endophyte. Endophytic bacteria are defined as bacteria that colonize healthy plant tissue without causing obvious symptoms or producing obvious injury to the host. Endophytic bacteria colonize a large number of plants, which include plant growth-promoting bacteria. Endophytic bacteria form associations with plants, at least in one phase in their life cycle. Endophytic bacteria normally live on intercellular spaces that contain carbohydrates, amino acids, and high amounts of inorganic nutrient.

The presence of fungal endophytes can cause higher rates of water loss in leaves. However, certain microbial endophytes may also help plants to tolerate biotic stress. The wide range of compounds produced by endophytes have been shown to combat pathogens and even cancers in animals including humans. Endophytes are also being investigated for roles in

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biofuels production. Some groups of endophytic microorganisms have been believed to be mutualists that protect plants against biotic stresses. Co-evolution may exist between endophytes and their host in resist to environmental stresses. (Tadych & White, 2001). Endophytic bacteria produce a wide range of phytohormones.The bioactive natural products from endophytes are promising resources for medicine kinds of Alkaloids are contributed to plant by endophytes. Some of these alkaloids raise plants‟ resistance to environmental stress, and some are growth-promoting compounds., agriculture and industry (Guo *et al*., 2008).

Many of those metabolites act as potential therapeutic agents against cancer and infectious diseases (Al *et al*., 2010). Endophytes are well known for the production of various classes of natural products and have been reported to exhibit a broad range of biological activity and are grouped into various categories, which include alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones and lignans. (Tan and Zou 2001 : Strobel and Daisy. 2003).Endophytes can be a promising source of bioactive compounds, and should be continuously isolated, characterized, and investigated for the discovery of lead bioactive compounds which can be employed in agriculture, medicine, and industries (Tiwari, *et, al,* 2013).

In this investigation, altogether twelve bacteria were isolated into pure culture by using nutrient agar medium. Twelve isolated strains are subjected into the physiochemical examinations concerning enzyme and other bioactive substances. This study was initiated to characterize the endophytes, in different parts of *Garcinia mangostana* L. This study was aimed to evaluate several isolated bacteria from *Garcinia mangostana* L. and to select best endophytic bacteria for further extraction partial identification of its metabolites.

Materials and Methods

Collection of Plant Materials

In the present research, the healthy leaf, bark and fruit of *Garcinia mangostana* L. Samples were collected from Mawlamyin Township.Samples were collected into clear plastic bags and brought to Botanical Lab of Dagon University .The identification of plant samples were done with the help of available literature as well as revised Handbook written by Wadhwa and Weerasooriya (1996) .The screening and isolation of endophytes have been performed in the Lab of Microbiology, Dagon University since July, 2006.

Pre-treatment and Surface Sterilization of Plant Parts

The leaf, bark and fruit of each plant *Garcinia mangostana* L. were washed separately under tap water to remove adhering soil particles and the majority of microbial surface epiphytes are a part of pre-treatment.

Surface sterilization

Freshly collected leaf, bark and fruit of *Garcinia mangostana* L. were washed under slow running tap water for 15 minutes followed by washing in Tween 20 (1 drop in 200 ml sterile distilled water) for 1 minute .Then they were rinsed three times with sterile distilled water in the chamber. Commonly used sterilizing agents are ethanol: 70-95% for 30 seconds. After drying, each sample segment was cut into approximately 0.5 cm and placed on Petri plates containing nutrient agar medium (NA). Petri plates were incubated at room temperature. They were monitored every day for growth of endophytic bacteria colonies. Bacterial growing out from the samples was subsequently transferred onto fresh NA media plates to isolate pure colonies. All selected isolates were subculture in NA slants and finally, all the purified endophytes were maintained at 4°C till further used.

Media for Isolating Endophytic Bacteria

The choice of the growth medium is crucial as it directly affects the number and type of endophytic bacteria that can be isolated from the leaves, bark and fruit. Nutrient Agar medium (NA) was used for the isolation of endophytic bacteria. Since there is no component in NA which can suppress the growth of endophytic fungi, so the media used for the isolation of endophytic bacteria were supplemented with antifungal agent, nystatin at a concentration of 100 μg/ml of each to suppress fungi growth.

