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INHIBITORY POTENTIALS OF OIL PEELS OF CITRUS VARIETIES ON PATHOGENIC BACTERIAL ISOLATES: *KLEBSIELLA AEROGENES AND LISTERIA MONOCYTOGENES*

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Abstract: Five methanoic extracts of citrus oil peel were screened for their inhibitory actions against bacterial isolates of *Klebsiella aerogenes* and *Listeria monocytogenes*. This investigation was conducted by inoculating the isolates into pure oil peel extract obtained after drying, blended to powder and thereafter mixed with the named alcohol placed in Soxhlet apparatus for the extraction of oil, using the wet-steam distillation technique. The received extract was placed onto nutrient agar media and observed for growth at appropriate standards. The disk diffusion method was performed to determine the sensitivity test and the minimum inhibitory concentration (MIC) was carried out to ascertain the lowest drug concentrations that prevented the bacterial growth. The sensitivity screening revealed that, the peel oil extracts of *Citrus pomela*, *Citrus citron* and *Citrus paradise* indicated no visible growths (-) of the test organisms; *L. monocytogenes* and *K. aerogenes*. However, high growths (++) of the test bacteria were observed in the extract of *C. reticulate*. The sensitivity test recorded the lowest and highest inhibition zones on *C. citron* (16.3 mm) and *C. pomela* (32.0 mm) respectively against the *K. aerogenes*; *C. paradise* (10.0 mm) and *C. sinensis* (35.3 mm) against *L. monocytogenes*. Meanwhile, the lowest minimum inhibitory concentration (MIC) value (1: 10000) was recorded in the extract of *C. pomela* and *C.citron* each against the test bacterial isolates. Therefore, the Citrus peel oil extracts from *C. pomela* and *C. citron* possessed the most optimum antimicrobial effects and should be considered as widely used therapeutics for infections and associated diseases caused by the test organisms.

Keywords: Bacterial isolates, oil extracts, growth rate, sensitivity screening and minimum inhibitory concentration .

INTRODUCTION

The efficacious and discovery rates of synthetic therapeutic drugs and insecticides are on a decline and have resulted in deleterious clinical health challenges in recent times. So, natural chemical constituents of herbs may provide alternate sources of antimicrobial and insecticidal properties with innate characteristic modes of activities (Edogbanya *et al.,* 2019).

The natural plants' parts; the leaves, roots, fruits, bark and seeds consist of essential oils with main chemical components (terpenoids) such as diterpenes, monoterpenes, sesquiterpenes. They are volatile secondary metabolites in plants that impart the usual aroma, taste and inhibitory effects on Citrus against antibiotic resistant bacteria and insect pests. However, the basic fulcrum and active molecular structures for drug synthetic fields are

supplied by the rich indigenous plant sources (Preeti *et al.*, 2010).

Herbs have been utilized for the treatment of numerous infectious and non infectious diseases due to their easy accessibility, reliability, low toxicity and general acceptance by all religions and traditions globally. Meanwhile the World Health Organization (WHO) stated that, 80 % of the world population depends on herbal remedies for some aspect of primary health-care service delivery (Nayan and Shukla., 2011; Tresle and Ressy, 2018). More so, a larger number of persons use plant derived concoction as the initial primary attempt of action to curb human ailments because of its reduced or insignificant side effects (Jia *et al.*, 2016).

Additionally, the other needs that promote herbal remedies include; resistance of certain organisms and insect pests to these synthetic drugs and insecticides, herbal preparations retaining life-giving nutrients such as vitamins and minerals contained in the original plant's composition and potential hazards associated with excessive intake and external application chemically synthesized chemical drugs.

The oil peel of Citrus fruit is very rich in alkaloids, flavonoids, glycerols, phenols and volatile oils with huge pharmacological potentials against increasing trends of fungal infections, cancer, ulcer, migraine, inflammation and cell body radicals. Specifically, the Citrus fruit peels usually considered as agricultural and allied industrial by-products, serves as valuable precursor of essential oils and secondary metabolites with inhibitory plants 'active principles (Lawal *et al.*, 2016; Preeti *et al.*, 2017).

The ubiquitous nature of citrus endowed it with numerous species (more than a thousand species) and entrusted as one of the world,s prominent fruit crops that are produced in several countries with tropical or non tropical environmental conditions.

Environmentally safe approaches for microbial control has been developed from plants that possess rich source of novel natural compounds. The effects of plants' extracts on microorganisms can be established in several aspects that include toxicity, growth inhibitor, suppression of metabolic process rates (WHO 2021).

