

Phytochemical Screening and *in vitro* Antibacterial Activity of *Kyllinga nemoralis* Aqueous and Methanolic Root Extracts

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Abstract

The present study focused to explore the phytochemical composition and *in vitro* antibacterial potential of *Kyllinga nemoralis* aqueous and methanolic root extracts. The antibacterial activities of *K. nemoralis* aqueous and methanolic root extracts were tested against five Gram positive and four Gram negative bacteria strains. Disk diffusion method was performed to evaluate antibacterial activities of the both extracts. *K. nemoralis* aqueous and methanolic root extracts showed a potent antibacterial activity against Gram positive and Gram negative bacteria strains. The methanolic root extract of *K. nemoralis* showed efficiency against *S. aureus*, MRSA, *S. pyogenes*, *S. sonnei*, *E. coli* and *K. pneumoniae* while the aqueous extract of *K. nemoralis* was found to possess antibacterial activity against *S. aureus* and MRSA. This activity seems to be related to the phytochemicals (saponins) detected into the aqueous extract and phytochemicals (terpenoid and steroid) in methanolic extract. The results confirm that root extracts of *K. nemoralis* can be used as a drug to fight infections caused by the broad-spectrum bacteria strains.

Keywords

Kyllinga nemoralis, Phytochemical Analysis, Disk diffusion, Antibacterial Activity, Minimum Inhibitory Concentration.

Introduction

Antibiotic resistance is one of the biggest public health challenges that need to be fight. The infections that caused by the antibiotic resistant microorganism is hard to treat and usually fatal to the patient. The treatment for this infection usually will require a longer treatment at the hospital, more follow up with the doctor and it will cost a lot more than the regular infections treatment. Besides that, the treatment for the antibiotic resistance infection will use agents with increased toxicity as an alternatives way to treat the patient [1]. Thus, the searching for the alternative antimicrobial must be find to solve this problem. The new antibiotic must have the same or better efficacy than the current used antibiotic and also cost less than the drugs that being use today. Moreover, the alternative antibiotic must also reduce the side effect of the antibiotic effect and also not toxic to the human cell. The property of the natural product is suitable and match the characteristic which is not toxic in right dosage and it is cost effective to produce in mass production than the synthetic substitutes [2].

The use of antibiotics is still questionable in the future due to the growing problem of microbial resistance. Therefore, actions should be taken to resolve this issue, such as limiting the use of antibiotics, conducting research to gain a deeper understanding of the genetic pathways that cause resistance, and conducting continuous research to develop new medicines, whether natural or synthetic. Providing the patient with the best antimicrobial medications is the ultimate goal [3]. Natural product with modern synthetic modifications approaches to develop new antibiotic can overcome the antibiotic resistance [4]. The medicinal values of plants are based on the presence of the phytochemical constituents which can give a physiological or pathological effect towards the human body. Phytochemical is defined as a bioactive compound that produce by the plants such as the fruit, vegetables and grains which can give health benefits and even reduce the risk of major chronic diseases [5]. Some of the bioactive substances that can be extract from the plant is flavonoids, alkaloids, tannin, phenol compound, saponin, glycoside and anthraquinones. Research of the phytochemical composition of plant is important to develop the product or drugs based on the natural products. The phytochemical tests will sort out which active compound is found in the plants and will exert the therapeutic effects [6].

