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Evaluation of Selected Essential Oils as Biocontrol Agents Against *Listeria monocytogenes*

Chinta Siva Swetha[1], Porteen Kannan[1*], Elango A.[2], Ronald B.S.M.[3], Senthil Kumar T.M.A.[4] and Sureshkannan Sundaram[1]

[1]Department of Veterinary Public Health and Epidemiology, Madras Veterinary College, Chennai, TN, INDIA
[2]Dean i/c, Veterinary College and Research Institute, Salem, Tamil Nadu, INDIA
[3]Department of Veterinary Microbiology, Madras Veterinary College, Chennai, Tamil Nadu, INDIA
[4]Central University Laboratory, CAHS, TANUVAS, Madhavaram Milk Colony, Chennai, Tamil Nadu, INDIA

**Corresponding author: P Kannan; E-mail: rajavet2002@gmail.com*

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ABSTRACT

The increased concern towards food safety led to application of natural extracts as antimicrobial agents to control food borne pathogens. The aim of the present study was to determine antimicrobial activity of the four essential oils *viz.,* cinnamon bark, cinnamon leaf, clove bud and garlic oils against *Listeria monocytogenes* by agar well diffusion method. All these essential oils were characterized by Gas chromatography-mass spectrophotometer (GC-MS) to know the chemical constituents present in them. Out of these four oils, *L. monocytogenes* was found to be sensitive to cinnamon bark oil, followed by cinnamon leaf oil, clove bud oil and garlic oil. Further, all these oils were evaluated to know minimal inhibitory concentration (MIC) and the cinnamon bark oil alone was found to be effective with a MIC of 1% against *L. monocytogenes.* The present study findings suggest that plant based natural extracts might be used as antimicrobial, flavouring and food biopreservative agent.

HIGHLIGHTS

- Cinnamon bark oil at 2% and 1% showed minimum inhibitory activity concentrations against *L. monocytogenes.*
- Organoleptic characteristics of foods were unaltered at 2% and 1% of Cinnamon bark oil.
- Essential oils from plant source can be employed for food preservation and to encourage 'green consumerism'.

Keywords: Agar well diffusion method, Antimicrobial, Essential oil, Minimum inhibitory concentration (MIC)

Food borne illnesses and food safety are the major public health issues still faced by people, inspite of being many improvements in slaughter hygiene, food production techniques and control programmes. This problem is aggravated by emergence of multi-drug resistance pathogens globally and is now recognized as global health challenge (Rai *et al.,* 2017). The antibiotic resistance is due to overuse of antibiotics which can be attributed to over prescription and haphazard patient compliance.

L. monocytogenes is a Gram positive, facultative anaerobe, rod shaped and able to survive at wide range of temperatures from -0.4ºC to 45ºC, pH range from 4.0 to 9.6 with an optimum of 6-8, at water activity (a_w) levels of 0.90 (Valimaa *et al.,* 2015). They have ability to form biofilms by adhering to different food contact surfaces like stainless steel, polystyrene at food processing facilities and persist for several months to years. *L. monocytogenes* biofilms can tolerate high concentrations of sanitizers, disinfectants and antimicrobials and can resist UV light, which results in contamination of food contact surface, in turn contaminating RTE foods, main

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cause of *L. monocytogenes* infection. As this organism has the ability to survive in low temperatures, both in aerobic and anaerobic conditions and in modified atmospheric packaging makes the food industry to take necessary actions and implement control measures along the food chain to prevent food borne listeriosis, which is a serious threat to public health (Valimaa *et al.,* 2015).

The common preservative techniques followed by food industries will only improve organoleptic quality of food, but food safety was not completely assured. In addition, most of synthetic preservatives were restricted to use in food industries, negative perception of consumers towards these preservatives and increase in the interest of consumer towards 'green consumerism' had made the food industries to use plant based natural extracts as they are safe and eco-friendly (Prakash *et al.,* 2018). From the past few years, research on essential oils and phytochemicals has shown that they can act as potential antimicrobials to prevent proliferation of pathogenic food bore microbes and further increases the shelf life of food products. Around 300 essential oils were considered as generally recognized as safe approval (GRAS) and can be used for flavouring or fragrance commercially and as biocontrol agent (Gray *et al.,* 2018).

