

Full Length Research Paper

Antimicrobial studies of *Cosmos caudatus* Kunth. (Compositae)

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Present study aimed to investigate crude *n*-hexane, diethyl ether (Et₂O), ethanol (EtOH) and phosphate buffered saline (PBS) extracts of *Cosmos caudatus* Kunth leaves for their antimicrobial potential against 5 microbial strains comprising 2 Gram positive bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, 2 Gram negative bacteria: *Escherichia coli*, *Pseudomonas aeruginosa* and 1 fungi: *Candida albicans* by the disc diffusion method. Preliminary antimicrobial screening showed inhibition by the *n*-hexane, diethyl ether, and ethanol extracts against all the tested microbes, however the phosphate buffered saline extract was inactive against *Bacillus subtilis* and *Staphylococcus aureus*. MIC values ranging from 6.25 - 25 mg/ml for the tested crude extracts were obtained by the microtiter plate method. This study showed that the crude extracts of the leaves of *C. caudatus* Kunth. could be potential source of new antimicrobial agents especially to treat infections caused by the tested microbial strains and confirms its utility in folk medicines.

Key words: *Cosmos caudatus* Kunth, antimicrobial activity, gram positive, gram negative bacteria, fungi, MIC.

INTRODUCTION

The increase in prevalence of multiple drug resistance has slowed down the development of new synthetic antimicrobial drugs which necessitates the search for new antimicrobials from alternative sources. In general, microbes have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents. One way to prevent antibiotic resistance is by using new compounds which are not based on the existing synthetic antimicrobial agents (Shah 2005). Antibiotics are sometimes associated with adverse effects on the host including hypersensitivity and immune-suppression thus necessitating the need for the development of novel

antimicrobials especially from plant sources (Sieradski et al., 1999).

Traditional medicinal practices have been known for centuries in many parts of the world for the treatment of various human ailments. Herbal medicine is still the mainstay of about 75 - 80% world's population, mainly in developing countries for primary health care because of better cultural acceptability, better compatibility with the human body and fewer side effects (Tomoko et al., 2002). However, the last few years have seen a major increase in their use especially in the developed world. There is a growing interest in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity (Costa et al., 2008; Al-Bayati and Al-Mola, 2008). Screening active compounds from plants has led to the discovery of new medicinal drugs which have shown efficient protective power and treatment roles against various chronic diseases (Fabricant and Fansworth, 2001).

C. caudatus Kunth (synonym: *Bidens caudatus* (Kunth) Sch. Bip.) (wild cosmos) belongs to the family asteraceae. It is an edible plant having 20 - 26 species worldwide and popularly known as Ulam Raja (King's

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Abbreviation: *n*-Hexane, normal hexane; Et₂O, diethyl ether, EtOH, ethanol; PBS, phosphate buffered saline; MIC, minimum inhibitory concentration.

Microorganisms: *S. aureus*, *Staphylococcus aureus*; *B. subtilis*, *Bacillus subtilis*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *E. coli*, *Escherichia coli*; *C. albicans*, *Candida albicans*.

salad) in Malaysia. It is an annual herb bearing purple, pink, or white ray florets, grows up to about 1 - 8 ft tall, hairless or sparsely hairy and leaves are finely dissected, 10 - 20 cm long. It flowers from June to November (Guanghou et al., 2005). It is found worldwide in tropical areas including Mexico, United States (Arizona and Florida), Central America, South America, Malaysia and Thailand (Samy et al., 2005). *Ulam*, a Malay word used to describe a preparation that combines food, medicine and beauty is widely popular Malay herbal salad. As a Malaysian delight, it is served throughout the country from major hotels for tourists to buffet lunches or dinners for the locals.

In Malaysia, the plant is used traditionally for improving blood circulation, as an anti-aging agent, reducing body heat, strengthening bone marrow (because of its high calcium content), to promote fresh breath and to treat infections associated with pathogenic microorganisms (Hassan 2006; Bodeker, 2009). The methanol extract of *C. caudatus* Kunth has been reported to show moderate antioxidant activity when tested using the xanthine-xanthine oxidase enzymatic assay (Norhanom et al., 1999).

Recently, antioxidative and radical-scavenging activities of compounds isolated from this plant have been reported (Faridah et al., 2003; 2006). Medicinal plants remain a rich source of novel therapeutic agents. Many plant species have not been tested chemically or biologically in order to ascertain their folk claims scrupulously. Keeping view of the presence of strong antioxidant property in ulam raja, presence of biological active phenolic compounds and folk claims in the treatment of infectious diseases, we were prompted to evaluate the antimicrobial activity of the leaves of *C. caudatus* Kunth against some pathogenic microorganisms *in vitro* to establish its claim as an effective folk remedy to treat infectious diseases.

