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## ***In vitro* antimicrobial properties of different solvent extracts from carissa fruts *Carissa carandas* L.**

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**Abstract** *In vitro* antimicrobial activities of various solvent systems (0%, 25%, 50%, 75% and 100% ethanol in water) of carissa fruit extracts (*Carissa carandas*) was reported. Agar well diffusion, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and killing time were determined. The results found that absolute ethanol and 75% ethanol extracts inhibited all 11 strains of both pathogenic and spoilage bacteria at the concentration of 100 mg/ml. The absolute ethanol extract was selected as an optimal extraction solvent for the following study showed the strongest antimicrobial activity. The MIC values of absolute ethanol extract to inhibit *Pseudomonas fluorescens* TISTR 358, *Staphylococcus aureus* TISTR 118, *Salmonella* Typhimurium TISTR 292, *Escherichia coli* TISTR 780 were 25, 25, 50, and 50 mg/ml, whereas the MBC values to inhibit these bacteria were 100, 25, 50, and 100 mg/ml, respectively. Moreover, *S. aureus* TISTR 118 and *S. Typhimurium* TISTR 292 were completely killed at 20 and 6 minutes exposure time by absolute ethanol extract from *C. carandas* fruits at the concentration of 25 mg/ml and 50 mg/ml, respectively.

**Keywords:** *Carissa carandas* fruit extract, antimicrobial

### **Introduction**

Synthetic antimicrobial compounds have been used directly in human life such as surfactants, cosmetics, medicines, especially food additives. (Huang *et al.*, 2011). However, they are strictly regulated due to toxicological concerns and some health problems (Wilson and Bahna, 2005). A variety of bacteria also lead the spoilage and illness that is encountered as one of the most important matter concerning the industry. Besides, numerous antimicrobial chemicals or drugs used to treat many infectious diseases has inevitably led multiple resistance in both human, food and plant pathogenic bacteria (Sokmen *et al.*,

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2004). Therefore, in the past few decades, the antibacterial and antioxidant properties of various medicinal plants are being investigated all over the world (Baba and Malik, 2015). To avoid human health problems and satisfy to the consumer concerns about safety and toxicity risks of synthetic compounds, there is a strong need to replace synthetic compounds with natural compounds from safe natural sources (Sokmen *et al.*, 2004). Numerous attempts are conducted to find natural alternatives means of chemical use.

Several plant extracts have demonstrated a great potential as natural inhibitors against food pathogenic and spoilage bacteria (Mith *et al.*, 2014; Negi, 2012). In the last decade, there has been much interest in the potential health benefits of plant polyphenols as antioxidant and antimicrobial properties (Pandey and Rizvi, 2009). A large number of bio-active compounds such as phenolic acids, flavonoids, stilbenes, and lignans were found in plant extracts (Bendaoud *et al.*, 2010). In extraction process, solvent extract plays an important role since with change in solvent polarity its ability to dissolve especial group of antioxidant and antimicrobial compounds (Singh *et al.*, 2014). Therefore, there is extremely necessary to study about polarity of solvent extract to justify the best solvent for polarity of polyphenols in antimicrobial activities.

In this study, *Carissa carandas* fruits were characterized for antimicrobial properties. *C. carandas* commonly known as Karanda belongs to family Apocynaceae. In recent years its botanical name was changed to *Carissa congesta* Wight (syn. *Carissa carandas* Auct. widely known as *C. carandas* L.) (Itankar *et al.*, 2011). This plant is native to India and distributed in Sri Lanka, Indonesia, Malaysia, Myanmar and different parts of Pakistan (Itankar *et al.*, 2011; Sumbul and Ahmed, 2012). Various part (fruits, leaves, barks and roots of *C. carandas* have been used traditionally for the treatment of human ailments, such as hyperpiesia, diarrhea, stomachic, anorexia, intermittent, and fever (Kumar *et al.*, 2013; Sumbul and Ahmed, 2012). Phytochemical studies revealed the presence of glycosides, terpenoids, flavonoids, tannins, saponins, unsaturated sterols, salicylic acid, proteins, vitamin C, phenolic acids, carissol, carissic acid and  $\beta$ -sitosterol as plant constituents (Mehmood *et al.*, 2014). In addition, antioxidant activities of this plant are also reported (Singh and Uppal, 2015). The present investigation was undertaken to find out the antibacterial potential of crude extracts of different parts of *C. carandas* against some Gram-positive and Gram-negative bacteria. Antimicrobial activity against *S. aureus*, *S. epidermidis*, *E. coli*, *A. niger*, *C. albicans* was seen in aqueous, ethanol, methanol, chloroform and acetone extract of *C. carandas* (Salar and Dhall, 2010). Antimicrobial activities of ethanolic extract of fruits of *C. carandas* have been reported against *S. aureus*, *S. epidermidis*, *S. pneumoniae*, *B. subtilis*,

