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Original article

# Antibacterial potency of the coat of *Citrus sinensis* (orange) on *Bacillus species*

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A B S T R A C T

**Background:** In other to curb the resurgence of drug resistance, there is need to find cheaper, environmental friendly and readily available alternative chemotherapy for all patients for all ages. Extracts from plants has proven to be potent against wide range of pathogen such as bacteria, viruses, fungi and parasites of which *Citrus sinensis* is an important example. Both the aqueous and ethanoic extracts of *Citrus sinensis* were evaluated on some *Bacillus* species which were isolated from vegetables in nearby farm around the Oke Ogun Polytechnic Saki. **Aim:** The aim of this study was to evaluate the phytocompounds and antimicrobial activities of the coat of *Citrus sinensis* on some *Bacillus species*. **Methods:** The aqueous and ethanoic extract of the dried plant coat was evaluated for its antimicrobial activities on the isolated organisms using Kirby bauer disc diffusion method. The isolates were subjected to molecular identification using the polymerase chain reaction and subsequent Sanger sequencing techniques. There was high correlation between the isolates' identity at all the levels and sequencing except in few. The FASTA format of the nucleotides sequences were submitted at the GenBank at NCBI database for accession numbers which were MW362290 (*Bacillus cereus*), MW362291 (*Bacillus cereus*), MW362292 (*Bacillus weidmanii*), MW362293 (*Bacillus cereus*), MW362294 (*Bacillus cereus*) and MW362295 (*Bacillus thuringiensis*). **Results:** The active phytocompounds present in the aqueous and ethanol solvent were saponin, alkaloid, flavonoid, tannin, coumarin, steroid, terpenoid, cardiac glycosides, quinones, anthraquinones and phenol while Anthocyanin was present only in aqueous extract. These extracts both showed high antibiotics activities on all the isolated organisms. All the extracts recorded considerable antibiotic potency against all the isolates making the extracts of *Citrus sinensis* coat a suspect containing active biocidal agents. **Conclusion:** The coat of *Citrus sinensis* showed high antimicrobial properties against some *Bacillus* species, the antimicrobial effects was as a result of active phytochemicals present in the coat. This study established biocidal activities in both aqueous and ethanolic extracts. Its utilization for its pharmaceutical would be of great advantage both economically and environmentally.

## Introduction

The use of antimicrobial drug in the treatment of diseases has lead to multiple drug resistance in human [1]. Drug resistance occurred at a very high rate and hence, there is a need to develop a cheaper, non resistant alternative drug to treat bacteria and other infectious pathogen with little or no toxicity [2]. Bacterial infection is one of the

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important causes of health problems, physical disabilities and mortality around the world. Medicinal plants are rich in antimicrobial constituents and it provides a safer and cost effective way of treating bacterial infection. The antibacterial constituents of natural products from medicinal plants are used for the treatment of bacteria, fungi and viral disease. In the last three decades, pharmacological industries have produced a number of new antibiotics even though there is an increased in microorganisms resistant [3]. Secondary metabolites such as phenols, flavonoids, alkaloids, terpenoids and essential oils have proved to contain antimicrobial activity of plants [4].

Orange; which is an evergreen tree which grow between 7.5-15 Metres high was originated from southern China and it belongs to the family *rutacae* and it is called *Citrus sinensis* botanically. It is a tasty, juicy fruit with high medicinal value. It is found in the tropics and subtropics climatic region with the production rate of about 120million tons globally. One of the most essential fruit crops is Citrus which was commercially cultivated in all continents of the world [5].

The coat of *Citrus sinensis* bears high amount of flavonoids and three flavonoids are reported to present in citrus fruits are flavones, flavonones and flavonolds. The main compounds of flavonoids present in Citrus *sinensis* are narirutin, hesperidium, naringin and eriocitrin [6]. In reference to epidemiological studies of human being Citrus flavonoids can reduce the risk of coronary heart disease [7].

The coat of *Citrus sinensis* extract had been reported of its medicinal importance in the treatment of cancer, diuretic, lmmuno enhancing, colic, upset stomach, skin, weight loss and prevention kidney stone. It was also reported to be useful in the treatment and prevention of vitamin deficiency, cold, flu, scurvy and fight viral and bacterial infection. Citrus peel is also used as fodder in fisheries, raw materials for traditional paper, activated carbon, and cosmetics product and bio-ethanol production [8]. Likewise essential oil of citrus coat has been identified to exhibit antibacterial activity [9] and was effective against some enteric pathogens and *Klebsiella pneumonia* [5].

