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ANTIMICROBIAL ACTIVITY, PHYTOCHEMICAL ANALYSIS AND TOXICITY TEST OF Cosmos caudatus Kunth EXTRACT

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ANTIMICROBIAL ACTIVITY, PHYTOCHEMICAL ANALYSIS AND TOXICITY TEST OF *Cosmos caudatus* Kunth EXTRACT

By

NOR ASMA HUSNA MOHAMED YUSOFF

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctor of Philosophy

ANTIMICROBIAL ACTIVITY, PHYTOCHEMICAL ANALYSIS AND TOXICITY TEST OF *Cosmos caudatus* Kunth EXTRACT

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December 2015

Chairman : Associate Professor Yaya Rukayadi, PhD
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The uses of natural products in the elimination of microorganism contaminations in food have increased nowadays. In this study, air-dried leaf methanolic extract of *Cosmos caudatus* Kunth was used. The antimicrobial activity of *C. caudatus* extract and time-kill curve analysis was evaluated against foodborne pathogens. The stability of extract at different pH and temperature also were evaluated. The identification and quantification of bioactive compounds were conducted using LC-MS/MS and HPLC. Three representative microorganisms were observed under transmission electron microscope (TEM). The effect of *C. caudatus* extract on natural microflora in selected food materials and its sensory acceptability were tested at different concentrations of extract for different exposure time. The toxicity level of extract was assessed using *Artemia salina* spp. Results showed that all tested foodborne pathogens were susceptible to *C. caudatus* extract at different range of MIC value from 6.25 to 50.00 mg/ml. Time-kill curve study shows that all tested foodborne pathogens can be killed by the extract with different time-killing curve. Generally, the antimicrobial activities of *C. caudatus* extract were not significantly affected on tested pH and temperatures. LC-MS/MS analysis identified the presence of rutin, quercetin 3-*O*-glucoside, quercetin 3-*O*-arabinoside, quercetin 3-*O*-rhamnoside and quercetin. HPLC analysis showed the highest concentration was quercetin rhamnoside (29.66 mg/g). All the identified active compounds proved possess antimicrobial activities. TEM observation showed changing on the pathogen cells's structure. Generally the significant reduction of natural microflora in raw food materials were started at 0.05% of *C. caudatus* extract for 10 min of exposure time, and the sensory acceptability at this concentration and time were accepted by the panellists. The toxicity study demonstrated that *C. caudatus* extract was not toxic to *A. salina* (LC_{50} = 3.54 mg/ml). In conclusion, *C. caudatus* extract exhibits antimicrobial activities, thus can be developed as natural sanitizer for washing raw food materials.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

AKTIVITI ANTIMIKROB, ANALISIS FITOKIMIA DAN UJIAN TOKSISITI OLEH EKSTRAK *Cosmos caudatus* Kunth

