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Isolation of *Streptomyces* with Broad Spectrum Antifungal Activity from Polyherbal Products

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Abstract: This study was conducted to determine if commercial herbal products harbour *Streptomyces* possessing broad spectrum antimicrobial activity. Samples of six polyherbal products were cultured for actinomycete and 4 products were found to contain actinomycetes with count between 1.06 $\log_{10}$ CFU and 2.33 $\log_{10}$ cfu g^{-1} sample. Seventeen morphologically *Streptomyces* species were isolated and 8 isolates (47%) were identified to the species level by 16S rRNA gene sequences analyses. Only 2 isolates were inhibitory to all test bacteria. Thirteen isolates (77%) were inhibitory to all four test *Candida* species and seven of these isolates were also inhibitory to *Geotrichum candidum, Trichosporon cutaneum* and *Aspergillus niger*. One isolate, identified as *S. rochei* was inhibitory to all 26 test microorganisms including aerobic bacteria, facultative anaerobic bacteria, anaerobic bacteria, microaerophilic bacteria and pathogenic moulds. On the condition of this study, it can be concluded that *Streptomyces* species associated with herbal plant can be isolated from plant materials sold as herbal products. Some *Streptomyces* species isolated from these herbal products have broad spectrum antimicrobial activity especially against clinically important fungi. A lot of herbal plants have been processed for herbal products but these products have not been intended for isolation of *Streptomyces* species. In order to search for new antimicrobials, herbal products in the market may serve as readily culturable materials for rapid isolation of *Streptomyces* species from herbal plants that have not been screened for *Streptomyces* species.

Key words: Antibacterial, antifungal, antimicrobial, herbs, actinomycetes

INTRODUCTION

A large number of pathogenic bacteria and fungi have become resistant to antibiotics in common use. These antibacterial and antifungal resistance are presently an urgent focus of research and new antibiotics are necessary to fight against these pathogens. The discovery of new antibiotics has declined since the 1990s due to a decrease in screening efforts (Jesse *et al*., 2009).

Actinomycetes, filamentous soil bacteria are widely recognised as important microorganisms because of their ability to produce many kinds of secondary metabolites such as antibiotics. Actinomycetes continue to be routinely screened for new bioactive substances (Kekuda *et al*., 2010). These screening have been remarkably successful and approximately two-thirds of naturally occurring antibiotics have been isolated from actinomycetes (Okami and Hotta, 1988) and majority of the antibiotics (55%) were obtained from the genus *Streptomyces* (Marinelli, 2009; Kekuda *et al*., 2010).

Streptomyces are ubiquitous in soil, water and their spores have been found in the air (Skorska *et al*., 2005). They are known to constitute a large part of the rhizosphere microbiota. *Streptomyces* are also found associated with plant tissues (endophytes) such as in roots (Sardi *et al*., 1992), seeds (Mundt and Hinckle, 1976) and leaves (Matsukuma *et al*., 1994; Matsumoto *et al*., 1998). Metabolites from some *Streptomyces* endophytes were found to be inhibitory to bacteria and it has been suggested that endophytes protect the host from fungi and pathogenic bacteria (Cao *et al*., 2003; Jesse *et al*., 2009). It is well documented that some *Streptomyces* form associations with medicinal plants (Strobel and Daisy, 2003; Thamchaipenet *et al*., 2010; Li *et al*., 2009). Thus, medicinal plants are potential sources of *Streptomyces* (Cragg *et al*., 1999; Qin *et al*., 2009; Hazalin *et al*., 2009; Zhao *et al*., 2010).

Researches on antimicrobial activity of Streptomyces associated with medicinal plants have been focused on isolation of these organisms from individual plants (Taechowisan *et al*., 2003). Fresh plant samples such as the roots and leaves were dried, cut to smaller sizes and then cultured onto actinomycete isolation media. These forms of samples are available readily as medicinal products sold at herbal medicine shops.

