JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2014. Vol. **8**(5), p. 3681-3688

Antimicrobial Activity of *Cosmos caudatus* Extract Against Foodborne Pathogens

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(Received: 07 May 2014; accepted: 21 July 2014)

The antimicrobial activity of *Cosmos caudatus* extract was evaluated against *Bacillus cereus*(ATCC 33019), *Bacillus subtilis*(ATCC 6633), *Proteus mirabilis* (ATCC 21100), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231) using the methods as recommended by the Clinical and Laboratory Standard Institute (CLSI). The antimicrobial tests were conducted in term of susceptibility, minimum inhibitory concentration (MIC), minimum bactericidal/fungicidal concentration (MBC/MFC) and killing-time curve. The results showed that *C. caudatus* extract was susceptible against all tested pathogens; the inhibition zone ranged from 8.60 mm to 9.83 mm. The MIC and MBC/MFC values were ranged from 6.25 mg/ml-12.50 mg/ml and 12.50 mg/ml-50.00 mg/ml, respectively. Mean while, killing-time curves showed that *C. caudatus* extract can killed the *B. cereus*, *B. subtilis*, *P. mirabilis*, *P. aeruginosa* and *C. albicans* at concentration of 8 MIC for 2 h, 4 MIC for 2 h or 2 MIC for 2 h, 8 MIC for 4 h, 4 MIC for 0.5 h and 4 MIC for 1 h, as respectively. Findings indicated that *C. caudatus* extract has the potentiality to develop as a natural antimicrobial agent.

Key words: Antimicrobial activity; *Cosmos caudatus*; Foodborne pathogen; Time-kill study; Plant extract.

Nowadays, food safety becomes a crucial problem worldwide which affects millions of people suffered from foodborne illness. In Malaysia the reported cases of foodborne outbreaks were increased from 6,930 to 17,320 cases in between 2006 to 2008 (Soon *et al.*, 2011).Same issues faced in United States, where the foodborne outbreaks had drastically increased (1990-2008), which it was estimated from 76 million illnesses, 325,000 of them were hospitalised and 5,000 weredead (Mead *et al.*, 1999). Meanwhile, a great consumer concern on the safety of foods containing synthetic preservatives has led the researchers to find the best alternative way to replace the synthetic preservatives with effective and non-toxic antimicrobial compounds.

Antimicrobial compounds from plants have been proved to have higher levels of food safety (Alzoreky and Nakahara, 2003). Numerous research has been reported on the antimicrobial potentiality of medicinal plants (Mahesh and Satish, 2008; Valgas *et al.*, 2007; Gutierrez *et al.*, 2009; Lucera *et al.*, 2012; Negi, 2012) and the research has increasingly reported until now. Medicinal plant also known as herbs which can promote several health benefits to human and has been used throughout human history. Some of them have been used by many cultures for food preservation also as food additives to enhance aroma and flavour (Hoque *et al.*, 2008). The

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consumption of them also can increase the activity of digestive enzymes which can provide some protection against gastrointestinal infection (Low Dog, 2006).

Cosmos caudatus or *Ulam raja* is one of the medicinal plant and has been famously been used in Malaysia to cure several health problems such as in improving blood circulation, anti-aging agent, reducing body heat and promoting fresh breath (Reihani *et al.,* 2012). This plant can be eaten freshly as salad especially by Malay communities. *C. caudatus* is originated from Central America and can be found worldwide in tropical areas including Malaysia, Thailand, Indonesia, Mexico and United States (Arizona and Florida) (Shui *et al.,* 2005). Usually it can grow up to 1 - 8 feet tall and starts flowering from June to November, with white floret, pink and purple flowers (Shui *et al.,* 2005).

Mediani *et al.* (2013) and Ragasa *et al.* (1999) found that *C. caudatus* contained quercetin rhamnoside, quercetin glucoside, rutin, stigmasterol, costunolide and lutein. The *C. caudatus* extract has strong antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis, Candida albicans*and *Saccharomyces cerevisiae* (Ragasa *et al.,* 1999). This antibacterial activity might be due to the viability of bioactive compounds including phenolics, flavonoids, carbohydrates, proteins, minerals and vitamins (Abas *et al.,* 2003).

