RESEARCH ARTICLE

Evaluation of the antibacterial activity of lemon fruit juice, *Mondia whitei* ethanolic extract, and their combination against *Streptococcus mutans*

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ABSTRACT

Dental caries has gained momentum as one of the main public healthcare concerns worldwide. Due to the ability to synthesize extracellular polysaccharides that facilitate the formation of plaque biofilm on the teeth enamel which is vital for carcinogenicity, *Streptococcus mutans* has been implicated as the major causative agent of dental caries. Although the occurrence of dental caries in Uganda is on the rise, little or no attention has been paid to promoting oral healthcare in the country. Thus, this study was aimed at evaluating the citrus lemon fruit juice and ethanolic extract of *Mondia whitei* root bark as candidate alternative therapeutic agents for *Streptococcus mutans* the causative agent of dental caries. In this study, the lemon juice, pulp lemon juice, and *Mondia whitei* ethanolic extract were screened for phytochemicals. Furthermore, the anti-*Streptococcus mutans* activity of the lemon juice, lemon pulp juice, and *Mondia whitei* ethanolic extract was determined by the agar well diffusion method while the minimum inhibitory concentration and minimum bactericidal concentration were determined by serial broth dilution. Phytochemical screening revealed the presence of alkaloids, flavonoids, terpenoids, and tannins in the ethanolic extract of *Mondia whitei* and lemon and lemon pulp juice, while glycosides were detected only in juices obtained from the lemon. The zones of inhibition of ethanolic extract of *Mondia whitei*, citrus lemon juice, citrus lemon pulp juice, and the cocktail were 13.67 ± 0.33, 18.67 ± 0.33, 18.33 ± 0.67 and 18.00 ± 0.58 respectively, The lemon juice and lemon pulp juice exhibited significantly lower MIC of 0.195 mg/ml, and 0.391 mg/ml respectively. The efficacy of the extract/juices increased with an increase in the concentration. Conclusively, the findings of this study indicated that the *Mondia whitei* ethanolic extract, lemon juice, and lemon pulp exhibited significant antibacterial activity against *Streptococcus mutans* the main causative agent of dental caries, and can be further explored for the formulation of herbal concoction for the prevention and treatment of oral cavities in resources-limited low-income communities.

Key Words: Oral healthcare; Dental caries; Lemon juice; *Mondia whitei*; Ethanolic extract

INTRODUCTION

Streptococcus mutans is a gram-positive bacteria belonging to phylum Firmicutes, Bacilli class, order lactobacillales, and family streptococcaceae. The bacterium mainly colonises the mouth and through carbohydrates metabolism an acidic medium is created that is responsible for causing dental caries. The bacterium synthesizes an Extracellular Polysaccharide (EPS) that facilitates the establishment of plaque biofilm which is the basis of carcinogenicity. Thus, *Streptococcus mutans* is the leading cause of dental caries worldwide and is considered the most pathogenic of all the eight species of oral streptococci.

Despite attempts to improve oral health worldwide, dental caries is still a major public health concern. Dental caries impacts the social life of people reducing the quality of life, restricted school time and work activities in adults while in children it reduces intake of food which affects growth and development. Indeed, the WHO termed a silent pandemic especially in school aged children and most adults with a prevalence of around 60-90%. The survey done in 2011 showed that 91% of American adults between 20 and 64 had suffered from dental caries and it affected their quality of life and social relations [1]. The incidence and severity of dental caries have been low in Uganda until recently where a marked change in the trend mainly attributed to poor oral health coupled with the growing consumption of sugars was reported. Despite the increasing prevalence and impact, dental caries has attracted less attention and investment from government; for instance, less than 0.1% of the gross domestic product is allocated for the direct oral healthcare and the dentist to the population ratio is 1:158,000 people in Uganda. With this insufficiency there is marked need to explore natural remedies for the prevention of dental caries.

Citrus lemon is a flowering plant species that belongs family Rutaceae native to Asia, primarily Northeast India and is commonly grown for commercial purposes across the world. Citrus lemon is a rich source of ascorbic acid, flavanones and flavones. Chemical analysis of citrus lemon juice shows the presence flavonoids, ascorbic acid, tocopherols, and citric acid while its microbiological study revealed its antibacterial effect on *Klebsiella pneumoniea*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*, *Staphylococcus aureus*. Furthermore, a study by Bhat showed that fresh lemon juice is most effective against *S. Paratyphi B*, and *S. sonnei*. Eating fresh lemon fruits in addition to taking juice from the fruits has been associated with overall gum health and protection form oral periodontal diseases [2].