Therefore, this research work aimed to evaluate the phytocompounds and antimicrobial activities of the coat of *Citrus sinensis* on some *Bacillus species.*

## Materials and Methods

### Collection of orange coat

Eighty Fresh oranges were brought from Sango a local market in Saki Oyo state in Nigeria. The oranges were washed thoroughly with clean water, dried with clean towel and the coat of the oranges were removed, air dried and ground into powder using electric blender and stored in a air tight bottle until required for use.

### Extraction process

Using cold maceration method, One hundred and fifty grams (150g) of the grounded sample was soaked in 300 ml of ethanol of analytical grade for 48hours. The solution was filtered using sterile muslin cloth and whatsman No1 filter paper. Also for the aqueous extraction; One hundred and fifty grams (150g) of the grounded sample was soaked in 300 ml of distilled water and boiled for 20 minutes at 35°C; the solution was allowed to cool and filtered using sterile muslin cloth and whatsman No 1 filter paper. The recovered filtrate was concentrated using rotary evaporator at 45°C. The extract was weighed and stored in well stopper bottles and kept in refrigerator at 4°C until require for use [10].

### Phytochemical screening

### Determination of phytochemical contents of the orange coat

#### *Tannin contents*

0.1 ml of the *Citrus sinensis* extract was introduced into a volumetric flask (10 ml) containing 7.5 ml of distilled water and 0.5 ml of *Folin-Ciocalteu* Phenol reagent, 1 ml of 35 % Na2CO3 solution and dilute to 10 ml with distilled water. The mixture was well agitated and kept at temperature of 25c for 30 mins. Sets of reference standard solutions of Gallic acid (20, 40, 60, 80 and 100µg/ml) were prepared as mentioned earlier. The test absorbance and standard solutions were measured against the space at 725 nm with an ultraviolet/Visible spectrophotometer. The tannin present was expressed in terms of mg of GAE /g of extract [11].

#### *Saponin contents*

Twenty gram (20g) of the grounded dried sample peel was measured into a conical flask containing 100 $cm^3$ of 20% aqueous ethanol. The samples were heated in a hot water bath for 4h with continuous stirring at the temperature of about 55°C. The mixture was filtered and the residue was re-extracted with another 200 ml 20% ethanol. The combined extracts were evaporated in order to be

reduced to 40 ml over water bath at about 90°C. The concentrate was later transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and mixed vigorously. The aqueous layer was retrieved while the ether layer was thrown away. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight; the saponin present was calculated as percentage [12].

***Alkaloid contents***

Five grams (5g) of the dried sample peel was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 hours. The mixture was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added in drop to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue was dried and weighed, and it was the alkaloid [13].

***Total flavonoid contents***

Determination of flavonoid was carried out by measuring the aluminum chloride colorimetric assay. The mixture consists of 1 ml of extract and 4 ml of distilled water was introduced in a 10 ml volumetric flask. To the flask, 0.30 ml of 5 % sodium nitrite was added and after 5 minutes, 0.3 ml of 10 % aluminum chloride was also added and mixed. After 5 minutes, 2 ml of 1M Sodium hydroxide was treated and diluted to 10 ml with distilled water. A set of reference standard solutions of quercetin (20, 40, 60, 80 and 100μg/ml) were prepared in the same manner as mentioned earlier. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with an UV/Visible spectrophotometer. The total flavonoid content was expressed as mg of QE/g of extract [11].

***Glycosides contents***

0.5g of the grinded *Citrus sinensis* dried coat was dissolved in 2ml of Glacial acetic acid which contains one drop of ferric chloride solution, in a test tube which previously contained 1 ml of concentrated Tetraoxosulphate IV acid. Formation of brown ring indicates the presence of glycosides [10].

***Determination of phytosterols contents***

Salkowski's test: Few drops of the extract was treated with chloroform in a test tube and filtered. Then few drops of concentrated sulphuric acid was added to the filtrate and allowed to stand for 2-3 minutes. Formation of golden yellow color indicates the presence of triterpenes [14].

***Protein and amino acids test***

**Xanthoproteic test:** 2ml of the extract was measured in a test tube, few drops of concentrated nitric acid was added to it. Formation of yellow color indicates the presence of proteins [14].

***Quinones test***

2ml of the extract was measured in a test tube, few drops of concentrated hydrochloric acid was added and was observed for yellowish precipitation [15].

***Terpenoids test***

2ml of the plant extract was measured in a test tube containing few drops of chloroform. Then, few drops of concentrated sulphuric acid was added. Formation of reddish brown precipitate indicates the presence of terpenoids [15].

