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Research Article
In vitro Evaluation of Antibacterial Potential of Combined Extract from *Kalanchoe pinnata* and *Citrus aurantifolia* on Otitis-media Pathogens

[1]Uzoamaka Ogechi George-Okafor, [2]Enuma Ekpereka Mike-Anosike and [1]Uju Felicia Nwachukwu

[1]Department of Applied Microbiology and Brewing, Enugu State University of Science and Technology (ESUT) Agbani, P.M.B. 01660, Enugu, Nigeria
[2]Department of Microbiology, Federal University of Technology, Owerri, Imo State, Nigeria

Abstract

Background and Objective: The advocacy for the alternative use of some combined therapy in herbal medicine is currently gaining more grounds in Nigeria than before due to incessant drug resistance phenomenon. Thus, the study was focused on the evaluation of the antibacterial property of the combined extract from *Kalanchoe pinnata* and *Citrus aurantifolia* for the inhibition of the otitis-media isolates. **Materials and Methods:** The test organisms were isolated from otitis-media samples by standard bacteriological methods and identified by numerical taxonomic procedures using Jaccard Simple Matching Coefficient tool. The Kirby-Bauer disk diffusion susceptibility method was adopted for their sensitivity to varied concentrations of the combined extract. The obtained data were considered significant at $p<0.05$ by using statistical package for social sciences (SPSS), version 15 for paired-t-test correlation analysis. **Results:** The predominant recovered isolates were *Staphylococcus aureus* (86.7%), *Streptococcus pneumoniae* (60%), *Streptococcus pyogenes* (80%) and *Proteus mirabilis* (56.7%). Relatively, the combined extract had greater antibacterial action especially on the Gram-ve *Proteus mirabilis* (119.3±0.14%) than the single extract (71.5±0.09%) and demonstrated above 70% sensitivity in line with the most sensitive reference antibiotics (Ampiclox, Gentamicin and Ciprofloxacin) at a significant level of $p<0.05$. There was significant dose-dependent efficacy of the extract on these isolates. The lower concentration (10 µg), significantly ($p<0.05$) exhibited stronger inhibitory actions (82.7±0.11-119.3±0.14%) than the higher (30 µg) concentration which achieved a range of 71.9±0.07-99.3±0.10% inhibition against the test isolates. **Conclusion:** The observed results indicated the strong antibacterial potential of the combined test extract against otitis-media pathogens and these will be supportive to scientific documentation for pharmacological utilization.

Key words: Combined extract, *Kalanchoe pinnata*, *Citrus aurantifolia*, antibacterial, otitis-media, isolates

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Corresponding Author: Uzoamaka Ogechi George-Okafor, Department of Applied Microbiology and Brewing, Enugu State University of Science and Technology (ESUT) Agbani, P.M.B. 01660, Enugu, Nigeria Tel: +2348035853812

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Otitis-media is an ear infection which involves the middle ear and it is more common in babies and young children than adults because of their narrow eustachian tubes[1]. The common cause of all forms of otitis-media is the blockage of eustachian tube which results from the swelling of the mucous membranes in the nasopharynx and the severe cases are associated with high risk of developing conductive and sensor-neural hearing loss[2,3]. The common cause of infantile otitis-media is bacteria and to some extent virus and this has led to their treatments with varying antibiotics. The use of antibiotics has been among the first-line treatment for otitis-media as its common pathogens include *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Proteus mirabilis.* However, long usage of antibiotics has been reported to have some adverse effects such as hearing loss in children[4]. In addition, the development of microbial resistance to these drugs is another factor. As a result of antibiotic resistance, many individuals especially in Nigeria resort to treating most infections including otitis-media with natural herbs including *Kalanchoe pinnata*.

