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EFFECT OF CITRUS FRUIT (*CITRUS SINENSIS, CITRUS LIMON AND CITRUS AURANTIFOLIA*) RIND ESSENTIAL OILS ON PRESERVATION OF CHICKEN MEAT ARTIFICIALLY INFECTED WITH BACTERIA

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ABSTRACT

Essential oils (EOs) obtained from a wide variety of plants have become popular with increased scientific interest as potential natural agents for food preservation. Two concentrations of rind EOs (400 mg/ml and 200 mg/ml) from three species of citrus fruit; *Citrus sinensis* (Sweet orange), *Citrus limon* (Lemon), *and Citrus aurantifolia* (Lime) were used to treat fresh chicken meat inoculated with *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 20971 and *Salmonella enterica* ATCC 14028 to evaluate their protective abilities on bacteria-contaminated meat The EOs were extracted from the ground rinds by hydro-distillation. Alongside the EOs, sodium nitrate ($NaNO_3$) was used as a positive control preservative. A viable count was carried out to determine the bacteria load reduction on the inoculated fresh chicken meat. After 24 hours of treatment, the results showed that the EOs had no adverse effect on the physical attributes of the meat: the color and smell of the chicken meat were unaltered compared with the negative control (None EO and $NaNO_3$ treated meat) that showed evidence of putrefaction through color change and foul smell. The two- lime rind EOs concentrations used to treat the *Escherichia coli* ATCC 25922 inoculated meat reduced the viable count of the organism by 7.9 log compared to the *Escherichia coli* ATCC 25922-inoculated meat which received no rind EOs or $NaNO_3$ treatment. Other results showed that sweet orange (SO) rind EOs (400 mg/ml and 200 mg/ml) treatment of meat inoculated with *Salmonella enterica* ATCC 14028 had similar but mild preservative effects as both treatments reduced the log of the bacteria by 1.1 and 0.8, respectively. In comparison with $NaNO_3$, the EOs treatment had a significant ($p<0.05$) preservative effect on the bacteria-inoculated meats. Findings from this study, therefore, suggest that *Citrus* spp. rind EOs have good potential as natural preservative for chicken meat. However, notwithstanding the relative positive organoleptic results observed in this study, further investigations on the prolonged preservation effect of the EOs on the physical attributes of fresh chicken meat need to be undertaken.

Key words: Bacteria, Chicken meat, Citrus rind, Essential oil, Preservation, Sodium nitrate

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INTRODUCTION

Food, either raw or processed, should be wholesome and free of contaminants. Notwithstanding, foods are often exposed to physical, chemical, or biological contaminants that can cause undesirable changes, thus reducing quality as well as causing food-borne illnesses. Meat and meat products are highly perishable if not properly handled and so require standard aseptic processing, storage, and distribution techniques. More importantly, meat that is not to be consumed immediately after processing should be preserved to avoid microbial contamination. Meat is rich in protein, fat, essential amino acids, minerals, and vitamins [1]. The breakdown by microbial enzymes of lipids and proteins and the growth of microorganisms are major problems causing deterioration in meat, decrease in shelf life, public health hazards, and enormous economic losses [2, 3]. For a long time, meat has been preserved by salting, smoking, drying, irradiation, vacuum packaging, and use of chemicals such as sulfites, benzoic acid, sorbic acid among many others. The search for novel, natural, toxic-free, and more efficient preservatives to replace synthetic preservatives used in food is on-going worldwide [4]. Consequently, plant materials which have found use in food processing and flavouring since prehistoric times [5, 6] are now being vigorously investigated for use in food preservation. Among the plant materials of interest are the essential oils from fruits. Most of these serve as good decontaminating agents due to some inherent essential active phytoconstituents they contain and because they are Generally Recognized as Safe (GRAS) [7]. Among the bioactive compounds present in fruits and fruit by-products are Vitamins C and E, carotenoids, phenolic compounds, and dietary fibre [8]. These compounds are commonly found in fractions and most of them have a wide spectrum of antimicrobial activity against food-borne pathogens [9]. Extracts from *Citrus* spp. peels are rich in these bioactive compounds in addition to being a good source for natural antioxidants as seen in apples and berries [10, 11].

