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FULL PAPER

Antimicrobial stability of *Cosmos caudatus* extract at varies pH and temperature, and compounds identification for application as food sanitiser

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Abstract

The occurrence of foodborne diseases and food poisoning due to the consumption of contaminated foods is increasing nowadays, thus become a major threat to food industries in particular. In order to overcome this problem, prevention must be taken at the early stages of food preparation like sanitization. Typically, chemically based antimicrobial sanitisers were used in food industries to remove dirt and microbial population on food surfaces or food equipment. However, the emergence of microbial resistance and consumer awareness on the formation of carcinogenic compounds and safety issues in long term effects has led researchers to find an alternative. Therefore, a study was conducted to find a natural food sanitiser that was able to minimize the number of harmful bacteria without a change in the food quality and safety. In this study, the stability of *Cosmos caudatus* extract at different pH (pH 3, pH 7, pH 8 and pH 11) and temperatures (25°C, 30°C, 50°C, 80°C and 121°C) were determined for suitability as food sanitiser. The identification and quantification of this plant extract also were performed using HPLC and LC-MS/MS analysis to detect the major compounds which contributed to the biological activity of *C. caudatus* extract. Generally, results showed that the antimicrobial activity of *C. caudatus* extract was stable after exposure to various pH and temperatures, in fact, the extract increased its antimicrobial activity at lower acidity (pH 3) and higher temperature (50°C) against most pathogens. Furthermore, quercetin 3-*O*-rhamnoside was identified as a major compound in *C. caudatus* extract with the relative amounts of 29.66 mg/g. It can be concluded that *C. caudatus* extract is stable when exposed to various pH and temperatures. These useful findings have proved the antimicrobial stability of *C. caudatus* extract after exposure to several pH and temperatures thus can be further developed as a food sanitiser in food industries.

1. Introduction

The problem of foodborne pathogens becomes a threat to both developed and developing countries worldwide. The major factors of food related outbreaks are caused by the unhygienic handling of food and lack of knowledge during food preparations and storage (Janjić *et al.,* 2016). Common foodborne pathogens include *Salmonella* spp., *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* and many more (Nguyen *et al.,* 2016). A study by Nerín *et al.* (2016) concurred that the bacteria contaminations and off-odours usually occurred during the storage period where food is stored in their raw form and are readily contaminated with pathogens originated from their grown environment (Rather *et al.,* 2017). Furthermore, once the attached bacteria have found their favourable growth conditions, they will multiply and deteriorate the food and cause foodborne illness to the consumer (Scallan *et al.,* 2011). Although most of the attached bacteria can be killed during the cooking process, some such as *B. cereus* are heat resistant and are capable to form spores in extreme conditions (Kramer and Gilbert, 1989). Hence, decontamination methods such as food sanitization are crucial to prevent the growth of harmful

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bacteria into the subsequent phase of food processing stages.