Due to the huge demand for free chemical preservatives in processed foods, people in food companies and researchers were pushed to find an alternative sources of natural antimicrobial compounds which can inhibit the growth of undesirable microorganisms and provide no toxic effects particularly to consumers. Hence, this study was conducted to evaluate the antimicrobial activity of *C. caudatus* plant against several foodborne pathogens namely *Bacillus cereus, Bacillus subtilis, Proteus mirabilis, Pseudomonas aeruginosa* and *Candida albicans.*

MATERIALS AND METHODS

Plant material collection

The sample of *C. caudatus* was purchased from Pasar Borong Selangor in Seri Kembangan, Selangor, Malaysia. The leaves of *C. caudatus* were separated from stems, cut into small pieces and dried under room temperature (27°C) until fully dried. Then sample was powdered using Waring blender (Sinclair and Campbell, Scotland, UK) until fine powder formed.

Plant extract preparation

A100 g of powdered sample was soaked in 400 ml of methanol (99.80%) for 3 days with occasionally shaken, as stated by Rukayadi *et al.* (2008), with slight modifications. Then the mixture was filtered using Whatman filter paper No. 1 (Whatman, Buckinghamshire, UK) and further concentrated by rotary evaporator (Buchi, Flawil, Switzerland) at 50°C to get the gummy-like crude extract. The *C. caudatus* crude extract was dissolved in 10% dimethylsulfoxide (DMSO) to get the stock solution. The final concentration of extract was standardized at 100 mg/ml or 10%.A 10% DMSO was used as negative control where it was found not to kill all foodborne pathogens tested in this study.

Bacterial strains

All microorganisms were obtained from American Type Culture Collection (Rockville, MD, USA); *B. cereus* (ATCC 33019), *B. subtilis* (ATCC 6633), *P. mirabilis* (ATCC 21100), *P. aeruginosa* (ATCC 9027) and *C. albicans* (ATCC 10231). All bacteria cultures were grown in Mueller-Hinton broth (MHB) or Mueller-Hinton agar (MHA) (Difco,Sparks, MD, USA) at 37°C for 24 hours, while fungal culture was grown in Saboured dextrose broth (SDB) or Saboured dextrose agar (SDA) (Difco, Sparks, MD, USA) at 35°C for 48 hours.

In vitro susceptibility test

Disc-diffusion method

The *C. caudatus* extract was tested for antimicrobial activity against five types of foodborne pathogens using disc-diffusion method. Overnight cultures of each type of pathogens were spread on MHA/SDA using sterile cotton swabs. After that, 6.00 mm sterile paper discs (Whatman, Buckinghamshire, UK) were impregnated in 10µl of 10% *C. caudatus* extract and were placed on MHA/SDA. The MHA/SDA plates then were incubated, at 37°C for 24 hours (bacterial) and 35°C for 48 hours (fungal). Results were observed based on the inhibition zones occurred surrounding the paper discs and expressed in unit millimeter (mm). Each test was

performed in duplicate for data validation. The 1% of chlorhexidine and 1% Amphotericin B (Merck, Darmstadt, Germany) were used as positive control for bacterial and fungal, respectively. The purpose of these antibiotics was to ensure the activity of standard antibiotics on the tested foodborne pathogens.

Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) evaluation

Minimum inhibitory concentration (MIC) of *C. caudatus* extract was determined based on the method described in the guidelines of Clinical Laboratory Standard Institute M7-A6 (2003). Test was performed using sterile 96-wells round bottom microtitre plates (Brandon, Malaysia) with an inoculum approximately 10^6 cfu/ml. Briefly, wells from the first row were filled with 200µl MHB/SDB (positive control) and for the second row were filled with 200µl bacterial/fungal inoculum (negative control). Then, a two-fold dilution of *C. caudatus* extract in the range of 100 mg/ml – 0.1mg/ml was performed starting from well (12) until well (3) and the remaining 100µl from well (3) were discarded. These made the highest extract concentration at well (12) and the lowest extract concentration at well (3). Microtiter plates then were incubated at 37°C for 24 hours (bacterial) and 35°C for 48 hours (fungal). The MIC value was visually determined by observing the well with completed inhibition of visible growth.

Minimum bactericidal/fungicidal concentration (MBC/MFC) was defined as the lowest concentration of the extract to demonstrate bactericidal/fungicidal activity. The test was performed by sub-culturing 10µl of the suspension from each well of microtiter plates(including positive and negative control) onto the MHA/SDA. Then plates were incubated and the growth colonies were recorded.

