

Original Research Article

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***In vitro* Antimicrobial Activity of Honey and Lemon Juice Mixture against Clinical Isolates from Patients with Respiratory Tract Infections, Bamenda-Cameroon**

Gerald Ngo Teke^{1*}, Flore Nguemaïm Ngoufo¹, Alexis Njiki Njiki²
and Henri Lucien Fouamno Kamga²

¹Department of Biomedical Sciences, ²Department of Medical Laboratory Science, Faculty of Health Sciences, The University of Bamenda, P.O. BOX 39 Bambili, Cameroon

*Corresponding author

ABSTRACT

Honey and lemon juice are ingredients used indigenously to treat a number of microbial infections. Our aim was to study the *in vitro* antimicrobial activity of honey and lemon fruit juice mixtures against clinical isolates from patients with respiratory tract infections. Mixtures of honey and lemon juice were prepared at various proportions (90:10, 75:25, 50:50, 25:75 and 10:90% v/v respectively) and tested for antimicrobial activity against clinical isolates using agar well diffusion method. MICs and MBCs of the most active test mixture proportion were determined using broth and agar media respectively. Cirprofloxacin (for bacteria) and ketoconazole (for yeast) were used as reference substances. Data were analysed with the help of statistical package for social sciences (SPSS), version 20. Student Newman-Keuls test was used to compare inhibition diameters. Generally, the diameter of microbial growth inhibition zones increased with decreasing proportion of honey in the mixtures. *Staphylococcus aureus* was most sensitive to the honey/lemon mixture of 25/75% (v/v) producing a growth inhibitory diameter of 14 mm. Two test mixture proportions (75/25 and 90/10% v/v) were not able to inhibit the growth of *S. aureus* and *Streptococcus pyogenes*. *Candida albicans* was susceptible at 25/75% (8.66 mm) and 10/90% (11.33) test concentrations. The MIC values for *S. aureus* and *S. pyogene* were recorded as 60% concentration of test mixture. *C. albicans* had a higher MIC value (80%). All the MBC values for the test microorganisms were considered greater than 80% of test mixtures. Appropriate mixtures of honey and lemon juice (lemon 50-75% for bacteria and 75-90% for yeast) could be a better alternative treatment against some infectious diseases.

Keywords

Honey/lemon juice mixtures, *In vitro*, Antimicrobial activity, Clinical isolates, Bamenda-Cameroon

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Introduction

Honey is a sweet food made by bees using nectar from flowers. The variety produced by honey bees (the genus *Apis*) is the one most

commonly referred to and is the type of honey collected by beekeepers and consumed by humans. There are many species including: *Apis andreniformis*, *A. florea*, *A. dorsata*, *A. cerana*, *A. koschevnikovi*, *A. mellifera*, *A.*

nigrocincta (Denise *et al.*, 2002). Most microorganisms do not grow in honey because of its low water activity of 0.6 (Molan, 1992). Hydrogen peroxide, methylglyoxal, bee defensin, pH, osmotic effect as well as leptosin were known to be responsible for the antimicrobial effects of honey (Mandal, 2011; Kato *et al.*, 2012).

Lemon fruit is an available citrus fruit, popular for its culinary and medicinal uses. Its fruit juice consists of about 5% citric acid that gives to it a sour taste (NPCS, 2012). It is an important medicinal plant of the family Rutaceae. It is a rich source of vitamin C and it is cultivated mainly for its alkaloids, which are having anticancer activities and the antibacterial potential in crude extracts of different parts (*viz.*, leaves, stem, root and flower) of lemon against clinically significant bacterial strains has been reported (Kawaii *et al.*, 2000). Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities (Burt, 2004). There are different varieties of lemon including: Bush lemon tree, Eureka, Lisbon, Meyer, Ponderosa, and Variegated pink (NPCS, 2012). The species *Citrus limon* was used in this study. This study was aimed to determine the antibacterial activity of honey and lemon juice mixtures on strains of *Staphylococcus aureus*, *Streptococcus pyogenes* and yeast (*Candida albicans*) isolated and characterized by standard microbial procedures from respiratory tract of patients consulting at the Regional Hospital Bamenda.

