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Evaluation of Antimicrobial Activity of *Rhinacanthus nasutus* (L.) Kurz and *Acanthus ilicifolius* L. Extracts

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Abstract

The aim of this research was to evaluate the antimicrobial properties of medical plants *Rhinacanthus nasutus* (L.) Kurz and *Acanthus ilicifolius* L. that were extracted with water/aqueous (AqER, AqEA) and ethanol (EtER, EtEA). The extracts were tested for activity and evaluated based on the effectiveness against three strains of microorganism: Gram-positive bacteria such as *Staphylococcus aureus,* Gram-negative bacteria such as *Escherichia coli,* and fungal such as *Candida albicans* by using the agar well diffusion and broth dilution method. The extracts from *Rhinacanthus nasutus* and *Acanthus ilicifolius* with ethanol showed the effect of inhibiting all microbes. The most effective against *Candida albicans* with the similar MIC and MFC values of 18.75 and 37.50 mg/mL. Meanwhile, extracts with water of *Rhinacanthus nasutus* and *Acanthus ilicifolius* with MIC values of 37.50 and 75 mg/mL and MFC values of 75 and 150 mg/mL, respectively. Conversely, these extracts showed no effect to inhibit *Escherichia coli.* This could be due to the capabilities of the solvents extractive and using part of the plant. Likewise, a combination of the extracts with ethanol of *R. nasutus* and *A. ilicifolius* to evaluate the efficacy of synergistic herbs can be considered from the MIC value. The antimicrobial synergy was evaluated in terms of FIC obtained from multiple-combination bactericidal/fungicidal assays. FICi value was interpreted as synergy only in ethanol extract *R. nasutus+A. ilicifolius* (EtRA) of 0.26.

Introduction

Rhinacanthus nasutus (L.) Kurz belongs to Acanthaceae family, having the common name "white crane flower" and is a medicinal shrub that is widely distributed in Southeast Asia. In Thailand the local name is also referred to as "Thong phan chang" (Puttarak et al., 2010; Brimson et al., 2020). Several reports have revealed that traditional medicinal uses for the treatment of diverse diseases that include diabetes, hepatitis, tuberculosis, hypertension, inflammation, psoriasis, eczema, ringworm, antioxidant, neuroprotective, aphrodisiac, anticancer, and antimicrobial (Puttarak et al., 2010) also showed significant larvicidal activity against

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four larvae of mosquitos (Komalamisra et al., 2005). The extract of *R. nasutus* (Rn) has shown to have an effective antibiotic activity against Gram-positive strain such as *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus* (Sendl et al., 1996; Puttarak et al., 2010; Kumar et al., 2021) and against Gram-negative bacteria such as *Klebsiella pneumoniae,* followed by *Enterobacter aerogenes, Proteus mirabilis* and *Escherichia coli* of ethanol extract (Kumar et al. 2011; Sheikh & Reshi 2020). The major bioactive active compound to antimicrobial was the naphthoquinone esters, namely rhinacanthin-C, -N, -Q from ethanol and aqueous extracts (Puttarak et al., 2010; Panichayupakaranant et al., 2021).