Plant compounds used as food additives for other reasons may also act as preservatives, preventing the growth of pathogens and spoilage microorganisms. Consumers are becoming more aware of the health benefits they provide by providing nutrients such as vitamins and natural antioxidants, and thus their use has become vital. [10, 11]

The citrus species are the most widely grown fruit crop worldwide, with the rind accounting for up to 50-60% of the total weight of the fruit [12]. Quite often, the rind is left unprocessed, thereby polluting the environment and constituting breeding grounds for insects [13, 14]. *Citrus spp*. rind has been extensively researched, from its use in livestock feed to the extraction of pectin and essential oils, the production of thickening and clouding agents, and its utility as a natural pigment for food or juice coloring [15, 16, 17, 18].

Thus, rinds from *Citrus* spp. are promising new sources of antimicrobial and antioxidant compounds and the EOs derived from them may be useful in food preservation.

Since not all synthetic food preservatives are GRAS for human consumption when used in food, the need to seek alternative sources of nontoxic food preservatives has become

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imperative. The artificial or synthetic food preservatives which may be man-made such as sodium benzoate or naturally occurring salts and sugars may act as antioxidants, make foods more acidic, reduce the food moisture content, slow down the ripening process and prevent the growth of microorganisms, hence, making the food last longer. International and national food regulatory agencies have recognized some food preservatives as causing unpleasant reactions such as diarrhea, abdominal flushing and pain, rashes, low blood pressure, asthmatic and anaphylactic shock in sensitive individuals and are potential causes of cancer [19].

In addition, the development of resistance by some food spoilage microorganisms to synthetic preservatives continuously used to preserve meat is now a major threat to food industries [20]. Synthetic preservatives have been linked as the cause of the mental disorder of the neurodevelopmental type, Attention Deficit Hyperactivity Disorder (ADHD), characterized by problems with paying attention, excessive activity or difficulty in controlling behavior which is not appropriate to a person's age [21]. Attention deficit hyperactivity disorder has been linked to the consumption of foods preserved with sodium benzoate, red food dye, and yellow food dye [22, 23].

Nitrate, a notable food preservative used in curing foods such as hotdogs and sausages, can destroy *Clostridium botulinum* but is known to cause chronic diseases like pancreatic and lung cancer [24]. These synthetic preservatives, among many others, are now known to be inimical to human health, including the risk of causing cancer, asthma, hives, and other allergic reactions, even though they can adequately preserve and extend the shelf life of foods. These observations make it imperative to continue with the search for new and nontoxic food preservatives, essential oils from plants being one of such.

This study evaluates the ability of EOs from various citrus rinds to protect meat from potential bacterial pathogens that cause gastroenteritis.

MATERIALS AND METHODS

Processing of Citrus Samples

Fresh *Citrus sinensis* (Sweet orange)*, Citrus limon* (Lemon), *and Citrus aurantifolia* (Lime) fruits were procured from noncommercial farms in Kwara State, Nigeria.

The fruits were thoroughly washed and rinsed twice in clean water and allowed to air dry. Rinds from each Citrus species, avoiding most of the mesocarp, were carefully cut and each species stored separately for further processing in clean sterile sealed jars to minimize dehydration.

Extraction of Essential oils (EOs) From the Rind

The rinds were pulverized into a pulp in a Waring electric blender (Eberbach Warring® Lab Blender 0379V66 Mfr No. E8120). The pulps obtained were transferred into sterile containers and labeled according to species. To aid extraction, 1g of pulverized rind was mixed in 10mls distilled water and allowed to stand for 6 hours before homogenizing by stirring in an orbital shaker (Stuart Orbital shaker – SSL1) at 150 rpm

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for 1hour. The homogenized pulps were packed in sterile clean muslin and pressed by wringing to obtain the crude filtrate. The crude extracts were then distilled at 60°C – 100°C in round- bottom flasks over a heating mantle attached to a condenser connected to a round- bottom flask to obtain the EOs. The collected EOs were stored in sterile bottles at 4°C – 8°C for use.

