

RESEARCH ARTICLE

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## Phytochemical Analysis and Antibacterial Activities of *Kyllinga nemoralis* Extracts against the Growth of some Pathogenic Bacteria

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### Abstract

This study aimed to screen the phytochemical contents and investigate antibacterial activities of the aqueous and methanolic extracts of *Kyllinga nemoralis*. Extraction was done using the whole plant of *K. nemoralis* except the root. The phytochemical screening was carried out on both aqueous and methanolic extracts of *K. nemoralis*. The aqueous extract showed the presence of saponin and high amount of steroid, while the methanolic extract showed high amount of terpenoid and steroid. The antibacterial activities of *K. nemoralis* extracts were tested against five Gram positive bacteria (*Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, *Streptococcus pyogenes*, and *Bacillus thuringiensis*) and four Gram negative bacteria (*Escherichia coli*, *Shigella sonnei*, *Salmonella Typhi* and *Klebsiella pneumoniae*). *K. nemoralis* extracts were subjected to testing of their antibacterial activities by the disk diffusion method. Furthermore, the minimum inhibitory concentrations of the extracts were determined. The results indicate that the aqueous extract of *K. nemoralis* exhibits more antibacterial activities than the methanolic extract. The aqueous extract of *K. nemoralis* showed efficacy against *S. aureus* and MRSA while the methanolic extract of *K. nemoralis* was found to exert antibacterial activity against MRSA. The results proved the potency of *K. nemoralis* extracts as natural antibacterial and supported the potential of use in the medication of the diseases caused by the tested bacteria.

**Keywords:** *Kyllinga nemoralis*, Phytochemical Screening, Antibacterial Activity, Medicinal Plants, Minimum Inhibitory Concentration

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## INTRODUCTION

In the past two decades, the discovery of new molecules (with antimicrobial properties) from microorganisms has declined drastically. Scientists began to seek potential novel antibacterial compounds within medicinal plants. There are a lot of methods that are used to screen the antibacterial properties of medicinal plants, with different types of principles. However, with the different types of principles that are used, the results become varied. The results can be influenced by the method selection, the microorganisms that are used, and the degree of solubility of the test compound.<sup>1</sup>

Medicinal plants have long been used in traditional and complementary medicine worldwide. The information that the ancient people used plant materials as medicinal sources has been discovered in archaeological finds and old literature. In the Malay Peninsula, the record shows that not less than 1,300 plants have been used in the traditional medicine practice. This is due to Malaysia's tropical rainforest which is rich with biological and chemical resources. The plants in the tropical rainforest are very diverse biologically, and they synthesize a lot of chemicals to protect themselves from pests, predators, and diseases.<sup>2</sup> However, Malaysia is yet to develop the rich plant resources to their full potential as pharmaceutical agents. This is due to the high cost of research and development, coupled with the lack of expertise and technology in the natural product research. Ostensibly, Malaysia needs to have a collaboration with international researchers or pharmaceutical companies to explore and develop the product from the rich medicinal plant resources that can be found in Malaysia.<sup>3</sup>

The medicinal plant that is used in this study is *Kyllinga nemoralis*. The common name for *K. nemoralis* is white water sedge, whitehead spike sedge, white *kyllinga*, white-flowered *kyllinga*, and poverty grass. The family of the *K. nemoralis* is Cyperaceae and the genus is *Kyllinga* Rottb. The description of this plant is grass-like in the habitat, and it propagates by seeds and creeping rhizomes. The plant is considered invasive because when the plant is dispersed in a new place, it will

reproduce and then out-compete the native plants. Moreover, this plant also has the characteristics of a successful invasive species because it has an asexual spreading and has positive respond to human-caused disturbance.<sup>4</sup>

*K. nemoralis* has been known to be widely used in the traditional medicine practice throughout the world. The parts that are usually used are the leaves and the rhizomes, which contain a lot of biologically active chemicals. The leaves are traditionally used to relief malarial chills, pruritis of the skin, diabetes, and thirst due to fever.<sup>5</sup> In India, the leaves are used as anti-venom for snake poisoning.<sup>6</sup> However, the mechanism of action of the plant is not direct inhibition of the action of the venom, but rather symptomatic relief by the anti-inflammatory, tranquilizing, or analgesic properties of the plant. The rhizomes of *K. nemoralis* has a sweet fragrant. The rhizomes are traditionally used as antidiarrhea, expectorant, refrigerant, stomachic, and diuretic. The rhizomes can also be used to treat worm infection by mixing the rhizomes paste with milk.<sup>7</sup> The current study was carried out to screen the phytochemical contents and investigate antibacterial activities of *K. nemoralis* extracts towards selected Gram positive and Gram negative bacteria.

