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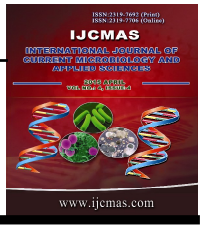

Original Research Article

Synergistic Antibacterial Potentials of *Citrus aurantifolia* (Lime) and Honey against Some Bacteria Isolated from Sputum of Patients Attending Federal Medical Center Umuahia

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Keywords

Synergism, Antibacterial Potential, *Citrus aurantifolia*, Honey, Sputum

A B S T R A C T

This research work was carried out to evaluate the synergistic antibacterial potentials of *Citrus aurantifolia* (Lime) and honey against some bacterial strains isolated from sputum. Methods adopted include streak method for inoculation, Agar well diffusion for sensitivity testing and Broth dilution method for the determination of the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration. The morphological and biochemical examinations reveal the bacterial isolates to belong to the genera *Staphylococcus aureus, Streptococcus spp, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Escherichia coli.* The results showed that the lime and honey mixture exhibited very strong inhibitory effect on the growth of the tested isolates at varying degrees. The 40% mixture had the highest zone of inhibition ranging from 23.00-24.33mm while the 10% mixture had the least zone of inhibition 14.67mm. Less activity was observed when lime and honey were used singly (inhibition zone diameter range = 14.33-21.33mm and 16.33-17.00mm respectively). 40% and 30% of the mixture exhibited a cidal effect against *Streptococcus spp* at a concentration of 25mg/ml and 50mg/ml respectively. The Minimum Inhibitory Concentration value at 40% and 30% mixture of lime and honey against S*taphylococcus aureus* and *Klebsiella pneumonia* is 25mg/ml concentration with the Minimum Bactericidal Concentration value 50mg/ml. At 20% and 10% mixture, the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration value against *Klebsiella pneumoniae* and *Streptococcus spp* was 200mg/ml concentration. The phytochemical screening showed presence of tannis, alkaloids, saponins and flavonoids. In conclusion, the mixture of lime and honey showed more activity than when they were used singly. This has scientifically proved that the use of lime and honey combination could be adopted in the treatment of people suffering from cough and that the mixture when refined could serve as a good tool in the pharmaceutical industry in manufacturing drug for the treatment of cough.

Introduction

Antimicrobial agents are essentially important in reducing the global burden of infectious disease. However, as resistant pathogens develop and spread, the effectiveness of the antibiotics is diminished.

This type of bacterial resistance to the antimicrobial agents poses a very serious threat to public health, and for all kinds of antibiotics, including the major last resort drugs, the frequencies of resistance are increasing worldwide (Levy and Marshall, 2004). Therefore, alternative antimicrobial strategies are urgently needed, and thus this situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as plants based products including honey (Mandal *et al*, 2010).

Currently, many researchers have reported the antibacterial activity of honey and lime and found that natural unheated honey has some broad spectrum antibacterial activity when tested against pathogenic bacteria, oral bacteria as well as food spoilage bacteria (Lusby *et al*, 2005).

Also lime and honey contain some bioactive components such as phenolics, flavonoids, hydrogen peroxide etc. these constitutes their phytocomponents responsible for their antibacterial activities. Several up to date research work and practical experience have shown that using medicinal plants and honey is better than allopathic drugs by being safer besides have synergistic effect (Ifra and Ahmed 2004)

According to Adedayo, during the last two decades, the plant has been subjected to extensive phytochemical, pharmacological and clinical investigations and many interesting findings in the areas of insecticidal activity (Adedayo *et al*, 2001). Investigation of the antimicrobial activity of lime extract alone and in combination with other substance mixture or herbs has been investigated (Rodriguez *et al*, 2002). Also according to Taylor, lime extract has been found to have high antimicrobial activity.

He stated that the potency of lime fruit is enhanced by the type of solvent used indicating that there are some active ingredients in lime which have antimicrobial /antifungal effect but which would not be released except when lime fruit is used in conjunction with a particular solvent (Taylor, 2004).

