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Chemical compositions and *in vitro* antimicrobial activity of *Cymbopogon nardus* on food borne microorganisms

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ABSTRACT

Aims: Food borne infections are routinely observed and frequently reported. *Cymbopogon nardus*, or commonly known as citronella is a tropical plant widely used for aromatherapy and insect repellant. This study aimed to evaluate the phytochemical compositions of *C. nardus* ethanol extract and its antimicrobial activity on food borne microorganisms.
Methodology and results: The plant materials were extracted with ethanol using maceration technique. The ethanol extract obtained was analysed using GC-MS and the most abundant compounds were 6-methyloctahydrocoumarin (31.5%) and 2,4-di-*tert*-butylphenol (28.7%). On Kirby Bauer assay, the extract showed wide spectrum inhibitory effect on one Gram-positive bacteria (*Bacillus coagulans*), two Gram-negative bacteria (*Proteus mirabilis* and *Escherichia coli*), and one yeast (*Candida albicans*). All the test microorganisms showed different susceptibility to the extract, where the minimal inhibitory concentrations were ranged from 63 to 250 µg/mL. The inhibitory effect of the extract was bactericidal. In addition, the time kill analysis of the extract exhibited inhibitory effect on *P. mirabilis* which was concentration-dependent. The *C. nardus* extract exhibited rapid bactericidal effect on *P. mirabilis* and lower bacterial counts were obtained with increased extract concentration. Besides, the extract did not exert significant toxicity effect to *Artemia salina* on brine shrimp lethality assay.
Conclusion, significance and impact of study: Therefore *C. nardus* can be a potential source of safe and effective antimicrobial agent to combat food borne infections.

Keywords: Antimicrobial activity, *Cymbopogon nardus*, toxicity.

INTRODUCTION

Food borne infections are caused by consuming food contaminated with disease-causing microorganisms such as viruses, parasites, bacteria and fungi (Scallan *et al*., 2011; Rajkowska and Kunicka-Styczynska, 2018). Food borne infections are important in both resource-rich and resource-poor countries as the problem causes considerable morbidity and mortality. Food and water are the common vehicles for the transmission of illness caused by microorganisms, or the microbial-origin toxins. There are more than 200 known microorganisms which can cause illness when ingested (Newell *et al*., 2010; Sharif *et al*., 2018). Food borne infections are routinely observed and reported. In each year, approximately 48 million people are diagnosed with food borne infections in United States, with 128,000 cases of hospitalization and 3000 deaths. Besides, food borne infections also cause annual medical expenses of 15.5 billion in United States (Hoffmann *et al*., 2015).

Botanical products are widely used in the treatment and prevention of food borne infections. The World Health Organization estimated that almost 80% of the world population is depending on herbal medicine for disease treatment and healthcare (Verma *et al*., 2010; Liu *et al.*, 2018). *Cymbopogon nardus* (Poaceae), is native to South India or Sri Lanka. It is commonly known as citronella, a perennial grass cultivated in South East Asia. The essential oil of *C. nardus* is traditionally used in aromatherapy to improve skin and hair conditions. Besides, the oil is widely used as a natural insect repellent in Asian countries. Today, the essential oil of *C. nardus* is popular for its commercial significance in flavours, fragrances, cosmetics, perfumery, detergents and pharmaceuticals (Silva *et al*., 2011). Previous studies shown that the *C. nardus* essential oil possess several useful bioactivities, such as anti-helminthic, allelopathic, anti-termite, cytotoxic, anticancer, anti-inflammatory and antioxidant activities (Ganjewala, 2009).

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Even though previous studies have revealed the essential oil of *C. nardus* exhibited antibacterial and antifungal activities, however, the antimicrobial activity of its ethanol extract is not reported. Therefore, in this study, we aim to evaluate the antimicrobial activity of *C. nardus* ethanol extract on food borne microorganisms along with its chemical compositions.