Food sanitization is one of the methods used in controlling and decontaminating the attached bacteria on food sources primarily after the post-harvesting period. Currently, the use of electrolyzed water containing low levels of chlorine (2-6 mg/L) was claimed to be able to reduce bacterial populations on food (Ding *et al.,* 2011). A combination of heating with acidic and alkaline electrolyzed water was also reported to reduce the bacterial load (Liu *et al.,* 2017; Xuan *et al.,* 2017). Other chemically-based sanitizations including sodium hypochlorite, organic acids, electrolyzed water and ozonation were reported to promote the microbial reduction in various food sources (Ding *et al.,* 2011; Upadhyay *et al.,* 2013; Botta *et al.,* 2018; Islam *et al.,* 2018; Liu *et al.,* 2019). Even though all these methods proved to be able to reduce microbial populations in fresh or raw food sources, most of them imparted some drawbacks such as health risks, processing costs, product stability, and customer preferences (Chen and Hung, 2017). Moreover, the safety of chlorine used in food or water treatment has been questioned due to its ability to form carcinogenic by-products once they react with organic matter such as decayed leaves (Fan and Sokorai, 2015). With all these obstacles, studies on natural food sanitisers particularly from plant-based materials become a demand. For instance, green tea at 10 mg/L had been reported to possess antiviral activity when applied as a sanitiser on *Cosmos caudatus* Kunth. or known as '*Ulam'* raja among the Malay community, widely found in several tropical areas including Malaysia, Thailand, Indonesia, South America, Mexico and United States (Arizona and Florida) (Bunawan *et al.,* 2014). This plant is classified under the family of Asteraceae, under the genus of *Cosmos* and species of *Cosmos caudatus* Kunth. Generally, *C. caudatus* grows up to 8 feet tall with an annual to short-lived perennial life cycle (Bunawan *et al.,* 2014). This *Cosmos caudatus* species is an edible plant and is traditionally consumed as a salad or used as a food flavouring agent (Perumal *et al.,* 2014). They are believed to have several medicinal values such as decreasing blood pressure, improve blood circulation and as an anti-ageing agent (Shui *et al.,* 2005; Abas *et al.,* 2006; Rasdi *et al.,* 2010; Mediani *et al.,* 2012). Besides that, *C. caudatus* plant has also been reported to possess several biological activities due to their beneficial active compounds which are mostly derived from the flavonoid group (Mustafa *et al.,* 2010; Rasdi *et al.,* 2010; Ajaykumar *et al.,* 2012; Mediani *et al.,* 2012). Due to all the beneficial values belonging to the *C. caudatus* plant, it was further selected for its potential to be developed as a food sanitiser. As food sanitisers are required to maintain their effectiveness in various food environments, their stability on various pH and temperatures were investigated. Moreover, identification and quantification of *C. caudatus* extract were also performed to determine the major compound found in its extract which influences its biological activity.

2. Materials and methods

2.1 Plant sample collection and extraction

Fresh samples of *C. caudatus* plant was purchased from Pasar Borong Selangor, Seri Kembangan, Selangor, Malaysia, and sent for taxonomic identification in the Institute of Bioscience, Universiti Putra Malaysia (UPM) under the specimen number of SK 2668/15. Only the leaves were used in this study, they were dried under room temperature (27°C), ground into fine powder and soaked into methanol solvent (99.8%) for 2 days before evaporated with a rotary evaporator at 50°C until a gummy-like extract is formed. The extraction method was performed as described by Rukayadi *et al.* (2008), with slight modifications. Then the crude extract was collected and stored in -20°C for further analysis.

2.2 Stability of Cosmos caudatus extract on different pH and temperatures

The stability of *C. caudatus* extract at different pH and temperature were determined as according to Durairaj *et al.* (2009). Briefly, the stability of *C. caudatus* extract on pHs was determined by adjusting the pH of extract at pH 3, pH 7, pH 8 and pH 11 using 0.1 M hydrochloric acid (HCl, Sigma Aldrich, United States) or 0.1 M sodium hydroxide (NaOH, Sigma Aldrich, United States). Meanwhile, for its stability of temperature, the extract was exposed to several temperatures starting from 24°C, 30°C, 50°C, 80°C and 121°C for 15 mins each. Then, their antimicrobial activities (MICs and MBCs) were determined right after the treatment. Untreated extract at pH 8 at room temperature (25°C) was served as a control.

2.3 Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) test

A total of 10 species of foodborne pathogens tested in this study includes *Bacillus cereus* (ATCC 33019), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* O157:H7 (ATCC 43895), *Klebsiella pneumoniae* (ATCC 13773), *Listeria monocytogenes* (ATCC 19112), *Pseudomonas aeruginosa* (ATCC 9027), *Proteus mirabilis* (ATCC 21100), *Salmonella* Typhimurium (ATCC 14028), *Staphylococcus aureus* (ATCC 29737) and *Candida albicans* (ATCC 10231). The MIC of *C. caudatus* extract against foodborne pathogens was

