Original Research Article

Phytochemical compositions and Antimicrobial activities of *Citrus sinensis* and *Citrus aurantifolia* peel on Selected Pathogenic Bacteria isolated from Jollof Rice

ABSTRACT

Aims: The aim of this study is to investigate the antimicrobial activities of aqueous and ethanolic extracts of orange (*Citrus sinensis*) and lime (*Citrus aurantifolia*) peels on some selected pathogenic bacteria isolated from jollof rice.

Study design: Antimicrobial analysis, phytochemical analysis

Place and duration of study: Biology Department Laboratory, Wesley University of Science and Technology, Ondo, Ondo State, Nigeria, between June 2009 and July 2017

Methodology: Antimicrobial analysis of aqueous and ethanolic extracts prepared from orange and lime peels were done by using the agar well diffusion method against the selected pathogenic bacteria. The extracts were screened for anti-nutrients such as alkaloids, tannins, oxalate, phytate and glycosides.

Results: The ethanolic extracts of orange peel showed a remarkable zone of inhibition against *Escherichia coli* (23.5 ± 0.1 mm) followed by *Staphylococcus aureus* (11.4 ± 0.0mm) and *Bacillus cereus* (9.8 ± 0 mm). Whereas, the aqueous extracts of orange showed no zone of inhibition against the tested pathogenic bacteria. In addition the ethanolic peel extract of lime showed maximum zone of inhibition against *Staphylococcus aureus* (15.5 ± 0.0 mm) followed by *Escherichia coli* (14.3 ± 0.1 mm) and *Bacillus cereus* (12.1 ± 0.2 mm), whereas its aqueous peel extract showed no zone of inhibition against *K. pneumonia, S. aureus, E. coli* and *B. cereus.* Both ethanolic extracts of orange and lime peels showed no zone of inhibition against *K. pneumonia.* Streptomycin, the reference antibiotic, had no zone of inhibition against *B. cereus* and *S. aureus* whereas it recorded maximum zone of inhibition against *E. coli* (24.0 ± 0.0 mm) and *K. pneumonia* (25.1 ± 0.1 mm). The phytochemical analysis showed presence of oxalate, alkaloids, phytate, tannins and glycoside in the aqueous and ethanolic extracts of lime and orange peels. The antimicrobial activities of ethanolic extracts of both lime and orange peels demonstrated inhibitory effect against the targeted organisms such as *B. cereus, S. aureus* and *E. coli*.

Conclusion: The exploration of novel antimicrobial agents from natural resources such as plant like Lime and sweet orange as food preservative is due to the presence of various secondary metabolites.

Keywords: Agar well diffusion, Phytochemical constituents, Antimicrobial activities, *Citrus sinensis*, *Citrus aurantifolia*, Pathogenic bacteria

Introduction

Sweet orange (*Citrus sinensis*), the tasty, juicy fruit, belonging to the family Rutaceae and subfamily *Aurantioideae* is a small evergreen tree 7.5 m high and in some cases up to 15 m (**Etebu and Nwauzoma, 2014**). It is commonly called orange and a major source of vitamins, especially vitamin C, sufficient amount of folacin, calcium, potassium, thiamine, niacin and magnesium (**Etebu and Nwauzoma, 2014**). Sweet orange is the second most important and widely grown fruit crop after banana, with total global production reported to be more than 180 million tons (**Etebu and Nwauzoma, 2014**). Economically, trades of orange fruits worldwide generate about 105 billion dollars per year all over the world. Orange is widely grown in Nigeria and many other tropical and subtropical regions for its nutritional and medicinal properties (**Etebu and Nwauzoma, 2014; Parle and Chaturvedi, 2012**). The major medicinal properties of orange have been reported to include anti-bacterial, anti-fungal, anti-diabetic, cardio-protective, anti-cancer, anti-arthritic, anti-inflammatory, anti-oxidant, anti-Tubercular, anti-asthmatic and anti-hypertensive (**Parle and Chaturvedi, 2012**). Oranges are generally available from winter through summer with seasonal variations depending on the variety.

