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In vitro antibacterial activity of mixed *Garcinia buchananii* B. and *Curcuma longa* L. ethanolic extracts against *Streptococcus pneumoniae*

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***Streptococcus pneumoniae* is the main bacterial cause of community-acquired pneumonia. In children, 22% of the deaths are due to pneumonia as the single leading cause of death. The local people in Uganda use herbs like *Curcuma longa* Linnaeus and *Garcinia buchananii* Baker to manage upper respiratory tract infections (URTIs). The ethanolic extracts of the *C. longa* rhizome and *G. buchananii* stem bark have individually demonstrated antimicrobial activity against bacteria, protozoa, and viruses. Crude extracts of *C. longa* rhizome powder and *G. buchananii* fresh back were obtained through maceration using ethanol. *In vitro* disc diffusion method and serial dilution method were used to determine antibacterial susceptibility and minimum inhibitory concentration (MIC), respectively of the plant extracts against *S. pneumoniae*. Both ethanolic extracts of *C. longa* rhizome and *G. buchananii* stem bark individually showed activity against *S. pneumoniae* and this antibacterial effect was largely dose-dependent. However, ceftriaxone had superior antibacterial activity ($p < 0.0001$) than all the individual extracts and combinations. The MICs of *C. longa* and *G. buchananii* ethanolic extracts were 3.125 and 1.5625 mg/mL, respectively. The 50:50 *C. longa* - *G. buchananii* combination showed superior activity compared to other combinations, though it was not statistically significant ($p > 0.05$). The Fractional Inhibitory Concentration Index (FICI) was 11.68. This study concluded that the ethanolic extracts of both the rhizome of *C. longa* and the stem bark of *G. buchananii*, when used singly and in combination, demonstrated antibacterial activity against *S. pneumoniae.* However, the combination of the ethanolic extracts of these two plants demonstrates antagonistic activity.**

Key words: *Curcuma longa*, *Garcinia buchananii*, *Streptococcus pneumoniae*, combined antibacterial activity.

INTRODUCTION

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia. It is one of the most common pathogens that cause invasive diseases such as sepsis, meningitis, and pneumonia, bacteremia, and

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sinusitis (Soto-Noguerón et al., 2016; Wessels et al., 2012). Pneumonia due to *S. pneumoniae* accounts for numerous hospitalizations and deaths among all age groups (Baldo et al., 2015). In children, acute respiratory tract infections account for 22% of deaths; with pneumonia as the single leading cause of death. The World Health Organization (WHO) estimates that about 14.5 million episodes of serious pneumococcal disease occur, resulting in about 826,000 deaths in children under five years. The highest number of deaths due to *S. pneumoniae* occurs in developing countries than in industrialized country settings (WHO, 2012). Seventy percent of the deaths occur in African and Asian countries (Krumkamp et al., 2012).

The first-line management of URTIs caused by *S. pneumoniae* is penicillins like amoxicillin. However, due to the emergence of penicillin-resistant bacterial strains, many other drugs are often used to manage these infections including fluoroquinolones, cephalosporins like ceftriaxone, cefotaxime. Other drugs used include clindamycin, doxycycline, vancomycin, and linezolid (UCG, 2016). However, resistance to penicillins, macrolides, fluoroquinolones, tetracycline, clindamycin, and trimethoprim-sulfamethoxazole combination is on the rise and occurs through various mechanisms (Cherazard et al., 2017).