As there are only few reports of investigation on essential oils to use as biocontrol agents in food systems, the present study was designed to study the antimicrobial effect of essential oils *viz.,* cinnamon bark oil, cinnamon leaf oil, clove bud oil and garlic oil against *L. monocytogenes* in foods.

MATERIALS AND METHODS

Procurement of essential oils

Four essential oils *viz.,* Cinnamon bark oil, Cinnamon leaf oil, Clove oil and Garlic oil were obtained from M/s Kancor essential oils, Ernakulam, Kerala. All these essential oils were stored in amber colored bottles at 4ºC for further use.

Characterization of essential oils

All the four essential oils were characterized by Gas Chromatography- Mass Spectrometry system (789ºA, Agilent Technologies Inc., Santa Rosa, CA, USA) to know the chemical constituents present in them. This system is equipped with a HP-5 MS capillary column (30 m × 0.25 mm, 0.25 mm, Agilent Technologies Inc., Santa Rosa, CA, USA) and injected volume of each sample was 1 µL. Helium gas was used as carrier gas which has flow rate of 1 mL/min. The temperature of the injection port was 250ºC and the temperatures programmed in the oven were: 50ºC for 2 min, then increased to 180ºC at a rate of 5ºC/min, followed by an increase to 270ºC at a rate of 20ºC/min and maintenance at 270ºC for 5 min. The mass spectra used – EI ion source temperature of 230ºC, ionization energy of 70eV and a mass scan range of 40-500 amu. The essential oil components were tentatively identified by comparing their mass spectra with the standards present in NIST library.

Preparation of bacterial culture

Four *L. monocytogenes* isolates obtained from milk, soil, beef, along with positive control (MTCC 1143) were used to determine antibacterial efficacy of essential oils. All these isolates were inoculated in Buffered Listeria Enrichment Broth (BLEB) and incubated at 37ºC for 24 hrs. After incubation, the inoculum was centrifuged at 8000 rpm for 10 minutes and the supernatant was discarded. Then the sediment was washed twice with normal saline and centrifuged at 8000 rpm for 10 min. Supernatant was discarded and the cells were mixed with normal saline to get 0.5 McFarland standard turbidity which indicates 1.5×10^8 cells/mL.

Dilution of essential oils

Essential oils were diluted a day prior to use with 1% of Tween 80 in requirement of concentrations of 2%, 1%, 0.5% and 0.25%. The diluted essential oils were stored at 4ºC for further use.

Screening of essential oils for antibacterial activity against *L. monocytogenes* by Agar well diffusion method

The antibacterial activity of essential oils was tested by agar well diffusion method (Gupta *et al.,* 2008). The standard *L. monocytogenes* isolate was inoculated in nutrient broth and incubated at 37ºC for 24 hrs. The inoculum was

adjusted to 10^6 CFU/ml with McFarland standards. A 100 µl of test bacterial culture (10^6 CFU/ml) was spreaded on dried Mueller Hinton agar plates and left for 10 minutes. Holes were punched on the agar plates with 6 mm cork borer and 50 µl each of four essential oils were poured in to different wells after sealing wells bottom with 1% agar. Sterile Tween 80 was kept as negative control. These plates were kept aside for one hour for proper diffusion of essential oils and incubated at 37ºC for 24 hrs. The plates were prepared in duplicate. After incubation, the zones of inhibition were measured in mm and the sensitivity of each oil was compared with standards was given by Ponce *et al.,* 2003 and Moreira *et al.,* 2005: < 8 mm-Non sensitive; 9 to 14mm-Sensitive; 15 to 19mm- Very sensitive; >20mm-Extremely sensitive.

