Efficiency of *Justicia gendarussa* Burm F. extracts against *Staphylococcus aureus*, and Acute Toxicity Test with Brine Shrimp Model

Jinta Prakob[1*], Pattaraporn Phanchai[1] and Sakchai Hongthong[2]

[1]Student, The Prince Royal's College, Chiangmai, Thailand
[2]Faculty of Science and Technology, Rajabhat Rajanagarindra University, Chachoengsao, Thailand
*Corresponding Author: E-mail: jinta28p@gmail.com

Abstract

Justicia gendarussa Burm F. is a medicinal herb that has been used since ancient times. Nowadays, the issue of drug resistance has become a top public health threat globally, and medicinal herbs have the potential to serve as raw materials for the synthesis of substitutions for drugs. *Justicia gendarussa* is a medicinally important herb used in the treatment of inflammatory disorders. This research was an investigation into the inhibitory effects on *Staphylococcus aureus* ATCC 25923 and the acute toxicity to brine shrimp of the *Justicia gendarussa* Burm extracts obtained from the extraction with hexane and ethanol solvents. The experimental results indicated that the ethanol extract had an inhibitory effect against S. aureus with an inhibition zone of 22.0 ± 0.3 millimeters, which was greater than the hexane extract with an inhibition zone of 14.5 ± 0.3 millimeters. Additionally, toxicity tests on brine shrimp analyzed by using probit regression analysis revealed that the ethanol extracts of the *J. gendarussa* Burm had a median lethal dose (LD_{50}) and hexane extraction of 1,786.48 µg/mL and 29.99 µg/mL, respectively. These results suggest that extract from the *J. gendarussa* herb using ethanol solvent is not toxic to brine shrimp and has properties as well as potentiality for the further development of herbal products in the future.

Keywords*: Justicia Gendarussa extracts, Antibacterial activity, Toxicity*

1. Introduction

The scientific name for *Justicia gendarussa* is *Justicia gendarussa* Burm F. It belongs to the Acanthaceae family and has a bush-like appearance. It typically grows to a maximum height of 1.5 -2 m. The branch has clear nodes with dark red to black and has a smooth, shiny surface. The leaves are white, green, and gray with a length of 7-14 cm and a width of 1 – 2.5 cm. The flowers are white with purple lines and spots on them. This plant is commonly found along streams in tropical rainforests or cultivated in gardens. (Paval et al., 2009; Chandra, & Lo, 2020). Many international studies have reported on the pharmacological properties of *Justicia gendarussa*, including its anti-inflammatory properties (Kavitha, Viji, Kripa, & Helen, 2011; Nirmalraj et al., 2015), antioxidant effects (anti-oxidant) (Kumar, Mary, & Kumari, 2017; Sivasakthi & Vijayalakshmi, 2014), antibacterial activity (Kumar et al., 2017; Sivasakthi & Vijayalakshmi, 2014), and hepatoprotective effects (Jakma et al., 2020; Kavitha, Sangeetha, Sujatha, & Umamaheswari, 2014; Phukan, Kakoti, Verma, & Kumar, 2014). Additionally, studies have highlighted the important phytochemicals found in *Justicia gendarussa* including alkaloids, anthraquinones, saponins, tannins, phenols, steroids, flavonoids, terpenoids, and glycosides (Subramanian, Jothimanivannan, & Moorthy, 2012; Krishna, Mruthunjaya, & Patel, 2010; Yadav et al., 2017).

At present, numerous bacteria cause diseases in both humans and warm-blooded animals, requiring antibiotic treatment. This leads to the problem of antibiotic resistance, affecting both human and economic aspects (Su et al., 2015). According to data from the Ministry of Public Health in 2010, approximately 100,000 people are found to be infected with drug-resistant bacteria annually, with over 30,000 deaths per year. In 2050, it is estimated that there could be up to 10 million people infected with drug-resistant bacteria, with half of them being in Asia (Duangkaew, & Rakying, 2018; Sheppard, 2023).

