



# *In Vitro* Antibiotic Activity of Red Shallot (*Allium ascalonicum*), Mulberry (*Morus indica*), and Marigold (*Tagetes erecta*) Extracts against *Streptococcus pyogenes*

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## ABSTRACT

Bacterial infection is a major global health concern. One of the critical problems is the widespread of antimicrobial-resistant bacteria from inappropriate and prolonged use of antimicrobial agents in both humans and animals. Plant extracts might afford the chance to replace antibiotic drugs and reduce the emerging of antimicrobial-resistant bacteria. This study aimed to examine the antibiotic activity of ethanolic crude extracts of some Thai medicinal plants grouping in their parts as whole onions of red shallot (*Allium ascalonicum*), petals of marigold (*Tagetes erecta*), mulberry leaves, and root barks (*Morus indica*) to inhibit the growth of *Streptococcus pyogenes*. The antibiotic activities of the crude extract of three Thai medicinal plants using absolute ethanol were trialed against *Streptococcus pyogenes* using the disk diffusion method. Erythromycin and Ceftriaxone discs were chosen to be positive control standards as the representative of antibiotic drugs. Each dried plant extracts was prepared to test the inhibition with a concentration at 25, 50, and 75 mg/mL stock solution. The results showed that three groups from all testing groups of Thai medicinal plant extracts had the potential of antibiotic activity against *S. pyogenes*. The highest antibiotic activity against *S. pyogenes* was detected from whole onion extract red shallot followed by the extract of the mulberry leaves and root barks of mulberry strain Nakhon Ratchasima 60 (Nak 60) while the extract of marigold petal did not present antibiotic activity. The results revealed that crude extract of those two Thai medicinal plants, including red shallot and mulberry, had antibiotic activity against bacterial growth of *S. pyogenes* in the experiment and these medical Thai plants had potential benefits for developing as alternative treatment agents for *S. pyogenes* infections in both humans and animals in the future.

**Keywords:** Antibiotic activity, Ethanolic crude extract, Thai medicinal plants, *Streptococcus pyogenes*

## INTRODUCTION

*Streptococcus pyogenes* (*S. pyogenes*) is known as an important Gram-positive bacteria which display oxidase negative, catalase-negative, and  $\beta$ -hemolytic on blood agar. It is a facultative anaerobe bacteria that usually grows in 5-10% carbon dioxide and forms colonies on blood agar. It is grouped as Group A Streptococci (GAS) by Lancefield serological grouping system (Spellerberg, 2016). In humans, *S. pyogenes* normally colonizes in the throat, skin epithelial surfaces, anus, genital mucosa, and pharynx (Cunningham, 2000), while it was reported to be found in the eye discharge of a dog with conjunctivitis, feces of a dog with colitis, free-living European hedgehog, free-ranging non-human primates and bovine mastitis (Vela et al., 2017). However, the spread of the bacteria to typically sterile body sites can induce invasive conditions that lead to high morbidity and mortality. Therefore, *S. pyogenes* is pathogenic to humans and animals with its contagious infections. *S. pyogenes* can transmit through various routes, including direct contact, droplets, nasal discharge, fomites or surfaces contaminated with this bacteria, skin contact with a contaminated lesion, and by oral route from contaminated foods (Vela et al., 2017).

Currently, the increasing antimicrobial resistance (AMR) is becoming the most important problem for both animal and human health as well as the potential zoonotic infection. The problem can challenge the public health system, cause economic loss, and increase the morbidity rate of infected cases and also the mortality rate of the patients who might not be cured by any antibiotic medications (Shuster et al., 2013). The increase of AMR in *S. pyogenes* has been observed worldwide (Lai et al., 2021). The highest rate of resistance is usually found in erythromycin which was reported in Europe, including Finland, Spain, Italy, Netherlands, Germany, and also the USA (Arvand et al., 2000).

