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Assessment of *in vitro* antibacterial activity and MIC of cinnamon bark powder ethanolic and aqueous extracts against bacteria

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ABSTRACT

The study was planned to evaluate the *in vitro* antibacterial activity and minimum inhibitory concentration (MIC) of cinnamon (*Cinnamomum zeylanicum*) bark powder ethanolic and aqueous extracts. Screening of cinnamon powder ethanolic and aqueous extracts for antibacterial sensitivity and MIC against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli* and *Pseudomonas aeruginosa* was carried out. ABST was performed by the disc diffusion method. The cinnamon powder ethanolic and aqueous extracts were suspended in a solution containing 10% dimethyl sulfoxide and 0.5% tween 80. Under aseptic condition, empty sterilized discs were impregnated with 50 µl of different concentrations (50%, 25%, 12.5%, 6.25% and 3.12%) of the cinnamon powder ethanolic and aqueous extracts and placed on the agar plate surface. Paper disc moistened with vehicle (DMSO plus tween 80) was placed on the seeded petri plate as a vehicle control. Standard disc containing antibacterial drugs (gentamicin, tetracycline, cefpirome and ampicillin) were used as reference control. The petri plates were incubated at 37°C for 18 h. After the incubation period, the zone of inhibition was measured. Among the tested bacteria, *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes* and *Escherichia coli* showed the sensitivity at different concentration of cinnamon powder ethanolic extract whereas *Streptococcus agalactiae* and *Pseudomonas aeruginosa* showed no zone of inhibition. Aqueous extract of cinnamon powder showed no antibacterial activities against all tested bacteria. The minimum inhibitory concentration of cinnamon powder ethanolic and aqueous extracts were determined by micro-broth dilution technique. The results of MIC revealed that both ethanolic and aqueous extracts showed various MIC against all tested bacteria. Ethanolic extract of cinnamon powder has lower MIC value against *Staphylococcus aureus* among tested bacteria.

Keywords: Cinnamon Bark Powder, Antibacterial Activity, MIC, Bacteria.

INTRODUCTION

Phyto biotics or Phytogenic feed additives (PFAs) are natural growth promoter which have several medicinal properties with no residual harmful effects and are the best alternatives to antibiotic growth promoters [1]. They are plant-derived products such as essential oils, herbs and oleoresins. They can be added to feed or water of commercial animals to improve productivity by increasing feed characteristics, boosting animal production performance, and improving the quality of goods obtained from these animals [2]. Globally, India is one of the recognized countries for spices and traditional medicine which have a wide range of physiological and pharmacological properties. A spice is a dried seeds, fruits, roots, bark or flowers of plants or herbs used in small quantities for flavoring, color or as a preservative [3]. The active compounds of spices have shown a number of vitamins, flavonoids, terpenoids, carotenoids, phytoestrogens and minerals, and have demonstrated their medicinal properties such as antioxidant, antibacterial, anti-inflammatory, anticancer, digestive stimulant and antipyretic [4,5]. Phytochemical components of photobiotic have been shown to exhibit high antibacterial action against Gram-positive [6,7]. and Gram-negative bacteria in both *in vivo* and *in vitro* environments [8].

Cinnamon (*Cinnamomum zeylanicum*) is an ancient spice which is member of the family lauraceous and belongs to genus Cinnamomum. It is commonly known as 'cinnamon' in English, 'dalchini' in Hindi, 'taj' in Gujarat and 'tweak' in Sanskrit. It is cultivated mostly in Sri Lanka, Mayan mar, Malabar coast of India, South America, Caribbean, Southeast Asia and the West Indies [9]. The chemical compounds found in various parts of cinnamon tree where leaves contain 70-95% eugenol and 1-5% cinnamaldehyde while bark contain 5-10% eugenol and 65-80% cinnamaldehyde [10,11]. The report on *in vitro* antimicrobial activity and minimum inhibitory concentration (MIC) of cinnamon powder against various bacteria was very limited. Therefore, the present study was planned to evaluate the *in vitro* antibacterial activity and MIC of cinnamon bark powder ethanolic and aqueous extracts at different concentrations (50%, 25%,

12.5%, 6.25%, 3.12%) along with standard antibacterial drugs against various gram positive and gram negative bacteria.

MATERIALS AND METHODS

Collection of Plant Materials

Dried cinnamon (*Cinnamomum zeylanicum*) bark was purchased from local market of Anand, Gujarat (certified by FSSAI, Ministry of Health and Family Welfare, Government of India) and then powdered by mechanical grinder and stored in air tight containers. The dried cinnamon powder was subsequently used for the preparation of ethanolic and aqueous extracts.

