SCREENING OF SELECTED ZINGIBERACEAE EXTRACTS FOR DENGUE-2 VIRUS PROTEASE INHIBITORY ACTIVITIES

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ABSTRACT

The crude extracts and the methanol and hexane partitioned fractions from the rhizomes of six Zingiberaceae comprising five Curcumas and one Zingiber were screened for dengue-2 (Den2) virus NS2B/NS3 protease inhibition. The percentage inhibition of Den2 virus NS2B/NS3 protease cleavage of the substrate showed linear dose-dependent increment for all the samples tested. The crude extracts were less potent than the best of the partitioned fractions. For all three concentrations studied, the methanol fractions of the extracts of Curcuma longa (L.) (CL), Zingiber zerumbet Smith (ZZ) and Curcuma rubescens Roxb. (CR) were much more inhibiting than the corresponding hexane fractions but the converse was true for Curcuma aeroginosa Roxb. (CA). However, the inhibiting activities of the two partitioned fractions of the extracts of Curcuma mangga Roxb. (CM) and Curcuma xanthorrhiza Roxb. (CX) were found to be similar. The CL methanol fraction exhibited the strongest inhibitory activity (91.3±3.1%, 300 ppm), followed closely by methanol fraction of ZZ (89.0±1.7%, 300 ppm). The results show that the methanol fractions of CL and ZZ, and both the methanol and hexane fractions of CM were most potent against Den2 virus NS2B/NS3 protease activity and may provide potential leads towards the development of anti-viral agents.

Key words: Dengue, Den2, NS2B/NS3, protease inhibition, Zingiberaceae.

INTRODUCTION

Dengue fever (DF) and dengue haemorrhagic fever (DHF)/dengue shock syndrome (DSS) are caused by viruses of the family Flaviviridae. Yellow fever, Japanese encephalitis and hepatitis viruses are also members of the Flaviviridae virus family. There are four closely related but antigenically distinct dengue virus serotypes (Den1, Den2, Den3, and Den4). All four serotypes can possibly cause DF, DHF and DSS. A mosquito-borne disease, dengue is characterized by excruciating pain and is also known as “bone-break disease” (Deem, 2006).

Typical of the Flaviviridae, dengue virus consists of a single positive-sense 1-kilobase RNA genome that encodes for a single polyprotein comprising three structural (C, prM, E) and seven non-structural (NS) proteins, of sequential order C-prM-E-NS1-NS2A-NS2B-

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NS3-NS4A-NS4B-NS5 (Chambers et al., 1990). The polyprotein precursor is cleaved by both host proteases and virus protease complex NS2B/NS3. The viral protease NS3 is highly conserved and multi-functional (Wengler et al., 1991; Li et al., 1999), catalyzing the polyprotein at several sites (Falgout et al., 1991). Together with its cofactor NS2B, NS3 catalyzes the cis cleavage of NS2A/NS2B and NS2B/NS3 (Arias et al., 1993; Jan et al., 1995), and trans cleavage of NS3/NS4A and NS4B/NS5 junctions of the polyprotein (Chambers et al., 1990; Preugusch et al., 1990; Zhang et al., 1992; Clum et al., 1997; Valle et al., 1998). Furthermore, NS3 and NS5 proteins are thought to be components of the putative viral replicase complex (Kapoor et al., 1995; Cui et al., 1998) since NS3 has been shown to be regulated by viral RNA polymerase and binds with NS5 both in vitro and in vivo. This characteristic, together with the fact that NS3 mediates for a variety of diverse functions, has made it a good target for therapeutic intervention of DF/DHF.