METHODOLOGY

Collection and preparation of plant material

C. caudatus Kunth was acquired from the medicinal garden of the Forest Research Institute Malaysia, KL., Malaysia (FRIM). Specimen sample was identified and authenticated by a Herbarium officer (Dr. Richard Chang, FRIM) and the plant sample (voucher specimen number: NMPC-KOS-018) was deposited in the Herbarium, Kulliyyah of Pharmacy, International Islamic University Malaysia (IIUM), Malaysia.

Leaves were carefully separated and cut into small pieces, dried in a protech laboratory dryer (LDD-720) at 40°C in the dark for 7 days and pulverized to powdered form using the Fritsch universal cutting mill. Leaves powder was stored in a dessicator in dark before being extracted.

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical Co. (USA) and Merck Chemical Co. (Germany). All chemicals and reagents were of analytical grade.

Extraction

100 g powdered leaves of *C. caudatus* Kunth was exhaustively extracted using soxhlet apparatus for 24 h in order to get maximum yield of soluble compounds (Castillo et al., 2007). The powdered leaves were sequentially extracted with *n*-hexane, diethyl ether and finally with ethanol according to increasing polarities, starting with the least polar solvent. Chronological extraction procedure was adopted based on the fact that different polarity of solvents facilitates the removal of desirable compounds soluble in particular solvents (Bazykinal et al., 2002). After successive and exhaustive extraction, the crude extracts were filtered and concentrated under vacuum and controlled temperature with a rotary evaporator (Büchi Rotavapor R-200), and residues were freeze dried using a freeze-drier (Christ alpha 1 - 4). All extracts were stored at -8°C in deep freezer until further use.

The dried leaves of *C. caudatus* Kunth were independently extracted with phosphate buffered saline (PBS) by the method adopted by Nor et al. (2008) with a little modification. 100g of the powdered leaves were mixed with PBS and kept at room temperature on a shaker (Multi-wrist shaker, Barnstead Lab Line) for 8 h. The mixture was centrifuged for 30 min at 27°C. Resultant supernatants were collected, filtered, rotary evaporated and eventually freeze dried.

All refined and freeze dried extracts were dissolved in dimethyl sulfoxide (DMSO) to obtain the desired concentrations of 1, 20 and 50 mg/ml. Extract samples were stored at -8°C in the deep freezer (Sanyo Medicoool) before being subjected to evaluate their meticulous antimicrobial activity.

Microorganisms

Five pathogenic microbial strains comprising 2 Gram-positive strains (*Staphylococcus aureus*, IMR S-277 and *Bacillus subtilis*, IMR B-140), 2 Gram-negative strains (*Pseudomonas aeruginosa*, IMR P-84 and *Escherichia coli*, IMR E-940) and a fungal strain (*Candida albicans*, IMR C-44) were used in the present study. All microbial strains were purchased from the Institute for Medical Research (IMR), Malaysia. All strains were carefully identified and grown using standard microbiological methods.

Inoculum preparation

Mueller-Hinton broth (MHB) and potato dextrose agar (PDA) were used for growing and diluting the microbial suspensions. Bacterial strains were grown to exponential phase in MHB at 37°C for 18 h and adjusted to a final density of 10⁸ CFU/ml by diluting fresh cultures and comparing with McFarland density. *C. albicans* was aseptically inoculated on petri dishes containing autoclaved, cooled and settled PDA medium. The petri dishes were incubated at 32°C for 48 h to give white round colonies against a yellowish background. These were aseptically subcultured on PDA slants. The colonies from PDA slants were suspended in a sterilized 0.9% NaCl solution (normal saline), which was compared with McFarland solution. 1 ml yeast suspension in normal saline was added to 74 ml of sterile medium, kept at 35°C, to give concentration of 10⁷ CFU/ml.

Antimicrobial assay

The agar disc diffusion method was employed for the determination of antibacterial activities of the leaves of *C. caudatus* Kunth extracts (NCCL, 2004). The inocula of the respective bacteria and fungus were streaked on to the Mueller-Hinton (MHA) and Sabouraud dextrose agar (SDA) plates. 6 mm in diameter disc's were punched,

impregnated aseptically with 10 µl concentration of each extracts (1, 20 and 50 mg/ml), allowed for 2 h to dry and placed on the inoculated MHA and SDA plates. The plates were then allowed to stay for 1 h at room temperature. Finally, the plates were incubated at 37 °C for 24 h for bacteria and at 32 °C for 48 h for fungi (Heraeus GmbH, D-6450, and Germany). The resulting diameters of zones of inhibition were measured and results were recorded in mm. Two antibiotics were used as positive control, tetracycline (10 µg/ml) for the bacterial strains while nystatin (30 µg/ml) for fungal strains. 10% Dimethyl sulfoxide (DMSO) was used as negative control. All the assays were carried out in triplicate.