Lime (*Citrus aurantifolia*) also belonging to the family Rutaceae, it is a small evergreen, shrubby and ever bearing tree, about 5 m tall, that is densely and irregularly branched and possesses short and stiff spines (thorns) (**Enejoh *et al.* 2015**). It is commonly called Lime (Nigeria), Key lime, Mexican lime, Sour lime, Dayap, bilolo, Indian lime, Egyptian lime (**Enejoh *et al.* 2015**). *C. aurantifolia* is widespread in tropical and subtropical regions around the World such as North America (Florida, Texas, California, Mexico, *etc*.), India, Egypt, and Central America (**Sandoval-Montemayor *et al.* 2012**). Lime is used in traditional medicine as an antiseptic, anthelmintic, mosquito bite repellent, tonic, antiscorbutic, astringent, diuretic, headache, arthritis, digestive and appetite stimulant, and for colds, coughs and sore throats (**Sandoval-Montemayor**

et al. **2012**). In addition, essential oils derived from lime are used as flavoring agents in beverages, foods manufacturing, pharmaceutical products and as ingredients in perfumes (**Sandoval-Montemayor *et al.* 2012**).

The demand for novel antimicrobial agent source from nature for food preservation has been on the increased worldwide (**Dabesor *et al.* 2017**). These antimicrobial agents with potential benefits over synthetic antimicrobials have been defined as the agent that kill or inhibit the growth of other microorganisms (**Dabesor *et al.* 2017**). The exploration of novel antimicrobial agents from natural resources such as plant or plant products and others has been used mainly for treating diseases, food safety and food preservation purpose (**Ashok *et al.* 2015**). In addition to the used of citrus in food industry for juice production, citrus processing by-products such as the peels are rich sources of secondary metabolites, which are able to exhibit inhibitory effect against the growth of most pathogens (**Ashok *et al.* 2015**). *Escherichia coli, Salmonella species, Shigella species, Klebsiella pneumonia, Vibrio species, Clostridium botulinum, Enterococcus species* are few examples out of many known food borne pathogens. Also there is a rapid increase in food borne illnesses caused by the presence of food borne pathogens in food either due to food contamination, food spoilage or mishandling of food. But use of natural antimicrobial agents may prevent or extend the time duration required for spoilage of food (**Dabesor *et al.* 2017**). Antimicrobial activities of solvent extracts and oils from citrus peel have been demonstrated in several literatures, but there are little or no knowledge of the antimicrobial activities of aqueous and ethanolic extracts of orange (*Citrus sinensis*) and lime (*Citrus aurantifolia*) peels on selected food borne pathogens. Therefore, the objectives of these studies are: to assess and compare antimicrobial activities of both aqueous and ethanolic extracts of *C. sinensis* and *C. aurantifolia* peels on selected pathogenic bacteria isolated from jollof rice, and to determine the anti-nutrients composition of both aqueous and ethanolic extracts of *C. sinensis* and *C. aurantifolia* peels.

Materials and Methods

Collection of plant materials and preparation of plant extracts

Fresh fruits of Orange (*Citrus sinensis*) and Lime (*Citrus aurantifolia*) used in this study were purchased from the local market in Ondo town, Ondo state, Nigeria.

Extracts were prepared as described by **Harbone** (**1998)** with slight modifications. The peels were removed and carefully washed under running water, followed by sterile distilled water. They were then air dried at room temperature for 14 days and pulverized to fine powder using a sterilized electric blender, then stored in air-tight bottles. The solvents used for the extraction were 98% ethanol and cold distilled water. Exactly 20g each of the dried powder of the two peels were separately soaked in 100 and 200 ml of (98%) ethanol and cold distilled water, respectively. Each solution was allowed to stand for 72 hours, after which they were sieved with a muslin cloth and filtered using No. 1Whatman filter paper. The filtrates were collected in a beaker and concentrated in a vacuum at a temperature below 40°C using a rotary evaporator (Heidolph, VE-11). The resulting crude extracts obtained were exposed to UV rays for 24 hrs to check for sterility on nutrient agar plates.

