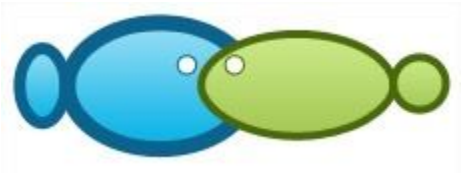

Effect of *Cosmos caudatus* extract on antibacterial activity and lethality activity of brine shrimp

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Abstract. Exact level of herb extracts to be applied as feeding additives or therapeutics are necessary to investigate. Therefore a preliminary in-vivo test and bioassay were performed to determine the inclusion level of *Cosmos caudatus* extract in animal feed. The inhibition of gram negative bacteria, *Aeromonas hydrophila* were tested with the methanolic extract of *C. caudatus* at concentrations of 25, 50, 100, 200, 400 and 800 µg mL^{-1}. The same concentrations of herb extract were applied for bioassay of brine shrimp nauplii. The results showed that all the tested concentrations inhibited the bacterial growth. Moderate activity of inhibition was observed at concentration lower than 200 µg mL^{-1}, while higher activity of inhibition was recorded at 400 and 800 µg mL^{-1}. Meanwhile for bioassay, 100% mortality of brine shrimp nauplii was observed at the higher concentrations (400 and 800 µg mL^{-1}) and LC_{50} was observed at concentrations between 100 to 200 µg mL^{-1}. Therefore, it is concluded that the effective and safe level of *C. caudatus* extract is lower than 100 µg mL^{-1}.

Key Words: *Cosmos caudatus*, *Aeromonas hydrophila*, inhibition, bacterial growth, bioassay, brine shrimp.

Introduction. Recently natural feed additives are becoming more popular and thus more studies have been conducted to investigate novel feed additives from natural sources (Abas et al 2006; Ji et al 2007; Citarasu 2010; Talpur & Ikhwanuddin 2013). It is not limited to human but also for animals and including fish. The knowledge of green aquaculture has been increasing thus leading to investigation of the function of natural products as alternative antibiotic. Usually, natural products like plants or herbs extracts contain secondary metabolites which have therapeutic and prophylactic effects on fish diseases (Talpur & Ikhwanuddin 2013).

Besides, some studies showed that the incorporation of herbs extract in diet can increase weight gain (Citarasu 2010; Talpur & Ikhwanuddin 2013), improves fatty acid utilization and digestibility (Ji et al 2007) and helps in stimulating appetite (Putra et al 2013). The beneficial properties and efficiency of plant products on fish however depends on the part of plant, extraction method and its concentration. According to Reverter et al (2014), little effort has been done to homogenize the extraction procedure, the extract concentration and the way of administration.

Cosmos caudatus, locally known as 'ulam raja' is a common herb in Malaysia. Traditionally, the leaf of this herb has been eaten raw or cooked. Although studies on *C. caudatus* are scarce in terms of application, it was noticed that this herb contains more than 20 antioxidants which might reduce oxidative stress and had potential in inhibiting pathogens (Shui et al 2005). The total phenolic compound (TPC), total flavonoids content (TFC), ascorbic acid, and diphenylpicrylhydrazyl (DPPH) are some active agents in *C. caudatus* (Shui et al 2005; Abas et al 2006; Mustafa et al 2010; Andarwulan et al 2012; Mediani et al 2012; Salehan et al 2013; Dian-Nashiela et al 2015). Moshawih et al (2017) also had reviewed comprehensively on *C. caudatus* in terms of pharmacology, ethnopharmacology and phytochemistry.

Aeromonas hydrophila is a gram negative bacteria, widely available in all kinds of environment and a common pathogen for freshwater, brackish water and seawater fish and shrimp. The presence of the colonies may also transfer to other animals and human being which in worst case may cause mortality (Janda & Abbott 2010; Sakar & Rashid 2012). An epidemic of virulence *A. hydrophila* was reported in United States where the bacteria were proven originated from Asia (Hossain et al 2014). For freshwater fish like catfish, carps and perch, the bacteria were highly virulent with external pathological symptoms (Banu et al 1999; Sakar & Rashid 2012).