At present, there are many attempts to reduce the usage and for the rational use of antibiotic drugs to treat bacterial infections. Then, the development of alternative agents, including effective natural agents is necessarily required to replace antibiotic drugs. Many studies have addressed the antibiotic activity of plant extracts against the growth of bacteria that could be developed into antibacterial agents or therapeutic drugs (Abiala et al., 2016; Elisha et al., 2017). Marigold petal (*Tagetes erecta*) has revealed a large amount of flavonoids biologically content, especially for patulitrin that is the dominant active substance in the flavonoids containing anti-inflammatory effect and also effective antibiotic

ORIGINAL ARTICLE  
pif: S232245682100058-11  
Received: 13 July 2021  
Accepted: 01 September 2021

activity to destroy bacterial cells (Rhama and Madhavan, 2011; Dasgupta et al., 2012). An earlier study has reviewed the bulbs of red shallot (*Allium ascalonicum* Hort.) entailing a high level of quercetin can inhibit bacterial growth (Fattorusso et al., 2002). The mulberry (*Morus alba* L.) extract was found to have bioactive components, such as flavonol quercetin 3-(6-Malonylglucoside) substance which could inhibit both Gram-negative and Gram-positive bacterial pathogens, including *Escherichia Coli*, *Bacillus subtilis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Salem et al., 2013; Grajek et al., 2015; Gunjal et al., 2015) and quercetin which could destroy the membranes of bacteria leading to growth inhibition (Wang et al., 2018). Therefore, the current research aimed to evaluate the antibiotic activity of ethanolic crude extracts from potential Thai medicinal plants, such as red shallot (*Allium ascalonicum*), mulberry leaves and root (*Morus indica*) strain Nakhon Ratchasima 60 (Nak 60), and marigold (*Tagetes erecta*) against *Streptococcus pyogenes* for the potential of usage as an antibiotic agent to reduce the problem of antibiotic resistance in the future.

## MATERIALS AND METHODS

### Ethical approval

Ethical approval was not required for this research. All experiments and procedures in the current study did not certainly interrupt both humans and animals. All of the experiments and procedures were prepared and processed One Health research unit and Veterinary Public Health (VPH) laboratory room, Faculty of Veterinary Sciences, Maharakham University, Maha Sarakham province, Thailand.

### Medicinal plant materials

All Thai medicinal plants in this study were identified, housed, harvested, and collected at Maharakham University, Maha Sarakham, Northeastern Thailand. Ethanolic crude extracts were prepared from three Thai medicinal plants as were shown their external characteristic in Table 1 and their antibiotic activities were investigated. The selected medicinal plants which used in this research included root barks and leaves of mulberry (*Morus indica*) strain Nakhon Ratchasima 60 (Nak 60), marigold petal (*Tagetes erecta*), and whole onions or bulbs of red shallot (*Allium ascalonicum*) as shown in Figure 1. All medicinal plants were weighed, washed with purified and distilled water, then, they were dried at room temperature for 5 days. After that, they were chopped into 1x1 cm pieces and desiccated in a drying oven for 24 hours at 65°C. The dried medicinal plants were weighed after drying and extracted by soaking with absolute ethanol in a plant-ethanol ratio of 1:5 for 24 hours at room temperature within the cabinet. The medicinal plant extracts were then sieved using a filter paper and concentrated to yield ethanolic crude extracts via evaporation by vacuum rotary evaporator (Heidolph, Germany) at 289 mbar, 60 rpm, and 60°C in a water bath as shown in Figure 2. Each Thai medicinal plant crude extract was then dissolved in dimethyl sulfoxide (DMSO) as solvent before measuring the antibiotic activity test. Each dried plant extracts in this study were used three different concentration tests (low, medium, and high) at 25 mg/ml, 50 mg/ml, and 75 mg/ml, respectively.