The evergreen spiny herb named *Acanthus ilicifolius* L. with the local name "Sea holly" is in the same family of Acanthaceae. It is widely found in mangroves of southern Thailand. It has been used to treat rheumatism, asthma, paralysis, psoriasis and leucorrhoea. The antimicrobial activity of alcoholic, butanolic and chloroform extracts of leaves and roots of *A. ilicifolius* is strong against Gram-positive bacteria such as *Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis* and fungi such as *Candida albicans*, *Saccharomyces cerevisiae, Aspergillus niger, Aspergillus fumigatus, Rhizopus oryzae* while moderate inhibitory action against Gram-negative bacteria such as *Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Escherichia coli* (Siripong et al., 2006; Bose & Bose 2008; Kumar et al., 2011; Govindasamy & Arulpriya 2013; Pothiraj et al., 2021). The major bioactive active compound to antimicrobial of *A. ilicifolius* reveals the presence of 2-benzoxazolinone, lignan glucosides, benzoxazinoide glucosides, flavone glycosides and phenylethanoid glycosides (Govindasamy & Arulpriya 2013). In currency, antibiotics are widely used that can cause many bacterial mutations such as *Salmonella* spp. (Onvimol et al., 2020). The use of medicinal herbs for infectious control has started to play an increasingly important role. But the use of antibiotics alone may not be as effective as the combination. The combination of herbal extracts with antibiotics was found to be able to increase the efficiency of infection prevention (Jiang et al., 2021). Clinical studies also exhibited that patients treated with antibacterial combination therapy can obtain good clinical effect and lower mortality rates (Ni et al., 2015). However, the results of this study may reduce the medical uses of antibiotics also in the food, pharmaceutical industry including replacing preservatives in cosmetic products. The aim of this research was to evaluate the antimicrobial properties of medical plants that include *R. nasutus* (L.) Kurz and *A. ilicifolius* L. and were extracted with water/aqueous (AqER, AqEA) and ethanol (EtER, EtEA).

Materials and methods

1. Plant material

The fresh sample of *Rhinacanthus nasutus* was collected from Tatum Subdistrict, Sangkha District, Surin Province. *Acanthus ilicifolius* was collected from Laem Sak Subdistrict, Ao Luek District, Krabi Province, Thailand. The samples were compared with specimens deposited in the Bangkok Forest Herbarium.

2. Preparation of plant extracts

The fresh leaves and stem of *R. nasutus* were extracted with two solvents; aqua and 95% ethanol. For water extraction, 50 g of herbs were boiled with 500 mL of distilled water at 90°C for approximately 2 h and allowed to cool at room temperature. After that, it was filtered through a straining cloth to separate the residue and filtered with filter paper (Whatman No.1). The filtrates were pooled and evaporated on a rotary evaporator under reduced pressure at 50°C to obtain dry powder. For the ethanol extraction, 50 g of the herb was soaked with 50 mL of 95% ethanol for 7 days subsequently filtered through a straining cloth and filtered with filter paper (Whatman No.1), concurrently. The filtrates were pooled and evaporated on a rotary evaporator under reduced pressure at 50°C to obtain a crude residue and concentrated at 45°C approximately 12 h. Similarly with the solvent and procedures for fresh leaves and stem of *A. ilicifolius*. These extracts were kept in the refrigerator until usage (applied from Santos et al., 2002; Puttarak et al., 2010).

3. Microorganisms tested

The standard strain of bacteria and fungus used to evaluate the antibacterial properties of medical herb plants were obtained from the Department of Medical Sciences, Ministry of Public Health, Nonthaburi that include *Staphylococcus aureus* (DMST 8840), *Escherichia coli* (DMST 7948) and *Candida albicans* (DMST 8684). Pure cultures of these bacteria and fungus strains were grown on Mueller Hinton agar (MHA) and Sabouraud dextrose agar (SDA) consequently and maintained on agar slant at 4°C until used.

4. Determination of antibacterial activity

Antimicrobial activity of 95% ethanol and aqueous

extracts of *R. nasutus* (EtER, AqER) and *A. ilicifolius* (EtEA, AqEA) were tested by agar well diffusion method (applied from Jeyaseelan et al., 2012; Chanda et al., 2013) using MHA and SDA medium for bacteria and fungus to screen the plant extracts for antimicrobial activities. Briefly, MHA or SDA plates were prepared by incorporating 1 mL of test bacteria or yeast (0.5 McFarland turbidity standards) into 20 mL of molten medium at 45°C. After solidification of the medium, wells were made using 8 mm diameter of sterile stainless steel cork borer, and 100 µL of each of the test extracts (300 mg/mL), the antibiotic tetracycline (125 mg/mL) for bacteria or ketoconazole (200 mg/mL) for yeast were used as a positive control and negative control (0.2% DMSO) were added into the wells separately. This was the initial concentration of the extract used to check the antimicrobial activities of the extracts from the plant. Plates were incubated at 35-37°C for 24 h. Finally, the antimicrobial activity of the test extracts were determined by measuring the diameter in millimeters of clear zone around the well. Each experiment was performed in triplicate and the average value of inhibition was calculated.