However, it has been reported that, there is a relationship between plants used for bacterial inhibitionl and medicinal plants (Tresle and Ressy, 2018). In addition, a good number of studies also revealed insecticidal potential of citrus oils extracted from different Citrus species and their constituents at separate periods, a few of which are produced to be applied by the consumers against insect pests. Essential oils extracted from Citrus peels contained insecticidal substances, but the most active and predominant is the limonene, that ranges between 95 to 98 % of the Citrus peel oil by weight (Preeti *et al.*, 2017). Bernhoft (2010) indicated that, it is a naturally occurring compound with low toxicity for warm blooded animals and high toxic affinity for household pests.

Subsequently, some of the key compounds for controlling microbes and insect pests definitely lie within the secondary metabolites that mainly occur in the diverse aromatic oil part of the plant. The aim of this study is to validate the claims by herbal medicine practitioners in their folklore, as to the efficacy of plants' materials in the treatment of various kinds of diseases.

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MATERIALS AND METHODS

Collection of plant materials

The Citrus fruits employed in this analysis were purchased from New Benin main market, Mission Road, Oredo Local Government Area, Edo State, Nigeria. Five different species of ripe Citrus fruits; *Citrus pomela* (maxima) *Citrus citron* (medica) *Citrus paradise* (grape) and *Citrus reticulate* (tangerin) and Citrus sinensis (sweet orange) were collected in clean state and immediately transported to the Microbiology laboratory, University of Benin, Nigeria.

Collection of test microorganisms

Sterile cultures of pathogenic bacterial isolates; *Listeria monocytogenes* and *Klebsiella aerogenes* were obtained from the Department of Clinical Pathology, University of Benin Teaching Hospital, Nigeria for the study. These test isolates were sub-cultured in nutrient agar and stored in nutrient agar slants in a refrigerator at 4 ℃ until required for use.

Extraction of essential oils from Citrus fruits

The collected Citrus peels were cut into pieces and dried in the oven for 24 hr at 60 ℃ and the dried peels were grind using an automated electric blender. The ground peel powder was placed in Soxhlet apparatus for the extraction of oil, using the wet-steam distillation technique as described by (Barrow and Feltham, 2003; Cassini *et al.*, 2016).

In vitro antimicrobial screening of test extracts

The antimicrobial tests were conducted using the test tube technique: Ten milliliter of each Citrus oil peel extract was measured into 2 test tubes, 1 ml each of the two test organisms was used to inoculate each of the test tubes and left for 24 hr. Sterile nutrient agar was poured onto pre - sterilized petri plates and allowed to set. These agar plates were then seeded with 0.5 ml of the test organism which was inoculated into the pure extract in the test tubes and spread evenly with a flamed, but cool glass spreader to derive effective growth of a smooth bacterial lawn. Finally, the plates were then incubated at 37 ℃ for 24 to 48 hr. However, control plates were prepared using distilled water and 2.5 % phenol as negative and positive controls respectively (Lawal *et al.*, 2016; Hans *et al.*, 2017).

Sensitivity test

The extracts with high spectra of activities against the test organisms obtained from the test tube approach of plants' screening were further confirmed for their extent of inhibiting the growth of prevailing organisms by employing the Disc diffusion method. This was conducted by sterilizing the filter paper soaked in an appropriate extract before each was inserted at middle of the plates previously flooded with test organisms. The plates were incubated at 37 ℃ at 24 to 48 hr and the cleared zones of inhibition produced around the epicenter of the plates were observed and measured in millimeter (Cassini *et al.* 2016; Hans, *et al.*, 2017).

Minimum inhibitory concentration (MIC)

Five milliliters (5 ml) of varying dilutions of the extract was prepared using peptone water as diluents; the serial dilution made ranged from 10^{-1} to 10^{-5} (1: 10 to 1: 100000). In preparing the dilution, 8 test tubes containing antibiotic concentration (Streptomycin 500 mg) as a clearing agent; was prepared and inoculated with standard quantity of each extract. These dilutions were aseptically inoculated with the test organisms and incubated at 37 ºC for 24 to 48 hr. Thereafter, 1 ml of each dilution was inoculated into the 8 nutrient

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media plates, incubated at 37 ℃for 24 to 48 hr (Nayan and Shukla, 2011).

RESULTS

The Citrus oil peel of all extracts: *C. pomela, C. citron, and C. paradise* completely inhibited the *Klebsiella aerogenes* growth (-) meaning that, no colonies were observed, while the extracts of *Citrus pomela and paradise* inhibited the growth of *L. monocytogenes.* High bacterial growth was observed in the extract of *C. reticulate* against the test isolates (++) (Table1).

Table 1: Effects of essential oil extracts on growth rate of bacterial isolates.