**Table 1.** Thai medicinal plants and their part used for antibiotic activity test

Thai medicinal plants		Part used	Weight before drying (g)	Weight after drying (g)
Common name	Scientific name			
Red shallot	<i>Allium ascalonicum</i>	Whole onions	3226	557.5
Mulberry	<i>Morus indica</i>	Root barks	345.65	286
		Leaves	340.45	101.78
Marigold	<i>Tagetes erecta</i>	Petals	557.5	201.31

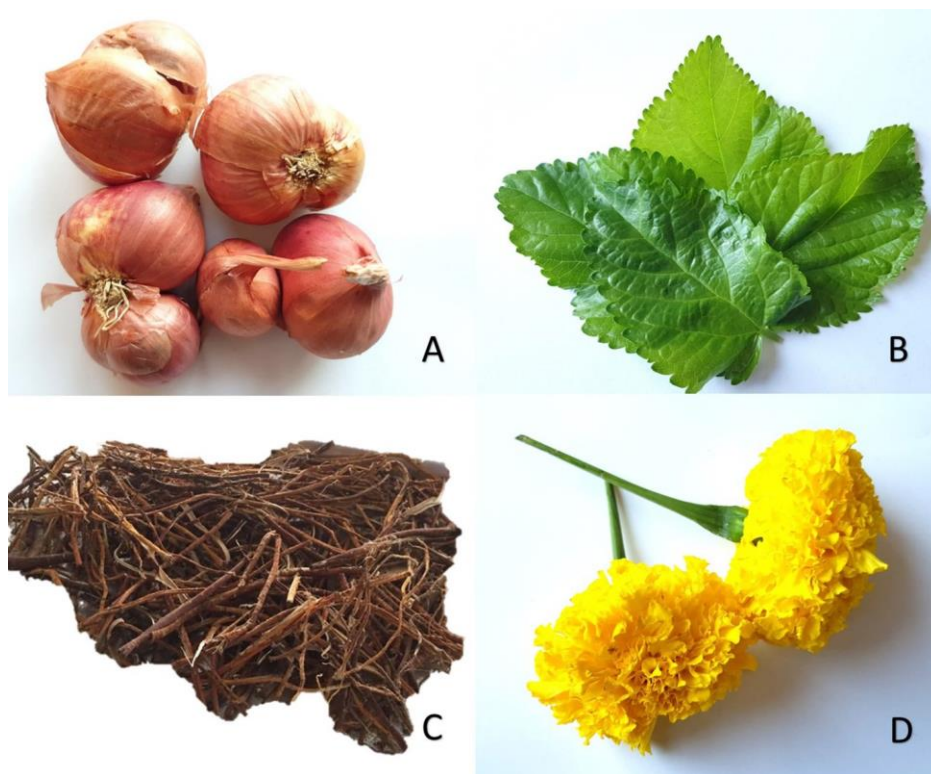
### Target bacterial strain

This study used a reference strain of *Streptococcus pyogenes* (ATCC19615) as a target microorganism. The bacterial strain was maintained in the Veterinary Public Health Laboratory at the Faculty of Veterinary Sciences, Maharakham University, Thailand. *S. pyogenes* was subcultured from the original bacterial culture, stored at -20°C, and streaked on nutrient agar plates (Oxoid, UK) to maintain at 4°C. It was used to test for antibiotic susceptibility by disc diffusion protocol. *S. pyogenes* was cultured on tryptone soy agar (Oxoid, UK) in aerobic incubation at 37°C for 24 hours. Antibiotic activity testing protocol was processed following the methods for antimicrobial dilution and disk susceptibility testing CLSI guidelines M45 (CLSI, 2017). The *S. pyogenes* colony had cultured overnight, then, it was suspended in sterile saline water and the suspension has adjusted the turbidity to equal that of a standard value of 1.5x 10<sup>8</sup> CFU/ml by 0.5 McFarland.