Drying the Extracted EOs

The extracted EOs were dried in small aliquots of 2mls to further remove residual water in the oils using the Lyotrap freeze dryer; LTE Scientific, Greenfield, UK for 12 hours. Dried EOs was later pooled together according to citrus species for use.

Preparation of *Citrus* Rind EOs and $NaNO_3$

Two concentrations of freeze-dried rind Eos were weighed and dissolved in 10 ml sterile distilled water in universal bottles to obtain concentrations of 200 mg/ml and 400 mg/ml, respectively.

A standard solution of 500 ppm/ml of $NaNO_3$ was prepared according to the method described by U.S. Food and Drug Administration for use as control [25].

Bacterial organisms

Freeze-dried bacteria organisms: *Escherichia coli* (ATCC 25922)*, Salmonella typhi* (ATCC 20971), *and Salmonella enterica* (ATCC 14028) used in this study were reconstituted in sterile physiological buffered saline (PBS) and sub-cultured in 2 ml amounts of nutrient broth (NB) contained in sterile 5ml plain bottles and incubated at 37°C for 24 hours. The bacteria organisms were then sub-cultured on blood and Mac-Conkey agar and incubated at 37°C for 24 hours for purity check. Following this, few colonies of each bacterium were emulsified in 10 ml PBS in 20 ml universal bottles and homogenised on a Vortex mixer (SA8, BioCote) until turbidity corresponded to McFarland turbidity standard of 10^3CFU/ml.

Processing of Chicken meat

A 3.1kg live weight, non-fatty chicken from a disease free flock was obtained and processed under controlled sanitary environment. Meat was cut from the nonfat blood-free flesh of the chicken breast quarter, drumstick, thigh, and wings mid-section aseptically in pieces weighing 50 g each. Some of the chicken parts used had the bones intact. These were surface sterilized using slow jets of sterile distilled water from a wash bottle to avoid injury to the meat. The washed samples were placed in clean sterile 100 ml beakers and covered with sterile aluminum foil paper.

Experimental Groups

Five experimental groups of chicken meat samples A, B, C, D, and E were adopted based on the three rind EOs extracts (lime, lemon, sweet orange), $NaNO_3$ (positive control) solution and PBS (negative control) treatments, respectively. Three replicates were used in the study.

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Bacteria Inoculation of Meat and Treatment with Rind Extracts, $NaNO_3$ and PBS
Meat samples in groups A, B, and C were prepared in duplicates to accommodate the two EO concentrations used for each citrus rind. The meat in each group was then inoculated with 10ml of 10^3CFU/ml in PBS of each bacteria species, respectively, and incubated at 37°C for 8 hours. After 8 hours, the excess inoculum was drained off the meats in the samples - A, B, C, D, and E. One of the subgroups of bacteria inoculated meat samples A, B, and C were sprayed with 10 ml; 200 mg/ml, and 400 mg/ml, respectively of the sterile PBS-reconstituted freeze-dried EO extracts of lime, lemon and sweet orange rinds.

The rind EO extract concentrations used were determined during preliminary studies that showed extract activity against the selected bacteria at concentrations ranging from 200 mg/ml to 500 mg/ml. The positive and negative control groups were sprayed with sterile PBS (pH 7.4) and 10 ml NaNO3 solution (500 ppm) correspondingly. After treatment with the relevant extracts, NaNO3, and PBS solutions, the tests and controls were re-incubated for 24 hours at 37°C. (Table 1).

Determination of Reduction of Bacterial Load
After incubation, meat from both test and controls were removed, carefully washed in sterile PBS (pH 7.4), wash-out, and decanted into clean sterile universal bottles. One milliliter (1ml) of each wash-out obtained was made up to 10 ml using sterile PBS solution.