*E. coli* (Israr *et al.*, 2012). Therefore, the objective of this study was to investigate *in vitro* studies of antibacterial properties of *C. carandas* fruits extracts for new natural ingredients that can be further use for food safety in the future.

## **Materials and Methods**

### ***The different solvent extracts for bio-active compound extraction from C. carandas fruits***

#### **Plant materials**

Mature fresh *C. carandas* fruits were collected from the local area at Samut Songkhram province, Thailand. The fruits were washed thoroughly under running tap water, the fresh weight was measured and the seeds were removed. The pitted fruits were cut to small pieces and dried in a hot-air oven at 45 °C until the weight was not changed. Ten gram of *C. carandas* fruits was soaked in each 90 ml of different solvent systems of (0%, 25%, 50%, 75% and 100% (v/v)) ethanol in water and kept for 3 days at 8 °C. Extraction was repeated thrice and the extracts were then combined following filtered through three layers of cheesecloth to remove large debris particles and re-filtered through Whatman No.1 filter paper. The filtrates were evaporated in a rotary evaporator (BUCHI Rotavapor R255), BUCHI, Lausanne, Switzerland) at 45 °C, to leave a sticky residue and stored at 4°C in refrigerator for further use. The sticky crude of each extract was dissolved in their extraction solvent, as stock solutions at appropriate concentrations for further use.

### ***In vitro antimicrobial activity of the extracts from C. carandas fruits***

#### **Microbial preparation**

Pathogenic strains of *Salmonella* Typhimurium TISTR 292, *Staphylococcus aureus* TISTR 118, *Escherichia coli* TISTR 780, *Aeromonas hydrophila* TISTR 1321 and spoilage strains of *Pseudomonas fluorescens* TISTR 358, *Lactobacillus plantarum* ATCC 14947<sup>T</sup>, *Lactobacillus sakei* TISTR 890, *Leuconostoc mesenteroides* subsp. *mesenteroides* TISTR 942, *Streptococcus* sp. TISTR 1030, *Lactococcus cremoris* TISTR 1344, *Bacillus coagulans* TISTR 1447 and were obtained from Thailand institute of scientific and technological research, Thailand; and American Type Culture Collection, Rockville, Md. The bacteria strains were grown and maintained in glycerol eppendorfs containing MRS broth for lactic acid bacteria and TSB-YE (Trypticase Soy broth with 0.6% Yeast Extract) for pathogenic bacteria. All the stock bacteria strains were stored at -80°C for further use.

### **Agar well diffusion**

Antibacterial property of the *C. carandas* fruit extracts against 11 strains of food pathogenic and spoilage bacteria was determined using the method of Biswas *et al.* (2013). Bacterial strains were cultured on petri plates of de Man, Rogosa and Sharpe (MRS; Merck, Germany) for lactic acid bacteria and Trypticase Soy Broth (TSB; Merck, Germany) with 0.6% Yeast Extract (YE; Merck, Germany) for pathogenic bacteria at 48 h to obtain single colony. The bacterial suspensions were adjusted with sterile 0.85% sodium chloride solution to contain  $10^8$  cfu/ml of tested bacteria according to 0.5 McFarland standard. Consequently, 25  $\mu$ l of these inoculums were transferred to 25 ml of proper media and poured into sterile petridish. Later, agar plates were allowed to become solid, wells were prepared in the plates with the help of a 6 mm sterile cork-borer. A total of 50  $\mu$ l of each stock extract solution (100 mg/ml) was added into the well. 10% of ethanol was used as negative control. The plates were incubated overnight at proper conditions for each strain. Microbial growth was determined by measuring the diameter of zone of inhibition (mm). The experiment was done three replicates and the mean values were observed.

