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Evaluation of the antimicrobial activities of *Cymbopogon schoenanthus*

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Medicinal plants have been widely used in folk medicine. They are considered today as an interesting source of new drug due to their bioactive components. The genus, Cymbopogon (Poaceae) seems to be the most promising source of several bioactive compound. This study aimed at evaluating the antimicrobial activities of *Cymbopogon schoenanthus* on several pathogenic bacteria, fungi and virus. Antibacterial and antifungal activities were evaluated using the agar well diffusion methods. The MIC was determined by micro-broth dilution methods. The cytotoxicity of *C. schoenanthus* extracts was evaluated using MTT assay. The results showed that methanol extract of *C. schoenanthus* exhibit an antibacterial effect on several Gram-positive and negative bacteria. All extracts tested (aqueous extract, methanol extract and ethyl acetate extract) were found to have an antiviral effect on HSV1; whereas, no antifungal effect was detected on both *Candida albicans* and *Aspergillus niger.*

Key words: Antiviral, antibacterial, bioactive properties, *Cymbopogon schoenanthus*, medicinal plant.

INTRODUCTION

Since ancient times, medicinal plants have been widely used as a valuable source of medicines in several cultures. Nowadays, according to the World Health Organization (WHO), about 80% of people worldwide are currently depending on traditional medicine for their primary health care needs (WHO, 2005, 2008). Medicinal plants are considered today as an interesting source of new drug due to their bioactive components (Cragg et al., 1997; 1999; Harvey, 1999, 2001; Ali et al., 2015).

Numerous scientists have reported an increased need for novel antimicrobial drugs due to the relatively high incidence of bacterial infection as well as the capacity of pathogenic bacteria to develop a type of resistance against classical antimicrobial drugs (Ganjewala, 2009).

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In fact, the random use of multiple antibiotic drugs is considered to be the principal cause of this resistance. For this reason, numerous researches have been conducted recently to find new sources of bioactive compound (Al-Ali and El-Badry, 2010). Currently, essential oils from *Cymbopogon* species of diverse origin have been studied extensively (Sidibe et al., 2001; Ganjewala et al., 2008).

In Saudi Arabia, there are two species of the Cymbopogon genus, which are the *Cymbopogon commutatus* (also known as Sakhbar) and the *Cymbopogon schoenanthus* (also known as El-Ethkher or camel grass) (Atyat, 1995; Hilo, 1996). *Cymbopogon commutatus* has reported presence in eastern region only (Al-Doussary, 1998), while *C. schoenanthus* can be found in several regions in Saudi Arabia including Makkah Al-mukarmah region (Migahid, 1996).

According to the Arabic folk medicine, the only manner to treat with the *Cymbopogon s.* is through drinking the herb aqueous extract as a tea, in other words, by preparing herbal tea from Ethkher leafs. The traditional treatment has been used as antihelminthic, antidiarrheal, antirheumatic, carminative, diaphoretic, stomachic, diuretic, emmenagogue, for treating fever and for treating jaundice and as tonic (Rizk, 1986; Atyat, 1995). The Saudi folk medicine uses the *C. schoenanthus* for treating people with kidney stone (Hilo, 1996; Al-Ghamdi et al., 2007). In fact, beside their traditional uses in folk medicine, many scientists have reported numerous useful biological activities of the essential oils and essential oil constituents of *Cymbopogon* species. Both *in vitro* and *in vivo* studies have demonstrated how *Cymbopogon* species shows anti-inflammatory, anticancer and allelopathic activities (Li et al., 2005; Santoro et al., 2007; Alitonou et al., 2006). There are few reports available describing other bioactivities of agricultural and ecological significance such as food packaging and insect repellent of essential oils of *Cymbopogon* species (Das and Ansari, 2003; Singh et al., 2006).

The bioactive potential of *Cymbopogon* essential oils and their constituents have been rapidly recognized in the past ten years, although the conventional bioactive properties against microbes have been known for decades. Essential oils of *Cymbopogon* species are easily available, have a pleasant aroma, are non-toxic and safe; the active principles are therefore becoming increasingly popular in pharmaceuticals and medicines.

