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Cosmos caudatus Kunth. extract reduced number of microflora in oyster mushroom *(Pleurotus ostreatus)*

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Abstract

Nowadays consumer is more demand on natural foodstuff instead of synthetic product due to their concern on health. The objective of this study is to investigate the effect of *C. caudatus* extract on the number of microflora in oyster mushroom at different concentration of *C. caudatus* extract and exposure time using dilution method. The results showed that the number of microorganisms (Log_{10} CFU/g) in oyster mushroom in term of Total Plate Count (TPC), *Bacillus cereus, Escherichia coli* and *Staphylococcus aureus* were 6.13 ± 0.04, 6.15 ± 0.09, 5.97 ± 0.04, and 6.46 ± 0.00, respectively. The effect of *C. caudatus* extract on microflora in oyster mushroom at concentrations of 0.00%, 0.05%, 0.5%, and 5.0% with exposure time of 0, 5, 10, and 15 min demonstrated that the reduction number of microflora in oyster mushroom was dependent on the concentration of *C. caudatus* extract and exposure times. The number of TPC (Log_{10} CFU/g) in oyster mushroom was significantly reduced after treated with *C. caudatus* extract at concentration of 0.05% for 15 min; 6.13 ± 0.04 reduced to 2.62 ± 0.07. Moreover, *B. cereus* (Log_{10} CFU/g) in oyster mushroom was significantly reduced by treatment of *C. caudatus* extract at concentration of 0.05% for 5 min; 6.15 ± 0.09 reduced to 3.77 ± 0.15. Meanwhile, the number of *E. coli* (Log_{10} CFU/g) in oyster mushroom was significantly reduced at concentration of 0.05% for 10 min; 5.97 ± 0.04 reduced to 3.21 ± 0.13. Lastly, the survival number of *S. aureus* in oyster mushroom was significantly reduced after treated with *C. caudatus* extract at concentration of 0.05% for 15 min; 6.46 ± 0.00 reduced to 4.83 ± 0.07. In conclusion, *C. caudatus* extract has potentiality to be developed as natural sanitizer for rinsing raw food materials such as oyster mushroom.

Introduction

The growth of bacteria, yeast, and mould in food products results in food contaminants which consequently affects consumer with food illness. Several food preservation techniques such as heating, refrigeration and addition of chemical preservatives can reduce food contaminants in food. However these techniques frequently have associated adverse changes in organoleptic characterizations and loss of nutrients (Valero and Frances, 2006). Moreover, the use of chemical preservatives has been questioned by consumer due to their safety and risk in long time use. Consumer demands on fresh food, safe and free from any chemical–additives also become a concern (Naidu, 2000).

Recently, there are interests in the development of natural preservatives from plant extracts (Singh *et al*., 2010). Plant antimicrobials which contained in plant extracts fulfill the needs for consumer who are looking for wholesome food without chemical preservatives (Sultanbawa, 2011). Many researcher have reported that plant produce a wide variety of secondary metabolites such as vitamins, terpenoids, tannins, flavonoids, and other metabolites, which are responsible for antimicrobial and antioxidants activity (Patil *et al.*, 2013). Plant antimicrobials such as phenolic compounds; phenolic acids, simple quinones, flavonoids and tannins have been reported possesses an antioxidant and antimicrobial properties (Balasundram *et al*., 2006). Valero and Salmeron (2003) have reported the ability of cinnamon essential oils to inhibit the growth of *Bacillus cereus* for at least 60 days in carrot model media. Other researcher also reported the antibacterial activity of lettuce leaf to inhibit *Listeria* sp. and other spoilage bacteria in beef and milk model media (Gutierrez *et al*., 2009). Oregano and thyme extract have been investigated to have antibacterial effect on *Listeria* sp. and other spoilage microorganisms (Gutierrez *et al*., 2008). Besides, the essential oils of ginger revealed a high antibacterial potential against strains of *B. subtilis*

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and *Staphylococcus aureus* (Malu *et al.*, 2008).