Determination of minimum inhibitory concentration (MIC) by agar well diffusion method

The Minimum Inhibitory Concentration (MIC) of Cinnamon bark oil, Cinnamon leaf oil, Clove bud oil, Garlic oil was also determined by agar well diffusion method (Gupta *et al.,* 2008). The MIC was defined as the lowest concentration of essential oils at which no visible growth of bacteria was observed within 24 hrs of incubation at 37ºC (Thongson *et al.,* 2004). Various concentrations of essential oils were prepared by two-fold dilution series to get concentrations of 2%, 1%, 0.5% and 0.25%. A 50 µl of various concentrations of essential oils were poured in to wells which were punched on Mueller Hinton agar plates spreaded with 10^6CFU/ml inoculum and incubated at 37ºC for 24 hrs. The tests were done for twice. After incubation, zones of inhibitions were measured and the lowest concentration at which zone of inhibition was clear can be considered as MIC value of that essential oil.

RESULTS AND DISCUSSION

Characterization of essential oils by GC-MS

The chemical constituents present in Cinnamon bark oil, cinnamon leaf oil, clove bud oil and garlic oil were represented in Fig. 1 to 4. The present study identified cinnamaldehyde (74.76%) as the major chemical constituent in cinnamon bark oil and this is similar to findings of Mareim *et al.,* 2020 and Jham *et al.,* 2005 who reported 74.56% and 75% of cinnamaldehyde as major component in their studies. Sun *et al.,* 2016 obtained a little high prevalence of cinnamaldehyde of 79.39% while Zhang *et al.* (2016) reported a very high percentage of cinnamaldehyde (92.4%) in their studies respectively. Shan *et al.,* 2007 in their study also identified 60-80% of cinnamaldehyde from the barks of *Cinnamon zeylanicum* which is almost similar to the present study. In contrary to this, other researchers identified 45.13% and 12.5% of cinnamaldehyde respectively (El-Baroty *et al.,* 2010; Bayoub *et al.,* 2010).