In order to evaluate the bioactive potential of essential oils and their constituents using animal models and cell systems, several species of *Cymbopogon* such as *Cymbopogon martini, Cymbopogon citrates, C. schoenanthus* and *C. winterianus* were studied recently. The obtained results demonstrated that *C. citratus* has been recognized as most promising since the essential oil of this species has exhibited a variety of fundamental and novel bioactivities. In contrast, *C. schoenanthus* essential oil demonstrated antioxidant activity, while the two other species (*C. martinii* and *C. winterianus*) showed outstanding bioactivities, particularly insect repellent and antihelmintic. The bioactive potential of other species of *Cymbopogon* have not yet been fully exploited (Das and Ansari, 2003; Li et al., 2005; Ketoh et al., 2005, 2006; Santoro et al., 2007).

In 2010, a new study was carried out by EL-Kamali and EL-Amir to exanimate the antibacterial activity of ethanol extracts obtained from eight Sudanese medicinal plants. In this study, scientists have demonstrated that *C. schoenanthus* showed relatively higher propensity to act on Gram- positive bacteria. Recently, the antibacterial activity of *C. schoenanthus* collected from the north-east area of Jeddah, Saudi Arabia has been studied by Hashim and co-workers in 2016. The *C. schoenanthus* essential oils were evaluated against Gram-positive and negative bacteria. Hashim and co-workers (Hashim et al., 2016) have demonstrated that *C. schoenanthus* essential oils represent an inhibitory effect against *S. aureus* methicillin sensitive (MSSA), *S. aureus* (MRSA), *Escherichia coli* and *Klebsiella pneumonia*.

Until now, few studies have been conducted to evaluate the anti-viral and anti-fungal activity of *C. schoenanthus* collected in Saudi Arabia.

The study was aimed at investigating the possible antifungal, antiviral and antibacterial activities of *C. schoenanthus* (L.) *Spreng* collected from Almadinah Almunawarah, in Saudi Arabia against several human pathogenic bacteria, fungi and viruses.

MATERIALS AND METHODS

Plant collection and extracts preparation

C. schoenanthus (L.) Spreng. was collected from Al Madinah Almounawarah, in the western area of Saudi Arabia. The collected plant was subjected to scientific identification using the scientific identification manuals, with the kind help of our botany specialized colleagues in the Faculty of Pharmacy at Taibah University. Voucher specimens have been deposited at the herbarium of the Faculty of Science, Taibah University, Saudi Arabia.

C. schoenanthus was rinsed twice with tab water. Leaves were separated and washed again using sterilized water and left in shade for drying. Plant material was pulverized using domestic blender to powder form. The air-dried powdered herb (1000 g) was extracted with 70% methanol (5 L × 5) till exhaustion and then concentrated under reduced pressure to yield a viscous gummy material. The residue was suspended in water H_2O (5 L) and defatted with n-hexane (4 L). The aqueous layer was then extracted with ethyle acetate (EtOAc) and butanol (1-BuOH) successively (4 L each). The ethyle acetate and butanol fractions were concentrated under reduced pressure to give EtOAc and 1-BuOH fractions, respectively.

Antibacterial and antifungal susceptibility testing

Antimicrobial susceptibility testing was done using the agar well diffusion method to evaluate the anti-bacterial and anti-fungal activities of this plant.

Bacterial and fungal cultures

Eleven bacterial pathogens and two fungal strains were used to evaluate the antimicrobial effect of *C. schoenanthus* extract. The pathogens included Gram-positive bacteria: *Enterococcus faecalis* isolate, *Staphylococcus saprophyticus* (ATCC49907), *Staphylococcus saprophyticus* isolate, *Staphylococcus aureus* (ATCC25923), *Staphylococcus aureus* isolate and *Streptococcus pyogenes* isolate) and Gram-negative bacteria: *E. coli (ATCC35218), Pseudomonas aeruginosa* isolate, *Klebsiella pneumonia* isolate, *Proteus mirabilis* and *Salmonella paratyphi B* isolates, as well as two fungal strains, *Candida albicans* and *Aspergillus niger*.