5. Minimum inhibitory concentration (MIC)

Antibacterial activity of *R. nasutus* (EtER, AqER) and *A. ilicifolius* (EtEA, AqEA) extracts were determined by the broth dilution method (Chic & Amom 2014). All dissolved extracts were serially diluted two-folds in Muller-Hinton broth/Sabouraud dextrose broth to give a final concentration ranging from 4.69-300 (4.69, 9.38, 18.75, 37.50, 75, 150 and 300) mg/mL. An aliquot (0.1 mL) of overnight broth culture of test microorganism (approximately concentration 1.5×10^8 cfu/mL of bacteria and 2.0×10^5 spores (yeast cell)/mL for fungal that were adjusted by using 0.5 McFarland standard) in sterile normal saline was introduced into each extract dilution. The mixtures in sterile test tubes were incubated (37ºC, 24 h) and observed for turbidity (signifying growth) or absence of it (signifying inhibition). Tetracycline (antibacterial drug) at the concentrations of 125 mg/mL and ketoconazole (antifungal drug) at the concentrations of 200 mg/mL were used as a positive control agent, and sterile normal saline as negative control. The MIC (Minimum Inhibitory Concentration) is the lowest concentration of an antimicrobial agent or extract solution that inhibits or prevents growth as determined visually after a standard incubation period of 18-24 h at 35-37°C.

6. Minimum bactericidal concentration (MBC)

A loopful (1 µL) of the test mixture was transferred from each MIC tube that showed no growth or no turbidity, inoculated onto Mueller-Hinton/Sabouraud dextrose agar plate, incubated at 37ºC for 24 h, and inspected for the presence of colonies indicating growth. The MBC (Minimum Bactericidal Concentration) and MFC (Minimum Fungicidal Concentration) is the lowest concentration of the antimicrobial agent or extract solution that shows no growth, defined as a 99.9% reduction in the initial inoculum, after a subculture of all the dilutions that showed no bacterial or fungal growth in the MIC test (Magaldi et al., 2004).

7. Fractional inhibitory concentration (FIC) and ΣFIC determinations

According to Doern (2014) and Tiwana et al. (2020) the extracts solution showing appreciable antibacterial activity were tested in combination with each other, to test whether interactions occurred between *R. nasutus* (EtER, AqER) and *A. ilicifolius* (EtEA, AqEA) extracts. Initially, 1:1 ratio of two different extracts (concentration 300 mg/mL) were tested based on the hypotheses of mutant selection window (MSW) and mutant prevention concentration (MPC) (Drlica, & Zhao 2007; Xu et al., 2018; Jiang et al., 2021). Interactions between extracts were examined by determination of the sum of fractional inhibitory concentrations (ΣFIC) for each combination. The FIC values for each component (a and b) were calculated using the following equations where a and b represent the two plant extracts being tested:

FIC (a) = MIC (a in combination with b) / MIC (a alone)

FIC (b) = MIC (b in combination with a) / MIC (b alone)

ΣFIC was then calculated using the formula ΣFIC = FIC (a) + FIC (b). The interactions were classified as synergistic ($\Sigma FIC \leq 0.5$), additive ($\Sigma FIC > 0.5$ to ≤ 1.0), indifferent ($\Sigma FIC > 1.0$ to ≤ 4.0) or antagonistic ($\Sigma FIC > 4.0$ The multiple-combination bactericidal/fungicidal assays were inoculated with the test microorganism to a final approximate concentration of 1.5×10^8 CFU/mL (using 0.5 McFarland standard) and incubated at 37°C for 24-48 h. The inspection for turbidity, and without visual evidence of growth were sub-cultured to MHA or SDA medium and assessed after overnight incubation for 99.9% killing.

8. Statistical analysis

Values are represented as mean ± SD. All analyses were done as three biologically independent experiments.

The student's t test was used to determine the statistical significance of differences between groups in EtER-AqEr and EtEA-AqEA expression. A value of p = 0.05 was considered statistically significant.