It has been a practice in herbal medicines to mix several plants in one product to form a polyherbal medicine (Mohd-Fuat *et al*., 2006). Although, roots are the

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main part of the herbs used, some formulations included leaves, seeds or a whole plant. These polyherbal products can serve as readily culturable materials for *Streptomyces*. There is little or no report of isolation of actinomycetes from these products or some plants used in the formulation of the products. The present work was carried out to isolate *Streptomyces* from some polyherbal products and to determine the spectrum of antibacterial and antifungal activities of the isolates.

MATERIALS AND METHODS

Herbal products: Six polyherbal products from different manufacturers were purchased in January 2008 from retail shops in Kuala Lumpur, Malaysia. The products, namely JAA, JCM, JGG, JT, JPL and JS, were finely ground herbal powder (not less than 140 mesh) packed in paper envelopes clearly labelled with the herbs used in the formulation of the products. Table 1 shows the formulation of the herbal products, products JAA, JCM, JGG and JT were polyherbal mixtures containing 4 different herbs while JPL and JS contained 10 and 7 herbs, respectively. In all products except JGG, roots (radix or rhizoma) were the most common part of the herbs used. Some formulations included leaves, seeds, flowers and the bark collected from the root, stem or branches of the herbal plants. All products were at least 6 months before the expiration date and were kept in 3±2°C until culture for actinomycetes were done.

Table 1: Herbal formulations of some polyherbal products purchased from retail shops in Kuala Lumpur, Malaysia

Herbal products	Constituents	Plant parts used*	% in the formulation
JAA	*Woodfordiae* species	Flos	10
	Parameriae species	Cortex	15
	Kaempferiae species	Rhizoma	20
	Curcuma species	Rhizoma	20
JCM	*Pipiris* species	Fructus	10
	Zingiberis species	Rhizoma	4
	Cinnamoni species	Cortex	3
	Curcuma species	Rhizoma	4
JGG	*Comus officinalis*	Fructus	20
	Tumera diffusa	Folium	20
	Ptychopetalum olaccides	Radix	20
	Piper retrofracti	Fructus	30
JPL	*Piperis nigri*	Fructus	4.1
	Boesenbergiae species	Rhizoma	8.2
	Curcumae species	Rhizoma	22
	Curcumae domestica	Rhizoma	20.5
	Languatis species	Rhizoma	20.5
	Zingiberis purpurei	Rhizoma	6.3
	Zingiberis aromatica	Rhizoma	4.1
	Foeniculi species	Fructus	4.1
	Alyxiae species	Cortex	5.1
	Glycyrrhizae species	Radix	5.1
JS	*Zingiberis* species	Rhizoma	30
	Myristicae species	Semen	10
	Linguatis species	Rhizome	13
	Saussureae lappae	Radix	10
	Curcumae domestica	Rhizoma	20
	Curcumae aeruginosae	Rhizoma	*15*
	Piper retrofracti	Fructus	2
JT	*Zingiberis* species	Rhizoma	n.i*
	Piper retrofracti	Fructus	
	Zingiberis zerumbet	Rhizoma	
	Alyxiae species	Cortex	

*Plant parts used: n.i, not indicated; Radix, the root of the plant; Rhizoma, rhizome or a creeping horizontal stem generally bearing roots on its underside; Flos, the flowers of plant usually consisting of a single flower or the entire inflorescences (i.e., head, umbel, panicle, spike, etc.), Fructus, the fruit or berry; Semen, the seed usually removed from the fruit and may or may not contain the seed coat; Cortex, the bark collected from the root, stem, or branches; Folium, Leaf.

Enumeration, isolation and identification of actinomycetes

Isolation of actinomycetes: One gram of the herbal powder was sprinkled on Actinomycete Isolation Agar plate (AIA, Oxoid) supplemented with glycerol (5%, w/v), cycloheximide (0.50%, w/v) and naladixic acid (0.02%, w/v) (Imada *et al*., 2007). The plate was incubated at room temperature (28±2°C) for 5 weeks and the actinomycetes colonies were counted. Based on colonial characteristics, representative colonies were picked out and stored on AIA slants for further identification.