Sources of Test Organisms for ABST and MIC

The test bacterial organisms were procured from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune. Bacterial strains of six bacterial species, namely, *Staphylococcus aureus* (ATCC 6538), *Streptococcus agalactiae* (ATCC 13813), *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111), *Escherichia coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 9027) were procured. Purity and viability of the organisms were checked by morphological, cultural and biochemical tests and maintained by periodical subculture.

Preparation of Ethanolic Extract

The dried plant material (Cinnamon bark powder) was subjected to repeated extraction in Soxhlet extraction apparatus using solvent ethanol. The extract was then concentrated using rotary evaporator under reduced pressure. The solvent was later separated from the extract with the aid of rotary evaporator at 40°C [12]. The ethanolic extract was stored in a labeled sterile screw capped glass bottle at 4°C for further experimental uses.

Preparation of Aqueous Extract

For this purpose, about 100 g of the cinnamon bark powder were soaked in 1 liters of distilled water for 72 hours with shaking thrice daily. The mixture was filtered through sterilized Whatman no.1 filter paper. After filtration, the extract was evaporated in water bath (50°C) until the solvent gets completely evaporated. The aqueous extract was labeled and stored in air tight glass containers in refrigerator at 4°C for further experimental use.

Determination of Antibacterial Sensitivity by Disk Diffusion Assay

Preparation of diffusion solution and different concentration

For the preparation of 10% dimethyl sulfoxide (DMSO), 10 ml of DMSO was dissolved in 90 ml of distilled water in measuring cylinder. For the preparation of diffusion solution, 0.5 ml of tween 80 was dissolved in 99.5 ml of 10% DMSO. The preparation of various dilutions of cinnamon powder ethanolic and aqueous extracts is shown in Table 1.

Table 1: Schedule for the preparation of cinnamon bark powder ethanolic and aqueous extracts dilution

Concentration	Dilution (%)	Cinnamon powder (g)	Diffusion solution (ml)
A	50	0.5	0.5
B	25	0.250	0.750
C	12.5	0.125	0.875
D	6.25	0.062	0.938
E	3.12	0.031	0.969

Procedure for disk diffusion assay

Screening of cinnamon powder ethanolic and aqueous extracts for antibacterial activity was done by the disc diffusion method. It was performed using an 18 h culture at 37°C in 10 ml of Mueller Hinton Agar. The test suspension was standardized to match the 0.5 McFarland turbidity standard which corresponds to approximately 1.5×10^8 CFU/ml with sterile saline solution. Five hundred microliters of the suspensions were spread over the plates containing Mueller-Hinton agar (for *Streptococcus agalactiae* 5% defibrinated sheep blood was added) using a sterile cotton swab to get a uniform microbial growth on both control and test plates. Various concentration of cinnamon powder extracts (50%, 25%, 12.5%, 6.25%, 3.12%) were prepared with diluent solution (99.5 ml 10% DMSO mixed with 0.5 ml tween 80). It was sterilized by filtration through a 0.45 μ m membrane filter. Under the aseptic condition, empty sterilized discs (6 mm) were impregnated with 50 μ l of different concentrations (50%, 25%, 12.5%, 6.25%, 3.12%) of the cinnamon powder ethanolic and aqueous extracts and placed on the agar surface [13]. Sterile disc moistened with diluent was placed on the seeded petri plate as vehicle control. Standard discs of Gentamicin, Tetracycline, Cefpirome and Ampicillin were used as reference control. All Petri plates were sealed with sterile laboratory parafilm to avoid eventual evaporation of the test samples. The plates were left for 30 minutes at room temperature to allow the diffusion of extracts and then they were incubated at 37°C for 18 h. After the incubation period, the zone of inhibition was measured with help of vernier caliper. All the dilutions of cinnamon powder ethanolic and aqueous extracts were tested in a triplicate manner against each bacterium.

Minimum Inhibitory Concentration

Minimum inhibitory concentration of cinnamon powder ethanolic and aqueous extracts was determined for different six gram positive and gram negative organisms by microbroth dilution technique with minor modifications [14].

Preparation of drug stock solution

The stock solution of cinnamon powder ethanolic and aqueous extracts (50%) was prepared using Brain heart infusion broth and 0.5% tween-20 as an emulsifying agent, mixed well by shaking vigorously. The concentration of cinnamon powder ethanolic and aqueous extracts was decided based on their density. Chloramphenicol stock solution (250 mg/ml) was prepared in sterile water to use as a positive control.