A Log dilution from the wash-out was prepared in PBS for both test and control and 0.1ml of each dilution was seeded on nutrient agar plates in three replicates. The inoculated nutrient agar plates were incubated at 37°C for 24 hours and from these, mean values of the viable bacterial counts were obtained for analysis.

Physical Observations on the Meat Inoculated with Bacteria
The samples of meat were observed for characteristic colour changes that could arise from the microbial effects leading to meat spoilage by adopting the standard colour space system (L*a*b*) procedure of the International Illumination Commission [26]. Briefly, the colour variance was measured using the CIE coordinates for differences in lightness and darkness, and colour changes as observed in the control and test meat samples. Each of the samples was examined for the presence of foul smell before and after incubation.

Statistical analysis
Results for the bacterial counts were analyzed statistically using the Paired samples t-test at 95% level of significance.

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RESULTS AND DISCUSSION

Curbing microbial contamination of meat is now a big public health and industrial concern. In recent times, the application of bioactive natural compounds that have preservative potential for foods and with antimicrobial properties, especially food poisoning microorganisms, has received wide attention.

Effect of Citrus Rind EOs on Meat Inoculated with Bacteria

The rind Eos, on the other hand, inhibited the three pathogenic bacteria species used in inoculating the fresh meat to varying degrees, indicating a preservation effect on the chicken meat. The mean Log viable counts from the negative controls for *Escherichia coli, Salmonella typhi,* and *Salmonella enterica* were 7.9, 8.3, and 8.0, respectively. Viable Log counts of 4.8, 8.1, and 6.8 for Escherichia coli, Salmonella typhi, and Salmonella enterica were obtained from the NaNO3 controls, respectively (Figure 1).

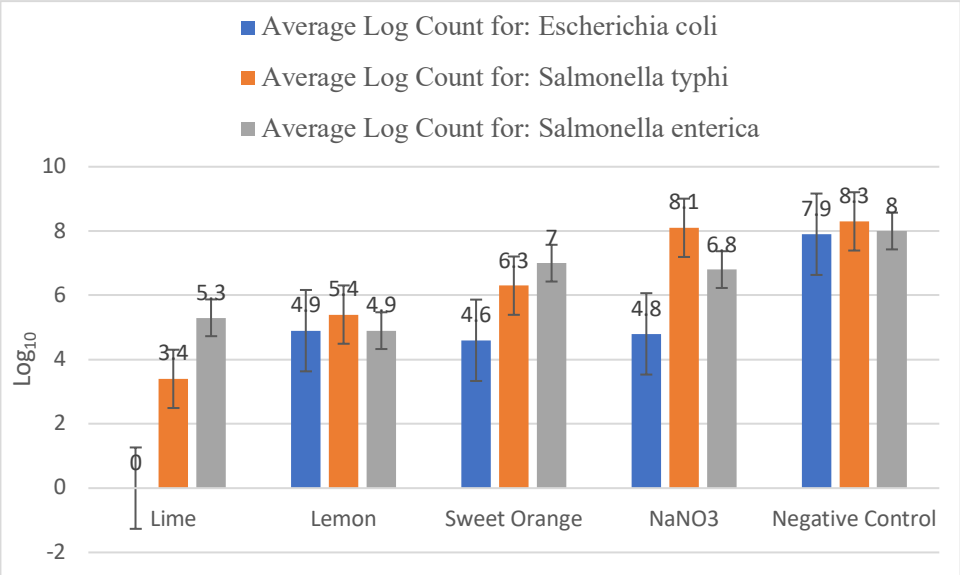

Figure 1: Mean log counts of bacteria organisms from citrus rind EOs (for the combined 400mg/ml and 200mg/ml) and sodium nitrate-treated infected meat

At the two concentrations of 400 mg/ml and 200 mg/ml lime rind EO treated infected meat for *Escherichia coli, Salmonella* typhi and *Salmonella enterica,* the mean log counts recorded were 0, 3.4, and 5.3, respectively; for lemon, mean Log counts of 4.9, 5.4, and 4.9 were observed, respectively for *Escherichia coli, Salmonella* typhi and *Salmonella enterica.* For the sweet orange rind EO-treated infected meat, average viable bacterial Log counts of 4.6, 6.3, and 7.0 for *Escherichia coli, Salmonella* typhi and *Salmonella enterica,* were recorded, respectively {Figure 1}.