determined by broth microdilution techniques (CLSI, 2012). The test was performed by using the round bottom 96-microtiter plate. Briefly, 200 µL of 10^5-10^8 CFU/mL bacteria/yeast inoculum were filled in the 2nd column, whilst 200 µL of MHB/SDB were filled in the 1st column. The inoculum was served as positive control while MHA/SDB was served as a negative control. Then, a 2-fold dilution technique was performed starting from 100 mg/mL, which is its highest concentration at column 12 (50 mg/mL) and the lowest at column 3 (0.1 mg/mL). Plates were incubated at 37°C for 12-24 hrs for bacteria, whilst yeast was extended for 24-48 hrs. The MIC value was determined by observing the well with complete inhibition growth (Rukayadi *et al.,* 2013). The MBC test was determined by subculturing 10 µL from each of the suspension in microtiter plates onto the MHA/SDA agar and incubated overnight at 37°C for bacteria and an extended 24-48 hrs for yeast. The MBC value was observed by determining no visible growth after incubation at the lowest concentration (Andrews, 2001).

2.4 Identification of active compound in Cosmos caudatus extract using LC-MS/MS analysis

Analysis was carried out using Dionex Ultimate 3000 Rapid Seperation LC system (ThermoFisher ScientificTM, San Jose, CA) linked simultaneously with Q Exactive Plus HRAM LC-MS/MS (ThermoFisher ScientificTM, San Jose, CA) equipped with both positive and negative ionization mode (ESI). Instrument control, data acquisition and data processing were performed using Xcalibur software version 1.2 (Thermo Finnigan, San Jose, CA, USA). Analyte separation was performed using Hypersil GOLD aQ column (100 × 2.1 mm ID) up to 30°C and the gradient program was tabulated as in Table 1.

Table 1. LC-MS/MS gradient elution for compound identification in *C. caudatus* extract

Time (min)	Solvent A: Deionized water with 0.1% formic acid	Solvent B: Acetonitrile with 0.1% formic acid	Flow rate (µL/min)
0.00	90	10	250
5.50	80	20	250
10.50	50	50	250
15.50	30	70	250
18.00	0	100	250
20.00	0	100	250
21.00	50	50	250
22.00	90	10	250
25.00	90	10	250

2.5 Quantification of quercetin compound in Cosmos caudatus extract using HPLC analysis

Quantification of the flavonoid content in *C. caudatus* extract was determined by using HPLC (Agilent, 1100 Series) as according to the method described by Mediani *et al.* (2012). The ZORBAX Eclipse Plus C18 (4.6 × 250 mm, 5µm) column (Agilent, USA) was used, utilizing deionized water with 2% acetic acid (solvent A-mobile phase) and pure acetonitrile (solvent B). The solvent gradient elution used as depicted in Table 2, the UV detection was at 360 nm and the flow rate was maintained at 1.0 mL/min. The standards used are rutin, quercetin and quercetin rhamnoside which were previously isolated or purchased from Sigma Chemicals (St. Louis, USA).

Table 2. HPLC gradient elution for determination of compound in *C. caudatus* extract

Time (min)	Solvent A: Deionized water with 2% acetic acid	Solvent B: Acetonitrile (100%)	Flow rate (µL/min)
0	90	10	1.0
5	90	10	1.0
10	40	60	1.0
15	10	90	1.0
20	90	10	1.0

Both samples and standards were prepared prior to use. A mass of 2 mg of *C. caudatus* extract was dissolved in 2 mL pure methanol, filtered using 0.2 µm nylon membrane into a 2 mL vial and 20 µL from them was injected into the HPLC system by using 1 mL injection loop. Detection of the phenolic peaks was performed at 360 nm wavelength and the same procedure was prepared for standards detection peak; whilst for concentrations of different peaks, 5 different concentrations of each standard were injected into the HPLC system and the standard curves were plotted.

2.6 Statistical analysis

All antimicrobial tests were conducted three times with two replications each and results were interpreted as means ± standard deviation (SD) of the replicate analysis.