Determination of minimum inhibitory concentration (MIC)

The microdilution technique using 96 well micro-plates (0.5 ml volume, Fisher Scientific) was used to determine the minimum inhibitory concentration (MIC) (Eloff, 1998). Plant extracts inhibiting the growth of the test strains by the disc diffusion test were serially diluted in two-fold from the highest concentration using 96 well microtiter plates. Wells of the first row (A) were filled with 200 µl of consecutive extracts and the remaining wells from row B to H were loaded with 100 µl MH (bacteria) or SD broth (fungi). 100 µl of extract from the first well was added and mixed into the second well of row B. Similar procedure was followed for the rest of wells in a particular column, and 100 µl of mixture at well of row H was discarded and 100 µl of the test organisms (bacteria and fungi separately) corresponding to 10^6 - 10^7 CFU/ml were added to the dilutions in the plates and control wells. After 24 h of incubation at 37 °C (bacteria) and 48 h at 32 °C (fungi), the MIC was visually determined based on the turbidity of the medium. The growth end-points were determined by comparing amount of growth in the well with extracts to that of turbid positive control well (Bin et al., 2008).

Phytochemical screening

TLC analysis of *n*-hexane, diethyl ether, ethanol and phosphate buffered saline extracts of the leaves of *C. caudatus* Kunth was carried out on silica gel 60 F₂₅₄, 0.2 mm thickness aluminium plates, with Toluene:Ethylformate:Formic acid (TEF) (5:4:1), Benzene:Pyridine:Formic, (BPF) (39:6:5) and Benzene: Acetone: Formic acid (BAF) (3:1:0.1), *n*-Hexane:Dichloromethane (DCM) (90:10) and *n*-Hexane:Ethylacetate (EtOAc) (97:03) to confirm the presence of alkaloids, flavonoids, saponins, tannins, and terpenoidal compounds by using different class of reagents to monitor the specific class of phytochemicals (Harborne, 1998).

Statistical analysis

Results are given as means ± SEM values. All the experiments *in vitro* were conducted at least three times, each time with three or more independent observations.

RESULTS AND DISCUSSION

All extracts of the leaves of *C. caudatus* Kunth showed varying degrees of antimicrobial activity against all microorganisms tested. The gram positive bacteria were more susceptible than gram negative bacteria and the bacterial strains were more susceptible than the fungal strain. These differences could be due to the nature and level of antimicrobial agents present in the plant, their mode of action and the typical differences in the microbial

cell walls between the strains (Tortora et al., 2001). EtOH extract exhibited a higher degree and broad spectrum antimicrobial activity as compared to the *n*-hexane, Et₂O and PBS extracts. Concentration effect was also observed with all the extracts as the highest activities were observed at the highest concentrations used to screen the test strains. This could be possible due to the fact that the compounds responsible for displaying their antimicrobial activities of the plant were present in each extract at different concentrations and were not enough to exhibit their antimicrobial action at a low concentration of each extract. This finding is correlated with the medicinal preparations that use rum and liquor to extract the active plant components (Jhon et al., 2006). The *n*-hexane, Et₂O, EtOH and PBS extracts of the leaves of *C. caudatus* Kunth exhibited different levels of antimicrobial activities against almost all the tested strains. Highest and least activities were exhibited by the EtOH extracts at 50 mg/ml and 1 mg/ml on *S. aureus* (forming inhibition zones of 10.00 ± 0.00 and 7.00 ± 0.00) respectively. However, *S. aureus* and *B. subtilis* were resistant to the PBS extract at all concentrations used. *C. albicans* and *B. subtilis* were resistant to the EtOH extract at 1mg/ml and *B. subtilis* was resistant to the Et₂O extract at 1mg/ml (all forming no zone of inhibition 6.00 ± 0.00 which is the size of the sensitivity disc used in the study). *E. coli*, *B. subtilis*, *P. auresginosa*, *S. aureus* and *C. albicans* were all susceptible to the extracts especially at high concentrations (Table 1).

The highest MIC value was 25 mg/ml exerted by the *n*-hexane extract on all strains and the least was 6.25 mg/ml exerted by PBS extract on *E. coli* and *B. subtilis*. Et₂O also exerted an MIC value of 6.25 mg/ml on all the strains tested (Table 1). All the tested strains were susceptible to the positive controls (Tetracycline and Nystatin) used (Table 1) and DMSO as a negative control did not produce any inhibitory activity against any microorganism tested. The preliminary phytochemical screening of the leaves of *C. caudatus* Kunth extracts explicitly showed the presence of terpenoids, fatty acids, flavonoids, alkaloids, tannins, and saponins on tlc plates in different solvent systems (Harborne 1998) and EtOH extract revealed the maximum number of components which could be responsible for the antimicrobial potential of the leaves of *C. caudatus* Kunth.

