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Antimicrobial Effects of Calcium Oxide Nanoparticles and Some Spices in Minced Meat

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Abstract: *The effect of calcium oxide nanoparticles (CaO NPs) was investigated comparing it to effects of some natural spices, in the refrigerated chilled minced meat to improve its shelf life. Also, the in vitro antibacterial effect was evaluated against E.coli ATCC 8739 and Salmonella typhimurium using agar well diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The results demonstrated that all treatments showed improved effects on pH, color and odor in contrast to control. CaO NPs and garlic powder had the best effect on the Aerobic plate count (APC) and yeast and mould count, while onion powder had the best effect on the Enterobacteriaceae count through the storage period. It was concluded that CaO NPs showed good antimicrobial activity and consequently had improved the shelf life when added to minced meat. Also, garlic powder and onion powder enhanced the shelf life. In addition, CaO NPs solution, sage, garlic and onion extracts demonstrated superior antibacterial effects against E.coli ATCC 8739 and Salmonella typhimurium than ginger extract. SO, CaO NPs can be added for meat products as a multifunctional food additive.*

Keywords: *Calcium oxide nanoparticles; spices; minced meat; Salmonella typhimurium; E.coli ATCC 8739*

1. INTRODUCTION

Food additives are used for improving the quality of food either by extending the shelf life of food, enhancing the appearance of food or by developing a good flavor. Food additives may be natural as spices and herbs or artificial as calcium oxide and calcium acetate which are approved newly as food additives used for acid regulator [1].

Spices have more than one function in food; it can be used as flavoring agent and as bio-preservatives due to its antimicrobial effect. So, it can be used to prevent both food spoilage and growth of pathogenic microorganisms. Additionally, spices have nutraceutical benefits, used as immunomodulators (garlic), lowering blood cholesterol (garlic, onion and turmeric), antimutaginic (garlic, ginger and turmeric), digestive stimulant (turmeric, cumin, ginger, onion, sage and mint) natural antimicrobials (garlic, sage and turmeric) and can be safely used especially against antibiotic resistant microorganisms [2]. Garlic and onion are widely used for flavoring of foods. Moreover, these spices contain antimicrobial compounds. There are higher quantities of sulphur compounds (allicin) in garlic than onion, whereas, onion contains higher amount of flavonol compounds. Moreover, ginger includes gingerol and shagelol[3].

Calcium oxide and calcium acetate are accepted as food additives. Calcium oxide FCC food grade helps as preservative, acid regulator, dough conditioner and prevents food elements from aggregation and increases its bioavailability [1]. In addition, metal oxides nanoparticles demonstrate antimicrobial in previous studies [4, 5], which may produced from its alkalinity and presence of active oxygen particles. Metal oxides generally considered safe for human and animals comparing to other organic materials. Also, the antibacterial effect of Calcium carbonate is related to CaO formed by heat treatment. Calcium oxide nanoparticles (CaO NPS) are low cost, easily accessed and biocompatible. So, metal oxides could be good substitutes in medical and food processing purposes [6]. Consider

Nanotechnology can be applied in food production and processing with applying of

nanoparicles which may be organic or inorganic. Organic nano-material as protein, fat, carbohydrates and etc. inorganic nanomaterial may be metals as calcium and magnesium or nonmetals as selenium and silicate which may be applied in food, food additives or food packaging. The term nano-food is used by applying nanoparticles or nanotechnology in any step of food production [7].Nano-particles can be applied in food as nano-sized food additives, which naturally produced or artificially. However, manufactured nano-sized particles may cause harm to human or environment specially if penetrate the cell membrane [8].There is a little knowledge about nanofood, although nanoparticle can be produced naturally during the manufacture of food products. Also, Nanofabrication helps in absorption and transportation of fat in our body [9].

The intend of the study is to assess the effect of four spices - garlic (*Allium sativum*) powder, onion (*Allium cepa*) powder, ginger powder (Zingiber officinale) and sage powder (Salvia officinalis) and calcium oxide nanoparticles on sensory and microbial quality of meat, and the in vitro antibacterial effect against *E. coli* ATCC 8739 and *Salmonella typhimurium* as food borne pathogens.

2. Materials and Methods

Source of spices used in the study

Spices powders were purchased from local markets in Assiut city.

Preparation of CaO nanoparticles

CaONPs nanoparticles were prepared from calcium nitrate supplied from Sigma-Aldrich. CaNO3 was dried at 150°C for two hours in oven and then using a SPEX8000 instrument with a vial and balls of tungsten carbide and a ball-to-powder weight ratio (BPR) of 10:1. Fritsch, Mini-mill- Pulverisette23.