According to most researchers, lime extract known to be very potent in treatment of infectious diseases and ailments when used alone and in combination with other herbs, solvent or extract, ranks fifth in antimicrobial potency when compared with the other forms and type of solvent in which, it is used locally.

This implies that there are still lot more to gain from the lime fruit as an antimicrobial agents when used in the other forms and in solvents such as honey and palm wine (Taylor, 2004). This result is a pointer to new source of noval drugs, which needs to be further investigated.

According to microbiological investigation on market survey, many plants are used in Nigeria in the form of crude extracts, infusions or plasters to treat common infections without any scientific evidence of efficacy. Whereas investigation done on lime fruit obtained in Nigeria have shown activity coherent with the use of this plant in folk medicine. (Onyeagba *et al*, 2004).

Honey as an ancient remedy has been recently rediscovered by the medical

profession, particularly where conventional therapeutic agents fails . According to Lusby *et al*, he stated that the healing properties of honey can be ascribed to the fact that it offers antibacterial activity, maintains a moist wound environment that promotes healing, and has a high viscosity which provides a protective barrier to prevent infection (Lusby *et al*, 2005)

There are many reports of honey being very effective as dressing of wounds, burns, skin ulcers and inflammations, the antibacterial properties of honey speed up the growth of new tissues to heal the wound (Lusby *et al* 2000).

This work reviews, the synergistic potential of lime and honey. And explore its efficacy as an antibacterial in respiratory tract infections as well as its bioactive compounds (phytocompounds) through scientific methods.

The antibacterial activity in most honey is due to the enzymatic production of hydrogen peroxide. Its mechanism may be related to the low pH level of honey and its high sugar content (High osmolarity) that is enough to hinder the growth of microbes (Al-waili *et al*, 2005). The medical grade honeys have potent *in vitro* bactericidal activity against antibiotic resistant bacteria.

The beneficial role of honey is attributed to its bacterial property with regards to its high osmolarity, acidity and hydrogen peroxide and non-peroxide components i.e the presence of photochemical components like methyl glyoxal (MGO) (Mavric *et al*, 2008). The antimicrobial aspect in honey are predominantly hydrogen peroxide of which the concentration is determined by relative levels of glucose oxidase, synthesized by the bee and catalyse originating from flower pollen (Weston,2000).

Materials and Methods

Collection of materials and samples

Pure honey was collected from National Root Crop Research Institute, Umudike Abia State. However, the Lime (*Citrus aurantifolia*) was obtained from Ndoro market, Umuahia. Samples of Sputum were collected from patients after seeking their consent. These were transported to the laboratory and processed.

Isolation and identification of bacteria from respiratory tract

Samples of sputum were collected and then inoculated on chocolate agar, MacConkey agar, nutrient agar using streak method of inoculation and the plates incubated at 37^0C for 24-48hours. After incubation, the colonial appearance of the colonies were observed.

Pure colonies were sub-cultured on nutrient agar further identification or confirmation was done using biochemical test such as catalase test, oxidase test, Indole test, citrate test.(Cheesebrough, 2010).

Antibacterial activity testing

Susceptibility testing of the bacterial isolates to honey and lime and the standard antibiotics.

Preparation of test samples

The honey was diluted with sterile distilled water to concentrations of between 25% (v/v) to 100% (v/v) (i.e 1ml of honey to 4mls of distilled water). The lime was washed with water to remove sand and other particles and rinsed with sterile distilled water. It was cut with sterile knife and boiled before the extract was squeezed out and sieved. The sieving was done to remove

the seeds and other particles. The extract was diluted with sterile distilled water to concentration of between 25% (v/v) to 100%(v/v) (i.e 1ml of lime to 4mls of distilled water). However, for the combination, Lime: Honey was diluted at 10:50, 20:50, 30:50, 40:50, (Using sterile distilled water 40%, 30%, 20%, 10% respectively to make it up to 100% (v/v) concentrations, 50:50v/v concentration)