Nutrient Agar Medium (Atlas, 1993)

Peptone	-	5.0 g
Beef extract	-	1.0 g
Yeast extract	-	2.0 g
Sodium chloride	-	5.0 g
Agar	-	20.0 g
Distilled water (DW)	-	1000 ml
pH	-	7.0

Nystatin was added to the medium after autoclaving

Biochemical Characteristic of Isolated Endophytic Bacteria

The biochemical tests of isolates were conducted according to Bergey's Manual of Determinative Bacteriology. For each strains , test include Gram Staining, Oxygen Requirement (Aerobic/Anaerobic), Hydrogen Sulfide Production Test, Salt Tolerant, Nitrate Reduction, Citrate Utilization Test, Methyl Red Test, Voges Prokaur Reaction, Urea Hydrolysis, Starch Hydrolysis and Utilization of Carbohydrate .(Prescott, 2002).

Test Organisms

All endophytic bacteria isolates were screened for antimicrobial activities. The test bacteria included *Agrobacterium tumefaciens, Bacillus subtilis, Candida albicans*, *Escherichia coli, Micrococcus* sp., *Pseudomonas aeruginosa, Saccharomyces cerevisiae* and *Staphylococcus aureus*.

Antimicrobial activity Estimation

The study of antimicrobial activity was performed by paper-disc diffusion method. Nutrient agar was prepared according to the method described by Cruickshank. 1975.

Extraction and Isolation of Crude Ethyl Acetate Extracts and Butanol Extract From Bacterial Fermentation Broths

Organic solvents used extraction and isolation of compounds in two days fermentation broths using three isolated bacteria. At the twelve isolated bacteria,B-2,B-7 and B-9 were selected for further investigation of antimicrobial activity based on the results of paper disc diffusion methods .They were subjected in the fermentation using nutrient broth as basal fermentation media , for two days at room temperature. After fermentations butanol and ethyl acetate were used in the extraction and isolation of crude compound from individual fermented broths.

Isolation Procedure of Crude Extract from Selected Bacteria

Isolation of useful metabolites in the crude extracts was conducted at the Laboratory of Chemical Engineering Department, Yangon Technological University. The screening of crude extracts using two solvents such as Butanol and ethyl acetate obtained after extraction were conducted according to the method reported by Harbone, (1973) and extraction and purification of crude extracts were performed by applying TLC plates coated with silica gel.

Results

Morphological Characteristics of Collected Plant Sample

Scientific Name - *Garcinia mangostana* L.

Myanmar Name - Mingut

Family - Guttiferae

In evergreen tree; younger stems cylindrical, glabrous, latex yellow. Leaves opposite, distichous, simple, exstipulate; petiolate; laminae elliptic-oblong, the margins entire. Inflorescences terminal cymes, bisexual flowers solitary. Flowers ebracteate, ebracteolate, peticellate, actinomorphic, tetramerous, hypogynous. Calyx aposepalous, the sepals 4, concave, decussate, yellowish persistent. Corolla apopetalous, the petals 4, imbricate, rosy pink, glabrous. Androecium polyandrous, stamens numerous, the filaments short, basifixed, dehiscence longitudinal. Pistil 1, ovary globose, 4 carpelled, syncarpous, the ovule solitary in each locule, the axile placentation, the stigma sessile, yellow.

Figure 1 Habit of the Plants, Leaves, Fruit and Bark

Table 1 Isolation of Endophytic Bacteria from *Garcinia mangostana* L

Used Basal Medium	Designated of bacteria		
	From leaves	From bark	From fruit
Nutrient Agar Medium (NA)	B-2, 4, 5, 10	B-1, 3, 6,7, 8	B-9,11,12

In the present works, twelve isolated bacteria designated as B-1 to B-12 were maintained into the pure culture for further studies.