Characterization of CaO nanoparticles

Transmission Electron Microscope (TEM)

The morphology and sizes of silver nanoparticles were determined by TEM micrographs using the JEOL TEM 100 CXII (Electron Microscope Unit, Assiut University, Egypt). The sample was prepared by placing a drop of synthesized calcium nanoparticles (dissolved in absolute ethanol) on a negative carbon coated copper grids and dried in air [10].

Preparation of meat samples

Frozen minced meat (1000grams) was left overnight in the refrigerator to be thawed and divided to 100grams portions. Five portions (each 100 grams) were used for the experiment. One gram of each spice (Onion powder (Allium cepa L.), garlic powder (Allium sativum L), ginger powder (Zingiber officinale) and sage powder (Salvia officinalis)) was added separately to one portion (100grams) of the minced meat to study the effect of each spice alone. Also, 100ppm of calcium oxide nanoparticles was added to 100 gram of the minced meat. All treatments were kept at refrigerator temperature (4°C) for 7 days. Samples of the minced meat from the five treatments were periodically analyzed at storage days 0, 1, 3, and7 for pH, visual color, odor and microbial load.

PH Measurement

PH was measured according to [11].Ten grams of the minced meat was homogenized in 100 mL distilled water, and the homogenate was filtered. The pH of the filtrate was detected using a waterproof pH meter AD11 (Adwa two points pH calibration, Romania).

Sensory evaluation

Sensory estimation for color and odor, applied by help of panelists from the Department of Food hygiene using the 9-point hedonic scale, where 9 = like extremely; 7= like moderately; 5 = neither like nor not dislike; 3 = dislike moderately; 1 = dislike extremely [12].

Microbiological analysis

Ten grams from each of the five treatments were homogenized aseptically into 90 ml of peptone water. Dilutions were prepared using 0.1% peptone water. Aerobic plate count (APC) were counted on duplicate of plate count agar (PCA, Hi-Media Laboratories, M 091, Mumbai, India) using spread method and incubated at 35±2°C for 48h; Enterobacteriaceae counts by spreading on Violet Red Bile Glucose Agar plates (VRBGA, Hi-Media, M 049) which were incubated at 35°C for 48 h. Yeast and mould counted by spreading on freshly prepared potato dextrose agar plates and after incubation at 25±2°C for 5-7 days. Colony counts were expressed as CFU/g [13].

Antibacterial activity of CaO Nps and spices extracts

Preparation of aqueous spices extract

Five grams of each dry ground spice were added to 100 ml sterile distilled water and left overnight at room temperature. The extracts were filtered by sterile cotton piece and by filter

paper. Then extracts were evaporated at 50°C till leave pasty material which weighed and stored at 4°C till used [14].

Microorganisms and inoculums preparation

Salmonella typhimurium previously isolated from chicken carcass meat and molecularly identified [15], and *Escherichia coli* ATCC 8739**.** Nutrient agar (NA) was used for growing and purification of microorganisms. The bacterial strains were grown in a nutrient broth for 12–18 h at 37°C. Dilutions were made from the overnight incubated tube till reach the dilution which contains $5x10^5$cfu/ml.

Agar –well diffusion technique

The preliminary antimicrobial susceptibility was tested using agar-well diffusion method. One hundred micro liters of the cell suspensions were spread on a Mueller-Hinton agar (Hi Media) and the agar medium was hollowed out with wells (6mm) and filled with 100 µl of each spices extract (500 mg/ml) and CaO nanoparticles (100 ppm) solutions in equal amounts (50 µl). The plates were observed for zone of inhibition after 24 h incubation at 37°C [4].

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

The antimicrobial activities were evaluated through the determination of the minimum inhibitory concentration (MIC) by the two-fold serial micro dilution method in 96-well microtiter plate. Hundered µl of dilution $5x10^5$CFU/g of the microorganisms and 100 µl of the spices or CaO NPs were pippeted in the wells (beginning with the concentration 250mg/ml for spices and 600µg/ml for CaO NPs). Positive and negative controls were also prepared. Then the plates incubated at 37°C for 24 hours. MIC is the concentration at which the antimicrobial agent visually inhibits the growth of the microorganism. MBC is the concentration at which the antimicrobial agent starts to have the bactericidal effect on the microorganism [16].