Identification of actinomycetes isolates: Characterization of isolates based on sporulating structures and spore arrangements were done using slide culture method as described in Bergeys's Manual of Systematic Bacteriology (Holt *et al*., 1994). For analysis of cellular diaminopimelic acid (DAP) and whole-cell sugars, actinomycetes isolates were grown in half-strength nutrient broth, shaken at 30°C for 1 week and harvested by centrifugation at 10,000 g for 10 min (White *et al*., 1986). The cells were washed twice with dionised water by centrifugation and then dried at 45°C overnight (Staneck and Roberts, 1974). The method of Schon and Groth (2006) was followed for DAP analysis and whole-cell sugars by descending paper chromatography. Extraction of the isolates DNA was carried out using DNA extraction kit (Bio Basic Inc., Canada) using the procedures recommended by the manufacturer. PCR was done with the universal 16S primers designed to amplify the region between positions 27 and 765 of the 16S rRNA gene in actinobacteria. The primers were designated 27f (5-AGAGTTTGATCMTGGCTCAG) and 765r (5'-CTGTTTGCTCCCCACGCTTTC). The PCR was carried out in 25 μL-reaction volumes with the following reagents: 1 μL of 27f (200 ng μ^{-1}), 1 μL of 765r (200 ng μL^{-1}), 2.5 μL of Taq buffer, 13 μL of water, 0.5 μL of Taq polymerase (2 U μL^{-1}), 1.5 μL $MgCl_2$, 0.5 μL dNTP and 5 μL of template DNA. The reactions were subjected to the following temperature cycling profile: 94°C for 8 min;

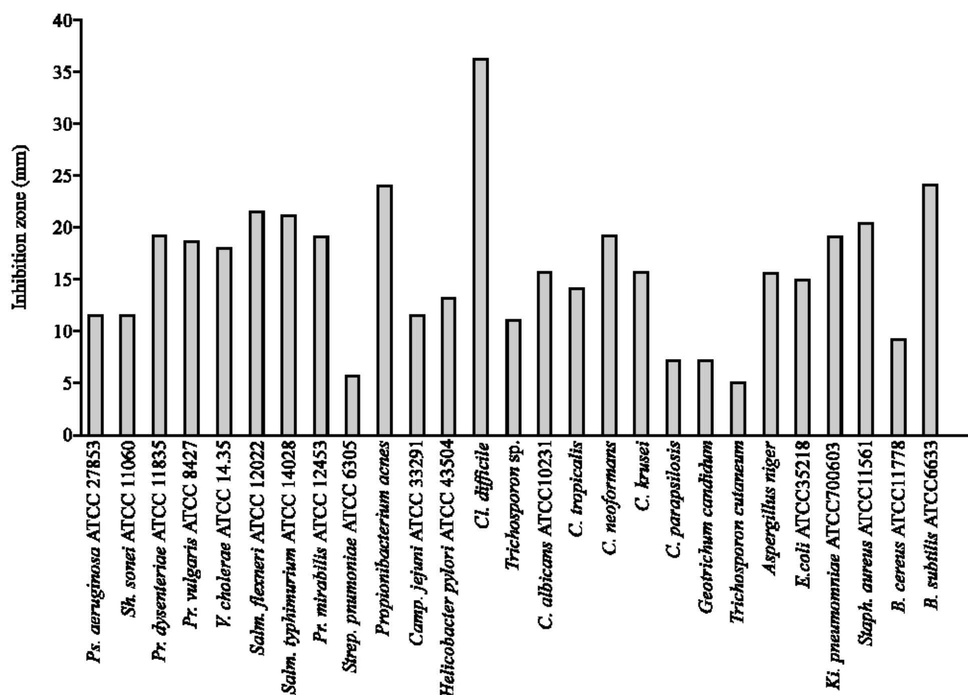

Fig. 1: Antibacterial and antifungal activities of isolate JPL1 (*S. rochei*) against 18 species of bacteria and 9 species of fungi