Results and discussion

1. Plant extracts

The profiles of *R. nasutus* and *A. ilicifolius* extracts are shown in Table 1. The highest yield of extract was with aqueous (7.66%), whilst the least yield was obtained with ethanol (4.15%).

Table 1 Yield and other physical properties of medical herb plants extracts

Plant name	Solvent extractant	Method to extraction	Yield (%)	Colour and consistency
R. nasutus	Ethanol	Maceration	4.15	Deep green, Gummy solid
	Aqueous	Digestion	6.89	Dark brown almost black, solid in form of dry powder
A. ilicifolius	Ethanol	Maceration	5.40	Deep green, Gummy solid
	Aqueous	Digestion	7.66	Dark brown almost black, solid in form of dry powder

2. Antimicrobial assay

All four extracts of *R. nasutus* (EtER, AqER) and *A. ilicifolius* (EtEA, AqEA) showed different degrees of activity against bacterial and fungal. Preliminary testing to confirm the antimicrobial activity of EtER and EtEA extracts by agar well diffusion method showed that both plant extracts had inhibitory activity against Gram-positive bacteria such as *Staphylococcus aureus* with mean clear zone of inhibition of 29.33 ± 1.15, 23.67 ± 0.58 mm., Gram- negative bacteria such as *Escherichia coli* with mean clear zone of inhibition of 22.33 ± 0.58, 18.00 ± 0.00 mm., and fungal such as *Candida albicans* with mean clear zone of inhibition of 17.67±0.58, 14.33±0.58 mm., respectively, as shown in Table 2. While the antimicrobial efficacy of AqER and AqEA extracts showed only inhibitory activity against *S. aureus* and *C. albicans* without ability to inhibit Gram-negative bacteria such as *E. coli* and had less of the zone of inhibition. It was shown that extraction of *R. nasutus* and *A. ilicifolius* by using 95% ethanol had more antimicrobial effect than aqueous extraction.

3. The MIC and MBC (MFC)

The antimicrobial activity of the four extracts (EtER, AqER, EtEA, AqEA) potency was quantitatively assessed by the MIC and MBC (MFC) values of the extracts. The MIC and MBC (MFC) values were between 18.75 to 37.50 mg/mL and 18.75 mg to 75 mg/mL for ethanol extract of *R. nasutus* and *A. Ilicifolius,* respectively, which were found to be better than the MIC and MBC (MFC) values of aqueous extracts (Table 2). The highest MIC and MBC (MFC) values were recorded of 150 mg/mL for the aqueous extract of both *R. nasutus* and *A. ilicifolius*. We found that the lowest MIC and MBC (MFC) were 18.75 mg/mL and 37.50 mg/mL for ethanol extract of *R. nasutus* (EtER), which showed the activity against Gram-positive bacterial and fungal while the lowest MIC and MBC was 37.50 and 75 mg/mL of both EtER and EtEA showed the activity against Gram-negative bacterial. Conversely, neither AqER nor AqEA were found to be effective against Gram-negative bacteria.

However, EtER and EtEA have shown antimicrobial efficacy with MIC and MBC (MFC) values between 18.75 to 75 mg/mL. Consistent with the experiment of Bose & Bose (2008) that studied antimicrobial activity of *A. ilicifolius* extract by using ethanol and results exhibited strong inhibitory action against Gram-positive bacteria, fungi and showed moderate activity in Gram-negative bacteria. While Govindasamy, & Arulpriya (2013) conclusion that aqueous extract of *A. ilicifolius* included a variety of bioactive components, including alkaloids, saponins, phenolics, flavonoids, steroids, cardiacglycosides, tannins, and terpenoids showed minimum activity against both bacterial and fungal species. Similarly, these phytochemical compounds are found in both *A. ilicifolius* and *R. nasutus* but the naphthoquinone rhinacanthin is not contained in *A. ilicifolius* while the glycosides are not found in *R. nasutus*. Conversely, Pothiraj et al. (2021) reported that the aqueous extract of *A. ilicifolius* was more effective against Gram-negative bacteria than against Gram-positive bacteria of inhibition zone being between 9.6 ±0.01-11.1±0.10 mm and that probably the extract of leaves of polar solvent inhibits Gram-negative bacteria in a dose-dependent manner. This consideration is also compatible, with the observations of Park et al. (2016) who recommended that the action could potentially be explained by changes in the quantities of phytoconstituents and bioactive components restrictive in the crude extract, and the capabilities of the solvent extractive.