Preparation of bacterial suspension

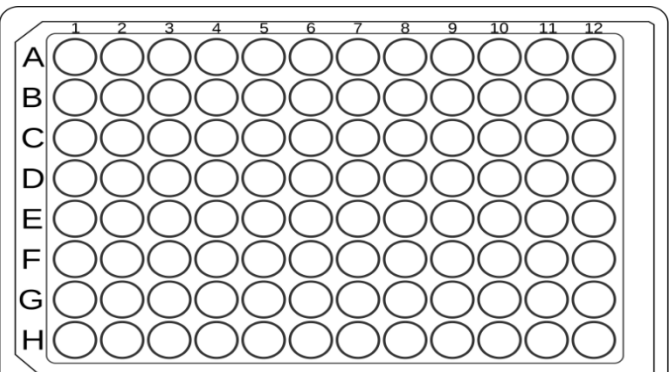
After overnight incubation, all bacterial cultures were prepared to McFarland 0.5 standard equivalent to 1.5×10^8 cfu/ml. The bacterial suspension's turbidity was adjusted to McFarland 0.5 by adding more bacterial culture if turbidity was found too low and by adding sterile broth if the turbidity was found too high. Final dispensing inoculums were prepared in sterile test tubes by adding 2 ml bacterial suspension (1.5×10^8 cfu/ml) of respective organisms into 198 ml sterile broth. Final dispensing inoculum concentrations were 1.5×10^6 cfu/ml.

Procedure for microbroth dilution technique

Sterile 96 well microtiter plate with sterile lid were used. Sterile broth of 100 μ l was added in each well except first well of row. Then, 200 μ l drug from stock solution was added in first well of first column. Followed by, 100 μ l drug from well number 1 was taken and added to well number 2 and then it was serially diluted (two-fold dilution) up to well number 10. From 10 number well, discarded 100 μ l drug. Chloramphenicol stock 100 μ l was added in column number 11 as a positive control. Serial dilution of drug kept starting concentration 25% in first well whereas 0.05 % in 10th number well. At lastly, 100

µl bacterial suspensions were dispensed in well number 1 to 12 of row while 12th well was kept as a growth control where the drug was not added. 'G' row of 96 well plate was kept as a negative control where only BHI broth was added and 'H' row of 96 well plate was kept as vehicle control where only vehicle added. Microtiter plate was incubated at 37°C for 16-20 hours. Details of microdilution technique of MIC using 96 well micro titer plate are shown in Table 2.

Table 2: Microdilution technique of MIC using 96 well micro titer plate

Row													
Column	Concentration of plant Extract (% W/V)	25	12.5	6.25	3.12	1.56	0.78	0.39	0.19	0.10	0.05	PC	GC
	No. of Wells	1	2	3	4	5	6	7	8	9	10	11	12
	A												
	B												
	C												
	D												
	E												
	F												
	G (NC)												
	H (VC)												

PC- Positive control (Standard drug); GC- Growth control (Bacterial suspension) NC- Negative control (BHI broth); VC- Vehicle control (DMSO + Tween 80); A- *Staphylococcus aureus*; B- *Streptococcus agalactiae*; C- *Bacillus cereus*; D- *Escherichia coli*; E- *Pseudomonas aeruginosa*; F- *Listeria monocytogenes*

Table 3: Antibacterial activity of cinnamon powder ethanolic extract and standard antibiotic discs against various bacteria by disk diffusion assay

Groups	Zone of inhibition (mm)					
	<i>Staphylococcus aureus</i>	<i>Streptococcus agalactiae</i>	<i>Bacillus cereus</i>	<i>Listeria monocytogenes</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
CN A (50%)	14.67±2.03	0.00	12.67±1.20	18.00±1.73	12.67±0.88	0.00
CN B (25%)	11.00±1.00	0.00	8.00±0.58	0.00	0.00	0.00
CN C (12.5%)	9.67±1.20	0.00	0.00	0.00	0.00	0.00
CN D (6.25%)	0.00	0.00	0.00	0.00	0.00	0.00
CN E (3.12%)	0.00	0.00	0.00	0.00	0.00	0.00
Gentamicin	24.33±0.88	27.67±0.88	19.33±0.33	24.00±0.58	18.33±0.33	25.00±0.58
Tetracycline	23.67±0.88	33.67±0.88	25.67±0.88	28.33±0.33	22.67±0.88	17.33±0.33
Cefpirome	17.00±0.58	17.00±0.58	13.67±0.88	30.00±1.15	29.00±0.58	29.33±0.33
Ampicillin	30.33±0.88	10.00±1.15	10.33±0.33	10.33±0.33	21.67±0.88	0.00