3. Results and discussion

In the present study, five peaks of compounds were identified in *C. caudatus* extract which includes rutin, quercetin 3-*O*-glucoside, quercetin 3-*O*-arabinoside, quercetin 3-*O*-rhamnoside and quercetin aglycon (Table 3). All these identifications were performed based on their mass measurement of the molecular ion $[M-H]^-$ and

Table 3. List of the identified compounds in *C. caudatus* extract using LC-MS/MS

Peak	RT	$[M-H]^-$	MS/MS	Compounds
1	7.18	609	463, 300, 179, 151	Rutin
2	7.47	463	300, 179, 151	Quercetin 3-*O-glucoside*
3	8.26	433	300, 179, 151	Quercetin 3-*O-arabinoside*
4	8.47	447	300, 179, 151	Quercetin 3-*O-rhamnoside*
5	10.03	301	151	Quercetin

RT: retention time, [M-H]: molecular ion peak (deprotonated ion)

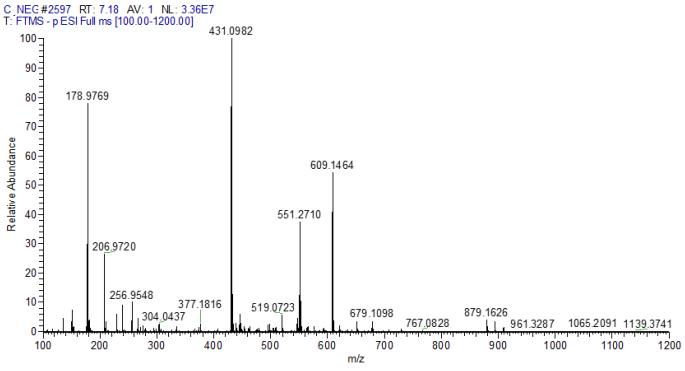

Figure 1. Rutin fragmentation ion

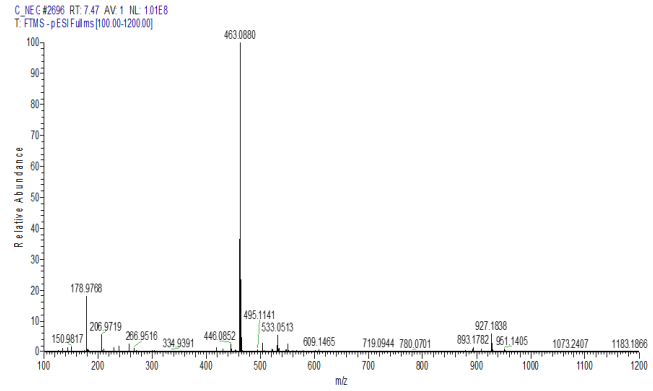

Figure 2. Quercetin 3-O-glucoside fragmentation ion

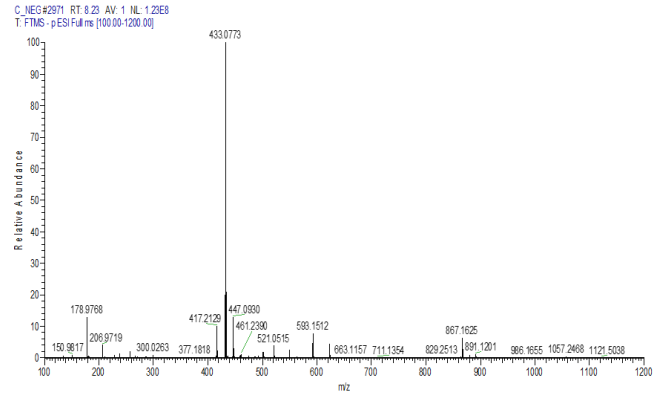

Figure 3. Quercetin 3-O-arabinoside fragmentation ion

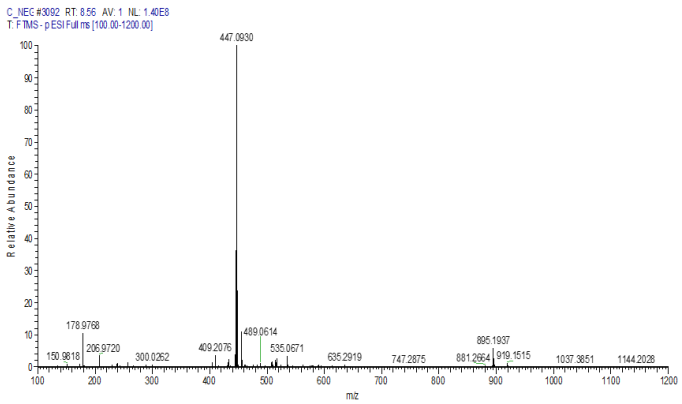

Figure 4. Quercetin 3-O-rhamnoside fragmentation ion

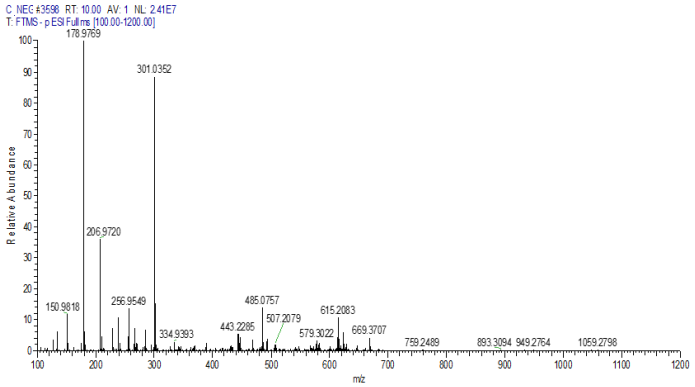

Figure 5. Quercetin fragmentation ion

their fragmentation ions obtained (Figure 1 to Figure 5). Identification of each compound was compared with previous studies (Shui *et al.,* 2005; Abas *et al.,* 2006; Mediani *et al.,* 2012; Rukayadi *et al.,* 2013) for confirmation. All these compounds were