Anti- nutrients composition of the Plant Extract

The extracts were screened for anti-nutrients such as alkaloids, tannins, oxalate, phytate and glycosides in accordance with **Trease and Evans** (**2004**).

Sources of Microorganisms

The microorganisms used for this study were isolated from food samples (Jollof rice). The food samples were obtained from three randomly selected restaurants in Wesley University Ondo, Ondo State, Nigeria in sterile plastic container (labelled with appropriate letters and numbers) and transported to the University's Microbiology laboratory within 60 minutes of collection and kept for 72 hours for microbiological analysis.

Isolation of Microorganisms

Preparation of Culture Media

The culture agar media used for the isolation were prepared according to the manufacturer's specification.

Culture Preparation

The samples were inoculated in triplicate on Eosin Methylene blue agar, Salmonella-Shigella agar, McConkey agar and Nutrient agar media as base media. The streak plate method was used for plating. Briefly, a grain of 72 hours old food samples (jollof rice) was picked and smeared over one corner of the solid medium. The wire loop was sterilized over a Bunsen flame, cooled

and used to make parallel streaking from the main inoculated plate. The plates were then incubated at 37ºC for 24 hours and analyzed.

Identification of Microorganisms

The isolates were identified by standard methods **Buchanan and Gibbon** (**1974**). Biochemical tests for sugar fermentation, starch hydrolysis test, catalase test, coagulase test, and indole test were carried out for further identification.

Evaluation of Antibacterial Activity by disc diffusion method

The antimicrobial activities of aqueous and ethanolic extract of the peel of *C. sinensis* and *C. aurantifolia* extracts were determined by the Agar Well diffusion method as described by **Esimore *et al.*** (**1998**). Nutrient agar plates were prepared to evaluate the Antimicrobial Activity of aqueous and ethanolic extracts of the peel of *C. sinensis* and *C. aurantifolia* against isolated pathogenic bacteria. 0.05ml inoculums of isolated bacteria in sterile distilled water was uniformly spread on nutrient agar plates with the help of glass spreader, after five minutes 8.0 mm diameter well was bored in the plates with the help of sterile cork borer. 0.05 ml of 20 mg/ml aqueous and ethanolic fruit extracts and standard antibiotic; streptomycin (1.5 mg) were poured into the well with the help of sterile syringe. The plates were allowed to diffuse for about 30 min and then transferred to the incubator. The plates were incubated at 37^{o}C for 24hr. and after incubation plates were observed for the zone of inhibition.

Results

Table 1 shows the isolated organisms and some biochemical characteristics of the isolates. From the results, the bacteria isolates were *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus.*

Table 1. Isolated microorganisms and some biochemical characteristics.

Biochemical test	Organisms			
	Escherichia coli	***Bacillus cereus***	***Klebsiella pneumoniae***	***Staphylococcus aureus***
Catalase	+	+	+	+
Coagulase	`+	+	-	+
Starch hydrolysis	+	+	+	-
Sugar fermentation	**AG**	**AG**	**AG**	**AG**
H_2S production	+	+	+	+
Voges proskauer	-	-	-	-
Indole	-	-	-	-
Gelatin hydrolysis	-	+	+	+
Gram reaction	**-ve**	**+ve**	**-ve**	**+ve**

Key: (+) = Positive reaction, (-) = Negative reaction, (-ve) = gram negative, (+ve) = Gram posiive, AG = Acid and gas production.

Quantitative screening of some extracted phytochemicals show that the extracts of orange and lime peels contained alkaloids, oxalate, tannins, phytate and glycosides. The values of ethanolic extracts of lime and orange for alkaloid, oxalate, tannins, phytate and glycosides were (11.65 mg/g, 1.22 mg/g, 2.91 %, 5.85 mg/g and 0.17 mg/g) and (12.20 mg/g, 0.84 mg/g, 1.34 %, 6.33 mg/g and 1.45 mg/g), respectively. The values of aqueous extracts of lime and orange for alkaloid, oxalate, tannins, phytate and glycosides were (9.10 mg/g, 1.54 mg/g, 3.48 %, 4.70 mg/g and 0.11 mg/g), respectively (Table 2).