Kyllinga nemoralis or well-known as white water sedge is a common grass that can be found in tropical countries, Australia and Pacific islands. In Malaysia it is known as 'rumpit butang' which is the wild plant (weed) that grow without cultivated. It often lives in the wet grassland or at the moist areas which up to 850m above the sea level. The family name for *K. nemoralis* is Cyperaceae [7]. The water sedge is easily identified by its three-sided stems and have a small green or white button-like flower at the top of the three frass-like leaves. *K. nemoralis* is use as a folk medicine in many countries for example Malaysia, India and China. Both of the white headed or green headed variety can be used as a traditional medicine. The usage of this plant is to treat the common colds, bronchitis, malaria, arthritis and some injuries. Other than that, the major usage of the *K. nemoralis* is to treat the digestion problem such as diarrheal, intestinal problems and dysentery. According to the [8], study shows that *K. nemoralis* essential oils has a moderate antibacterial activity towards the Gram negative bacteria such as *E. coli* and *Pseudomonas aeruginosa* and have antimalarial and anticancer properties. Research by [9] showed that *K. nemoralis* aqueous extract showed direct virucidal activity against HSV-1. This result revealed the strong extracellular interaction between HSV-1 and extract. In addition, *K. nemoralis* roots aqueous extract exhibited more effective when administered as pre-treatment in HSV-1 infection compare when the treatment is given in pre-treatment assay and virucidal assay [10]. The problem of using the broad spectrum of antibiotic such as chloramphenicol is the spread of resistance in the multiple bacterial species. Besides that, the broad antibiotic can also harmful to the microbiome in the human body [11]. Thus, the rationale of this study is to analyse the phytochemical that presence in the aqueous and methanol extract of *K. nemoralis* and also measure the antibacterial activity of the aqueous and methanol extract of *K. nemoralis* against the Gram positive and Gram negative bacteria.

Materials and Methods

Plant Material

K. nemoralis plant samples were collected in Kuala Nerus, Terengganu, Malaysia. Roots part of fresh *K. nemoralis* (500 g) were rinsed with flowing water, sun dried until it completely dried. Dried roots were powdered in an electric blender.

Preparation Aqueous and Methanol Extracts

Aqueous and methanol root extracts of *K. nemoralis* were prepared based on guidelines from [12] with modifications. For producing *K. nemoralis* aqueous root extract, dried root powder (100 g) was dissolved with 1.5 L of deionized water. The concentrate was boiled for 20 minutes before it was filtered. Then, the filtrate was frosted, -20°C, seven hours and was freeze dried using a freeze dry machine. The resulting extract was kept in the chiller at 4°C until it was used for subsequent tests.

The methanolic extract was processed by sinking dried root powder (100 g) with methanol (1L) for 3 days at room temperature. Then, the precipitate was re-sunk in methanol (1L) for another 3 days and this procedure was continual for three times. Lastly, rotary evaporator was used to evaporate the extract before it was stored at 4°C in a brown bottle until use.

Phytochemical Screening

Test for Alkaloid

1 mL of ammonia (25%) was mixed with the 10 mg/mL of extract. The mixture was put aside for 1 minute. Consequently, the chloroform (5 mL) was added to the mixture and shaken for 3 minutes. The formation of creamy white coloured precipitate after Mayer's reagent were put in to the mixture indicating the presence of the alkaloids [13].

Detection of Saponin

The formation of soap-like foam layer after 10 mg/mL of extract was diluted with 1 mL of distilled water and shaken for 5-15 minutes indicating the presence of saponins in the extract solution [12].

Determination of Steroid

10 mg/mL of extract was mixed the chloroform (2 mL) and 100% sulphuric acid (2 mL). If there was existence of sterols steroid, the chloroform layer would resemble red or reddish-brown in a few minutes after the mixture was shaken [12].

Test for Terpenoid

The extract (10 mg/mL) was mixed with the chloroform (2 mL) and followed 100% sulphuric acid (3 mL). The presence of terpenoids in the extract indicated by the formation of a reddish-brown layers [14].

Antibacterial Activity

Bacteria Strains

Staphylococcus aureus (ATCC 11632), clinical isolate methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pyogenes* (ATCC 12344), *Streptococcus epidermidis* (ATCC 12228), *Bacillus thuringiensis* (ATCC 10792), *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 10031,) *Shigella sonnei* (ATCC 25931) and clinical isolate *Salmonella* Typhi were obtained from the Microbiology Laboratory, Faculty of Medicine, Universiti Sultan Zainal Abidin were used as test organisms.