MATERIALS AND METHODS

Plant materials

Fresh sample of *C. nardus* was obtained from Malaysian Agriculture Research and Development Institute (MARDI), Kuala Linggi, Negeri Sembilan. A representative accession (MC0771) was deposited at herbarium of Universiti Kuala Lumpur. The collected plant samples were washed under tap water. The samples were then dried at 50 °C until a constant weight was obtained. The dried samples were powdered with food blender. Then, the powdered samples were kept in desiccator until further use.

Extraction

The extraction of plant material was done according to the method described by Urmi *et al*. (2013). The plant samples were soaked in ethanol at ratio of 1:40 (w/v) for four days. The occasional shaking and stirring was done to increase the extraction yield. Then, the extract was filtered with Whatman No. 1 filter paper. The filtrate was collected and dried under reduced pressure using rotatory evaporator to obtain the extract paste. The extract was kept at 4 °C until further use.

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis was performed using an Agilent Technologies 7890A equipped with mass spectrometer (Agilent Technologies 5975C inert XL MSD with Triple Axis Detector). HP-5 MS column (Agilent Technologies Inc., Santa Clara, CA), (5%-phenyl)-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness, 0.25 µm was employed. The operating conditions of the column were as follows: initial oven temperature, 100 °C for 1 min, then to 250 °C at 5 °C/min and held for 5 min; injector port, 260 °C; detector temperatures, 280 °C; carrier gas, 1.0 mL/min He; injection volume, 1.0 µL; split ratio, 50:1. The detected compounds were identified by direct comparison of their mass spectra with NIST 08 Library.

Test microorganisms

The test microorganisms used in this study were previously isolated from food samples purchased from local fresh markets. The test microorganisms used include four Gram-positive bacteria (*Bacillus cereus*, *B. coagulans*, *Staphylococcus aureus*, *B. spizizenii*), four Gram-negative bacteria (*Proteus mirabilis*, *Klebsiella pneumoniae*, *Yersinia enterocolitica*, *Escherichia coli*), two yeasts (*Candida albicans*, *C. utilis*) and two filamentous fungi (*Penicillium expansum*, *Rhizopus stolonifer*). The microbial inoculums were prepared as per protocols described by Tong *et al*. (2014).

Extract preparation

For all the bioassays, the ethanol extract was dissolved in 10% Tween-80 solution to the desired concentration and filtered with membrane filter (PTFE, pore size 0.25 µm) prior to use.

Kirby Bauer assay

The antimicrobial activity of the ethanol extract was screened with Kirby Bauer assay as described by Sasidharan *et al*. (2010). Mueller Hinton agar (Merck) plates were used for test bacteria and Sabouraud dextrose agar (Merck) plates were used for test yeasts and fungi. Firstly, 100 µL of the microbial inoculum was inoculated on surface of agar medium by using sterile cotton swab. Then, 10 µL of 100 mg/mL extract (equivalent to 1 mg of extract per disc) was pipetted onto sterile Whatman A disc. 40 µg/mL of chloramphenicol was included as antibiotic control for test bacteria and yeasts whereas 40 µg/mL of ketoconazole was used for test fungi. For test bacteria and yeasts, the plates were incubated at 37 °C for 24 h whereas for test fungi, the plates were incubated at 30 °C for 72 h. After the incubation period, the diameter of clear zone surrounding the paper disc was measured. The experiments were performed thrice in separate occasions. The results were expressed in average diameter of clear zone ± standard deviation.

Broth microdilution assay

Broth microdilution assay was performed according to Tong *et al*. (2014), with some modifications. A volume of microbial inoculum was diluted 1:10 (v/v) in sterile double strength Mueller Hinton broth (Merck). Doubling dilutions of the extract ranging from 2000 to 63 µg/mL was prepared in 96-wells microtitre plate. After the addition of 100 µL of diluted microbial inoculum, the plates were incubated at 37 °C for 24 h. The final extract concentrations ranging from 1000 to 31 µg/mL were achieved. Growth controls consisting of diluted microbial inoculum and 10% Tween-80 solution was included for each test microorganism. After the incubation period, 20 µL of 0.2 mg/mL tetrazolium violet salt (Sigma) was added into each well. The plate was incubated for 30 min at 37 °C. The color changed from yellow to purple indicates the microbial growth in the well. The minimal inhibitory concentration (MIC) of the extract was defined as the lowest concentration of the extract that completely inhibits the microbial growth. Then, the minimal bactericidal concentration (MBC) was determined by spot inoculating 10 µL from each well onto Mueller Hinton agar plates. The plates were then incubated for 24 h at 37 °C.