Table 2: Quantitative analysis of phytochemicals of *C. sinensis* and *C. aurantifolia* peels

Extract	Alkaloid (mg/g)	Oxalate (mg/g)	Tannin (%)	Phytate (mg/g)	Glycoside (mg/g)

AEL	**9.10 ± 0.03**	**1.54 ± 0.01**	**3.48 ± 0.01**	**4.70 ± 0.05**	**0.11 ± 0.02**
AEO	**14.25 ± 0.03**	**0.56 ± 0.0**	**0.86 ± 0.01**	**5.95 ± 0.0**	**2.18 ± 0.0**
EEL	**11.65 ± 0.41**	**1.22 ± 0.03**	**2.91 ± 0.12**	**5.85 ± 0.03**	**0.17 ± 0.03**
EEO	**12.20 ± 0.15**	**0.84 ± 0.05**	**1.34 ± 0.09**	**6.33 ± 0.03**	**1.45 ± 0.03**

Values are means ±standard deviation for three samples. Legend: SD = Standard deviation; AEL= Aqueous extract of lime peel; AEO = Aqueous extract of orange peel; EEL= Ethanolic extract of lime peel; EEO = Ethanolic extract of orange peel.

Table 3 shows the antimicrobial activity of aqueous and ethanolic extracts of *C. sinensis* peel (orange). Aqueous peel extract of orange showed no inhibitory effect against all the tested microorganisms. Meanwhile, its ethanolic peel extract resulted in a remarkable inhibition zone against *E. coli* (23.5 ± 0.1 mm) followed by *S. aureus* (11.4 ± 0.0 mm) and *B. cereus* (9.8 ± 0.0 mm). No inhibitory effect was recorded against *K. pneumoniae*.

Table 3: Antimicrobial activity of aqueous and ethanolic extracts of *C. sinensis* (Orange) peel on the tested microorganisms

	Zones of inhibition zone (mm)		
Microorganisms	**Streptomycin (1.5mg/ml)**	**Aqueous extract**	**Ethanolic extract**
B. cereus	**-**	**-**	**9.8 ± 0.0**
E. coli	**24.0 ± 0.0**	**-**	**23.5 ± 0.1**
K. pneumoniae	**25.1 ± 0.1**	**-**	**-**
S. aureus	**-**	**-**	**11.4 ± 0.0**

Observations are expressed as means ± standard deviation (SD) for three samples, (-) represents no inhibition.

Table 4 shows the antimicrobial activity of aqueous and ethanolic extracts of *C. aurantifolia* peel (lime). Aqueous peel extract of orange showed no inhibitory effect against all the tested microorganisms. Ethanolic peel extract of lime resulted in a remarkable inhibition zone against *S. aureus* (15.5 ± 0.0 mm) followed by *E. coli* (14.3 ± 0.1 mm) and *B. cereus* (12.1 ± 0.2 mm). No inhibitory effect was recorded against *K. pneumoniae*.

Table 4: Antimicrobial activity of aqueous and ethanolic extracts of *C. aurantifolia* (Lime) peel on the tested microorganisms

Microorganisms	Zones of inhibition zone (mm)		
	Streptomycin (1.5mg/ml)	Aqueous extract	Ethanolic extract
B. cereus	-	-	**12.1 ± 0.2**
E. coli	**24.0 ± 0.0**	-	**14.3 ± 0.1**
K. pneumoniae	**25.1 ± 0.1**	-	-
S. aureus	-	-	**15.5 ± 0.0**

Observations are expressed as means ± standard deviation (SD) for three samples, (-) represents no inhibition.

Discussion

Microorganisms isolated from the jollof rice samples in this study have been earlier reported by **Okolie *et al.*** (**2012**). The biochemical test performed on the isolated microorganism reveals that out of the four isolates two were gram positive (*B. cereus* and *S. aureus*) while the other two are gram negative (*E. coli* and *K. pneumoniae*). These pathogenic organisms in addition to others release toxins, which are the agents responsible for illnesses such as diarrhea, dysentery, nausea and vomiting, caused by these organisms upon consumption of the contaminated foods (**Okolie *et al.* 2012**).