RESULTS

Antibacterial Sensitivity Test of Ethanolic and Aqueous extract

The anti-bacterial activity of ethanolic extract of five different concentrations (50%, 25%, 12.5%, 6.25%, 3.12%) of cinnamon powder have been presented in Table 3. There was no inhibition in growth of bacteria with the vehicle control. Four antibacterial drugs (Gentamicin, Tetracycline, Cefpirome and Ampicillin) were found active against test bacteria.

Ethanolic extracts of cinnamon powder at 50%, 25% and 12.5% concentrations showed antibacterial activities against *Staphylococcus aureus*. Cinnamon powder ethanolic extract at 50% and 25% concentrations showed antibacterial activity against *Bacillus cereus* whereas *Listeria monocytogenes* and *Escherichia coli* were sensitive at 50% concentrations of ethanolic extracts of cinnamon powder. No antibacterial activity of cinnamon powder ethanolic extract was found

After incubation, freshly prepared 30 µl of Iodonitrotetrazolium chloride (INT) dye (1 mg/ml) was dispensed in all wells of micro titer plate. Plate was again incubated for 30 minutes for development of purple color, which indicated presence of live bacteria. Minimum inhibitory concentration for each bacterial species was observed as absence of color development. This protocol was done in triplicate to minimize the standard error.

against *Streptococcus agalactiae* and *Pseudomonas aeruginosa*. Representative photographs of the zones of inhibition against test bacteria for ethanolic extract are shown in Figure 1 to 4. Aqueous extract of cinnamon powder at 50%, 25%, 12.5%, 6.25% and 3.12% concentration showed no antibacterial activities against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli* and *Pseudomonas aeruginosa*.

MIC of Ethanolic and Aqueous Extracts

MIC of cinnamon powder ethanolic and aqueous extracts at 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9, 0.95 and 0.47 mg/ml concentration with two-fold serial dilution in six different gram positive and gram-negative organisms in triplicate manner was determined. The MIC values (Mean ± S.E.) of ethanolic and aqueous extracts of cinnamon powder against different test bacteria was presented in Table 4. Representative photographs of 96 well plates used for determination

of MIC for ethanolic and aqueous extracts of cinnamon powder against different bacterial species are presented in Figure 5 and 6. The mean values of MIC for ethanolic extract were 5.20 ± 1.30 , 10.40 ± 2.60 , 10.40 ± 2.60 , 16.92 ± 7.92 , 11.70 ± 3.90 and 31.25 ± 0.00 mg/ml against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus cereus*, *Listeria monocytogenes* and *Pseudomonas aeruginosa*, respectively and MIC for aqueous extract were 125.00 ± 0.00 mg/ml against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Listeria monocytogenes* and *Pseudomonas aeruginosa* while 83.33 ± 20.83 and 62.50 ± 0.00 mg/ml against *Bacillus cereus* and *E. coli*, respectively.



Figure 1: Antibacterial sensitivity test of ethanolic extract of cinnamon powder against *Staphylococcus aureus* (A=50%, B=25%, C=12.5%, D= 6.25% and E =3.12%)



Figure 2: Antibacterial sensitivity test of ethanolic extract of cinnamon powder against *Bacillus cereus* (A=50%, B=25%, C=12.5%, D= 6.25% and E =3.12%)

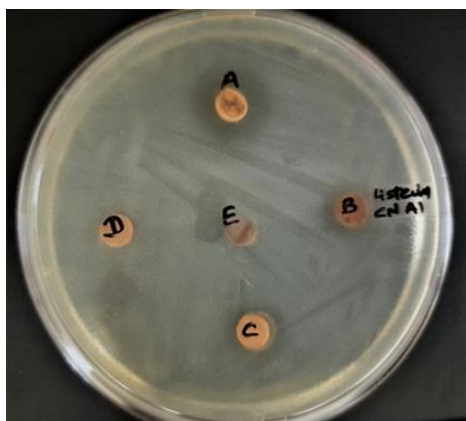


Figure 3: Antibacterial sensitivity test of ethanolic extract of cinnamon powder against *Listeria monocytogenes* (A=50%, B=25%, C=12.5%, D= 6.25% and E =3.12%)