Extracts	***K. aerogenes***	***L. monocytogenes***
C. *pomela*	_	_
C.citron	_	+
C.paradise	_	-
C. reticulate	++	++
C.sinensis	+	+
Sterile water	++	++
2.5% phenol	_	_

Key: No bacterial growth (-) Slight growth (+) High growth (++)

The peel oil extracts that indicated high potential of antimicrobial properties: *C. pomela*, *C. citron*, *C. paradise and C.sinensis* were further screened to determine the level of clearing zones of test bacterial isolates using an antibiotic drug (Streptomycin 500 mg) and measured in millimeters (mm).

Table 2 Inhibition zones (mm) of the potential essential oil extracts on test bacterial isolates

Extracts	*K. aerogenes*	***L.*** *monocytogenes*
C. pomela	32.0	23.2
C. citron	16.3	29.1
C. paradise	25.4	10.0
C. sinensis	26 4	35.3
Distilled water	9.00	15.0
2.5 % Phenol	40.3	33.0

The minimum inhibitory concentration (MIC) of an extract or chemotherapeutic agent is the lowest concentration of the chemical substance present in plant, that prevents or stops visible growth of an

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organism. At the lowest extract dilution factor, the MIC was reported as followed: C. *pomela*: 1:10000; 1:10000, *C. citron*: 1:1000; 1:1000, C. paradise 1: 1000; 1: 100 (Tables 3 -5)

Table 3: Minimum inhibitory concentration (MIC) of essential oil extract (C. pomela) for test isolates

Extracts	***B. aerogenss***	***L. .monocytogens***
1: 10	–	–
1: 100	–	–
1: 1000	–	–
1: 10000	_	–
1: 100000	++	+
MIC	1: 0000	1: 0000

Key: No growth (–) moderate growth (+) extreme growth (++)

Table 4: Minimum inhibitory concentration (MIC) of essential oil extract (*C.citron*) for test isolates

Extract dilutions	*K. aerogenes*	*L.monocytogenes*
1: 10	–	–
1: 100	–	–
1: 000	–	–
1: 10000	+	+
1: 100000	+	++
MIC	1: 1000	1: 1000

Key: No growth (–) moderate growth (+) extreme growth (++)

Table 5: Minimum inhibitory concentration (MIC) of essential oil extract (*C. paradise*) for test isolates

Extract dilutions	***K. aerogenes***	***L.monocytogens***
1: 10	–	–
1: 100	–	–
1: 1000	–	+
1:10000	++	+
1: 100000	++	++
MIC	1: 1000	1: 100

Key: No growth (–) moderate growth (+) extreme growth (++)

DISCUSSION

Improper use of drugs for the cure of infectious diseases has resulted in the increase of several multi drug-resistant bacteria, which has facilitated the alarming health challenges that have fostered or culminated the desire to search for new plant active prinviples in order to articulate recent compounds and inhibitory constituents naturally contained in plants (Badawy and Abdelgaleil, 2014).

It had long been known even before the advent of microbes by mankind; that certain plants possessed curing prowess and they indeed composed what

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has presently been classified as antimicrobial or inhibitory compounds (Jia *et al.,* 2016; Hans *et al.*, 2017).

Muhammed (2013) indicated that; in determining the antimicrobial effects of Citrus varieties on *Pseudomonas aeruginosa*, the mean $LD_{50,}$ ranged between 16.7 to 39.6 µl indicating that, at lowest concentration of 16.7 µl, the extracts of *C.pomela* and *C.citron* exhibited more inhibitory properties than the extract of *C. paradise* (39.6 µl). More so, the oil peel of *C. pomela* showed more toxicity (15.3 and 10.1 µl) at exposure time of 18 and 24 hr respectively against tested bacterium, than that of *C. paradise* (33.4 and 45.3 µl) which indicated less toxicity.

Antimicrobial activities of several Citrus peel essential oil extracts have been investigated and revealed potent antibacterial effects against; *Listeria monocytogenes, Klebsiella aerogenes, Pseudomonas aeruginosa, Staphylococcus epidermidis, Bacillus cereus and Escherichia coli.* (Preeti *et al.*, 2010) analyzed the antimicrobial activity of *Citrus aurantifolia* (lime) and *Citrus sinensis* (sweet orange) methanoic and ethyl acetate fruit peel extracts. It was however found that, the ethyl acetate extract indicated more effect against several pathogenic bacterial isolates such as *Escherichia coli, Salmonella typhi* and *Streptococcus faecalis.*

In reference to the antimicrobial actions of the five varieties of Citrus essential oils investigated in this study, the *C. pomela, C. citron and C. paradise* exhibited the most potent extract against the tested organisms *Klebsiella aerogenes* and *Listeria monocytogenes* because, there were no observed growths. Slight or moderate growth was noticed in the extract of *C. sinensis* and profuse or high bacterial growth was observed in the *C. recticulate* extract. (Table 1).

