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Research Article

PRELIMINARY PHYTOCHEMICAL STUDY AND ANTIMICROBIAL ACTIVITY FROM VARIOUS EXTRACT OF *CYNODON DACTYLON* (L.) PERS. (BERMUDA) AGAINST SELECTEDPATHOGENS

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ABSTRACT

Cynodon dactylon(L.) Pers. is a type of perennial grass that possesses great medicinal values. In this study, seven different solvents (acetone, chloroform, diethyl ether, ethanol, ethyl acetate, methanol, and n-pentane) were used to investigate the phytochemical constituents of the plant. The antimicrobial activity of the plant crude extract from the selected solvents was investigated against some pathogens (*Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella spp., Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes,* and *Streptococcus pneumoniae*) using disc diffusion method. Crude extraction showed that ethanolic extraction produced highest yield (7.065%) followed by methanolic (5.420%) and chloroform (3.550%) extraction. The lowest yield obtained from n-pentane extraction (0.500%). Phytochemical studies confirmed the plant contains many bioactive compounds such as alkaloids, cardiac glycosides, terpenoids and steroids, saponins, phenolic compounds, flavonoids, tannins, carbohydrates and proteins. Ethanol was more effective to resolves almost all the plant constituents while n-pentane was the least. Antimicrobial study revealed that ethanol (7.0-10.0±0.0-1.0mm) and ethyl acetate (7.0-12.0±0.0-1.0mm) extracts showed broad spectrum activity to all of the tested pathogens. Both methanol and acetone extracts showed activity to *B. cereus* (8.0±0.0mm) and *B. subtilis* (7.0±0.0mm) while chloroform extract showed activity to *B. subtilis* (7.0±0.0mm) and *S.pyogenes*(8.3±0.6mm) respectively. Diethyl ether extraction showed activity only on *S. pyogenes* (7.3±0.6mm) while no activity observed for n-pentane extraction.

Keywords: *Cynodon dactylon*, Crude extract, Phytochemical, Antimicrobial, Pathogens

INTRODUCTION

Over thousand years plants have been utilised traditionally to treat many diseases before it potentials in medicine were being realized by researchers. For decades researchers have done numerous works to explore the medicinal potential from plants which help to boost the pharmaceutical development[1]. Medicinal plants play a very important role in pharmaceuticals industry in developing alternative drugs to overcome the pitfalls possessed by the synthetic drugs[2]. The development of drug resistant pathogens which mostly involved in nosocomial infection has raised concern among medicinal practicers[3]. It was believed that the intense used of a number of synthetic antimicrobial drugs which contributed to the development. Beside of the tougher jobs to search for more effective drugs against the pathogens, it also created the problem in controlling the growth of infectious diseases caused by the pathogens. Meanwhile, most of the synthetic drugs possess side effect to the consumers[4]. Given the alarming incidence of antibiotic resistance in pathogens raise concern among the medical practitioners, there is a constant need for new and effective therapeutic agents. Hence, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants. Plants produce large number of organic compounds such as alkaloids[5], flavonoids[6], glycosides[7], tannins[8], terpenoids[9] and phenolics[10,11,12,13,14] as secondary metabolites which used as defensive mechanism against respective pathogens. These compounds possess medicinal values and become an attractive subject for researchers to develop new antibiotics[15]. Previous studies show that many plant secondary metabolites act as bioactive compounds, chemotherapeutic, bactericidal, and bacteriostatic agents[16,17,18]. As a result, considerable amount of attention of antimicrobial substances derived from higher plants are immerging in recent years. However, the assessments on plant-derived antibiotics are still under investigation.

Cynodon dactylon is a perennial, pan tropical species of grass which belongs to the family Poaceae. It is found almost everywhere in tropical, sub-tropical and even in semi arid climates[19]. It is not only widespread, but is also widely used by human. Scientifically, it is tested to have anti-diabetic effect, diuretic activity, antioxidant, anticancer potentials, anti-ulcer activity, right heart failure protective activity, and allergic effect[20, 21, 22, 23, 24, 25, 26]. Traditionally, *C. dactylon* is used as a rejuvenator and for wound healing[27]. Besides used in remedy, the plant also used as forage for animals[28] and as turfgrass[29]. In this report, the phytochemical constituents of *C. dactylon* were presented along with its antimicrobial activity against some common pathogens such as *Escherichia coli, Staphylococcus aureus*and *Pseudomonas aeruginosa* which are frequently reported responsible for nosocomial infection.