Oleh

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Sekarang ini, penggunaan produk semula jadi dalam penghapusan pencemaran mikroorganisma dalam bahan makanan meningkat. Dalam kajian ini, sampel daun pengeringan-angin metanolik ekstrak *Cosmos caudatus* Kunth digunakan. Aktiviti antimikrob ekstrak *C. caudatus* dan analisis keluk masa-pembunuhan dinilai terhadap patogen bawaan makanan. Kestabilan ekstrak pada pH dan suhu yang berbeza juga dinilai. Mengenalpasti dan mengukur kandungan sebatian bioaktif dalam ekstrak dilakukan menggunakan LC-MS/MS dan HPLC. Tiga wakil mikroorganisma diperhatikan di bawah mikroskop elektron transmisi (TEM). Kesan ekstrak *C. caudatus* terhadap mikroflora semulajadi dalam bahan makanan dan penerimaan deria diuji pada kepekatan ekstrak berbeza untuk masa berbeza. Tahap ketoksikan ekstrak dinilai menggunakan *Artemia salina* spp. Keputusan menunjukkan semua patogen yang diuji dipengaruhi oleh ekstrak *C. caudatus* pada nilai MIC yang berbeza bermula dari 6.25 hingga 50.00 mg/ml. Kajian keluk masa-pembunuhan menunjukkan bahawa semua patogen bawaan makanan boleh dibunuh oleh estrak pada keluk masa-pembunuhan yang berlainan. Secara umumnya, aktiviti antimikrobial ekstrak *C. caudatus* terhadap semua patogen bawaan makanan yang diuji tidak terjejas dengan ketara oleh pH dan suhu yang diuji. Analisis LC-MS/MS menentukan kehadiran rutin, kuersetin 3-*O*-glukosida, kuersetin 3-*O*-arabinosida, kuersetin 3-*O*-ramnosida dan kuersetin. Analisis HPLC menunjukkan kepekatan tertinggi adalah kuersetin ramnosida (29.66 mg/g). Semua sebatian ini telah dibuktikan mempunyai aktiviti antimikrob. Pemerhatian TEM menunjukkan perubahan kepada struktur sel. Secara keseluruhannya, jumlah pengurangan mikroflora semulajadi dalam bahan makanan mentah yang dirawat adalah ketara bermula pada kepekatan ekstrak *C. caudatus* 0.05% selama 10 min masa pendedahan, dan penerimaan deria pada kepekatan dan pendedahan masa ini telah diterima oleh ahli panel. Kajian ketoksikan meunjukkan bahawa ekstrak *C. caudatus* tidak toksik kepada *A. salina* (LC_{50} = 3.54 mg/ml). Kesimpulannya, ekstrak *C. caudatus* mempamerkan aktiviti antimikrob, dengan itu boleh dimajukan sebagai pembersih semulajadi untuk mencuci bahan makanan mentah.

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I certify that a Thesis Examination Committee has met on 3 December 2015 to conduct the final examination of Nor Asma Husna bt Mohamed Yusoff on her thesis entitled "Antimicrobial Activity, Phytochemical Analysis and Toxicity Test of *Cosmos caudatus* Kunth Extract" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

amu	-	atomic mass unit
cfu/g	-	colony forming unit/gram
DCD	-	developmental coordination order
EDTA	-	ethylene diamine tetra acetic acid
FRAP	-	ferric reducing ability of plasma
HPLC	-	high performance liquid chromatography
H_2O_2	-	hydrogen peroxide
LC	-	lethality concentration
LC-MS	-	liquid chromatography - mass spectrometry
MAP	-	modified atmosphere packaging
Mg^{2+}	-	magnesium ion
MRSA	-	methicillin resistant *Staphylococcus aureus*
OH	-	hydroxyl radical
O_2	-	Oxygen
O_2^-	-	superoxide anion radicals
Ppm	-	parts per million
ROS	-	reactive oxygen species
USFDA	-	united state of food and drug administration
WHO	-	world health organization

CHAPTER 1

INTRODUCTION

1.1 General Introduction

An illness caused by the consumption of food contaminated with pathogens has a wide economic and public health impact worldwide (Gandhi & Chikindas, 2007). The spoiled foods include lipid oxidation, color changes, off-flavours and off-odours are mainly caused by the growth of microbes and excess of their metabolisms in the food products (Gram et al., 2002). In England and Wales, it has been estimated that there are around 1.3 million reported cases of foodborne illnesses which have caused up to 21,000 people got hospitalized and 500 deaths annually (Adak et al., 2002). Meanwhile, in Australia, about 5.4 million cases were reported with 15,000 people got hospitalized and 120 deaths annually (Soon et al., 2011). In the United States, the reported foodborne cases has shown an increase of approximately 48 million illnesses with 128,000 hospitalizations and 3,000 deaths each year (Scallan et al., 2011). The same trend has been shown in Malaysia where the reported cases of foodborne illnesses had increased from 17.76 cases in 2005 (MOH, 2006) to 47.79 per 100,000 populations in 2013 (MOH, 2014).