Sensitivity testing

Agar well diffusion technique was used to determine the antibacterial activities of honey, lime and the combination of both and a standard antibiotics was used as control. (Udobi *et al*, 2008). About 20mls of Mueller Hinton agar were poured into sterile petridishes, and allowed to set. Overnight culture of the test organism which was diluted in sterile normal saline was spread thinly with sterile bent glass rod on the surface of the agar. There, after, holes (6mm) were bored using sterile cork borer to make uniform wells on the inoculated agar. (Samie *et al*, 2005)

The wells were filled with test samples with the help of sterile droppers. The standards antibiotics were placed in another inoculated plate after boring the wells. The standard antibiotics used were Doxycycline (100mg), Brylhromycin (500mg) and Amozine (250mg). it was diluted with distilled water at 100mg/10mls, 500mg/50mls and 250mg/25mls (ie mg/mls). The pre-incubation diffusion time (45minutes to 1 hour) was allowed, after which the petri dishes were incubated at 37^0C for 18-24hrs.

After the incubation period, the diameters of the zones of inhibition were measured in millimeters. The diameter of the clear zone around the wells were measured. The sensitivity test was done in triplicate

Determination of minimum inhibitory concentration (mic)

The MIC was carried out using the broth dilution method(Kabir *et al*, 2005). A stock solution of each (Lime and honey) and combination of both was prepared by dissolving 1ml of each extract in 5mls of the distilled water. Different concentrations of the extract were obtained by two fold serial dilution of the stock solution of the different extracts. For each extract, sterile five test tubes labeled 200mg/mls 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml respectively were used.

5mls of distilled water was pipette into the first test tube labeled 200mg/ml, while the other four test tubes, 2.5mls of distilled water was pipette in them.

Thereafter 2.5mls of the stock solution of the test sample (honey and lime extract) was aseptically transferred into the first test tube containing 2.5mls of distilled water and then gently mixed. 2.5mls of the mixture in the first tube was transferred to the second test tube labeled 100mg/ml which contains 2.5mls of distilled water. This was also mixed by shaking and the procedure serially repeated until the final concentration (12.5mg/ml) is reached in the fifth test tube. Finally, 0.1ml inoculum of the test organism was added to each of the test tubes and the tubes were tightly plugged and incubated at 37^0C for 24hours. After 24hours, the tubes were observed for turbidity or growth of the organisms. The MIC was noted as the concentration that prevented visible growth when compared with the control.

Determination of minimum bactericidal concentration

From the tube not showing any visible growth on MIC test, an inoculums was picked using a wire loop and sub-cultured

unto a nutrient agar plate. The plates were incubated at 37^0C for 24 hours after which it was observed for the presence or absence of growth. The least concentration of the test sample without any growth on the agar surface is recorded as MBC.

Determination of phytochemical components of lime extract

Preparation of Extracts

The fruits of *Citrus aurantifolia* were collected and cleaned. The fruit of citrus was boiled and passed through a mesh sieve. A watery juice was extracted with acetone, ethanol, petroleum ether, chloroform, hydro alcohol using cold maceration method. The extraction was done for 72 hours at room temperature with mild shaking. The extracts were filtered and concentrated. The *Citrus aurantifolia* were subjected to preliminary phytochemical screening for the presence or absence of various active metabolites. The preliminary screening of C*itrus aurantifolia* reveals the presence of flavonoids, tannis, alkaloids. Tannis and saponins are well known since they are important plant metabolites responsible for antimicrobial activity

Detection of alkaloids: Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.(Ncube *et al*, 2008)

Detection of tannins

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.(Handa *et al*, 2008)

Detection of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids. (Das *et al*, 2010)

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids. (Parekh *et al*, 2006)

Detection of saponins

Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins. (Eloff, 1998)

Foam Test: 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.(Lapornik *et al*, 2005)

