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NEW PERSPECTIVE OF *DENDROBIUM CRUMENATUM* ORCHID FOR ANTIMICROBIAL ACTIVITY AGAINST SELECTED PATHOGENIC BACTERIA

UMA MAGESWARY SANDRASAGARAN[1], SREERAMANAN SUBRAMANIAM[2*] AND VIKNESWARAN MURUGAIYAH[1]

[1]*School of Pharmaceutical Sciences, Universiti Sains Malaysia (USM), 11800 Gelugor, Penang, Malaysia*
[2]*School of Biological Sciences, Universiti Sains Malaysia (USM), 11800 Gelugor, Penang, Malaysia*
[*]*Corresponding author's e-mail: sreeramanan@usm.my / sreeramanan@gmail.com*

Abstract

The present study was undertaken to investigate the potential anti-microbial activity from different parts of *Dendrobium crumenatum* (leaf, stem, root and pseudo-bulb) against 8 pathogenic bacteria. The antimicrobial activities were determined by using disc diffusion assay, microdilution test for determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The methanolic extracts of stem, root and pseudo-bulb displayed antimicrobial activity comparable to that of the standard antibiotics. Stem extract of *D. crumenatum* had the most potent antimicrobial activity against *Staphylococcus aureus, Klebsiella pneumoniae* and *Enterobacter aerogenes* with MIC values of 0.39, 0.195 and 0.195mg/mL, respectively. Root and stem extracts were found to be active against *Streptococcus pneumoniae, Shigella dysentriae* and *Saccharomyces cerevisiae* with MIC values of 0.78 mg/ml compared to 0.00312mg/mL, 0.025mg/mL and 0.0125mg/mL of standard antibiotics of amoxcillin, chloramphenicol and kanamycin. Stem and root extracts yield MBC values in the range of 0.78 mg/mL to 6.25 mg/mL against *Staphylococcus aureus, Enterobacter aerogenes, Klebsiella pneumoniae* and *Saccharomyces cerevisiae*. The present study showed that *D. crumenatum* exhibited potential antimicrobial activity which could be due to the presence of alkaloid and flavonoid compounds and this is a first report on South East Asia region's wild orchid.

Introduction

Dendrobium species of *Orchidaceae* family has been credited as a traditional medicine over the centuries in Asia, Europe and Australia countries with more than 1100 species (Rosa, 2010). There are records of some species of *Dendrobium* used for medicinal purposes during ancient China during 2800 B.C (Hedge & Ingalhalli, 1988). Currently, total of 74 species of *Dendrobium* plants found in China and about 30 species of them are used in traditional or folk medicine as antipyretic, eyes remedy, immunoregulator and as anti aging agent (Commission of Chinese Pharmacopoeia, 2005).

The pigeon orchid (*Dendrobium crumenatum*) is a tropical epiphytic orchid that demonstrates a flowering process associated with temperature changes. The developed meristem enters a phase of dormancy. Flowering of this plant is triggered by a drop in temperature. This flower is believed to develop in nine days after a drop of 5.5^{o}C or more, like when a sudden heavy and long downpour occurs after a period of hot weather. The white flowers consists of sepals (elliptical dorsal and two lateral sepals), petals (two lateral petals similar to dorsal sepal and one labellum of three lobes), and the column (pistils and stamens) (Seidenfaden & Woods, 1992).

The pharmacological profile of species of *Orchidaceae* family should be reviewed more intensively. Throughout the ages, several health-promoting benefits, including diuretic, anti-rheumatic, anti-inflammatory, anticarcinogenic, hypoglycemic activities, antimicrobial, anticonvulsive, relaxation, neuroprotective, and antivirus, activities have been attributed to the use of orchids extracts (Rosa, 2010). *Dendrobium crysanthum* which is widely found in China is aid to use the leaves part dried and grounded to function as antipyretic agent (Li *et al*., 2001). In Japan, *Dendrobium fimbriatum* leaves paste is used to promote body fluid. The paste is applied to the fractured area to set the bone (Bi *et al*., 2003).

One way to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents. Thus, drug resistance in human pathogens developed the necessity to search antimicrobial compounds as an alternative. Therefore, screening of medicinal plants is vital to overcome these emerging problems (Monica *et al.*, 2013). For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. However, inappropriate and excessive use of antibiotics, has led to the emergence of pathogenic bacterial strains that are highly resistant to most current antibiotics.