Earlier reports indicated that in the wide spectrum of EOs, those of citrus plants are particularly interesting because they can be used in food as preservatives, antioxidants, and flavoring compounds. In addition, the antimicrobial properties of C*itrus* spp. rind EOs against some selected food poisoning bacterial and fungal organisms have been

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established, thus making them prospective food preservatives against spoilage microorganisms [30]. Findings from this study which investigated the citrus rind EO effect on meat preservation support these assertions. However, it may be difficult to draw any conclusions based on the apparent protective effect of the EO from the citrus rind on meat inoculated with *E. coli*.

What is encouraging is that this activity may be a pointer to the fact that more highly infectious *E. coli* strains and other enteric bacteria, often reported in food- associated disease outbreaks, may also be liable to destruction by lime rind EO.

In comparison to the negative control, the EOs from the citrus rind reduced the viable log counts of all three bacterial organisms used to infect the chicken meat, significantly by two concentrations (400 mg/ml and 200 mg/ml) of the citrus rind EOs: lime (*E. coli, Salmonella typhi, Salmonella enterica*) (p<0 [.05); lemon (*E. coli, Salmonella typhi, Salmonella enterica*) p< 0.05); Sweet orange (*E. coli, Salmonella typhi*) p<0.05). A similar result was obtained for $NaNO_3$ (*E.coli*) p<0.05) after 24 hours. Notably, bacterial log counts from the 200mg/ml citrus rind EOs treated inoculated meats were generally slightly higher than counts recorded from the 400 mg/ml citrus rind EOs treated infected meat (Figure 2). This gives an indication that citrus rind EO concentrations can be 200 mg/ml and possibly lower, for use in effective meat preservation.