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MBC was defined as the lowest concentration of extract resulting in no growth on subculture.

Time kill analysis

To study the effect of extract concentration of the bacterial growth, a time kill analysis was performed on *P. mirabilis* according to May *et al*. (2000). A volume of 100 µL of bacterial inoculum was transferred into 20 mL of sterile Mueller Hinton broth. The experiments were performed by culturing *P. mirabilis* in the medium with extract concentrations MIC, MBC and 2× MBC at 37 °C with rotational speed of 120 rpm. A growth control was included by replacing the extract with 10% Tween 80 solution. Sampling times for the bacterial culture included 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 h. Samples (1 mL) were taken from each flask at designated time point and were diluted 1:10 in sterile distilled water until a bacterial concentration was obtained that could be visually quantified on agar plates. 100 µL of samples were plated on Mueller Hinton agar plates. Plates were incubated for 24 h at 37 °C. Subsequently, the number of colony forming units (CFU) on each plate was counted. A graph of bacterial count (CFU/mL) versus time was plotted.

Brine shrimp lethality assay

This assay was conducted according to Urmi *et al*. (2013) with modifications to determine the toxicity of the ethanol extract. *Artemia salina* eggs (Ocean stars, USA) were hatched in artificial seawater (38 g/L of sea salt) at room temperature, with the aeration from air stone pump. After 48 h of incubation, 20 *A. salina* nauplii were transferred to 6 wells plates containing 5 mL of artificial seawater. The ethanol extracts were tested at final concentration ranging from 0.2 to 1.2 mg/mL by serial double dilution. A well containing 10% Tween-80 solution was included as solvent control. The numbers of survived nauplii in each well were determined at 6 h (acute toxicity) and 24 h (chronic toxicity) of incubation, with the aid of light microscope. A graph of percentage mortality against extract concentration was plotted. The median lethal concentration (LC_{50}) was then calculated from the graph.

RESULTS AND DISCUSSION

The selection of extraction method is crucial to ensure the antimicrobial compounds were successfully extracted from *C. nardus*. The dried plant materials were used in this study to ensure a high extraction yield obtained from the plants. Ethanol was selected as it is one of the most efficient solvent used to extract bioactive compounds from plant materials (Baylac and Racine, 2003; Piana *et al*., 2018). In addition, ethanol interferes with the lignocellulosic components of *C. nardus*, which allows the release of bioactive compounds from the plant materials. However, it is crucial to ensure that all ethanol content was removed by rotatory evaporator in order to avoid false positive results in antimicrobial assays.

In this study, GC-MS was employed to identify the bioactive compounds present in *C. nardus* extract. In total, seven compounds were detected in the ethanol extract of *C. nardus*, which may contribute to its biological activities (Table 1). Two major compounds, 6-methyl-3,4,4a,5,6,7,8,8a-octahydrochromen-2-one and 2,4-di-tert-butylphenol were identified in the extract. 6-methyl-3,4,4a,5,6,7,8,8a-octahydrochromen-2-one or known as 6-methyloctahydrocoumarin (PubChem CID 556977) was found to be present as a major constituent (31.5%) at the retention time 19.07 min. This compound is a derivative from coumarin, a fragrant compound normally found in the benzopyrone chemical class (Mayer *et al*., 2014). Compound methyloctahydrocoumarin was previously identified from *Terminalia catappa* ethanol extract which exhibited antimicrobial activity against a wide spectrum of microorganisms (Krishnaveni *et al*., 2015). On the other hand, 2,4-di-*tert*-butylphenol (PubChem CID 7311), accounting for 28.7% of the sample, is an alkylated phenol that known for its antioxidant activity. This compound is widely used in industry as UV stabilizer (Varsha et al., 2015). Padmavathi *et al*. (2015) reported the inhibitory activity of this compound on growth and biofilm formation of *C. albicans.*

Table 1: GC-MS analysis of *C. nardus* ethanol extract.