Materials and Methods

Collection of test materials and microbial samples

Pure colonies of *S. aureus*, *S. pyogenes* and yeast (*C. albicans*) isolated from throat and

mouth was collected at the Microbiology Unit of the Regional Hospital Bamenda, Cameroon. Honey and ripen lemon fruits (*Citrus limon*) were purchased from the Bamenda food market. The honey was extracted from Mbengwi, a subdivision in the same Region of the North West of Cameroon.

Verification of bacterial isolates from throat infections

The microbial isolates from mouth and throat collected at the Microbiology Unit of the Regional Hospital Bamenda were sub-cultured on Blood agar and Sabouraud Dextrose agar for confirmation and were further subjected to Gram stain techniques, catalase test, coagulase test and lance field grouping (Cheesbrough, 2006). The bacterial and fungal strains were grown at 35°C and maintained on nutrient agar (NA, Conda, Madrid, Spain) and Sabouraud Dextrose Agar (SDA, Conda) respectively.

Preparation of honey and lime mixture at various proportions

The commercial honey obtained from Bamenda food market was kept in sterile screwed cap containers and stored at 5°C in the Microbiology Laboratory of the Regional Hospital Bamenda prior to use.

Lemon fruits were obtained from the same market, washed with tap water and vinegar and then allowed to air dry. It was sterilized with 70% alcohol and cut using a knife which was first sterilized with 70% alcohol and passed over flame. The lemon juice was squeezed, filtered using sterile cotton and glass funnel and placed in a sterile screwed cap container before refrigerating for later use.

The honey and lemon juice were removed from the fridge and the honey placed in warm water to bring it to the liquid state. Then both honey

and lemon juice were allowed on the slap to attain room temperature. Using a 1000 µl micropipette, the following five (5) mixtures were prepared at different proportions of each substance to a final volume of two milliliters (2 ml):

90% (v/v) honey +10%(v/v) lemon juice (900 µl honey+100 µl lemon juice)

75% (v/v) honey + 25% (v/v) lemon juice (750 µl honey +250 µl lemon juice)

50% (v/v) honey + 50% (v/v) lemon juice (500 µl honey +500 µl lemon juice)

25% (v/v) honey + 75% (v/v) lemon juice (250 µl honey +750 µl lemon juice)

10% (v/v) honey + 90%(v/v) lemon juice (100 µl honey +900 µl lemon juice)

Preparation of antimicrobial discs from test mixtures

Antimicrobial discs of 6mm of diameter were made using Whattmann filter paper^o3. These discs were sterilized under ultraviolet light for 2hrs then submerged in the test mixtures for 30 minutes (Vineetha *et al.*, 2015) such that each disc could absorb a quantity of about 20 µl of prepared mixture.

Preparation of microbial suspensions

The microbial isolates were first activated by streaking on nutrient agar and incubating for 24 and 48 hours respectively for bacteria and yeast. A pipette with a sterile pipette tip was used to transfer 5 ml of sterile saline distilled water into labeled sterile plain tubes. A wire loop was heat flamed, cooled and used to touch 3 to 5 well isolated similar colonies of the test organisms from the pure cultures and emulsified in the sterile saline distilled water in the labeled sterile plain tubes with each representing a particular isolate. Each microbial suspension in test tube was mixed vigorously, compared visually and adjusted to the turbidity standard of McFarland 0.5 (Cheesbrough, 2006).

Antimicrobial susceptibility testing

The various honey and lime fruit juice mixtures were tested for antimicrobial activities using agar disc diffusion technique to determine the diameter of growth inhibition zones.

Agar disc diffusion test

Briefly, a quantity of 100µl of suspension of the test microorganisms (prepared as mentioned above) was spread on Mueller Hinton agar and Sabouraud dextrose agar medium respectively for bacteria and yeast. Prepared filter paper discs such as to separately absorb 20 µl of the different concentrations of honey/lemon fruity juice mixtures (90/10, 75/25, 50/50, 25/75 and 10/90% v/v respectively) were placed on the seeded plates. This was done using sterile forceps under aseptic conditions. Ciprofloxacin (for bacteria) and ketoconazole (for yeast) were used as reference substances.