### ***Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)***

#### **Minimum inhibitory concentration (MIC)**

The MIC of the extracts from *C. carandas* fruit extracts was achieved by a broth microdilution method (Sen and Batra, 2012). Two-fold serial dilutions of the crude extract from 100 to 6.25 mg/ml were individually mixed with TSB with 0.6% YE (Merck, Germany) in sterile 96 - well microliter plate. 10% ethanol was used as a negative control. Each of 20  $\mu$ l of the tested bacterial suspension was added in each well. Subsequently, the micro plates were incubated at the proper condition for each bacterium.

#### **Minimum bactericidal concentration (MBC)**

The MBC of the extracts from *C. carandas* fruit extracts was examined by comparing the number of remaining viable bacteria with the internal number of bacteria. All wells from the MIC experiments that showed no visible turbidity were serially diluted and spread on TSB with 0.6% YE (Merck, Germany) plates for viable cell count. The plates were incubated at the proper temperature of each bacterium for 24 h and then recorded as log cfu/ml. The results are presented as MIC (minimum inhibitory concentration), MIC<sub>90</sub> (minimum inhibitory concentration which inhibits 90% of the isolates of the species) and MBC (minimum bactericidal concentration).

### ***Determination of bacteria killing time by C. carandas fruit extracts***

Bacteria killing time was determined by the method of Saritha *et al.*, (2015). The exposure time of *C. carandas* fruit extracts to inhibit and kill pathogenic bacteria were carried out using MBC values. This study, stressed cells and non- stressed cells were also calculated. Total culturable cells and cultureable cells were counted in different contacting time and recorded as log cfu/ml. The total culturable cells and culturable cells of *S. Typhimurium* were investigated by plating on Mueller Hilton broth (Merck, Germany) and Hekoten (Merck, Germany) whereas total culturable cells and culturable cells of *S. aureus* were examined by plating on MHA (Merck, Germany) and Baird parker (Merck, Germany).

Stressed cells = Total culturable cells – Culturable cells

Non-stressed cells = Culturable cells

### ***Statistical analyses***

The experimental design was carried out as a Completely Randomized Design (CRD) with three replications and was repeated thrice using one-way analysis of variance (ANOVA). Analysis of variance was performed using raw data with the mean values and standard errors of the means (SEM) were calculated by Statistical Analysis System (SAS, version 9.0, Cary, NC, USA). Differences among the means were analyzed using the Tukey's multiple range tests with a significance defined at  $P < 0.05$  level and also mean differences were separated by the least significance difference (LSD) procedure

## **Results**

### ***In vitro antimicrobial properties of the extracts from C. carandas fruits***

#### **Agar well diffusion**

The antimicrobial activity of *C. carandas* extracts using different solvents was evaluated according to their clear zone of inhibition against pathogenic and spoilage bacteria (Table 1). Among extract solvent system, the extract from absolute ethanol solvent showed more effective than other solvents and all extracts showed varying degrees of antimicrobial activity against all the tested bacteria including pathogenic and spoilage bacteria. The inhibiting activity of absolute ethanol extract were 28.78 mm (*S. Typhimurium* TISTR 292), 28.11 mm (*A. hydrophila* TISTR 1321), 27.33 mm (*S. aureus* TISTR 118), 26.55 mm (*B. coagulans* TISTR 1447), 25.44 mm (*E. coli* TISTR 780), 23.55 mm (*P. fluorescens* TISTR 358), 20.78 mm (*L. sakei* TISTR 890), 19.00 mm (*L. mesenteroides* subsp. *Mesenteroides* TISTR 942), 16.89 mm (*L. plantarum*

ATCC 14947<sup>T</sup>), 12.78 mm (*Streptococcus* spp. TISTR 1030), and 11.78 mm (*L. cremoris* TISTR 1344). The lowest inhibition activities was observed in water extract which inhibit only 6 species of tested strain including *S. Typhimurium* TISTR 292, *S. aureus* TISTR 118, *E. coli* TISTR 780, *A. hydrophila* TISTR 1321, *P. fluorescens* TISTR 358 and *B. coagulans* TISTR 1447.

***Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)***

As previous studies (Table 1) showed that the highest antimicrobial activity against pathogenic and spoilage bacteria was 100% ethanol extract. This study continued using 100% ethanol solvent to determine the MIC and MBC. MIC and MBC results of *C. carandas* extract against spoilage bacteria (*P. fluorescens* TISTR 358) and pathogenic bacteria (*S. Typhimurium* TISTR 292, *S. aureus* TISTR 118, *E. coli* TISTR 780) were shown in Table 2. The results showed that *C. carandas* ethanol extract showed MIC value at 25 mg/ml against *P. fluorescens* TISTR 358, *S. aureus* TISTR 118 and 50 mg/ml for *S. Typhimurium* TISTR 292, and *E. coli* TISTR 780. While, MBC value at 100 mg/ml against *P. fluorescens* TISTR 358 and *E. coli* TISTR 780, 25 mg/ml against *S. aureus* TISTR 118, 50 mg/ml for *S. Typhimurium* TISTR 292.

***Bacteria killing time by C. carandas extract***

Bacteria killing time of the ethanol extract from *C. carandas* fruits was determined by using the MBC values against *S. aureus* TISTR 118 (25 mg/ml) and *S. Typhimurium* TISTR 292 (50 mg/ml). The results showed that the *C. carandas* extract completely killed *S. aureus* TISTR 118 and *S. Typhimurium* TISTR 292 (0 % tress cells) when exposed to extract at 20 and 6 mins, respectively (Figure 1). Stress cell percentage of *S. aureus* TISTR 118 was 88.95% to 89.33% and 86.49% to 0% at the end of tested time (25 mins) for the control and *C. carandas* extract, respectively. Whereas, stress cell percentage of *S. Typhimurium* TISTR 292 was 84.43% to 83.34% and 84.83% to 0% at the end of tested time (10 mins) for the control and *C. carandas* extract, respectively.

**Table 1.** Screening of antimicrobial activity of the extracts from *C. carandas* fruits against spoiled and pathogenic bacteria using agar well diffusion method

Cultures	Media, Temp. (°C)	Inhibiting zone of the extracts at 100 mg/ml (mm)				
		100% ethanol	75% ethanol	50% ethanol	25% ethanol	Water extract
<b>Pathogenic bacteria</b>						
<i>Salmonella</i> Typhimurium TISTR 292	TSB-YE, 37	28.78 ± 0.69 <sup>a</sup>	28.73 ± 0.23 <sup>a</sup>	20.00 ± 0.67 <sup>b</sup>	20.00 ± 0.34 <sup>b</sup>	19.00 ± 0.34 <sup>b</sup>
<i>Staphylococcus aureus</i> TISTR 118	TSB-YE, 37	27.33 ± 1.20 <sup>a</sup>	26.11 ± 0.19 <sup>ab</sup>	25.39 ± 0.10 <sup>b</sup>	17.33 ± 0.33 <sup>d</sup>	20.33 ± 0.88 <sup>c</sup>
<i>Escherichia coli</i> TISTR 780	TSB-YE, 37	25.44 ± 0.51 <sup>a</sup>	25.22 ± 0.19 <sup>a</sup>	20.33 ± 0.33 <sup>b</sup>	18.33 ± 0.67 <sup>c</sup>	17.00 ± 1.00 <sup>c</sup>
<i>Aeromonas hydrophila</i> TISTR 1321	TSB-YE, 37	28.11 ± 0.19 <sup>a</sup>	27.66 ± 0.58 <sup>a</sup>	21.00 ± 0.00 <sup>b</sup>	20.00 ± 0.00 <sup>bc</sup>	19.33 ± 0.33 <sup>c</sup>
<b>Spoilage bacteria</b>						
<i>Pseudomonas fluorescens</i> TISTR 358	TSB-YE, 37	23.55 ± 0.39 <sup>a</sup>	21.66 ± 0.88 <sup>b</sup>	18.66 ± 0.34 <sup>c</sup>	20.33 ± 0.33 <sup>b</sup>	18.22 ± 0.38 <sup>c</sup>
<i>Lactobacillus plantarum</i> ATCC 14947 <sup>T</sup>	MRS, 30	16.89 ± 0.77 <sup>a</sup>	16.77 ± 0.51 <sup>a</sup>	12.00 ± 1.00 <sup>b</sup>	Ni	Ni
<i>Lactobacillus sakei</i> TISTR 890	MRS, 30	20.78 ± 0.39 <sup>a</sup>	20.00 ± 1.00 <sup>a</sup>	15.00 ± 0.50 <sup>b</sup>	Ni	Ni
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> TISTR 942	MRS, 30	19.00 ± 1.00 <sup>a</sup>	18.66 ± 0.67 <sup>a</sup>	15.61 ± 0.09 <sup>b</sup>	Ni	Ni
<i>Streptococcus</i> sp. TISTR 1030	MRS, 30	12.78 ± 0.69 <sup>a</sup>	12.55 ± 0.69 <sup>a</sup>	12.00 ± 1.00 <sup>a</sup>	Ni	Ni
<i>Lactococcus cremoris</i> TISTR 1344	MRS, 30	11.78 ± 0.69 <sup>a</sup>	11.39 ± 0.34 <sup>a</sup>	11.00 ± 1.00 <sup>a</sup>	Ni	Ni
<i>Bacillus coagulans</i> TISTR 1447	MRS, 30	26.55 ± 0.51 <sup>a</sup>	25.28 ± 0.25 <sup>a</sup>	20.27 ± 0.54 <sup>b</sup>	16.00 ± 1.00 <sup>d</sup>	18.11 ± 0.19 <sup>c</sup>