MATERIALS AND METHODS

Plant collection (*Cynodon dactylon*)

Wild ecotype of *C. dactylon* were collected near the Universiti Malaysia Sabah's area in Kota Kinabalu, Malaysia, sowed and maintained in a mini-nursery in campus. Voucher (jgobilik 1090/2011) was kept at School of Sustainable Agriculture, UMS and a duplicate was submitted to BORH Herbarium, Institute of Tropical Biology and Conservation, UMS. Samples were collected in the evening during the daylight time. Prior to extraction, the plant samples were cleaned thoroughly with distilled water to remove soil and dirt.

Plant extraction

The whole part of *C. dactylon* which cleaned by distilled water were shade-dried for 24 hours in a drying chamber at 40-50^{0}C and powdered using a mechanical blender (Waring® Commercial Blender). Approximately 100g of plant powder later was soaked into 200mL of different solvents (acetone, chloroform, diethyl ether, ethanol, ethyl acetate, n-pentane, and methanol - Merck) and shaken on a platform shaker (LabCompanion™) at 150 rpm with temperature of 25 ^{0}C to obtain various plants extracts. The soaking process was repeated three times for each extraction to obtain a complete extraction. The extracts obtained were then evaporated and concentrated under reduced pressure (768mmhg to 7mmhg) using Rota Vapor™ (BUCHI) to obtain 1 mL of extract per 10g of plant sample. Aliquot were then kept in -20^{0}C temperature for further use. Extraction yield was determined using the following formula:

$$\text{Yield (\%)} = \frac{\text{Dry weigth of extract}}{\text{Dry weight of plant powder}} \times 100$$

Phytochemical study

The studies were done to identify the presence of bioactive chemical constituents such as alkaloids, cardiac glycosides, phenolic

compounds, flavonoids, tannins, terpenoids, saponins, carbohydrates and proteins according to the standard protocols described previously[30, 31, 32].

Test Microorganisms

In this study, pure cultures of eight different pathogens (*Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella spp., Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes,* and *Streptococcus pneumonia*) were obtained from Queen Elizabeth Hospital, Kota Kinabalu, Malaysia. The microbial cultures were preserved in 30% glycerol stock solution at -85^{0}C freezer. Prior to the antimicrobial activity study, the test microorganisms were sub-cultured on Nutrient Agar (NA) media, incubated at 37^{0}C for 24 hours then inoculums of the test microorganisms were grown into Nutrient Broth (NB) and adjusted according to Mac Farlands Standard to achieve approximately 1x10^{8} CFU/ml before introduced into the test media.

Antimicrobial activity study

Antimicrobial activity of the plant extracts was evaluated using disc diffusion method according to Kirby-Bauer method described previously[33]. Sterile discs of Whatman® No.3 paper (6mm diameter) were prepared for the disc diffusion bioassay. All the extracts concentration was standardised to 100mg/mL which 60µL (6mg) of the extracts were loaded into the discs. For the negative control discs, 60µL of the respective solvents were loaded into separate discs. Chloramphenicol antibiotic discs (10mg/mL, 30µg/disc) were used as positive control to compare the antimicrobial activity. Muller-Hinton Agar (MHA) medium was prepared and sterilised in an autoclave at 121^{0}C for 15 minutes at 15 psi then it was transferred into sterilised petri dish. Approximately 0.1 mL of culture of bacterial pathogens adjusted according to Mac Farlands Standard were placed on the MHA media and spread throughout the plate using spread plate technique. The discs loaded with test extracts, their corresponding solvents and the antibiotic disc was placed with the help of a sterile forcep carefully with adequate spacing between each other. The plates were kept at room temperature for 30 min, which helps to diffuse the extract on the medium. Later the plates were incubated at 37^{0}C for 24 hrs in an incubator to determine the antibacterial activity of the respective solvent extraction of *C. dactylon*. After incubation, zone of inhibition in diameter was measured and recorded.

RESULTS

Extraction yield

Different solvent have different resolving strength towards the plant constituents which resulted in different yield as shown in table 1.From all of the extractions, ethanolic extraction produced the highest yield (7.065%) followed by methanolic extraction (5.420%) and chloroform extraction (3.550). Most of the extractions using non-polar solvents produced lower yield. The lowest yield was obtained using n-pentane extraction with only 0.500% of yield. The other extractions yield results are as shown in table 1.

