ARTÍCULO ORIGINAL

Antibacterial effect of crude methanol *Carica papaya* L. (papaya) extract and amoxicillin combination

Efecto antibacteriano de la combinación del extracto metanólico crudo de *Carica papaya L*. (papaya) y amoxicilina.

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ABSTRACT

Background: the emergence of multi-drug resistant bacteria and the diseases caused by them are a serious threat to global health necessitating an urgent need for new approaches to combat them. Synergy studies of conventional antimicrobial drugs and medicinal plants with antibacterial effects are important to establish whether it is prudent to recommend their concurrent administration to get successful treatments.
Objective: evaluate the antibacterial effect resulting from the combination of *Carica papaya* (papaya) and amoxicillin.
Methods: the papaya methanol extract was obtained from seeds and phytochemical screening was done. Checkerboard assay was used to determine the Minimum Inhibitory Concentration. Combined effect of both *Carica papaya* methanol extract and amoxicillin was determined by calculating the Fractional Inhibitory Concentration index. Strains of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used in the tests.
Results: phenols and tannins were found in the *Carica papaya* seed methanol extract. The minimum inhibitory concentration of *Carica papaya* extract was 100 µg/mL for both microorganisms studied which was higher than the Minimum Inhibitory Concentration of amoxicillin being 3.12 µg/mL for *Escherichia coli* and 0.2 µg/mL for *Staphylococcus aureus.* The Fractional Inhibitory Concentration of the combination of drugs was 0.99 for *Escherichia coli* and 2.51 for *Staphylococcus aureus.*
Conclusions: the antibacterial effect of *Carica papaya* extract may be attributed to the presence of phenolic compounds. There was no interaction between amoxicillin and *Carica papaya* extract on *Staphylococcus aureus,* but the antimicrobial activity against *Escherichia coli* of both drugs can be potentiated by their additive interaction.

Key words: additive interaction, *Carica papaya*, amoxicillin, minimum inhibitory concentration, fractional inhibitory concentration index.

RESUMEN

Introducción: la creciente multi-resistencia bacteriana y emergencia de enfermedades causadas por estas bacterias, constituyen un serio problema global, por lo que es importante y urgente el desarrollo de nuevas propuestas terapéuticas para combatirlas. Estudios sinérgicos sobre la combinación de antimicrobianos convencionales y plantas con efectos antibacterianos son importantes para determinar si es aconsejable la administración concomitante de los mismos.
Objetivo: evaluar el efecto antibacteriano de la combinación de *Carica papaya* (papaya) y amoxicilina.
Método: fueron usadas semillas de papaya para obtener el extracto alcohólico de papaya y realizado el estudio fitoquímico. La Concentración Mínima Inhibitoria fue determinada por el método del "tablero de ajedrez". La Concentración Inhibitoria Fraccionada se calculó para medir el posible efecto sinérgico de la combinación entre el extracto alcohólico de *Carica papaya* y la amoxicilina. Cepas de *Staphylococcus aureus* ATCC 25923 y *Escherichia coli* ATCC 25922 fueron usadas.
Resultados: en el extracto alcohólico de papaya fueron encontrados fenoles y taninos. La Concentración Mínima Inhibitoria del extracto de papaya coincidió para ambos microorganismos (100 µg/mL), la cual fue mayor que la Concentración Mínima Inhibitoria de la amoxicilina, siendo 3.125 µg/mL para *Escherichia coli* y 0.2 µg/mL para *Staphylococcus aureus.* La Concentración Inhibitoria Fraccionada de la combinación de drogas, fue 0.99 para *Escherichia coli* y 2.51 para *Staphylococcus aureus*.
Conclusiones: los compuestos fenólicos presentes en el extracto de papaya pueden ser responsables de su efecto antimicrobiano. No existe interacción entre la amoxicilina y el extracto metanólico de papaya contra *Staphylococcus aureus*. Sin embargo, la actividad antomicrobiana contra *Escherichia coli* puede ser potenciada por su interacción aditiva.

Palabras clave: Interacción aditiva, *Carica papaya*, amoxicillin, concentración mínima inhibitoria, concentración inhibitoria fraccionada.