***Determination of total phenolic contents***

The concentration of phenols in plant extracts was determined using spectrophotometric method. *Folin-Ciocalteu* assay method was used for the determination of the total phenol present. The reaction mixture consists of 1 ml of the extract and 9 ml of distilled water in a volumetric flask (25 ml). One milliliter of *Folin-Ciocalteu* phenol reagent was treated to the mixture and well agitated. 5 minutes later, 10 ml of 7 % Sodium carbonate ($Na_2CO_3$) solution was treated to the mixture. The volume was made up to 25 ml. Sets of standard solutions of Gallic acid (20, 40, 40, 60, 80 and 100μg/ml) were prepared in the same manner as mentioned earlier. It was incubated for 90 min at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with an Ultraviolet (UV /Visible spectrophotometer). Total phenol content was expressed as mg of GAE/gm of extract [11].

**Preparation of inoculums**

One gram (1g) of cow dung and soil sample gotten from the farm was measured in a sterile container and taken to the laboratory in appropriate analytic condition. Serial dilution was made to disperse the load of the micro organism evenly in numbered test tubes. After that, it was introduced on to a nutrient

agar plate using spread plate method. The plates were then incubated at 37°C for 24hrs.
After the incubation, the growth on nutrient agar was subsequently sub cultured into selective and differential media (MacConkey agar, Salmonella Shigella agar and Eosin Methylene Blue Agar) until pure colonies were achieved.

**Biochemical analysis for identification of the isolates**

***Indole test***

Nutrient broth was prepared aseptically and 10 ml each was poured into several test tubes. Using a sterile inoculating loop, a loopful of the bacterial isolates were taken into the test tubes leaving one as control. The tubes were all incubated at 37°C for 24 hours. After incubation, 1.0 ml of Kovacs's reagent was added to the test tubes. Formation of red color at the top layer indicated positive (+) result which implies production of indole while a negative (-) result gave a green coloration [16].

***Catalase test***

A loopful of the bacterial isolates was placed on a glass slide. A drop of 3% hydrogen peroxide ($H_2O_2$) was added to the isolate on the glass slide. Production of gas bubbles indicated positive result while no bubbles indicated negative result [16].

***Oxidase test***

A few drops of oxidase reagent was added to a clean filter paper. An inoculum of each isolate was smeared onto the impregnated filter paper and observed for color change. A purple coloration indicated positive result i.e. oxidase positive while no color change indicated oxidase negative result [16].

***Citrate utilization test***

This was carried out by streaking the isolate on a sterile and solidified Simon's Citrate Agar plate, leaving one plate uninoculated to serve as control and incubated at 37°C for 24 hours. Color changes from green to blue indicate positive result while original green color indicated a negative result [17].

***Sugar fermentation test (glucose, sucrose and lactose)***

Triple sugar iron agar was prepared and distributed into sterile test tubes aseptically. After cooling, the bacterial culture was inoculated into the test tubes and incubated at 37°C for 24 hours. Glucose fermentation showed a red slant and yellow butt, lactose and sucrose fermentation showed a yellow slant and yellow butt [16].

***Starch hydrolysis test***

Using a sterile inoculating loop, the bacterial isolates were streaked on the prepared and solidified starch agar plates and incubated at 37°C for 24 hours. After incubation, the plates were flooded with Iodine solution. The formation of transparent zone around the colony indicated positive reaction for the test while negative is indicated as a reaction that is otherwise [17].

***Motility test***

A motility agar medium was prepared aseptically into test tubes and was stabbed inoculated at 37°C for 24 hours. After, incubation they were examined and recorded. Non motile bacteria grew only on the stab line leaving the surrounding medium clearly transparent while motile bacteria grew throughout the medium making it slightly opaque [17].

**Gram staining reaction**

The bacterial isolates were smeared on glass slide and allow to dry. After drying, the glass slide is flooded with primary stain called crystal violet and allows to stand for 1 minute before rinsing with water. It was allowed to dry after rinsing; mordant called Lugos Iodine was added and allowed to stand for 1 minute before rinsing in order to fix the primary stain. Decolourizer which is 70% Alcohol was added but was rinsed after few seconds. Lastly counter stain safranin was added and allowed to stand for 1 minute before rinsing. After rinsing it was air dried and view under the microscope using × 100 oil immersion lens for viewing.