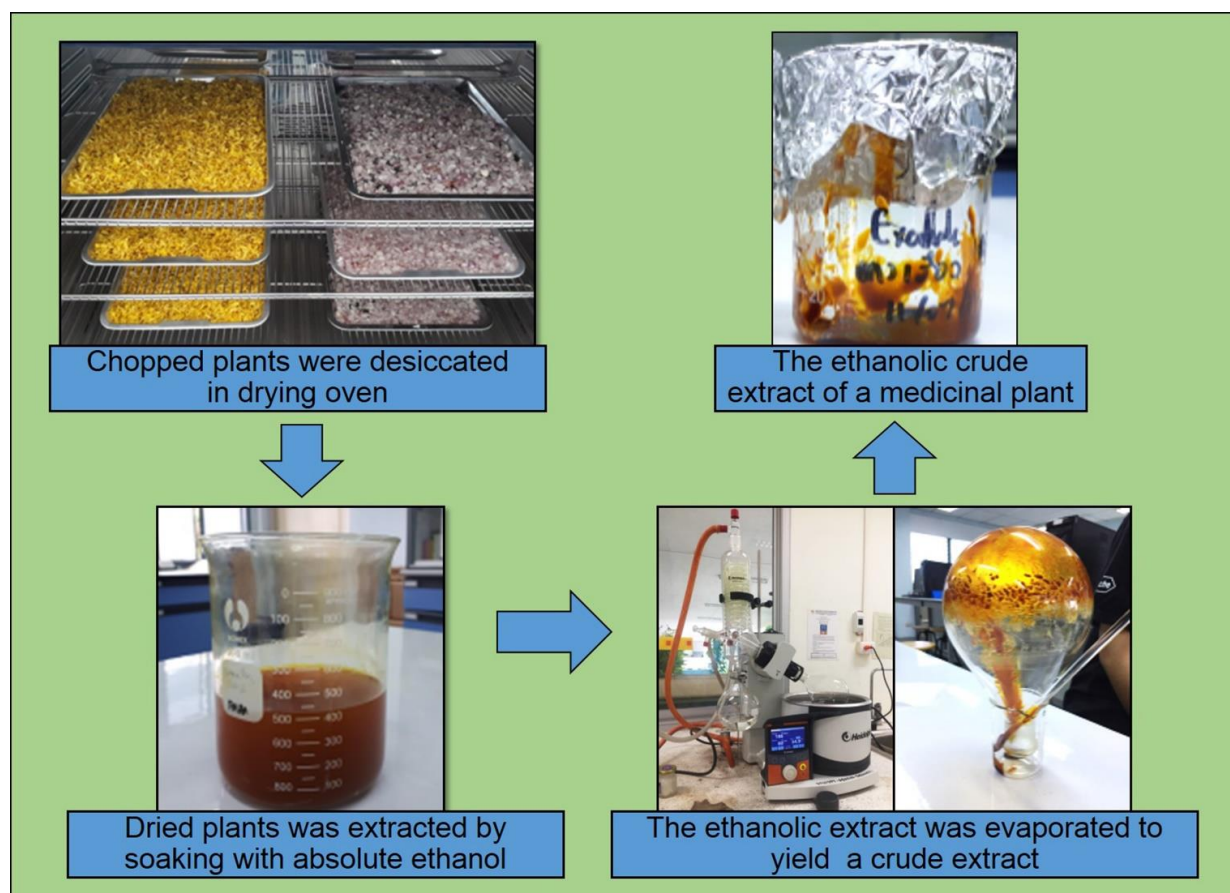
### Evaluation of antibiotic activity

Disk diffusion method was processed in triplicate for root barks of mulberry, leaves of mulberry, marigold petal, and whole onions of red shallot ethanolic crude extracts in each concentration. The bacterial suspension was spread onto the surface of the Muller Hinton agar; MHA (Oxoid, UK) that was used to determine the antibiotic activity by sterile cotton swabs. Each dried plant extracts was prepared a concentration at 25, 50, and 75 mg/mL stock solution in DMSO. Wells which were used to place the medicinal plant extracts testing were cut into MHA. The volume of stock solutions

about 5  $\mu$ l in each concentration was loaded to the wells in MHA plates which the target bacterial strain was spread along with representative antibiotic drugs as selected antibiotic assay discs including erythromycin and ceftriaxone (Oxoid, UK) which used as the positive control, then incubated the plates in aerobic incubation for 24 hours at 35°C together with DMSO which was utilized as the solvent control or negative control in this trial. The zone of the inhibition diameters in each plate was interpreted following the recommendation of CLSI guidelines M100 (CLSI, 2020).



