Research Article

Antimicrobial activity of certain herbal plant extracts against pathogenic microbes and their application in sterilized milk medium

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Citation
Farzana Siddique, Aaayisha Latif, Muhammad Arshad and Mushtaq Hussain Lashari. Antimicrobial activity of certain herbal plant extracts against pathogenic microbes and their application in sterilized milk medium. Pure and Applied Biology. Vol. 10, Issue 2 pp378-387. http://dx.doi.org/10.19045/bspab.2021.100041

Received: 07/03/2020 Revised: 01/10/2020 Accepted: 02/10/2020 Online First: 08/10/2020

Abstract
The antibacterial activities of the rosemary, oregano, clove and cinnamon extracts against *Listeria monocytogenes*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were evaluated in agar diffusion test medium and in sterilized milk medium at 37 °C within 24 hr time interval. All tested herbal extracts exhibited an inhibitory effect against pathogens. Gram-negative bacteria *E. coli* and *P. aeruginosa* were less susceptible to the inhibitory activity of all herbal extracts used in the experiment as compared to Gram-positive bacteria *Staph. aureus*, *L. monocytogens* and *B. subtilis* in nutrient agar medium as well as in sterilized milk medium. Oregano showed significant effect against all pathogens as compared to clove, cinnamon and rosemary. Whereas, results from rosemary showed significant decrease in microbial activity against pathogens as compared to other tested herbal extracts. These results suggest the prospect of using these herbal extracts in milk and milk products as natural antimicrobials.

Keywords: Antimicrobial activity; Herbs; Pathogens; Sterilize milk

Introduction
Prevention of food spoilage and food poisoning pathogens is usually achieved by use of chemical preservatives which have negative impacts including: human health hazards of the chemical applications, chemical residues in food & feed chains and acquisition of microbial resistance to the used chemicals. Because of such concerns, the necessity to find a potentially effective, healthy safer and natural alternative preservative is increased. Within this text, Plant extracts have been used to control pathogens causing diseases and spoilage to milk and milk products. Bacterial contamination is one of the major reasons that causes loss of quality of food and reduce shelf life of food products during
transportation, refrigeration and storage [1]. Many plants have antimicrobial action due to their vital oil elements present in their extracts. Several scientists have described the antimicrobial action of vital oils from oregano, thyme, sage, rosemary, clove, cinnamon, garlic, cayenne, pepper, celery, chives, ginger, savory and onion against microbes [2-4]. Various naturally derived constituents derived from herbs and spices are utilized as preservatives such as essential oils, flavonoids, phenolic compound, microbial metabolites and antimicrobial constituents of other foods that possess the preserving action. The structural and functional groups of the oils showed a significant role as antimicrobial action [5, 6]. Some main antimicrobial components have been documented as aldehydes, ketones, polyphenols, ethers, alcohols, and hydrocarbons [7]. Some spices oils are utilized in food industry as natural food preservatives. It possesses important characteristics to inhibit wide spectrum of pathogens due to presence of antimicrobial components [8, 9]. These natural preservatives are gaining significance in recent years as they have little or no harmful effects. Greater consumer responsiveness and awareness about synthetic chemical additives directed researchers and food processors to look for natural food preservatives with a wide series of antimicrobial agent.

Milk is mostly produced in small units in remote areas. It gets spoiled in short time due to its perishable nature and conventional unhygienic handling practices mainly in summer season. Raw or processed milk is a good medium for growth of a number of microbes and consumption of the spoiled product results into infections/ intoxications in consumers [10, 11]. Dairy cows suffering from sub clinical or clinical mastitis are source of direct entry of microbes into raw milk from the farm environment, mostly the water source and utensils used for the storage of milk on dairy farm or for the duration of transportation. The dairy industry is developing minimum processing techniques [12, 13], that can be used to extend the shelf of fluid milk. Soto preserve milk there is need to develop bio-preservation instead of chemical preservatives by utilization of plant extracts. The current study is conducted to evaluate the antibacterial activities of the plant extracts of rosemary, oregano, clove and cinnamon extracts against Listeria monocytogenes, Bacillus subtilis, Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa in through agar diffusion test medium and milk medium.

**Materials and methods**

**Herbal plant samples**

The description of herbal plants used in this study is given in (Table 1). Rosemary, clove, cinnamon and oregano were collected from local market, Islamabad, Pakistan.

**Table 1. Herbal plants used in the experiment**

| Common name of Plants | Botanical name                  | Plant parts used |
|-----------------------|--------------------------------|------------------|
| Rosemary              | *Rosmarinus officinalis* L.    | Leaf             |
| Clove                 | *Eugenia caryophylata*         | Bud              |
| Cinnamon              | *Cinnamomum cassia*            | Bark             |
| Oregano               | *Origanum vulgare*             | Leaf             |

**Preparation of aqueous extracts of rosemary, clove, cinnamon and oregano**

Extraction was carried out using method described by [14] with some modification. 100 g crushed powder from each part of...
plant was individually immersed using 500 ml of 95 percent ethanol taken in a flask of 1 liter capacity, the flask was kept shaking for 4 days at 150 rpm and room temperature. The solutions were first passed through a muslin cloth and filtrate collected were filtered again by using a filter paper i.e. Whatman’s filter paper No.1 to achieve a particle free solution. The filtrates were evaporated using rotary evaporator and kept at -80°C to be frozen before subjecting to freeze drying. The prepared aqueous extracts were collected in sample bottles and stored in refrigerator at 4°C before using.