No.	Retention time (min)	Constituents	Percentage of area (%)
1	14.24	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	5.3
2	17.48	2-Ethyl-(dimethyl)silyloxybutane	2.9
3	18.19	2-Ethyl-6,6-dimethylbicyclo[3.1.1]heptan-3-one	9.7
4	19.07	6-Methyl-3,4,4a,5,6,7,8,8a-octahydrochromen-2-one	31.5
5	23.44	2,4-Di-tert-butylphenol	28.7
6	30.19	2-((2R,4aS,8R)-4a,8-Dimethyl-2,3,4,4a,5,6,7,8-octahydronaphthalen-2-yl)propan-2-ol	11.3
7	30.58	Undec-10-enyl pentanoate	10.06

The ethanol extract showed significant inhibitory effect on one Gram-positive bacteria (*B. coagulans*), two Gram negative bacteria (*P. mirabilis* and *E. coli*) and one yeast (*C. albicans*). The extract exhibited a broad spectrum of antimicrobial activity (Table 2). Among all the test microorganisms, the largest inhibition zone was observed on *B. coagulans* and *P. mirabilis*, with a diameter of 20.0 mm. The results revealed a high susceptibility of these food borne bacteria to the extract. It has been reported that the *C. nardus* essential oil showed antimicrobial activity against pathogenic *E. coli* and *S. aureus* (Rocha *et al*., 2011). In this study, it is worthy to note that the crude ethanol extract exhibited anti-yeast activity against *C. albicans.* The result is similar with Bona da Silva *et al*. (2008), even though they reported an excellent anti-*Candida* activity of *C. nardus* using its essential oil, with an inhibition zone larger than 40 mm on Kirby Bauer

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assay. The anti-*Candida* activity of the ethanol extract may be due to the presence of phenolic compound, notably, 2,4-di-*tert*-butylphenol detected by GC-MS analysis. However, the extract did not exhibit inhibitory activity against filamentous fungi.

Table 2: Antimicrobial activity of *C. nardus* ethanol extract on Kirby Bauer assay.

Test microorganism	Diameter of inhibition zone (mm)		
	Ethanol extract	Positive control	Negative control
Gram-positive bacteria			
B. cereus	-	25.6 ± 0.6	-
B. coagulans	20.0 ± 1.4	23.6 ± 1.2	-
S. aureus	-	10.0 ± 1.0	-
B. spizizeni	-	-	-
Gram-negative bacteria			
P. mirabilis	20.0 ± 1.4	25.0 ± 1.4	-
K. pneumoniae	-	18.0 ± 1.2	-
Y. enterocolitica	-	23.2 ± 1.6	-
E. coli	15.0 ± 2.9	20.0 ± 1.4	
Yeasts			
C. utilis	-	16.3 ± 1.4	-
C. albicans	17.0 ± 1.4	19.0 ± 0.7	-
Filamentous fungi			
P. expansum	-	18.2 ± 1.6	-
R. stolonifer	-	15.1 ± 1.4	-

(-) No inhibitory activity

All the test microorganisms showed different susceptibility to the ethanol extract (Table 3). The MIC were ranged from 63 to 250 µg/mL. A low MIC indicates a high susceptibility of the test microorganism to the extract. The lowest MIC (63 µg/mL) was recorded on *B. coagulans* and *P. mirabilis*, mirroring the results recorded of Kirby Bauer assay where the largest inhibition zones were observed on these test bacteria. For all test microorganisms, the MBC obtained were higher than the MIC, revealing that a higher concentration of extract is needed in order to exhibit bactericidal effect to the bacterial cells. The low MBC/MIC ratios of the extract indicate the high sensitivity of the test bacteria to the extract.

Table 3: The susceptibility of test microorganisms to *C. nardus* ethanolic extract on broth microdilution assay.