Various phenolic secondary metabolites such as bibenzyls, phenanthrenes, fluorenones, as well as alkaloids and sesquiterpenes have been isolated from *Dendrobium* orchid's species. The compounds are found in various plant parts such as stems, roots, leaves, bark, flowers or fruits and seeds (Rosa, 2010). Gigantol compound found in *Dendrobium nobile* displayed higher antioxidant activity than standard Vitamin C (Zhang *et al*., 2007). New compounds found in *Dendrobium nobile* such as dendroside D, dendroside E, dendroside F and dendroside G showed immunomodulatory activity (Ye *et al*., 2012).

Hence the aim of this study is to identify the phytochemical constituents and investigate it antimicrobial properties of *Dendrobium crumenatum* against various types of pathogenic microbes by determining the MIC and MBC which are more accurate, efficient and faster method.

Materials and Methods

Plant material: *D. crumenatum* was collected from Alor Star, Kedah. A voucher specimen number 11091 was deposited at herbarium of School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia.

Preparation of plant extracts: The plants were separated into four different parts as in leaves, pseudo-bulbs, stems and roots. The various parts were rinsed with distilled water to remove dirt's and dried in an oven at 50ºC for 48 hours. The dried plant materials were powdered using grinder and the powdered materials were extracted using methanol by using the maceration method. The extracts were filtered and dried using rotary evaporator. The semi-solid extracts were subjected to freeze - drying in order to obtain powdered extract (Bhattacharjee *et al*., 2006). The methanol extracts were kept in sterile bottles at 4ºC until further use.

Phytochemical screening: Phytochemical screening for major constituents of the plant was done using standard procedures described by Sofowora (1993). Nine (9) types of test were carried out in order to confirm the presence of alkaloids (Dragendorff's test), saponin (Frothing test), terpenoid (Lieberman-Burchard's test), flavonoid (Ferric chloride test), cardiac glycosides (Keller-Kiliani test), anthraquinones (Borntrager's test), tannins (Potassium hydroxide test), reducing sugar and phlobatannins.

Microbial strains and growth media: Eight pathogenic microorganisms species were used: *Streptococcus pneumoniae* (lab strains), *Staphylococcus aureus* (ATCC 25933), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella flexeneri* (ATC 12022), *Klebsiella pneumoniae, Enterobacter aerogenes* (lab strains) and *Saccharomyces cerevisiae* (lab strain). Stock cultures were maintained at 4°C on Mueller-Hinton Agar (MHA) and Potato Dextrose Agar (PDA) plates were used for bacteria and fungal respectively. Prior to use, stock was sub-cultured into new MHA plates to ensure active growth of the microbes that were incubated at 37°C overnight. The cultures were diluted with fresh Mueller–Hinton Broth (MHB) or Potato Dextrose Broth (PDB) to achieve optical densities corresponding to 1.5 x 10^8 CFU/mL (McFarland 0.5) for microbes.

Disc diffusion assay: Approximately 20mL of MHA were poured into each sterilized Petri dishes and left to solidify at room temperature about 30 minutes prior to inoculation. Bacterial suspension containing approximately 1.5×10^8 CFU / mL which is equivalent to standard Mc Farland No 0.5 was spread on the agar using cotton swab over the entire surface of the medium three times. Sterile filter paper disc impregnated with 20µL of extract diluted with methanol (1mg/mL per disc) were placed on the agar using sterile forceps. The plates were then inverted for incubation at 37°C for 24h for bacteria and at 30°C for 48h for the fungal strains. The inhibition zones were measured in millimeters (mm). The experiment was carried out in triplicate. Chloramphenicol (30µg), gentamycin (10µg), penicillin G (10µg), tetracycline (30µg), vancomycin (10µg), micanazole (30µg) and kanamycin served as positive control and 100% methanol was used as negative control.

Minimum inhibitory concentration assay: Minimum Inhibitory Concentration (MIC) of *D. crumenatum* extract was assessed using the 96 well – plate method. An inoculum of the microorganism was prepared from 16 hours Mueller Hinton Broth (MHB) cultures and suspensions were adjusted with a turbidity equivalent to that of a McFarland No. 0.5 standard to achieve inoculums of approximately 1.5×10^8 CFU/mL. Stock solutions of plant extracts prepared in dimethyl sulfoxide (DMSO) were diluted with MHB. Total of 100µL culture suspension was added to each well containing 100µL of extracts in concentration range between 25 to 0.39 mg/mL. After incubated overnight, 50 µL of freshly prepared para-iodonitrotetrazolium (INT) or 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) were added into each well. The MIC provides a quantitative measurement of the lowest concentration of extracts inhibiting the microbes. As for MTT, a yellow colour in the well is interpreted as no growth of microbes and a purple colour is scored as presence of growth. Whereas for INT, a yellow colour indicates inhibition of microbes and pink colour indicates presence of microbes. Chloramphenicol, vancomycin, kanamycin, amoxicillin and micanazole served as positive controls for bacteria and fungi while 50% DMSO served as negative control.