Most herbs are commonly used as medicines without undergoing testing or proving their toxicity. When consumed continuously, they may become toxic to living organisms, especially humans and animals.

Therefore, it is essential to conduct toxicity tests before using them (Widiyanti, Prajogo, & Hikmawati, 2016; Erhirhie, Ihekwereme, & Ilodigwe, 2018; Obakiro et al., 2024). From the literature review, it was found that there is no existing research on the acute toxicity of the *Justicia gendarussa* plant, either internationally or in Thailand. Consequently, the research team is interested in assessing the toxicity of *Justicia gendarussa* by using the brine shrimp lethality assay (BSLA). BSLA is a rapid and highly efficient method for testing the acute toxicity of bioactive compounds. It is widely used as a preliminary toxicity screening test of plant extracts (Ghosh, Banik, Islam, 2015; Kibiti, & Afolayan, 2016; Syahmi et al., 2010; Vanhaecke, Cooreman, & Sorgeloos, 1981; Moshi et al., 2010; Sarah, Anny, & Misbahuddin, 2017).

For these reasons, the research team is interested in studying the extraction of compounds from the *Justicia gendarussa* plant, which is an easily accessible and cost-effective herb in the local area and can serve as a new alternative to avoid the use of antibiotics to inhibit bacterial growth. It can also be utilized in the development of products for disease treatment. Importantly, these products must be safe and non-toxic when used by consumers or other living organisms.

2. Objectives

1) To study the extraction methods of compounds from *J. gendarussa* with organic solvents such as hexane and ethanol
2) To investigate the efficiency of *J. gendarussa* extracts against *S. aureus*
3) To study the acute toxicity of the extracts of *J. gendarussa* on brine shrimp

3. Materials and Methods

The research project was done with the following scope:

1) The plant used in the study: *Justicia gendarussa* Burm f. leaves were obtained from a cultivated crop from the village of Bankao, Tambon Sopmaekha, Hangdong, Chiangmai, Thailand.
2) Organic solvents used for extraction: hexane and ethanol
3) Bacterial strain used for Testing: *Staphylococcus aureus* ATCC 25923.

Note: As this involves testing pathogenic bacteria, the analysis was conducted at the Molecular Microbiology and Biotechnology Laboratory, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang

3.1 Extraction of substances by using solvents such as hexane and ethanol

Dried *J. gendarussa* plants consisting of leaves and stems weighing 400 grams were blended. The plant sample was divided into two parts, each weighing 150 grams, and placed into 500 ml beakers. Then, a graduated cylinder was used to measure the volume of the solvent mixture, hexane, and ethanol at a volume of 200 ml. Afterward, the plant powder was divided into two equal parts. One part was extracted using hexane, while the other part was dissolved using ethanol. Next, both beakers were covered with foil paper to prevent evaporation, and the plant material was soaked at room temperature for 7 days. After 7 days, the solution obtained from the extraction was filtered using filter paper, and the solvent was evaporated using steam distillation. The remained plant powder was re-extracted twice or more. After that, the obtained samples were combined to use as a crude extract. After drying, the hexane crude extract (2.80 grams) and ethanol crude extract (4.77 grams) were obtained for further analysis.

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(A) (B) (C)

Figure 1. (A) *Justicia gendarussa* (B) Dried *J. gendarussa* stems (C) dried *J. gendarussa* leaves

3.2 Evaluating the efficacy of J. gendarussa herb extracts against Staphylococcus aureus using hexane and ethanol solvent extraction via the disc diffusion method

The antibacterial activity against Staphylococcus aureus (ATCC25923) was performed by the disc diffusion method and was tested at the Faculty of Science, King Mongkut's Institute of Technology Ladkrabang in Bangkok, Thailand. For antibacterial activity, the maximum tested concentration was done at 50 µg/mL. The disc diffusion test was performed. The inoculum suspension of each microbial strain was swabbed on the entire surface of Mueller-Hinton agar (MHA, Difco) for bacteria. Sterile 6 mm filter paper discs were aseptically placed on MHA and SDA surfaces. Twenty µL of the sample was immediately added to discs.