Time-kill curve

Time-kill assay of *C. caudatus* extract against *B. cereus, B. subtilis, P. mirabilis, P. aeruginosa*and *C. albicans*were performed according to the method of Lorian (2005) and Pankey and Ashcraft (2009), with slight modifications. Firstly, bacterial/fungalsuspension was prepared in the range of (10^6-10^8cfu/ml) by diluting with the MHB/SDB medium. Then, the mixing procedure between *C. caudatus* extract (100 mg/ml) and inoculum suspensions were done at different length time (0 h, 0.25 h, 0.5 h, 1 h, 2 h and 4 h). Then, 10µl of each mixture (extract with inoculum suspension) at 0 h, 0.25 h, 0.5 h, 1 hour, 2 hours and 4 hours were serially diluted for 10^{-2}, 10^{-4} and 10^{-6}. After that, diluted mixtures were pipetted onto the MHA/SDA plate with replications, separately. The plates were incubated at 37°C for 24 hours (bacterial) or 35°C for 48 hours (fungal) and colonies were counted. The same procedures were performed for OMIC (control), 0.5 MIC, 1 MIC, 2 MIC, 4 MIC and/or 8 MIC, for each of the tested pathogens.

RESULTS AND DISCUSSIONS

In this present study, the antimicrobial activity of *C. caudatus* extract was evaluated against five species of food borne pathogens including *B. cereus* (ATCC 33019), *B. subtilis* (ATCC 6633), *P. mirabilis* (ATCC 21100), *P. aeruginosa* (ATCC 9027) and *C. albicans* (ATCC 10231). Results were analysed based on the inhibition zone appeared, MIC and MBC/MFCas presented in Table 1. In this study, methanol was used as the organic solvent for the extraction due

Table 1. Inhibition zone, minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of *Cosmos caudatus* extract against foodborne pathogens.

Foodborne pathogens	Inhibition zone (mm)	MIC (mg/ml)	MBC/MFC(mg/ml)
Bacillus cereus(ATCC 33019)	9.83±0.29	6.25	50.00
Bacillus subtilis(ATCC 6633)	9.83±0.58	12.50	25.00
Proteus mirabilis(ATCC 21100)	9.67±0.58	6.25	50.00
Pseudomonas aeruginosa(ATCC 9027)	8.67±1.15	12.50	50.00
Candida albicans(ATCC 10231)	8.60±0.00	12.50	12.50

to its ability to extract most of the polyphenolic compounds in plant-based extract. Polyphenolic compounds such as flavonols and other bioactive compounds are generally having high solubility in polar solvents like methanol (Parekh and Chanda, 2008). Abdullah *et al*. (2012) also reported the significance of antibacterial activity when plant materials were extracted using methanol compared to hexane and water. Besides, the use of methanol solvent also can increase the total yield of the extract (Caunii *et al.*, 2012).

From all the pathogen tested, bacteria species exhibited larger inhibition zone which was in the range of (8.67 mm -9.83 mm) compared to fungal species which was 8.60 mm. These findings proved that fungal species was more resistant on *C. caudatus* extract than bacterial species. Results were supported by Singh *et al*. (2006) who found that fungal species were hard to be killed since their ability to have the same structure and metabolism with their eukaryotic host once disease happened. Frequent disease caused by the fungi