For the extractive values as shown in Table 2, the ethanol extract of *R. nasutus* (EtER) and *A. ilicifolius* EtEA indisputably showed antibacterial activity against Gram-positive *S. aureus* more aqueous extract with an inhibition zone of 29.33 ± 1.15 and 23.67 ± 0.58 mm, respectively. Followed by Gram-negative *E. coli* with an

Table 2 Antimicrobial activity of leaf and stem extracts of *R. nasutus* and *A. ilicifolius*

Plants	Extracts	*Staphylococcus aureus*			*Escherichia coli*			*Candida albicans*		
		Inhibition zone (mm)	MIC (mg/mL)	MBC (mg/mL)	Inhibition zone (mm)	MIC (mg/mL)	MBC (mg/mL)	Inhibition zone (mm)	MIC (mg/mL)	MFC (mg/mL)
R. nasutus	EtER	29.33 ± 1.15	18.75	37.50	22.33 ± 0.58	37.50	75.00	17.67±0.58	18.75	37.50
	AqER	14.33 ± 0.58	75.00	150.00	NA	NA	NA	16.67±1.15	37.50	75.00
	EtER - AqEr	15.00 ± 0.82*	-56.25*	-112.5*				1.00±0.67*	-18.75*	-37.5*
A. ilicifolius	EtEA	23.67 ± 0.58	37.50	75.00	18.00 ± 0.00	37.50	75.00	14.33±0.58	18.75	37.50
	AqEA	12.00 ± 1.00	150.00	150.00	NA	NA	NA	14.00±0.00	75.00	150.00
	EtEA- AqEA	11.67 ± 0.74*	-112.50*	75.00*				0.33±0.28*	-56.25*	-112.5*
Control										
Tetracycline		41.00 ± 1.15	31.25	62.50	38.33 ± 0.58	31.25	62.50	NT	NT	NT
Ketoconazole		NT	NT	NT	NT	NT	NT	43.00±1.15	12.50	25.00
DMSO		NA	NA	NA	NA	NA	NA	NA	NA	NA

Remark: EtER= ethanol extract of *R. nasutus,* AqER= aqueous extract of *R. nasutus,* EtEA= ethanol extract of *A. ilicifolius,* AqEA= aqueous extract of *A. ilicifolius,* NA = No activity, NT = Not tested; *Significance level (p = .05)

inhibition zone of 22.33 ± 0.58 and 18.00 ± 0.00 mm, respectively. While EtEA and AqEA showed similar antifungal activity against *C. albicans* with an inhibition zone of 14.33±0.58 and 14.00±0.00 mm, respectively. Although in both plants the alcohol extract showed antimicrobial activity, it was significantly less than that of tetracycline except for EtER. Therefore, this study shows that the extraction of *R. nasutus* and *A. ilicifolius* with alcohol has a greater inhibition effect on microorganisms than the extraction with aqueous.