Minimum bactericidal concentration assay: Minimum bactericidal concentration (MBC) assay was carried out to determine the lowest concentration of drug that kills microbes. Total of 25µL of culture treated with extract was pipetted out from each well of the 96 well plates which was assessed for MIC assay. The suspension is then dropped onto MHA plates, a drug-free solid medium which is then incubated further for 18 to 24 hours. Plates that yielded zero or less than 10 single colonies is accounted as for MBC value determination (Kabir *et al*., 2005).

Bioautography assay: Modified method of Khurram *et al.*, (2011) was used for this assay. Merck® TLC plates size 20 cm × 20 cm and 1mm of thickness was used for bioautography assay. Plant extracts (1mg/mL) were spotted 15 times and the chromatogram was developed using hexane: ethyl acetate (1:2) as solvent system. TLC plates were run in duplicate and one set was used as reference. Spots and bands were visualized by UV irradiation (254 and 365 nm). The other set was run for bioautography. Inoculum of microbes was adjusted to Mc Farland standard No. 0.5 containing 1.5×10^8 CFU/mL. The TLC plate was sprayed with the inoculums and was incubated overnight at 37ºC. Subsequently the bioautogram were sprayed with INT and incubated at 37ºC for 4 hours. Inhibition zones indicated presence of active compounds.

Results

The result of phytochemical test shows that bioactive compounds such as saponin, terpenoid, alkaloid, reducing sugar and flavonoid were present in most of the parts of *Dendrobium crumenatum*. Terpenoid and reducing sugar were however absent in leaves extract. Flavanoid was absent in both stem and root extract (Table 1).Cardiac glycosides were only detected in leaves whereas phlobatannins, anthraquinones and tannins were not detected in any of the plant extracts. Alkaloids have numerous functions and among them are most is analgesic, anti–inflammatory and antibacterial effects. The presence of terpenoid may contribute to analgesic and anti– inflammatory activity. This screening could be a useful tool for comparative studies of bioactive principles present in different parts of this plant.

The *In vitro* antimicrobial activity of methanolic extract of *D. crumenatum* leaves against the microorganisms was assessed qualitatively and quantitatively inhibition zones. The extracts showed antimicrobial activity against tested microorganisms (*Escherichia coli, Klebsiella pneumoniae, Streptococcus pneumonia, Shigella flexeneri, Pseudomonas aeroginosa, Enterobacter aerogenes, Staphylococcus aureus* and *Saccaromyces cerevisiae*) (Table 1). In our studies, methanol was chosen as the solvent. Plant extracts in organic solvent (methanol) provide more consistent antimicrobial activity. This can be rationalized in terms of the polarity of the compounds being extracted by the solvent and in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media used in the assay (Jigna & Sumitra, 2006). The methanolic extracts of stem, root and pseudo bulb were found to exhibit comparable antimicrobial activity to that of the standard antibiotics against all tested microorganisms with inhibition zone in range of 7 to 10 mm (Table 2). The antimicrobial activity of leaf extract against all tested microorganisms was generally low. Whereas the antimicrobial activity of stem and root extracts were slightly better than leaf extract. In comparison of the extracts and antibiotics, there was a major difference in terms of their inhibiting zone diameters. When the extracts exhibited only slight antimicrobial activity, the antibiotics showed clear and strong antimicrobial activity against all test microorganisms (Table 3).

Table 1. Phytochemistry of the plant extracts.

Plant/ Secondary metabolites	Stem	Root	Pseudobulb	Leaves
Saponin	++	++	+	+
Terpenoid	+++	++	+	-
Alkaloid	+	++	+++	+++
Reducing sugar	+++	++	+++	-
Phlobatannins	-	-	-	-
Flavonoid	-	-	++	+++
Anthraquinones	-	-	-	-
Cardiac glycosides	-	-	-	+
Tannins	-	-	-	-

+++ : Abundantly present ++ : Moderately present + : Weakly present - : Absent

Table 2. Antimicrobial activity of *Dendrobium crumenatum* samples (1mg/disc) against selected microbes using disc diffusion method.