**Figure 1.** Thai medicinal plants used in the experiment, Whole onions of red shallot (A), Mulberry leaves (B), Root barks of mulberry (C), Petals of marigold (D)



**Figure 2.** The extraction methods of Thai medicinal plants

## Statistical analysis

The experimental data were statistically analyzed using differentiation of disk diffusion results by descriptive statistics analysis and one-way analysis of variance (ANOVA) and the least significant difference (LSD) post hoc tests at 95% confidence level. The significance statistical difference was set at  $p < 0.05$ . The IBM SPSS statistic version 21.0 was used for analysis .

## RESULTS

The experimental result from the antibiotic activity trial for inhibiting the growth of *S. pyogenes* by the ethanolic crude extracts of Thai medicinal plants is demonstrated in Table 2 .Ethanolic crude extracts of red shallot and mulberry showed the antibiotic activities on the reference strain of *S. pyogenes*. From the result of inhibition zone performed by the lowest concentration at 25 mg/ml, the average diameters of inhibition zone of whole onions of red shallot extract, root barks of mulberry extract, leaves of mulberry extract, and marigold petal extract were 7.1, 5.17, 5.33, and 2 mm, respectively. At 50 mg/ml concentration, the average diameters of the inhibition zone were 7.83, 6.17, 6.67, and 2.5 mm, respectively. At least, the average diameters of the inhibition zone of the highest concentration at 75 mg/ml were 8.67, 7, 7.83, and 3 mm, respectively.

Thai medicinal plant extracts comprising red shallot, root bark, and leaves of mulberry showed good antibiotic activity against *S. pyogenes*, while marigold extract rarely had antibiotic activity. Red shallot ethanolic extracts appeared the most antibiotic activity following by the crude extract of mulberry leaves and root bark of mulberry extract, respectively. The inhibition zones of erythromycin and ceftriaxone which were used as the positive control in this trial were 15.67 mm and 24.42 mm in diameter, respectively. There were statistically significant differences among the four categories of Thai medicinal plant extracts in all concentration tests when analyzed through one-way ANOVA and the least significant difference (LSD) post hoc tests at 95% confidence level. The obtained results from LSD post hoc test indicated that the ethanolic extracts of red shallot bulbs were the most effective against *S. pyogenes*, while the ethanolic extracts of marigold showed the lowermost antimicrobial activity at all concentration tests.

**Table 2.** Antibiotic activity of Thai medicinal plant ethanolic crude extracts demonstrated as the diameter of inhibition zone

Thai medicinal plants extract	Zone of inhibition diameter (mm)		
	Plant extracts concentration (mg/ml)		
	25	50	75
Red shallot	7	7.5	8.5
	7.5	8	8.5
	7	8	9
Average	7.16	7.83	8.67
Mulberry (root bark)	5	6	7.5
	5	6	6.5
	5.5	6.5	7
Average	5.17	6.17	7
Mulberry (leaves)	6	7	8
	5	6	7.5
	5	7	8
Average	5.33	6.67	7.83
Marigold	2	2.5	3
	2.5	3	3.5
	1.5	2	2.5
Average	2	2.5	3

## DISCUSSION

The outcomes of the study revealed the potential antibiotic activity derived from ethanolic crude extracts of Thai medicinal plants comprising red shallot, mulberry leaves, and mulberry roots strain Nakhon Ratchasima 60 (Nak 60) inhibiting the growth of *S. pyogenes*. Ethanolic crude extracts of bulbs or whole onions part of red shallot expressed the highest potential inhibitory activity against *S. pyogenes* which is consistent with a previous study from Mekvimol et al. (2020) that mentioned the high antibiotic activity of red shallot and mulberry leaves extracts against the growth of Gram-positive bacteria, such as *Streptococcus agalactiae*. The findings of the current research indicated that the bulbs or whole

