P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia
Vol.25 No.3 July - September 2020
Page: [127-130]
Original research article

The Effects of Stress Conditions on Antibacterial Activities of *Zanthoxylum rhetsa* Fruit Extract

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Received 3 October 2019; Received in revised form 4 July 2020
Accepted 4 August 2020; Available online 3 November 2020

ABSTRACT

Zanthoxylum rhetsa (Ma-kwaen) fruit is widely distributed in the northern part of Thailand. It is used for food flavoring and treating diseases of the oral cavity by folk doctors in the same manner as an antibiotic. In our previous study, 50% ethanolic extract of *Z. rhetsa* fruit exhibited antibacterial activities against oral pathogens *Streptococcus mutans*, *Streptococcus pyogenes,* and *Staphylococcus aureus*. The aim of this study was to investigate the effects of degradative stress conditions (thermolysis, hydrolysis, acid hydrolysis, alkaline hydrolysis, and oxidation) on *Z. rhetsa* antibacterial activity, in order to obtain relevant data on the extract before product formulation. The extract was treated with H_2O, 3N HCl, 3N NaOH, 30% H_2O_2 and heated to 80°C on a water bath for 3 hours. All extracts were tested for antibacterial activity by the determination of minimal inhibitory concentration (MIC). The extract, which had gone through thermolysis, hydrolysis, and oxidation, as well as the control, showed antibacterial activities against *S. mutans*, *S. pyogenes* and *S. aureus* with the same MIC values of 0.078, 0.156, and 0.313 mg/mL, respectively. The acid-hydrolysed extract showed MIC values of 1.25, 0.625, and 2.5 mg/mL and the alkaline-hydrolysed extract showed MIC values of 0.313, 0.625, and 0.625 mg/mL, respectively. These results indicated that acid and alkaline hydrolyses lowered the extract's antibacterial activity. Therefore, pH should be considered when Ma-kwaen extract preparation is formulated, i.e. it should be adjusted to neutral to maintain its antibacterial activity.

Keywords: Stress test; Antibacterial activity; *Zanthoxylum rhetsa* fruit extract; Ma-kwaen

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doi: 10.14456/scitechasia.2020.41

1. Introduction

Zanthoxylum rhetsa (syn. *Z. limonella*) is widely distributed in the northern part of Thailand such as in the Chiang-rai province, where it is locally known as "Ma-kwaen", and is used for food flavoring and, globally in ethno-medicine, for the treatment of toothaches, dental caries, and periodontitis [1]. Antimicrobial activity of the active compounds from the plant extract may combat the diseases through inducing leakage of the microbes' cytoplasmic membrane , damaging their outer and inner membranes [2]. In our previous study, we found that 50% ethanolic extract of *Z. rhetsa* fruit exhibited antibacterial activities against oral pathogens: *S. mutans*, *S. pyogenes,* and *S. aureus* with minimal inhibitory concentrations (MIC) of 0.078, 0.156, and 0.313 mg/mL, respectively [3].

In this study, we investigated the effect of stress degradation on the antibacterial activity of *Z. rhetsa* extract. The stress conditions studied were thermolysis, hydrolysis, acid hydrolysis, base hydrolysis, and oxidation. Results of this study were expected to provide relevant information required for the development of suitable product formulations containing 50% ethanolic extract of *Z. rhetsa* fruit.

2. Materials and Methods

2.1 Plant materials

The dry fruits were harvested in its natural habitat of Chiang-rai, Thailand, in 2018. The voucher specimen BKF NO. 193834 was deposited at The Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Thailand. The dry fruit was ground to coarse powder and macerated with 50% ethanol for three days, then filtered and evaporated to dryness in a vacuum evaporator.

2.2 Microbial strains and reagents

The bacteria used in this study were standard strains of *S. mutans* ATCC 25175, *S. pyogenes* ATCC 19615, and *S. aureus* ATCC 25923. Nutrient agar (NA; Difco, Detroit, MI) and brain heart infusion agar (BHI agar; Difco) were used as subculture media for all tested bacteria. The bacteria were stored in 20% glycerol broth at -70°C and sub-cultured on the appropriate fresh agar plates for 24 hours prior to antibacterial activity assay. Ampicillin and vancomycin (Sigma-Aldrich, Welwyn Garden City, UK) were used as positive controls.

2.3 Stress test of the 50% ethanolic extract of *Z. rhetsa* fruit

Degradative stress conditions (stress test) [4], including thermolysis, hydrolysis, acid hydrolysis, alkaline hydrolysis, and oxidation, were applied to the bacteria before testing for their antibacterial activity.

Thermolysis: The extract was accurately weighed to 50 mg and placed in a vessel. It was heated at 80°C in a water bath for three hours and dried by vacuum evaporator.

Hydrolysis: The extract was accurately weighed to 50 mg and placed in a vessel. Three drops of deionized water (DI water) was added and heated at 80°C in a water bath for three hours and dried by vacuum evaporator.

Acid hydrolysis: The extract was accurately weighed to 50 mg and placed in a vessel. Three drops of 3N hydrochloric acid (HCl) was added and heated at 80°C in a water bath for three hours. Sodium hydroxide (NaOH) was then added to adjust to neutral pH and then dried by vacuum evaporator.

