Antimicrobial compounds from Alpinia conchigera

Ahmad Nazif Aziza, Halijah Ibrahimb, Devi Rosmy Syamsirb, Mastura Mohtarc, Jaya Vejayand, Khalijah Awange,*,

A Department of Chemical Sciences, Faculty of Science and Technology, Universiti Malaysia Terengganu (UMT), 21030 Kuala Terengganu, Terengganu, Malaysia
B Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
C Medicinal Plants Programme, Biotechnology Division, Forest Research Institute Malaysia (FRIM) 52109, Kepong, Selangor, Malaysia
D School of Medicine and Health Sciences, Monash University Sunway Campus, Jalan Lagoon Selatan, 46150 Bandar Sunway, Selangor, Malaysia
E Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
F Centre for Natural Products and Drug Discovery (CENAR), Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

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Abstract

Ethnopharmacological relevance: The rhizome of Alpinia conchigera has been used as a condiment in the northern states of Peninsular Malaysia and occasionally in folk medicine in the east coast to treat fungal infections. In some states of Peninsular Malaysia, the rhizomes are consumed as a post-partum medicine and the young shoots are prepared into a vegetable dish. This study aimed to investigate the chemical constituents of the pseudostems and rhizomes of Malaysian Alpinia conchigera and to evaluate the antimicrobial activity of the dichloromethane (DCM) extracts of the pseudostems, rhizomes and the isolated compounds against three selected fungi and five strains of Staphylococcus aureus.

Materials and methods: The dried and ground pseudostems (0.8 kg) and rhizomes (1.0 kg) were successively extracted in Soxhlet extractor using n-hexane, dichloromethane (DCM) and methanol. The n-hexane and DCM extracts of the pseudostem and rhizome were subjected to isolation and purification using column chromatography on silica gel using a stepwise gradient system (n-hexane to methanol). Briefly, a serial two fold dilutions of the test materials dissolved in DMSO were prepared prior to addition of 100 μl overnight microbial suspension (108 cfu/ml) followed by incubation at 37 °C (bacteria) or 26 °C (dermatophytes and candida) for 24 h. The highest concentration of DMSO remaining after dilution (5%, v/v) caused no inhibition to bacterial/candida/dermatophytes' growth. Antibiotic cycloheximide was used as reference for anticandidal and antidermatophyte comparison while oxacilin was used as reference for antibacterial testing. DMSO served as negative control.

Turbidity was taken as indication of growth, thus the lowest concentration which remains clear after macroscopic evaluation was taken as the minimum inhibitory concentration (MIC).

Results: The isolation of n-hexane and DCM extracts of the rhizomes and pseudostems of Alpinia conchigera via column chromatography yielded two triterpenes isolated as a mixture of stigmasterol and β-sitosterol: caryophyllene oxide, chavicol acetate 1, p-hydroxy cinnamaldehyde 2, 1,5'-hydroxy-1,5'-acetoxyeugenol acetate 3, trans-p-coumaryl diacetate 4, 1,5'-acetoxyeugenol acetate 5, 1'-hydroxychavicol acetate 6, p-hydroxycinnamyl acetate 7 and 4-hydroxybenzaldehyde.

The DCM extract of the rhizome of Alpinia conchigera indicated potent antifungal activity against Candida albicans, Microsporum canis and Trychophyton rubrum with MIC values of 625 μg/ml, 156 μg/ml and 156 μg/ml, respectively. It also showed significant inhibitory activity with MIC values between 17.88 and 35.75 μg/ml against the mutant Staphylococci isolates MSSA, MRSA and Sa7.

Amongst the isolated compounds, the lowest inhibition observed were of 1,5'-acetoxyeugenol against the dermatophytes (MIC 313 μg/ml) followed by trans-p-coumaryl diacetate against both dermatophytes and candida (MIC 625 μg/ml). The compound p-hydroxycinnamyl acetate strongly inhibited Staphylococcus aureus strain VISA (MIC 39 μg/ml) followed by trans-p-coumaryl diacetate and 1'-hydroxychavicol acetate with MIC value of 156 μg/ml.