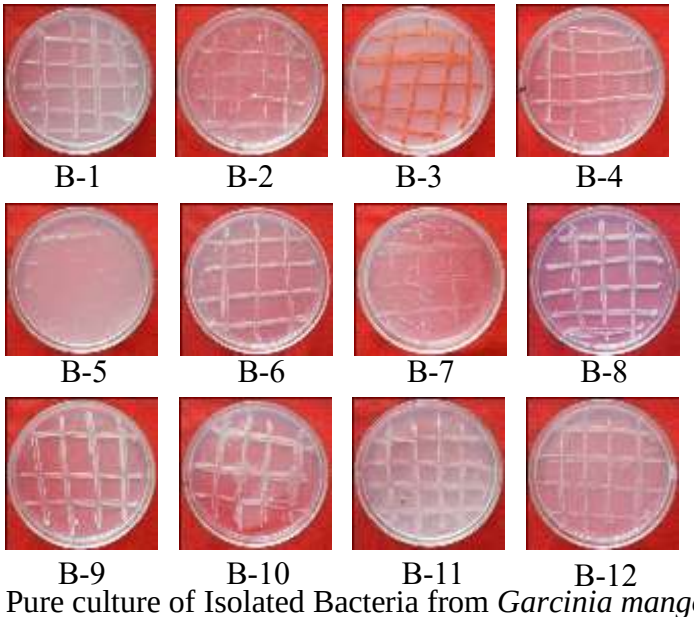
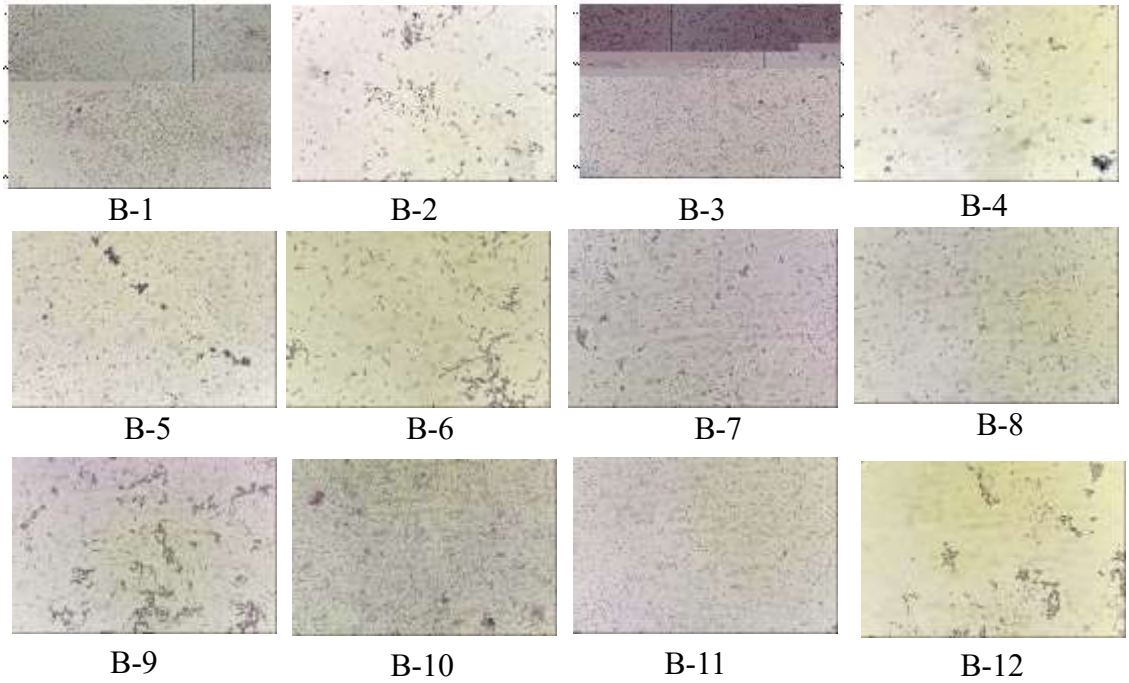


Figure 2 Pure culture of Isolated Bacteria from *Garcinia mangostana* L.



All isolated strains are small rod to rod. The scale bar of all photomicrographs is 50 µm.

Figure 3 Morphology Characters of Isolated Bacteria From *Garcinia mangostana* L.

Identification of isolates to possible Genus Level According to Bergey's Manual of Determinative Bacteriology Eighth Edition (1974)