Drugs used by people in ancient time are mostly prepared by extraction with water, because they do not usually had access to more lipophilic solvents (**Hussain *et al.* 2015**). This is of concern, as all the active compound(s) that are present in the plant are not all extracted by most healers and consequently the prepared drug might not contain all the pharmacologically active compounds. The obtained results of phytochemical analysis indicated the presence of alkaloid, oxalate, tannins, phytate and glycosides. Phytochemicals are secondary metabolites produced by plants that fight against microorganisms in their environment (**Dabesor *et al.* 2017**). There are variations in the phytochemical constituents; this may be due to its solubility in the solvents used for extraction. **Ngele *et al.*** (**2014**) stated that phytochemical constituents of the extracts are known to be biologically active and therefore aid in the antimicrobial activities.

In this study, the ethanolic extracts of the peels of orange and lime fruits showed greater antibacterial activity as compared to their water extracts with no antibacterial activity against the tested food borne microorganisms. *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumonia* and *Staphylococcus aureus* were found to be resistant with aqueous extracts of both lime and orange fruits peels, but showed antibacterial activity against *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus* with ethanolic extract. *Klebsiella pneumoniae* was found to be also resistant with the ethanolic extract of both lime and orange fruits peel. The ethanolic extract of orange fruit peel exhibited a remarkable zone of inhibition against *E. coli* (23.5 ± 0.1 mm) followed by *S. aureus* (11.4 ± 0.0 mm) and *B. cereus* (9.8 ± 0.0 mm) compared to *K. pneumoniae* with no zone of inhibition. While the ethanolic extract of lime fruit peel also showed remarkable zone of inhibition against *S. aureus* (15.5 ± 0.0 mm) followed by *E. coli* (14.3 ± 0.1 mm) and *B. cereus* (12.1 ± 0.2 mm) compared to *K. pneumoniae* with no zone of inhibition. This research work is in agreement with Nisha *et al*. (**Nisha *et al.*, 2015**) and Nair *et al.* (**Nair *et al.* 2005**) who also reported better antibacterial activity with orange peel extract prepared in organic solvent. Nisha *et al*. reported that the potency of citrus fruit peel is enhanced by the type of solvent used indicating that there are some active ingredients in orange peel which have high antimicrobial effect but which would not be released except when orange fruit peel is used in conjunction with a particular solvent(**Nisha *et al.* 2015**).

Notably, the zone of inhibition of the ethanolic extracts of orange and lime fruits peel against *S. aureus* and *B. cereus* are higher than the control (Streptomycin) with no zone of inhibition, these findings corroborates the potentials of plant extracts for antibacterial activity. In this study the antimicrobial activity against gram negative (*E. coli* and *K. pneumoniae*) and gram positive (*B. cereus* and *S. aureus*) bacteria is an indication of the broad spectrum activity of the orange and lime peel extracts.

The variation in the antimicrobial activity of the two extracts (Tables 3 and 4) showed that different extracts may have varying antimicrobial agents with different modes of action and bacteria susceptibility or that not all phytochemicals that are responsible for antibacterial activity are soluble in a single solvent (**Dabesor *et al.* 2017**).

Conclusion

The study suggested that the ethanolic extract of *C. sinensis* and *C. aurantifolia* peels have varying degrees of antimicrobial activity against some tested food borne pathogen such as *E. coli*, *S. aureus* and *B. cereus*. This suggests that the ethanolic extracts of both fruit peels can be of beneficial effect in developing a preservant that can be used in preserving food against food borne pathogens. The results also revealed the presence of bioactive phytochemicals in the peels of both fruits, in which evidences gathered in earlier studies have confirmed to be medicinally important. Therefore, the peels of orange and lime fruits could be used as a good source of antibacterial agent against food borne pathogens.

Conflict of interest: The authors declared no conflicts of interest.

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