In general, foodborne illnesses occur are due to improper food handling and poor hygienic practices, especially during food preparation and storage period (Scott, 2003). Common examples of widely reported foodborne pathogens include *Bacillus cereus*, *Campylobacter*, *Clostridium*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* and *Staphylococcus aureus* (Singh et al., 2003). Food contamination can occur during post-harvest processing or even earlier which is during pre-harvesting period due to the contamination from a farm environment, water irrigation systems, manure and so on (Doyle & Erickson, 2006). Without proper decontamination techniques, pathogens will keep on growing especially during food storage period and during food preparation caused by cross contamination (Scott, 2003).

In fresh produce foods such as fruits and vegetables, washing with tap or chlorinated water is the common decontamination practice used. The washing treatment is to ensure the removal of soil and other plant debris to improve the appearance of the product (Delaquis et al., 2004) and reduce the microbial proliferation during subsequent storage (Simons & Sanguansri, 1997). As recommended by United States of Food and Drug Administration (USFDA) (1998), the total chlorine concentration allowed for the washing treatment was about 50 - 200 mg/l (pH 6.0 - 7.5) with contact time of 1 - 2 minutes. Chlorinated water is reported to have disinfectant effect on fresh produce (Delaquis et al., 2004; Baur et al., 2004). However, the practice of using chlorinated water is being questioned due to the formation of carcinogenic by-products (trihalomethanes) when chlorine reacts with organic matter such as decayed leaves (Chang et al., 2000). In addition, water treated with high chlorine residues will also produce some odour which might affect the food flavour and smell.

Moreover, food sanitization on meat and poultry products such as cattle, pork, beefs and chicken carcasses have also become a concern. Some bacteria associated with poultry products such as *B. cereus*, *Campylobacter*, *Clostridium*, *L. monocytogenes* and *Salmonella* have been widely reported to cause food spoilage (Hinton Jr et al., 2004). All these bacteria usually come from the farm or during slaughtering process (Solomon et al., 2002). Even though the bacteria's population is at a safe level earlier, they will rapidly multiply especially during storage period if no proper decontamination treatment is applied. Some people believe by chilling these poultry can stop and kill the foodborne pathogens attached, without realizing that the pathogens have a special ability to adjust their intracellular cells in a stress environment. As reported by Capita et al. (2002), even though these poultry foods are refrigerated or frozen prior to cooking, the survival of psychrotrophic pathogens such as *L. monocytogenes* is a food safety concern. A study reported by Al-Nehlawi et al. (2014) revealed the ability of *L. monocytogenes* to spoil a poultry sausage during refrigeration due to their ability to grow anaerobically during refrigeration, thus contaminating the food products.

There are many food decontamination treatments that have been applied to eliminate or substantially decrease bacterial populations both in fresh produce and raw poultries. A number of antimicrobial treatments have been studied including chlorine, organic acids, bacteriocins, hydrogen peroxide, ozonation, irradiation, UV light and many more. All these techniques were applied alone or sometimes through the combination called as hurdle technology. However, those treatments seemed to be ineffective since there were many drawbacks reported such as the deterioration in food quality, health risks, unattractive cost implication and consumer preference (Leistner, 2000). In addition, the use of advanced methods for food treatment may also cause loss of organoleptic properties of food which in turns reduce consumer acceptability (Negi, 2012). Others have also reported that by applying too much techniques will somehow affect the food quality in terms of their loss of nutrient contents, poor appearance and at the same time increase the cost of food processing (Fisher & Phillips, 2006). The application of hurdle technologies in food preservation also initiate food safety issues even though the technique is sometimes successful in controlling the growth of foodborne pathogens (Leistner, 2000). Moreover, reportedly that the long-term use of chlorine sanitizer may cause several carcinogenic effects (Neo et al., 2013).