followed by 40 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min; followed by 45°C for 1 min and finally 72°C for 10 min. The PCR products were purified by using a QIAquick Spin PCR purification kit (Qiagen 28106, Germany) before automated sequencing with the 27f and 765r primers. The resultant 16S rDNA sequences were compared to bacterial gene sequences in GenBank by using the BLAST software at the National Center of Biotechnology Information (NCBI) web site (http://www.ncbi.nih.gov).

Antimicrobial activity

Test bacteria and fungi: Test bacteria were *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus aureus* (ATCC 11561), *Bacillus cereus* (ATCC 11778) and *Bacillus subtilis* (ATCC 6633). All test fungi except *Candida albicans* (ATCC 10231) were clinical isolates obtained from the Culture Collection Centre, Bacteriology Unit, Institute for Medical Research, Malaysia. Isolates that showed activity against all test bacteria and fungi were further tested on additional strains of bacteria and fungi (Fig. 1).

Antibacterial and antifungal testing: Actinomycetes were cultured on Mueller-Hinton (MH) agar or MH incorporated with sheep blood (5% v/v) by a single line streak of the spores down the centre of the MH plate extending to the plate edges. The culture was incubated at 30°C for 5 days. The test bacterial cultures were suspended in normal saline to match turbidity of 0.5 McFarland standards. Yeasts and moulds were grown on Sabouraud dextrose agar slant for 2 and 7 days, respectively. Yeast cells were suspended in normal saline to match the turbidity of 0.5 McFarland standards. Slants of *Aspergillus niger* and other moulds were covered with 1 mL saline and were gently probed with a sterile loop. Heavy particles were allowed to settle and the upper homogenous suspension was removed and the turbidity was adjusted to 1.0 McFarland standards. A loop of bacterial or fungal cell suspension was streaked perpendicular to the actinomycete line of growth adjacent to this line and extended to the plate's edge. Cultures of anaerobic bacteria, *Helicobacter pylori* and *Campylobacter jejuni* were incubated in anaerobic jar with appropriate gas generator (Becton Dickinson). All bacterial cultures were incubated at 37°C for 3 days while all fungal cultures were incubated at 30°C for 7 days. Antagonism was determined by the size (length) of zone of inhibition as measured from the actinomycete growth line.

RESULTS

Four of the six polyherbal products contained actinomycetes with counts between 1.06 $\log_{10}$ cfu and 2.33 $\log_{10}$ cfu g^{-1} sample (Table 2). Seventeen morphologically

Table 2: Total Actinomycetes counts and actinomycetes isolated from herbal products

Polyherbal products	Total count ($\log_{10}$cfu g^{-1})*	Actinomycete isolated	Species
JAA	NG	NI	-
JCM	1.20	JCM1	*Streptomyces* species
		JCM2	*S. flavidofuscens*
JGG	1.73	JGG1	*Streptomyces* species
		JGG2	*Streptomyces* species
JPL	1.06	JPL1	*S. rochei*
		JPL2	*Streptomyces* species
JS	NG	NI	-
JT	2.33	JT1	*S. sampsonii*
		JT2	*Streptomyces* species
		JT3	*Streptomyces* species
		JT4	*S. griseus*
		JT5	*S. violaceans*
		JT6	*Streptomyces* species
		JT7	*Streptomyces* species
		JT8	*S. champaratii*
		JT9	*S. vineaceous*
		JT10	*Streptomyces* species
		JT11	*S. diastaticus*

NG: No growth was obtained; *Values were calculated from the mean of two sample replicates

Table 3: Antifungal activity of actinomycetes isolated from some polyherbal products purchased from retail shops in Kuala Lumpur, Malaysia