Ni = No inhibition

MRS = de Man, Rogosa and Sharpe media

TSB-YE = Trypticase soy broth with 0.6% yeast extract media

TISTR = Thailand Institute of Scientific and Technological Research, Thailand

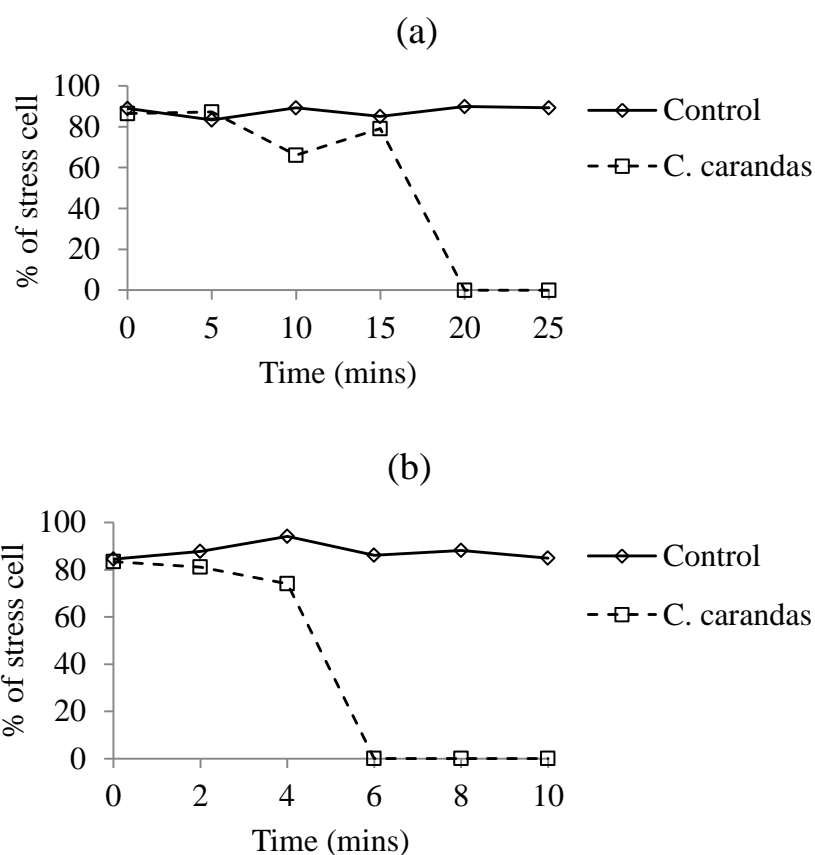
ATCC = American Type Culture Collection, Rockville, Md

<sup>a-b</sup> Means sharing different letters in the same row are significantly different (P < 0.05).

**Table 2.** Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of ethanol extract from *C. carandas* fruits against *P. fluorescens*, *S. aureus*, *S. Typhimurium* and *E. coli*

Cultures	Concentration of ethanol extract (mg/ml)		
	MIC	MIC <sub>90</sub>	MBC
<i>Pseudomonas fluorescens</i> TISTR 358	25	100	100
<i>Staphylococcus aureus</i> TISTR 118	25	25	25
<i>Salmonella</i> Typhimurium TISTR 292	50	50	50
<i>Escherichia coli</i> TISTR 780	50	100	100