Since *C. caudatus* had potential to inhibit pathogen, efforts for documenting the safety and efficacy should be conducted. Therefore, an experiment was conducted to investigate the inhibition activity of using different levels of herb extract against *A. hydrohila*. Brine shrimp lethality assay was also conducted to determine the toxicity of the extract.

Material and Method

Herb preparation and extraction. *C. caudatus* leaves were bought from local market. The herb was cleaned and dried until they reached a stable weight using oven for 2-3 days at 35-40^{o}C. The samples were then weighed and grinded to obtain a powder form (size about 0.70 mm) and finally stored at -20^{o}C until use.

The blended herbs were soaked until homogenization with pure methanol for 24 hours in dark. The extract was then filtered through filter paper before extracted and concentrated in vacuum at 40^{o}C using rotary evaporator (Buchi, Switzerland). The sample then was freeze dried and stored at -20^{o}C until use. The yield was determined in percent of weight. This method was implemented from Mediani et al (2012) with slight modification:

Percentage extraction yield = [weight after extraction/weight before extraction] x 100

Preparation of herbs and bacterial concentration. Various concentrations of *C. caudatus* extract (50, 100, 200, 400 and 800 µg mL^{-1}) were prepared. For antimicrobial study of *C. caudatus*, the methods as described by Khanam et al (2015) were applied with slight modification. Gram negative bacterial pathogen (*A. hydrophila* KF146350) was obtained from Universiti Malaysia Terengganu, Terengganu, Malaysia. Nutrient agar plates (Merck) were prepared before the pure culture of pathogen streak onto it and incubated for 24 hrs at 37^{o}C.

After the optimum growth, the fresh cultures were transferred into nutrient broth (Merck) and incubated again for 24 hr at 37^{o}C. Optical density of pathogen was then measured using a UV spectrophotometer (Shimadzu, Japan) at 600 nm wavelength. The optical density of desired pathogen concentration was 10^{8} CFU mL^{-1} represented 0.5 McFarland standards (Thermo Fisher Scientific©). Adjustment of turbidity of pathogen by dilution was done to meet the optical density of the standards (Valgas et al 2015).

Disc diffusion assay. The extract of herb was screened for antimicrobial activity on tryptic soy agar (TSA). These methods were based on Khanam et al (2015) and Laith (2013). Briefly, the disc was impregnated with the extract (20 µg disc^{-1}) for few minutes. The disc then was air dried 2-3 min and placed onto the agar. The plate was incubated at 28^{o}C for 24 to 48 hrs and the bacteria growth observed. Antibiotic disc of control was methanol. The results were assessed in diameter (mm) and expressed in the terms of zone of inhibition (ZI) as follows: for low activity, 1-6 mm; moderate activity, 7-10 mm; high activity, 11-15 mm, very high activity, 16-20 mm; and no activity, 0 mm (Khanam et al 2015).

Brine shrimp lethality bioassay. Bioassay was implemented as described by Pisutthanan et al (2004) and Laith (2013). The brine shrimp eggs were incubated in seawater at 28^{o}C for 24 hrs. After the brine shrimp hatched, the nauplii in 100 µL of seawater were collected. There were 15 nauplii in 100 µL of seawater.

For the extract solution preparation, 10 mg of extract were dissolved in 50 µL dimethyl sulphoxide (DMSO) prior to adding seawater up to 2 mg mL^{-1}. Serial dilutions were conducted to prepare 25, 50, 100, 200, 400 and 800 µg mL^{-1} of extract. All the extract solutions were prepared in quadruplicate.

One hundred microlitres of solutions (extract and control) were placed in vials and added with 100 µL of nauplii. After incubation for another 24 hours at 28°C, the content of the vials were transferred onto petri dish and examined under binocular microscope (Nikon, Japan). The number of dead nauplii in each treatment was counted and recorded. Methanol (100 µL) was added into the tubes to immobilize the nauplii. The total number of the nauplii was taken after 15 minutes.