Phytochemical study

Phytochemical study showed that *C.dactylon* contains many bioactive constituents such as alkaloids, cardiac glycosides, terpenoids and steroids, saponins, phenolic compounds and flavonoids, tannins, carbohydrates and proteins as shown in table 2. Most bioactive constituents were detected in ethanol extract except for the alkaloid and protein while the least were detected in more non-polar solvent such as in n-pentane extract. Alkaloids were detected only in diethyl ether extract while cardiac glycosides were detected in most of the plant extracts except in n-pentane. Phenolic compounds (based on magnesium-HCl reduction test) were positive in ethanol extract while flavonoids (based on gelatin test) were positive both in ethanol and methanol extracts. Tannins were present in both acetone and ethanol extracts while saponins were detected in chloroform, ethanol, and methanol extracts. Acetone, diethyl ether, and methanol extracts showed negative result for terpenoids and steroids. Most of the plant extracts showed negative result for carbohydrates test except for the alcoholic extraction while proteins test showed positive result only in the chloroform extract.

Antimicrobial activity

Ethanol and ethyl acetate extracts of the plant exhibited broad spectrum of antimicrobial activity. From table 4, both ethanol and ethyl acetate extracts showed significant activity toward almost all the tested pathogens with the size of inhibition ranging from 7.0±0.0mm to 10.0±1.0mm for ethanol extract and 7.0±0.0 to 12.0±1.0mm for ethyl acetate extract. The greatest activity observed was against *S. pyogenes* (10.0±1.0mm) for ethanol extract while *B.cereus* (12.0±1.0mm) for ethyl acetate extract. The lowest activity observed was against *B. subtilis* (7.0±0.0mm) for ethanol extract while *S. pneumonia* (7.0±0.0mm) for ethyl acetate extract. Methanol extract showed very weak activity against *S. pneumoniae* (7.0±0.0mm) while both methanol and acetone showed weak activity against *B. cereus* (8.0±0.0mm) and *B. subtilis* (7.0±0.0mm). Chloroform extract showed activity towards two pathogens tested, *B. subtilis* (7.0±0.0mm) and *S. pyogenes* (8.3±0.6mm). Diethyl ether extract exhibited the least activity with only against *S. pyogenes* (7.3±0.6mm). Meanwhile, n-pentane extract did not exhibit any antimicrobial activity against all the tested pathogens. In the present study, *B. subtilis* (except for the diethyl ether and n-pentane extracts) and *S. Pyogenes* (except for acetone and n-pentane extracts) were more sensitive toward most of the extracts. Meanwhile, *E. coli*, *P. aeruginosa*, *S. aureus, Klebsiella spp.,* and *S.pneumoniae* only sensitive to ethanol and ethyl acetate extracts. No growth or activity observed on any negative disc. Growth inhibition observed on standard disc (chloramphenicol) proved none of the microorganisms tested were chloramphenicol resistant.

Table 2: Phytochemical study of the *C. dactylon*extract using different solvents

Phytochemical constituents	Test	Result for the respective solvents						
		Acetone	Chloroform	Diethyl ether	Ethanol	Ethyl acetate	Methanol	n-pentane
Alkaloid	-Dragendorff'stest	--	--	++	--	--	--	--
	-Wagner's test	--	--	++	--	--	--	--
	- Mayer's test	--	--	++	--	--	--	--
Cardiac glycoside	-Keller-kiliani	++	++	++	++	++	++	--
Phenolic compound and flavonoid	-Magnesium-HCl reduction	--	--	--	++	--	--	--
	-Gelatin test	--	--	--	++	--	++	--
Tannin	-$FeCl_3$ test	++	--	--	++	--	--	--
Terpenoid and steroid	-Salkowski test	--	++	--	++	++	--	++
Saponin	-Frothing test	--	++	--	++	--	++	--
Carbohydrate (Glucose)	-Benedict test	--	--	--	++	--	++	--
Protein	-Biuret test	--	++	--	--	--	--	--

+ Present; - absent; no. of +/- represent the no. of test replicate(s)

Table 3: Solvent polarity index and no. of phytochemical test which show positive result for the respective solvents

Solvent	n-Pentane	Diethyl-ether	Ethyl acetate	Acetone	Chloroform	Methanol	Ethanol
Polarity index*	0.0	2.8	4.4	5.1	4.1	5.1	5.2
No. of phytochem. test which shows positive result	1	2	2	2	4	4	7

*Phenomenex™appendices: Solvent miscibility table, page 366.