Within 30 minutes of applying the discs, the culture plates were inverted and incubated aerobically at 35°C for 24 hours for bacterial strains and 48 hours for yeast. This was done for each isolate and each assay in this experiment was repeated thrice. The diameters of zones showing complete microbial growth inhibition by gross visual inspection around each disc was measured in millimeters using a transparent meter rule over the back of the inverted plate and across each disc. A Clinical and Laboratory Standards Institute (CLSI) reference chart was used to report microbial sensitivity as follows: Resistant (0-8mm); Intermediate (9-13mm); Sensitive (14mm and above).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The broth macrodilution technique was used for this assay (Barchiesi *et al.*, 1994). This

was done with the proportion of the mixture which with a majority of higher zones of microbial growth inhibition. I.e. 25% honey: 75% (v/v) lemon fruit juice for *Staphylococcus aureus* and *Streptococcus pyogenes*; and 10/90% (v/v) for *Candida albicans* using broth dilution method. These mixtures were prepared and considered mother test samples from which a certain quantity was obtained (x ml) and transferred separately into 5 ml test tubes previously containing Mueller Hinton broth or Sabouraud dextrose broth (y ml).

Freshly prepared microbial suspension (20µl) was then separately added to the tubes making a final volume of 3 ml and the test concentrations were: 0, 20, 40, 60, 80 and 100%. A sterility control tube containing the test mixture and broth medium but no microorganisms was equally made. The tubes were vortexed and incubated at 35°C for 24 hours for bacterial strains and 48 hours for yeast. The turbidity of each test tube was visually inspected and compared with the control (tube with 0% test mixture). The lowest of the honey/lemon fruit juice mixture test concentration in clear test tubes was considered as the MIC (Keizo *et al.*, 2012).

For the MBC determination, all the clear test tubes indicating no visible sign of microbial growth or turbidity in the MIC assay, were further sub-cultured on sterile Mueller Hinton agar/Sabouraud Dextrose agar plates by streak plate method.

Then the plates were incubated at 35°C for 24 hours for bacterial strains and 48 hours for yeast. The least concentration that did not show growth of test organisms was considered as the MBC.

Data management and analysis

Data on inhibition zone diameters were analysed using statistical package for social

sciences (SPSS), version 20. Student Newman-Keuls test was used to compare inhibition diameters at different test concentrations. MICs and MBCs were expressed as % of honey/lemon fruit juice mixtures.

Ethical considerations

Ethical clearance was obtained from the Institutional Review Board of The University of Bamenda (2018/0022/UBa/IRB). Administrative authorization was obtained from the Regional Hospital Bamenda.

Results

Agar disc diffusion assay

A total of four mixtures at different proportions of honey and lemon fruit juice were tested against microbial isolates causing throat infections: *Staphylococcus aureus*, *Streptococcus pyogenes* and *Candida albicans*. Globally, the diameter of microbial growth inhibition zones increased with decreasing proportion of honey in the mixtures. *S. aureus* was most sensitive to the mixture of 25/75% (v/v) honey/lemon fruit juice producing a growth inhibitory diameter of 14 mm (Fig. 1).

Two proportions (75/25 and 90/10% (v/v) of honey/lemon fruit juice mixtures were not able to inhibit the growth of *S. aureus*. This similar situation was observed for *S. pyogenes* (Fig. 2) in the presence of the test mixtures.

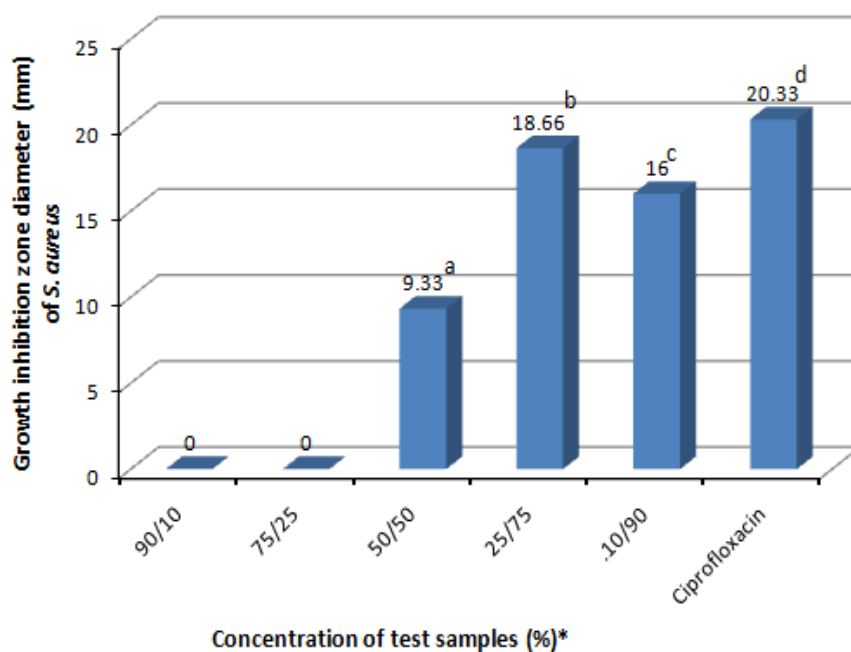
The honey and lemon fruit juice mixtures tested on *Candida albicans* isolate was active only at two test concentrations. The smaller diameter of growth inhibition zone was 8.66 mm at 25% (v/v) honey + 75% (v/v) lemon juice while the least honey content (10%) in the mixture gave 11.33 mm inhibition zone diameter (Fig. 3).