The sample was dissolved with the respective solvent and then a sample solution volume of 20 µL was dropped onto the disc with a centerline diameter of 6 mm and allowed to dry before being used for testing. Next, Staphylococcus aureus ATCC 25923 was prepared by cultivating it on Muller Hinton agar at a temperature of 37 ºC for 24 hours and at a temperature of 30 ºC for 48 hours. The following step involved diluting the bacterial suspension in a 0.85% NaCl solution to achieve McFarland No. 0.5 (1.5x108 CFU/ml). Next, the bacterium was inoculated onto Muller Hinton agar for bacteria using a cotton swab, and a disc was placed on the agar surface. The agar plate was incubated at a temperature of 37 ºC for 18-24 hours. Penicillin G (10 units/mL) was used as a positive control. The results were evaluated by measuring the diameter of the inhibition zone, and the percentage of inhibition was calculated by using the formula below:

$$\% \text{ inhibition} = [(A - B)/ A] \times 100$$

A = The average value of the diameter of the inhibition zone of bacterial colonies on the microbial culture medium in the comparative set.

B = The average value of the diameter of the inhibition zone of bacterial colonies on the culture medium with the incorporated plant extract.

3.3 Testing for toxicity to marine organisms

The toxicity analysis of marine organisms using the Brine Shrimp Lethality Assay (BSLA) was conducted following the method of Meyer and colleagues (Meyer et al., 1982, cited in Hamidi, Jovanova, & Panovska, 2014). Brine shrimp eggs (*Artemia salina* L.) were cultured in artificial seawater (with a seawater salt ratio of 33.33 grams per liter of distilled water) and left exposed to light with aeration for 48 hours. This resulted in the hatching of brine shrimp larvae at instar stage III, which were ready for use in the experiment (Vanhaecke et al., 1981). The plant extracts were prepared at concentrations of 10, 100, 500, and 1,000 µg/mL using 1% 1% dimethyl sulfoxide (DMSO) in artificial seawater as the solvent. Each concentration was repeated three times and carried out in triplicate. Afterward, each trail extract tube containing the prepared concentrations was placed with 10 brine shrimp (nauplii) and left at room temperature for 24 hours. The number of survivors and number of dead nauplii in each tube were counted to assess the effectiveness of the

extract. In this experiment, Potassium dichromate solution ($K_2Cr_2O_7$) and 1% DMSO in seawater were used as positive and negative control sets, respectively (Naidu, Ismail, & Sasidharan, 2014). The mortality percentage was calculated by the equation below:

$$\text{Mortality (\%)} = [1- (A1 - A2) / A1] \times 100$$

A1 = The control groups consist of live nauplii without the test substance.

A2 = The number of dead nauplii with the test substance

The collected data were analyzed with probit regression analysis to determine the quantity of the chemical given to the test animals all at once that resulted in a 50 % mortality rate among the experimental group (Median Lethal Dose (LD_{50})), which is the concentration of a chemical substance that causes death in half of the tested animals.

3.4 Statistical analysis

The acute toxicity testing on brine shrimp was repeated three times (n=3). The results are presented in terms of the mean values and standard deviation (S.D.). The LD50 (Median Lethal Dose) was calculated as the concentration of the chemical substance that causes death in half of the total tested brine shrimp. Probit regression analysis was utilized for this analysis.

4. Results

The results from extracting substances from the leaves and stems of the dried and finely grounded J. gendarussa herb using the solvents hexane and ethanol, with a total weight of 400 grams which were divided into two parts, each weighing 150 grams. The crude extracts obtained from the leaves and stems had thick, sticky, greenish liquid characteristics. The weight of the hexane-extracted substance was 2.80 grams, and the weight of the ethanol-extracted substance was 4.77 grams. The calculated percentage yields for hexane and ethanol extracts were 1.87% and 3.18%, respectively, based on the weight shown in Table 1.