Hence, an alternative method to eliminate and reduce microbial populations needs to be investigated. With a great consumer concern regarding on synthetic antimicrobials has lead the researchers and food processors to look for more on natural decontamination agents with a broad spectrum of antimicrobial activity (Marino et al., 2001). In this context, plant antimicrobials are gaining wide interest as most of them are classified as GRAS (Generally Recognized as Safe) status which has been discovered to have higher levels of food safety (Alzarokey & Nakahara, 2003). Plants or leafy green vegetables are widely consumed by people worldwide, either eaten fresh or cooked using various methods. These vegetables contain a large amount of phytochemicals such as phenolic acids, flavonoids and aromatic compounds and have been proven to possess a wide range of biological functions including as antioxidant and having antimicrobial activity (Tzortzakis & Economakis, 2007; Gutierrez et al., 2008; Moon et al., 2013).

Cosmos caudatus Kunth. are widely distributed in tropical countries and have long been used in folk medicines. Several bioactive compounds have been isolated from leaves of *C. caudatus* including flavonoids, phenols and alkaloids with reference to several biological properties such as antioxidant, antifungal, antibacterial and anticancer activities (Abas et al., 2006; Pebriana et al., 2008; Salehan et al., 2013). However, in terms of antimicrobial activities, only a few of antifungal compounds were isolated from roots of *C. caudatus* extract have been reported. One of them was hydroxyeugenol, which was proved to inhibit *C. albicans* (Fuzzati et al., 1995). Thus, study was undertaken to discover the antimicrobial activity of *Cosmos caudatus* Kunth. extract against several foodborne pathogens namely *Bacillus cereus, Bacillus subtilis, Candida albicans, Escherichia coli* O157:H7*, Klebsiella pneumoniae, Listeria monocytogenes, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella* Typhimurium and *Staphylococcus aureus.* This study is important to assess the ability of plant extract to decrease, inhibit or eliminate microbial activity which can provide basic knowledge on the development of *C. caudatus* extract as a natural food sanitizer. Moreover, this research was also believed to significantly contribute to the increase in application of other natural plant extracts as food sanitizer or perhaps, in combination with other food preservation methods to ensure the safety of preserved foods.

1.2 Objectives

Therefore, the objectives of this study are:

1. To determine the susceptibility of *Bacillus cereus, Bacillus subtilis, Escherichia coli* O157:H7*, Klebsiella pneumoniae, Listeria monocytogenes, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella* Typhimurium, *Staphylococcus aureus* and *Candida albicans* by *C. caudatus* extract in term of disc diffusion test, MIC, MBC/MFC and killing time curves.

2. To evaluate the stability of *C. caudatus* extract at different pH and temperature against *Bacillus cereus, Bacillus subtilis, Escherichia coli* O157:H7*, Klebsiella pneumoniae, Listeria monocytogenes, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella* Typhimurium, *Staphylococcus aureus* and *Candida albicans* in term of MIC and MBC/MFC.
3. To analyse the mode of actions of antimicrobial activity of *C. caudatus* extract on representative foodborne pathogens using light sheet electron microscope (LSEM) and transmission electron microscope (TEM).

4. To identify and quantify the bioactive compounds in *C. caudatus* extract which are responsible for antimicrobial activity using Liquid Chromatography Mass-Spectrometry (LC-MS/MS) and High Performance Liquid Chromatography (HPLC), respectively.

5. To analyse the effect of *C. caudatus* extract on foodborne pathogens in raw food materials including beef meat, chicken meat, shrimp and oyster mushroom.

6. To evaluate the sensory acceptability of raw food materials; beef meat, chicken meat, shrimp and oyster mushroom after treated with *C. caudatus* extract at different concentration and exposure time.

7. To evaluate the toxicity effect of *C. caudatus* extract on eukaryotic cells using brine shrimp (*Artemia salina* spp.) lethality assay.

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