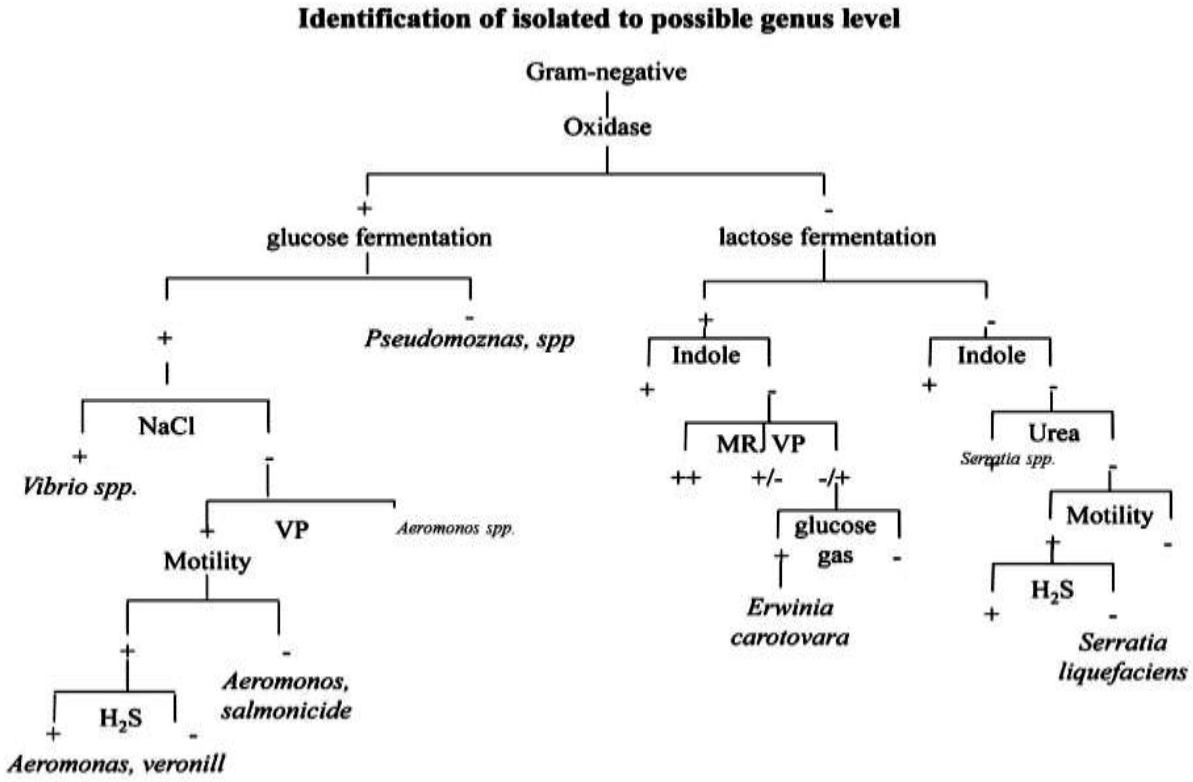


Figure 4 Flow Diagram of Identification of Isolated Bacteria to Possible Genus Level

Table 2 Results of Biochemical Tests of Isolated Bacteria from *Garcinia mangostana* L.

Test		B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
Cell Morphology		rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod
Glucose	fer	-	++	++	++	++	++	+	+	++	++	+	++
	gas	-	-	+	-	+	-	-	+	-	-	-	+
Lactose	fer	-	-	-	-	-	-	-	+	-	-	+	-
	gas	-	-	-	+	+	++	-	++	-	++	++	+
Ribose	fer	-	-	++	-	++	-	-	++	++	++	-	++
	gas	-	-	+	-	+	-	-	+	+	+	-	-
Hydrogen sulphide		-	-	-	-	-	+	-	-	-	-	-	-
Aerobic / Anaerobic		A	A	A	A	A	A	A	A	A	An	A	A
Moile / Non motile		nm	m	m	m	m	m	nm	m	m	m	m	m
Soluble Starch Hydrolysis		+	+	-	-	-	+	-	+	+	-	+	-
Tapioca Hydrolysis		+	+	+	-	+	+	-	+	+	-	+	-
Sticky Rice Hydrolysis		+	+	-	-	-	+	-	+	+	-	-	-
Wheat hydrolysis		-	+	-	+	-	+	+	+	+	-	+	-
Urease Test		-	+	-	-	+	-	-	-	+	-	+	-
Citrate utilization		++	++	++	+	++	+	++	++	+	++	++	++
Methyl red (MR)		-	-	-	-	-	-	-	-	-	-	-	+
Voges-Proskaver (VP)		-	-	+	+	+	+	+	+	+	+	+	+
Nitrate Test		+	-	+	-	-	+	+	-	-	+	-	-
Gram Stain		-	-	-	-	-	-	-	-	-	-	-	-
Catalase		+	+	+	+	+	+	+	+	+	+	+	+
NaCl 6%		+	+	-	+	+	+	-	+	+	+	+	-
NaCl 1%		+	+	+	+	+	+	+	+	+	+	+	+
Oxidase Test		++	+	++	++	++	++	+	++	++	-	-	+
Indole Test		-	-	-	-	-	-	-	-	-	-	-	-
Growth on Potato		+	+	+	+	+	+	-	+	+	-	+	+

A= aerobic A n = anaerobic m= motile nm= non-motile + = positive reaction - = negative reaction