Actinomycete isolates	Antifungal activity (inhibition zone)*							
	Ca	Ct	Ck	Cp	Cn	Gc	Tc	An
JCM1	++	+	++	+	++++	+	+	++
JCM2	+	-	++	-	++++	-	-	+
JGG1	-	-	++	-	+++	-	-	+
JGG2	++	+	+++	+	+++	-	-	+++
JPL1	++++	+++	+++	+++	++++	++	++	++++
JPL2	+	+	+	++	+	+	+	++
JT1	+++++	+++	+++	++	+++++	-	++	++
JT2	++++	++	+++++	++	+++++	+	+	++
JT3	+	+	+	+	++	-	+	++
JT4	++++	++	++++	++	++++	-	++	++
JT5	+++++	+++	+++++	+++	+++++	+	+++	++
JT6	++	-	++	-	++	-	-	++
JT7	+++++	+++	+++++	+++	+++++	+	+++	++
JT8	++++	++	+++++	++	+++++	+	++	++
JT9	+++++	+++	+++++	++	+++++	+	+	++
JT10	+	+	++	+	+++	+	-	+
JT11	++	+	+	+	+++	+	-	+++

*No inhibition zone; +: 1-5 mm; ++: 6-10 mm; +++: 11-15 mm; ++++: 16-20 mm; +++++: >20 mm. Ca: *C. albicans*; Ct: *C. tropicalis*; Ck: *C. krusei*; Cp: *C. parapsilosis*; Cn: *C. neoformans*; Gc: *G. candidum*; Tc: *T. cutaneum*; An: *A. niger*

different actinomycetes strains were isolated and 65% of these isolates were obtained from herbal product JT. All isolates were assigned to the genus *Streptomyces* based on the presence of substrate hyphae and relatively abundance aerial hyphae, short to long chain of conidia on the aerial hyphae, the presence of L-DAP and absence of any diagnostic sugar in analysis of the whole cell hydrolysate. Analyses of 16S rRNA gene sequences confirmed that all isolates belong to the genus *Streptomyces* and 8 isolates were identified to the species level (Table 2). Eleven isolates (65%) were inhibitory to Gram-positive bacteria *S. aureus* and *B. subtilis* but only 2 of these isolates were also inhibitory to both Gram-negative bacteria *E. coli* and *K. pneumoniae.* All isolates were inhibitory to *Cryptococcus neoformans* and *A. niger* and 67% of these isolates were inhibitory to all test *Candida* (Table 3). Nearly half of the isolates (47%) were inhibitory to all eight test fungi. One isolate, JPL1, identified as *Streptomyces rochei* was inhibitory to all 27 test bacteria and fungi (Fig. 1).

DISCUSSION

Presence of *Streptomyces* in four out of six herbal products cultured in the present study suggests that actinomycetes could be common filamentous bacteria harboured in the polyherbal products. In one study, counts of actinomycetes in some medicinal plants were found to be between 1.5 $\log_{10}$ cfu and 2.2 $\log_{10}$ cfu g^{-1} sample (El-Shatoury *et al*., 2006) and these counts were comparable to the counts of *Streptomyces* found in the present study. This result suggests that *Streptomyces* species were the main actinomycetes found in the polyherbal products and this supports the findings of other studies elsewhere (Taechowisan *et al*., 2003; Li *et al*., 2009). Other common actinomycetes isolated from medicinal plants such as *Microbispora* species, *Nocardia* species and *Micromonospora* species (Taechowisan *et al*., 2003; Khamna *et al*., 2009) were not isolated from the herbal products.

Streptomyces species commonly produce abundant spores (Ochi and Inatsu, 1995; Lee and Rho, 1993; Cruz *et al*., 2000) and this could be a reason why they were the main actinomycetes isolated in this study. Herbal plants used in herbal products are normally dried to less than 10% water (Mahanom *et al*., 1999) and at this level of water content vegetative cells lost viability but the spores survived. Vegetative cells are also affected by the powdering process that cut the herbal material shorter than 105 μm in order for the plant materials to pass through the 140 mesh-sieve.