Conclusion: In conclusion, the observed antibacterial, anticandidal and antidermatophyte activity of the extracts and compounds obtained from the rhizome confirm the traditional use of Alpinia cochigera rhizome in the treatment of skin infection.
1. Introduction

*Alpinia conchigera* Griff. is a herbaceous perennial, 2 to 5 ft. tall, found in eastern Bengal and southwards to Peninsular Malaysia and Sumatera. In Malaysia, it is also locally known as *lengkuas ranting*, *lengkuas kecil*, *lengkuas padang*, *lengkuas genting* or *chengkenam* (Burkill, 1966). This species is semi-wild, common in open wet grounds such as edges of rice fields, streams as well as under the shade of palm oil and rubber trees. The rhizome is used as a condiment in the northern states of Peninsular Malaysia and occasionally in folk medicine in the east coast to treat fungal infections (Ibrahim et al., 2000). In some states of Peninsular Malaysia the rhizomes are consumed as a post-partum medicine and the young shoots are prepared into a vegetable dish (Ibrahim et al., 2009).

There were few studies on the chemical constituents of *Alpinia conchigera* reported to date. Yu et al. (1988) reported the presence of nonacosane, β-sitosterol, 1'-acetoxychavicol acetate and 1'-acetoxyeugenol acetate in the fruit, the two phenylpropanoid derivatives showing anti-inflammatory activity. Later, Athamaprasangsara et al. (1994) communicated the detection of four known phenylpropanoids: chavicol acetate, 1'-hydroxychavicol acetate, 4-acetoxyxannamyl alcohol and 4-acetoxyxannamyl acetate from the aqueous layer obtained from the hydro-distillation of the fresh rhizomes of *Alpinia conchigera* Griff. from Thailand. They also reported five diarylheptanoids: 1,7-diphenyl-3,5-heptadione, 1,7-diphenyl-5-hydroxy-3-heptanone, 5-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-1-phenyl-3-heptanone, 1,7-diphenylethyp-4-en-3-one and 7-(4-hydroxy-3-methoxyphenyl)-1-phenylethyp-4-en-3-one and two flavonoids: 3,5,7-trihydroxyflavone and 3,5,7-trihydroxy-4'-methoxyflavone from the n-hexane and DCM extracts of the dried rhizomes.

Recent studies on chemical constituents of *Alpinia conchigera* by Phan et al. (2005) reported β-sitosterol, stigmasterol and three flavonoids: cardamomin, alpinetin and naringenin 5-Me ether isolated from the rhizomes of *Alpinia conchigera* Griff. from Vietnam. Further study on the methanolic extract of the rhizomes resulted with β-sitosterol, stigmasterol, cardamomin, chalconaringenin 2'-O-Me ether, alpinetin, and naringenin 5-O-Me ether (Le et al., 2007). In addition, Giang et al. (2007) isolated flavokawin B, β-sitosterol, stigmasterol, alpinetin, (3S,5S)-trans-3,5-dihydroxy-1,7-diphenyl-1-heptene, β-D-fructopyranose, β-D-fructofuranose, and 2-O-Me β-D-fructofuranose from the fruits of *Alpinia conchigera* Griff. (Zingiberaceae) from Vietnam. 1'S-1'-acetoxychavicol acetate and 1'S-1'-acetoxyeugenol acetate obtained from *Alpinia conchigera* were found to induce apoptosis in oral squamous carcinoma cells (HSC-4) and human breast cancer cells (MCF-7), respectively (Awang et al., 2010; Hasima et al., 2010). Ibrahim et al. (2009) communicated the essential oil constituents of *Alpinia conchigera*.

All of the above mentioned studies involved the constituents from the rhizomes and fruits of *Alpinia conchigera* from China, Thailand, Vietnam and Malaysia and fruits of ract of the rhizomes. In this study, the chemical constituents from the pseudostems and rhizomes of Malaysian *Alpinia conchigera* will be investigated and discussed. In addition, investigation on the antimicrobial activity of the DCM extracts of the pseudostems, rhizomes and the isolated compounds against three selected fungi and five strains of *Staphylococcus aureus* will be carried out.

2. Material and methods

2.1. Plant material

The pseudostems and rhizomes of wild *Alpinia conchigera* Griff. were collected from Jeli province of Kelantan, Malaysia with herbarium series number KL 5049. The samples were authenticated by Professor Dr. Halijah Ibrahim of the Institute of Biological Sciences, University of Malaya and the voucher specimens were deposited in the herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur.

2.2. Extraction and isolation

The dried and ground pseudostems (0.8 kg) and rhizomes (1.0 kg) were extracted in Soxhlet extractor using n-hexane, dichloromethane (DCM) and methanol (MeOH) successively. The pseudostem and rhizome extracts were dried in vacuo. The n-hexane and DCM extracts were further subjected to chemical compound isolation and antimicrobial activity (antibacterial and antifungal activity) studies. The n-hexane and DCM extracts of the pseudostems and rhizomes were subjected to column chromatography (CC) on silica gel using a stepwise gradient system (n-hexane to MeOH). All compounds were successfully isolated using CC with a combination of n-hexane-ethyl acetate (EtOAc) as the solvent system.