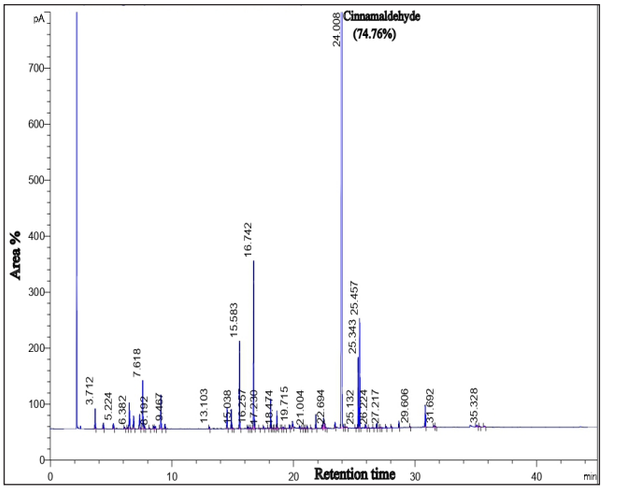

Fig. 1: GC-MS chromatogram of Cinnamon bark oil

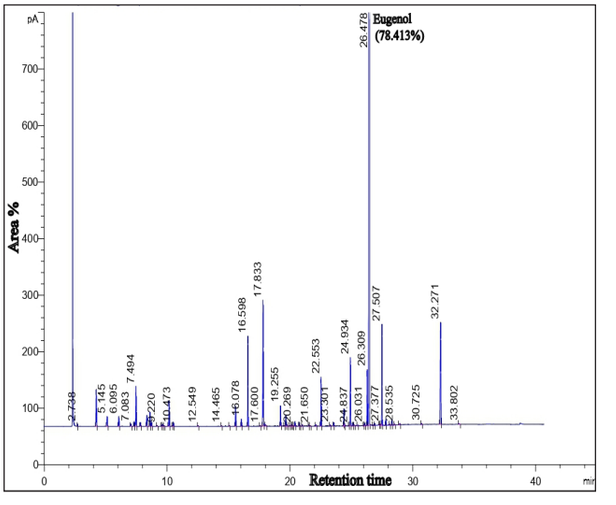

Fig. 2: GC-MS chromatogram of Cinnamon leaf oil

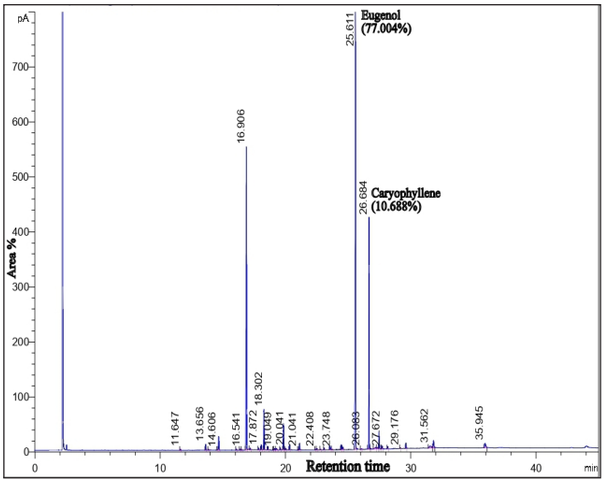

Fig. 3: GC-MS chromatogram of Clove bud oil

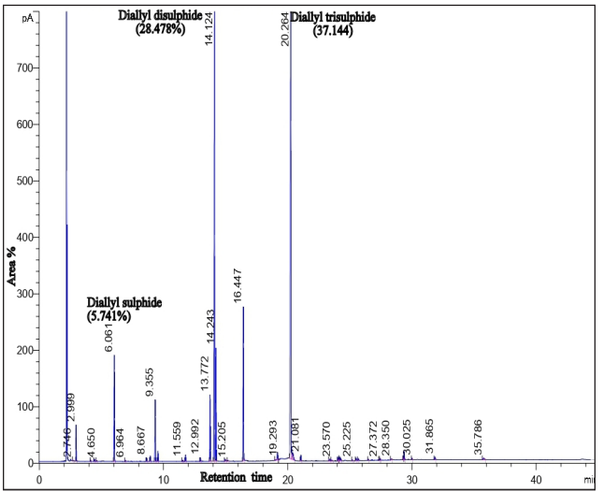

Fig. 4: GC-MS chromatogram of Garlic oil

Eugenol (78.413%) is the main component found in cinnamon leaf oil in the present study which is similar to the findings of Bayoub *et al.,* 2010 (76.91%). Singh *et al.* (2007) also reported eugenol (85%) as predominant component in cinnamon leaf oil which is slightly higher than the present study.

The main components identified in clove bud are Eugenol (77.004%) and Caryophyllene (10.688%) were similar to the Mith *et al.* (2014) who found same compounds with little high percentages - Eugenol (84.75%) and beta caryophyllene (4.60%). The studies of Cava *et al.* (2007) also determined cinnamaldehyde (67%) and eugenol (4%) as the important components in cinnamon bark oil while 4% cinnamaldehyde and 77% eugenol in cinnamon leaf oil; 86% eugenol and <0.1% cinnamaldehyde in clove essential oils respectively.

On characterization of garlic oil in the present study, it was found that diallyl trisulphide (37.144%), diallyl disulphide (28.478%) and diallyl sulphide (5.741%) were the main constituents. Mareim *et al.* (2020) recorded presence of diallyl trisulphide and diallyl disulphide in garlic oil with different percentages of 25.13 and 22.74. Razavi Rohani *et al.* (2011) also identified 27.33% of diallyl trisulphide, 24.67% of diallyl disulphide, 19.11% of methyl allyl trisulphide, 8.59% propenyldithiopropanoate, 2.18% dimethyl trisulfide, 2.13% diallyltetrasulfide, 1.49% of 3-vinyl-[4H]-1,2 dithin (3-VDT) and 1.25% 2-vinyl-[4H]-1,2 dithin (2-VDT). Similarly, El-Sayed *et al.,* 2017 and Zhang *et al.,* 2016, have also found similar compounds in garlic oil but with different concentrations.