INTRODUCTION

Infectious diseases are among the major diseases of public health concern and account for almost 50.000 deaths every day. This situation has further been complicated by the rapid development of multidrug resistant organisms and emergence of new pathogenic microorganisms.[1,2]

Antibiotics are one of the most important drugs in fighting bacterial infections and have greatly improved the health-related quality of human life since their introduction in the medical practice. However, over the past few decades these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not only because many of them

produce toxic reactions but also due to emergence of drug resistant bacteria,[3] thus resulting into their non-responsiveness to treatment with mostly a single drug regimen and, consequently in therapeutic failure.[4]

The situation of resistance to previously effective antibiotics that have emerged globally in recent years is especially dire in Africa where irrational antibiotic practices are common. According to a study conducted in Northern Uganda in 2013, there was widespread resistance among all uropathogens tested to cotrimoxazole, amoxicillin, nitrofurantoin and nalidixic acid. Among those microorganisms were included *S*almonella species and *E. coli*.[5]

The current edition of the National Treatment Guidelines in Uganda recommends the use of amoxicillin for many conditions like typhoid fever, laryngitis, pneumonia, infective endocarditis, cellulitis and other skin diseases, ear, nose and throat conditions, genito-urinary diseases, obstetric and gynecological conditions, zoonotic diseases and oral and dental conditions[6] as supported by most of the updated Pharmacological books.[7,8]

However, evidence based medicine as well as studies conducted in Ugandan setting, have suggested a high resistance to amoxicillin.[5,9] Therefore, continued use of this antibiotic may increase chances of treatment failure, leading to unnecessary patient suffering and increased health care costs in the long run.

In rational drug therapy, it is often essential to concurrently administer two or more agents in order to reduce development of resistance and minimize side effects. However, the drug interaction may have different effects on the host as well as the infecting organism and can increase or decrease potency, as well as increase adverse effect or toxicity.[10]

The combination of known antimicrobial agents and bioactive plant extracts is a novel concept and has been reported to be profitable for patients with serious infections caused by drug-resistant pathogens. Plants antimicrobials have been found to be synergistic enhancers; they may not have any antimicrobial properties when used alone, but taken concurrently with standard drugs they can enhance the effect of them.[3]

Considering the surfacing of resistant species of *S. species* and *E. coli* to therapeutically available agents like amoxicillin, probably due to antibiotic misuse by poorly trained health workers and by domiciliary self-medication practices that have become commonplace in Uganda,[5] it has verified a clear and emerging need to introduce new antimicrobial alternatives in the therapeutic arsenal. In this view arises the possibility to investigate the interactive effects of conventional compounds and natural products, which can promote greater effectiveness of each drug.[11]

Carica papaya L. (*C. papaya*) specie, known as papaya, which belongs to Caricaceae family, grows in tropical and subtropical countries,[12] Uganda being one of them. Considering the known antimicrobial activity of this plant,[2,13,14] the purpose of this study was to assess the antibacterial effect resulting from the combination of *C. papaya* and amoxicillin on strains of *S. aureus* and *E. coli*.

METHOD

The antibacterial effect resulting from the combination of *C. papaya* and amoxicillin was assessed determining the Minimum Inhibitory Concentration. Finally, the combined effect of both was determined by calculating the Fractional Inhibitory Concentration index.

Plant material

C. papaya seeds were collected from a garden in Mbarara, Uganda, identified by the University botanist and given a voucher Number Bridge Mwesigwa 001.

Preparation of the extract

The *C. papaya* fruits, commonly known as pawpaw or papaya, were cut open to obtain the seeds; the seeds were air dried under a shade for five consecutive days to preserve the activity of the active constituents and later pulverized in a blender to obtain the surface area for optimal drug extraction, to obtain a desirable yield.

The cold maceration method of extraction was used to macerate 250.8 g of the powder (70 % methanol w/v). The extract was filtered and concentrated to a solid residue of plant extract.

The plant extract was obtained at a percentage yield of 4.54 % after evaporating off the methanol to dryness using an oven (MEMMERT 2000) at 60 ^{o}C.[15]

hytochemical screening

Two grams of 4.54 % of *C. papaya* extract were dissolved in 20 mL of distilled water to form a solution which was used for phytochemical screening.