Kalanchoe pinnata which is a succulent perennial plant of Crassulaceae family and other herbs have been used in traditional medicine for self-medication against many ailments such as earache, wound infections, diarrhoea, inflammations, convulsion and general debility[5,6]. The presence of bufadienolide compounds and other active ingredients in *K. pinnata*[6,7] may be responsible for its efficacy in the treatment of microbial infections. Although there were claims on the efficacy of *K. pinnata* in the treatment of various diseases including earache, yet there was limited or no evidence regarding its effectiveness on acute otitis media. This generated the interest of the authors in studying its effect on otitis media pathogens in combination with *Citrus aurantifolia* (Lime) which is rich in vitamin C. The choice of *C. aurantifolia* was because vitamin C has been associated with the boosting of the body immunity during and after treatment[8,9] and it has powerful antioxidant property which has been reported to aid in the reduction of acute otitis media and tonsillitis[10,11]. The sought for this combination practice is also due to several reports including that of Duval *et al.*[12] which demonstrated more bactericidal activity and less resistance development in the use of combined drugs than the single ones. The present research therefore, was aimed at studying the antimicrobial potential of combined extract of *K. pinnata* and *C. aurantifolia* against otitis-media pathogens; with a view of recommending it to pharmacology unit for processing if found very effective.

MATERIALS AND METHODS

The 5 month study which commenced in May, 2018, was conducted at Biotechnology Centre of ESUT. The reference organisms for identification and the antibiotics were sourced prior to extraction of the test plant.

Source of test organism: The organisms used for the study were isolates from otitis media samples (30) collected from 5-10 year old children that attended Enugu state University Teaching Hospital within the period of the study. The isolates were recovered following standard bacteriological methods and identified by comparing the isolates with the reference organisms from ESUT Teaching Hospital Laboratory. The identification was carried out by numerical taxonomic procedures using Jaccard Simple Matching Coefficient tool as described by Dalirsefat *et al.*[13]. The isolates confirmed as *Streptococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Proteus mirabilis* were maintained in nutrient agar at 4°C. Prior to sensitivity test, each stock culture of the isolates (1% w/v) was reactivated with peptone water, incubated at 37°C/24 h and subsequently diluted with sterile peptone water to obtain a working concentration of 1.0×10^8 cfu mL^{-1} using 0.5 McFarland turbidity standard.

Collection of the test samples: The fresh leaves of *K. pinnata* (Fig. 1) and the fresh fruits of *C. aurantifolia* were collected from the botanical garden belonging to Prof. J.C. Okafor, an adjunct lecturer in the Department of Applied Biology and Biotechnology of Enugu State University of Science and Technology, Nigeria. The leaves of *K. pinnata* were identified by him and deposited for use with the specimen number of JCO, 05:51.

Fig. 1: External features of *K. pinnata* leaves

Preparation and extraction of plant materials: The fresh leaves of *K. pinnata* and fruits of *C. aurantifolia* (300 g each) were washed thoroughly with microbial-free-water and drained to free them from water. The leaves and the seedless fruits with their pericarps were homogenized with sterile home-use blender, respectively. Thereafter, 10 g of the mixed fresh homogenates (1:1w/w) were finally extracted with 200 mL of methanol (as the best solvent after series of extractions with other solvents: ethanol, petroleum ether, hexane, ethyl acetate). The extraction was facilitated by agitation (80 rpm/24 h) at room temperature using Griffin and George rotary shaker (British). The recovered filtrate was concentrated by evaporation under water-bath (60°C/8 h) and reconstituted (1:1 w/v) with sterile Tween 80. The reconstituted extract was further diluted with Tween 80 to obtain concentration of 10-30 µg similar to the concentrations of the control samples used (i.e., Nigerian manufactured antibiotics: Gentamicin (10 µg), Ciprofloxacin (10 µg), Refampin (10 µg), Streptomycin (30 µg), Erythromycin (30 µg), Ampiclox (30 µg)). The single extract of *K. pinnata* used as second control sample was also prepared as earlier described.