Culture Media and Inoculums Preparation

All of the bacteria strains were cultured in nutrient agar and nutrient broth. Nutrient broth was used to prepare bacterial inoculums from bacteria isolates culture. All of the bacteria strains used in agar well diffusion assay was originally sub-cultured in nutrient agar media and incubated at 37 °C for 24h. MIC assay were determined by the broth dilution method using Muller Hinton broth which the bacteria were grown at 37 °C for 24h. Plant extract and broth inoculated with particular bacteria strains were used as positive and negative controls in MIC assay. While for antibacterial assay, chloramphenicol (30 µg/mL) was used as the positive control and methanol 10% as the negative control.

Antibacterial Test

The bacterial suspension was lawn on the Mueller-Hinton agar (MHA) petri plates. The antibacterial activities of the extract were measured by using the disk diffusion method. 100 µL of the extract with different concentration (500 mg/mL, 250 mg/mL and 125 mg/mL) was drop on each disk respectively. The plates were incubated at 37 °C for 18-24 hours. After that, the plates were analyzed for inhibition zones, and the results were correlated to the positive control, chloramphenicol.

Determination of Minimum Inhibitory Concentration (MIC)

Antibacterial capability of *K. nemoralis* aqueous and methanolic root extracts were determined by using macro-broth dilution technique by estimating the observable bacterial growth in MHB. For MIC evaluation in MHB, serial dilutions of the extract at the several concentrations (600 to 0.98 mg/mL) with an adjusted concentration of bacterial strains equal to 0.5 McFarland standard. The positive control was inoculated broth and the negative control was only plant extract at which incubated at 37°C, 24 h. The MIC was defined as the least concentration of extracts in consequence the tubes did not show any observable bacterial growth. In MIC determination, the test tubes were evaluated for their turbidity after pre-incubation and post-incubation [15].

Results

Phytochemical Analysis

The phytochemical analysis of aqueous root extract of *K. nemoralis* exhibited the presence of saponin and steroid, meanwhile methanolic root extract of *K. nemoralis* exhibited steroid and terpenoid. However, the results for the alkaloid test are negative for both extracts. It is observed that extraction of the bioactive compounds from *K. nemoralis* root using aqueous and methanolic was have the same efficiency.

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

| Solvent | Phytochemical tests | | | |
|------------|---------------------|---------|---------|-----------|
| | Alkaloid | Saponin | Steroid | Terpenoid |
| Aqueous | - | + | + | - |
| Methanolic | - | - | + | + |

Indicator: - : absence, +: presence

Antibacterial Activity

Varying antimicrobial activities were shown by the aqueous and methanolic root extracts of *K. nemoralis* against tested bacteria strains (Tables 2 and 3). The findings showed six over nine tested bacteria showed sensitivity against aqueous and methanolic root extracts of *K. nemoralis* at concentration of 500mg/mL. The aqueous root extract shown a consistent trend of bacterial inhibition with the highest zone of inhibition towards MRSA (11 mm), *S. pyogenes* (11 mm), *S. sonnei* (11 mm), *S. aureus* (10.5 mm), followed by *E. coli* (7 mm) and *K. pneumoniae* (7 mm) as well as with methanolic root extract MRSA (13 mm), *S. pyogenes* (12.5 mm), *S. sonnei* (12 mm), *S. aureus* (11 mm), followed by *E. coli* (8 mm) and *K. pneumoniae* (7 mm). Due to the significance antibacterial effect in agar well diffusion analysis against the tested bacterial strains, the MIC was determined in the both extracts respectively. MIC values for aqueous root extract of *K. nemoralis* are ranged from 31.25-250 mg/mL towards tested bacterial strains. Meanwhile, the MIC values for methanolic root extract of *K. nemoralis* are ranged from 0.98-15.63 mg/mL. These results indicated the potency of *K. nemoralis* root in both aqueous and methanolic extracts. The potent antibacterial effect could be associated to the existence of bioactive compounds in the extracts.