Microbes extracts	*Escherichia coli*	*Klebsiella pneumoniae*	*Streptococcus pneumoniae*	*Shigella flexeneri*	*Pseudomonas aeruginosa*	*Enterobacter aerogenes*	*Staphylococcus aureus*	*Saccharomyces cerevisiae*
Leaves		~						~
Stems	~	~	~	~	~	~	~	+
Roots	~	~	~	~	~	~	~	~
Pseudo bulb			~				~	~
Ref activity	Chloramphenicol + + +	Gentamycin + + +	Penicilin G + + +	Tetracycline + +	Chloramphenicol + + +	Tetracycline + + +	Vancomycin + +	Micanazole + +

= No antimicrobial activity

~ = Slight antimicrobial activity (of sample 1–3 mm)

+ =Moderate antimicrobial activity (i.z. of sample 3–4 mm)

+ + = Clear antimicrobial activity (i.z. of sample 4–10 mm)

+ + + = Strong antimicrobial activity (i.z. of sample > 10 mm)

*Diameter of inhibition zone excluding disc diameter of 6.0 mm

Table 3. Antimicrobial activity of *Dendrobium crumenatum* samples using minimum inhibitory concentration (MIC).

Microbes extracts	***Escherichia coli***	***Klebsiella pneumoniae***	***Streptococcus pneumoniae***	***Shigella dysentriae***	***Pseudomonas aeruginosa***	***Enterobacter aerogenes***	***Staphylococcus aureus***	***Saccharomyces cerevisiae***
Stems	12.5	0.195	0.78	0.78	1.56	0.195	0.39	0.78
Roots	12.5	1.56	1.56	1.56	3.125	0.39	0.78	0.78
Pseudo bulb	12.5	3.125	6.25	3.125	6.25	1.56	6.25	3.125
Ref activity	Chloramphenicol 0.025	Kanamycin 0.025	Amoxicilin 0.00312	Chloramphenicol 0.025	Chloramphenicol 0.025	Chloramphenicol 0.05	Vancomycin 0.0125	Micanazole 0.0125

Subsequent MIC determination found that the stem extract of *D. crumenatum* had good antimicrobial activity against *Staphylococcus aureus, Klebsiella pneumoniae* and *Enterobacter aerogenes* with MIC values of 0.39, 0.195 and 0.195 mg/mL, respectively. On the other hand, root and stem was found to be active against *Streptococcus pneumonia, Shigella flexeneri* and *Saccharomyces cerevisae* with MIC value of 0.78 mg/mL compared to 0.00312, 0.025 and 0.0125 mg/mL for amoxcillin, chloramphenicol and kanamycin,respectively. However, the pseudo bulb did not produce a higher MIC value compared to those of stem and root extracts. From MIC results, and 2 extract (stem and root) were further tested for minimal bactericidal concentration (MBC) against 4 microbes. MBC values of 0.78 and 6.25 mg/mL were obtained for stem and root respectively against *Klebshilia pneumonia*. MBC value for both stem and root against *Enterobacter aerogenes* was 1.56mg/mL. On the other hand, MBC value for both stem and root is 1.56 mg/mL against *Saccharomyces cerevisae* which the same as its MIC, where it was MBC values against *Staphylococcus aureus*'s were 0.78 and 1.56mg/mL for stem and root extracts, respectively. These extracts were bacteriostatic at lower concentrations and bactericidal at higher concentrations as revealed by MIC and MBC values (Table 4).

Figure 1 presents the result of TLC bioautography assay. Figure 1a shows the standard TLC chromatogram of plant extracts. Figure 1b visualizes TLC plates after being sprayed with bacterial inoculums of *S. aureus* indicating bacterial inhibition zones at *Rf* values of 0.62 (i) and 0.65 (ii) for stems and roots. This spots indicate the presence of active compounds that are responsible for the antimicrobial activity observed. The compounds corresponds to the phytochemical screening and were grouped into alkaloid compound in comparison to their *Rf* value.

Discussion

Dendrobium crumenatum is easily being planted and grows well especially in Malaysia's climate. Orchids are often looked at as ornamental illustration but its biological importance was overlooked. Pharmacological studies conducted on orchids indicate the potential of this plant in treating diseases such as diabetic, convulsive and cancer. The studies gap needs to be bridged in order to exploit full medicinal potential of orchids. *Dendrobium* species with over 60 structures of alkaloid and nitrogen compounds identified, of which have demonstrated pharmacological activities such as antitumor, anti-angiogenic, anti-platelet aggregation, anti-inflammation and immunoregulatory activities. Bibenzyl and phenanthrene are most characteristic as chemical marker for genus *Dendrobium*, as the presence of over 40 compounds of those types were reported from this genus (Chapman & Hall, 2005).