reported to previously possess antimicrobial activity on various microbial strains (Nitiema *et al.,* 2012; Pimentel *et al.,* 2013). The hydroxyl group and phenolic ring in the flavonoid structure reported being able to disrupt membrane structure by complexing with protein and microbial cell wall, thus leading to cell death (Zongo *et al.,* 2011).

The quantification of quercetin rhamnoside, rutin and quercetin in *C. caudatus* extract was performed by spiking and comparing the retention time of the spiked compound with the unspiked *C. caudatus* extract chromatogram. All these three compounds were selected for quantification based on their possibility to exhibit high antimicrobial activity in other plants (Betts *et al.,* 2015; Vanaraj *et al.,* 2017; Jesus *et al.,* 2018; Silva *et al.,* 2018). Results showed that the relative amounts of quercetin rhamnoside, rutin and quercetin were 29.66 mg/g, 1.85 mg/g and 0.95 mg/g, respectively. The value of $R^2 = 0.9996$ obtained from the regression line in the flavonoid standard curve represents a good linear relationship within the test range (Figure 6). Mediani *et al.* (2012) had reported the amount of quercetin in *C. caudatus* extract which was about 1.16 mg/g. The amount of these metabolites differs among plants due to several environmental changes (Shuib *et al.,* 2011). In fact, Tajkarimi *et al.* (2010) reported that each metabolite amounts in plants highly affect their biological activities.