onions part of red shallot and the leaves and root barks part of mulberry ethanolic crude extracts have the possibility to inhibit the growth of *S. pyogenes* in all concentrations. Some previous studies reported that the shallot bulbs and mulberry leaves contain a high level of active phenolic compound which demonstrated to have antimicrobial activities, including flavonoid substance which can inhibit the growth of bacteria (Butkhup et al., 2013; Mozin et al., 2015). The major active ingredient of flavonoid substances from these herb extracts is quercetin with a bactericidal effect by demolishing bacterial cell membranes (Mozin et al., 2015). Therefore, this result is consistent with many previous studies indicating that quercetin was a crucial bioactive compound and plays a role in inhibiting Gram-positive and Gram-negative bacteria by prohibiting the activity of extracellular proteins (Koo and Jeon, 2009; Mozin et al., 2015; Fredotović et al., 2021). Nevertheless, some reports revealed that quercetin could not inhibit the growth of some Gram-negative organisms include *E. coli* and *Salmonella typhi* (Souza et al., 2010; Mozin et al., 2015). The bioactivity compounds of red shallot and mulberry which were revealed the potential antibiotic activity on *S. pyogenes* were likely more effective to inhibit bacterial growth, while ethanolic extract of marigold seemed to have no effect on the bacterial growth. The *in vitro* study of antibiotic activity from Thai medicinal plant extracts is a crucial phase for the alternative treatment strategy as surrogate antibacterial agents replacing antibiotic drugs for several bacterial pathogens, including *S. pyogenes*. The findings of the current *in vitro* study can be well used for alternative medicine. These medicinal plants in urban communities can be developed into therapeutic drugs as plant-based antimicrobial agents. This evidence is in accordance with many previous studies that revealed the success of herbal medicine in curing bacterial infectious diseases (Palombo, 2011; Abiala et al., 2016; Elisha et al., 2017). More than that, the current research seems to be the first investigation that discovered ethanolic crude extracts from various Thai medicinal plants, including whole onions of red shallot, root bark, and leaves of mulberry had antibiotic activity against the growth of *S. pyogenes*.

## CONCLUSION

The current *in vitro* study revealed that red shallot (*Allium ascalonicum*), leaves part and root barks part of mulberry (*Morus indica*) strain Nakhon Ratchasima 60 (Nak 60) ethanolic extracts expressed the antibiotic activity against *S. pyogenes*. Thus, the future development of red shallot and mulberry leaves ethanolic extracts as an alternative treatment for *S. pyogenes* infections in both humans and animals are recommended for resolving the antimicrobial resistance in the prospect. Ethanolic crude extract of red shallot exhibited strong activity against *S. pyogenes* reference strain ATCC19615. Further investigation should be aimed to analyze the quantity of active ingredients and action mode of these plant extracts against bacterial clinical strains. It would be interesting to evaluate *in vivo* trial for developing and applying these medicinal plants as natural therapeutic products for both humans and animals for medical and veterinary use.

## DECLARATIONS

### Competing interest

The authors declare that they have no competing interests.

### Authors' contribution

Natapol Pumipuntu designed and supervised the research project, supported in the experimental study and data analysis. Tanawadee Mekvimol, Chayanit Chaipunna, and Gannika Poonthong designed the research experiment and collected the data. All authors interpreted the data, wrote the manuscript, and approved the final version.

### Ethical consideration

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

### Acknowledgments

This research project was financially supported by Mahasarakham University (Fast Track 2021). The authors are grateful to the Faculty of Veterinary Sciences, Mahasarakham University for the use of the laboratory to carry out this experiment. The authors would like to thank Asst. Prof. Dr. Prasoborn Rinthong, Faculty of Pharmacy, Mahasarakham University for her advice. All authors are also grateful to Miss Thamonphan Wimonrinarachai, a veterinary student at the Faculty of Veterinary Sciences, Mahasarakham University for the graphical abstract design and illustration.

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