Results and Discussion

The morphological and biochemical examination of the isolates reveals them to be *Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus spp, Klebsiella pneumoniae, and Escherichia coli.*

The diameter of the zone of inhibition of lime, honey and lime and honey combinations against the isolates are shown in Table 2. As observed from the table, 40% Mixture of lime and honey produces the highest zone of inhibition with the range of 23.00mm – 24.33mm, followed by 30%

(22.67mm) while the least was recorded with 10% mixture (range = 14.67mm). For the individual organism *Klebsiella pneumoniae* was mostly inhibited by the 40% mixture (24.33mm) while lime alone and 30% mixture had the same effect (21.33mm). The least activity was observed with 10 % mixture (14.67mm). *Staphylococcus aureus* was mostly inhibited by the 40% and 30% mixture (23.00mm and 22.67mm) respectively

This was followed by lime alone and 20% mixture (18.33mm and 21.00mm) respectively. The least activity was observed with honey alone (16.67mm) *Streptococcus spp* was mostly inhibited by the 40% mixture (23.67) followed by 30% mixture (22.64mm), lime alone (17.00mm) and 20% mixture (16.67mm) in that order. The control antibiotics exhibited inhibitory effect against the organism with the range of (22.67mm and 32.33mm).

Table 3 Shows the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of lime, honey and the mixture of both against the test isolates. The least concentration of lime that inhibited the growth of *Staphylococcus aureus* and *Klebsiella pneumoniae* was 50mg/ml with an MBC value of 50mg/ml and 100mg/ml respectively. The MIC and MBC of lime against *Streptococcus spp* was at 100mg/ml.

At 25mg/ml, Honey inhibited the growth of *Staphylococcus aureus* with an MBC value of 50mg/ml. for *Streptococcus spp* and *Klebsiella pneumoniae* the minimum inhibitory concentration of honey against them was at 100mg/ml and 50mg/ml respectively. Honey was able to kill these 2 organisms at a concentration of 100mg/ml.
The MIC concentration of 40% mixture of lime and honey was 25 mg/ml for all the isolates. At 50mg/ml, 30% mixture of lime and honey inhibited *Staphylococcus aureus and Klebsiella pneumoniae* with MBC value of 100mg/ml. For 20% mixture of both, *Staphylococcus aureus* was inhibited at concentration of 50mg/ml while *Klebsiella pneumoniae* and *Streptococcus spp* had MIC and MBC value of 200mg/ml. 10% had no inhibitory effect on *Staphylococcus aureaus* but only inhibited *Klebsiella pneumoniae* and *Streptococcus spp* at concentration of 200mg/ml. The phytochemical screening test of lime extract shows the presence of Saponin, Alkaloids, Flavonoids and Tannis. This is as shown in table 4.

The activity of any agent against microorganisms is as a result of the presence of substances that are capable of inhibiting the growth of the microorganism (Molan, 1992). As observed from this study, the lime and honey singly and in combination exhibited an antibacterial effect against the isolates, indicating that there are some bioactive agents present in them. This activity could be tied down to the active nature of honey and lime besides other antimicrobial properties they were known to possess.

Nevertheless, the combination of lime and honey showed greater activity than when they were used singly. This suggest a synergistic activity between the lime and honey, indicating that there were components in either honey or lime which when alone has lesser activity but coming together enhances the activity of each other, thereby, resulting to higher activity as observed. This finding is in agreement with what was reported by Beringer (1999) that most extract or bioactive agent produces greater activity when combined together.