Cosmos caudatus Kunth. belongs to the family of Asteraceae. It is an edible plant which having 20 – 26 species worldwide. In Malaysia, it is famously known as *Ulam raja* (King's salad) and usually eaten raw by Malay communities or as a side dish. It can grow about 1 to 8 feet tall in tropical area and starts flowering from June to November with purple, pink or white florets (Guanghou *et al*., 2005; Rasdi *et al.*, 2010). *C. caudatus* can be found worldwide in tropical areas including Mexico, United States, South America, Malaysia and Thailand (Salehan *et al*., 2013). It is a very good source of natural antioxidants, antimicrobial, anti-inflammatory, antifungal and rich in minerals such as calcium, phosphorus, iron, magnesium, and potassium (Shui *et al*., 2005). Various bioactive compounds have been identified in *C. caudatus* including quercetin 3-0-rhamnoside, quercetin 3-0-β-arabinofuranoside, quercetin3-0-β-glucoside, quercetin, proanthocyanidin, crypto-chlorogenic acid, neo-chlorogenic acid, chlorogenic acid, catechin, epicatechin, myricetin and naringenin (Mediani *et al.*, 2012). *C. caudatus* extract has been reported to have antimicrobial activity against 5 microbial strains including *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Candida albicans* (Rasdi *et al.*, 2010).

The family of Asteraceae has been well studied by several researchers possesses antimicrobial properties (Asghari *et al*., 2012). However, to best of our knowledge, no study has reported on the use of *C. caudatus* extract as a natural sanitizer to treat microbial pathogen in oyster mushroom. Hence, this study was conducted to determine the effects of *C. caudatus* extract on microbial population in oyster mushroom at different concentration of *C. caudatus* extract with different exposure time. This result might be very useful as basic information in applying *C. caudatus* extract as a natural food sanitizer.

Materials and Methods

Sample collection of C. caudatus *and oyster mushroom*

The *C. caudatus* sample was purchased from vendors at the Pasar Borong Selangor, Seri Kembangan, Malaysia. Leaves were removed from stalk and washed thoroughly under tap water and left to dry at room temperature (27°C) for 2-3 days until fully dried. Then, dried sample was grinded, kept in sealed plastic bag and stored in fridge (-80°C) until further used. While, the fresh oyster mushroom sample was purchased from Pusat Jualan Taman Pertanian Universiti (TPU), Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. Sample was kept in fridge while waiting for analysis.

Extraction of C. caudatus

The extraction of *C. caudatus* was conducted according to the method described by Rukayadi *et al*. (2008) with some modification. A 100 g of dried grinded *C. caudatus* was soaked in 500 ml of absolute methanol (99.8%)(Sigma-Aldrich, Saint Louis, MO, USA) for 2 days with occasionally shaken. The resulting slurry was vacuum-filtered through Whatman filter paper No. 2 (Whatman International Ltd, Middlesex, England) by aspirator pump attached with Buchner funnel and flask to get the liquid solution of the mixture. The liquid solution was further concentrated by using rotary evaporation (Heidolph VV2011, Schwabach, Germany) at 50°C with the speed of 150 rpm until the gummy-like extract formed. In order to obtain methanol free extracts, the temperature was increased up to 65°C by two times for 30 seconds during the concentration process. The crude extract was then stored at 4°C prior to use.

Preparation of C. caudatus *extract for treatment*

C. caudatus extract was diluted in 10% of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, MO, USA) and sterile deionized water (DIW) (B. Braun Medical Industries, Penang, Malaysia) to make three different concentrations of extract (0.05%, 0.50%, and 5.00%). A 5 g of *C. caudatus* extract was diluted in 50 ml of 10% DMSO resulting to 10% concentration of *C. caudatus* extract. Further dilution was made up by diluting 12.5 ml of 10% concentration with 12.5 ml of DIW to make into 5.00% of concentration. Next, 2.5 ml of 5.00% of concentration was diluted again with 22.5 ml of DIW resulting into 0.50% of *C. caudatus* extract. The final concentration was prepared by diluting 2.5 ml of 0.50% concentration diluted with 22.5 ml of DIW to make into 0.05% of *C. caudatus* extract. Each concentration solutions were prepared in total of 25 ml.

Preparation of selective media agar

Four types of selective media agar were used for the enumeration of bacteria in oyster mushroom; Plate count agar (PCA), Typtic soy agar (TSA), mannitol egg yolk polymyxin agar (MYP) and sorbitol Mac Conkey (SMAC). All of them were prepared as according to the label attached on each bottle. The importance of the selective media agar is to make a selection for the growth of desire microorganisms only and prevent others. This special characteristic

will make the detection of selected bacteria easier.