Statistical analysis

The data of pH, sensory and microbiological evaluation were analyzed by ANOVA using SPSS (version 14). P value < 0.05 was considered significant and on the very limits of significance (0.1>p>0.05), respectively.

3. Results And Discussion

Spices used as natural antimicrobial agents. Powders of spices were preferred than essential oils for application in minced meat, for evaluation of the microbial count and shelf life, as mostly consumer applied spices in powder form ,and essential oils could be affected by presence of fat in meat or meat products [17].

PH and Sensory analysis

Spices or calcium oxide nanoparticles have enhanced effects on pH and sensory parameters (color and odor) when compared with control (figures 1, 2 and 3). PH values were significantly increased ($p<0.05$) by increasing storage days. All treatments statistically had improved result on pH than control during storage days, in the order of onion= CaO NPs> garlic>sage> ginger; in particular there were on the very limits of significance (0.1>p>0.05) between control and each of onion, garlic and CaO Nps. Chemically, the addition of CaO Nps increases pH due to its alkalinity, which is favored as an antimicrobial factor [18].Onion had the least pH at 7th day of storage, which in accordance with Park *et al.*, [19] who found that the pH when garlic was added to pork belly and lion increased from 6.13 to 6.27, while as onion was added, pH was increased from 5.76 to 5.93.

By the end of storage period, there was general significant decline in color and odor score during storage, which attributed to liberation of fatty acids, lipid oxidation, subsequent browning of minced meat and increased microbial count as prescribed by Kandeepan *et al.* [20]. Additives produced less decline than control, and the order of treatments affecting color was CaO NPs> ginger> onion> sage= garlic, while for odor was CaO NPs> sage = garlic = onion> ginger. Previous study by Javed *et. al.* [3] reported that addition of garlic in beef sausage improved flavor, taste, color and pH of sausage**.**