**Antibiotics susceptibility testing**

Using [18] method was used for the assessment of antibacterial activity. A sterile cotton swab was used to take broth culture of the test bacteria aseptically. A prepared and sterile Muller Hinton plate is swabbed with the test culture. Swabbing is done to cover the entire plate. Using a sterile forceps antibiotics disc containing ceftazidime, cefuroxime, gentamicin, ciprofloxacin, Ofloxacin, amoxicillin/cavulanate, nitrofurantoin and ampicillin was placed on the swabbed agar surface and incubated at 37°C for 24 hours. Following incubation, plate was taken out and examined. Diameter of growth inhibition zone was measured using a ruler. Zone less than 15 is resistance while zone greater than 15 is susceptible. The commercially produced antibiotic discs the antibiotics discs prepared from the extract were used as the positive control, while distilled water was used as negative control.

**Results**

Results obtained for the qualitative comparative screening of phytochemicals found in the coat of *Citrus sinensis* are represented in **table (1)**: out of the twelve phytochemicals screened for eleven were found present in various extracts. They are saponin, alkaloids, flavonoids, tannin, coumarin, steroid, terpenoid, cardiac glycosides, quinines, anthraquinone and phenol. While anthocyanin was found only in aqueous extract. According to [14], the factors affecting the choice of solvent are; the toxicity of the solvent in the bioassay process, potential health hazard of the extracts, ease of subsequent handling of the extracts, diversity in inhibitory compounds extracted, rate of extraction and quantities of phytochemicals to be extracted.

**Table 2** shows the results obtained from quantitative analysis of both extracts which indicate the analysis of the aqueous extract greater than the ethanoic extract. The aqueous extract of saponin shows 6.80% while the ethanoic extract shows 4.20%, the aqueous extract of flavonoid shows 7.015mg/g while ethanoic extract shows 5.940mg/g, the aqueous extract of alkaloid shows 9.40% while ethanoic extract shows 7.60%, the aqueous extract of tannin shows 1.286mg/g while ethanoic extract shows 0.638mg/g and the aqueous extract of phenol shows 5.462mg/g while ethanoic extract shows 3.752mg/g.

**Table 3** shows the biochemical characteristics of the organisms indicating that all the organisms were motile, positive to gram staining reaction, starch hydrolysis test, citrate utilization test and were negative to oxidase test.

**Figure 1** shows the potency of some commercially produced antibiotics disc on the organisms which indicated that the organisms were resistant to ceftazidime, cefuroxime, amoxycillin/clavulanate, nitrofurantoin and ampicillin but were susceptible to gentamicin, ciprofloxacin and ofloxacin with zone of inhibition ranging from 15mm to 28mm.

**Figure 2** shows the potency of *Citrus sinensis* coat extract using disc diffusion method on the isolated organisms and it inhibited their growth at the range of 13mm to 22mm. that is; the organisms were susceptible to both the aqueous and ethanoic extracts. Similar results were obtained by [19] indicated that it was sensitive against *Escherichia coli, Salmonella typhi and Staphylococcus aureus*. Also [3] who indicated that it was effective against *Staphylococcus aureus, Escherichia coli, Pseudomonas aerogenes, Bacillus cereus, and Candida albicans*.

**Table 1.** Qualitative comparative results of phytochemical screening**.** The phytochemical compounds in Citrus peel ethanol and aqueous extract

| | Parameters | Aqueous | Ethanol |
|---|---|---|---|
| 1 | Saponin (Froth’s Test) | + | + |
| 2 | Alkaloid (Hager’s Test) | + | + |
| 3 | Flavonoid (Lead acetate Test) | + | + |
| 4 | Tannin (Braymer’s Test) | + | + |
| 5 | Coumarin (Reaction with 10 % NaOH) | + | + |
| 6 | Steroid (Salkowaski’s Test) | + | + |
| 7 | Terpenoid (Salkowaski’s test) | + | + |
| 8 | Cardiac Glycosides (Legal’s Test) | + | + |
| 9 | Anthocyanin | + | **-** |
| 10 | Quinones | + | + |
| 11 | Anthraquinone | + | + |
| 12 | Phenol | + | + |