The toxicity level of *C. caudatus* on survival of brine shrimps (LC_{50}) were determined as suggested by Moshi et al (2010) where: LC_{50} < 1.0 µg mL^{-1}, highly toxic; LC_{50} 1.0-10.0 µg mL^{-1}, toxic; LC_{50} 10.0-30.0 µg mL^{-1}, moderate toxic; LC_{50} > 30 < 100 µg mL^{-1}, mildly toxic; LC_{50} > 100 µg mL^{-1}, non-toxic.

Statistical analysis. All the data were expressed as mean±standard deviation from the data presented from quadruplicate value (n = 4), unless stated otherwise. The data were analyzed using one-way analysis of variance using SPSS ver. 21.0 (SPSS Inc., Chicago, IL). Level of significance between individual treatments ($p > 0.05$) was evaluated by Duncan's test.

Results

Leaves and extraction yields. Using methanol as solvent, we could extract 31.23 g of *C. caudatus* from 2.035 kg of fresh leaves. Therefore the percentage yield of the herb was 1.53%.

Disc diffussion assay. The effectiveness of different concentrations of *C. caudatus* extract on inhibition of *A. hydrophila* is shown in Table 1. Significant differences were found among different concentrations of *C. caudatus* extract against inhibition zone of *A. hydrophila* ($p < 0.05$). The values for inhibition zone of bacteria tended to increase with the increasing concentrations of *C. caudatus* extract. The significantly lowest inhibition zone was detected at 25 µg mL^{-1} concentration of *C. caudatus* extract while the highest ($p < 0.05$) values were found at 400 and 800 µg mL^{-1}. There were no significant differences ($p > 0.05$) in inhibition zone within the concentrations of *C. caudatus* extract at 25-200 µg mL^{-1}. Therefore, inhibition activity of methanolic *C. caudatus* extract is considered moderate at concentration 25-200 µg mL^{-1}, high at 400 µg mL^{-1}, and extremely high at 800 µg mL^{-1} respectively.

Table 1

Effect of different concentration of *Cosmos caudatus* extract on inhibition of *Aeromonas hydrophila* using TSA*

Parameters	*Concentration (µg mL^{-1})*					
	25	*50*	*100*	*200*	*400*	*800*
Inhibition zone (mm)	7.75±0.50[a]	9.25±2.63[a]	10.00±2.58[a]	10.50±1.92[a]	14.50±1.29[b]	15.75±1.26[b]

*Values are means of quadruplicate groups±SD. Within a row, means with the same letters are not significantly different ($p > 0.05$).

Brine shrimp lethality test. Brine shrimp lethality assay was performed to determine the lethal concentration of *C. caudatus* extract. All the brine shrimps died at the level of 400 and 800 µg mL^{-1}. On the other hand, the mortality was 13.33, 20.00, 33.33 and 93.33% at 25, 50, 100 and 200 µg mL^{-1} of *C. caudatus* extract, respectively (Table 2). The LC_{50} value was estimated at the concentrations between 100-200 µg mL^{-1} extract which considered as non-toxic.

Table 2

Brine shrimp lethality test of *Cosmos caudatus* methanolic extract

Concentration of extract (µg mL^{-1})	*Initial number of nauplii*	*Number of nauplii survive*	*Number of nauplii died*	*% mortality / lethality*
800	15	0	15	100.00
400	15	0	15	100.00
200	15	1	14	93.33
100	15	10	5	33.33
50	15	12	3	20.00
25	15	13	2	13.33

Discussion

Leaves extraction yield. The present study showed that the percentage yield of *C. caudatus* leaves was 1.53%. This value is higher than recorded by Huda-Faujan et al (2009) where the yield of *C. caudatus* was 1.13% using the same solvent with longer soaking time. The yield also different is probably due to part of herb used in current study. Different plant parts (leaf, root and stem) usually resulted in various levels of antioxidant agents. In this experiment, young leaves of *C. caudatus* were used because younger plant shows strong antioxidant and free radical scavenging agents (Abas et al 2006; Dian-Nashiela et al 2015).