Bacterial and fungal cultures were prepared as follows. The bacterial cultures were sub-cultured in nutrient broth for 24 h at 37°C while the fungal cultures (*C. albicans* and *A. niger*) were sub-cultured in liquid Sabouraud dextrose medium for 48 h at 25°C. The turbidity of the broth cultures was equilibrated with 0.5 McFarland standard. Mature cultures of *C. albicans* and *A. niger* were inoculated unto Sabouraud dextrose broth to prepare the test inocula which were similarly equilibrated with 0.5 McFarland standard (Baker et al., 1983).

Agar plates preparation and inoculation

Antibacterial tests were carried out in triplicate. Petri dishes were prepared with 20 ml of Mueller Hinton agar. After autoclaving, the agar was allowed to cool down (45 to 50°C). Then, 0.1 ml of the diluted culture was poured on each plate using a mixer (Whirli Mixer, Fisher brand, England), and Petri dishes were left to dry for 30 min at 37°C (Bauer, 1966). Wells of 6 mm diameter were cut with sterile cork borer in the inoculated agar. Then, wells were filled with plant extract. Two positives control were used. Norfloxacin (10 µg/ml) was used as positive control-1 and Augmentin (30 µg/ml) as positive control-2. Distilled water was used as the negative control. Petri dishes were incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the diameter of zones of inhibition excluding the hole size by using an antibiotic zone reader (Fisher-Lilly, USA).

Antifungal tests were performed by the same method in triplicate. Petri dishes with 20 ml of Sabouraud Dextrose agar were used. Amphotericin B (10 µg/ml) was used as positive control and distilled water was used as negative control. Petri dishes were incubated at 25°C for 48 h then antifungal activity was evaluated by measuring the diameter of zones of inhibition.

Minimum inhibitory concentration (MIC) test

The MIC was determined by micro-broth dilution methods (Chambers, 2001). The tested extracts were serially diluted in Mueller-Hinton broth (Oxoid) medium. Duplicate tubes of each dilution (100, 50, 25, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4 and 0.2 mg/ml) were inoculated with 100 µl (5×10^5 CFU/ml) of appropriate bacterial and fungal suspension. Then, cultures were incubated at 37°C for 24 h. MICs were considered as the least concentration of each extract with no visible bacterial growth in terms of turbidity (Demarsh et al., 2001).

Antiviral activity examination

Propagation of Vero cell line by enzyme treatment

African green monkey kidney (Vero) cells incubated into culture bottle were checked using inverted microscope for its proper physical conditions. Healthy cells were propagated as follows: the media overlaying cell monolayer was poured off. Cells were released from tissue culture flask by treating with 5 ml pre-warmed trypsin-EDTA solution. EDTA chelates metal ions are involved in cell adherence, the flask was rocked so that trypsin completely cover the cell monolayer. The trypsin was aspirated with a pipette, then 2 ml of trypsin were dispensed, the bottle rocked and were incubated at 37°C. Cells were examined from time to time to avoid trypsin over action. The bottle was struck with hand to completely dislodge the cells from the bottle surface. Cells were suspended in 8 ml of growth media. 10 ml pipette was used to disperse cell aggregates by sucking up and expelling the cells about 4 times to ensure that no clumps of cells were present. After that, cells were counted using haemocytometer using trypan blue vital stain.

Ten milliliters ($2x10^5$) of Vero cells suspension were transferred to 50 cm^3 TC bottle (Falcon) tightly closed. Vero cell suspension was then incubated at 37°C and cells were sub-cultured once weekly. For seeding 96 well plate, 0.1 ml (2×10^5) of cells were transferred to each flat bottomed well and incubated at 37°C for 48 h to develop a complete monolayer sheet.

Cytotoxicity examination

Vero cells monolayer were treated with various concentrations of *C. schoenanthus* extracts for three days and the cytotoxicity of the extracts was evaluated by direct count using Neubauer hemacytometer to indicate their replication rate and checked for any physical signs of toxicity morphologically, e.g. partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation by daily observation by using optical inverted microscope. Then, the maximum non-toxic concentration (MNTC) of three extracts (aqueous extract, methanol extract and ethyl acetate) was determined.