These variations in the composition of essential oils from the present study to other studies may due to species, subspecies and variety of plants selected for preparation of essential oils, various agroclimatic conditions *viz.,* climatic, seasonal and geographic of the regions, harvesting seasons (Mith *et al.,* 2014), maturity stage and adaptive metabolism of plants, distillation conditions and the plant part analyzed (El-Baroty *et al.,* 2010). The antimicrobial activity these essential oils also depends on the concentration of the phenolics compounds present in them, where at low concentrations, these phenolics compounds affects enzyme activity related to energy production and at high concentrations, causes protein precipitation (Prindle and Wright, 1997).

Screening of essential oils for antibacterial activity against *L. monocytogenes* by agar well diffusion method

All the four essential oils *viz.,* cinnamon bark, cinnamon leaf, clove bud and garlic oils have shown antibacterial activity against *L. monocytogenes*. Among the four essential oils, cinnamon bark oil has shown with widest zone of inhibition (27±0 mm), followed by cinnamon leaf oil (17±0 mm), clove bud oil (14±0 mm) and garlic oil (12±0 mm).Similar to the present study, Ghabraie *et al.,* 2015 in their agar diffusion assay studies, found that cinnamon bark oil and clove bud oil has shown highest

inhibition diameters of approximately 27.8±0.6 mm and 14.8±0.3 mm among 32 essential oils used against *L. monocytogenes*. Babu *et al.,* 2011 also observed that cinnamon oil (25±0.06) has shown more zone of inhibition followed by clove (21±0.08) and garlic (17±0.18) oils in their studies. Hoque *et al.,* 2008 also found 33 mm and 14 mm inhibition zones by testing the antimicrobial activity of cinnamon and clove essential oils against *L. monocytogenes.* In contrary to the present study, Mareim *et al.*(2020) recorded high zone of inhibition by garlic oil (31±1.7 mm) than cinnamon oil (12.3±0.5) against *L. monocytogenes*. While, Smith and Palmer *et al.*(1998) have found 6.8 mm, 9.2 mm, 8.4 mm and 4 mm zones of inhibition showed by cinnamon bark, cinnamon leaf, clove and garlic oils against *L. monocytogenes* respectively. Bayoub *et al.* (2010) observed more zone of inhibition for clove oil (25±10 mm) than cinnamon oil (22±0 mm) against *L. monocytogenes* in their preliminary screening of essential oils. Similarly, in a study conducted by Shan *et al.,* 2007, clove bud oil (13.7 mm) showed more zone of inhibition than cinnamon oil (8.9 mm) against *L. monocytogenes* by agar well diffusion method.

Determination of Minimum Inhibitory Concentration (MIC) by agar well diffusion method:

The minimum inhibitory concentration (MIC) of the four essential oils *viz*., cinnamon bark oil, cinnamon leaf oil, clove bud oil and garlic oil were given in Table 1.

Table 1: Minimum Inhibitory Concentrations (MIC) of essential oils against *L. monocytogenes*

Test isolate	Cinnamon bark oil	Cinnamon leaf oil	Clove bud oil	Garlic oil
Milk isolate	1%	NA	NA	NA
Soil isolate	1%	NA	NA	NA
Beef isolate	NA	NA	NA	NA
Positive control (MTCC 1143)	1%	NA	NA	NA

Among four essential oils, Cinnamon bark oil alone was found to be sensitive against *L. monocytogenes* at tested concentrations (0.25%, 0.5%, 1% and 2%) with MIC of 1%. These findings are similar to the studies of Gupta *et al.* (2008) who recorded 1.25% MIC of cinnamon bark oil. In contrary, Mith *et al.* (2014) observed antilisteria activity of cinnamon oil against *L.monocytogenes* with a MIC of 0.125 µl/ml; Bayoub *et al.*(2010) had MIC of 0.4 mg/ml for cinnamon oil against *L. monocytogenes*.