Test microorganism	MIC (µg/mL)	MBC (µg/mL)
E. coli	125	250
B. coagulans	62.5	250
P. mirabilis	62.5	125
C. albicans	250	1000

P. mirabilis is a member of the Enterobacteriaceae family of Gram-negative bacillus that resides as normal flora of human guts. However, it causes opportunistic infections via urinary tract and wounds, that resulting in septicemia (Daly *et al*., 2016). A few cases of food poisoning caused by *P. mirabilis* have been reported in last decade (Cock, 2017). The control time kill curve shows a significant exponential and stationary phase. High CFU counts were obtained throughout the incubation period (Fig. 1). The time kill curve revealed a 10% of Tween-80 solution did not exhibit any inhibitory effects on the bacterial growth. From 0 to 24 h, the extract did not exhibit any bactericidal effect, at any of the test concentration. However, the significant lower CFU counts were obtained for all the extract concentrations, relative to the growth control. The time kill curve of concentration MIC is in agreement with the result of broth microdilution assay, where the extract concentration of MIC was only sufficient to inhibit the bacterial growth, without exerting any killing effect. A partial regrowth of bacterial cells was observed after 36 h of incubation. However, at extract concentration of MBC, a noticeable reduction of bacterial counts was obtained throughout the incubation period. The extract caused 99.9% reduction in bacterial count at 32 h of incubation, with no regrowth observed. The antimicrobial activity of *C. nardus* extract was therefore concentration dependent. At the concentration of 2× MBC, the extract achieved 99.9% of bacterial cells reduction at 22 h. The bacterial counts obtained also significantly lower than the MBC throughout the incubation period. The time kill analysis showed that *C. nardus* extract exhibited rapid bactericidal effect on *P. mirabilis*. This property makes it a promising agent to combat food borne microorganisms.

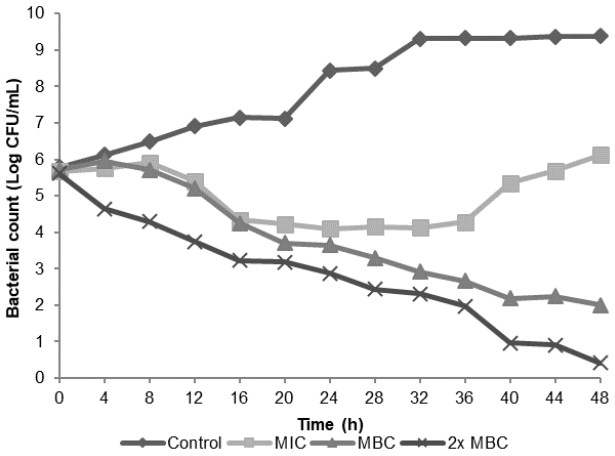

Figure 1: Time kill curve of *C. nardus* ethanol extract on *P. mirabilis*.

Brine shrimp lethality assay is commonly used to determine the potential toxicity effect of the plant extracts (Pham *et al*., 2014). The LC_{50} is defined as the dose of a substance to kill half the members of a test population (Doumbia, 2014). The dead shrimp was determined by observing the mobility of the shrimps (Jamil *et al*., 2016). Based on the results, *C. nardus* extract showed a LC_{50} value of 1.585 mg/mL for acute toxicity, and 1.262 mg/mL for chronic toxicity. Generally, there was no effect of toxicity effect caused by the extract on *A. salina*, at both 6 and 24 h, as the LC_{50} values obtained were greater than 1

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mg/mL. However, the toxicity effect of the extract was time dependent, as the LC_{50} value decreased with time. The obtained results are in agreement with Kpoviessi *et al*. (2014) where the essential oils of *Cymbopogon* species did not exhibit any toxicity effects on the cell lines tested.

In short, the ethanol extract of *C. nardus* exhibited significant inhibitory effect on several food borne microorganisms. However, the extract did not show any toxicity effects on *A. salina*. *C. nardus* may be a potential source of antimicrobial agent to combat food borne infections. Further investigations should be conducted to determine the *in vivo* antimicrobial efficiency of the extract on animal models.

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