Figure 4: Antibacterial sensitivity test of ethanolic extract of cinnamon powder against *Escherichia coli* (A=50%, B=25%, C=12.5%, D= 6.25% and E =3.12%)

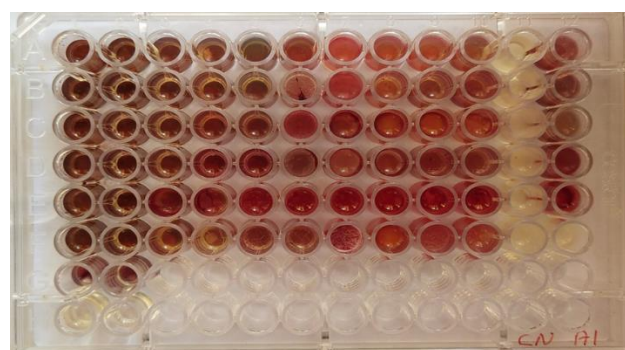


Figure 5: MIC test of ethanolic extract of cinnamon powder against (A= *Staphylococcus aureus*, B= *Streptococcus agalactiae*, C= *Bacillus cereus*, D= *Escherichia coli*, E= *Pseudomonas aeruginosa*, F= *Listeria monocytogenes*)

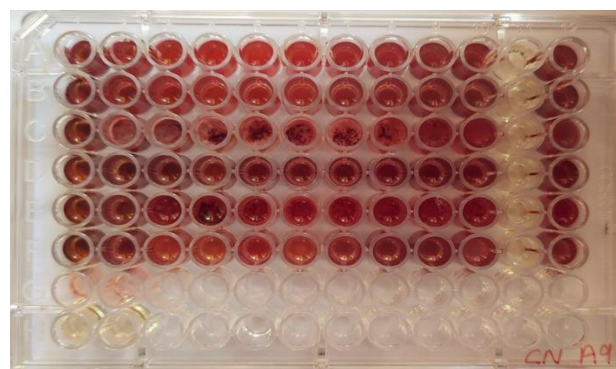


Figure 6: MIC test of aqueous extract of cinnamon powder against (A= *Staphylococcus aureus*, B= *Streptococcus agalactiae*, C= *Bacillus cereus*, D= *Escherichia coli*, E= *Pseudomonas aeruginosa*, F= *Listeria monocytogenes*)

Table 4: MIC of cinnamon powder ethanolic extract against various bacteria by microbroth dilution technique

Sr.no	Bacterial species	MIC (mg/ml)	
		Ethanolic extract	Aqueous extract
1	<i>Staphylococcus aureus</i>	5.20 ± 1.30	125.00 ± 0.00
2	<i>Streptococcus agalactiae</i>	10.40 ± 2.60	125.00 ± 0.00
3	<i>Bacillus cereus</i>	10.40 ± 2.60	83.33 ± 20.83
4	<i>Listeria monocytogenes</i>	16.92 ± 7.92	125.00 ± 0.00
5	<i>Escherichia coli</i>	11.70 ± 3.90	62.50 ± 0.00
6	<i>Pseudomonas aeruginosa</i>	31.25 ± 0.00	125.00 ± 0.00