Table.1 Microbial susceptibility, MICs and MBCs of honey and lemon fruit juice mixtures

Microbial isolates	Mean diameter ^a value ± SD	Susceptibility ^b	Honey-lemon fruit juice mixture	
			MIC (%)	MBC (%)
<i>S. aureus</i>	17.33 ± 0.28	Sensitive	60	>80
<i>S.pyogenes</i>	13.33 ±0.57	Intermediate	60	>80
<i>C. albicans</i>	9.99 ± 0.57	Intermediate	80	>80

^atwo test concentrations with high inhibition zones were considered; ^bResistant (0-8 mm); Intermediate (9-13 mm); Sensitive (14 mm and above); SD: standard deviation

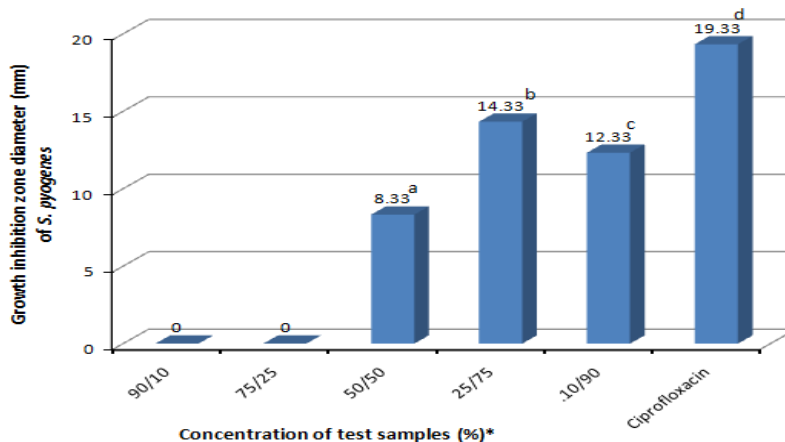
Fig.1 *Staphylococcus aureus* growth inhibition at various proportions of honey/lemon fruit juice mixtures



^aValues with different superscripts are significantly different; Student Newman Keuls at $p < 0.05$.

*The proportion of honey decreases while that of lemon fruit juice increases

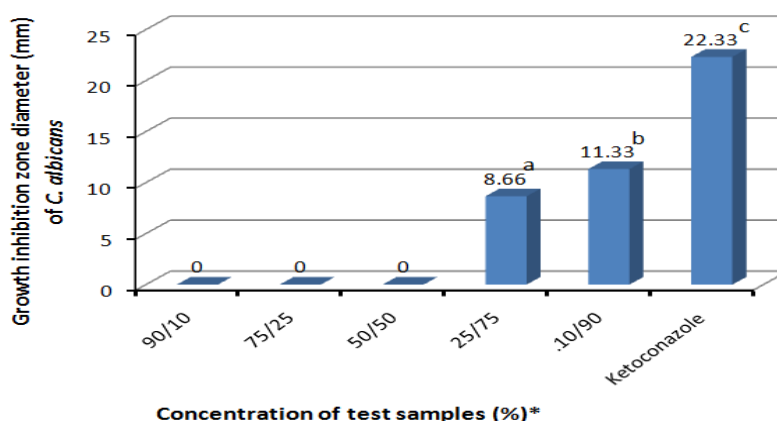
Fig.2 *Streptococcus pyogenes* growth inhibition at various proportions of honey/lemon fruit juice mixtures



^aValues with different superscripts are significantly different; Student Newman Keuls at $p < 0.05$.