Medicinal plants in Africa constitute a large but still largely untapped pool of natural product remedies. World Health Organization (WHO) estimates that 80% of the population in developing countries still relies on plant-based medicines for some part of primary health care (Ekor, 2014). In parts of East Africa like the Lake Victoria basin, numerous medicinal plants including *Garcinia buchananii* B. and *Curcuma longa* L. are used individually or in combination to manage upper respiratory tract infections (URTIs). In Africa, *G. buchananii* (family Clusiaceae) bark extract is widely used traditionally for the management of gastrointestinal diseases like diarrhea, and dysentery (Balemba et al., 2010), and other conditions such as respiratory tract infections, eye diseases, hypertension, and diabetes (Okullo et al., 2014). *G. buchananii* contains an isoprenylated benzophenone derivative garcinol as one of its phytochemicals (Schobert and Biersack, 2019; Stark et al., 2015). Garcinol has been reported to have antibacterial activity against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis,* and *Enterobacter aerogenes* (Varalakshmi et al., 2010). *C. longa* L. (Tumeric) belongs to the Zingiberaceae family and is used largely as a coloring agent, as a spice, and also as a medicine (Gurning, 2020). As a medicine, it is used widely as an antimicrobial, anti-inflammatory, anticancer, antidiabetic, and antioxidant agent (El-Kenawy et al., 2019; Teow et al., 2016). Rhizome extracts of *C. longa* have been found to possess broad-spectrum antibacterial activity (Kumar et al., 2020), with this activity attributed to its major constituent curcumin (Teow et al., 2016). Studies on *C. longa* rhizome extracts have shown activity against *S. aureus*, *Klebsiella pneumonia, E. coli,* and *Staphylococcus epidermidis* (Feghali et al., 2018; Singh et al., 2017).

Mixtures of different plants extracts are widely used to manage various diseases. The rationale of the use of combinations is to benefit from the possible synergistic or potentiating effects (Ozioma and Okaka, 2019). There is however a great need to evaluate whether the different combinations of the crude plant actually achieve the desired benefits. In various studies, the antibacterial activity of individual extracts of *C. longa* rhizome and *G. buchananii* stem back extracts have been established for various micro-organisms but not *S. pneumoniae.* This study aimed at establishing the antibacterial activity of individual and combination of ethanolic extracts of *C. longa* rhizome and *G. buchananii* stem bark against *S. pneumoniae.*

MATERIALS AND METHODS

Reagents and chemicals

Ethanol (70%), distilled water, concentrated sulfuric acid, ferric chloride, sodium hydroxide, hydrochloric acid, iodine, potassium iodide, chloroform, dimethylsulfoxide (DMSO), and ceftriaxone standard.

Collection and identification of plant

Fresh forms of turmeric rhizome were obtained from a garden in Rakai-Uganda, while fresh forms of the stem bark of *G. buchananii* were obtained from a garden in Kisaasi, Uganda in February 2018. The plant materials were transported to the pharmacognosy laboratory at the Department of Pharmacy, Makerere University. Herbarium specimens were submitted to the Makerere University herbarium for authentication by a botanist.

Preparation of plant extracts

The fresh rhizomes of *C. longa* were washed using distilled water. The rhizomes were then dried in the open air under shade for 10 days after which, they were ground using a motor and pestle. The obtained powder was macerated in ethanol (70%) in a closed glass container; in a ratio of 1:6 at room temperature, for 5 days with occasional agitation. The obtained mixture was then filtered using Whatman filter paper number 1. The filtrate was concentrated by evaporation in a rotary evaporator at 90°C. This process was also repeated for the fresh bark of *G. buchananii*. The percentage yield for the plant materials was then calculated using the formula:

Percentage yield of extract = (Mass of dried crude extract / Mass of powdered plant material macerated) ×100%

Phytochemical analysis

Phytochemicals screening for tannins, alkaloids, saponins, flavonoids, steroids, and phenols was done following standard methods (Evans, 2009) (Table 1).

Test for tannins (Ferric chloride test)

Ferric chloride (1 mL) was added to the extract. The formation of a blue-green precipitate confirmed the presence of tannins.

Test for alkaloids

Extracts (10 g) were dissolved in dilute hydrochloric acid and then filtered. To 2 mL of the filtrate was added Wagner's reagent (Iodine in Potassium iodide). The formation of a reddish-brown precipitate indicated the presence of alkaloids.

Test for saponins

The extract was added to distilled water (20 mL) in a measuring cylinder and agitation was done for 15 min. The formation of a 1 cm layer of foam confirmed the presence of saponins.

Test for flavonoids

Sodium hydroxide (2 mL) was added to the extract. The formation of a yellow color, that became colorless once diluted acid was added confirmed the presence of flavonoids.