Table 1. Quantity and percentage yields of the extracted substances obtained from the leaves and stems of *J. gendarussa* (150 grams)

Plant	Extract	Weight of extracted substance (grams)	% yields (by weight)
leaves and stems of the *J. gendarussa*	Hexane	2.80	1.87
	Ethanol	4.77	3.18

Penicillin G (10 units/mL) was used as a positive control with zone inhibition 33.7 ± 0.1 mm.

The study investigated the inhibitory efficacy against *Staphylococcus aureus* ATCC 25923 of extracts from the leaves and stems of *J. gendarussa* using two types of solvents, including hexane and ethanol. The evaluation was based on the size of the inhibition zones by using the disc diffusion method. The results showed that the extracts from the leaves and stems of *J. gendarussa*, extracted with both ethanol and hexane, inhibited the growth of *S. aureus*. However, there were differences in the size of the inhibition zones. The ethanol extract demonstrated an inhibition zone size of 22.0 ± 0.3 millimeters, while the hexane extract had an inhibition zone size of 14.5 ± 0.3 millimeters, as shown in Table 2.

Table 2. Anti-bacterial activity (*Staphylococcus aureus* ATCC 25923) of *J. gendarussa* extracts in the hexane and ethanol extracts

Plant	Extract	Zone of inhibition (mean ± S.D., mm)
leaves and stems of the *J. gendarussa*	Hexane	14.5 ± 0.3
	Ethanol	22.0 ± 0.3

The study investigated the acute toxicity (Lethal Dose; LD_{50}) of *J. gendarussa* using the Brine Shrimp Lethality Assay. Brine shrimp eggs were cultured in artificial seawater and exposed to light with aeration for 48 hours. Afterward, concentrated solutions were prepared including a concentration range of 10, 100, 500, and 1,000 micrograms per milliliter using 1% DMSO in artificial seawater as the solvent. Next, ten brine shrimp larvae were placed in each test tube with the prepared concentrations and left for 24 hours. The numbers of living or dead shrimps were then counted. The research found that the LD_{50} value for the hexane extract of the *J. gendarussa* plant was 29.99 µg/ml. In contrast, the LD_{50} value for the ethanol extract was greater than 1,000 µg/ml. In summary, the ethanol extract with the highest LD_{50} value was considered to have lower acute toxicity to brine shrimp, while the hexane extract with a lower LD_{50} value was suggested to have higher acute toxicity to brine shrimp, as shown in Table 3.

Table 3. Acute toxicity LD50 value of *J. gendarussa* against Brine shrimp mortality

***J. gendarussa* extracts**	**% brine shrimp mortality (concentration in µg/mL)**				**LD_{50} (µg/mL)**
	10	**100**	**500**	**1,000**	
Hexane	40	60	76.66	96.66	29.99
Ethanol	0	3.33	13.33	33.33	>1,000
$K_2Cr_2O_7$	76.66	100	100	100	<10

5. Discussion

From the study of the effectiveness against *Staphylococcus aureus* ATCC 25923 and the acute toxicity on brine shrimp of the extracts from The *J. gendarussa* herb, the results can be discussed and summarized as follows:

First, the results of the extracts from *J. gendarussa* leaves and stems were gained using the solvents (hexane and ethanol), and then crude extracts were obtained. These extracts appeared as viscous green liquids. The weight of the hexane extract was 2.80 grams, while the ethanol extract weighed 4.77 grams. The percentage yield of the extracts was found to be 1.87% for hexane and 3.18% for ethanol, respectively. It can be summarized that the extraction yields from ethanol extract provided a higher yield than hexane extract did and the solvent polarity significantly affected the extraction yield. The result of this study was in line with studies by Kerdsiri et al. (2020) and Lai et al. (2009), which reported that the extraction yield was increased with increasing polarity value, and the extract yield varied with the solvent used (Kerdsiri et al., 2020; Lai, Li, Lu, & Chen, 2009).