2.3. General experimental procedure

All NMR spectra were recorded on a JEOL LA-400 spectrometer (400 MHz), in CDCl₃. The mass of the compounds were obtained from GC–MS (Hewlett Packard 5989 A) on HP-5 column.

2.4. Antimicrobial activity

2.4.1. Test isolates

Microbes selected for this study are those implicated in skin infections (Williams et al., 1999; Pereira Gonzales and Maisch, 2012). The crude extracts and the isolated compounds were individually tested against five strains of *Staphylococcus aureus* (each differing in their antibiotic resistance profile) namely Meticillin sensitive *Staphylococcus aureus* ATCC 29213 (MSSA), Meticillin resistant *Staphylococcus aureus* ATCC 33591 (MRSA), Vancomycin intermediate resistant Staphylococcus aureus ATCC 700699 (Sa7), MRSA with intermediate resistance to vancomycin (VISA24) and MRSA with complete resistance to vancomycin (VRSAS156), a candida: *Candida albicans* ATCC 10231, two dermatophytes: *Microsporum canis* (ATCC 36299) and *Trichophyton rubrum* (ATCC 28188). All isolates were purchased from American Type Culture Collection (ATCC) except two mutant isolates namely VISA24 and VRSAS156 that were obtained through a step wise lab-passage procedure as described previously (Mohtar et al., 2009).

2.4.2. Inoculum preparation

Media was sterilized by autoclaving at 120 °C for 15 min and all subsequent manipulations were carried out in a class 2 bio-hazard cabinet. The *Staphylococcus aureus* strains (coded as Sa 2, Sa 3, Sa 7) were cultured in Mueller Hinton Broth (MHB), VISA and VRSAS in Tryptic soy broth (TSB) overnight (24 h) at 37 °C while the candida and dermatophytes were cultured in Potato dextrose broth (PDB) overnight at 26 °C. The resulting inoculum was further adjusted to obtain a turbidity comparable to that of McFarland standard tube No. 0.5 (Vandepitte et al., 1991) prior to use.

2.4.3. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) value determination assay was carried out to evaluate the potential compounds as inhibitory agent against test isolate using double-broth micro-dilution method involving 96-wells microtitre-plates as described
previously (Saiful et al., 2008). Briefly, a serial two fold dilutions of the test compounds dissolved in DMSO were prepared prior to addition of 100 μl overnight microbial suspension (10⁸ cfu/ml) followed by incubation at 37 °C (bacteria) or 26 °C (dermatophytes and Candida) for 24 h. The highest concentration of DMSO remaining after dilution (5%, v/v) caused no inhibition to bacterial/candida/dermatophytes’ growth. DMSO served as negative control. Antibiotic cycloheximide was used as reference for antifungal/candidal and antidermatophyte comparison while oxacillin was used as reference for antibacterial testing.

Turbidity was taken as indication of growth, thus the lowest concentration which remains clear after macroscopic evaluation was taken as the minimum inhibitory concentration (MIC). For further reconfirmation, 20 μl of MTT reagent (1 mg/ml) was added to the suspension in the selected wells, followed by 20 min incubation at 37 °C. The reagent-suspension colour will remain clear or yellowish indicating complete inhibition (cidal) activity as opposed to dark blue for growth (Eloff, 1998). The MIC was recorded as the most repeatable minimum concentration of triplicate.

### 3. Results and discussion

Soxhlet extraction of the pseudostems yielded 5.89 g (0.74% w/w) of n-hexane extracts, 14.70 g (1.84% w/w) of DCM extracts and 72.30 g (9.03% w/w) of MeOH extracts while the rhizomes produced 22.68 g (2.27% w/w) of n-hexane extracts, 15.87 g (1.59% w/w) of DCM extracts and 150.36 g (15.04% w/w) of MeOH extracts.