Table.1 Zone of inhibition diameter (mm) of samples against the isolates

Bacterial isolates	Lime	Honey	40% L/H	30% L/H	20% L/H	10% L/H	Antibiotics		
							Doxy	Bry	Amo
Staphylococcus aureus	18.33	16.67	23.00	22.67	21.00	18.00	22.67	24.00	27.67
Streptococcus spp	14.33	17.00	23.67	22.64	16.67	15.67	22.67	24.00	24.00
Klebsiella pneumonia	21.33	16.33	24.33	21.33	17.33	14.67	32.33	23.33	25.67

Keys: L/H = lime/honey; Doxy = doxycycline; Bry = Brylhromycine; Amo = Amozine

Table.3 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of lime, honey and the mixture of lime and honey against the isolates

Sample	Bacterial	Conc (mg/ml)	Turbidity	Growth on plates	MIC(mg/ml)	MBC(mg/ml)
Lime	*Staphylococcus aureus*	200 100 50 25 12.5	- - - + +	- - - 	50	50
	Klebsiella pneumonia	200 100 50 25 12.5	- - - + +	- - + 	50	100
	Streptococcus spp	200 100 50 25 12.5	- - + + +	- - + 	100	100
Honey	*Staphylococcus aureus*	200 100 50 25 12.5	- - - - +	- - - + 	25	50
	Streptococcus spp	200 100 50 25 12.5	- - + + +	- - 	100	100
	Klebsiella pneumonia	200 100 50 25 12.5	- - - + +	- - + 	50	100

40% L/H	*Staphylococcus aureus*	200 100 50 25 12.5	- - - - +	- - - +	25	50
	Klebsiella pneumonia	200 100 50 25 12.5	- - - - +	- - - +	25	50
	Streptococcus Spp	200 100 50 25 12.5	- - - - +	- - - -	25	25
30% L/H	*Staphylococcus aureus*	200 100 50 25 12.5	- - - + +	- - +	50	100
	Klebsiella pneumonia	200 100 50 25 12.5	- - - + +	- - +	50	100
	Streptococcus spp	200 100 50 25 12.5	- - - - +	- - - +	25	50
20% L/H	*Staphylococcus aureus*	200 100 50 25 12.5	- - - + +	- + +	50	100
	Klebsiella pneumonia	200 100 50 25 12.5	- + + + +	-	200	200
	Streptococcus spp	200 100 50 25 12.5	- + + + +	-	200	200

10% L/H	*Staphylococcus aureus*	200 100 50 25 12.5	+ + + + +		>200	>200
	Klebsiella pneumonia	200 100 50 25 12.5	- + + + +	-	200	200
	Streptococcus spp	200 100 50 25 12.5	- + + + +	-	200	200

Keys: + = Turbidity/growth
= No turbidity
L/H = lime/ honey

Table.3 Phytochemical screening result of the lime sample

Sample	Phytocomponents tested	Remarks
Lime sample (*Citrus aurautifolia)*	Saponin	+
	Alkaloids	+
	Flavoniods	+
	Tannins	+

Keys: + = presence

More so, the mixture of lime and honey exhibited greater activity at higher percentage i.e. 40% better than 30%, 30% better than 20% in that order. This could be due to the fact that at 40%, the mixture (lime and honey) had a higher concentration of the components, indicating that the higher the concentration of any bioactive agent present in an agent, the greater the activity. This resembles the findings made by *Mshelia et al,* (2014) where activity were shown to be greater at a higher percent than at lower percent.

However, two gram positive organism (*Staphylococcus aureus and Streptococcus spp*) and one gram negative (*Klebsiella pneumoniae)* were isolated from sputum sample of patients suffering from cough and were used for the study. As observed, the agent (lime and honey) tested showed greater activity against *Klebsiella pneumoniae* which is a gram negative organism than the gram positive organism. These findings, disagree with what was reported by *Hugo et al, (1991)* that gram negative organisms are mostly resistant to bioactive agents (due to the nature of their cell wall) than the gram positive.

Therefore, as vividly demonstrated in this work; honey, lime and honey and lime combination were found to possess antibacterial activity at varying degrees.

Since lime extract and honey were able to produce effective inhibitory activity against the isolates. When refined/purified, they

could serve as a good pharmaceutical tool in the production of drugs that could be used to combat infections affecting the respiratory tract.

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