Secondly, the results of studying the antibacterial activity of extracts from *J. gendarussa* leaves and stems using organic solvents including hexane and ethanol were determined by the disc diffusion method. It was found that the ethanol extract and hexane extract could inhibit the growth of S. aureus. However, there were differences in the sizes of the inhibition zones. The ethanol extract had an inhibition zone of 22.0 ± 0.3 millimeters, while the hexane extract had an inhibition zone of 14.5 ± 0.3 millimeters. These experimental results are consistent with the findings reported by Subramanian et al. (2012), which found that an aqueous extract of *J. gendarussa* stem could inhibit *S. aureus* with an inhibition zone of approximately 26.33 millimeters. This finding was also in line with the study of Sugumaran, Kowsalya, Karthic, and Seshadri (2013) studying biomass production and antibacterial activity of *Justicia Gendarussa*: a valuable medicinal plant, which found that the maximum inhibition zone against *S. aureus* was observed in stem extracts using methanolic extract (Sugumaran et al., 2013), following the study by Jain et al. regarding a review of the pharmacology activities of *Justicia gendarussa*. It was reported that the plant is rich in chemical constituents such as alkaloids, flavonoids, tannins, saponins, phenolics, and essential oils. Furthermore, these alkaloids possess various biological activities and their phytochemical constituents have demonstrated potential as new drug leads for the treatment of various diseases. However, further studies are needed to fully understand its mechanisms of action, safety, and efficacy before being used by humans (Jain, Singh, Bhardwaj, & Gohil, 2024).

Finally, research revealed that the ethanol extract of *J. gendarussa* had LD_{50} values of 1,786.48 µg/mL. This indicates that this extract is highly non-toxic to brine shrimp. As for the hexane extract of *J. gendarussa*, it had LD_{50} values of 29.99 µg/ml, which was considered highly toxic to brine shrimp. According

to Meyer's toxicity index, extracts with LD_{50} < 1000 µg/ml are considered toxic, while extracts with LD_{50} > 1000 µg/ml are considered non-toxic (Meyer et al., 1982). Although testing for toxicity to brine shrimp is a convenient, rapid, low-quantity, accurate, and widely applicable method for assessing toxicity to living organisms in general laboratory settings, scientific literature searches in databases such as SciFinder and Scopus, which are reputable scientific databases, revealed no previous studies concerning the acute toxicity to brine shrimp of extracts from *J. gendarussa*. Interestingly, a study was conducted by Patel and Zaveri (2012) regarding *J. gendarussa* being screened for cytotoxicity using the brine shrimp lethality test. The leaf and root of *J. gendarussa* were extracted using different solvents (n-hexane, methanol, and water) and tests were conducted using the leaves and roots separately. The findings revealed the methanolic fraction of the leaf and root showed significant cytotoxic activity with LD_{50} values of 48.71 µg/ml and 93.25 µg/ml, while other fractions exhibited no significant cytotoxic activity (LD_{50} >1000) (Patel, & Zaveri, 2012).

6. Conclusion

Based on the findings of this study, ethanol extract showed a higher percent yield than hexane extract. As for the antibacterial activity of extracts from J. gendarussa, both ethanol and hexane were active against *S. aureus*. However, ethanol extract had more of an inhibition zone than hexane. Regarding acute toxicity to brine shrimp, the hexane extract of J. gendarussa also showed high acute toxicity to brine shrimp. On the contrary, ethanol extract was not toxic to brine shrimp. However, further studies are needed to compare various methods of extracting compounds from J. *gendarussa* as well as find extraction methods that are easy and cost-effective. Additionally, experiments should be conducted to compare the harvest of J. gendarussa in different seasons for studying and identifying bioactive compounds with the highest efficacy.

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