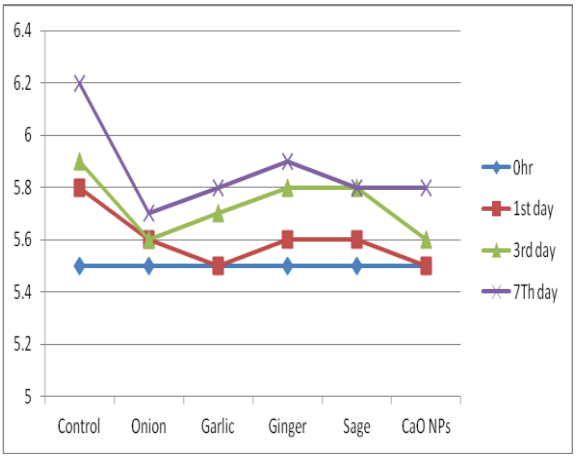

Figure1. *Effect of different treatments on pH of the stored minced meat*

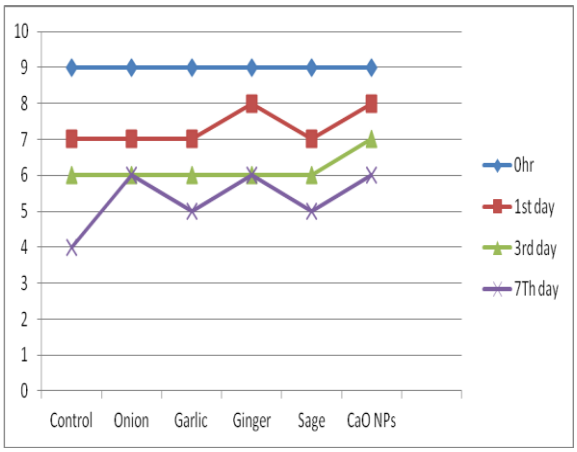

Figure2. *Effect of different treatments on color of the stored minced meat*

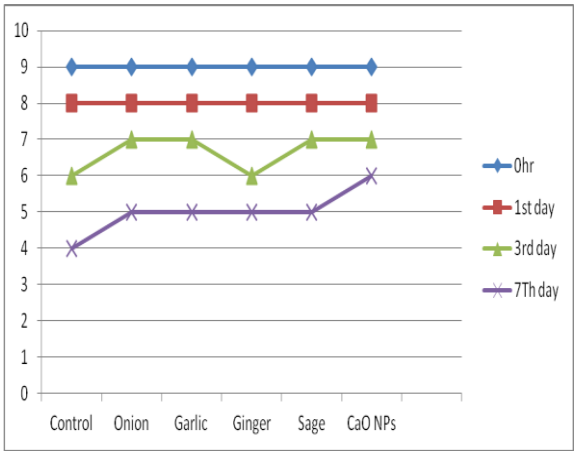

Figure3. *Effect of different treatments on odor of the stored minced meat*

Antimicrobial effect of CaO nanoparticles and spices in minced meat

Aerobic plate count (APC) often used for the prediction of the shelf life of the meat. It is concluded from figures 4, 5 and 6 that APC of the control reach $3x10^7$CFU/g, on the 7^{th} day, which exceed the permissible limit by Egyptian standard [21] for frozen minced meat, whereas, the other treated samples still below this limit. Therefore, all used spices and CaO NPs could be used to expand the shelf life of minced meat. Also, it is noticed that all treatments could reduce Enterobacteriaceae and yeast and mould counts comparing with the control.

All treatments had significantly ($p \leq 0.05$) reduction effects throughout the storage period on APC (garlic= CaO NPs> onion= ginger> sage), and marginal significant reduction effect on Enterobacteriaceae count (onion> ginger> garlic= CaO NPs> sage) in contrast to control. On the other hand, CaO NPs, garlic and sage had significant reduction ($p \leq 0.05$) effect on the count of yeast and mould, when compared with control, following this order CaO NPs=garlic>sage >onion> ginger.

Application of spices powders or CaO oxide nanoparticles in minced meat had excellent effect in reduction of aerobic plate count (APC), Enterobacteriaceae count and yeast and mould count in relation to the control (figures 4,5 and 6). So, these additives can be applied in minced meat to expand its shelf life. This previously reported by many studies that spices and herbs have antimicrobial effect when applied in food [2, 22, and 23]. They also established that adding of fresh garlic and garlic powder to meat products gave enhanced reduction in APC than garlic oil. Muthia *et al.* [24] established that using of garlic powder in duck sausage provided enhanced microbial reduction than fresh garlic, and they owed that to organosulphur compositions (allicin).As well, Javed *et. al.* [3] recommended that garlic in beef sausage cause reduction in APC due to allicin. Krisch *et. al.* [25] demonstrated that dried garlic achieved a reduction in the total cell count when added to the minced pork, and adding of salt could enhance the reduction of the count. In addition to Park *et al.* [19], who found that onion and garlic had good effects in declining microbial counts, after had been mixed with pork belly and lion.

Calcium oxide is newly approved as food additive [1]. The TEM images as shown in Figure7 particles of the prepared CaO NPs with a varying size range from 15.5 to 41.7nm. The novelty in this research is the application of CaO nanoparticles in minced meat. Results demonstrated a reduction in APC, Enterobacteriaceae and yeast and mould counts when compared with control, which indicated that CaO nanoparticles have antimicrobial effect when added to minced meat. Therefore, addition of calcium oxide nanoparticles manages the microbial growth and improves the shelf life of minced meat. The reason of antibacterial effect of CaO Np is owed to the active oxygen and the alkaline pH when dissolved in water, which in turn cause destruction to the bacterial cell membrane and consequently death [18]. Oyster shell was employed in previous studies as a source of CaO to extend the durability of fried chicken and sardine balls [26], and pork sausage [27].

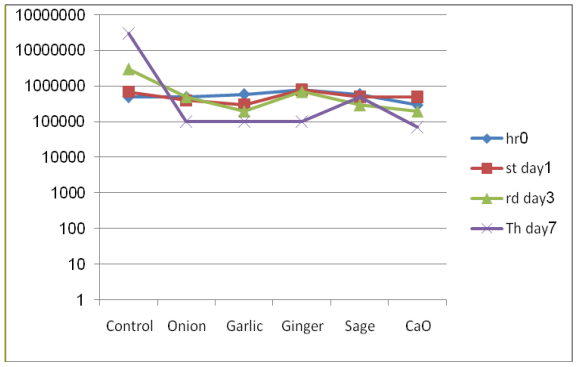

Figure4. *Effect of different treatments on APC of the stored minced meat*

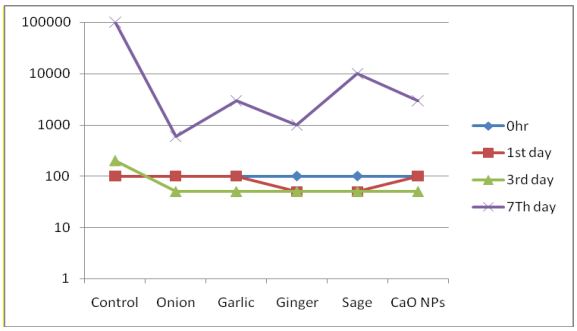

Figure5. *Effect of different treatments on Enterobacteriaceae count of the stored minced meat*