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

| Tested bacterial strain | Inhibition zone (mm) | | | | |
|-------------------------|----------------------|-------------|------------|-----------------------------|------------------------------------|
| | 500 mg/mL | 250 mg/mL | 125 mg/mL | Methanol (Negative control) | Chloramphenicol (Positive control) |
| <i>S. aureus</i> | 10.5 ± 0.57 | 10.0 ± 0.15 | 9.0 ± 0.17 | 6.0 ± 0.0 | 25.5 ± 0.12 |
| <i>S. epidermidis</i> | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 32.0 ± 0.30 |
| <i>B. thuringiensis</i> | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 31.5 ± 0.22 |
| MRSA | 11.0 ± 0.11 | 7.5 ± 0.22 | 6.0 ± 0.0 | 6.0 ± 0.0 | 24.5 ± 0.10 |
| <i>S. pyogenes</i> | 11.0 ± 0.15 | 9.0 ± 0.61 | 6.0 ± 0.0 | 6.0 ± 0.0 | 31.5 ± 0.17 |
| <i>S. sonnei</i> | 11.0 ± 0.37 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 31.5 ± 0.21 |
| <i>E. coli</i> | 7.0 ± 0.23 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 22.5 ± 0.32 |

| | | | | | |
|----------------------|------------|-----------|-----------|-----------|-------------|
| <i>K. pneumoniae</i> | 7.0 ± 0.17 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 31.0 ± 0.19 |
| <i>S. Typhi</i> | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 28.5 ± 0.33 |

*Data are means of three replicates (n = 3) ± standard error

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

| Tested bacterial strain | Inhibition zone (mm) | | | | |
|-------------------------|----------------------|-------------|-----------|-----------------------------|------------------------------------|
| | 500 mg/mL | 250 mg/mL | 125 mg/mL | Methanol (Negative control) | Chloramphenicol (Positive control) |
| <i>S. aureus</i> | 11 ± 1.8 | 10.5 ± 0.45 | 9.0 ± 1.4 | 6.0 ± 0.0 | 25.5 ± 0.31 |
| <i>S. epidermidis</i> | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 32.0 ± 0.55 |
| <i>B. thuringiensis</i> | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 31.5 ± 0.08 |
| MRSA | 13 ± 0.35 | 8.5 ± 0.31 | 6.0 ± 0.0 | 6.0 ± 0.0 | 24.5 ± 0.03 |
| <i>S. pyogenes</i> | 12.5 ± 1.4 | 9.0 ± 1.25 | 6.0 ± 0.0 | 6.0 ± 0.0 | 31.5 ± 0.21 |
| <i>S. sonnei</i> | 12 ± 0.22 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 31.5 ± 0.31 |
| <i>E. coli</i> | 8.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 22.5 ± 0.14 |
| <i>K. pneumoniae</i> | 7.0 ± 0.25 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 31.0 ± 0.40 |
| <i>S. Typhi</i> | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 6.0 ± 0.0 | 28.5 ± 0.37 |

*Data are means of three replicates (n = 3) ± standard error

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

| Tested bacterial strain | MIC values of extract (mg/mL) | |
|-------------------------|-------------------------------|-------------------------|
| | Aqueous root extract | Methanolic root extract |
| <i>S. aureus</i> | 62.5 | 15.63 |
| <i>S. epidermidis</i> | 250 | 7.81 |
| <i>B. thuringiensis</i> | 250 | 1.95 |
| MRSA | 31.25 | 7.81 |
| <i>S. pyogenes</i> | 125 | 0.98 |
| <i>S. sonnei</i> | 31.25 | 1.95 |
| <i>E. coli</i> | 125 | 1.95 |
| <i>K. pneumoniae</i> | 62.5 | 1.95 |
| <i>S. Typhi</i> | 125 | 7.81 |