Various tests, mainly chemical reactions identification by color change or precipitated formations, were used to determine the presence of secondary metabolites: saponins (Foam), proteins (Biuret), free amino acid/ amines (nihydrin), isoflavones (Shinoda), steroids and triterpenoids (Libermann-Buchard), phenols and tannins (ferric chloride $FeCl_3$), fats and oils (Sudan III), lactones and coumarins (Baljet), alkaloids (Dragendorff), glycosides (Keller-killiani), and reducing sugars (Fehling).[16]

Microbiological Assay

The organisms used were standard strains of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 which were obtained from MUST Microbiology Laboratory and Epicenter Laboratory. The checkerboard assay was used to determine the individual Minimum Inhibitory Concentration (MIC) of *C. papaya* methanol extract and amoxicillin, as well as the MIC of the combination of these two drugs/compounds.

The MIC results were used to calculate the Fractional Inhibitory Concentration Index (FIC index) to determine the possible synergistic effects of the association between amoxicillin and *C. papaya* methanol extract.

Determination of MIC by checkerboard assay

· *Preparation of Rezasurin solution 0.01 %*: Resazurin solution 0.01 % was prepared from Resazurin powder and filtered through the 0.2 µm pore filter. If not

immediately used, the solution was preserved at 4 °C for a maximum period of one week and protected from the light. Resazurin solution which is an oxy-reduction indicator was employed as MIC indicator.[17]

· *Preparation of the bacteria culture suspension*: Upon sub-culturing the reference strains on Nutrient agar, a colony of each organism was emulsified in 1.5 mL of Muller-Hinton broth and incubated overnight at 37 ^{0} C. The density of the bacteria culture suspension to be used for the tests was adjusted, using the broth, to McFarland standard 0.5 (1.5 x 10^8 Colony Forming Units/ml) using a Turbidometer (DENSIMAT).[17]

· *Preparation of the antibiotic working solutions:*[17] Both amoxicillin and *C. papaya* stock solutions with a concentration of 1mg/mL were each diluted 1 in 5, giving a concentration of 200 µg/mL. This was filtered through a 0.2 µm membrane pore into separate sterile well-labeled 2 mL cryo-vials. Sterility of the suspensions was tested by inoculating a drop of each on a blood agar plate and incubating at 37 ^{0}C for 48 h and observing for growth.

· Amoxicillin with a concentration of 1 mg/mL (1000 µg/mL) was diluted 1 in 5 to easily filter through the millipore filter, giving a concentration of 200 µg/mL, then was serially diluted 9 fold in Muller-Hinton broth on the microtiter plate (neat, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512; giving concentrations of 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL 6.25 µg/mL, 3.12 µg/mL, 1.5 µg/mL, 0.8 µg/mL, 0.4 µg/mL, 0.2 µg/mL, respectively.

· *C. papaya* with a concentration of 1 mg/mL (1000 µg/mL) was serially diluted 9 fold in Muller-Hinton broth on the microtiter plate (neat, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, giving concentrations of 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.12 µg/mL, 1.5 µg/mL, 0.8 µg/mL, 0.4 µg/mL, 0.2 µg/mL, respectively.

· *Preparation of the microtiter plates:* [17] 100 µL of sterile water were pipetted in all the outer wells of a 96-well microtitre plate to provide moisture for the test.

A total of 50 µL of Mueller-Hinton broth was distributed into each well of the micro-dilution plates. The first drug (*C. papaya*) working solution of the combination was serially diluted along the first column, while the second drug (amoxicillin) solution was serially diluted along the first row. Dilutions were started from the last well with a higher concentration (neat) towards the first well for the two drugs. These concentrations were then diluted along the ordinate (vertical axis) and abscissa (horizontal axis) to obtain varying concentrations of the combined drugs (Range: 200 µg/mL to 0.2 µg/mL). The resulting checkerboard contained each combination of the two antimicrobial agents, with wells that contain the highest concentration of each antibiotic at opposite corners.

To each of the wells, 50 µL of the bacterial suspension was added. The plates were incubated aerobically at 37 ^{0}C for 24 h. After 24 h of incubation, 20 µL of 0.01 % reassuring solution were added to each of the well and incubated for another 24 hours under the same conditions.

The wells that showed a colour change, by visual inspection, from blue to pink were considered to have growth. The colour change was a result of reduction of reassuring by live organisms. The MIC was taken as the lowest concentration of each antibiotic alone or in combination that prevented bacterial growth.