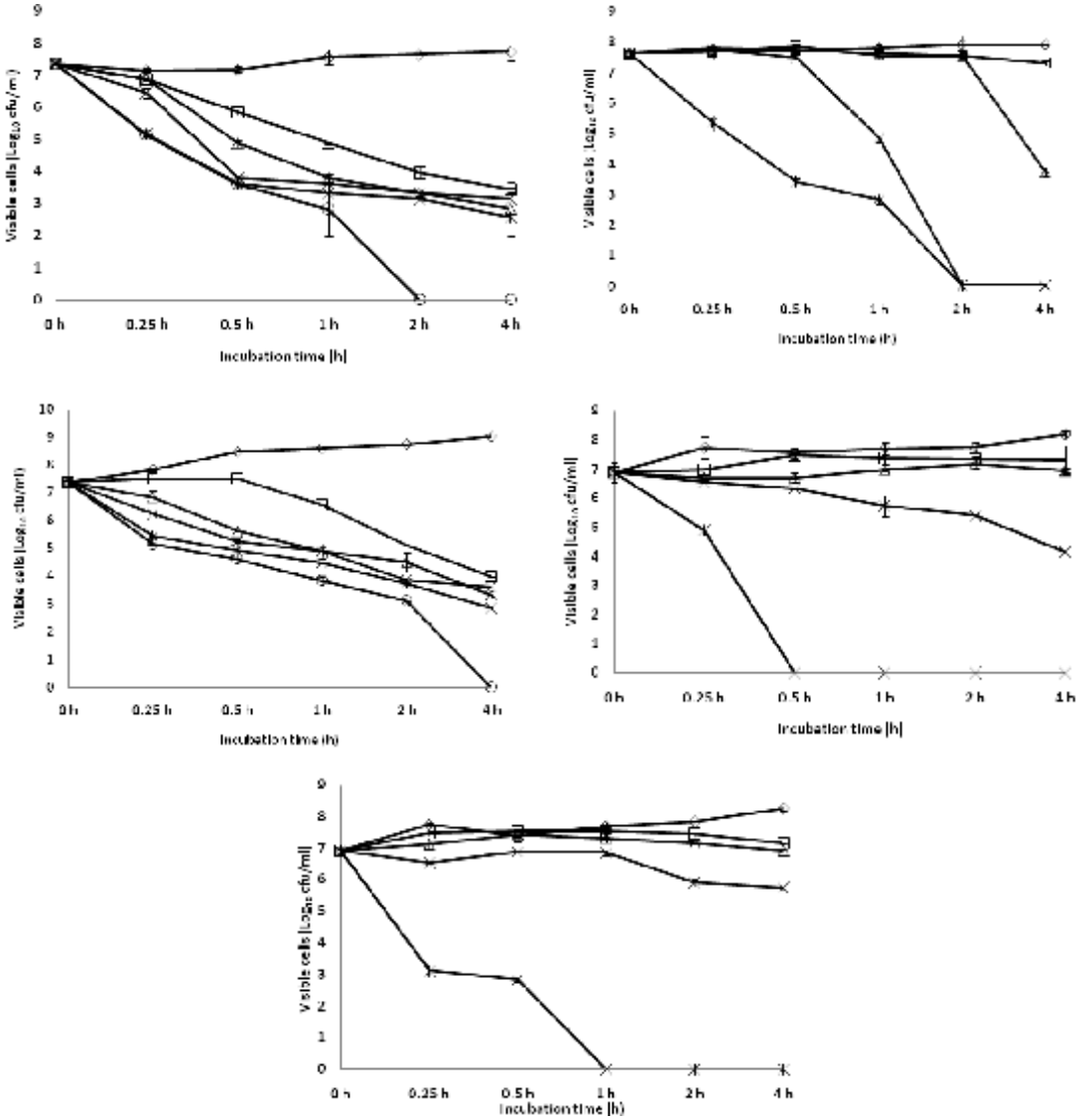

Fig 2. Time kill plots for five types of foodborne pathogens following exposure to *C. caudatus* extract. (a) *B. cereus* (0, 3.13, 6.25, 12.5, 25, 80); (b) *B. subtilis* (0, 6.25, 12.5, 25, 50); (c) *P. mirabilis* (0, 3.13, 6.25, 12.5, 25, 80); (d) *P. aeruginosa* (0, 6.25, 12.5, 25, 50); and (e) *C. albicans* (0, 6.25, 12.5, 25, 50). Values given in the brackets after species are 0 MIC, 0.5 MIC, 1 MIC, 2 MIC, 4 MIC and/ or 8 MIC, as respectively.

including respiratory illness and nosocomial infections (Tournas, 2005; Chiu *et al.,* 2007; Perlroth *et al.,* 2007; Alangaden, 2011).

While, among the bacteria, *B. cereus* and *B. subtilis* exhibited larger inhibition zones (9.83 mm) compared to *P. aeruginosa* and *P. mirabilis* which were in the range of (8.67 mm and 9.67 mm), respectively. These differences might be due to the different cell wall structure since *B. cereus* and *B. subtilis* are in the group of Gram positive bacteria while *P. aeruginosa* and *P. mirabilis* are Gram negative bacteria. Findings were consistent with Rasdi *et al.* (2010) who also reported the resistance of Gram negative than Gram positive bacteria. In general, the major difference is due to the presence of an outer-membrane permeability barrier in Gram negative bacteria which limits the access of antimicrobial agent into their cytoplasmic area (Hendra *et al.,* 2011).