Mondia whitei is an aromatic soft wood climber of drier forest with pale greenish-white or cream flowers and its roots and root bark have a vanilla-like scent. The roots are valuable as aphrodisiac, to prevent premature ejaculation, increase sperm production and generally, to treat sexual weakness while a decoction or infusion of the roots is widely used to treat malaria, gastro-intestinal problems, pains and as restorative and appetite stimulant. It is also effective in the treatment of gonorrhoea and paediatric asthma. Earlier studies demonstrated that the ethanolic extract of *Mondia whitei* roots is effective against several oral *Streptococci* like *S. pyogenes*, *S. sobrinus* and *S. mitis*. *Mondia whitei* and citrus lemon are being used by the local communities of Bushenyi district and many other areas of western Uganda to treat and prevent dental caries, but their efficacy is yet to be documented [3]. Therefore the purpose of this study was to evaluate the antibacterial activity of the citrus lemon juice, ethanolic extract of *Mondia*

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whitei root bark and the cocktail of citrus lemon juice and *Mondia whitei* root bark ethanolic extract against *Streptococcus mutans*.

MATERIALS AND METHODS

Study design and site

The study was an *in vitro* quantitative experimental study where the antibacterial activity of citrus lemon juice, citrus lemon pulp juice, *Mondia whitei* ethanolic root extract and combination of *Mondia whitei* ethanolic root extract and juice were evaluated. The study was carried out from the Institutional Biomedical Research Laboratory, Kampala International University Western Campus.

Plant collection and identification

Fresh fruits of citrus lemon were bought from Ishaka Market while *Mondia whitei* fresh roots were obtained from Rukararwe medicinal Gardens, Bushenyi District. The plants were identified by a botanist at the Department of Botany Makerere University.

Preparation of citrus lemon juice

The fresh citrus lemon fruits were rinsed under clean tap water and their surfaces wiped with cotton wool soaked in 70% alcohol. The pericarp was peeled off using a spatula followed by straining off the juice from the fleshy pulp into a sterile 100 ml beaker. Approximately 100 g of the pulp was crushed in distilled water using an electric blender and the obtained extract was sieved and dispensed into a sterile 100 ml beaker. Both the citrus lemon juice and citrus lemon pulp juice were evaluated for antibacterial activity on the same day of preparation.

Preparation of the ethanolic root extract of *Mondia whitei*

The fresh roots of *Mondia whitei* were washed under running tap water to remove entrapped soil on its surface. The roots were then being air dried to a constant weight and then later grounded into a fine powder using mortar and pestle. The resultant powder was weighed and stored in an air tight container until use [4]. The total powder yield was 137 gm. The ethanol root extract was obtained using a ratio of 1:10 of the powder material to 80% ethanol in soxhlet extractor incubated at 78°C for a period of one hour. The obtained extract was collected in a reagent bottle and later concentrated using a rotary evaporator. The percentage extract yield was estimated using the formula below:

$$\text{Mondia whitei ethanolic extract percentage yield} = \frac{\text{Weight of Extract obtained}}{\text{Mondia whitei powder initial weight}} X\ 100$$

Phytochemical screening

Phytochemical screening of the ethanolic extract of *Mondia whitei* root barks and citrus lemon juice was carried out to determine the presence of alkaloids, glycosides, terpenoids, flavonoids and tannins. The analysis was carried out according to the standard method described by Nitika et al, outlined below:

Alkaloids: To test for alkaloids three drops of hydrochloric acid were added to 5 ml of the extract and citrus juice while shaking to obtain uniform solutions. This was followed by adding Meyer's reagent (potassium mercuric iodide solution). Formation of a yellow precipitate indicated the presence of alkaloids in the samples.

Glycosides: The presence of glycosides was evaluated by adding the *Mondia whitei* ethanolic extract and citrus juice to equal volumes of distilled water (5 ml) followed by 0.5 ml of lead acetate solution. The mixture was then shaken and filtered. The filtrate was then extracted with equal volume of chloroform and the solution evaporated to dry. The remainder was then dissolved in 3 ml of glacial acetic acid plus 3 drops of iron (III) chloride solution and 4 ml of concentrated sulphuric acid. A reddish blown layer that turned to bluish green indicated the presence of glycosides.

