

**ANTIMICROBIAL EFFECT OF ROSELLE (*HIBICUS SABDARIFFA* LINN.)
EXTRACT AGAINST BACTERIAL PATHOGENS ASSOCIATED IN THAI
FERMENTED MEAT (NHAM)**

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Roselle, a Thai medicinal plant, has been known to exhibit antioxidant and antimicrobial activities. The study of antimicrobial activities of roselle extract (RE) at different concentrations (25, 50, 100, 150 and 200 mg/ml) were tested against 6 strains of bacteria associated in retailed meat and Thai fermented meat (Nham) [Four strains of gram-positive : *Staphylococcus aureus*, *Listeria innocua*, *Lactobacillus plantarum* and *Pediococcus pentosaceus*, and two strains of gram-negative : *Escherichia coli* and *Salmonella anatum*]. The results revealed that RE at 25 mg/ml showed an inhibitory effect only on *Lis. innocua*, while at 50 mg/ml inhibited *Lis. innocua* and gram-positive pathogen of *Staph. aureus*. The RE extract at 100 mg/ml exhibited wide range of inhibitory effect on both of gram-positive and gram-negative tested strains except two strains of lactic acid bacteria associated in the fermentation of Nham, while RE at 150 and 200 mg/ml could inhibited all the tested indicator strains. In order to confirm the inhibitory effects were not due to the lower pH of RE, three different concentrations of citric acid solution (CAS) with pH corresponding to the pH of RE at 100, 150 and 200 mg/ml were used to evaluate the inhibition of aforementioned 6 indicators. Wider inhibition zones were observed for RE compared to CAS at all concentrations evaluated. In addition, no inhibition zone for 2 LAB indicator strains was found with CAS. The concentration of RE at 25 – 50 mg/ml (w/v) in trypticase soy broth + 0.6 % yeast extract (TSBYE) exhibited a bactericidal effect on the cells of *Staph. aureus*, *Salm. anatum*, *Lis, innocua* and *E. coli*. RE concentration at 12.5 mg/ml showed a bactericidal effect only on *Staph. aureus* and *Salm. anatum*, while this same concentration implied a bacteriostatic effect on *Lis, innocua* and *E. coli*. This study implied that RE contain other components, in addition to organic acids, with antimicrobial activity. Moreover, the concentration of RE at 12.5 - 50 mg/ml in collaboration with important LAB as starter for Nham production may potentially be used to produce a safety of this Thai fermented meat product.

Introduction

Roselle (*Hibiscus sabdariffa* Linn.), one of Thai medicinal plants, is known to contain abundant phenolic compounds such as anthocyanin. The efficacy of this substance in Roselle extract against some gram-positive and gram-negative bacteria is well established when using in foods [1-3].

Nham, Thai traditional fermented meat, is produced when *Pediococcus cerevisiae*, *Pediococcus* spp. and heterofermentative lactobacilli reproduce rapidly during the first 3 days of the natural fermentation process, particularly when the incubation temperature is about 25-30°C [4]. The product is normally consumed after 3 days of fermentation without cooking. The incidence of salmonellae in the product [5 – 8] is therefore a serious public health concern. Since all ingredients to produce Nham cannot be pasteurized and, therefore, the fermentation flora has to compete with the fortuitous microorganisms. The application of starter cultures in this fermented meat product has become a means of controlling the fermentation process [9-10]. The fermented products have a safe reputation but, nevertheless, the process technology has to be directed to the prevention of the growth or even the elimination of food pathogens such as *Staph. aureus*, *Lis. monocytogenes* and *Salmonella*. Thus, the use of RE is one of the process technology to prevent the growth of associated food pathogens and lead to produce a safety of this Thai fermented meat product.

Objective

The study was to evaluate the antimicrobial activity of Roselle extract (RE) against some gram-positive and gram-negative bacteria associated in raw meat and Nham (Thai fermented meat). In order to study the potential use of RE for the safety prospect of Nham production, the effect of appropriate amount of RE on opportunistic food pathogens was also determined in an *In-vitro* trypticase soy broth + 0.6 % yeast extract (TSBYE).