## MATERIALS AND METHODS

### Plant Material

In this study, the plant samples were collected in Kuala Nerus, Terengganu, Malaysia. Whole plants of *K. nemoralis* except root (500 g) were washed with water and then drained at ambient temperature until it dried completely. The plants were further oven-dried for three days at 60°C. After the drying process, the plants were cut into small pieces and ground using the electric blender (Kenwood Ltd, Havant, United Kingdom). The coarse powder was kept in air-tight container. The coarse powder of the plants was split into two portions which were used for the aqueous and methanolic extracts.<sup>8</sup>

### Extraction Methods

For preparing aqueous extract of the *K. nemoralis*, 100 g of the coarse powder of the

plants was mixed with 1.5 L of distilled water and stirred using glass rod to dissolve the powder properly. The mixture was boiled on the hot plate for 20 minutes in a glass beaker. Then, the mixture was cooled off to room temperature before being filtered with the filter paper. The filtering process was repeated twice to finely filter the powder. After that, the filtrate was frozen at -20°C for seven hours. Then, the frozen filtrate was transferred into the freeze dry machine (SP VirTis Genesis Pilot Lyophilizer, USA). The freeze dry process was to stabilize and preserve the chemical structure of the plants extract; the process took 31 hours to complete. The resulting extract was weighed using analytical balance and kept in the chiller at 4°C.<sup>9</sup>

The methanolic extract of the *K. nemoralis* was prepared by immersing 100 g of the coarse powder of the plants with 1 L of methanol for 96 hours at ambient temperature. Then the mixture was filtered using filter paper. After that the powder was re-immersed in 1 L of methanol for another 96 hours and this process was repeated three times. All of the three repeated filtrates were separately collected. The filtrates were then evaporated using rotary evaporator (Eyela N-1110, Japan) until almost dry. The crude extract was oven dried at 50°C for three days to completely dry the methanol solvent. After that, the extract was weighed and the extract was preserved at 4°C.<sup>10</sup>

### Phytochemical Screening

#### Test for Alkaloid

One mL of 25% ammonia was added into 10 mg of extract in a test tube and the mixture was set aside for a minute. After that, 5 mL of chloroform was added into the mixture and the test tube was shaken for three minutes. Then, three drops of Mayer's reagent were added to the mixture. The development of creamy white-colored precipitate indicated the presence of alkaloid in the mixture.<sup>11</sup>

#### Detection of Saponin

10 mg of extract was dissolved in 1 mL of distilled water. Then the mixture was shaken for 5-15 minutes. The formation of soap-like foam layer shows that there was presence of saponin in the extract solution.<sup>12</sup>

Test for terpenoid. 10 mg of extract was added to 2 mL of chloroform followed with 3 mL

of concentrated sulphuric acid. The formation of a reddish-brown indicated the existence of terpenoid in the extract.<sup>13</sup>

#### Determination of Steroid

10 mg of extract was added to 2 mL of chloroform. Then the mixture was added with 2 mL of concentrated sulphuric acid and shaken well. If there was presence of steroid, the chloroform layer would appear red or reddish-brown after a few minutes.<sup>14</sup>

### Antibacterial Activity

#### Test Bacteria

The bacterial strains used as test organisms were *Staphylococcus aureus* (ATCC 11632), clinical isolate methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus epidermidis* (ATCC 12228), *Bacillus thuringiensis* (ATCC 10792), *Streptococcus pyogenes* (ATCC 12344), *Shigella sonnei* (ATCC 25931), *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 10031) and clinical isolate *Salmonella* Typhi. The first five organisms are Gram positive, while the rest are Gram negative. All the stock cultures were obtained from the Microbiology Laboratory, Faculty of Medicine, Universiti Sultan Zainal Abidin.