It is known that the stability of phytochemical

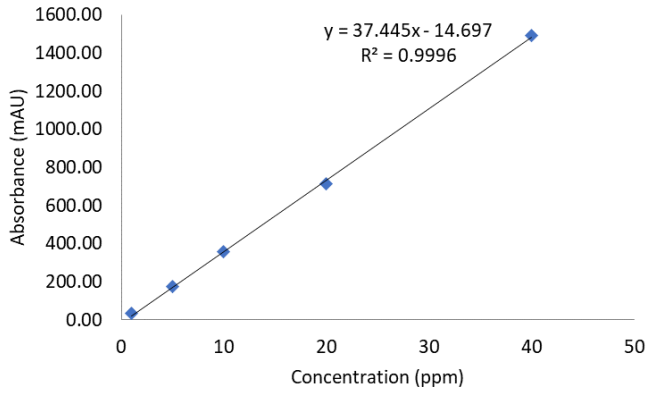

Figure 6. Standard curve of the flavonoids in *C. caudatus* extract

compounds in plant extract is highly influenced by the processing temperature, pH and storage condition (Negi, 2012). Various studies on the stability of plant extract had been previously reported (Deri and Rajkumar, 2013; Anees *et al.,* 2015; Anees *et al.,* 2015; Ramli *et al.,* 2020). In this present study, the stability of *C. caudatus* extracts at various pH and temperature were evaluated as depicted in Tables 4 and 5. The original pH of *C. caudatus* extract was pH 8. After changes in various pH conditions, the antimicrobial activity of the extract was found to be the most stable in acidic conditions. However, with no statistical difference with the original pH (pH 8). In general, the MIC and MBC values of *C. caudatus* extract improved when in acidic pH (pH 3) against most of the tested pathogens. Results were supported by Molan (1992) who stated that the plant phytochemical compounds expressed their phytochemical properties utmost in acidic condition. Gunduz (2013) had reported the reduction of numbers of *Salmonella* spp. after being treated at pH 3.3 - 3.4. Similar results were shown by Anees *et al. (*2015) when the garlic and *Zingiber officinale* extract improved their antimicrobial activities in acidic conditions, respectively. In contrast, the MIC and MBC of *C. caudatus* against *S. aureus* were optimum in alkaline condition (pH 8). These results were in line with Srinivasan *et al.* (2009) who also showed the optimum antimicrobial activity *of Allium sativum* in alkaline condition. In addition, Ramli *et al.* (2020) also reported the stability of *Syzgium polyanthum* to treat pathogenic bacteria in alkaline condition. Although the antimicrobial activity of *C. caudatus* was the best when in acidic condition compared to their original pH, however, their MIC and MBC activities were still sustained in the range of 6.25-12.5 mg/mL in most of the treated pathogens which indicate the stability of this *C. caudatus* extract after being exposed to various pH ranges.

The stability of *C. caudatus* extract at different temperature was tabulated in Table 5. Generally, the antimicrobial activity of *C. caudatus* extract was similar in most of the treated pathogens before and after exposure to various temperatures, particularly on *E. coli*

Table 4. Stability of *C. caudatus* extract at different pH on the MIC and MBC of pathogens

Bacteria / *Candida spp.*		pH 3	pH 7	pH 8[a]	pH 11
B. cereus	MIC (mg/mL)	6.25	6.25	6.25	6.25
(ATCC 33019)	MBC (mg/mL)	50.00	50.00	50.00	50.00
B. subtilis	MIC (mg/mL)	6.25	6.25	12.50	12.50
(ATCC 6633)	MBC (mg/mL)	25.00	25.00	25.00	50.00
E. coli O157:H7	MIC (mg/mL)	6.25	12.50	12.50	12.50
(ATCC 43895)	MBC (mg/mL)	6.25	12.50	12.50	25.00
K. pneumoniae	MIC (mg/mL)	12.50	25.00	12.50	12.50
(ATCC 13773)	MBC (mg/mL)	25.00	50.00	50.00	50.00
L. monocytogenes	MIC (mg/mL)	6.25	12.50	12.50	25.00
(ATCC 19112)	MBC (mg/mL)	12.50	50.00	50.00	50.00
P. mirabilis	MIC (mg/mL)	6.25	6.25	6.25	12.50
(ATCC 21100)	MBC (mg/mL)	6.25	12.50	50.00	50.00
P. aeruginosa	MIC (mg/mL)	12.50	12.50	12.50	12.50
(ATCC 9027)	MBC (mg/mL)	12.50	25.00	50.00	50.00
S. enterica ser. Typhimurium	MIC (mg/mL)	6.25	6.25	12.50	12.50
(ATCC 14028)	MBC (mg/mL)	6.25	6.25	50.00	50.00
S. aureus	MIC (mg/mL)	12.50	6.25	6.25	6.25
(ATCC 29737)	MBC (mg/mL)	50.00	12.50	12.50	50.00
C. albicans	MIC (mg/mL)	12.50	12.50	12.50	25.00
(ATCC 10231)	MBC (mg/mL)	25.00	25.00	12.50	50.00

[a]: control

Table 5. Stability of *C. caudatus* extract at the different temperature on the MIC and MBC of pathogens