Antiviral assay

The cytotoxicity of *C. schoenanthus* extracts was evaluated by MTT assay kit. $1x10^4$ of Vero cells were plated in 96-well plates (each well contain 200 µl of media), eight wells were left empty for blank controls. Cells were incubated overnight at 37°C in a humidified incubator with 5% CO_2. Equal volume (1:1 v/v) of non-lethal dilution of Herpes simplex virus-1 suspensions (HSV1) was incubated for one hour. 100 µl of viral suspension was added and placed on a shaking platform at 150 rpm for 5 min. Then, viral suspension was incubated at 37°C in a humidified incubator with 5% CO_2 for one day to allow virus to take effect. 2 ml of MTT solution were made per 96 well plates at 5 mg/ml in PBS. 20 µl of MTT solution were added to cells, then incubated for 4 h. The media was clearly removed and MTT metabolic product was resuspended in 200 µl DMSO and thoroughly mixed on a shaking platform at 150 rpm for 5 min. Optical density (OD) was measured at 560 nm (Denizot and Lang, 1986; Hu et al., 1999).

RESULTS

Antibacterial and antifungal activity

Agar well diffusion test

The antifungal and antibacterial activities of four extract (methanol, aqueous, acetone and dichloromethane extract) of *C schoenanthus* were evaluated by using Agar well diffusion test. The antimicrobial effects were tested

Table 1. Antibacterial activity of several *C. schoenanthus* extract by measuring zones of growth inhibition (mm) using agar well-diffusion assay.

Microorganism	Methanol extract	Aqueous extract	Acetone extract	Dichloromethane extract	Augmentin[a] 30 µg/ml	Norfloxacin[b] 10 µg/ml
S. saprophyticus ATCC49907	14.7±0.22	0	0	0	32.2±0.47	20.2±0.67
S. aureus ATCC 25923	14.0±0.33	0	0	0	44.0±0.33	18.7±0.22
S. saprophyticus isolate	9.7±0.47	0	0	7.3±0.58	9.3±0.75	0
S. aureus isolate	19.3±0.58	0	0	0	38.8±0.46	16.8±0.37
S. pyogenes isolate	11.6 ±0.58	0	0	0	44.7±0.94	0
E. faecalis isolate	12.0±0.58	0	0	0	21.2±0.97	11.2±0.38
E. coli ATCC 35218	14.7±0.43	0	0	0	22.3±0.47	30.8±0.13
P. aeruginosa isolate	7.7±0.85	0	0	0	0	23.6±0.74
K. pneumoniae isolate	13.67±0.58	0	0	0	11.3±0.47	27.5±0.76
P. mirabilis isolate	20.67±0.58	0	0	0	22.6±0.48	28.8±0.37
S. paratyphi B isolate	22±1	0	0	18.6±0.58	0	23.2±0.89

[a]Positive control 1; [b]positive control 2. Values represent means ± standard deviations for triplicate experiments ($p < 0.05$). Differences between means of the zones of inhibitions were analyzed by student's t-test (SPSS version 22.0).

Table 2. Anti-fungal activity of several *C. schoenanthus* extract by measuring zones of growth inhibition (mm) using agar well-diffusion assay.

Microorganisms	Methanol extract	Aqueous extract	Acetone extract	Dichloromethane extract	Amphotericin B[a] 10 µg/ml
C. albicans	0	0	0	0	21.8±0.47
A. niger	0	0	0	0	7.8±0.47

[a]Positive control. Values represent means ± standard deviations for triplicate experiments ($p < 0.05$). Differences between means of the zones of inhibitions were analyzed by student's t-test (SPSS version 22.0).