Sensitivity test and statistical analysis: Kirby-Bauer disk diffusion susceptibility method as described by Pierce-Hendry and Dennis[14] was adopted. The prepared disks (5 mm) from Whatman No. 1 filter paper were impregnated with 1 mL of different concentrations of both the combined and single extracts of *K. pinnata.* Thereafter, each disk was subjected to sensitivity test with 0.5 mL of 1.0×10^8 cells of each test organism. The susceptibility strengths of the organisms to the combined extract, displayed by the presence of haloes around the colonies were determined and compared with both the single and the standard antibiotics using statistical package for social sciences (SPSS), version 15 for paired-t-test correlation analysis. The values were presented as Mean+SEM and then considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Percentage occurrence of identified isolates from the otitis-media samples: The conducted analysis on samples of otitis-media produced bacterial species with the predominant otopathogens identified as *Staphylococcus aureus Streptococcus pneumonia*e, *Streptococcus pyogenes* and *Proteus mirabilis* (Table 1). The percentage recovery of these organisms (>50%) from the samples was high (Fig. 2). A high percentage of these isolates had consistently been associated with either acute or chronic otitis-media in children less than

Table 1: Identification of otitis-media isolates

	Test organisms			
Parameters	*Staphylococcus aureus* (R1)	*Streptococcus pneumoniae* (R2)	*Streptococcus pyogenes* (R3)	*Proteus mirabilis* (R4)
Gram reaction	+cocci in clusters	+cocci in chains	+cocci in chains	-rod -rod
Catalase	+ +	- -	- -	- -
Coagulase	+ +	- -	- -	- -
Oxidase	- -	- -	- -	- -
Indole	- -	- -	- -	- -
Methyl red	- -	- -	- -	+ +
Voges proskauer	+ +	- -	+ +	- -
Citrate	+ +	- -	- -	+ +
H_2S production	- -	- -	- -	+ +
Urease activity	+ +	- -	- -	+ +
Starch hydrolysis	- +	- -	- -	- -
Gelatin liquefaction	- -	- -	- -	+ +
Lipid hydrolysis	+ +	- -	- -	- -
Acid from glucose	+ +	+ +	+ +	+ +
Acid from sucrose	+ -	+ +	+ -	- -
Acid from lactose	- -	- -	- -	+ +
Capsule staining	- -	+ +	+ +	- -
Growth in MSA	+ +	- -	- -	- -
Growth in 6% NaCl	+ +	- -	- -	- -
Mortility	- -	- -	- -	+ +
Bile solubility test	- -	+ +	- -	- -
Lysis of blood	βh βh	αh αh	βh βh	- -
Optochin resistance	- -	- -	+ +	- -
*Matching coefficient	95.7%	100%	95.7%	100%

βh: Beta- Haemolysis; αh Alpha-Haemolysis, R1, R2, R3 and R4 were codes for the reference organisms for the identification of the test isolates, *Matching Coefficient >75% depicts resemblance i.e. similarity in characters[14]

Table 2: Relative antibacterial activity of the test extract (10 µg) to the test organisms

	Test sample sensitivity		Control samples (10 µg) sensitivity Ref. antibiotics (%)		
Test organisms	Combined extract	Single extract	CPX	GNT	RFP
Staphylococcus aureus	98.5±0.09	68.2±0.21	67.6±0.12	100±0.16*	49.8±0.13
Streptococcus Pneumoniae	87.7±0.07	65.9±0.18	100±0.22*	70.1±09	53.8±0.08
Streptococcus pyogenes	82.7±0.11	48.4±0.14	100±0.08*	79.8±0.13	39.2±0.24
Proteus mirabilis	119.3±0.14	71.5±0.09	74.2±0.15	100±0.11*	48.8±0.22

CPX: Ciprofloxacin, GNT: Gentamicin, RFP: Refampin, Ref.: Reference, *: The most sensitive reference antibiotic at 10 µg was taken as 100% and utilized to compare the activities of other extracts

Table 3: Relative antibacterial activity of the test extract (30 µg) to the test organisms