To date, there is neither known cure nor approved vaccine for dengue although vaccination is available for yellow fever, tick-borne encephalitis and Japanese encephalitis. The current WHO control strategy emphasizes predominantly on vector control, surveillance and preparedness. The complexity of the antibody-dependent enhancement and original antigen sin (McMichael, 1998) makes development and efficacy of vaccine challenging. Previous exposure to one of the four serotypes, either by prior infection or by vaccination, makes people much more likely to develop the potentially lethal DHF/DSS if they are infected later by one of the other three serotypes. Mongkolsapaya et al. (2003) reported recovering few dengue-responsive CD8+ T cells during acute infection and most present were undergoing programmed cell death. In the same report, Mongkolsapaya et al. (2003) also found that many dengue-specific T cells were of low affinity for the infecting virus and showed higher affinity for other, probably previously encountered strains. Patients who were given a tetravalent-serotype vaccine wound up only being protected against one or two serotypes at most (Deem, 2006).

There is a need to develop anti-viral agents, both as prophylactic and treatment. A therapy similar to the ‘triple combination therapy’ using anti-retroviral drugs for HIV/AIDS can be developed eventually for DF/DHF if research is intensified. The typical HIV/AIDS triple combination therapy consists of two reverse transcriptase inhibitors and a protease inhibitor. A preliminary study of Ritonavir, an inhibitor of HIV-1 protease, has shown that the viral loads can be reduced by 99% (Markowitz et al., 1995).

The Zingiberaceae consists of about 1200 species of which 1000 are found in tropical Asia (Larsen et al., 1999). Zingiberaceae rhizomes are widely used in Asia as both traditional medicine and spices. The extracts of 13 Zingiberaceae species (Alpinia, Costus and Zingiber) were shown to exhibit antibacterial and antioxidant activity (Habsah et al., 2000). A compound, zerumbone, isolated from Zingiber zerumbet Smith exhibited HIV-inhibitory and cytotoxic activities (Dai et al., 1997). In addition, zerumbone has also been reported to suppress cancer-cell proliferation (Hoffman et al., 2002). The cyclohexenyl chalcone derivatives of a Zingiberaceae species, Boesenbergia rotunda (L.) Mansf., are reported to be anti-inflammatory (Tuchinda et al., 2002; Yun et al., 2003), strongly anti-mutagenic (Trakoontivakorn et al., 2001) and Den2 virus protease inhibiting (Tan et al., 2006). Further, curcumin from tumeric (Curtuma longa L.) has been widely studied and reported to exhibit anti-bacterial (Bhavanishankar and Murthy, 1985), anti-inflammatory (Rao et al., 1982) and hypcholesteremic effect (Rao et al., 1970; Patil and Srinivasan,
In this paper, we describe the Den2 virus NS2B/NS3 protease inhibitory properties of six selected Zingiberaceae species comprising five Curcumas and one Zingiber.

**MATERIALS AND METHODS**

**Expression and Purification of Den2 Virus NS2B/NS3 Protease**

The expression and purification of Den2 virus NS2B/NS3 protease are as reported by Tan et al. (2006). The protein precursor consisting of N-terminal hexahistidine tag fused sequentially to 40-residue NS2B cofactor, a linker of 10 residues and the first 185 amino acids of NS3 was expressed using transformed competent *Escherichia coli* strain XL1-Blue MRF; then harvested, purified and refolded following established procedures to yield proteolytically active protease complex, Den2 virus NS2B/NS3 protease (Murthy et al., 1999; Yuso et al., 2000). Large quantity of competent *Escherichia coli* strain XL1-Blue MRF was cultured in Luria-Bertani broth (LB) medium in the presence of ampicillin (100 µg/ml) at 37°C. Isopropyl-β-D-thiogalactoside (IPTG) was used to induce protease expression. The Den2 virus NS2B/NS3 protease was isolated using Ni²⁺-nitritotriacetic acid (NTA)-agarose resin affinity column (Qiagen, Chadowrth, CA) and subsequently purified using Sephadex G-75 gel filtration column (Amersham, Pharmacia Biotech, Piscataway, NJ). The ammonium sulfate precipitated proteins were then refolded by successive dialysis to yield the active Den2 virus NS2B/NS3 protease which was then stored at -70°C until used. A 12% SDS-polyacrylamide gel electrophoresis (PAGE) was used to track the protease-containing fractions both after Ni²⁺-NTA and Sephadex G-75 gel filtration, and the purity of the enzyme determined after renaturing by dialysis. Protein concentration was determined by the Bradford method using a Shimadzu UV-160 A spectrophotometer (Shimadzu Corp., Japan).