#### Culture Media and Inoculums Preparation

The Gram positive and Gram negative bacteria were cultured in both nutrient agar and broth. The bacteria inoculums were prepared from the 24 hours, 37°C culture of bacteria isolates in nutrient broth.

#### Antibacterial Test

Antibacterial test is to measure how much the aqueous and methanolic extracts of *K. nemoralis* can inhibit the bacteria. The antibacterial test was the agar disk diffusion method. The Mueller-Hinton agar plates were inoculated with a bacteria strain with 0.5 MacFarland standard. Then filter paper disc (6 mm in diameter) containing the extracts with concentrations of 500, 250 and 125 mg/mL were placed on the agar surface. Chloramphenicol was used as a positive control and 10% methanol was used as a negative control. The Mueller-Hinton agar plates were incubated aerobically at 37°C for 24 hours. Subsequently, the

**Table 1.** Phytochemical content of aqueous and methanolic extract of *K. nemoralis*

Extract	Phytochemical tests			
	Alkaloid	Saponin	Terpenoid	Steroid
Aqueous extract of <i>K. nemoralis</i>	-	+	-	+
Methanolic extract of <i>K. nemoralis</i>	-	-	+	+

Indicator: - : absence, +: presence

diameter of the zone of inhibition surrounding the extract disk, positive control, and negative control were measured and the data were recorded.<sup>15</sup>

### Minimum Inhibitory Concentration (MIC)

The MIC test was performed in 96-well microtiter plate. The aqueous and methanolic extracts of *K. nemoralis* were subjected to two-fold serial dilutions to a final volume of 100 µL at final concentrations of 125, 250 and 500 mg/mL. Next, 100 µL of stock solutions of *S. aureus*, *S. epidermidis*, *B. thuringiensis*, MRSA, *S. pyogenes*, *S. sonnei*, *E. coli*, *K. pneumoniae* and *Salmonella* Typhi (0.5 MacFarland) was added to each well to obtain a final volume of 200 µL. Wells containing MHB with aqueous and methanolic extracts of *K. nemoralis* were used as negative control while wells containing MHB and bacteria inoculum were used as viability control. Wells containing MHB alone acted as sterility control. Well with two-fold serial dilutions of 1 mg/mL to 512 mg/mL chloramphenicol were used as positive control. All tests were performed in triplicates and incubated at 37°C for 24 hours. The determination of MIC was based on the clear appearance of the MHB indicated that there is no visible growth after being incubated with the tested microorganisms.<sup>16</sup>

## RESULTS

The phytochemical screening of aqueous extract of *K. nemoralis* showed the existence of saponin and steroid, while methanolic extract of *K. nemoralis* showed terpenoid and steroid. The results for the alkaloid test are negative for both extracts, as given in Table 1.

A varying antimicrobial activities was shown by both extracts when tested against the nine pathogenic bacteria (Tables 2 and 3). Aqueous extract of *K. nemoralis* was active against Gram positive bacteria, while totally inactive against

Gram negative bacterial strains. The methanolic extract of *K. nemoralis* exhibited the highest zone of inhibition on MRSA.

MIC values for aqueous extract of *K. nemoralis* ranged from 15.63-500 mg/mL against tested bacterial strains and MIC values for methanolic extract of *K. nemoralis* ranged from 0.98-3.91 mg/mL against tested bacterial strains (Table 4).