	Test sample sensitivity		Control samples (30 µg) sensitivity Ref. antibiotics (%)		
Test organism	Combined extract	Single extract	SPT	AMX	EYT
Staphylococcus aureus	89.6±0.13	67.4±0.08	48.6±0.09	100±0.01*	51.7±0.09
Streptococcus pneumoniae	71.9±0.07	50.6±0.14	36.5±0.13	100±0.02*	52.4±0.06
Streptococcus pyogenes	75.2±0.07	46.8±0.08	22.4±0.05	100±0.05*	32.1±0.07
Proteus mirabilis	99.3±0.10	69.7±0.12	78.8±0.05	100±0.05*	68.9±0.07

SPT: Streptomycin, AMX: Ampiclox, EYT: Erythromycin, *: The most sensitive reference antibiotic at 30 µg was taken as 100% and used to compare the activities of other extracts

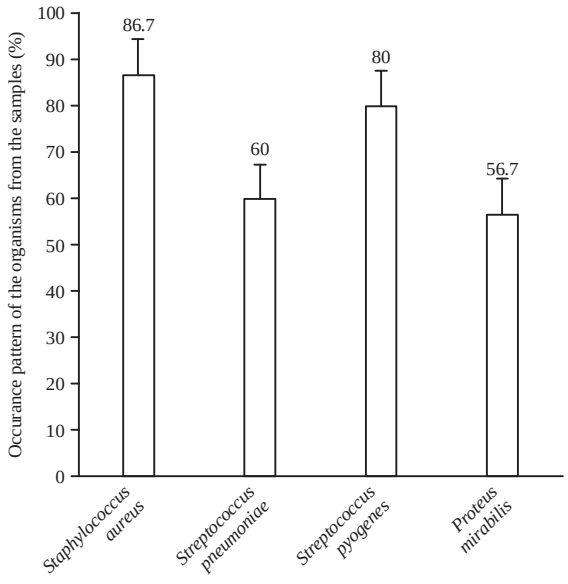

Fig. 2: Occurrence (%) of the bacterial isolates from the otitis-media samples

8 years old[15,16]. The utilization of the isolates from the study samples as test organisms was to give a clear indication on the efficacy of the test extract as samples were from patients who had been earlier exposed to some of the reference antibiotics during treatment.

Relative antibacterial activity of the combined extract on isolates: The observation on the antibacterial pattern of the combined extract revealed a significant higher activity on *Proteus mirabilis* (Gram-ve bacteria) than other test organisms (Table 2, 3) at $p<0.05$. High susceptibility of *Proteus mirabilis* could be due to its lesser content of peptidoglycan than the Gram+ve test organisms. It could also be that the patients from whom the samples containing *Proteus mirabilis* were recovered might not have been severely treated or abused with the in-use antibiotics.

Relative activity of combined extract to single extract: The combined extract of *K. pinnata* and *C. aurantifolia* also demonstrated significantly ($p<0.05$) stronger antibacterial action (<119.3±0.14%) than the single extract of *K. pinnata* (<71.5±0.09%) (Table 2). The combination process seemed to produce synergistic activity that promoted inhibitory actions. This can be supported by some other reports that also indicated more effective treatment with combined therapy than with single therapy[12,17]. The obtained result directly indicated the stimulatory impact of *C. aurantifolia* on the inhibition of the pathogens. Reports by Aibinu *et al.*[18] and Onyeagba *et al.*[19] also gave a support to it. The inhibitory potential of the combined extract could be linked to the presence of phenolic compounds, bufadienolide alkaloids flavonoids and carotenoids as stated by Narang and Jiraungkoorskul[20]. However, other bioactive compounds of the extract might have also played their inhibitory roles.