Number of plants species used in the product formulation may not be the major determinant of the counts and strains of *Streptomyces* found in the product. Despite having only 4 plants in its formulation (2 roots, 1 bark and 1 fruit berry), 11 strains of *Streptomyces* were isolated from JT compared to 2 strains isolated from JPL which was made-up of 10 plants (7 roots, 1 bark and 1 fruit berry). Although roots (rhizome or radix) were reported to be associated with more diverse actinomycetes (Tian *et al*., 2004), use of roots from various plants, as in the cases of JPL and JS, did not increase the varieties of actinomycetes strains isolated from the product. Low count of actinomycetes in JGG may partly be explained by

the use of mainly fruits which have been reported to have less association with actinomycetes compared to the roots (Tian *et al*., 2004). Three species of plants used in the formulation of JT (*Zingiberis zerumbet*, unknown species of *Alyxiae* and unknown species of *Zingiberis*) were not indicated in the formulation of JPL and the rest of the herbal products. These plants could have been associated with varieties of actinomycetes present only in JT as reports elsewhere showed that some endophytes microorganisms have preference for some species of plants (Arnold *et al*., 2000).

Results of antibacterial activity of the 17 isolates showed that the metabolites produced by the *Streptomyces* species were generally less inhibitory to the Gram-negative bacteria as compared to the Gram-positive bacteria. These results have similarly been observed elsewhere in both actinomycetes and herbal plant extracts (Sahin and Ugur, 2003; Oskay *et al*., 2004; Lopez *et al*., 2001). The different susceptibility between Gram-positive bacteria and Gram-negative bacteria has been attributed to differences in the composition and structure of the cell surface. In addition to cell wall, Gram-negative bacteria have an outer layer cell membrane composed of phospholipid bilayer which may be a protective barrier against antimicrobials (Kandler, 1982).

Fungi possess cell wall almost similar to the Gram-positive bacteria and this may partly explains why they are susceptible to the actinomycetes metabolites. The results showed that more than half of the isolates produced inhibitory effect on yeasts *C. albicans*, *C. krusei* and yeast-like fungi *T. cutaneum*. These results show that *Streptomyces* species associated with herbal plants are potentially to be explored as sources of metabolites against fungi reported to be increasingly resistant to the current available antifungals (Capoor *et al*., 2005; Walsh *et al*., 1990).

Streptomyces rochei strains isolated in the current study showed a broad spectrum antimicrobial activity. Metabolites of this strain were inhibitory to both fungi and bacteria. Strains of *S. rochei* isolated elsewhere were also inhibitory to varieties of aerobic Gram-positive bacteria and aerobic Gram-negative bacteria, *Candida* species and some pathogenic filamentous fungi (Kavitha and Vijayalakshmi, 2007; Augustine *et al*., 2005). However, this could be the first report on inhibitory effect of *S. rochei* on anaerobic bacteria (*Clostridium difficile* and *Propionibacterium acnes*), microaerophilic bacteria (*C. jejuni* and *H. pylori*) and yeast-like fungi of clinical importance (*Trichosporon cutaneum* and *Geotrichum candidum*). Apart from *S. rochei*, isolates JT11, identified as *S. diastaticus* was also showed broad spectrum antimicrobial activity and limited number of reports proved that *S. diastaticus* produced antibiotics (Zuniga *et al*., 2004; Shiba and Mukonoki, 1975). Thus, *S. diastaticus* is a promising actinomycete from which some useful antibiotics could be isolated.

CONCLUSIONS

Some polyherbal products harboured *Streptomyces* species which produced inhibitory metabolites especially on fungi. *Streptomyces rochei* isolated in this study was inhibitory to varieties of medically important fungi and species of physiologically different bacteria including aerobic, anaerobic and microaerophilic bacteria. Thus, polyherbal products in the markets are readily culturable materials for rapid isolation of *Streptomyces* associated with herbal plants.

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