## DISCUSSION

The presence of phytochemical constituents seen in this study like saponin, terpenoid, and steroid was in good agreement with a previous report,<sup>17</sup> while the presence of carbohydrates, phenols, flavonoids, flavones, tannins, saponins, alkaloids, steroids, terpenoids, coumarins, quinones, lignins, and fats and oils in all the plant parts have been reported.<sup>18</sup> This difference could be because of the geographical difference of the plant material. Phytochemical constituents such as saponins might potentially be antimicrobial, antiviral, antioxidant, and anti-inflammatory.<sup>19</sup>

Saponins are one of the most diverse and numerous groups of plant natural products. The function of saponins in plants is as the defense against disease, herbivores, and can be also allelopathic agents in interactions with other plants. The saponin compounds are present in the aqueous extract of *K. nemoralis*, thus this extract has antibacterial activity towards Gram positive bacteria. The result from our study is supported by the results of another study of saponin substances that have showed suppressive effect on Gram positive organism nevertheless not on Gram negative.<sup>20</sup>

Terpenoids are the substantial and also constitutionally most varied group of the secondary metabolites extracted from

**Table 2.** Zone of inhibition (mm) for disc diffusion method of aqueous extract of *K. nemoralis* against selected bacteria

Tested bacterial strains	Inhibition zone (mm)				
	500 mg/mL extract	250 mg/mL extract	125 mg/mL extract	Distilled water (negative control)	Chloramphenicol (positive control)
<i>S. aureus</i>	11	11	8	6	28.5
<i>S. epidermidis</i>	6	6	6	6	31.5
<i>B. thuringiensis</i>	6	6	6	6	31.0
MRSA	10	8	6	6	23.5
<i>S. pyogenes</i>	6	6	6	6	31.5
<i>S. sonnei</i>	6	6	6	6	30.0
<i>E. coli</i>	6	6	6	6	27.5
<i>K. pneumoniae</i>	6	6	6	6	33.0
<i>Salmonella</i> Typhi	6	6	6	6	28.5

Tests were performed in triplicate

**Table 3.** Zone of inhibition (mm) for disc diffusion method of methanolic extract of *K. nemoralis* against selected bacteria

Tested bacterial strains	Inhibition zone (mm)				
	500 mg/mL extract	250 mg/mL extract	125 mg/mL extract	Distilled water (negative control)	Chloramphenicol (positive control)
<i>S. aureus</i>	6	6	6	6	25.5
<i>S. epidermidis</i>	6	6	6	6	33.5
<i>B. thuringiensis</i>	6	6	6	6	30.5
MRSA	9	8	7	6	23.5
<i>S. pyogenes</i>	6	6	6	6	30.5
<i>S. sonnei</i>	6	6	6	6	30.0
<i>E. coli</i>	6	6	6	6	25.0
<i>K. pneumoniae</i>	6	6	6	6	29.5
<i>Salmonella</i> Typhi	6	6	6	6	25.0

Tests were performed in triplicate

plants. Previous studies on plant methanolic extracts showed terpenoids might potentially be responsible for the therapeutic value of the plant material extract.<sup>21</sup> It is known that terpenoids have various therapeutic effects which includes antifungal, antihyperglycemic, anti-inflammatory, antimicrobial, antiviral, antiparasitic, antioxidants, and immunomodulatory.<sup>22</sup> The presence of terpenoid in the methanolic extract of *K. nemoralis* may influence the antibacterial activity of the extract. From a previous study, the terpenoids have been shown to have a strong antibacterial

effect.<sup>23</sup> In another study, terpenoids were capable to suppress the essential activities needed for microbial viability which is oxidative phosphorylation and oxygen intake.<sup>24</sup>

In plants, steroids play a lot of roles that cannot be replaced by other substances such as controlling fluidity, sustaining membrane semi-permeability, and also serve as biosynthetic precursors for steroid hormones.<sup>25</sup> In one study, stigmasterol, the plant-based steroid, showed an antibacterial activity against *S. aureus* even at low concentrations. The antibacterial effect of the

**Table 4.** MIC value of aqueous and methanolic extracts of *K. nemoralis*

Tested bacterial strains	MIC values of extract (mg/mL)	
	Aqueous extract	Methanolic extract
<i>S. aureus</i>	62.5	3.91
<i>S. epidermidis</i>	125	3.91
<i>B. thuringiensis</i>	125	0.98
MRSA	15.63	3.91
<i>S. pyogenes</i>	>500	1.95
<i>S. sonnei</i>	15.63	1.95
<i>E. coli</i>	62.5	3.91
<i>K. pneumoniae</i>	15.63	1.95
<i>Salmonella</i> Typhi	31.25	3.91

Tests were performed in triplicate

steroid and cholesterol derivatives may come from the disruption of cell integrity and permeability.<sup>26</sup> In our study, steroids are present in both extracts. The steroids are soluble in both polar and non-polar solvents. However, polar solvents such as methanol and water are more preferred for steroid extraction.<sup>27</sup>

Saponin, terpenoid, and steroid contents in aqueous and methanolic extracts of *K. nemoralis* may lead their antibacterial property. The significant antibacterial activity against Gram positive bacteria is perhaps because of a single or joined effect of these secondary metabolites.