Treatment of oyster mushroom by C. caudatus *extract*

A 10 g of fresh oyster mushroom samples was undergoes the treatments in 25 ml of different concentration of *C. caudatus* extract (filter tap water, 0.00% (DIW), 0.05%, 0.50% and 5.00%) at different exposure time (0, 5, 10 and 15 min). Then, sample was homogenised in stomacher bag (BAGLIGHT, BagSysytem, Interscience, France) using stomacher machine (BagMixer 400-P, Interscience, France) for 2 min followed by a serial dilution; 1 ml from each treatment series was diluted into 9 ml of saline solution (0.85%) to create 10^{-1}, 10^{-2} and 10^{-3} dilution. A 25 µl from each dilutions series was spread on TPC agar, TSA agar, MYP agar, and SMAC agar and incubated at 37˚C for overnight. Data was analysed a day after by counting the presence of colonies on the agar. All analysis was performed in duplicate for data verification. The experiments were done two times with duplicates (n = 2 × 2).

Statistical analysis

The means value of microbial populations (Log_{10} CFU/g) from each treatment series was calculated from two times with duplicates (n = 2 × 2) on each experiment. MINITAB application software (version 16.0) was used to analyze the data for the variance (One-Way ANOVA). In addition, Turkey's test was used to determine the significant different ($P < 0.05$) between those treatments. Result was expressed as mean ± standard deviation (SD) of duplicate analyses.

Results and Discussion

Over the past 20 years, there has been a lot of interest in the investigation of natural materials as a source of new antimicrobial agents. Different extract from plants were tested and some natural products were approved as a new antimicrobial agent. Active constituents such as alkaloids, terpenoids and flavonoids usually give the antimicrobial properties to the plant (Daglia, 2012). In this experiment, *C. caudatus* extract was obtained by extracting with methanol solvent as reported by Rukayadi *et al.* (2008). Methanol was used as a soaking solvent since their ability to give consistent yield and suitable for extracting polyphenols compound in plant based material (Addai *et al.*, 2013). Mohamad *et al.* (2011) reported the effectiveness of methanol solvent to extract antimicrobial substances from plant materials compared to ethanol, hexane, and water. Dimethyl sulfoxide (DMSO) was used in the preparation of stock solutions to enhance the solubility of the crude extract when dissolved in solution. While, in the preparation of *C. caudatus* extract at different concentration, deionized water (DIW) was used in order to remove any ions which could be interfered the chemical reactivity and result analysis. Filtered tap water and 0.00% (deionized water, DIW) were performed as positive and negative control, respectively. Treatments of *C. caudatus* extract were done at different series of extract concentration (0.05%, 0.50% and 5.00%) with different exposure time (0, 5, 10 and 15 min).

Table 1. Number of microorganism isolated from oyster mushroom using different selective media agar (Log_{10} CFU/g)

TPC or Species	Number of microorganism (Log_{10} CFU/g)
TPC	6.13 ± 0.04
Bacillus cereus	6.15 ± 0.09
Escherichia coli	5.97 ± 0.04
Staphylococcus aureus	6.46 ± 0.00

TPC and selective media agar for *B. cereus, E. coli, Pseudomonas sp.* and *S. aureus* were used to enumerate the number of natural microflora in oyster mushroom. Results showed that the number of total plate count of *B. cereus, E. coli* and *S. aureus* were 6.13 ± 0.04, 6.15 ± 0.09, 5.97 ± 0.04 and 6.46 ± 0.00 (Log_{10} CFU/g), respectively. The presence of *B. cereus* and *S. aureus* in oyster mushroom also reported by Lim *et al.* (2008), while other bacteria perhaps due to the contamination from farm-land area.

Table 2 shows the number of total plate count after treated with a series of *C. caudatus* extract concentrations at different exposure time. The number of growing microorganisms was reduced slowly after treated with tap water and DIW but with no significance difference. The reduction was starting to reduce significantly after treated with *C. caudatus* extract at 0.05% for 5 min of exposure time (5.44 ± 0.02). The growth of microorganisms was reduced proportionally with the increasing of *C. caudatus* extract and exposure time. At the treatment of 5.00% concentrations, the growth number of total plate count was reduced almost half until 3.10 ± 0.07 Log_{10} CFU/g at similar exposure time; 5 min.