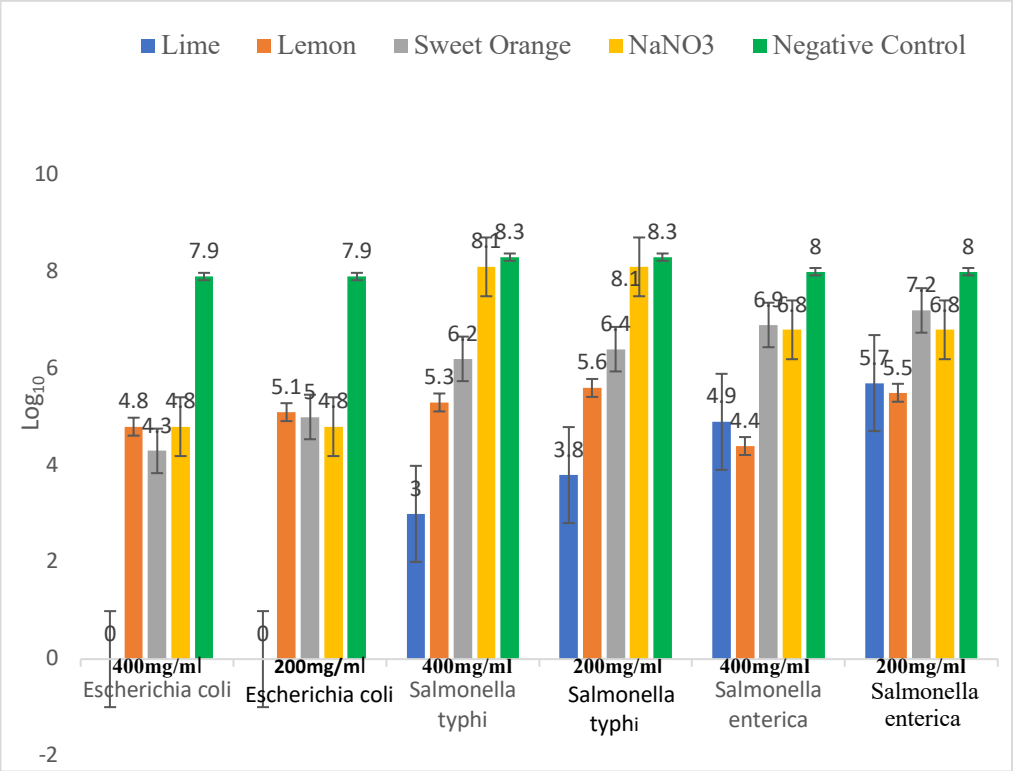

Figure 2: Mean log counts for bacteria organisms treated with 400mg/ml and 200mg/ml respectively of lime, lemon, sweet orange rind EOs and sodium nitrate

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The EOs from the rind extracts and $NaNO_3$ significantly ($p< 0.05$) reduced the number of *Escherichia coli* in the meat when compared against the negative control. The lime rind EO was significantly more inhibitory ($p<0.05$) on *Escherichia coli* than the EOs from lemon, sweet orange, and $NaNO_3$. All three had similar antimicrobial effects on the organism (Figure 2).

Only the lime and lemon rind EO extracts had a significant ($p<0$ .05) inhibitory effect on *Salmonella typhi* compared to the negative control. In addition, when compared to $NaNO_3$ control, the lime and lemon rind extract EOs showed a significant ($p<0.05$) inhibitory effect on *Salmonella typhi*, a known human pathogen (Figure 2).

Generally, the inhibitory activity of citrus rind EO, and $NaNO_3$ on *Salmonella enterica* was low compared to their effects on *E. coli* and *Salmonella typhi*. However, the lime and lemon rind extract EOs had a significant ($p<0.05$) inhibitory effect on the bacteria compared to the $NaNO_3$ (Figure 2).

The safety and quality of meat is evidently affected by the growth of microbes such as bacteria, molds, and yeasts in the meat. The prime effect is in the health hazard posed by such affected meat to human health. Additionally, microbial-contaminated meat results in significant economic losses [31].

As observed from this study, which did not test the antibacterial effect of rind EOs in chicken meat broth, citrus rind EOs, particularly from lime and lemon, significantly reduced the bacterial load of the infected meat. This finding is similar to other reports which showed that the EO from *Origanum vulgare* L (wild marjoram or sweet marjoram) when tested against *Staphylococcus aureus* showed mild anti-Staphylococcus activity on meat than in meat broth, as well as remarkably prevented spoilage in different types of food such as fish, meat, chicken, fruit and vegetables, dairy products and confectionery [32, 33]. Another interesting observation from this work was that the lime and lemon rind EOs were more efficacious than $NaNO_3$, an already known and widely used meat preservative. Overall, the rind EOs at concentrations of 400 mg/ml showed a better preservative effect than at 200 mg/ml. Notwithstanding, lime rind EO exhibited high preservation properties even at the lower concentration of 200 mg/ml, particularly against *E. coli*. The already established preservative $NaNO_3$, is generally used in the food industry to inhibit spoilage of meat by *Clostridium botulinum,* a pathogenic Gram-positive bacterium. This means that the mechanism of action of Gram positive bacteria may be different from that of Gram-negative bacteria such as those used in this research. The three bacterial organisms on which the rind EOs were tested are Gram negative bacteria and are potential causes of gastroenteritis. The ability of citrus rinds to inhibit their survival and multiplication on fresh chicken meat is therefore useful information. Furthermore, extensive studies on how to utilize these essential oils for meat preservation must now be undertaken to fully harness their inherent potential. Findings from this study also indicate that the use of citrus rind EOs has positive multiplier effects: from environmental decontamination of citrus rind waste to the fact that the EOs are organic and may be relatively safe to use as antimicrobial.