Relative activity of combined extract to single reference antibiotics: The relative antibacterial activity of the combined extract in relation to the reference antibiotics varied

(Table 2, 3). A higher activity was significantly ($p<0.05$) achieved by the combined extract in relation to Refampin, Streptomycin and Erythromycin while a close range of antibacterial activity was observed between the combined extract (71.9±0.07- 119.3±0.14%) and the other stronger antibiotics (68.1±0.09- 100±0.03%) viz., Ampiclox, Gentamicin and Ciprofloxacin used in the study. The result is encouraging as some reports have also shown that combination therapy improves survival in the high risk life threatening disease compared with monotherapy[21].

Effect of concentration of combined extract on inhibition: A significant higher inhibitory action was achieved at lower concentration (10 µg) than at 30 µg (Table 3). This observation is similar to the results obtained from other extracts on various pathogens[22,23]. It therefore indicated the presence of effective active ingredients in the combined extract which at higher concentration might not have fully dissociated to extensively penetrate the cell's membrane to lyze the cells. Generally, drugs at low concentration with high activity always stand as the choice-drug for *in vivo* use. Interestingly, the test extract possesses this property.

CONCLUSION

The results from the investigation have produced valid information on the synergistic property of the combined extract from *K. pinnata* and *C. aurantifolia* for the significant inhibition of otopathogens at a low concentration. However, there is still need for further research on its most effective proportional combinations for optimal inhibitory activity *in vivo.*

SIGNIFICANCE STATEMENT

This study discovered the significant antibacterial potential of the combined extract from *K. pinnata* and *C. aurantifolia* that can be utilized for the treatment of otitis-media and this will help the researchers to uncover the critical area of the combined extract that many researchers were not able to explore. Thus a new theory on bacterial inhibition by the combined extract may be arrived at.

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REFERENCES

1. Karunanayake, C.P., W. Albritton, D.C. Rennie, J.A. Lawson and L. McCallum *et al*., 2016. Ear infection and its associated risk factors in first nations and rural school-aged Canadian children. Int. J. Pediatr., Vol. 2016. 10.1155/2016/1523897
2. Perera, R., P.P. Glasziou, C.J. Heneghan, J. McLellan and I. Williamson, 2013. Autoinflation for hearing loss associated with otitis media with effusion. Cochrane Database Syst. Rev., Vol. 31, No. 5. 10.1002/14651858.CD006285.pub2
3. Browning, G.G., M.M. Rovers, I. Williamson, J. Lous and M.J. Burton, 2010. Grommets (ventilation tubes) for hearing loss associated with otitis media with effusion in children. Cochrane Database Syst. Rev., Vol. 6, No. 10. 10.1002/14651858.CD001801.pub3
4. Frei, H. and A. Thurneysen, 2001. Homeopathy in acute otitis media in children: Treatment effect or spontaneous resolution? Br. Homeopath. J., 90: 180-182.
5. Nayak, B.S., D.D. Ramdath, J.R. Marshall, G.N. Isitor, M. Eversley, S. Xue and J. Shi, 2010. Wound-healing activity of the skin of the common grape (*Vitis vinifera*) variant, cabernet sauvignon. Phytother. Res., 24: 1151-1157.
6. Nguelefack, T.B., P. Nana, A.D. Atsamo, T. Dimo and P. Watcho *et al*., 2006. Analgesic and anticonvulsant effects of extracts from the leaves of *Kalanchoe crenata* (Andrews) Haworth (Crassulaceae). J. Ethnopharmacol., 106: 70-75.
7. Adenike, K. and O.B. Eretan, 2004. Purification and partial characterization of a lectin from the fresh leaves of *Kalanchoe crenata* (Andr.) Haw. J. Biochem. Mol. Biol., 37: 229-233.
8. Carr, A.C. and S. Maggini, 2017. Vitamin C and immune function. Nutrients, Vol. 9, No. 11. 10.3390/nu9111211
9. Aprioku, J.S. and A.W. Obianime, 2014. Evaluation of the effects of *Citrus aurantifolia* (lime) juice in lead-induced hematological and testicular toxicity in rats. Pharmacologia, 5: 36-41.
10. Cemek, M., S. Dede, F. Bayiroglu, H. Caksen, F. Cemek and K. Yuca, 2005. Oxidant and antioxidant levels in children with acute otitis media and tonsillitis: A comparative study. Int. J. Pediatr. Otorhinolaryngol., 69: 823-827.
11. Karabaev, K.E., V.F. Antoniv and R.U. Bekmuradov, 1997. [Pathogenetic validation of optimal antioxidant therapy in suppurative inflammatory otic diseases in children]. Vestnik Otorinolaringol., 1: 5-7, (In Russian).
12. Duval, X., S. van der Werf, T. Blanchon, A. Mosnier and M. Bouscambert-Duchamp *et al*., 2010. Efficacy of oseltamivir-zanamivir combination compared to each monotherapy for seasonal influenza: A randomized placebo-controlled trial. PLoS Med., Vol. 7. 10.1371/journal.pmed.1000362