*The proportion of honey decreases while that of lemon fruit juice increases

Fig.3 *Candida albicans* growth inhibition at various proportions of honey/lemon fruit juice mixtures



^aValues with different superscripts are significantly different; Student Newman Keuls at $p < 0.05$.

*The proportion of honey decreases while that of lemon fruit juice increases

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Only one proportion of the mixtures with larger zones of growth inhibition on bacteria/yeast was used to determine the MIC and MBC. The MIC values for *S. aureus* and *S. pyogene* were recorded as 60% concentration of test mixture (Table 1). *C. albicans* had a higher MIC value (80%). All the MBC values

for the test microorganisms were considered greater than 80% of test mixtures.

In a study, bacteria and yeast isolated from patients presented with respiratory infections were tested for susceptibility in the presence of honey/lemon juice mixtures. The honey/lemon juice mixtures were more active in inhibiting bacterial growth at higher lime proportions. Moderate microbial susceptibility was observed at 50% mixture

concentration. This finding is similar to that reported by Mshelia *et al.*, (2017) in the susceptibility of similar microbes to this mixture.

In our study, the microbial inhibition zones for the mixture at 50:50 were slightly lower (less than 20 mm) compared to their findings (greater than 20mm). Considering the effects of honey and lemon juice mixtures at appropriate concentrations, the observed antimicrobial potential was slightly higher compared to those of honey and lemon juice tested singly as reported elsewhere (Mshelia *et al.*, 2017). It is worth mentioning that the microbial specimen and test materials may respond with slight differences regarding their sources and environmental conditions as suggested by Allison and Jennifer (2008).

From our findings, *C. albicans* exposed to the honey/lemon juice mixtures was susceptible only at low honey but high lemon juice proportions (25/75% and 10/90% respectively). Moreover, the highest test concentration (80%) could not kill the fungal agent. Similar studies with honey showed that it had antifungal activity against *C. albicans* (Shayeste *et al.*, 2013; Somnath *et al.*, 2017; Koc *et al.*, 2008) showed that honey/lemon juice mixture was efficient in inhibiting the growth of some fungal agents including *C. albicans*.

Other researchers have reported that honey inhibited the growth of *Candida* even up to 100% concentration (AL-Waili *et al.*, 2005). This fungal growth inhibition was not observed to be fungicidal as had equally been mentioned in other studies (Mayer and Williams, 2004; Bogdanov *et al.*, 2008).

Many phytochemical compounds with antibacterial components have been reported separately in honey and lime juice. According to Sohn *et al.*, (2004) and Soetan *et al.*,

(2006), some components like phenolic, flavonoids, alkaloids, saponins, tannins, steroids, glycosides and triterpenoids in lemon juice have antibacterial activity against gram positive and negative bacteria. In addition, some of these components like phenols may interfere with the active transportation of substances across the cytoplasmic membrane (Davidson and Naidu, 2000), thereby provoking bacterial death.

Honey has been reported to be acidic (Teke and Betie, 2016; Ball, 2007) and rich in hydrogen peroxide (produced by glucose oxidase originating from the bees) and methylglyoxal (Ramalivhana *et al.*, 2014; Mavric *et al.*, 2008; Paulus *et al.*, 2008). Lemon juice is equally known to be acidic (Kwakman *et al.*, 2008). Hence the acid nature of the mixture and the presence of hydrogen peroxide could be contributory factors to bacterial growth inhibition (Manisha and Shyamapada, 2011; Amal, 2014) beside other antimicrobial properties they are known to possess.

The results of our study showed that appropriate mixtures of honey and lemon juice (lemon 50-75% for bacteria and 75-90% for yeast) exhibit antibacterial and antifungal activities.

Inadequate anti-infective treatment mostly due to financial hardship is a contributing factor among others to the emergence of microbial resistance. Hence the use of honey and lemon, which can easily be obtained, and whose efficacy has been proven could be a better alternative treatment against some infectious diseases.

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