Though the cinnamon leaf oil, clove bud and garlic oils have shown antibacterial activity, but they didn't shown any zone of inhibitions at required concentrations (2%, 1%, 0.5% and 0.25%) while determining MIC. In contrast to this, Wafaa *et al.* (2019) investigations showed the MIC of cinnamon leaf and bark oils against *L. Monocytogenes* was 0.5% (v/v) and also reported that these oils can be used as potential antimicrobial agents to keep fresh produce safe from *Listeria* for human consumption.

In contrary to the clove bud oil findings in the present study, Menon and Garg (2001), Gupta *et al.* (2008), Barbosa *et al.* (2009) and Bayoub *et al.* (2010) have found the minimum inhibitory action of clove oil at a concentration of 1%, 5%, 0.09% and 0.25 mg/mL against *L. monocytogenes.* Hoque *et al.* (2008) reported MIC values for cinnamon and clove essential oils were 1.25 mg/ml and 2.5 mg/ml respectively against *L. monocytogenes*.

Cava *et al.* (2007) also observed antimicrobial effect of cinnamon bark, cinnamon leaf and clove oils against *L. monocytogenes* in pasteurized milk stored at 7ºC and 35ºC. They also observed strongest antimicrobial activity by cinnamon bark oil while cinnamon leaf and clove oils required higher concentrations for complete inhibition of growth of *L. monocytogenes* in pasteurized milk. They detected MIC value of 500 ppm for cinnamon bark oil and 3000 ppm of each for cinnamon leaf and clove oils respectively.

Similar to the present study, Indu *et al.* (2006) studies also found that garlic oil didn't show any inhibitory effect against growth of *L. monocytogenes* by agar well method. Kim *et al.* (2004) also found that garlic oil and its constituents have weak antibacterial activity with MIC value of 300 µg/mL and Razavi *et al.* (2011) had found 100 µg/mL MIC values against *L. monocytogenes.* Kumar and Berwal (1998) had found 85% inhibition of *L. monocytogenes* at 10% garlic concentration and also determined MIC value at 8.8% respectively. Allicin or diallylthiosulphinic acid or diallyl disulphide was responsible for antimicrobial activity of garlic oil (Indu *et al.,* 2006). Till now, only few works has been carried out on antibacterial activity of garlic oil due to rapid decomposition of the allicin, the major antibacterial compound in garlic, during the

preparation of garlic essential oil (Razavi Rohani *et al.,* 2011). The results may vary from one researcher to others due to selection of bacterial strains, volume of inoculum, incubation time and temperatures used in the experiments (Mith *et al.,* 2014).

Our present study was mainly aimed to know the concentration of essential oils at which antibacterial activity was shown without change in the organoleptic characteristics of foods. In the present study, cinnamon bark oil only has shown activity against *L. monocytogenes* at 2% and 1% respectively while the remaining three essential oils didn't show any activity at required concentrations. The results obtained from the present study highlights the use of cinnamon bark oil in food industries as food preservative in foods against *L. monocytogenes*.

CONCLUSION

The results of the present study showed that all the four essential oils *viz*., cinnamon bark oil, cinnamon leaf oil, clove bud oil and garlic oils were sensitive against *L. monocytogenes*. Out of these four essential oils, cinnamon bark oil alone shown minimum inhibitory activity at 2% and 1% concentrations against *L. monocytogenes* without change in organoleptic characteristics of foods. The results of this study might be useful to introduce cinnamon bark oil as food preservative in food industries at low cost without altering nutrients and organoleptic characteristics. Many studies are required in future, to know antilisteria activity of these essential oils by changing the extraction procedure, increasing the concentrations of usage without compromising organoleptic characteristics of food in food industry to ensure food safety and public health and to minimize alter chemicals usage with plant based natural extracts for food preservation and to encourage 'green consumerism'.

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