Flavonoids: To detect the presence of flavonoids, five drops of concentrated hydrochloric acid were added to 5 ml of ethanolic extract of *Mondia whitei* root bark and citrus lemon juice. Immediate development of the red colour indicated the presence of flavonoids.

Terpenoids: About 2 ml of chloroform was added to 0.5 ml of the ethanolic extract of *Mondia whitei* root bark and citrus lemon juice followed by 3 ml of conc. Sulphuric acid which formed a layer. Reddish blown discoloration of the interface indicated the presence of terpenoids.

Tannins: To identify tannins, 2 ml of 5% iron (III) chloride solution was added to 5 ml of the ethanolic extract of *Mondia whitei* root bark and citrus lemon juice. A greenish-black precipitate indicated the presence of tannins.

Saponins: To confirm the presence of saponins, 1 ml of the ethanolic extract of *Mondia whitei* root bark and citrus lemon juice was diluted with 20 ml of distilled water and shaken in a graduated cylinder for 15 minutes. The formation of a one-centimetre foam indicated the presence of saponins [5].

Preparation of different concentrations of the extracts

Preparation of different concentrations of the plant extracts for antimicrobial activity evaluation was performed using methods outlined by Eve et al. Briefly, 2 g of the ethanolic extract of *Mondia whitei* was mixed with 2 ml of 100% freshly prepared citrus lemon juice on a clean sterile glass plate and the mixture was stirred for complete dissolution, then covered with sterilised aluminium foil. This gave a concentration of 1000 mg/ml of *Mondia whitei* and 100% v/v of citrus lemon in the extract. Additionally, 1 ml of the combination was added to 1 ml of sterile distilled water, to obtain a 500 mg/ml of *Mondia whitei* ethanolic extract and 50%V/V of citrus lemon juice. The same procedure was used to prepare 1000 mg/ml and 500 mg/ml *Mondia whitei* ethanolic extract alone. Furthermore, 50% V/V of fresh citrus lemon juice was prepared by adding 2 ml of fresh citrus lemon juice of 100% v/v to 1 ml of sterile distilled water [6]. The same procedure was applied to obtain 50% V/V pulp juice preparation. Ampiclox 30 µg/ml was used as a control since it is the first choice antibiotic used to treat dental caries.

Experimental microorganisms

Streptococcus mutans isolates were obtained from the microbiology laboratory, Kampala international university. The samples were then inoculated on blood agar base using a sterile swab and incubated overnight for 18 hours at 37.8°C in order to obtain multiple colonies. On a separate blood agar plate the bacteria was inoculated again by streak method in order to obtain a pure culture and then stored at 4°C till when used [7].

Antimicrobial screening

Standardized test *Streptococcus mutans* suspension (0.5×10^8 McFarland standard) was inoculated uniformly on the entire surface of freshly prepared blood agar by streaking and four wells measuring 6 mm in diameter were made using a sterile cork borer on each plate. The following volumes and concentrations were used; 50 µl of concentrations (1000 mg/mL and 500 mg/mL)/100% V/V and 50%) of the combination extract, 50 µl of citrus lemon juice extract (100% V/V and 50% V/V), 50 µl of citrus lemon pulp juice with concentrations of (100% v/v and 50% v/v), 50 µl of *Mondia whitei* ethanolic root extract (1000 mg/ml and 500 mg/ml) and 50 µl of 30 µg/ml ampiclox was used as the positive control while 50 µl of sterile distilled water as the negative control [8]. The experiments were performed in triplicates. The inoculated plates were left to stand at room temperature for 30 min for the extract to diffuse and later, the plates were incubated at 37.8° C for 22 hours. After the incubation period, the diameters of zones of inhibition were measured in millimeters (mm) using a ruler, and results were interpreted according to guidelines.