Tests for steroids

To extract (1 mg) in a test tube was added chloroform (10 mL). Concentrated sulfuric acid (10 mL) was added slowly on the sides of the test tube. The formation of a brown ring at the intersection of the two layers and with the upper layer turning green confirmed the presence of steroids.

Test for phenols

The extract was dissolved in distilled water (2 mL) and then a few drops of ferric chloride were added. The formation of a deep blue or greenish colour confirmed the presence of phenols.

Antimicrobial susceptibility testing

The disc diffusion method was used to screen for antibacterial activity. *S. pneumoniae* ATCC 49619 (Provided by Microbiology department-Makerere University) was inoculated on blood agar plates by streaking. Sterile Whatman filter paper discs measuring 6 mm in diameter were sterilized by autoclaving at 121°C and a pressure of 15 psi for 15 min. These filter disks were then impregnated with crude plant extracts of different concentrations 3.125, 6.25, 12.5, 25, 50, 100 mg/mL, and 25:75, 50:50 and 75:25 fractional combinations of the extracts under aseptic conditions. The positive control used was ceftriaxone (100 mg/mL) and the negative control was DMSO (1.5%). This was done in triplicate for all the extracts and the controls. DMSO was the solvent used to make all the different concentrations of the extracts and positive control. Impregnated filter paper discs for both controls and those with plant extracts were then placed on the surfaces of the blood agar media, onto which *S. pneumoniae* had been inoculated, and incubated at 37°C for 24 h. After incubation, the plates were removed from the incubator and the diameters of the zones of inhibition were measured using a Vernier caliper.

Determination of the MIC and FICI

The minimum inhibitory concentration (MIC) of the ethanolic extracts was determined by the serial dilution method. Ethanolic extracts of both *C. longa* and *G. buchananii* were prepared using Brain Heart Infusion (BHI) agar to make different concentrations ranging from 0.78125 to 200 mg/mL in their respectively labeled test tubes. *S. pneumoniae* (strain ATCC 49619) was inoculated into the BHI with different concentrations of both the individual and with the most potent combination of *C. longa* and *G. buchananii* (50:50). Controls containing only the nutrient broth without the extracts were included. The test tubes were then incubated at 37°C for 24 h. The presence of turbidity after the incubation period denoted the presence of *S. pneumoniae,* while the absence of any turbidity indicated inhibition of microbial growth. The concentration of the extract in the test tube with the lowest dilution with no detectable growth by visual inspection was considered the MIC. Due to the colored nature of the plant extracts, which made the observation for turbidity difficult, the samples were subsequently sub-cultured at 37°C in an incubator for 24 h to observe for any growth. The Fractional Inhibitory Concentration Index (FICI) was calculated using the following standard formula. The effects of the combinations were then classified as: synergistic, additive, indifference and antagonistic, if the FICI is <1, =1, >1 ≤2, and >2, respectively.

FIC *(G. buchananii* extract) = MIC (*G. buchananii* extract in combination) / MIC (*G. buchananii* extract alone)

FIC (*C. longa* extract) = MIC (*C. longa* extraction in combination) / MIC (*C. longa* extract alone)

FICI = FIC (*G. buchananii* extract) + FIC (*C. longa* extract).

Data management and analysis

Graphpad Prism Ver.7.0 was used to compute descriptive statistics of the mean and standard deviation (SD) inhibition zone diameter. The data was then subjected to one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests to compare the antibacterial activities of the different extract concentrations and the controls. A value of $p<0.05$ was considered significant.

RESULTS

Extraction yield and phytochemicals

The percentage yield of ethanolic extracts of *C. longa* rhizome and *G. buchananii* stem bark was 14.3 and 9.4%, respectively. Both ethanolic plant extracts contained alkaloids, saponins, tannins, flavonoids, and phenols and lacked steroids (Table 1).

Antimicrobial susceptibility of *S. pneumoniae* to *C. longa* and *G. buchananii* ethanolic extracts

Ceftriaxone 100 mg/mL (positive control) had the largest zone of inhibition of 43.33 (± 3.05) on the *S. pneumoniae* strain ATCC 49619 used when compared with all other test substances. Among the 3.125 to 100 mg/mL ethanolic plant extracts of *G. buchananii* and *C. longa*, the highest dose concentrations of 100 mg/mL had the largest zones of inhibition of 26.67 (± 1.15) and 25.33 (±

Table 1. Phytochemical yields and composition of the *C. longa* rhizome and *G. buchananii* stem bark ethanolic extracts.