Materials and Methods

Indicator strains and media

Two strains of gram-positive bacteria (*Staphylococcus aureus* and *Listeria innocua*) and 2 strains of gram-negative bacteria (*Escherichia coli* and *Salmonella anatum*) occasionally found in meat and Nham were cultured overnight at 35-37° C in trypticase soy broth + 0.6 % yeast extract (TSBYE). Two gram-positive of lactic acid bacteria (LAB) associated during Nham fermentation were cultured overnight at 35-37° C in MRS broth. Trypticase soy agar + 0.6 % yeast extract (TSAYE) and MRS agar were used for susceptibility test of RE against all indicators.

Roselle extract (RE) preparation

To prepare Roselle extract (RE), the dried flower of *Hibiscus sabdariffa* Linn. (purchased from local markets in Bangkok, Thailand) was ground to powder. A 25 g portion of powder was extracted by stirring with 250 ml of 80 % ethanol for 4 h and then filtered through filter Whatman No. 1 paper. The residue was re-extract

overnight with 250 ml of 80 % ethanol. The filtrates were pooled and subjected to the evaporation in a rotary evaporator under reduced pressure at 40° C. Concentrated RE was then freeze-dried. The dried extract was redissolved in water to obtain sample solution at various studies concentration (mg/ml). Citric acid solution was prepared at different concentrations with pH corresponding to the pH of RE solution at 100, 150 and 200 mg/ml.

Determination of antimicrobial activity of Roselle extract

The antimicrobial activity of RE was evaluated by the agar well diffusion method using the overnight culture suspension approximately $10^5 - 10^6$ Colony forming unit (CFU) [11 -12]. Melted agar media (TSAYE and MRS agar) were poured into a plate containing with overnight cultured suspension ($10^5 - 10^6$ CFU/ml). The wells then were made in each media using a sterile metallic borer. An aliquot of 150 µl from each test concentration of RE, solvent control (negative control) and 30 mcg Chloramphenicol (positive control) were added in the wells. All plates were incubated at 35 - 37° C for 24 h. Results were expressed in terms of the inhibition zone surrounding the well. (Inhibition zone = diameter in mm. of the zone from the center of well – diameter in mm. of well). The experiment was performed in triplicate for each sample concentration.

In order to investigate the inhibition zone appeared to all tested bacteria by each concentration of RE were not due to the low pH, three different concentrations of citric acid solution (CAS) with pH corresponding to the pH of RE at 100, 150 and 200 mg/ml were used to evaluate the inhibitory effect on all tested strains by the agar well diffusion method as described above.

Determination of inhibitory effect of Roselle extract on tested opportunistic pathogens in trypticase soy broth plus 0.6 % yeast extract (TYBYE)

RE were filter-sterilized through a sterile 0.45 µm membrane filter. The sterile RE were then dissolved in TSBYE at the final concentrations of 50, 25, 12.5, 6.25 and 3.125 mg/ml (w/v). Each concentration RE was tested in triplicate for the inhibitory effect on *Lis. innocua*, *Staph. aureus*, *E. coli* and *Salm. anatum* (An initial load of each tested strain was $10^6 - 10^7$ CFU/ml) at 35 – 37° C for 24 h compared to the control TSBYE broth without RE.. After each tested strain inoculation in the broths, the cells counts were analyzed every 3 h interval during the incubation using spread technic on TSAYE and incubated overnight at 35 – 37° C.

Results and Discussion

Determination of antimicrobial activity of Roselle extract

The results in Table 1 revealed that RE at 25 mg/ml showed the inhibitory effects against only *Lis. innocua*, while at 50 mg/ml inhibited all gram-positive pathogens studied. Moreover, the RE at 100 mg/ml could inhibit both gram-positive and gram-negative pathogenic bacteria tested. However, the concentration of RE at 150 and 200 mg/ml inhibited not only the growth of pathogens but also that of lactic acid bacteria (LAB). The results indicated that the tested gram-positive (C and D) except LAB (E and F) were more sensitive to RE than gram-negative (A and B) bacteria.