on six Gram-positive bacteria (*E. faecalis, S. saprophyticus* ATCC49907, *S. saprophyticus* isolate, *S. aureus* ATCC 25923, *S. aureus* and *S. pyogenes* isolate) and five Gram-negative bacteria (*E. coli, P. aeruginosa, K. pneumonia, P. mirabilis and S. Paratyphi* B) as well as two fungal species (*C. albicans* and *A. niger*). Means of zones of inhibition are shown in Table 1. Results obtained showed that methanol extract of *C. schoenanthus* exhibit an antibacterial effect on all pathogenic bacteria tested .The largest inhibition zones were exhibited by Gram negative *Salmonella paratyphi B* isolate (22±1 mm) followed by *P. mirabilis* isolate (20.67±0.58mm) and Gram positive *S. aureus* isolate (19.3±0.58 mm). The lowest inhibition effect was detected on *P. aeruginosa* (7.7±0.85 mm). In contrast with the results of methanol extract, aqueous extract and acetone extract did not show any antibacterial activity on the 11 pathogenic bacteria tested. Moreover, dichloromethane extract was only active on two pathogenic bacteria. The zone of inhibitions was as follows: *Salmonella paratyphi B* isolate (18.6±0.58 mm) and *S. saprophyticus* isolate (7.3±0.58 mm). Regarding the antifungal effect of *C. schoenanthus*, the four extract showed no antifungal activity against *C. albicans* and *A. niger* (Table 2).

Determination of minimum inhibitory concentration (MICs)

Minimum inhibitory concentration was performed on eight species of pathogenic bacteria (Table 3). The obtained results demonstrated that methanol extract has an antibacterial activity against both. Gram positive and negative bacteria, MICs ranged from 0.8 to 25 mg/ml. Interestingly, methanol extract showed significant inhibitory effect on two Gram negative bacteria: *Proteus mirabilis* isolate (25 mg/ml) and *S. paratyphi* B isolate (12.5 mg/ml), while dichloromethane extract showed an antibacterial activity only against *S. paratyphi* B isolate ($P<0.05$).

Antiviral activity

Maximum non-toxic concentration

The maximum non-toxic concentration (MNTC) of three extracts (aqueous extract, methanol extract and ethyl acetate) was determined. The obtained results demonstrated that there was no significant difference

Table 3. Determination of minimum inhibitory concentration (MICs) of methanol and dichloromethane extracts.

Microorganism	Methanol extract	Dichloromethane extract
	MIC (mg/ml)	MIC (mg/ml)
E. coli ATCC 35218	1.6±2.71	0
S. saprophyticus ATCC49907	0.8± 1.36	0
S. aureus ATCC 25922	3.2± 5.43	0
S. saprophyticus isolate	1.6± 2.71	0
S. aureus isolate	6.25± 0.00	0
S. pyogenes isolate	3.75±0.02	
E. faecalis isolate	6.25± 0.00	0
K. pneumoniae isolate	3.2± 5.43	0
P. mirabilis isolate	25.00± 0.00	0
S. paratyphi B isolate	12.5.0±0.00	3.2± 5.43

Values represent means ± standard deviations for triplicate experiments ($p < 0.05$). Differences between means of the MICs were analyzed by student's t-test (SPSS version 22.0).

between cells control and the three extracts tested ($P>0.05$). The concentrations of extracts that exhibited cytotoxic effect were excluded. Results obtained are shown in Table 4.

Antiviral activity of C. schoenanthus extracts

All extracts were found to have an antiviral activity against HSV-1 at a concentration non-lethal to the cell line (Vero cells) used (Table 4). Aqueous extract showed significant antiviral effect on HSV-1, viral activity was decreased from 100 to 9.61%. Both methanol and ethyl acetate extracts have also exhibited antiviral effect on HSV-1, viral activity was decreased from 100 to 32.69 and 33.70%, respectively. Results obtained are shown in Table 5.

DISCUSSION

Medicinal plants have been widely used in folk medicine. They are considered today as an interesting source of new drug due to their bioactive components (Cragg et al., 1997, 1999; Harvey, 2001; Ali et al., 2015).

Currently, the genus, Cymbopogon (Poaceae) is renowned for their essential oils of immense commercial significance in flavours, fragrances, cosmetics, perfumery, soaps, detergents and pharmaceuticals (Ganjewala et al., 2008). The genus Cymbopogon comprises about 140 species of which most are aromatic and yield an essential oil upon the steam distillation of their aerial parts (Khanuja et al., 2005). Semi-chemical properties of the Cymbopogon essential oils have been promising in integrated pest management programme since this property may lead to development of alternatives to synthetic chemical pesticides (Kumar et al., 2008).