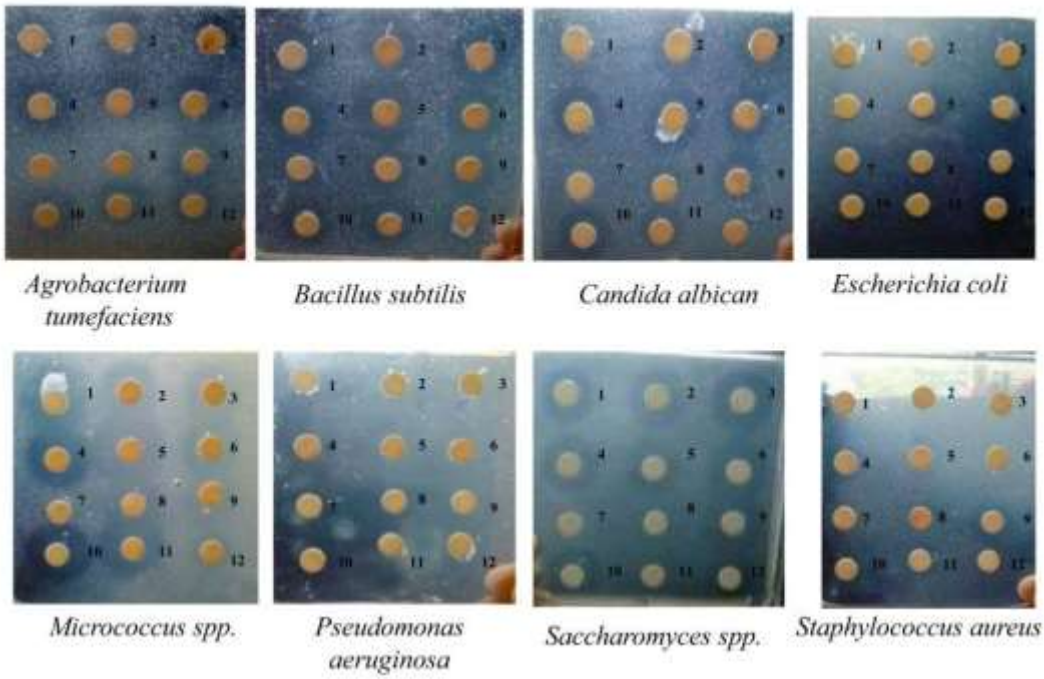


Figure 5 Antimicrobial Activity of Isolated Endophytes B-1 to 12 Against Eight Test Organisms

Screening of endophytic bacteria from different parts of *Garcinia mangostana* was done by using nutrient broth as basal isolation medium. To isolate the bacteria, the Nystatin 100 µg/ml was put into the nutrient medium for the bacteria only. Twelve isolated bacteria were obtained in the present screening. The isolated bacteria from different parts of Mangosteen were designated as B1 to 12. The colonies of isolated strains were shown in Fig. 2. The results of morphological cultural and biochemical test were shown in Table 2 and Fig. 3. One of the most important biochemical character the procedure of starch hydrolyzing activity was also experimented by using soluble starch, tapioca, wheat, sticky rice. The possible genus of isolated bacteria was estimated according to the Bergeys Manual of Determination Biology (1974). Based on the results of morphological, cultural and biochemical tests, all isolated bacteria may be possible geneus level *Pseudomonas spp,(*B- 1*)Vibrio spp,(*B- 2,4,5,6,8,9*), Aeromonas spp.* (B-3,7) , *Erwinia spp.(*B-11) and *Serratia* (10) . The antimicrobial activity of all isolates against eight test organisms was indicated by size of clear zone was shown in Table 3.

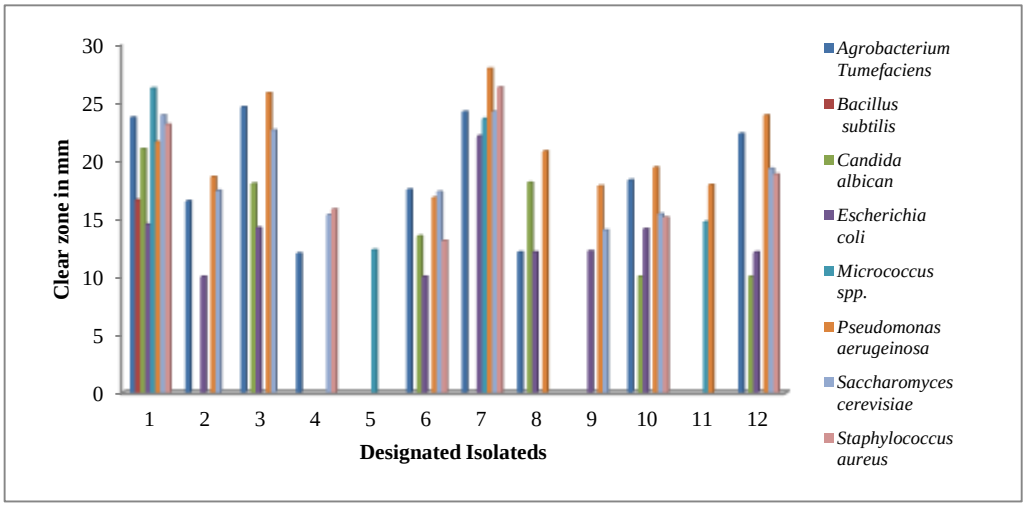


Figure 6 Antimicrobial activity of all Isolated Endophytic Bacteria Strains (B 1 to 12)