Table 1. Antimicrobial property of Roselle extracts (RE) against some pathogenic bacteria

Test sample	A	B	C	D	E	F
RE 25 mg/ml pH 2.35	-	-	1.47	-	-	-
RE 50 mg/ml pH 2.25	-	-	2.56	2.83	-	-
RE 100 mg/ml pH 2.05	1.74	4.57	8.73	9.22	-	-
RE 150 mg/ml pH 2.02	5.87	6.22	11.53	12.56	1.43	1.49
RE 200 mg/ml pH 1.97	9.41	7.64	11.90	14.28	3.07	3.06
chloramphenicol	6.69	8.41	11.48	11.84	15.94	15.42
solvent control	-	-	-	-	-	-

Notes : - no inhibition, A = *E. coli*, B = *Salm. anatum*, C = *Staph. aureus*, D = *Lis. innocua*, E = , *Lb. plantarum*, F = *P. pentosaceus*

The results to investigate whether the inhibitory effects were due to the low pH of RE, three different concentrations of citric acid solution (CAS) with pH corresponding to the pH of RE at 100, 150 and 200 mg/ml were used to evaluate the inhibition of aforementioned 6 indicators. It was confirmed that, wider inhibition zones were observed for RE compared to CAS at all concentrations evaluated (Table 2). In addition, no inhibition zone for LAB was found with CAS. By these results, it is confident to inform that RE contain other components, in addition to organic acids, with antimicrobial activity.

Determination of minimum inhibitory concentration (MIC) of Roselle extract on opportunistic pathogens in trypticase soy broth plus 0.6 % yeast extract (TSBYE)

The results for MIC of RE on opportunistic pathogens in TSBYE (Figure 1) revealed that the best concentration of RE to diminish the cells of *Salm. anatum* (b) and *Staph. aureus* (c) in TSBYE within 24 h is upto and more than 12.5 mg/ml. The higher concentration of RE upto and more than 25 mg/ml is the most appropriate to be used for *E. coli* (a) and *Lis. innocua* (d) diminishment in TSBYE within 24 h. It is implied that the concentration of RE at 12.5 - 50 mg/ml collaborated with important LAB as starter for Nham production may potentially be used to produce a safety of this Thai fermented meat product.

Table 2. Antimicrobial property of Roselle extract (RE) compared to citric acid solution (CA) at pH corresponding RE to against some pathogenic bacteria.

Test sample	Zone of inhibition (mm)					
	A	B	C	D	E	F
RE 100 mg/ml pH 2.05	1.74	4.57	8.73	9.22	-	-
CA 2 % (v/v) pH 2.05	3.42	2.84	5.48	4.08	-	-

RE 150 mg/ml	pH 2.02	5.87	6.22	11.53	12.56	1.43	1.49
CA 2.25 % (v/v)	pH 2.02	4.06	3.36		6.64	4.61	-
RE 200 mg/ml	pH 1.97	9.41	7.64	11.90	14.28	3.07	3.06
CA 3.3 % (v/v)	pH 1.97	7.08	4.13	8.67	6.05	-	-
chloramphenicol		6.69	8.41	11.48	11.84	15.94	15.42
solvent control		-	-	-	-	-	-

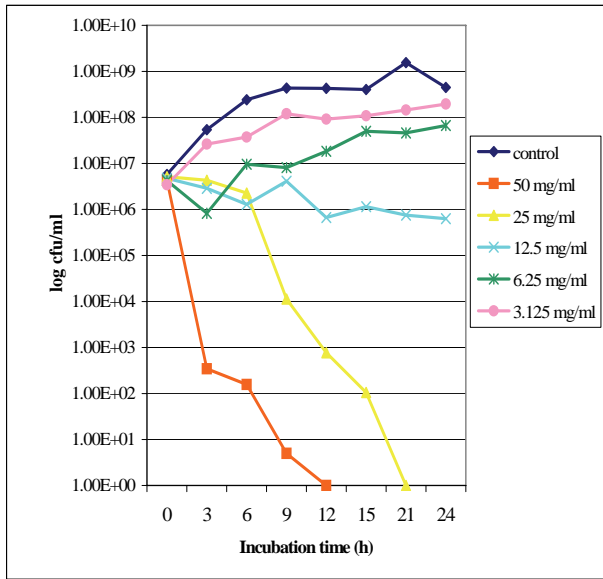
Notes : - no inhibition, A = *E. coli*, B = *Salm. anatum*, C = *Staph. aureus*, D = *Lis. innocua*, E = , *Lb. plantarum*, F = *P. pentosaceus*