**Table 2.** Quantitative analysis of phytochemicals.

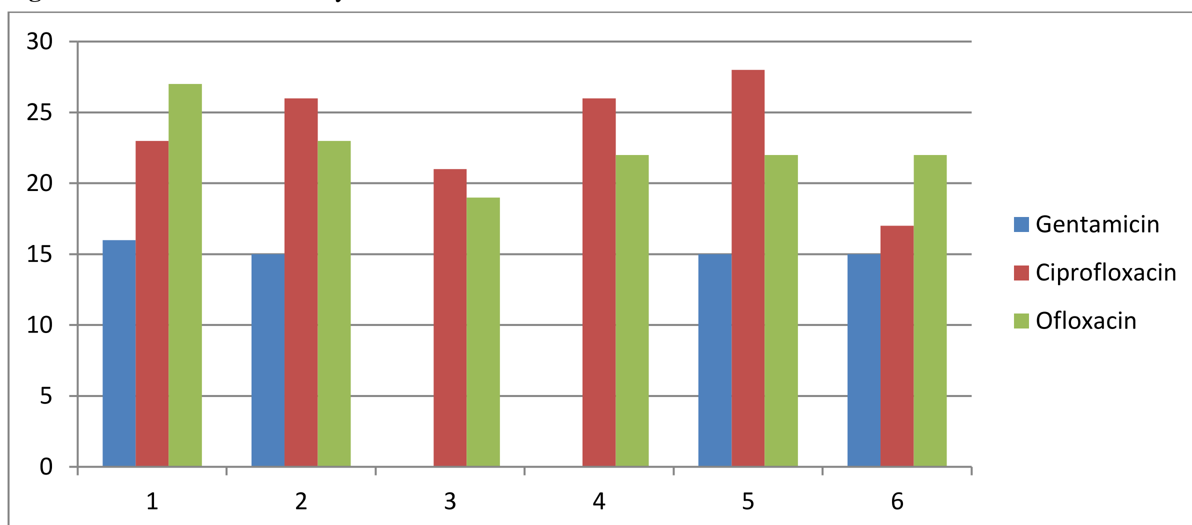
| Sample | Saponin (%) | Flavonoid (mg/g) QE | Alkaloid (%) | Tannin (mg/g) GAE | Phenol (mg/g) GAE |
|---|---|---|---|---|---|
| Aqueous | 6.80 | 7.015 | 9.40 | 1.286 | 5.462 |
| Ethanol | 4.20 | 5.940 | 7.60 | 0.638 | 3.752 |

**Table 3.** The biochemical tests on the colonies.

| Biochemical Test | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Indole Test | -ve | -ve | +ve | -ve | -ve | +ve |
| Catalase Test | +ve | +ve | -ve | +ve | +ve | +ve |
| Oxidase Test | -ve | -ve | -ve | -ve | -ve | -ve |
| Citrate Utilization Test | +ve | +ve | +ve | +ve | +ve | +ve |
| Starch hydrolysis Test | +ve | +ve | +ve | +ve | +ve | +ve |
| Gram staining reaction | +ve | +ve | +ve | +ve | +ve | +ve |
| Motility Test | Motile | Motile | Motile | Motile | Motile | Motile |

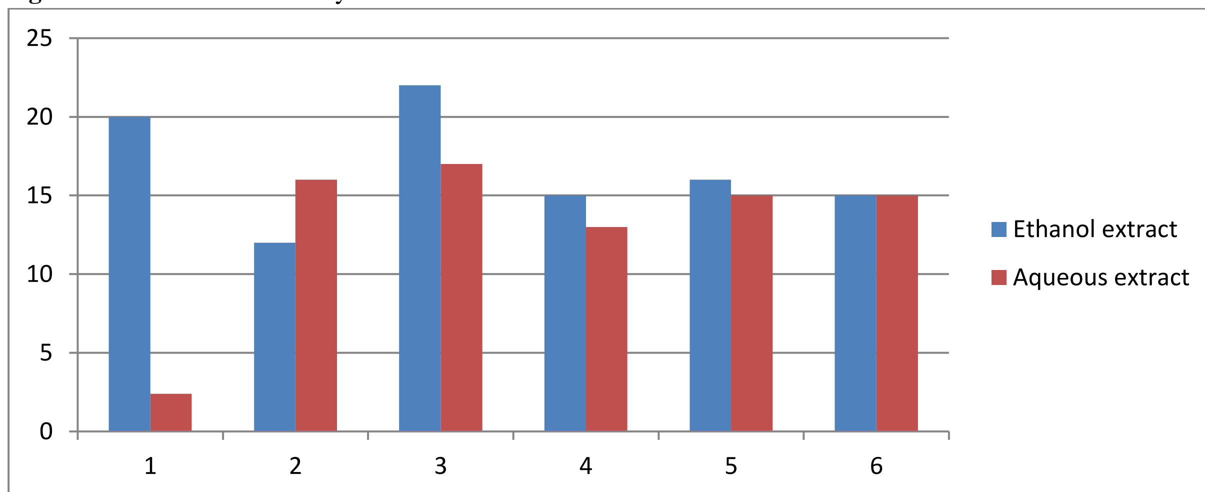
+ve positive reaction, -ve negative reaction. 1= *Bacillus cereus, 2= Bacillus cereus, 3=Bacillus weidmanni,* 4= *Bacillus cereus, 5=Bacillus cereus,6=.Bacillus thuringiensis.*