Determination of the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the ethanolic *Mondia whitei* roots extract, citrus lemon juice, citrus lemon pulp juice and the combination extract of lemon juice and *Mondia whitei* ethanolic extract were determined for *Streptococcus mutans* using a two-fold serial microbroth dilution method on 96 well microtiter plates using the Brain Heart Infusion nutrient Broth (BHIB) [9]. All tested extracts/juices exhibited potent antimicrobial activity at all concentrations. Therefore, a cocktail of 1000 mg/ml of ethanolic *Mondia whitei* extract and 100% V/V of lemon juice (preparation A), 100% of citrus lemon juice (preparation B), 100% of citrus lemon pulp juice (preparation C), 1000 mg/ml of *Mondia whitei* ethanolic root extract (preparation D), and 30 µg/ml Ampiclox (preparation E) were used for the determination of MIC. Briefly, 100 µl of sterile BHIB was pipetted into 14 wells of microtiter plates labelled preparation A, B, C, D and E. A two-fold serial dilution was carried out by pipetting 100 µl of the preparation A into the first well of the plate labelled preparation A. The BHIB and preparation A were thoroughly mixed into a uniform solution. This was followed by the transfer of 100 µl of the mixture from the first well to the second well. This step was repeated until the 14th well. This was followed by dispensing 1.0 µl of fresh bacterial culture of *Streptococcus mutans* at 0.5 McFarland's turbidity standard and 20 µl of resazurin into the 14 wells. This procedure was repeated for preparations B, C, D and E. The 14 wells of plate labelled positive control was filled with 200 µl of BHIB. The plates were then incubated at 37.8°C for 24 hours followed by plating the samples from each well onto the blood agar. The plates were left to stand all night at 37.8°C under aerobic conditions. The lowest concentrations of each preparation at which there was the least growth was taken as the MIC [10-12]

Determination of Minimum Bactericidal Concentration (MBC)

The MBC of the extract/juice preparations was estimated using the MIC microtiter plates with no visible bacteria growth following 24 hours incubation. The samples were inoculated on blood agar base plates by the streak method and then incubated at 37.8°C for 24 hours. The plates were later observed for any bacterial growth. MBC was taken as the concentration of the extract/juice preparation in the well before the one with growth [13].

Statistical analysis

Data was then analysed with graph pad prism 6.01 versions. Comparison of mean zone bacterial growth inhibition, MIC, MBC, of the different extract/juice preparations was done by the one-way Analysis Of Variance (ANOVA). Statistical significance was considered at $P \leq 0.05$.

RESULTS

Mondia whitei ethanolic extract percentage yield

About 21.72 g of the ethanolic extract was obtained from 130.7 g of *Mondia whitei* powder giving a yield of 15.85% [14].

Phytochemical profiling

Phytochemical screening revealed that both the *Mondia whitei* ethanolic extract and citrus lemon juice had alkaloids, flavonoids, terpenoids and tannins. However, glycosides were detected only in citrus lemon and pulp juice whereas saponins were not found in any.

Antimicrobial susceptibility

The antimicrobial activity of the *Mondia whitei* ethanolic extract, citrus lemon juice, citrus lemon pulp juice and the cocktail of the ethanolic extract and the lemon juice at different concentrations are shown in Table 1 and Figure 1 [15].

Table 1: Antimicrobial activity of Mondia whitei ethanolic extract and citrus lemon juice represented by mean zone of growth inhibition, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Plant extract/juice extract/ juice type	Concentration	Mean zone of growth inhibition (mm)	MIC (mg/ml or %v/v)	MBC (mg/ml or %v/v)
Citrus lemon juice	100% V/V	18.67 ± 0.33^{A}	0.195	3,125
	50% V/V	17.00 ± 0.00^{A}	-	-
Citrus lemon pulp juice	100% V/V	18.33 ± 0.67^{A}	0.391	3,125
	50% V/V	14.33 ± 0.67^{B}	-	-
Mondia whitei ethanolic extract	1000 mg/ml	13.67 ± 0.33^{B}	15.63	62.5
	500 mg/ml	11.00 ± 0.00^{B}	-	-
Mixture of *Mondia whitei* efhanolic extract and citrus lemon juice	1000 mg/ml/100% V/V	18.00 ± 0.58^{A}	3.906/0.391	62.5/6.25
	500 mg/ml/50% V/V	12 ± 0.00^{B}	-	-
Ampiclox (positive)	30 ug/ml	28.67 ± 0.00^{C}	0.015	0.059
Sterile water (negative)	-	0	-	-