The bacterial growth was determined by the color of the reassuring indicator:[17]

· No Growth: the color remained blue.

· Growth: the color changed from blue to pink.

Calculation of the Fractional Inhibitory Concentration (FIC) Index

Interaction between the two study drugs was assessed algebraically by determining the FIC index which was calculated as follows:[11]

$FIC = FIC_A + FIC_B$

Where:

FIC_A was calculated through the ratio MIC_A combined / MIC_A alone, $_A$ being amoxicillin.

FIC_B was calculated through the ratio MIC_B combined / MIC_B alone, $_B$ being *C. papaya* methanol extract.

This index was interpreted as follows:[11]

· Synergy: FIC ≤ 0.5

· Addition: 0.5 < FIC < 1

· Indifference or No interaction: 1 < FIC < 4

· Antagonism: FIC ≥ 4

Quality control

Media was controlled for sterility and viability using the reference strain**.** Positive and negative controls were used along with the extract and drug. The expiry dates of the reagents were checked and Good Clinical Laboratory Practices were observed.

RESULTS

Phytochemistry

The phytochemical screening of the *C. papaya* methanol extract found a positive reaction to phenols and tannins, which were present in high quantities. Is flavones, glycosides and free amino acids were found in moderate concentrations (table 1).

Table 1. Phytochemical constituents of *C. papaya* crude methanol extract

Constituents	Results
Phenols	+++
Tannins	+++
Isoflavones	++
Glycosides	++
Free amino acids	++
Lactones and coumarins	+
Alkaloids	+
Reducing sugars	+

Key:
High (+++).
Moderate (++).
Low (+).

MIC of *C. papaya* and amoxicillin on *E. coli* and FIC index

The MIC of *C. papaya* extract and amoxicillin on *E. coli*, determined by checkerboard assay, was 100 µg/mL and 3.12 µg/mL respectively; while the MIC for the combination of both drugs was 3 µg/mL. Results are shown in table 2.

The FIC index was calculated using the MICs obtained and the result was 0.99 demonstrating that there is an additive interaction between amoxicillin and *C. papaya* methanol extract on *E. coli.*

MIC of *C. papaya* and amoxicillin on *S. aureus* and FIC index

The MIC of *C. papaya* extract and amoxicillin on *S. aureus*, also determined by checkerboard assay, are shown in table 3. Results indicated that for the plant extract the MIC was 100 µg/mL while for the conventional drug was 0.4 µg/mL. The MIC for the combination of both drugs was 1 µg/mL.

The FIC index was calculated using the MIC obtained and the result was 2.51 indicating no interaction between amoxicillin and *C. papaya* methanol extract on this microorganism.

Table 2. Checkerboard Technique Results for *E. coli* (ATCC 25922)

Carica papaya ug/mL	0.0	0.2	0.4	0.8	1.5	3.12	6.25	12.5	25	50	100	200
200												
100	MIC_B											
50												
25												
12.5												
6.25												
3.12												
1.5					MIC_C							
0.8												
0.4												
0.2												
0.0						MIC_A						
amoxicillin µg/mL												

Plate 1: *Escherichia coli standard* (ATCC 25922)

KEY:
MIC_A MIC of amoxicillin.
MIC_B: MIC of *C. papaya*.
MIC_C: MIC of the drugs combination.

Table 3. Checkerboard Technique Results for *Staphylococcus aureus* (ATCC 25923)

Carica papaya ug/mL	0.0	0.2	0.4	0.8	1.5	3.12	6.25	12.5	25	50	100	200
200												
100	MIC_B											
50												
25												
12.5												
6.25												
3.12												
1.5												
0.8												
0.4												
0.2				MIC_C								
0.0			MIC_A			MIC_A						
amoxicillin µg/mL												

Plate 2: *Staphylococcus aureus* (ATCC 25923)

Key:
MIC_A: MIC of amoxicillin.
MIC_B: MIC of *C. papaya*.
MIC_C: MIC of the drugs combination.