Table 3 Antimicrobial activity of All Isolated Endophytic Bacteria Strains (B-1 to 12)

Paper Disc Size = 10 mm

Strain No.	*Agro-bacterium Tumefaciens*	*Bacillus subtilis*	*Candida albican*	*Escherichia coli*	*Micrococcus spp.*	*Pseudomonas aeruginosa*	*Saccharomyces cerevisiae*	*Staphylo-coccus aureus*
B-1	12.3 mm	18.3 mm	-	18 mm	23.4 mm	18 mm	20.1 mm	-
B-2	-	19.2 mm	19.8 mm	17 mm	22.5 mm	18.2 mm	22.2 mm	-
B-3	-	19.3 mm	18.2 mm	17 mm	22.5 mm	18.1 mm	22.2 mm	-
B-4	13.5 mm	21.1 mm	18.3 mm	20 mm	22.6 mm	20 mm	21 mm	18 mm
B-5	-	22.3 mm	19.2 mm	20 mm	24.5 mm	23 mm	20 mm	19 mm
B-6	-	20.1 mm	18.3 mm	20 mm	22 mm	20 mm	20 mm	-
B-7	-	-	-	22 mm	-	20 mm	-	-
B-8	-	18.3 mm	-	21 mm	18 mm	20 mm	-	-
B-9	12 mm	18.3 mm	18.3 mm	20 mm	20 mm	20 mm	22.2 mm	20 mm
B-10	13 mm	18 mm	19 mm	12 mm	22.7 mm	20.1 mm	20 mm	-
B-11	12 mm	19.8 mm	18.1 mm	20 mm	22.7 mm	20.2 mm	-	-
B-12	12 mm	-	-	19 mm	13 mm	20 mm	19.8 mm	-

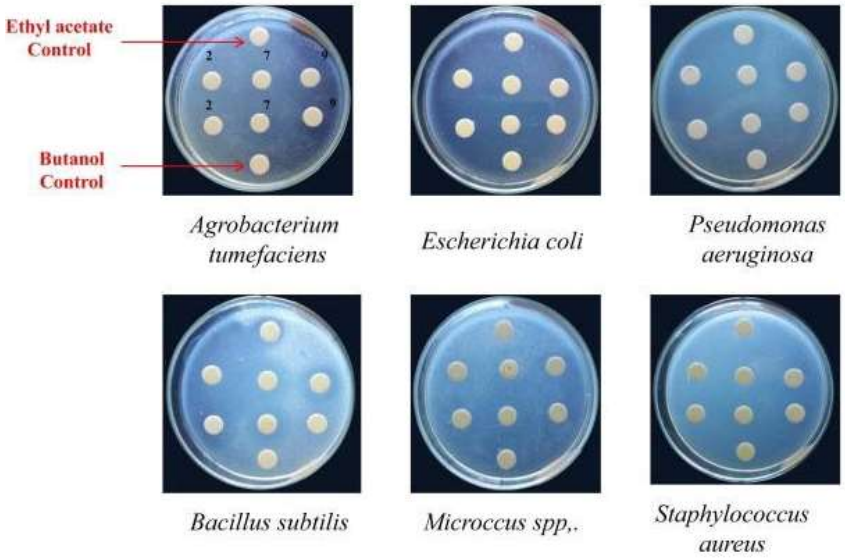


Figure 7 Antimicrobial Activity of Ethyl acetate and Butanol Extract of B -2,7 and 9

Table 4 Antibacterial activity of three crude extract from Isolated Bacteria Strains

Extracted solvents / Test organisms	Ethyl acetate			Butanol		
	B2	**B7**	**B9**	**B2**	**B7**	**B9**
Agrobacterium tumefaciens	25 mm	22 mm	23 mm	20 mm	20 mm	19.2 mm
Bacillus subtilis	32.3 mm	23 mm	20 mm	30 mm	23 mm	-
Escherichia coli	-	-	-	-	-	-
Micrococcus spp.	33 mm	25 mm	25 mm	35 mm	25 mm	23 mm
Pseudomonas aerugeinosa	33 mm	20 mm	20 mm	-	18 mm	18 mm
Staphylococcus aureus	18 mm	18 mm	18 mm	18 mm	20 mm	20 mm