Table 3, Table 4 and Table 5 show the total number of surviving bacteria for *B. cereus, E. coli* and *S. aureus,* respectively. The growth of *B. cereus* was significantly reduced at concentration of 0.05% of *C. caudatus* extract after 5 min of exposure time (3.77 ± 0.15). The growth was kept decreased as the concentration and exposure time increased. The

Table 2. Treatment of oyster mushroom with *C. caudatus* extract at different concentration of extract and exposure time on TPC (Log_{10} CFU/g)

ET / [C]	Filtered tap water	0.00%	0.05%	0.50%	5.00%
0 min	6.13 ± 0.04[a]	5.99 ± 0.06[a]	5.56 ± 0.13[a]	4.97 ± 1.66[b]	3.65 ± 0.07[c]
5 min	6.12 ± 0.01[a]	5.92 ± 0.11[a]	5.44 ± 0.02[b]	5.23 ± 0.01[b]	3.10 ± 0.07[c]
10 min	6.10 ± 0.03[a]	6.00 ± 0.07[a]	4.85 ± 0.05[b]	3.31 ± 0.06[c]	2.86 ± 0.05[d]
15 min	5.45 ± 0.03[a]	5.42 ± 0.03[a]	2.62 ± 0.07[c]	2.16 ± 0.09[d]	1.49 ± 0.13[e]

[a, b, c, d, e] Showing there is significant different between results at $P < 0.05$ if the row do not sharing the same lowercase letter between each other.
Mean values ± standard deviation with same lowercase letter in the same row show have no significant different between untreated and treated sample. ($p > 0.05$)
[C]: Concentration of *C. caudatus* extract used in this experiment.
ET: Exposure time interval.

Table 3. Treatment of oyster mushroom with *C. caudatus* extract at different concentration of extract and exposure time on *B. cereus* (Log_{10} CFU/g)

ET / [C]	Filtered tap water	0.00%	0.05%	0.50%	5.00%
0 min	6.15 ± 0.09[a]	5.84 ± 0.01[a]	4.94 ± 1.54[a]	3.58 ± 0.05[b]	3.44 ± 0.04[b]
5 min	5.89 ± 0.01[a]	5.46 ± 0.11[a]	3.77 ± 0.15[b]	2.64 ± 1.40[b]	1.65 ± 0.04[c]
10 min	5.71 ± 0.08[a]	5.64 ± 0.07[a]	1.77 ± 0.04[c]	1.41 ± 0.04[c]	0.00 ± 0.00[d]
15 min	5.91 ± 0.05[a]	5.87 ± 0.04[a]	1.61 ± 0.02[c]	0.00 ± 0.00[d]	0.00 ± 0.00[d]

[a, b, c, d, e] Showing there is significant different between results at $P < 0.05$ if the row do not sharing the same lowercase letter between each other.
Mean values ± standard deviation with same lowercase letter in the same row show have no significant different between untreated and treated sample. ($P > 0.05$)
[C]: Concentration of *C. caudatus* extract used in this experiment.
ET: Exposure time.

growth number of *B. cereus* started to reach 0.00 ± 0.00 (no colony growth) at concentration 0.50% during 15 min of exposure time.

Table 4 shows the total surviving number of *E. coli.* Initially, the detected number of *E. coli* was 5.97 ± 0.04 (Log_{10} CFU/g). The reduction number of *E. coli* become significant starting at concentration 0.05% where the value was reduce to 5.00 ± 0.17 during 0 min of exposure time and continually reduce until 2.90 ± 0.03 during 15 min of exposure time. At treatment 5.00% of *C. caudatus* extract shows the highest reduction number of *E. coli* where the value was reduced until 1.72 ± 0.04 and 0.00 ± 0.00 (Log_{10} CFU/g) for 0 min and 15 min exposure time, respectively.

Lastly, the surviving number of *S. aureus* was tabulated in Table 5. Results show that the number of surviving *S. aureus* was started to reduce significantly after treated with 0.05% of *C. caudatus* extract for 0 min of exposure time (6.05 ± 0.02). The growth was continuously reduced until 15 min of exposure time (4.83 ± 0.07). The reduction number was increased as the concentration of *C. caudatus* extract and the exposure time increased. The highest reduction was detected at 5.00% of *C. caudatus* extract after treated for 15 min of exposure time (2.78 ± 0.03).