DISCUSSION

Phytochemical screening of *C. papaya* extract showed the presence of phenols, tannins, amino acids, reducing sugars, flavonoids, lactones, coumarins and alkaloids, which match with those components reported by Augustine Ocloo in 2012. Concentration of phenols and tannins in *C. papaya* extract was high which are believed to be responsible for the antimicrobial activity.[1]

The phenolic components, for example tannins in *C. papaya* work by different proposed mechanisms to exert antimicrobial activity against microbes which include inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation. A further mechanism involving iron deprivation has been also proposed.[18]

The MIC of *C. papaya* seed extract was the same on *E. coli* (Gram negative bacterium) and *S. aureus* (Gram positive bacterium) meaning that the papaya extract shows the same activity against both types of bacteria. Earlier studies have been conducted with similar objectives and also similar results. However, those researchers reported slight differences compared to the present findings. In Nigeria in 2012, investigators found that dried and fresh leaves extracts of *C. papaya* tested at 25, 50 and 100 mg/mL concentrations have a potent activity against Gram-positive and Gram-negative bacteria including *S. aureus* and *E. coli*, with differences in relation to the type of extract used; the dried sample was equally effective against both types of bacteria while the fresh sample was more effective against Gram-negative bacteria.[14] In 2013 Nirosha and Mangalanayaki, from India, tested papaya leaf and stem extracts at 150, 200 and 250 mg/mL against *S. aureus* and *E. coli* among other bacteria. They reported both leaf and root extracts more active against gram-negative than gram-positive bacteria.[2] The referred differences may be related to the use of different strains of microorganisms under study, as well as the use of different parts of the plant, the types and concentrations of the extracts and the geographical region where *C. papaya* is cultivated and obtained from, since environmental factors like climate, altitude, season and soil can all influence the plants metabolism leading to differences in the composition of secondary metabolites.

The MIC of *C. papaya* was found to be higher than the MIC of amoxicillin on both microorganisms, which implies that amoxicillin is more active than *C. papaya* against both *E. coli* and *S. aureus* standard strains.

When amoxicillin is used alone, it has a higher activity against *S. aureus* compared to *E. coli*. This β-lactam antibiotic inhibits the last step in the peptidoglycan synthesis, heteropolymer that provides rigid mechanical stability to the bacterial cell wall by virtue of its highly cross-linked structure. One of the mechanisms of β-lactam bacterial resistance results from the inability of the drug to penetrate to its site of action. In gram-positive bacteria, the peptidoglycan polymer is very near to the cell surface and the antibiotic penetration is easy, but in gram-negative bacteria, the inner membrane is covered by the outer membrane, lipopolysaccharides and capsule, which block the access of some antibiotics. Some small hydrophilic antibiotics, including β-lactam, diffuse through aqueous channels in the outer membrane formed by proteins called *porins,* which vary among different gram-negative bacteria, thereby providing greater or lesser antibiotics access to the site of action.[7]

After conducting a deep search looking for published data on antibacterial effects of combination between plants and amoxicillin, none of the accessed by the research

team was aimed to evaluate the antibacterial effect of the combination between this conventional drug and *C. papaya*. However, the association of other natural products to conventional antibiotics has been reported by some contemporary authors,[11,19-25] what reflects an increasing interest for this type of theoretical and methodological approach.

Several techniques measure the effects of drug combinations. One of the simplest and well-known protocols is the «checkerboard» test, which provides a two-dimensional array of different concentrations of the substances evaluated and allows the calculation of FIC index.[11]

When the two antimicrobials where combined in different concentrations, the FIC index showed different interaction between the study drugs according to the microorganism against which the combination was used.

In the case of *S. aureus*, was found that *C. papaya methanol extract* does not modify the amoxicillin´s total activity showing an indifference interaction; therefore, it is not significant to combine *C. papaya* with amoxicillin against *S. aureus* since amoxicillin effect is not enhanced. However, it is important to combine the two antimicrobials against *E. coli* since the mixture has an additive interaction meaning that the resultant effect is the sum of the individual antimicrobial effects obtained when each drug is used alone.

Taking into account the results of this study can be concluded that *Carica papaya* has antibacterial effect which may be attributed to the presence of phenolic compounds. There was no interaction between amoxicillin and *Carica papaya* extract on *Staphylococcus aureus,* but the antimicrobial activity against *Escherichia coli* of both drugs can be potentiated by their additive interaction increasing the susceptibility of this microorganism when they are used concomitantly.

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