**Microbial inoculum preparation**

Strains of milk pathogenic bacteria *Listeria monocytogenes*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were obtained from the culture collection of laboratory of Microbiology, department of Food Technology, PMAS Arid Agriculture University Rawalpindi. During the entire research period, the fresh bacterial inoculums were cultured and used. The nutrient broth (NB) was prepared and 5ml was poured into each tube and sterilized. Inoculation in tubes was done by picking few colonies of tested bacteria with help of sterilized loop. The incubation of tubes was carried out at 37°C for 24 hours. The presence of inhibition zones were measured by Vernier caliper, recorded and considered as indication for antibacterial activity.

**Testing on isolated milk pathogenic strains**

The sterilized milk medium was inoculated at the rate of initial inoculum of 3.5, 5.54, 4.47, 4.3 and 7.62log CFU/ml of *L. monocytogen*, *Staph. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* respectively. The growth of microbes was observed at 37°C for 24 h in sterilized milk medium supplemented with clove, cinnamon, oregano and rosemary extracts at concentration of 5% (v/v).

**Statistical analysis**

The data obtained throughout the entire research was statistically analyzed using analysis of variance (ANOVA) by the help of Statistics 8.1 software to compare the means. Tukey’s Test was used for means comparison. Graphical representation of data was done on Microsoft excel.

**Results and Discussion**

**Antimicrobial screening of herbal extracts using agar well diffusion method**

In general, the obtained data in the present study showed that extracts of rosemary, cinnamon, clove and oregano confirmed the antibacterial activity against *Staph. aureus*, *L. monocytogens*, *B. subtilis*, *E. coli* and *P. aeruginosa* in agar well diffusion method. (Table 2) reviewed that inhibition of the oregano extract was stronger than that of the others, showing inhibition zones ranging from 23–30 mm. On the other hand rosemary extract showed less inhibitory effect with 15–22 mm diameter inhibition zones against all pathogenic bacteria compared to the same levels of oregano, clove and cinnamon extracts. Gram-negative bacteria *E. coli* and *P. aeruginosa* were less susceptible to the inhibitory activity of all herbal extracts used in the experiment as compared to Gram-positive bacteria *Staph. aureus*, *L. monocytogens* and *B. subtilis*. Clove and cinnamon showed bactericidal
effect with inhibition zones, diameter 18-27mm and 17-25mm, respectively against all four pathogenic bacteria. Previous researchers confirmed that natural antimicrobial compounds present in plants were found to possess antimicrobial activity. In order to use plants to control pathogens in milk products, it is essential that investigation of antibacterial activity of plant extracts carried out against pathogens [16]. *S. aureus*, *P. aeruginosa*, *E. coli* as were inhibited with extracts of cinnamon, cloves, and cumin with inhibition zones ranges between <10 and >30 mm estimated by the disc diffusion method. Researchers screened out the antimicrobial activity and total phenolic contents of dietary spices and medicinal herbs extracts including cinnamon, rosemary, oregano and clove against five foodborne bacteria (*B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli*, and *Salmonella anatum* [16].

Table 2. Antibacterial properties of rosemary, cinnamon, clove and oregano extracts against pathogenic strains in agar well diffusion method.

| Treatment  | *L. monocytogen* | *Staph. aureus* | *E. coli* | *B. subtilis* | *P. aeruginosa* |
|------------|------------------|-----------------|-----------|--------------|----------------|
| Control    | 0                | 0               | 0         | 0            | 0              |
| Rosemary   | 21.00±0.46       | 22.50±0.20      | 19.53±0.31| 21.00±0.40   | 15.50±0.20     |
| Clove      | 23.00±0.95       | 24.00±0.30      | 20.90±0.70| 27.00±0.40   | 18.23±0.72     |
| Cinnamon   | 24.00±0.42       | 25.0±0.1        | 18.07±0.43| 22.83±0.21   | 17.00±0.42     |
| Oregano    | 25.40±0.34       | 27.5±0.31       | 28.00±0.28| 31.87±0.85   | 21.00±0.42     |

*well diameter, 6 mm, included and results presented as a mean value of three replicates

Antimicrobial effects of herbal extracts against pathogenic strains in sterilized milk medium