Besides methanol, ethanol, dichloromethane (DCM) and n-hexane are usually used for herbs extraction. Salehan et al (2013) stated that ethanolic extraction with sonication yielded 128.2 g of crude herb from 3.06 kg fresh leaves (percentage yield is 4.19%). It is higher compared to this study. However, Pisutthanan et al (2004) suggested the most active fraction of plant extracts were mainly distributed in methanol fraction. Thus, methanolic extraction has advantages over the other procedure in partitioning and eliminating a large portion of inactive compounds. In the present study, methanol was used as solvent without sonication which might explaine comparatively lower percentage yield than other studies. Optimum extraction method is important especially for large scale industries in order to save operational cost and time.

Disc diffusion assay. Chemical components and its level in crude herb extracts related with antibacterial actions. From this study, there was increasing patterns of *C. caudatus* extract with the increasing of inhibition zone. The inhibition activity of *C. caudatus* extract is considered moderate (25-200 µg mL^{-1}), high (400 µg mL^{-1}) and very high (800 µg mL^{-1}). Therefore, concentrations of extractions can affect the effectiveness of herb against pathogen (Sekar et al 2012).

A study by Shui et al (2005) reported that *C. caudatus* contains L-ascorbic acid. It also contains TPC, TFC and DPPH where the compounds can inhibit the bacteria with the presence of free radical (Abas et al 2006; Salehan et al 2013; Dian-Nashiela et al 2015). The free radicals work by precipitating microbial protein (Iqbal et al 2015) then will hinder bacterial growth. The inhibition of *C. caudatus* extract against *A. hydrophila* was detected at all concentration level even at 25 µg mL^{-1} for this study. This finding was in agreement with Abas et al (2006) in which *C. caudatus* extract had strong free radical scavengers, mostly phenolic compounds and can exhibit pathogen as lower as 30 µg mL^{-1} by using ferric thyiocyanate (FTC) method.

Usually the younger plants show high inhibition ability due to high content of the secondary metabolites (Mediani et al 2012). So, selection of development stages of leaves may give an impact to antibacterial study. They suggested the best harvesting time of *C. caudatus* is during the dry season at the age of eight weeks and before the flowering stage to provide better productivity and beneficial medicinal value. Besides bacteria, *C. caudatus* extract is also effective to control fungus, *Phytophthora palmivora* in plants (Salehan et al 2013). A study by Moshawih et al (2017) has validated the application of this herb in many fields but the benefit still need to be fully optimized for other different fields such as neurological, allergy, immunity and depression.

Brine shrimp lethality assay. The bioassay of brine shrimp nauplii is practical for a variety of toxic substances. It is capable to detect a board spectrum of bioactivity in the crude extract (Pisutthanan et al 2004). Solis et al (1993) stated the responds of brine shrimp towards toxicity or biological active compounds are similar to mammalian systems. In the methodology, the nauplii of brine shrimp used were 24 hrs old and the observation of the mortality of the bioassay took for another 24 hrs. The young nauplii were used because of the sensitivity of the nauplii are higher in second and third stage of instar compared to first instar stage due to the exposure of epithelium of digestive tract (Sorgeloss et al 1978).

Based on the result, LC_{50} of *C. caudatus* extract was found in the range of 100-200 µg mL^{-1} and indicates non-toxic to brine shrimp (LC_{50} > 100 µg mL^{-1}). The range is higher than the study conducted by Ogugu et al (2012) on *Calliandra portoricensis* using the same solvent (LC_{50}; range of 0-100 µg mL^{-1}). This is because the toxicity of plant extract is distinct individually even the plants are from the same family (Pisutthanan et al 2004).

Conclusions. Based on the present experimental condition, it can be concluded that *C. caudatus* suppressed *A. hydrophila* growth. The effectiveness however depends on its concentration (25-200 µg mL^{-1}, moderate; 400 µg mL^{-1}, high; 800 µg mL^{-1}, very high). Meanwhile for bioassay using brine shrimp showed that LC_{50} for this herb extract was 100-200 µg mL^{-1}. Thus, *C. caudatus* extract is considered non-toxic at lower than 100 µg mL^{-1}. This study offered as a front line screen to establish *C. caudatus* extract in environment or as supplement in feed. Based on both inhibition and bioassay, *C. caudatus* extract recommended an effective alternative as antioxidant, supplement or food additive. However, further investigation on biological response of the herbs application in future is required to elucidate full toxicity profile.

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