Extracts	*C. longa* rhizome	*G. buchananii* stem bark
Percentage yield	14.3 %	9.4%
Alkaloids	+	+
Saponins	+	+
Tannins	+	+
Flavonoids	+	+
Phenols	+	+
Steroids	-	-

(+)- Present, (-) – absent
Source: Authors

Table 2. Antibacterial activity of ethanolic extracts of *G. buchananii* and *C. longa* against *Streptococcus pneumoniae*.

Test substances and concentrations	Mean zone of inhibition (± SD) (mm) (N=3)
Ceftriaxone 100 mg/mL (positive control)	43.33 (±3.05)[‡]
DMSO 156.26 mg/mL (negative control)	0 (±0)
G. buchananii 100 mg/mL	26.67 (±1.15)
G. buchananii 50 mg/mL	24.6 (±1.15)
G. buchananii 25 mg/mL	20.67 (±3.05)
G. buchananii 12.5 mg/mL	18.67 (±1.15)
G. buchananii 6.25 mg/mL	17. 33 (±1.15)
G. buchananii 3.125 mg/mL	16 (±2.1)
C. longa 100 mg/mL	25.33 (±2.31)
C. longa 50 mg/mL	22 (±5.29)
C. longa 25 mg/mL	20 (±4.1)
C. longa 12.5 mg/mL	19.33 (±3.05)
C. longa 6.25 mg/mL	16.67 (±1.15)
C. longa 3.125 mg/mL	14 (±2.3)
75% *C. longa* + 25% *G. buchananii*	16 (±1.2)
50% *C. longa* + 50% *G. buchananii*	20 (±2.15)
25% *C. longa* + 75% *G. buchananii*	17 (±1.82)

[‡]The positive control (Ceftriaxone) had extremely superior activity ($p < 0.0001$) on *Streptococcus pneumoniae* in comparison to the individual extracts of *G. buchananii* and *C. longa* at concentrations of 3.125-100 mg/mL and when in combination.
Source: Authors

2.31), respectively. Of the *C. longa* and *G. buchananii* extracts combinations, the 50% *C. longa* + 50% *G. buchananii*, had the largest zone of inhibition (Table 2). There was no significant difference ($p > 0.05$) in activity between the different *G. buchananii* and *C. longa* extracts combinations, and between similar concentrations of *G. buchananii* and *C. longa* when used singly.

The MICs for ethanolic extracts *C. longa* rhizome and *G. buchananii* stem bark against *S. pneumoniae* were 3.125 and 1.5625 mg/mL, respectively. The most potent combination of extracts (50% *C. longa* and 50% *G. buchananii*) had a MIC of 12.162 mg/mL and a Fractional Inhibitory Concentration Index (FICI) of 11.68.

DISCUSSION

The percentage yield of ethanolic extract of *C. longa* from this study was lower than that observed in a previous study that reported 17.39% (Tanvir et al., 2017). Variations in yields and phytochemical composition of plant extracts are reported to be caused by variations in climatic and agronomic factors, and as well as differences in the extraction procedures used (Borges et al., 2018; Dhanani et al., 2017). Both *C. longa* and *G. buchananii* contained significant amounts of alkaloids, saponins, tannins, flavonoids, and phenols. These findings are similar to those in previous studies (Gurning,

2020; Tanvir et al., 2017) and the anti-bacterial inhibitory effects identified could be attributed to some or all of these phytochemicals in the plant extracts (Oghenejobo et al., 2017; Stark et al., 2015). For instance, Garcinol which is a flavonoid in *G. buchananii* has antibacterial activity against *S. aureus, E. coli, Bacillus subtilis,* and *Enterobacter aerogenes* (Varalakshmi et al., 2010). Curcumin, a phenolic compound in *C. longa* has been reported to be responsible for its broad antibacterial effects (Oghenejobo et al., 2017; Teow et al., 2016), acting through reducing bacterial membrane integrity resulting in membrane leakages in both Gram-positive and Gram-negative bacteria (Tyagi et al., 2015).