In addition, many scientists have evaluated the potential use of *C. schoenanthus* against gastrointestinal nematodes**.** Results obtained showed that *C. schoenanthus* essential oil had the best activity against ovine trichostrongylids **(**Katiki et al., 2011, 2012). Other studies were also performed in order to investigate the antistress properties of *C. schoenanthus*. Results obtained showed that ethanol extract has a significant antistress effect at *in vitro* and *in vivo* levels (Mahmoud Ben Othman et al., 2013). However, the bioactive potential of other species of *Cymbopogon* have not yet been fully exploited (Das and Ansari, 2003; Li et al., 2005; Ketoh et al., 2005, 2006; Santoro et al., 2007).

Conclusion

This study illustrates the importance of *C. schoenanthus* as promising sources of novel antimicrobial drugs. The results showed that methanol extract of *C. schoenanthus* exhibit an antibacterial effect on several Gram-positive and negative bacteria. Furthermore, all extracts tested (aqueous extract, methanol extract and ethyl acetate extract) were found to have an antiviral effect; whereas, no antifungal effect was detected on both *C. albicans* and *A. niger.* In conclusion, drug discovery can be significantly improved through the use of the knowledge gained from research in natural products (Gad, 2005).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Table 4. Maximum non-toxic concentration [MNTC] of aqueous, methanol and ethyl acetate extracts.

Sample	Dilution 1:2	O.D	Mean O.D	Cell viability	Selected dilution
Cell control	-	0.165 0.172 0.159 0.141	0.159	100%	
Aqueous extract	1	0.028- 0.030 - 0.022	0.026	16.35	
	2	0.037 -0.028 -0.028	0.031	19.49	
	3	0.019- 0.025-0.029	0.024	15.09	
	4	0.037- 0.026- 0.040	0.034	21.38	
	5	0.045- 0.040- 0.042	0.042	26.41	
	6	0.051- 0.049- 0.049	0.049	30.81	
	7	0.061 - 0.062- 0.070	0.064	40.25 (LD-50)	
	8	0.137- 0.148- 0.154	0.146	91.82	10^{-8} non lethal conentration
Methanol extract	1	0.017- 0.012- 0.013	0.014	8.80	
	2	0.018- 0.012- 0.015	0.015	9.43	
	3	0.028- 0.024- 0.013	0.021	13.20	
	4	0.067- 0.020- 0.057	0.048	30.18	
	5	0.059- 0.060 0.059	0.059	37.10	
	6	0.061-0.060-0.072	0.064	40.25	
	7	0.075-0.069-0.072	0.072	45.28	
	8	0.091- 0.083-0.085	0.086	54.08 (LD-50)	
	9	0.089-0.092-0.089	0.090	56.60	
	10	0.095-0.100-0.098	0.097	61.00	
	11	0.128-0.122-0.130	0.126	79.24	
	12	0.141-0.152-0.149	0.147	92.45	10^{-12} non lethal conentration
Ethyl acetate	1	0.017- 0.013- 0.013	0.014	8.80	
	2	0.024- 0.033 - 0.059	0.038	23.89	
	3	0.067- 0.078- 0.037	0.060	37.73	
	4	0.083- 0.090- 0.094	0.089	55.97 (LD-50)	
	5	0.150 - 0.160- 0.163	0.157	98.74	10^{-5} non lethal conentration
	6	0.166- 0.170- 0.145			
	7	0.167- 0.154- 0.162			
	8	0.159-0.166-0.160			

Table 5. Antiviral activity of the studied extracts against HSV 1.

Extract	Test	O.D	Mean O.D	viability	toxicity	Viral activity
	Vero cells[a]	0.154/0.155/0.156	0.155	100%	0%	0%
	HSV 1[b]	0.040/0.063/0.055/0.066/0.033	0.051	32.90	67.1	100%
Aqueous		0.141 / 0.146 / 0.148	0.145	93.54	6.45	9.61
Methanol		0.131 / 0.116 / 0.118	0.121	78.06	21.94	32.69
Ethyl acetate		0.129/ 0.111 / 0.120	0.120	77.41	22.58	33.70

[a]Negative control; [b]Positive control.

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