TISTR = Thailand Institute of Scientific and Technological Research, Thailand



**Figure 1.** Percentage stress cell of *S. aureus* TISTR 118 (a) and *S. Typhimurium* TISTR 292 (b) treated absolute ethanol extract from *C. carandas* fruits at the concentration of 25 mg/ml and 50 mg/ml, respectively



## Discussion

Different solvents of *C. carandas* extract displayed antimicrobial activity. The highest inhibitory activity against both pathogenic and spoilage bacteria demonstrated in 100% ethanol extract. These results were in the agreement to many studies such as Salar and Dhall (2010), who reported that acetone, methanol and ethanol extracts of *C. carandas* was the most effective against the tested microorganisms closely followed by *T. cordifolia*, *C. dichotoma*, *P. cineraria* and *C. decidua*; Rojas *et al.* (2006) reported that ethanol extract exhibited a higher degree of antimicrobial activity as compared to water and hexane extracts fractions in screening ten medicinal plants used in Colombian folkloric medicine against *S. aureus*; Israr *et al.* (2012) investigated that antimicrobial activities of ethanolic extract from *C. carandas* fruits have been reported against *S. aureus*, *S. epidermidis*, *S. pneumoniae*, *B. subtilis*, *E. coli*. Sokmen *et al.* (2004) and Taie *et al.* (2010) also found the phenolic compounds found in numerous plant species appear to protect against pathogenic bacteria. Nychas (1995) proposed that phenolic could react with the phospholipid component of the cell membrane of *P. aeruginosa*, *P. flagi* and *P. fluorescence* thereby an increase in the permeability of the cell membrane or could cause significant changes in the fatty acid composition and phospholipid content of these organisms. In addition, Fullerton *et al.* (2011) who noted that phenol attacked the cytoplasmic membrane releasing intracellular constituents.

Determination of MIC and MBC is important in diagnostic laboratories because it helps in confirming resistance of microorganisms to an antimicrobial agent and it monitors the activity of new antimicrobial agents (Sen and Batra, 2012). As previous studies (Table 1) showed that the highest antimicrobial activity against pathogenic and spoilage bacteria was 100% ethanol extract. Moreover, Rawat (2015) reported that *P. fluorescens* which caused spoilage in food, decompose the food and cause changes in the taste/smell, which affect the quality of the products. Böhme *et al.* (2012) demonstrated that *E. coli* O157:H7, *Salmonella* spp., *S. aureus* are on the top of the lists of bacterial pathogens related to food responsible for foodborne illnesses. Therefore, this study continued using 100% ethanol solvent to determine the MIC and MBC. MIC and MBC results of *C. carandas* extract against spoilage bacteria (*P. fluorescens* TISTR 358) and pathogenic bacteria (*S. Typhimurium* TISTR 292, *S. aureus* TISTR 118, *E. coli* TISTR 780).

The killing time and stress cell of 100% ethanol extract from *C. carandas* were performed. Stressed or injury cell of microorganisms in term of food microbiology perspective occurred typically by physical, chemical, or insufficient nutrition, resulting in sublethally injured microbes. These can take

place either during processing treatments or environmental condition such as expose to antimicrobial compounds, heating, freezing and thawing etc. The presence of injured microorganisms in food poses significant public health concerns (Wesche *et al.*, 2009). Microorganism cells exposed to different physical and chemical treatments which suffered injury could be reversible from lethal microbes in food materials during storage (Bozoglu *et al.*, 2004). The important is stressed cells or injured cells may initially go undetected during routine quality control checks. They subsequently may repair and allow for growth, resulting spoilage toxins production and other virulence factors. *Salmonella* spp. and *S. aureus* are importance pathogenic bacteria know to experience reversible injury from sublethal stresses in food system (Wesche *et al.*, 2009).

In this conclusion, this study was determine *in vitro* antimicrobial activity of *C. carandas* fruit extract. It was found that 100% ethanol was the most appropriate solvent for extraction of antimicrobial property. The highest antibacterial activity against both pathogenic bacteria and spoilage bacteria was observed in 100% ethanol extract. In addition, 100% ethanol extract concentration at 25 and 50 mg/ml completely killed *S. aureus* and *S. Typhimurium* at 20 and 6 min, repectively. Therefore, the findings of this study suggested that *C. carandas* extract have a potential source as natural antioxidant and antimicrobial sources. Additional studies should be conducted to determine the composition and structure of the active polyphenolic compounds of the extracts.

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