The result of the present study also revealed that the extracts of *D.crumenatum* exhibited antimicrobial activities in different degrees. Bioactive compounds such as saponin, terpenoid, alkaloid, reducing sugar and flavonoid which was detected in most parts of medicinal plants have been reported to exhibit antimicrobial potency (Sofowora, 1993). Hence, the presence of the secondary metabolites in *Dendrobium crumenatum* may be responsible for its potential use as a drug against pathogenic bacteria. According to Cushnie & Lamb (2005), both alkaloids and flavonoids had antimicrobial activities. Saponins are a special class of glycolsides which have soapy characteristic and facilitate the absorption of foods and medicine. In addition, saponin is also said to be playing a role in inflammation management, where as flavonoids have activities like anti-allergy (Yebpella *et al*., 2011). Moreover, flavonoids were also known to be synthesized by plants in response to microbial infection. Hence, it does correlate their effectiveness as antimicrobial substance against a wide array of microorganisms. Presence of terpenoids could be linked to their mechanism which is speculated to involve membrane disruption at the phospholipid bilayer. Although studies of antimicrobial activity associated with *Dendrobium* genus are insufficient, it seemed reasonable to believe that this species has shown sufficient result to proceed with more studies.

Table 4. Antimicrobial activity of *Dendrobium crumenatum* samples using minimum bactericidal concentration (MBC).

Microbes		Stems	Roots	Gentamycin	Micanazole	Vancomycin
Klebsiella pneumoniae	MBC	0.78	6.25	0.0125		
	FOLDS	2	1	4		
Enterobacter aerogenes	MBC	1.56	1.56	0.2		
	FOLDS	3	2	4		
Saccaromyces cerevisiae	MBC	1.56	1.56		0.0125	
	FOLDS	0	0		1	
Staphylococcus aureus	MBC	0.78	1.56			0.025
	FOLDS	1	1			4

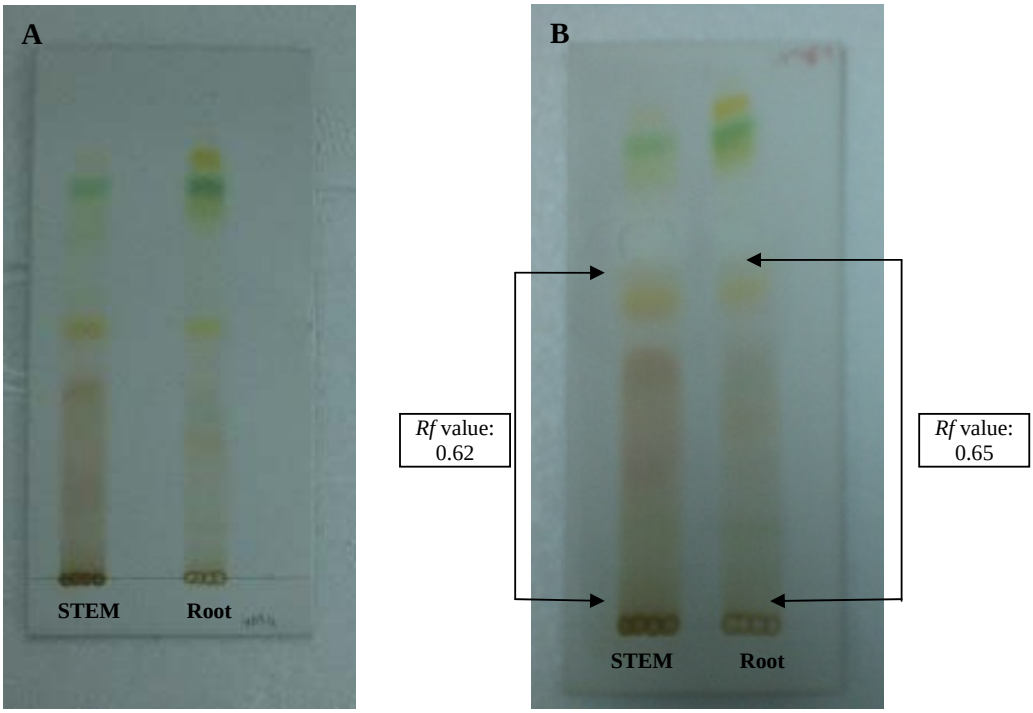

Fig. 1. TLC chromatogram for stem and root of *Dendrobium crumenatum*. A, Standard TLC chromatogram of plant extracts. B, Bacterial inhibition zones at *Rf* values of 0.62 (stem) and 0.65 (root).

Conclusion

Dendrobium crumenatum has great potential as antimicrobial agent against selected pathogenic microorganisms due to the presence of selected alkaloid and flavonoid compounds. Consequently, the antibacterial effects can be further investigated by assay such as time killing profile and scanning electron microscopy observation to understand the morphological changes produced by the microorganisms.

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