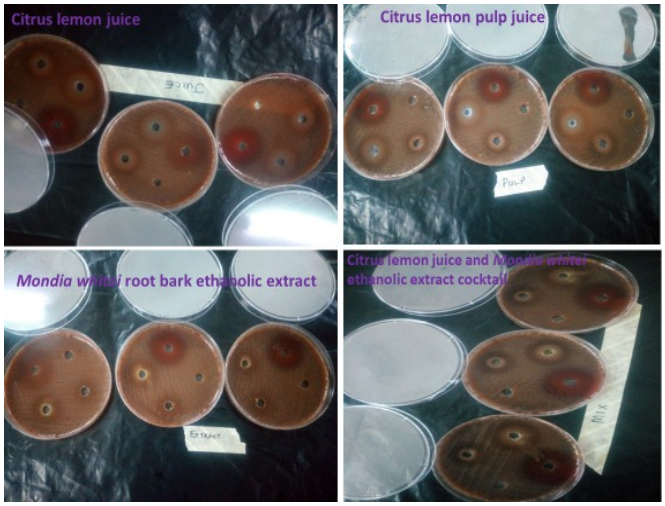

Figure 1) *Zones of growth inhibition of the different plant extracts*

The findings revealed that all the extracts/juice exhibited potent antimicrobial activity against *Streptococcus mutans*. Susceptibility of *Streptococcus mutans* to extract/juices significantly differed (P<0.05) [16]. Inhibition activity of the citrus lemon juice, citrus lemon pulp juice and a cocktail of citrus lemon juice and *Mondia whitei* ethanolic extract was considerably higher than that of *Mondia whitei* ethanolic extract alone and lower than ampiclox (positive control). The antimicrobial activity citrus lemon juices, a cocktail of citrus lemon juice and *Mondia whitei* and *Mondia whitei* ethanolic extract significantly increased with increase in concentration (Table 1 and Figure 2). Two-way ANOVA using side's multiple comparison test was revealed that the antibacterial activities between the lemon Juice, lemon pulp juice, ethanolic extract of *Mondia whitei*, the combination of *Mondia whitei* extract and lemon juice and ampiclox were considerably different (P ≤ 0.05), (Figure 2).

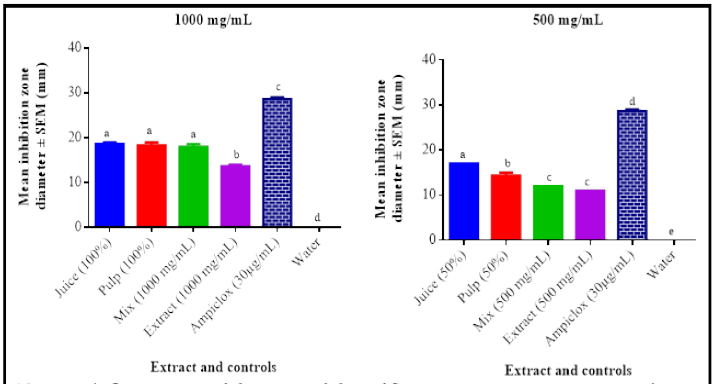

Figure 2) *Comparison of the susceptibility of Streptococcus mutans to extract/juices of different concentrations. Mean growth inhibition of each bar accompanied by the same letter are not significantly different (p>0.05) (Tukey Multiple Comparison), and values accompanied by letter (s) which are not similar are significantly different (P<0.05)*

Minimum inhibitory and bactericidal concentrations

The MIC and MBC of *Mondia whitei* ethanolic extract, citrus lemon juice and the cocktail of the ethanolic extract and the lemon juice are summarized in Table 1. The MIC and MBC for the citrus lemon juice and citrus lemon pulp juice were significantly (P<0.001) lower than the MIC and MBC for the *Mondia whitei* ethanolic extract; thus, lemon juices exhibited superior anti-*Streptococcus mutans* activity [17].

DISCUSSION

Approximately 60-90% of children and the vast majority of adults are affected by dental caries). Less priority is given to dental infections in relation to other communicable diseases in Africa. In Uganda, the oral health services are highly distributed in urban centres leaving the rural areas under served. In most low-income countries, the cost of treating dental decay alone could easily exhaust a country's total healthcare budget. Thus, the Ugandan government with limited resources has prioritized funding several pressing health issues giving limited attention to the less life-threatening conditions like oral health. Furthermore, the use of artificial tooth pastes is associated with exposure to fluoride which may cause side effects like osteoporosis, tooth decay, kidney damage, and fluorosis (change in appearance of the colour of the enamel) especially in children who swallow the commonly used tooth pastes. Furthermore removal of one's tooth affects the mastication process and further exposes to a risk of systemic infection in addition to intraoperative complications that include bleeding, destabilising surrounding teeth and injury to tissues. With that background, the search for effective, safer, and affordable alternatives for low-income communities like herbal medicines against *Streptococcus mutans* the dental caries causative organism is called for [18].