The *Listeria mocytogenes* in contact with foods such as meat, poultry, sea-foods, vegetables, refrigerated and ready - to eat foods, unpasteurized milk and dairy prodults results in Listeriosis and the symptoms include fever, meningitis, muscle ache, stiff neck, nausea and diarrhea (gastrointestinal). Similarly, the *K. aerogenes* is an environmental, water and urinary tract pathogen transmitted through person to person contact, hospital acquired pathogen in a health care facility, contaminated hands of health-care workers, use of urinary catheters and water distribution system and has the potential of forming biofilm. It has been implicated in gastroenteritis, pneumonia, sepsis wound and urinary tract infections (Lawal *et al*., 2016; Preeti *et al*., 2017; WHO 2021).

The results of this study differ from the work of similar research as stated by Lawal *et al.*, (2016) that *C. sinensis* oil showed the most killing impact than *C. pomela, C. citron* on the tested organisms of the five essential oils used, but in diadem with the report revealed by Badawy and Abdelgaliel (2014) which enunciated that *C. citron* and *C. paradise* oil are more effective as antimicrobial agents than the *C. reticulate* and extracts. The discrepancy in result could be due to difference in the environmental conditions of the different location sources, as clearly reported by Edogbanya *et al.*, (2019) that, location causes variation in the chemical composition of essential oils.

In Table 2; the growth inhibition zone measured ranged from 16.3 to 32.0 mm for the K. aerogenes with the lowest cleared area recorded in the extract of *C. citron* (16.3 mm) and the highest was in the extract of *C.pomela* (32.0 mm). Similarly, the

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inhibition cleared zone of the tested extracts against the L. monocytogenes ranged between 10.0 and 35.3 mm; the lowest cleared zone was recorded in the extract of *C. paradise,* while the highest killed zone was observed in the extract of *C. sinensis (35.3 mm).*(Table 2).

These very high cleared zones reported in *C. pomela* and *C. sinensis* showed the rich potent chemical principles contained in extracts of these essential oils, as the most active agents in preventing the growth and transmission of the tested isolates. However, the low and high inhibition levels obtained from the controls: sterile water (negative control) and 2.5 % Phenol (positive control) relatively confirmed the presence of the active compounds in plants to have been responsible for antimicrobial activities. This finding further revealed that, *C. pomela* and *C. citron are* considered extracts, with high potential antimicrobial properties against the tested bacterial isolates

The minimum inhibitory concentration (MIC) described as the lowest concentration of chemical (drug) that prevents visible growth of an organism is considered that; lower MIC values meant that, less drug concentration is required to inhibit microbial growth. Therefore, drugs with lower MIC scores are more effective in the control of infectious agents (Shyla *et al.*, 2017).

Consequently, the minimum inhibitory concentrations (MICs) for *C. pomela* against the tested isolates , *K. aerogenes* and *Listeria monocytogenes* was 1:10000 each; (Table 3). This showed that, this extract concentration (lower MIC value) is more potent on the *K. aerogenes* and *L. monocytogenes.* Subsequently, the MIC values of 1: 1000 was each recorded for *C. citron* and *C. paradise* on the tested isolates; while the MICs for *C. paradise* were 1: 1000 and 1: 100 respectively on the organisms. (Tables 4 and 5). This outcome revealed that *C. paradise* with high MIC-value (1: 100) is relatively not too effective against the *L. monocytogenes.*

However, the sensitivity difference between the Gram negative (*K. aerogenes*) and the Gram positive (*L. monocytogenes*) could be as a result of differences in their cell wall compositions, as the latter contained rigid peptidoglycan layer in its cell wall, which functions as a permeability barrier; whereas, the former possessed an outer less rigid cell membrane (Edogbanya *et al.*, 2019). More so, the inhibitory activity of the Citrus essential oils may be a combined effect of D- Limonene, as main chemical constituent with other compounds such as flavonoids, phenolics and terpenoids, that have been revealed to inhibit the growth of numerous pathogenic bacterial isolates (Shyla *et al*., 2017).

CONCLUSION

The use of Citrus peel oil extracts from *C. pomela*, *C. citron* should be considered as therapeutics for infections and associated diseases caused by the test organisms. Apparently, these extracts being cost effective, availability, eco-friendly with less toxicity may be recommended as options to the use of synthetic therapeutics. Hence, this study has validated the claimed viability and efficacious nature of the plants' materials in the herbal system of health care delivery services to humanity

Conflict of interest

No conflict of interest declared

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