Previous research (Panichayupakaranant et al., 2009; Maheshu et al., 2010; Puttarak et al., 2010; Bukke et al., 2011; Jayapriya 2015; Antonysamy 2017; Lukiati et al., 2019; Mondal et al., 2021) reported that the phytochemical compounds of *R. nasutus* contained from steroids, alkaloids, tannins, saponins, flavonoids, triterpenes, naphthoquinone rhinacanthin-C, D, N. These chemical compounds have antimicrobial activity that are extracted from different kinds of solvents such as chloroform, methanol, ethanol, ethyl acetate and aqueous, of which ethyl acetate extracts of *R. nasutus* showed maximum metabolites occurrence followed by alcoholic extract. The mechanism of polyphenol which is contained in *R. nasutus* and in *A. ilicifolius* as antibacterial were able to disrupted cell membrane permeability while hydroxyl group could form a hydrogen bond with positively charged nitrogen and oxygen in protein of cell membrane. Hydroxyl group also formed an ionic bond with positively charged amine in protein of cell membrane. The damage of membrane caused increase permeability and cell leak which are followed by intracellular material discharge that causes obstructed cell growth or cell death in microorganisms (Wink, 2015; Singh, 2017). In the same way, this study of 95% ethanol of *R. nasutus* showed the potency of antimicrobial activity more than aqueous and indicates that ethanol could be extracted from the phytochemical compounds of *R. nasutus* greater. Similarly in *A. ilicifolius* no naphthoquinone rhinacanthin was found in this plant. For this reason, *R. nasutus* exhibited more efficacy antimicrobial activity than *A. ilicifolius.* Although in this study the activity of each phytochemical compounds was not studied.

Likewise, Puttarak et al. (2010) found that rhinacanthin-C, -D, and -N in *R. nasutus* extract with ethyl acetate are active to kill *Streptococcus mutans* with MIC and MBC of 4 mg/mL, and potent activity against *Staphylococcus epidermidis, S. aureus* and *Cutibacterium acnes* (*P. acnes*), with the MICs of 8 to 16 mg/mL. Moreover, its extract showed no activity against *C. albicans* at concentration up to 2000 mg/mL. However, Panichayupakaranant et al. (2009) showed that the activity of *R. nasutus* against pathogenic fungal of *Trichophyton* spp., *Microsporum* sp. and *Candida albicans*. The ethanolic crude extract of *A. ilicifolius* (stem bark) dominates many important secondary metabolites, including alkaloids, flavonoids, terpenoids, phenols, glycosides, steroids and tannins. The extract showed moderate antibacterial activity against Gram-positive *Bacillus subtilis* and *B. megaterium* MIC value of 46.875 µg/mL, and Gram-negative *E. coli* MIC value of 750 µg/mL, this result was reported in 2021 by Mondal et al. Similarly, in this study ethanolic crude extract of *A. ilicifolius* showed activity against *E. coli,* though the MIC value had a higher mean of 37.50 mg/mL. This could be due based on the capabilities of the

solvents extractive and using part of the plant.

Based on Table 2, the mean difference of statistics test with t-test, found that the values between EtER-AqEr and EtEA-AqEA were significantly different ($p<0.05$), with EtER-AqEr being higher than EtEA-AqEA. This indicated that that the potency of antimicrobial activity from *R. nasutus* extracts were better than *A. ilicifolius,* in accordance with previous studies as explained above. While water/aqueous is a solvent with higher polarity than ethanol, enabling the extraction of phytochemicals to be greater, which is a bioactive substance that can inhibit many microorganisms as mentioned above. Moreover, aqueous has also been used as a safety or green solvent with optical extraction for polyphenols compared to organic solvents and for different classes of chemical compounds like flavonoid, organic acid, and alkaloid. Dielectric constant or polarity of water can be manipulated by the application of temperature making it suitable for extraction of polar, moderately polar and nonpolar compounds.

4. Fractional inhibitory concentration (FIC) determinations

The antimicrobial synergy between *R. nasutus* and *A. ilicifolius* extract was evaluated in terms of FIC obtained from multiple-combination bactericidal/fungicidal assays. Antagonism or synergism is a negative or positive effect observed when the combined effect of the drugs or agent substance is significantly less/more than expected (Renneberg 1993; Doern 2014; Gan et al., 2020). Interpretation of the fractional inhibitory concentration index (FICi) was followed as described by Doern (2014) and Gopal et al., (2014). We considered a synergistic effect for FICi < 0.5; partial synergy for $0.5 \leq$ FICi < 1; additive for FICi = 1; indifferent for 1 < FICi < 4; antagonism for FICi ≥ 4. In this study, the combination *R. nasutus* and *A. ilicifolius* extract with ethanol and aqueous found that the FICi value showed in Table 3 were interpreted as synergy only in ethanol extract *R. nasutus*+*A. ilicifolius* (EtRA). Our result may be the first FICi report of the combination between *R. nasutus* and *A. ilicifolius.* For the calculation of FICi value was applied from van Vuuren and Viljoen (2011).