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A major concern that has been noted in the past is the toxicity of some compounds present in some EOs. Fortunately, however, a variety of EOs components have been registered by the European Commission for use as flavorings in food. Furthermore, meticulous toxicology studies were carried out on foods coated with *Citrus* spp. EOs support the safety of their application in food with no risk to human health. They have been considered GRAS by the U.S Food and Drug Agency (FDA) [34, 35]. It may, therefore, be safe to conclude based on their modes of action and from the observations made in this study that the *Citrus* spp. peels EOs are good novel preservatives which can be used to preserve chicken meat against potential food-borne bacterial pathogens and good replacements for cytotoxic synthetic preservatives such as $NaNO_3$ [32, 33].

The simple interaction involving the cell structure of organisms and microbial sensitivity to EOs is yet to be established; in addition, the possible antagonistic or synergistic effects the various active constituents of the oils express in living cells are yet to be understood [36].

Observations on Meat Inoculated with Bacteria

This study, which evaluated the preservative activity of EOs of rinds from *Citrus sinensis*, *Citrus lemon* and *Citrus aurantifolia* on chicken meat inoculated with *Escherichia coli* ATCC 25922*, Salmonella typhi* ATCC 20971 *and Salmonella enterica* ATCC 14028 showed that all the tested *Citrus* spp. peel's EOs had no adverse physical discoloration effect on the chicken meat. No foul smell from the meat was perceived from the citrus rind, EOs, and $NaNO_3$ treated meats when compared to the negative control samples, which showed obvious evidence of putrefaction through the International Illumination Commission (CIE) colour change and foul smell.

One of the major concerns with the use of plant EOs for food preservation are the changes they cause to the organoleptic properties of the food. Several authors have reported unpleasant changes to the organoleptic properties of foods coated with EOs of plants [25, 27, 28, 29]. However, as seen from this study, the EOs from the rind extracts did not impact negatively on the colour and odour of the meat 24 hours after treatment. Whether this may be time or concentration related is yet to be determined.

Acceptance of food preserved by chemicals or organic materials is often first determined by visual perception. With the visual perception of the preserved meat, there is the need for further work that may include a longer incubation time in the use of EOs from citrus rinds. This will provide more information that may help to assuage the concerns often raised due to changes in colour arising from the preservative use.

CONCLUSION

Due to the growing preference by consumers for natural ingredients over synthetic ingredients for food preservation, the EOs from peels of citrus fruits, which are widely available and always disposed of as waste in the environment, hold some promise in food preservation. The application of the active principles in citrus rind essential oils in

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functional food development and in other biological materials that may be useful in food preservation will be of immense benefit.

Competing Interest

The authors declare no competing interest.

Authors' contributions

Irokanulo Emenike O conceived and designed the study, interpreted the data and prepared the final draft; Oluyomi Bankole W contributed to the initial draft and wrote the results, Nwonuma Charles O carried out the data analysis. All authors approved the final draft.

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Table 1: Protocol for Bacterial Inoculation of Chicken Meat and Treatment with Rind EOs Extract, NaNO3, and PBS

	Treatment with Citrus Rind EOs, $NaNO_3$ and PBS (Negative Control)							
	Lime (A)		Lemon (B)		Sweet Orange (C)		$NaNO_3$ (D) (500 ppm)	PBS (E)
Bacteria organisms	200 mg / 400 mg		200 mg / 400 mg		200 mg / 400 mg			
E.coli	**	**	**	**	**	**	*+	*-
S.typhi	**	**	**	**	**	**	*+	*-
S.enterica	**	**	**	**	**	**	*+	*-

** Meat inoculated with bacteria and treated with Rind EO Extracts
*+ Meat inoculated with bacteria and treated with $NaNO_3$ (500 ppm) only (Positive control)
*- Meat inoculated with bacteria and treated with PBS (Negative control)

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