Bacteria / *Candida spp.*		24°C[a]	30°C	50°C	80°C	121°C
B. cereus	MIC (mg/mL)	6.25	6.25	3.13	12.50	12.50
(ATCC 33019)	MBC (mg/mL)	25.00	25.00	25.00	50.00	50.00
B. subtilis	MIC (mg/mL)	12.50	12.50	6.25	12.50	12.50
(ATCC 6633)	MBC (mg/mL)	25.00	25.00	25.00	50.00	50.00
E. coli O157:H7	MIC (mg/mL)	12.50	12.50	12.50	12.50	12.50
(ATCC 43895)	MBC (mg/mL)	12.50	25.00	25.00	25.00	50.00
K. pneumoniae	MIC (mg/mL)	12.50	12.50	12.50	12.50	12.50
(ATCC 13773)	MBC (mg/mL)	25.00	25.00	25.00	50.00	50.00
L. monocytogenes	MIC (mg/mL)	12.50	12.50	12.50	25.00	25.00
(ATCC 19112)	MBC (mg/mL)	25.00	25.00	50.00	50.00	50.00
P. mirabilis	MIC (mg/mL)	6.25	12.50	12.50	12.50	12.50
(ATCC 21100)	MBC (mg/mL)	25.00	25.00	25.00	25.00	50.00
P. aeruginosa	MIC (mg/mL)	12.50	6.25	6.25	12.50	12.50
(ATCC 9027)	MBC (mg/mL)	25.00	12.50	12.50	12.50	25.00
S. enterica ser. Typhimurium	MIC (mg/mL)	12.50	12.50	12.50	12.50	25.00
(ATCC 14028)	MBC (mg/mL)	50.00	25.00	25.00	25.00	50.00
S. aureus	MIC (mg/mL)	6.25	6.25	12.50	12.50	12.50
(ATCC 29737)	MBC (mg/mL)	12.50	25.00	25.00	25.00	50.00
C. albicans	MIC (mg/mL)	12.50	12.50	12.50	12.50	25.00
(ATCC 10231)	MBC (mg/mL)	12.50	25.00	25.00	50.00	50.00

[a]: control

O157:H7 and *K. pneumoniae* with their similar MIC value (12.5 mg/mL). Surprisingly, the antibacterial activity of *C. caudatus* extract was improved at higher temperature (50°C) as compared to a lower temperature (23°C) against *B. cereus* and *B. subtilis*; where the value of MIC had decreased from 6.25 mg/mL to 3.125 mg/mL and 12.5 mg/mL to 6.25 mg/mL, respectively. A similar finding was observed in *P. aeruginosa* where the antibacterial activity was improved at temperatures of 30°C and 50°C with the MIC value ranging from 12.5 mg/mL to 6.25 mg/mL. A similar finding was reported by Ahmed *et al.* (2015) who found the stability of garlic extract when treated at a higher temperature. However, the finding was found to be contradicted with Arabshahi-D *et al.* (2007) who stated the decrease in the biological activity of *Moringa oleifera* extract after exposure to heat processing system. In fact, the stability of plant extract differs between plant species as their active compounds might be different, thus having different target sites on the microorganisms' cell membrane (Mikusanti *et al.,* 2009).

4. Conclusion

The identification and quantification analysis proved the presence of quercetin 3-*O*-rhamnoside as a major compound in *C. caudatus* extract in the amounts of 29.66 mg/g, which might contribute to the antimicrobial activity of the plant extract. In addition, the antimicrobial activity of *C. caudatus* extract was depicted to be stable when exposed to various pH and temperature. Based on these findings, it can be concluded that *C. caudatus* extract could potentially be developed as a natural food sanitiser, thus can be an alternative for the replacement of chemically used food sanitiser in food industries which might have an adverse effect in the long-time use. However, further study on the real food systems might be an advantage to confirm their extract stability. Nevertheless, the mode of actions of these activities with regards to each of the isolated active compounds can be further investigated in order to determine the specific actions for each of the single compound in the *C. caudatus* extract.

Conflict of interest

The authors declared no conflict of interest present in this study.

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