FIC of *R. nasutus* (ethanol extract) = MIC of *R. nasutus* in combination with *A. ilicifolius*/MIC of *R. nasutus* alone = (10/18.75 = 0.53)

FIC of *A. ilicifolius* (ethanol extract) = MIC of *A. ilicifolius* in combination with *R. nasutus*/MIC of *A. ilicifolius* alone = (10/37.5 = 0.27)

Table 3 Fractional inhibitory concentration (FIC) value combine of *R. nasutus* and *A. ilicifolius*

Solvent	Plant	MIC (300 mg/mL) alone	MIC (300 mg/mL) combine	*FICi value	Interpretation
95%Ethanol	*R. nasutus*	18.75 (EtER)	10 (EtRA)	0.53	Additive
	A. ilicifolius	37.50 (EtEA)	10 (EtRA)	0.26	Synergy
Aqueous	*R. nasutus*	75 (AqER)	150 (AqRA)	2	Indifference
	A. ilicifolius	150 (AqEA)	150 (AqRA)	1	Additive

Remark: EtER= ethanol extract of *R. nasutus,* AqER= aqueous extract of *R. nasutus,* EtEA= ethanol extract of *A. ilicifolius,* AqER= aqueous extract of *A. ilicifolius,* EtRA= ethanol extract of *R. nasutus*+*A. ilicifolius,* AqRA= aqueous extract of *R. nasutus*+*A. ilicifolius,*
*FICi value: ≤0.5 = Synergy, ≥0.5-1 = Additive, 1-4 = Indifference, >4= Antagonism

Nevertheless, sometimes the use of a single antibiotic or substance does not produce the desired or effective inhibitory effect, and to overcome this, treatment with a combination of drugs or substance may be attempted, their synergistic effect often surpasses their individual execution. In this study, the synergistic effect resulting from the combination of two plant extract both potency antimicrobial with crude plant extract with different solvent was verified against all tested microorganisms and consistent with the experiment above though augmentative research is required to extract and identify bioactive chemicals in order to create new antimicrobial substance from Thai medical plants. The FICi values 0.26 of EtEA+EtRA had shown a synergistic effect, this result confirms that some phytochemicals contained in *A. ilicifolius* may have a better ability against microorganisms and more active compounds than *R. nasutus.* Although MIC alone of EtER is better than EtEA and needs to be investigated in depth.

Conclusion

The medicinal plants *R. nasutus* and *A. ilicifolius* were extracted with aqueous and 95% ethanol. The antimicrobial activity of the four extracts (EtER, AqER, EtEA, AqEA) potency was quantitatively assessed by the MIC and MBC (MFC) values of the extracts. The MIC and MBC (MFC) values were between 18.75 to 37.50 mg/mL and 18.75 mg to 75 mg/mL for ethanol extract of *R. nasutus* and *A. Ilicifolius,* respectively, which were found to be better than the MIC and MBC (MFC) values of aqueous extracts. The lowest MIC and MBC (MFC) was 18.75 mg/mL and 37 mg/mL for ethanol extract of *R. nasutus* (EtER) and showed activity against Gram-positive bacterial and fungal. While the lowest MIC and MBC was 37.50 and 75 mg/mL of both EtER

and EtEA and showed activity against Gram-negative bacterial. Conversely, neither AqER nor AqEA were found to be effective against Gram-negative bacteria. As a result, the action could potentially be explained by changes in the quantities of phytoconstituents and bioactive components restrictive in the crude extract, and the capabilities of the solvents extractive. The antimicrobial synergy between *R. nasutus* and *A. ilicifolius* extract was evaluated in terms of FIC obtained from multiple-combination bactericidal/fungicidal assays. FICi value were interpret as synergy only in ethanol extract *R. nasutus*+*A. ilicifolius* (EtRA) of 0.26. Furthermore, augmentative research is required to extract and identify bioactive chemicals in order to create new antimicrobial substance from Thai medical plants.

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