Antibacterial behavior of clove, cinnamon, oregano and rosemary extracts against of *L. monocytogen*, *Staph. aureus*, *B. subtilis*, *P. aeruginosa* and *E. coli* was shown in (Figs. 1-5), respectively. The sterilized milk medium was inoculated at the rate of initial inoculums of 3.5 log CFU/ml, 5.54 log CFU/ml, 4.47log CFU/ml, 7.62log CFU/ml and 4.3 log CFU/ml of *L. monocytogen*, *Staph. aureus*, *B. subtilis*, *P. aeruginosa* and *E. coli* respectively, supplemented with clove, cinnamon, oregano and rosemary extracts at concentration of 5% (v/v).Concentration of plant extracts utilized in foods could vary from 2 to 100 fold of that used in the in vitro assays depending on the herb type and food system used [17, 18]. In sterilized milk medium the addition of herbal extracts led to reduction of the viability of all five pathogens. Cinnamon and clove showed statistically similar results in case of *L. monocytogen* and *B. subtilis* and reduction was upto 2.5 log cycle and 2 log cycles for both organisms, respectively. Reduction of *S. aureus* by cinnamon and clove was upto 3 log cycles and 2.5 log cycles respectively. *P. aeruginosa* and *E. coli* reduced upto 2 log cycles by clove and upto 2.4 log cycles by cinnamon. In the present study, the antimicrobial activity exhibited by the cinnamon extract against different pathogenic strains of bacteria may be due to the presence of its active aromatic aldehydic compound i.e. cinnamaldehyde. Cinnamon bark contain 50.5%cinnamaldehyde which is highly electronegative and interferes in biological processes involving electron transfer and react with nitrogen containing components (proteins and nucleic acids) and therefore reduce the growth of the microorganisms [19]. It also cause energy deprivation in the cell which leads to microbial cell death due
to inhibition of the amino acid decarboxylation activity in the cell. Therefore cinnamaldehyde was proved as an active agent against many pathogenic bacteria [20]. On the other hand, strong antibacterial effects of clove and its antimicrobial activity is due to its principal active constituent’s eugenol, eugenol acetate carvacrol and thymol that are phenylpropanoides. Eugenol having phenolic structure is highly active against the pathogens [21, 22]. Mixtures of clove and cinnamon essential oils inhibited the growth of *L. monocytogenes* and *E. coli* in pasteurized milk.

![Figure 1](image1.png)

**Figure 1.** Behavior of *L. monocytogenes* in sterilized milk incubated at 37°C for 24hr with addition of plant extracts

![Figure 2](image2.png)

**Figure 2.** Behavior of *S. aureus* in sterilized milk incubated at 37°C for 24hr with addition of plant extracts
Figure 3. Behavior of *B. subtilis* in sterilized milk incubated at 37°C for 24hr with addition of plant extracts.

Figure 4. Behavior of *P. aeruginosa* in sterilized milk incubated at 37°C for 24hr with addition of plant extracts.
Reduction of *L. monocytogens*, *Staph. aureus*, *B. subtilis* was up to 2-2.3 log cycles by rosemary while *P. aeruginosa* and *E. coli* decreased up to 1.0 log cycles. Oregano has more pronounced effect than all other herbal extracts as it reduced the all pathogens up to 2.7-3 log cycles showing greater antibacterial activity than all other herbal extracts. Rosmarinic acid and carnosic acid were considered to be the most effective antimicrobial compounds against bacterial species present in the rosemary plant. It was found by [23], that ethanol extract of rosemary exhibited wide-ranging activity against Gram-positive and gram-negative bacteria as compared to methanolic and aqueous extract. Antimicrobial property of oregano exhibited in the current study is due to its major active component carvacrol. Other constituents of oregano alpha pinene, linalol, gamma cariofilene, canfor, rhocymenene, limonene and thymol also play some role in its antibacterial activity [24] also confirmed the antibacterial activity of oregano against food borne pathogens.

The reduction in the number of *L. monocytogens*, *Staph. aureus* and *B. subtilis*, was more pronounced (P > 0.05) compared to the culture of *P. aeruginosa* and *E. coli*. Above results demonstrate that Gram-positive bacteria was more sensitive to all herbal extracts than Gram-negative bacteria. This was in agreement with the earlier studies on other herbal extracts [25, 26]. A possible reason for these observations may be due to the significant differences in the outer layers of Gram-negative and Gram-positive bacteria. An outer membrane and a unique periplasmic space present in the Gram-negative bacteria while Gram-positive bacteria lack of these outer layers make it easy for herbal components to enter in cell [27]. Several researchers reported the antibacterial actions of herbs like, vervan, cinnamon, oregano, rosemary and savage carrots in culture media [28-32]. Plant oils exerted only a limited action in food substrates [33]. Reported that microbes were neither eradicated nor completely inhibited, while clove oil was able to restrict the
propagation of *L. monocytogenes* in both milk and mutton.

**Conclusion**
The results of current study indicate that the use of these plant extracts in milk as natural antimicrobials. These herbs are utilized and consumed all over the world and have potential to be used as natural bio-preservative to increase shelf life of food products. However, great potential of these four plants for utilization in milk as well as milk products as antimicrobial possibly increase the safety of the product during its shelf life, provided that the initial microbial load in the milk was low. For further research, different combination of plant extracts would be suggested and their synergistic effects would possibly enhance the bactericidal effect.

**Authors’ contributions**
Conceived and designed the experiments: F Siddique, Performed the experiments: A Latif, Analyzed the data: M Arshad Contributed materials/ analysis/ tools & Wrote the paper: MH Lashari.

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