13. Dalirsefat, S.B., A. da Silva Meyer and S.Z. Mirhoseini, 2009. Comparison of similarity coefficients used for cluster analysis with amplified fragment length polymorphism markers in the silkworm, *Bombyx mori*. J. Insect Sci., Vol. 9, No. 1. 10.1673/031.009.7101
14. Pierce-Hendry, S.A. and J. Dennis, 2010. Bacterial culture and antibiotic susceptibility testing. Compend. Contin. Educ. Vet., 32: E1-E5.
15. Van Dyke, M.K., J.Y. Pircon, R. Cohen, S.A. Madhi and A. Rosenblut *et al*., 2017. Etiology of acute otitis media in children less than 5 years of age: A pooled analysis of 10 similarly designed observational studies. Pediatr. Infect. Dis. J., 36: 274-281.
16. Cilveti, R., M. Olmo, J. Perez-Jove, J.J. Picazo and J.L. Arimany *et al*., 2017. Epidemiology of otitis media with spontaneous perforation of the tympanic membrane in young children and association with bacterial nasopharyngeal carriage, recurrences and pneumococcal vaccination in catalonia, Spain-the prospective HERMES study. PLoS ONE, Vol. 12. 10.1371/journal.pone.0170316
17. Son, M.J., Y.E. Kim, Y.I. Song and Y.H. Kim, 2017. Herbal medicines for treating acute otitis media: A systematic review of randomised controlled trials. Complement. Ther. Med., 35: 133-139.
18. Aibinu, I., T. Adenipekun, T. Adelowotan, T. Ogunsanya and T. Odugbemi, 2007. Evaluation of the antimicrobial properties of different parts of *Citrus aurantifolia* (lime fruit) as used locally. Afr. J. Tradit. Complement. Altern. Med., 4: 185-190.
19. Onyeagba, R.A., O.C. Ugbogu, C.U. Okeke and O. Iroakasi, 2004. Studies on the antimicrobial effects of garlic (*Allium sativum* Linn), ginger (*Zingiber officinale* Roscoe) and lime (*Citrus aurantifolia* Linn). Afr. J. Biotechnol., 3: 552-554.
20. Narang, N. and W. Jiraungkoorskul, 2016. Anticancer activity of key lime, *Citrus aurantifolia*. Pharmacogn. Rev., 10: 118-122.
21. Tamma, P.D., S.E. Cosgrove and L.L. Maragakis, 2012. Combination therapy for treatment of infections with gram-negative bacteria. Clin. Microbiol. Rev., 25: 450-470.
22. Obeidat, M., M. Shatnawi, M. Al-Alawi, E. Al-Zu'bi and H. Al-Dmoor *et al*., 2012. Antimicrobial activity of crude extracts of some plant leaves. Res. J. Microbiol., 7: 59-67.
23. Mostafa, A.A., A.A. Al-Askar, K.S. Almaary, T.M. Dawoud, E.N. Sholkamy and M.M. Bakri, 2018. Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. Saudi J. Biol. Sci., 25: 361-366.