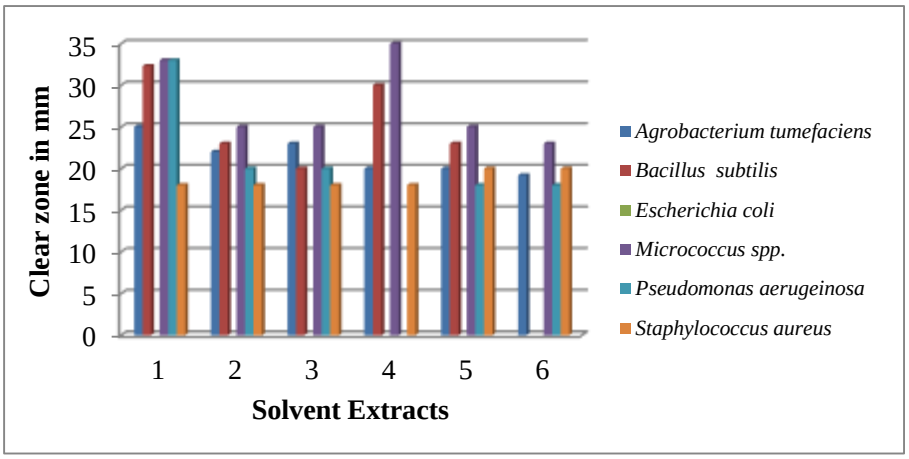


Figure 8 Antibacterial activity of three crude extract from Isolated Bacteria Strains

Antibacterial Activity of Three Crude Extract Bacteria B-2, 7and 9 Against Six Test Organisms

The second part of the present work mainly depends on extraction of metabolites by using ethyl acetate and butanol. According to the results of antimicrobial activity the isolated bacteria strains B 2, 7 and 9 were selectively used in extraction of metabolites. The isolated bacteria were grown in the 200 ml of nutrient broths. In the case of isolated bacteria the fermentation periods were checked up to three days and the best antimicrobial activity was detected in the 2 days. After each fermentation period, crude extract from fermented broth by butanol was also applied in the clear zone tests. In the metabolite extraction by bacteria, the isolate B-2 which found to give best antibacterial activity on *Micrococcus spp.* It was recorded that the ethyl acetate provide 33mm clear zone against *Micrococcus spp.* and *Pseudomonas aureginosa*, 32.3 mm against *Bacillus subtilis*. Similarly ethyl acetate extract of B-7 also showed 25 mm clear zone on *Micrococcu spp.*. The butanol extracts of B-9 also provided equally high antimicrobial activity, such as 19.2 mm on *Agrobacterium tumefaciens*, 23 mm on *Micrococcus spp.* and 1 8 mm on *Pseudomonas aeruginosa*. The results were shown in Table 4

Thin layer Chromatography of Crude Extract from Bacteria

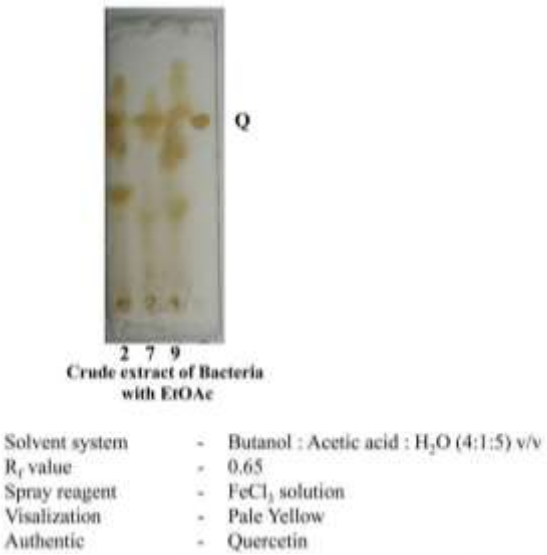
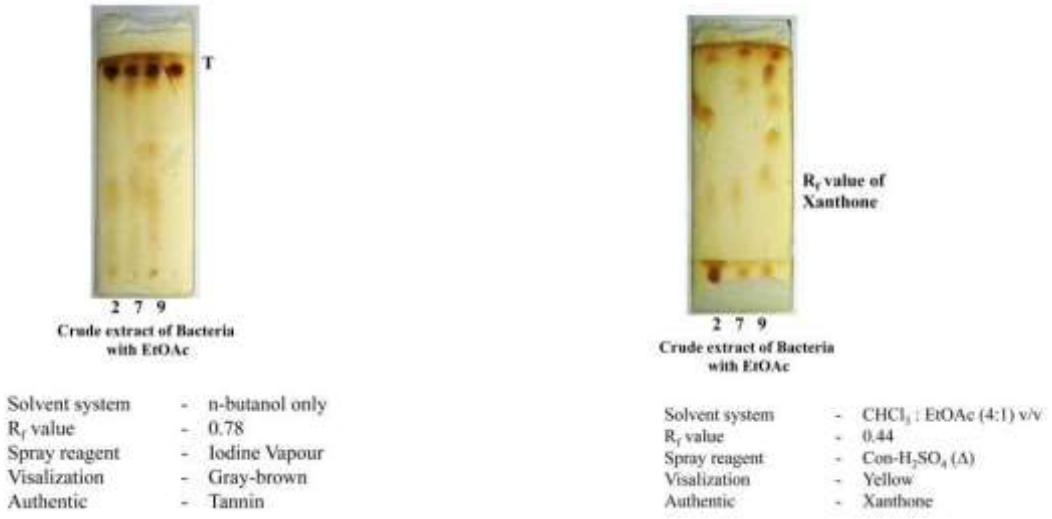