After the susceptibility test ,*C. caudatus* extract was subjected to MIC test using the standard two-fold dilution method against all the tested pathogens. Minimum inhibition concentration (MIC) is defined as the minimum concentration needed to inhibit at last 99% of bacterial/fungal growth. From the results, the least MIC value was detected both on *B.cereus* and *P. mirabilis* (6.25 mg/ml). The MIC value of *C. caudatus* extract was high compared to methanolic extract of *P. guajava* which only needed 10 mg/ml (Preethi *et al.,* 2010). Negi *et al.* (2012) also reported the MIC value of *Bauhunia purpurea* on *Bacillus* species which was at 1.5 mg/ml and lemon extract was less than 2 mg/ml (Sudhir *et al.,* 2012). Even though the amount of the *C. caudatus* extract needed to cause the inhibition was high compared to other plants, but it still can be considered as one of the potential sources as natural antimicrobials, perhaps with some method modification to improve the activity. Other plant extracts which contributed to the inhibition of *Bacillus* species also reported by Jun *et al.* (2013); Ababutain (2011); Nascimento *et al.* (2000) and Cock(2007). Even though the *Bacillus* species seems to be easier to be inhibited or killed, their growth also becomes a concern since their ability to produce spores (Agata *et al.,* 2002).

Minimum bactericidal/fungicidal concentration (MBC/MFC) is defined as the minimum concentration of the plant extract required to kill at least 99% of the bacteria/fungi. From the MBC/MFC results, *C. caudatus* extract showed the ability to kill all the tested pathogens; where *Candida* species exhibited the least MFC value (12.50 mg/ml) which indicated the most susceptible species towards the extract. These results showed some contradiction between the previous test (susceptibility and MBC/MFC) value where there wasno association between them. This situation usually happened especially when we tested on different microorganisms species which certainly possessed different ways of adaptation. In relation to this, Zainol *et al.* (2003) has concluded that it might be due to the adaptive ability of the species where they said to be easier to get inhibited but hard to be killed. The variation in susceptibility of the microorganisms also caused by several intrinsic factor like the permeability of the cell surface to the extract (Khan *et al.,* 2009).

The adaptive ability of microorganisms were also depends on the presence of bioactive compounds in the plant extract. Several bioactive compounds in plant extract have been reported among others including flavonoids, tannins, alkaloids, terpenoidsand saponins(Weerakkody *et al.,* 2010; Sohn *et al.,* 2004; Klausmeyer *et al.,* 2004, Alma *et al.,* 2003). Some of them were detected by using bio-guided method such as bio-autography and even of them were isolated through the isolation and elucidation process. Vital and Rivera (2009) proved the presence of flavonoids and tannins were contributed to antimicrobial activity. Hendra *et al.* (2011) showed the antimicrobial activity of various parts of *Phaleriam acrocarpa* (MahkotaDewa) which consists of flavonoids compounds; kaempherol, myricetin, rutin, quercetin and naringenin. *Cantella asiatica* (Pegaga) also showed antibacterial activity against various food and human pathogens, which comprises with high flavonoids content; kaempherol, quercetin, catechin, rutin, apigenin and naringenin (Pitella*et al.,* 2009). Cushnie and Lamb (2005) stated that there are some mechanisms occurred behind the significant antimicrobial activity such as the inhibition of nucleic acid synthesis, cytoplasmic membrane functions and energy metabolisms. All these mechanisms or at least one of them will contribute to the inhibition or killing of the microorganisms.

In the time-kill assay (Figure 1), *C. caudatus* extract indicated the ability to kill all the tested foodborne pathogens. For *B. cereus* and *B. subtilis*, the bactericidal endpoints were achieved at 8 MIC after 2 h and 4 MIC after 2 h or 2 MIC after 2 h, respectively. As reported by Cowan (1999), these bacteria were hard to be killed due to their ability to form spore coats at certain growth stage. They can cause food poisoning through the ingestion of thermostable enterotoxin or thermo sensitive enterotoxin. While for *P. mirabilis* and *P. aeruginosa*, the bactericidal endpoints were reached at 8 MIC after 4 h and 4 MIC after 0.5 h, respectively. For fungal, *C. albicans* reached the fungicidal endpoint at 4 MIC after 1 h. This study indicated the potentiality of *C. caudatus* methanolic extract to kill the *B. cereus, B. subtilis, P. mirabilis, P. aeruginosa* and *C. albicans*.

CONCLUSION

From these studies, it can be concluded that *C. caudatus* has the potentiality in treating several foodborne pathogens. Thus, they can be used as a basis guideline for the investigation and discovery of new natural bioactive compounds. However it is also necessary to determine their toxicity and the side effects caused by the active constituents inside the plant.

ACKNOWLEDGEMENTS

This research project was supported by Research University Grant Scheme (RUGS), Universiti Putra Malaysia (UPM) under grant vote number: 9329600.

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