This study obtained *Mondia whitei* ethanolic extract yield of 15.85%; however, there is not data from previous studies for comparison purposes. Phytochemical profiling revealed the presence of alkaloids, flavonoids, terpenoids and tannins in both the citrus lemon juice and *Mondia whitei* ethanolic extract but negative for saponins. Citrus lemon juice further demonstrated the presence of glycosides which were absent in *Mondia whitei* ethanolic root bark extract. This was consistent with results from a study conducted by Nitika et al, which reported the presence of alkaloids, flavonoids, terpenoids, and cardiac glycosides in citrus lemon and citrus lemon pulp juice but negative for tannins. Another study conducted by Danlami et al., on lemon juice documented the presence of flavonoids in high concentrations that were classified as flavonols limocitrin and spinacetin; flavones-orientin vitexin and eriocitrin [19]. Additionally, the presence of flavanones-eriodictyol, hesperidin, hesperetin, naringin; flavones-apigenin, diosmin, flavanols-quercetin in citrus lemon fruits was observed by Alani. Furthermore, Ngbolua, and Inkoto et al., identified all the phytochemicals detected in the *Mondia whitei* root ethanolic extract by this study in addition to phenols and saponins, coumarins, irridoids, anthocyanins, tannins, and anthraquinones. The observed differences in the phytochemicals may be attributed to differences in soil content and climatic conditions in the different geographical regions.

The study evaluated the antibacterial activity of lemon juice, pulp lemon juice, *Mondia whitei* root bark ethanolic extract and a cocktail of *Mondia whitei* ethanolic root bark extract and lemon juice against *Streptococcus mutans*. There is no available *Mondia whitei* ethanolic extract antibacterial data for comparison purposes but for citrus lemon juices, the results are consistent with a study conducted by Alani, who found that the juice at concentrations of 10%, 5%, 3.3% and 2.5% (V/V) had potent inhibitory activity against *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella sonnei* and *Streptococcus mutans*.

Furthermore, the lemon juice, lemon pulp and the cocktail of lemon juice and *Mondia whitei* root extract exhibited significantly higher antimicrobial activity against *Streptococcus mutans* than *Mondia whitei* ethanolic extracts but substantially lower than that of ampiclox the preferred antibiotic for treatment of dental caries as revealed by the ANOVA analyses. The antimicrobial activity in plant extracts is mainly attributed to flavonoids. However, in this study phytochemical screening exhibited presence of flavonoid in all plant extracts [20]. Therefore the significantly superior antibacterial activity in the lemon juice and pulp lemon may be due to the presence glycosides. Studies by Tagousop et al., and Kannan et al., fractionated individual phytochemicals of *Graptophyllum grandulosum* using Column chromatography and found that flavonoids conjugated to glycosides were responsible for the potent antibacterial activity exhibited by the extract and the mode of action is owed to bacteriolysis with remarkable irreversible leakage of the cytoplasmic content. Furthermore, the antibacterial activity of flavonoid glycosides is more pronounced at high concentrations as there was remarkable increase in the antibacterial activity with increase in the concentration of the plant extracts/juices.

CONCLUSION

The ethanolic extract of *Mondia whitei* root bark extract and citrus lemon juice demonstrated a potent antibacterial activity against *Streptococcus mutans*

the causative agent of dental caries individually and in combination. The combination thus represents a potential remedy for both the treatment and prevention of dental caries and further supports the use of this herbal concoction for oral healthcare in the local communities of Bushenyi district in Uganda. However, there is need to study stability and toxicity profile of the two plant extracts before formulating it for general public use.

AUTHOR CONTRIBUTION STATEMENT

Kenneth Ssekatawa and Silivano Niwagaba conceptualized the study; Markarius Katuromunda performed the experiments and wrote the first draft. All the authors analyzed and interpreted the data and managed revisions and contributed to the purchase of reagents and other consumables.

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DATA AVAILABILITY STATEMENT

Data associated with this study has been incorporated in this manuscript.

DECLARATION OF INTEREST'S STATEMENT

The authors declare no competing interests

ETHICAL APPROVAL AND CONSENT TO PUBLISH

The study was approved by the Scientific Institutional Ethical Review board of Kampala International University Western Campus, consisting of biomedical scientists who approved the study for implementation. *Streptococcus mutans* were obtained from the department of microbiology, Kampala International University archives hence no need for approval for patients' consent to participate.

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