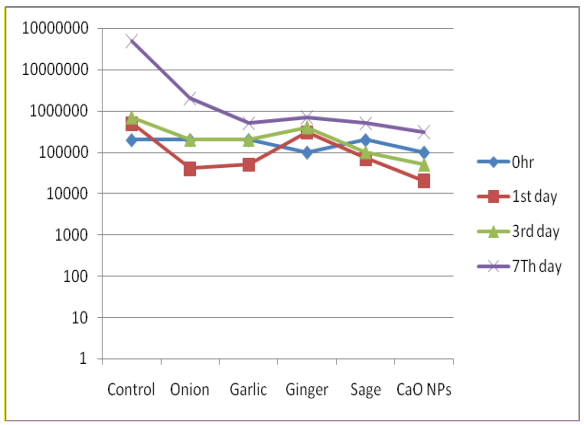

Figure6. *Effect of different treatments on yeast and mould count of the stored minced meat*

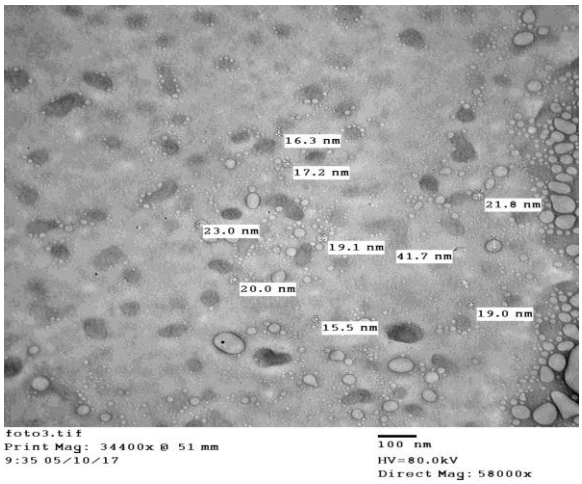

Figure7. *TEM image of prepared CaO NPs (CaO NPs in the range from 15.5-41.7nm)*

In vitro antibacterial effects of CaO nanoparticles and spices extracts on *E.coli* ATCC 8739 and *Salmonella typhimurium*

Agar immunodiffusion method was applied as a qualitative test for the antimicrobial susceptibility tested. The preliminary qualitative inhibition activities of sage, onion and garlic against *E.coli* ATCC 8739 were superior than ginger (15, 13, 12and 5 mm, respectively), while the diameter of the inhibition zone (DIZ) shown against *Salmonella typhimurium* were 12, 11 and 3 mm for onion, garlic and sage powders extracts, respectively (table 1). The minimum inhibitory concentration of onion, garlic, ginger and sage against *E.coli* ATCC 8739 were 31.25, 31.25, 62.5 and 15.63, while MBC were 62.5, 62.5, 125, and 31.25, respectively. MIC of onion, garlic and sage were 62.5, 62.5 and 15.63 mg/ml against *Salmonella typhimurium*, and MBC were125, 125 and 31.25 mg/ml, respectively, while ginger extract didn't provide any effect. Thus, Sage demonstrated the best result by microdilution broth method against both organisms. This result was in accordance with Witkowska *et. al.* [28].

This mean that agar diffusion test showed preliminary antimicrobial results, except for sage, agar diffusion test was indicative for *E.coli*, but wasn't indicative for *Salmonella typhimurium*, because microdilution broth method demonstrated acceptable result for sage against *Salmonella typhimurium*. Similarly, Witkowska *et. al.* [28] verified that the inhibitory actions of aqueous extracts of sage, onion and garlic were superior than ginger, and declared that heat treatment or autoclaving of the extract reduces the antimicrobial effect. On the contrary, Onyeagba *et. al.* [29] found that aqueous extract of garlic or ginger didn't show any inhibition to *E.coli* or *Salmonella*. Higher inhibition zone (16.5 ± 2.3 mm) for garlic powder aqueous extract was recorded by Krisch *et. al.* [25] against *E.coli*, but there was not any inhibition exposed by onion extract. Also, MIC values found by Panutat and Vatanyoopaisarn [30] for water extract of garlic against *E.coli* and *Salmonella*, were nearly with parallel inhibition zone diameters for both organisms, but they verified that the aqueous extract of onion showed zone of inhibition(only at MIC of 500 mg/ml) against *Salmonella sp.* Lesser diameters (ranged from 7 to 12 mm) of inhibition shown by Abdel-Salam *et. al.* [31] who stated that *Salmonella typhimurium* was more susceptible to aqueous extracts of red onion than *E.coliO157:H7* at concentrations of 40 and 60