Discussion

Natural antimicrobial bioactive constitution from plants acts as therapeutics that can suppress the growth of the pathogenic microorganisms and have been used to overwhelm the complications related with the synthetic or manufactured antimicrobial agents [16]. The findings of the study reported that the aqueous and methanol extracts of *K. nemoralis* showed remarkable inhibitory recreations against six from nine different pathogenic bacteria. The inhibitory recreations of the plant extract could be associated to the existence of bioactive compounds such as phenols because it controls the growth and proliferation of bacteria by perturbing the roles of bacterial cell membranes. The systemic phytochemical analysis of plant extracts is an essential approach to discover the new novel compounds of therapeutic properties [17]. Phytochemical analysis of aqueous and methanolic root extracts of *K. nemoralis* proved that the plant is rich in bioactive compounds such as saponins, steroids and terpenoids which could be responsible for its antibacterial effects. Phytochemicals display antimicrobial effect via varying mechanisms as an example, ruination of cell membrane, intercalation of DNA, obstruction of microbial adhesions and inactivation of microbial enzymes activities [18].

Methanolic extract was identified to be more strong than aqueous extracts against the bacterial strains. The recent research proved that methanol and ethanol solvent were more effective than water in isolating phenolics from medicinal plants [19,20,21]. Extraction solvents have an effect on the extraction yield and the total content of bioactive compounds, thus considerably affecting the biological activities of the extract

[22,23,24]. In addition, different studies have reported that the type of solvent used has a significant function in defining the activity of the extract [25,26,27]. This is because of the distinction in the relative solubility of different phytochemicals in the solvents with dissimilarity polarities. The negative control used in this test was 10% methanol because methanol was the solvent used as compound diluents that could dissolve *K. nemoralis* aqueous and methanolic extracts and had no antibacterial activity [28]. The positive control used in the antibacterial activity test was chloramphenicol. The selection of chloramphenicol as a positive control was due to chloramphenicol having a broad spectrum of activity against Gram positive and Gram negative bacteria [29].

Results of antimicrobial activity of the aqueous and methanolic root extracts of *K. nemoralis* extracts can suggested that *S. epidermidis*, *B. thuringiensis* and *S. typhi* was the most resistant bacteria strains to plant extracts. MRSA was the most susceptible strains to the aqueous root extract of *K. nemoralis* followed by *S. pyogenes*, *S. sonnei*, *S. aureus*, *K. pneumoniae*, and *E. coli* respectively. Moreover, MRSA also was the most susceptible strains to the methanol root extract of *K. nemoralis* followed *S. pyogenes*, *S. sonnei*, *S. aureus*, *E. coli*, and *K. pneumoniae*. Based on Davis and Stout, the classification of antibacterial strength activity are based on the diameter of inhibition zone: very strong (>20 mm), strong (10–20 mm), medium (5–10 mm), and weak (<5 mm) [30]. Therefore, both the aqueous and methanolic root extracts of *K. nemoralis* included in the strong category towards *S. aureus*, MRSA, *S. pyogenes* and *S. sonnei* at concentration of 500 mg/mL. The concentration of 250 mg/mL, both aqueous and methanolic root extracts of *K. nemoralis* were in the strong category towards *S. aureus*. This plant extracts, on the other hand at 125 mg/ml concentration were in the medium category in relation to *S. aureus*. Aqueous and methanolic root extracts of *K. nemoralis* at concentration of 250 mg/mL were included in the medium category towards MRSA and *S. pyogenes*. Meanwhile, aqueous and methanolic root extracts of *K. nemoralis* at concentration of 500 mg/mL were included in the medium category towards *E. coli* and *K. pneumoniae*. Extracts at all concentrations were in the very weak category towards *S. epidermidis*, *B. thuringiensis* and *S. Typhi*. The results of this study showed that the *K. nemoralis* root extract possessed potential antibacterial activity against MRSA and hence can be further explored for pharmaceutical applications as a natural antibacterial agent. *S. aureus* is a highly pathogenic bacteria that causes a wide range of diseases. The methicillin-resistant strains of this bacteria pose a serious health threat. Thusly, there is a high demand to develop antibiotics from natural sources based on medical plant extracts in an effort to support the effectiveness and potency of conventional antibiotics [31].