Conclusions

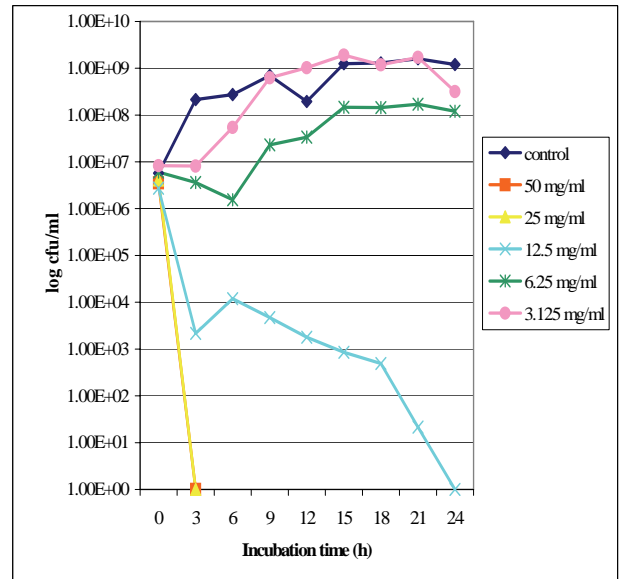
The study concludes that :

1. Roslle extract contains other components, in addition to organic acids, with antimicrobial activity.
2. *Salm. anatum* and *Staph. aureus* 2 opportunistic pathogens associated in Nham product were more sensitive to RE extract than *E. coli* and *Lis. innocua*
3. The concentration of RE at 12.5 upto 100 mg/ml implied to be used in collaborated with important LAB as starter to reduce some associated pathogens during Nham fermentation.

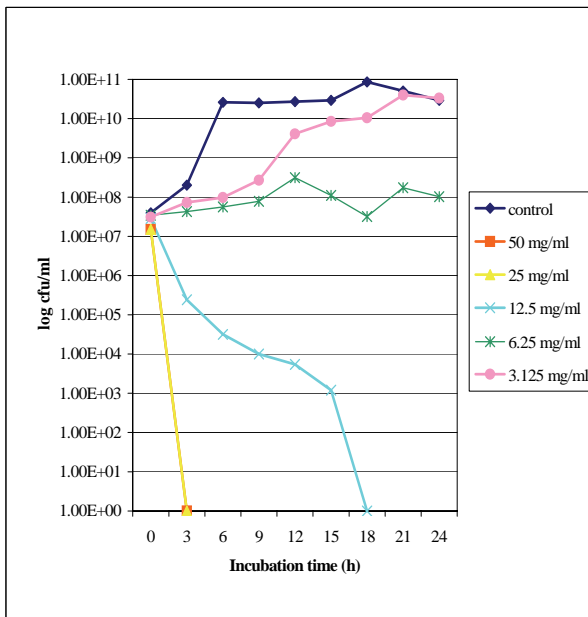
Figure 1 : Effect of various RE concentrations on *E. coli* (a), *Salm. anatum* (b), *Staph. aureus* (c) and *Lis. Innocua* (d) in TSBYE at 35-37° C



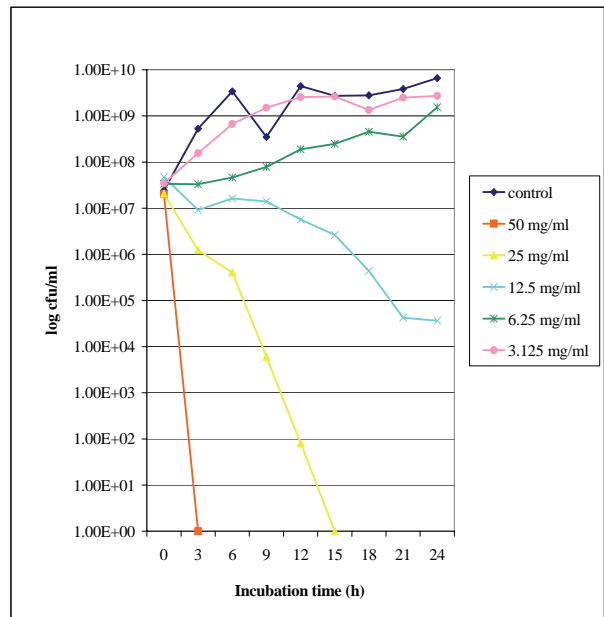
(a)



(b)



(c)



(d)

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