**Extraction**

All rhizomes, purchased from a local traditional herb supplier, were thoroughly flushed and rinsed in multiple changes of water. The rhizomes were sliced thinly and air-dried until constant mass. The dried slices were pulverized using a blender and the powder was soaked in methanol for several days. The methanol extract obtained after filtration was evaporated at 35°C and the resultant slurry liquid was partitioned with equal volume of ethyl acetate and water. The ethyl acetate layer was evaporated. The resultant thick brown liquid was further partitioned with equal volume of methanol and hexane and the solvents were then evaporated. The crude extract and methanol fractions were bio-assayed in methanol, and the hexane fractions in hexane.

**Den2 Virus NS2B/NS3 Protease Inhibition Bioassay using Fluorogenic Peptides**

NS2B/NS3 inhibition bioassay protocol was as reported by Tan et al. (2006). The standard 100 µL reaction mixtures comprised 100 µM fluorogenic peptide substrate Boc-Gly-Arg-
Arg-MCA (Peptide Institute, Inc., Osaka, Japan), 2 µM Den2 NS2B/NS3 protease, and with or without Zingiberaceae extract of varying concentrations buffered at pH 8.5 by 200 mM Tris-HCl. Every sample of extract was assayed at three different concentrations; 100, 200 and 300 ppm. Each test was done in quadruplicate. After enzyme addition, the reaction mixtures were incubated for 30 min at 37°C. The reaction was terminated with 1.9 mL of 125 mM ZnSO₄ (Brenner et al., 1992). The resultant precipitate was removed by micro-centrifugation for 3 min at 13,000 rpm and the supernatant was fluorometrically determined for the cleaved product (7-amino-4-methyl-coumarin) concentration at excitation λ = 385 nm and emission λ = 465 nm using a Shimadzu spectrofluorometer (model RF-5301PC; Shimadzu Corp., Japan). Four readings were taken each at time interval of 5 s per test and the three most consistent readings (percentage standard deviation <5%) were accepted.

RESULTS AND DISCUSSION

Den2 Virus NS2B/NS3 Protease Inhibitory Properties of Zingiberaceae Extracts

The percentage inhibition of Den2 virus NS2B/NS3 protease cleavage of the substrate showed linear dose-dependent increment for all the samples tested (Table I). A 2-way ANOVA with Tukey test as the post hoc test showed interaction between concentration and extract type. Consequently, a 1-way ANOVA with Tukey test as the post hoc test was carried out to check significance of inhibition between different extracts of each species at a given concentration. The test showed that all mean percentage inhibition of the crude and fractions for a given extract concentration of each species differed at 5% level of probability. The mean percentage inhibition of the crude and the fractions were significantly different at each extract concentration for each species. It suggests that the combined activity of the more polar compounds in methanol was significantly different from those of the less polar compounds in hexane. Similarly, the mean percentage inhibition of the crude extract differed significantly from those of the two partitioned fractions at all three concentrations for each species.