The isolation of n-hexane extracts of the rhizomes of *Alpinia conchigera* via column chromatography yielded two triterpenes isolated as a mixture of stigmasterol (Forgo and Kővér, 2004; Xu et al., 2005) and β-sitosterol (De-Eknamkul and Potduang, 2003); one sesquiterpene; caryophyllene oxide (Silverstein and Webster, 1998), seven phenyl propanoids; chavicol acetate 1 (Yu et al., 1988; Mitsui et al., 1976), p-hydroxy cinnamaldehyde 2 (Leem et al., 1999), 1’-5’-1’-acetoxychavicol acetate 3 (Janssen and Scheffer, 1985; Noro et al., 1988; Yang and Eilerman, 1999), trans-p-coumaryl diacetate 4, 1’-S-1’-acetoxycoumarin acetate 5 (Noro et al., 1988; Yang and Eilerman, 1999), 1’-hydroxychavicol acetate 6 (Janssen and Scheffer, 1985; Yang and Eilerman, 1999), p-hydroxybenzaldehyde 7 (Kiuchi et al., 2002), and one phenolic compound; 4-hydroxybenzaldehyde (Noro et al., 1988).

The DCM extract of the rhizome afforded nine compounds which were similar to that of the compounds found in the n-hexane extract, but deprived of chavicol acetate 1 and caryophyllene oxide. As for the purification of the extracts from the pseudostem, seven compounds were found in the n-hexane crude extract including a mixture of stigmasterol and β-sitosterol, 2, 3, 4, 6, 7 and 4-hydroxybenzaldehyde while 2, 3, 4, 6, 7 and 4-hydroxybenzaldehyde were successfully isolated from the DCM extract (Table 1, Fig. 1).

The dicrolomethane (DCM) crude extract of *Alpinia conchigera* and the nine isolated compounds obtained were evaluated for their antimicrobial activity against three fungi (Table 2) and five strains of *Staphylococcus aureus* (Table 3) using minimum inhibitory concentration (MIC) assay. Cycloheximide and oxacillin were included as comparison for the antifungal and antibacterial potential, respectively. The results from the antimicrobial assay exhibited a distinct inhibitory pattern in the major targeted groups namely the candida, dermatophytes and staphylococci, hence will be discussed accordingly.

The DCM extract of the rhizome of *Alpinia conchigera* indicated potent antifungal activity against *Candida albicans*, *Microsporum canis* and *Microsporum gypseum*. The DCM extract of the rhizome of *A. conchigera* showed potent antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*.

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**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pseudostem (% yield)</th>
<th>Rhizome (% yield)</th>
</tr>
</thead>
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<tr>
<td>Extract</td>
<td>n-hexane</td>
<td>DCM</td>
</tr>
<tr>
<td>Stigmasterol and β-sitosterol (mixture)</td>
<td>0.31</td>
<td>Ni</td>
</tr>
<tr>
<td>Chavicol acetate 1</td>
<td>Ni</td>
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<tr>
<td>Caryophyllene oxide</td>
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<td>0.57</td>
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<td>p-Hydroxy cinnamaldehyde 2</td>
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<td>0.18</td>
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<tr>
<td>1’-S-1’-Acetoxychavicol acetate 3</td>
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<td>9.08</td>
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<tr>
<td>trans-p-Coumaryl diacetate 4</td>
<td>0.68</td>
<td>0.95</td>
</tr>
<tr>
<td>1’-S-1’-Acetoxybenzaldehyde 5</td>
<td>0.37</td>
<td>0.88</td>
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<tr>
<td>1’-Hydroxychavicol acetate 6</td>
<td>0.37</td>
<td>0.15</td>
</tr>
<tr>
<td>4-Hydroxybenzaldehyde 7</td>
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<td>0.48</td>
</tr>
<tr>
<td>p-Hydroxybenzaldehyde 7</td>
<td>0.20</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Ni: not isolated.

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**Fig. 1.** The structures of compound 1–7 isolated from the pseudostems and rhizomes of *Alpinia conchigera*.
the dermatophytes (MIC: 313 g/ml) followed by 1,7-diyraylheptanoid from Alpinia conchigera (ATCC 25923) (Whiffen, 1948, 1950; Barbary et al., 2010). Amongst the isolated compounds, the lowest inhibition observed were of 1α-1′-acetoxycinnamoyl acetate 25. In this regard, replacement of a hydroxyl group (7) with an acetoxyl group (4) had resulted in a four fold reduction in the inhibitory activity. VRSA on the other hand was most affected by 1α-1′-acetoxycinnamoyl acetate 3, the major compound present in the DCM rhizome extract with inhibitory activity value of 313 μg/ml.

In conclusion, the observed antibacterial, anticandidal and antidermatophyte activity of the DCM extract and compounds obtained from the rhizome support the traditional use of Alpinia conchigera rhizome in the treatment of skin infection. In addition, our previous study has shown that the essential oils were devoid of this activity which is not surprising as the oils did not contain the active compounds; 1α-1′-acetoxycinnamoyl acetate and 3 α-1′-acetoxyeugenol acetate, discovered in this study.

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References