DISCUSSION

The results with respect to antibiotic sensitivity test were in accordance with Sofia *et al.* (2007) who found no antibacterial activity at 0.5 and 1% concentration of cinnamon aqueous extract against *Staphylococcus aureus* and *Bacillus cereus* [15]. Gupta *et al.* (2008) reported antibacterial activity of cinnamon powder alcoholic extract against *Escherichia coli* with 11mm zone of inhibition whereas *Pseudomonas aeruginosa* found resistant [16]. Hoque *et al.* (2008) also found *Pseudomonas aeruginosa* resistant to ethanolic extract of cinnamon powder whereas *Staphylococcus aureus* sensitive with 10-11 mm inhibitory zone [17]. Shafique *et al.* (2010) also found *Staphylococcus aureus* sensitive with inhibitory zones (9.2±0.28 mm) whereas no activity against *Pseudomonas aeruginosa* [18]. Ababutain (2011) found ethanolic extracts of cinnamon powder had no antibacterial activity against *Pseudomonas aeruginosa* [19]. Mukhtar and Ghorri (2012) found *Bacillus subtilis* and *E.coli* were resistant at 10, 20, 40% concentration of aqueous extract of cinnamon powder [20]. Likewise, Kapila (2015) noticed antibacterial activity of ethanolic extract of cinnamon against *Bacillus cereus* with 7 mm zone of inhibition while no inhibitory zone with aqueous extract of cinnamon bark against *B. cereus*, *S. aureus*, *E. coli* and *P. aeruginosa* [21]. Upadhyaya *et al.* (2018) also reported ethanolic extract of cinnamon showed 14 mm and 11 mm inhibitory zone against *Staphylococcus aureus* and *Escherichia coli*, respectively [22]. Similarly, Budhathoki and Basnet (2018) noted the 10 mm inhibitory zone with ethanolic cinnamon extracts against *Staphylococcus aureus* [23]. Keloth *et al.* (2018) observed no inhibitory effect of cinnamon aqueous extract against *E. Coli* and *S. aureus* [24]. Abdul Rasheed *et al.*, (2019) also observed no zone of inhibition against *E. coli*, *S. aureus* and *P. aeruginosa* at 20% concentration of cinnamon powder aqueous extract [25]. Moreover, Islam *et al.* (2019) suggested that *Staphylococcus aureus* (14.5 mm) and *Escherichia coli* (12.25 mm) sensitive to ethanolic extract of cinnamon at 50 µl concentration [26]. Prajapati *et al.* (2018) also reported cinnamon oil at different concentration inhibited the growth of *Streptococcus agalactiae* (ATCC13813), *Listeria Monocytogenes* (ATCC 1911), *Staphylococcus aureus* (ATCC 6538P), *Pseudomonas aeruginosa* (ATCC 19154) and *Escherichia coli* (ATCC 10799) with different zone of inhibition [27]. Kavitha *et al.* (2020) also found no inhibitory zone with aqueous extract of cinnamon bark powder against *Staphylococcus aureus* and *Escherichia coli* [28].

There are several pathways by these phytochemicals exist their actions in various bioactivities. They may inhibit microorganism proliferation, interfere with some biological metabolic processes, or may modulate signal transduction and gene expression pathways [29,30,31]. Phytochemicals primarily act by collapsing bacterial cell walls and membranes, resulting in cell component leakage, disruption of proton motive force, dysfunction of efflux pump and enzyme all these leads cytolysis [32].

The results with respect to MIC of ethanolic and aqueous extracts of cinnamon powder were in accordance with Hoque *et al.* (2008) who reported the minimum inhibitory concentration of cinnamon ethanolic extracts against *Staphylococcus aureus* (2 to 3.5 mg/ml) [17]. Bayoub *et al.* (2010) also reported that cinnamon ethanolic extracts showed 0.4 mg/ml minimum inhibitory concentration against *Listeria monocytogenes* [33]. Similarly, Ismail *et al.* (2012) found minimum inhibitory concentration of 2, 2, 3 and 4 mg/ml to cinnamon ethanolic extracts against *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa*, respectively whereas no determined MICs (mg/ml) of aqueous extract of cinnamon powder found against *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* [34]. Karleshia *et al.*, (2012) who reported 60 mg/ml MIC value of aqueous extract of cinnamon powder against *Bacillus cereus* and *Escherichia coli* [35]. Liang *et al.* (2019) also found 20 mg/ml MIC of cinnamon powder ethanolic extract for *L. monocytogenes* and *E. coli* [36].

The main phenolic component of cinnamon bark is cinnamaldehyde which has antibacterial properties. Cinnamaldehyde cause

disintegration of the bacterial cell membrane resulting release of cell contents and ion leakage which affect pH homeostasis and equilibrium of inorganic ions [37]. These phenolic compounds are capable of further cellular destruction and inhibition by establishing the hydrophobic and hydrogen bonding of these degradative phenolic compounds to membrane proteins resulting in portioning of the lipid bilayer [19]. The presence of cinnamaldehyde inhibits the amino acid decarboxylation activity in the cell which leads to energy deprivation and microbial cell death [38]. Alcoholic solvents might have exhibited better solubility of active ingredients of cinnamon than water therefore it showed better antibacterial activity [20].

CONCLUSION

Cinnamon powder ethanolic extract evinced antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes* and *Escherichia coli* whereas aqueous extract of cinnamon powder showed no antibacterial activity against all tested bacteria. Ethanolic extract of cinnamon powder has lowest MIC (5.20 ± 1.30 mg/ml) against *Staphylococcus aureus* among tested bacteria.

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Conflict of Interest

None declared.

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