These findings show that, TPC, *B. cereus, E. coli* and *S. aureus* were susceptibility to *C. caudatus* extracts. The decreasing of microflora growth in oyster mushroom samples was inversely proportional with the increasing of *C. caudatus* extract and the exposure time. The reduction was probably caused by the antimicrobial compounds present in *C. caudatus* extract especially at treatment with high concentrations. As reported by Weerakkody *et al.* (2010), bioactive compounds such as flavonoids, tannins, alkaloids, terpenoids and saponins possess an antimicrobial activity. Vital and Rivera (2009) have proved the antimicrobial activity of flavonoids and tannins. This antimicrobial activity could be

Table 4. Treatment of oyster mushroom with *C. caudatus* extract at different concentration of extract and exposure time on *E. coli* (Log_{10} CFU/g)

ET / [C]	Filtered tap water	0.00%	0.05%	0.50%	5.00%
0 min	5.97 ± 0.04[a]	5.70 ± 0.02[a]	5.00 ± 0.17[b]	3.11 ± 0.02[c]	1.72 ± 0.04[d]
5 min	5.59 ± 0.17[a]	5.42 ± 0.16[a]	4.79 ± 0.03[b]	2.41 ± 1.13[c]	1.61 ± 0.01[d]
10 min	5.58 ± 0.00[a]	5.31 ± 0.04[a]	3.21 ± 0.13[c]	1.87 ± 0.47[c]	1.34 ± 0.02[d]
15 min	5.43 ± 0.07[a]	5.38 ± 0.04[a]	2.90 ± 0.03[c]	1.45 ± 0.02[d]	0.00 ± 0.00[e]

[a, b, c, d] Showing there is significant different between results at $P < 0.05$ if the row do not sharing the same lowercase letter between each other.
Mean values ± standard deviation with same lowercase letter in the same row show have no significant different between untreated and treated sample. ($P > 0.05$)
[C]: Concentration of *C.caudatus* extract used in this experiment.
ET: Exposure time.

Table 5. Treatment of oyster mushroom with *C. caudatus* extract at different concentration of extract and exposure time on *S. aureus* (Log_{10} CFU/g)

ET / [C]	Filtered tap water	0.00%	0.05%	0.50%	5.00%
0 min	6.46 ± 0.00[a]	6.36 ± 0.01[a]	6.05 ± 0.02[b]	5.46 ± 0.01[c]	4.14 ± 0.05[c]
5 min	6.16 ± 0.15[a]	5.94 ± 0.09[a]	5.91 ± 0.01[a]	5.30 ± 0.03[b]	3.44 ± 0.05[c]
10 min	5.99 ± 0.18[a]	5.91 ± 0.02[a]	5.56 ± 0.02[b]	5.27 ± 0.03[b]	3.30 ± 0.06[c]
15 min	5.59 ± 0.02[a]	5.33 ±0.04[a]	4.83 ± 0.07[b]	3.85 ± 0.03[c]	2.78 ± 0.03[d]

[a, b, c, d, e] Showing there is significant different between results at $P < 0.05$ if the row do not sharing the same lowercase letter between each other.
Mean values ± standard deviation with same lowercase letter in the same row show have no significant different between untreated and treated sample. ($P > 0.05$)
[C]: Concentration of *C. caudatus* extract used in this experiment.
ET: Exposure time.

due the disruption of cell membrane through the partitioned of protein, lipid or carbohydrates which in turns inhibit or kill the microorganisms (Wariska *et al.*, 2014). Cushnie and Lamb (2005) stated that there are some mechanisms occurred during the inhibition or killing of microorganisms such as by the inhibition of nucleic acid synthesis, cytoplasmic membrane functions and energy metabolisms.

Most of the results show that the higher concentration of *C. caudatus* extract proved better activity than the lower. However, the application of plants extract at higher concentration is not recommended even though the reduction number is high. As advised by Gutierrez *et al*. (2009), the higher plant extract used in the application in food system will result in poor appearance and sometimes with smelly effect caused by some of the bioactive compounds. Furthermore, the use of plant extract in low concentration also can reduce their possibility of toxic happened.

In conclusion, the lowest concentration of *C. caudatus* extract with the significant reduction of pathogen was at 0.05% of *C. caudatus* extract for 5 min of exposure time is more recommended as it likely gives the more good effect towards human admissibility. Results explicitly express that leaves of *C. caudatus* extract have potential antimicrobial property against *B. cereus, E. coli* and *S. aureus.* Studies show the potentiality of *C. caudatus* extract to be developed as a natural sanitizer in rinsing oyster mushroom.

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