The results of measurements of inhibitory zone diameters generally showed that aqueous and methanolic root extracts of *K. nemoralis* had more antibacterial activity against Gram positive bacteria compared to Gram negative bacteria. This can be attributed to the differences in cell wall structure between Gram positive and Gram negative bacteria [32]. The outer membrane and cytoplasmic membrane are both part of the cell membranes of Gram negative bacteria, while Gram positive bacteria have only a cytoplasm. The antibacterial activity can be affected by the difference in cell wall composition and structure between Gram negative and Gram positive bacteria. The cell wall of *S. aureus*, which includes Gram positive bacteria, is composed mostly of a water-soluble polymer with peptidoglycan as its main component. The characteristic indicates that the walls of bacterial cells are polar. *K. nemoralis* root extracts that are both aqueous and methanolic are also polar, which makes it easier to break through the polar peptidoglycan layer compared to the non-polar lipid layer [33]. Gram negative bacteria contain more lipids and less peptidoglycan, and have an outer membrane in the form of a bilayer that serves as a selective defense [34]. The ineffectiveness of *K. nemoralis* extracts against Gram-negative bacteria can be attributed to this reason.

The presence of phytochemical compounds in *K. nemoralis* extract revealed from this study such as saponin, steroid and terpenoid was in agreement with that of an early study in the previous report, [12] while the unrepresented of alkaloids in *K. nemoralis* extract also have been reported [35]. Phytochemical compounds like saponins might possibly be antimicrobial. Saponins are one of the most various and many groups of

plant natural products. The saponins compound also have the antimicrobial and anti-herbivore activity which shows the function of the saponin as a plant defence substance [36]. The saponins are existent in the aqueous root extract. Thus, it shows why the aqueous root extract have antibacterial activity on some bacteria.

Terpenoids are the greatest and also structurally most varied group of the secondary bioactive compounds isolated from natural products. The plants synthesize a lot of the terpenoids compound even several hundred terpenoid compound with their own role which include antioxidants, phytohormones, protein modification reagents and others [37]. In human, there are also a lot of benefits that can gain from the terpenoids. It is known that the terpenoid have a various therapeutic effect including antimicrobial, antiviral, antifungal, antiparasitic anti-inflammatory, and antioxidants [38]. The existence of the terpenoid in the methanolic root extract may influenced the antibacterial effects of the extract. From the recent study, it shows that the terpenoids compound have a strong antibacterial effect [39]. The factors that determined the activity of antibacterial is the composition, functional groups and the synergistic interactions that present in the active components.

Steroids are also important in medically active organic compound that can be found in the natural products. In plants, the steroid plays a lot of functions that can't be changed by other constituents for example in maintaining membrane semi-permeability, regulating fluidity and also acts as steroid hormones biosynthetic precursors [40]. In the other study, the plant-based steroids such as stigmasterol show an antibacterial effect against *S. aureus* even though it is at low doses. The antibacterial activity of the cholesterol derivatives and steroid possibly result from the disturbance of cell integrity and permeability [41]. The steroids are presence in both of the aqueous and methanol root extracts. The steroids are resolvable in each of polar and non-polar solvents. The polar solvents such as methanol and water are more recommended for steroid extraction [42]. Basically, these results propose that methanol is the solvent of choice for extracting phytochemical constituents from *K. nemoralis* roots.

Conclusion

The finding of this study presented that *K. nemoralis* roots extracts consists of bioactive phytochemical components. Perhaps, these bioactive compounds have the most important approaches against human pathogens, which includes those that are multi drug resistance bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA). The current research provides evidence in favor of the traditional medicinal use of *K. nemoralis* and emphasizes its potential as a natural source of antibacterial compounds that could be used as natural antibiotics in the future to combat the prevalence of MRSA.

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Conflict of Interest Disclosure

None to declare.

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