Table 4: Antimicrobial activity of *C.dactylon* with the respective solvents against pathogens

Tested microbial pathogens	Size of inhibition zone (mm) of the plant extract with the respective solvents*							
	Acetone	Chloroform	Diethyl ether	Ethanol	Ethyl acetate	Methanol	n-pentane	CHL
Bacillus cereus	8.0±0.0	n.d	n.d	9.0±0.0	12.0±1.0	8.0±0.0	n.d	24.0±1.0
Bacillus subtilis	7.0±0.0	7.0±0.0	n.d	7.0±0.0	8.0±0.0	7.0±0.0	n.d	22.3±0.6
Escherichia coli	n.d	n.d	n.d	8.3±0.6	8.0±0.0	n.d	n.d	23.3±0.6
Klebsiella spp.	n.d	n.d	n.d	8.3±0.6	8.0±0.0	n.d	n.d	20.0±1.0
Pseudomonas aeruginosa	n.d	n.d	n.d	8.0±0.0	9.3±0.6	n.d	n.d	24.0±1.0
Staphylococcus aureus	n.d	n.d	n.d	9.0±1.0	10.0±0.0	n.d	n.d	22.3±0.6
Streptococcus pyogenes	n.d	8.3±0.6	7.3±0.6	10.0±1.0	8.0±0.0	7.0±0.0	n.d	24.0±0.0
Streptococcus pneumoniae	n.d	n.d	n.d	7.3±0.6	7.0±0.0	n.d	n.d	21.0±1.0

*Values presented are means of three replicates, ± stand. dev; each disc loaded with approx. 60uL or 12mg/disc of plant extract.

n.d= not detected; C CHL= Chloramphenicol (10mg/mL, 30µg/disc)

DISCUSSION

Higher plants consist of wide range of bioactive compounds, such as alkaloids, terpenoids, flavonoids, saponins, tannins, etc. which were utilised by the plants itself as a defensive mechanism and to maintain the plant biological activities[5]. From the extraction yield in table 1, polar solvents were able to produce higher yield. This implies that most of the plant constituents are polar compounds such as saponins and the phenolics. In table 4, many of the phytochemical tests are positive for the polar extraction in comparison to less polar extraction as indicated by the polarity index (table 2). This is accordance to the extraction yield's result where most of the polar solvents able to resolve most of the plant bioactive constituents. Phytochemical study indicates that most of the tested plant bioactive constituents were present in the plant. Biological actions are primarily due to these components in a very complicated concert of synergistic or antagonistic activities. Mixtures of such chemicals show a broad spectrum of biological effects and pharmacological properties. To a large extent, the age of the plant, percentage humidity of the harvested material, situation and time of harvest, and the method of extraction are possible sources of variation for the chemical composition, toxicity and bioactivity of the extracts[34]. From the phytochemical study, cardiac glycosides were present in almost all of the extracts. Vast diversity and abundance of glycosides in the plant might explain why most of the cardiac glycosides tests with the respective solvents are positive. Cardiac glycosides from *C. dactylon* were previously studied to possess antiarrhythmic activity against ischemia or reperfusion induced arrhythmias and cardioprotective properties in tested rat[23,35]. A large number of constitutive plant compounds have been reported to have antimicrobial activity. Due to the great extent of pharmacological effects exert by the plant glycoside, the antimicrobial activity exhibited by the plant in the present study could be induced by the other derivatives from this carbohydrate constituent. Carbohydrate and fatty acid derivatives from natural source have been proven to possess broad spectrum antimicrobial activity[36]. The antibacterial activity of ethanol extract was believed due to the presence of active principle in the extracts such as saponins, phenolics and terpenoids which might responsible for the broad spectrum of antibacterial activity compared to the other extracts[37, 38]. Higher resolving strength of ethanol in regards to its yield percentage consequently enables it to resolves comparatively more bioactive compounds which might explain the considerable antimicrobial activity compared to the other solvents. Meanwhile, other solvents such as ethyl acetate was able to resolve others trace bioactive constituents which are not being able to be resolved by ethanol in greater amount, explaining the significant antimicrobial activity which leveled to the ethanolic extraction. Generally, gram-negative bacteria were more resistant to antibiotics than gram positive bacteria. The resistance is due to the differences in their cell wall composition. In gram-negative bacteria the outer membrane acts as a great barrier to many environmental substances including antibiotics. Presence of thick murine layer in the cell wall prevents the entry of the entry of the inhibitors[39]. In the present study revealed that there is no significance between gram-negative and gram positive bacteria in term of susceptibility to the crude extracts although for the extracts other than ethanol and ethyl acetate extracts, the activity was mostly observed against gram positive bacteria. However, no significant different of antimicrobial activity between gram positive and negative bacteria were observed for both ethanol and ethyl acetate extracts. It may be due to the presence of broad spectrum of antibiotic compounds present in *C. dactylon* which able to penetrate or deteriorate the defensive or growth mechanism of the microorganisms. The potential as antimicrobial compounds sources from the plant extract may be further explored by identifying the bioactive constituents responsible for the action.

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