Figure 9 Thin layer Chromatography of Crude Extract by Ethyl acetate from Isolated Bacteria B-2, B-7 and B-9

Thin layer Chromatography of Crude Extract From Bacteria



Crude extract with Butanol

Figure 10 Thin layer Chromatography of Crude Extract by Ethyl acetate from Isolated Bacteria, B-2, B-7 and B-9

The third part of the present work concerned with identification of antimicrobial metabolites extracted with EtOAc using TLC. In the TLC techniques silica gel plates (3x7 cm) and butanol: acetic acid: H_2O (4:1:5) solvent system was used in the ascending methods as shown in Fig.9.After the treatment with butanol: acetic acid: water (4:1:5), the TLC plates were sprayed with $FeCl_3$ solution and pale yellow spots were recorded. The TLC of metabolic extract with butanol and developed with the solvent system of butanol: acetic acid: water ,it was observed that pale yellow spots with R_f values 0.65 was detected as shown in Fig.9. The R_f values were compared and suggest being Quercetin. When the fermented broth of bacteria extracted with EtoAc and developed the TLC with n-butanol, it was observed that tannin was contained after placing in the iodine vapor. The grey-brown spots with 0.78 R_f value were visualized Fig.10 compared with authentic tannin spot. Finally, the above extracts were subjected in the TLC with different solvent system of Chloroform and ethyl acetate (4:1 v/v). In TLC of bacteria extract using chloroform: EtOAc the spot of R_f0.44 were only detected in B-2,B-7 and B-9 it was observed pale yellow spots with R_f values 0.44 was observed as shown in Fig.10. According to the literature with same solvent system, it may be the spot of Xanthone.

Discussion and Conclusion

In the present work, the presence of entophytic microorganisms in the surfaces of leaves, bark and fruits of a famous nutraceutical fruit plant was verified by Lee (1997). Morphological, cultural, biochemical characteristics of 12 isolated bacteria were studied aiming to provide some valuate information concerning the further study of *Garcinia mangostana l.* After investigation the antimicrobial activity of 12 isolated bacteria B-2, B-7 and B-9 were selected for further research work, based on their clear zone conditions. The selected bacteria were subjected in preliminary fermentation using nutrient broth as basal medium. After fermentation at room temperature for two days the fermented broths were extracted with butanol and ethyl acetate. TLC analysis after extraction the results tentatively indicated the presences of quarcetin and tannin in all the fermented broths of bioactive crude extracted. But xanthone was estimated on the broth of B-2, B-7. Strobel *et.al* 2004 suggest that bioactive natural compounds assemble by endophytes have been promising potential usefulness in safety and human health concerns. Tan and Zou (2001) were proved that

endophytes presuming a broad variety of bioactive secondary metabolites with unique structure, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and others .Such bioactive metabolites find wide-ranging user as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, and anticancer agents by Gunatilaka (2006).

Acknowledgements

We are grateful to Dr. Nu Nu Yee and Dr Nay Thwe Kyi Pro- Rector, Dagon University for the use of research facilities related to the research work. We would like to express our deepest gratitude to Dr Myat Myat Moe Professor and Head, Department of Botany, Dagon University, for her invaluable guidance, continuous advice and for providing information and reference books. We want to express our gratitude to Rector (Retired) Dr. U Win, for his advice and encouragement. Our thanks go Dr. Sander Hlaing, Professors, Department of Botany, Dagon University, for her kind advice and suggestions. We also give our thanks to all of our teachers from whom we have learnt since our childhood and to all our colleagues for their valuable assistance.

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