Generally, the crude extracts were less inhibiting than the best of the partitioned fractions. For all three concentrations studied, the methanol fractions of Curcuma longa (L.)(CL), Zingiber zerumbet Smith (ZZ) and Curcuma rubescen Roxb (CR) were consistently more inhibiting than the corresponding hexane fractions. However, the hexane fraction (79.4±3.4%, 300 ppm) of Curcuma aeruginosa Roxb.(CA) was more inhibiting than its methanol fraction (44.8±2.4%, 300 ppm) while the inhibiting activities of the two partitioned fractions of Curcuma mangga Roxb.(CM) and Curcuma xanthorrhiza Roxb.(CX) were found to be similar. The CL methanol fraction at 300 ppm exhibited the strongest inhibitory activity of 91.3±3.1% whereas its hexane fraction was only weakly inhibiting (18.3±1.4%). ZZ methanol fraction negated 89.0±1.7% (300 ppm) but its hexane fraction negated only 46.6±3.1% (300 ppm) of the Den2 virus protease activity. The difference in the inhibitory activities of the methanol fraction over the hexane fraction was less contrasting in the case of CR, nevertheless its methanol fraction was twice as inhibiting as
Table 1. Inhibition of Dengue-2 Virus Protease NS3 Cleavage of Fluorogenic Peptide Substrate Boc-Gly-Arg-Arg-MCA

<table>
<thead>
<tr>
<th>Extract</th>
<th>Mean Percentage Inhibition ± S.D. (Standard Deviation)</th>
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<tbody>
<tr>
<td></td>
<td>CA</td>
</tr>
<tr>
<td>Crude extract/ppm</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>34.9±1.4</td>
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<tr>
<td>200</td>
<td>42.2±1.7</td>
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<tr>
<td>300</td>
<td>55.1±2.8</td>
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<tr>
<td>Methanol fraction/ppm</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>26.4±0.7</td>
</tr>
<tr>
<td>200</td>
<td>35.8±0.3</td>
</tr>
<tr>
<td>300</td>
<td>44.8±2.4</td>
</tr>
<tr>
<td>Hexane fraction/ppm</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>43.5±2.1</td>
</tr>
<tr>
<td>200</td>
<td>61.9±1.7</td>
</tr>
<tr>
<td>300</td>
<td>79.4±3.4</td>
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</table>

For each given extract concentration of a species, all the mean values between extract and different fractions differed at 5% level of probability by Tukey test.
CA: Curcuma aeroginosa Roxb.; CL: Curcuma longa L.; CM: Curcuma mangga Val.;

hexane fraction (67.8 ±1.0 % and 30.3±2.1 % respectively, 300 ppm). The methanol and hexane fractions of CM had comparable inhibition of 84.3±1.7% and 87.6±2.8% respectively at 300 ppm. Both partition fractions of CX extract were similarly inhibiting but not as potent as those of CL, ZZ, CA and CM. The CR hexane fraction was weakly inhibiting unlike its methanol fraction (67.8±1.0%, 300 ppm).

The results show that the methanol fraction of CL and ZZ, and both methanol and hexane fractions of CM were most potent against Den2 virus NS2B/NS3 protease. These results suggest that the active compounds of CL and ZZ were probably adequately polar in character to be partitioned into methanol. However, the active compounds of CA were possibly more lipophilic in character. The CM extract probably had at least two different active compounds of considerable different polarity since both its hexane and methanol fractions were of comparable activities. Further activity-guided isolation and purification of the most active fraction of CL, ZZ, CA and CM followed by spectroscopic analysis are needed to establish the nature of the active compounds in these fractions.

However, the best of the inhibitory activities of these partitioned fractions were lower than those reported for pure Zingiberaceae compounds such as the anti-HIV-1 protease activity of cardamonin (Tewtrakul et al., 2003) and the Den2 virus NS2B/NS3 protease inhibition by the cyclohexenyl chalcone derivatives (Tan et al., 2006). Both sets of compounds were extracted from a Zingiberaceae species, Boesenbergia rotunda (L.). Nevertheless, it is reasonable to suggest that the purified compounds of these fractions will show much higher inhibitory activities towards Den2 virus NS2B/NS3 protease since the activities were already good even for extract partitioned fractions. When isolated and
identified, the structures of the compounds in the methanol fractions of CL, ZZ and CM and the hexane fractions of CM and CA may provide insights to the mechanism of inhibition, and possibly lead to the development of effective anti-viral agents.

ACKNOWLEDGEMENT

The authors would like to thank Moy Tow Yoon of Sunway University College for her assistance in the statistical analysis.

REFERENCES