mg/ml, but both organisms showed similar sensitivity to garlic extracts. Iwalokun et. al., [32] studied the effect of aqueous extract of garlic and found higher diameter of inhibition zones ranged from 19.8 to 24.5 mm for gram-negatives and MIC values varied from 22.9 to 37.2 mg/ml. Ahmad *et. al.,* [33] indicated that MIC of the garlic aqueous extract was 5% against *E. coli*, but the organism was resistant to the aqueous extracts of onion.

Ginger has the least antibacterial effect on *E. coli* ATCC 8739 and *Salmonella*. This agreed with Zaki *et. al.* [34] who found that crude extract of ginger has good antibacterial effect, but the effect is declined when the extract diluted by using agar well diffusion method. Nearly similar results recorded by Sofia *et. al.* [35] who found that MICs of aqueous extracts of garlic were ranged from 5 to 30 mg /ml against *E. coli*. Also, Ortiz [36] documented that onion has more obvious antibacterial effect than ginger against *E.coli* using MIC, MBC, disc diffusion and agar well diffusion methods.

In contrast to, Jolly and Menon [37] established that ginger has improved antibacterial effect than garlic.

In this study Garlic and sage have obvious antibacterial effects against *E.coli* ATCC 8739, and *Salmonella typhimurium* than ginger. This result also reported by Tarik *et. al.* [2]. On the other hand Sethi et al. [38] found that methanolic extracts of garlic and ginger gave inhibition zones of 12 and 11 mm, respectively and MIC were 55 and 65 mg/ml, respectively, using agar dilution method. They stated that the antimicrobial activity of spices is reduced in food due to fat and protein contents, and it is preferred to use spice combined with preservatives.

Calcium oxide nanoparticles showed equal DIZ (5mm) against *E.coli* ATCC 8739, and *Salmonella typhimurium* by agar diffusion method, whereas, MIC and MBC against *E.coli* were 250µg/ml and 300µg/ml, and against *Salmonella typhimurium* were 25µg/ml and 50µg/ml, respectively (table1). It is reported before that calcium oxide has antibacterial effect against *E. coli* and *S. typhimurium* at concentration of 0.05 % CaO solution for 10 minutes, and it is attributed to its alkaline pH [39]. Jeong *et. al.* [40] found that $CaCO_3$ nanoparticles converted to CaO nanoparticles after heat treatment, which provided bactericidal effects against *E. coli* and *S. typhimurium*. Gedda et al. [41] used shrimp shell as a source of calcium oxide nanoparticle, and they found that these nanoparticles have successful antibacterial effect against *E.coli.* , and the DIZ was 19 ± 2mm, MIC and MBC were 10µg/ml and 50µg/ml, respectively.

Table1. *Antibacterial effect of spices extracts and CaO NPs on E. coli ATCC 8739And Salmonella typhimurium*

Treatment	*E.coli* ATCC 8739			*Salmonella typhimurium*		
	DIZ (mm)	MIC (mg/ml)	MBC (mg/ml)	DIZ (mm)	MIC (mg/ml)	MBC (mg/ml)
Onion	13	31.25	62.5	12	62.5	125
Garlic	12	31.25	62.5	11	62.5	125
Ginger	5	62.5	125	-	-	-
Sage	15	15.63	31.25	3	15.63	31.25
Cao NPs	5	250 (µg/ml)	300 (µg/ml)	5	25 (µg/ml)	50 (µg/ml)

DIZ: diameter of inhibition zone

(-) Not detected

4. Conclusion

CaO NPs and garlic powder showed the best reduction effects on APC and yeast and mould count, but onion show the best reduction activity on Enterobacteriaceae count. It was concluded that CaO NPs had improved the shelf life when added to minced meat. Also, garlic powder and onion powder enhanced the shelf life. Moreover, CaO NPs solution, sage, garlic and onion extracts showed enhanced antibacterial effects against *E.coli* ATCC 8739 and *Salmonella typhimurium* than ginger extract. CaO NPs can be added for meat products as a multifunctional food additive.

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