The natural products from the plants have been reported to target and act on a few bacterial targets such as the bacterial cell wall, bacterial cell division, pyruvate kinase, DNA topoisomerase, and efflux pump.<sup>28</sup> In one study, the antibacterial activities of the natural products were in a positive correlation with the amount of the phenolic compounds that can be found in the plants.<sup>29</sup> Thus, *K. nemoralis* might have an active phenolic compound that can inhibit the growth of the *S. aureus* and also MRSA. However, antibacterial activities of medicinal plants were observed by a researcher to be least effective against the Gram negative bacteria compared to Gram positive bacteria.<sup>30</sup> This is unsurprising because the Gram negative bacteria have been shown to be more resistant to antibiotics.<sup>31</sup> Probably due to the characteristic of their cell walls, Gram positive

bacteria were more susceptible to the plant substances than Gram negative bacteria. Gram negative bacteria have phospholipid membranes containing lipopolysaccharide elements that make their cell wall impassable to certain natural or synthetic antimicrobial drugs.

The results show that the methanolic extract exhibited antibacterial activities on more bacteria than the aqueous extract. Even though the freeze dry process can preserve phytochemical substances better than any other extraction method, the extraction yield and the biological activity of the extracts are also affected by the extraction solvent. The phytochemical processing of the raw plant materials is important to optimize the concentration of the constituents and to maintain the antibacterial activities.<sup>32</sup>

There are a few solvents that are usually used in the plant extraction such as distilled water, aqueous mixture of ethanol, ethanol, methanol, and acetone. The selection of the suitable extraction method and the solvent are important, and the selection is based on the sample chemical properties of the analytes, efficiency, desired properties, matrix properties and matrix-analyte interaction.<sup>33</sup> Besides that, the chemical characteristics and polarities of the substances in the natural products may be or may not be dissolved in a certain solvent.

Methanol, acetone, and ethyl acetate are examples of polar solvent frequently used in the process of recovering polyphenols from the natural products. Production of lower molecular weight of polyphenols is known to be more effective by using methanol as solvent; while aqueous acetone is known to extract higher molecular weight of flavanols.<sup>34</sup> However, distilled water is still useful in extracting other molecules. In a study, distilled water was found to be a better solvent in extracting tea catechins than methanol or ethanol.<sup>35</sup>

MIC is the minimal concentration of antimicrobial drug that inhibit the growth of microorganisms after 24 hours incubation. The MIC is used to estimate the antimicrobial efficacy of potential antimicrobial drug by estimating the impact of descending antimicrobial doses. Antimicrobial drug with lower MIC is thought to be more potent. The MIC values derived from the

current study proved that *K. nemoralis* aqueous extract was more potent against *S. aureus* and MRSA, which comply with the agar disk diffusion method result. Potent antibacterial activity was also recognized against *S. aureus* and MRSA at low concentrations of *K. nemoralis* extracts. The dissimilarities in bacterial susceptibility are perhaps due to differences in essential strength of bacteria, or the physicochemical effects of phytochemicals contained in the plant materials.<sup>36</sup>

## CONCLUSION

The aqueous and methanolic extracts of *K. nemoralis* exhibited a significant antibacterial activity. *K. nemoralis* extracts exhibited more effective antibacterial properties against Gram positive bacteria than Gram negative at low concentrations, which indicates that *K. nemoralis* contain special bioactive substances that may provide some benefits in the treatment of infectious diseases caused by Gram positive bacteria.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

## ETHICS STATEMENT

This article does not contain any studies

on human participants or animals performed by any of the authors.

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