The results obtained from our study vividly suggest that the non-polar and polar extracts of the leaves of *C. caudatus* Kunth possess significant antimicrobial properties. All the extracts showed varying degrees of antimicrobial activity on the microorganisms tested. The chance to find out potent antimicrobial agents is more promising in EtOH, Et₂O and *n*-hexane extracts than the PBS extracts of the plant. Results explicitly express that the leaves extracts of *C. caudatus* Kunth have great antimicrobial potential against the tested microorganisms.

Hence, they can be used in the treatment of infectious diseases caused by tested strains and the plant could

Table 1. Antimicrobial activities and minimum inhibitory concentrations of the leaves of *C. caudatus* Kunth extracts against bacterial and fungal strains based on the disk diffusion and microdilution methods. Numbers indicate the mean diameters of inhibition of triplicate experiments \pm standard.

Bacterial / fungal strains	Zones of inhibition (mm)				
	<i>S. aureus</i> (IMR S-277)	<i>B. subtilis</i> (IMR B-140)	<i>P. aeruginosa</i> (IMR P-84)	<i>E. coli</i> (IMR E-940)	<i>C. albicans</i> (IMR C-44)
Extracts (mg/ml)					
<i>n</i> -Hexane 1	7.00 \pm 0.00	7.00 \pm 1.00	6.67 \pm 0.58	7.33 \pm 0.58	6.67 \pm 1.15
20	8.33 \pm 0.58	8.67 \pm 1.15	7.33 \pm 0.58	7.67 \pm 0.58	7.00 \pm 1.00
50	9.33 \pm 1.53	9.00 \pm 2.00	8.33 \pm 0.58	6.33 \pm 0.58	6.67 \pm 0.58
Et ₂ O 1	6.67 \pm 1.15	6.00 \pm 0.00	7.00 \pm 1.00	7.67 \pm 0.58	6.67 \pm 0.58
20	8.33 \pm 1.53	7.67 \pm 1.53	7.33 \pm 1.15	8.00 \pm 1.00	7.33 \pm 0.58
50	9.33 \pm 2.31	9.00 \pm 1.00	7.67 \pm 0.58	8.00 \pm 1.00	8.00 \pm 1.00
EtOH 1	6.33 \pm 0.58	6.00 \pm 0.00	7.33 \pm 1.15	7.67 \pm 0.58	6.00 \pm 0.00
20	8.67 \pm 0.58	9.33 \pm 1.13	8.33 \pm 0.58	7.67 \pm 0.58	8.67 \pm 0.58
50	10.00 \pm 0.00	9.67 \pm 1.52	9.33 \pm 0.58	8.67 \pm 0.58	10.00 \pm 1.00
PBS 1	6.00 \pm 0.00	6.00 \pm 0.00	7.33 \pm 1.15	6.33 \pm 0.58	6.00 \pm 0.00
20	6.00 \pm 0.00	6.00 \pm 0.00	8.00 \pm 1.73	7.00 \pm 1.00	6.00 \pm 0.00
50	6.00 \pm 0.00	6.00 \pm 0.00	8.67 \pm 2.31	7.00 \pm 1.73	6.00 \pm 0.00
Tetracycline (30 μ g)	16.00 \pm 0.40	17 \pm 0.70	19 \pm 1.20	22 \pm 0.58	-
Nystatin (30 μ g)	-	-	-	-	16 \pm 1.73
MIC (mg/ml)					
	<i>n</i> -Hexane		Et ₂ O	EtOH	PBS
<i>S. aureus</i>	25		6.25	6.25	-
<i>B. subtilis</i>	25		6.25	12.5	-
<i>P. aeruginosa</i>	25		6.25	6.25	6.25
<i>E. coli</i>	25		6.25	6.25	6.25
<i>C. albicans</i>	25		6.25	6.25	-

be a source of new potent antibiotic agents. However, further sincere efforts are still needed to isolate the biological active compounds from the extracts studied in order to know specific active principles responsible for the antimicrobial activity of *C. caudatus* Kunth and its toxicity profile. This report further suggests that a number of the local traditional plants might have beneficial antimicrobial-preventive effects in addition to providing potential new sources of natural antimicrobial agents. This *in vitro* study further confirms the utility of this plant in folk medicines to treat diseases associated with the aforementioned pathogenic microorganisms and demonstrate that the folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. The millenarian use of the plant in folk medicine suggests that it represents an economic and safe alternative to treat infectious diseases efficaciously.

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