Alkaline hydrolysis: The extract was accurately weighed to 50 mg and placed in a vessel. Three drops of 3N Sodium hydroxide (NaOH) were added and heated at 80°C for three hours. HCl was then added to adjust to neutral pH and then dried by vacuum evaporator.

Oxidation: The extract was accurately weighed to 50 mg and placed in a vessel. Three drops

of 30% hydrogen peroxide (H_2O_2) were added and heated at 80°C in a water bath for three hours and then dried by vacuum evaporator.

2.4 Determination of the minimal inhibitory concentration (MIC)

The MIC was determined by the broth micro-dilution method as previously reported, with some modifications [5]. The inoculum was adjusted to 0.5 McFarland standard (1.5 x 10^8 CFU/mL) and diluted with sterile Mueller Hinton Broth (MHB; Difco) for *S. aureus* and BHI broth for *S. mutans* and *S. pyogenes* at 1:200 to give a final concentration of microorganism of 5 x 10^5 CFU/mL. The 50% ethanolic extract was added with 2% (v/v) dimethylsulfoxide (DMSO; RCL Labscan, Bangkok, Thailand). Serial two-fold dilution of the extract was performed. Then, 50 µL of each concentration of the extract solution, and 50 µL of the inoculum were added into 96 well micro-plates. Ampicillin and vancomycin, in serial two-fold dilutions, were used as positive controls. The plate was agitated in a plate incubator at 37°C for 24 hours for all tested bacteria.

MICs of the samples were determined after adding 10 µL of resazurin (blue compound, 7-hydroxy-3H-phenoxazin-3-one 10 oxide) and incubating at 37°C for two hours. The results were indicated by the change in color of resazurin. The MIC value is determined as the lowest dilution with unaltered color. The assay was repeated in triplicate. Positive control, negative control, and viable control of the microorganisms were included and the results of the MIC test were recorded.

3. Results and Discussion

The 50% ethanolic extract of *Z. rhetsa* fruit had a yield of 10.89%. It was treated with different degradative stress conditions, which included thermolysis, hydrolysis, and oxidation. The results of antibacterial activity testing showed that the extract under stress conditions exhibited the same effect on the tested bacteria as that of the untreated extract with MIC values of 0.078, 0.156, and 0.313 mg/mL. On the other hand, the acid-hydrolyzed extract showed higher MIC values of 1.25, 0.625, and 2.5; similarly, the alkaline-hydrolyzed extract also showed higher MIC values of 0.313, 0.625, and 0.625 mg/mL for *S. mutans*, *S. pyogenes* and *S. aureus,* respectively (Table 1). *Z. rhetsa* has been reported to have active alkaloid compounds which have antifungal activity [6]. The characteristics of alkaloid compounds may be changed in acidic conditions to alkaloid salt and in alkaline conditions to alkaloidal base [7]. This indicated that the extract is not stable in either acidic or alkaline conditions, but heat and moisture did not affect its antibacterial activity. The active components of the extract, consisting mainly of terpenes, have a complex structure which is resistant to damage by either dry or wet heat as in the thermal or hydrolytic degradation [8]. Likewise, the oxidative degradation had no effect on the antibacterial activity of the extract. The limonene moiety in the terpenes may be decomposed to limonene epoxide and limonene oxide which also possess antibacterial activity against the tested oral pathogens [9]. Therefore, in the development of products against oral pathogens using *Z. rhetsa* fruit, the products should be formulated in such a way as to avoid acidic or alkaline conditions.

4. Conclusion

We tested the 50% ethanolic extract of *Z. rhetsa* fruit according to the guidelines provided for the analysis of the stability of pharmaceutical products and found that it could withstand degrative stresses. These results provide relevant information for the future development of oral hygiene products from this fruit. The data provided necessary information for choosing a suitable formula, packaging, storage conditions, and expected

shelf life [10]. It is crucial that the pH of the products must be kept neutral to maintain their antibacterial activities.

Acknowledgements

The authors would like to thank The Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Thammasat University, Thailand, for financial and facility support, and The Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Thailand, for identifying plant materials.

Table 1. Minimal inhibitory concentrations (MICs) of the untreated extract and extracts under different degradative stress conditions against oral pathogens.

Extracts	MIC (mg/mL) (mean±SD)		
	Streptococcus mutans	***Streptococcus pyogenes***	***Staphylococcus aureus***
Untreated	0.078 ± 0	0.156 ± 0	0.313 ± 0
Thermolyzed	0.078 ± 0	0.156 ± 0	0.313 ± 0
Hydrolyzed	0.078 ± 0	0.156 ± 0	0.313 ± 0
Oxidation	0.078 ± 0	0.156 ± 0	0.313 ± 0
Acid hydrolyzed	1.25 ± 0	0.625 ± 0	2.5 ± 0
Base hydrolyzed	0.313 ± 0	0.625 ± 0	0.625 ± 0
Ampicillin	0.0002 ± 0	0.0002 ± 0	0.0002 ± 0
Vancomycin	-	-	0.0004 ± 0

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