**Figure 1.** Antibiotics sensitivity test.




*1. Bacillus cereus, 2. Bacillus cereus 3.Bacillus weidmann, 4.Bacillus cereus, 5.Bacillus cereus, 6.Bacillus thuringiensis*

**Figure 2.** Antibiotics sensitivity test.




*1. Bacillus cereus, 2. Bacillus cereus 3.Bacillus weidmann, 4.Bacillus cereus, 5.Bacillus cereus, 6.Bacillus thuringiensis*

## Discussion

The ethanoic extract has proven to be more effective than the aqueous extract. The result is in correlation with [10]. The introduction of heat to the aqueous extract could be responsible for its lower antimicrobial effects. The phytochemical presents such as the phenolic compounds especially flavonoids, tannin, coumarin and quinones are responsible for the antimicrobial activities. They also contribute to delay of aging process and decrease of the inflammation and oxidative stress risk related with chronic diseases [20]. They are also responsible for chemo- preventive properties and contribute to inducing apoptosis by arresting cell cycle, regulating carcinogen metabolism and ontogenesis expression, inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation and blocking signaling pathways [20].

There is a more controversial effects in human, because the intra and inter variability in absorption, metabolism and reported effects of flavonoids is large. Furthermore, the flavonoids solubility is affected and often low by micro biota present in the gut [21]. Thus, from whole citrus the amount of flavonones maybe very different from those that have beneficial effects in animal models studies using extracts. Nevertheless, in a study in adults with metabolic syndrome, the consumption of 500 mg hesperetin daily as a supplement for three weeks resulted in improved bronchial artery flow-Mediated dilation and reduced makers of inflammation (C-reactive protein, serum amyloid A protein, soluble E- selection) [22].

*Limonoids* can be attributed to the bitter taste in citrus. The plant *Rutacae* (Citrus) and *melicae* (neem, mahogany) families are the plant in which terpenes (limonoids) occurs [23]. Glycosides of limonin and nimilin are the most abundant limonoids in citrus [23] 100g of citrus fruit can contain 150-300mg of limonoids where as 100g of citrus fruit peels and flesh can contain 500mg.

In the cell lines of animals and humans, limonoids have been shown to help reduce the risk of cancer of the pancreas, stomach, colon and breast. In animal studies, skin tumors are also reduced by limonoids [24]. There is an evidence which indicates the antiviral and antibacterial properties of limonoid [24]. Most studies on limonoids have used extracted compounds rather than citrus as a whole food making translation of the dose used in cell culture and animal studies to intake in human difficult. However, with high concentration of limonoids in citrus, it may be highly participatory in providing the health benefits described [25]. The present study also corroborated several earlier studies where citrus peels proved its bactericidal activities against *Bacillus cereus* [26] and other pathogenic organisms [27]. In concordance with earlier studies; the phytochemical analysis [qualitative and quantitative] of the components of the citrus [26, 28-31]

## Conclusion

Following accepted customs and properties, of extract of *Citrus sinensis* peel using different solvents and it yielded different positive result in this study. Hence the solvent used are ethanol and aqueous. The antimicrobial properties of the coat of *Citrus sinensis* were analyzed in in vitro study by disc diffusion method against some *Bacillus species.*

The coat of *Citrus sinensis* showed high antimicrobial properties against some *Bacillus* species, the antimicrobial effects could be as a result of active phytochemicals which was dissolved in suitable solvents. Flavonoids and other phytochemicals are bioactive component on human health as they are antioxidant, anti-inflammatory, free radical scavenger and immune system modulator.

Although coats of fruits and vegetables are normally tossed away as waste product. But its pharmaceuticals importance has been reported in different studies. The components that were documented to be antimicrobial, antioxidant and anti-inflammatory.

The coat of *Citrus sinensis* is term as a waste causing environmental pollution could be re-cycled for its pharmaceutical purposes because it is more economical, eco-friendlier, non-toxic and could be well tolerated by human system.

**Conflict of interest:** None.

**Financial disclosures:** None

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