Both *C. longa* rhizome and *G. buchananii* stem bark extracts have been reported to have activity against various microbes (Afrose et al., 2015; Moghadamtousi et al., 2014; Wise et al., 1998). In this study, ethanolic extracts of *C. longa* at a concentration of 100 mg/mL showed sensitivity against *S. pneumoniae* while its concentrations of 50 and 25 mg/mL showed intermediate antibacterial activity. For *G. buchananii*, ethanolic extracts of concentration 50 and 100 mg/mL showed sensitivity against *S. pneumoniae,* while the 25 mg/mL had intermediate activity. *S. pneumoniae* was sensitive to ceftriaxone (positive control) with a mean diameter of the zone of inhibition of ≥ 23 mm (CLSI, 2013). There was no significant difference ($p > 0.05$) in the antibacterial activity of ethanolic extracts of *C. longa* rhizome and *G. buchananii* stem bark of the same concentrations on *S. pneumoniae.* Furthermore, the antibacterial effect of the two ethanolic plant extracts on *S. pneumoniae* was largely dose-dependent. The dose-dependent antibacterial property of *C. longa* rhizome extracts has been reported in other studies (Izui et al., 2016). There were however no significant differences in activity between *G. buchananii* concentrations of 50 and 100 mg/mL or *C. longa* concentrations of 12.5, 25, 50, and 100 mg/mL used in our study. Ceftriaxone 100 mg/mL was more active ($p < 0.0001$) on *S. pneumoniae* when compared with all the different ethanolic extracts of *C. longa* rhizome and *G. buchananii* stem bark at the different combinations used.

In this study, the MICs for ethanolic extracts *C. longa* rhizome and *G. buchananii* stem bark against *S. pneumoniae* were 3.125 and 1.5625 mg/mL, respectively. In previous studies, the MICs of *C. longa* ethanolic and methanolic extracts against other micro-organisms such as *S. aureus, S. epidermidis, E. coli*, and *K. pneumoniae,* have been reported to be in the range of 0.2 to 16 mg/mL (Niamsa and Sittiwet, 2009; Raji et al., 2018; Wise et al., 1998). Cephalosporins like ceftriaxone and cefotaxime, as well as β-lactams such as penicillin G, have MICs ≤ 2 mg/L on *S. pneumoniae* strains, and are effective treatments for pneumococcal bacteraemia and pneumonia caused by *S. pneumoniae* (Kaplan and Mason, 1998). Basing on the MIC ranges obtained in this study and in related studies, the plant extracts of *C. longa* rhizome and *G. buchananii* stem bark are potential treatments for infections caused by *S. pneumoniae* strains such as URTIs, pneumonia, otitis media, and sinusitis.

Synergistic antibacterial activity has been reported for extracts of *C. longa* when used in combination with various conventional antibiotics such as ampicillin, ciprofloxacin, gentamycin, amikacin, and cefepime among others (Moghadamtousi et al., 2014; Teow et al., 2016). Furthermore, mixtures of various plant extracts are widely used to benefit from potential synergistic or additive effects; however, combining plant extracts can also result in antagonism. Antagonistic effects from combination of plant extracts are reported to be a result of the masking of the active principles by other components in the complex mixture (Caesar and Cech, 2019).

Conclusion

In this study, the Fractional Inhibitory Concentration Index (FICI) of the most potent combination of extracts (50% *C. longa* and 50% *G. buchananii*) was 11.68. A calculated FICI greater than 2 indicates antagonism (Ofokansi et al., 2012). This indicates that combining *C. longa* and *G. buchananii* ethanolic extracts using earlier combination ratio provides no advantage to the antibacterial activity of each of these plant extracts on *S. pneumoniae*. The use of a broader range of *C. longa* and *G. buchananii* ethanolic extracts concentrations ratios is required to confirm this reported antagonism. Variations in concentration ratios of different plant extracts can result in either synergism or antagonism (van Vuuren and Viljoen, 2011).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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