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IN VITRO ANTIMICROBIAL ACTIVITY OF COMBINATIONS OF *Callistemon citrinus* AND *Eriobotrya japonica* AGAINST CARIOGENIC *Streptococcus mutans*

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ABSTRACT

Eriobotrya japonica (EJ) and *Callistemon citrinus* (CC) have been reported to separately have antimicrobial activities against *Streptococcus mutans* (the aetiological agent for dental caries), but their potential for beneficial synergistic, additive, or potentiative effects, when used in combinations has never been reported. The current study explored the possibility of *in vitro* antimicrobial interactions between EJ and CC ethanolic leaf extracts against the bacterium. The leaves of both plants (EJ and CC) were shade-dried and pulverized into a coarse powder. These were cold macerated using ethanol (60 %) for 24 h and phytochemical screening was conducted. Various combinations of the extracts (CC: CJ – 1:0, 3:1, 1:1, 1:3, and 0:1) were subjected to minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) determinations against *Streptococcus mutans*. Ciprofloxacin and 2.5 % Dimethyl sulfoxide (DMSO) was used as the positive and negative controls respectively. Antimicrobial interactions between the two extracts were evaluated using Fractional Inhibitory and Bactericidal Concentration Indices (FICI and FBCI). The extraction method yielded 20.05 % and 15.45 % crude extracts of EJ and CC respectively. Both extracts demonstrated almost similar phytochemical profiles revealing the presence of flavonoids, saponins, and tannins, and the absence of alkaloids and volatile oils. The combination CC: EJ (1:0) having only CC exhibited the lowest MIC and MBC comparable to that of the standard drug at $P < 0.05$. The FICI/FBCI values obtained for the combinations were between 1.5 and 3.917 indicating no antimicrobial interactions between the two plant extracts. Therefore, a combination of *Calistermon citrinus* and *Erybotrya japonica* hydro-ethanolic leaf extracts may not have any synergistic, additive, or potentiative *in vitro* antimicrobial activity against *Streptococcus mutans*. Instead, the *Calistermon citrinus* leaf extract is more efficacious against the cariogenic bacteria when used singly than its mixture with *Erybotrya japonica* leaves extract.

KEYWORDS: **Antimicrobial; *Streptococcus mutans; Callistemon citrinus*; *Eriobotrya japonica*; Hydro-ethanolic; interactions**

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INTRODUCTION

The use of herbs in combinations to achieve beneficial synergistic, additive, and potentiative effects in traditional herbal practice exists in many societies over the years [1]. The Indian ayurvedic medicine in the text of *Sarangdhar Samhita* considers this practice as one offering better therapeutic efficacy with holistic body rejuvenation [2]. Chinese Traditional Medicine (CTM) which has existed for thousands of years, also encourages the use of multiple herbs for better pharmacological effects and reduction of toxicity [3]. Ethnobotanical surveys and efficacy studies in Africa have also reported the use of more than one herb in traditional herbal preparations for the management of ailments as a common practice by community herbalists [4, 5, 6].

Despite the agreement among the various societies in the world concerning the benefits of using herbs in combinations, there is a need to scientifically validate them. Not all the interactions among the herbs are beneficial, cases of lost therapeutic efficacy or even increased toxicity are possible with this practice [7]. With the current approach of industrialization, the manufacture of herbal medicines is shifting from contemporaneous compounding to hi-tech production on large scale requiring a high level of scientific evidence. This necessitates a scientific evaluation of these combinations and a proper understanding of the role each herb is playing in the combination. Otherwise, some ingredients may not be performing beneficial effects in a product as expected or even increase toxicity and lower therapeutic activity, thus adding unnecessary costs to production. Optimization studies which are important in explaining the relationships between dependent and response (independent) factors are key in understanding the interactions among various herbs in one preparation. They do not only provide scientific information on the contribution of each herb in the total product efficacy or toxicity but also on the most effective dose combination [8, 9]. But for antimicrobial interactions between two drugs or herbal extracts, fractional inhibitory concentration index (FICI) and fractional bactericidal concentration index (FBCI) are commonly used for determination of synergism, antagonism, potentiation [10, 11]

Eriobotrya japonica (Loquat) and *Callistemon citrinus* (Bottlebrush) are used locally in Uganda for the management of tooth-related conditions such as tooth decay associated with pain. The plants have shown promising activity against oral microbes responsible for dental carries with zones of inhibition of 4 mm and 8 mm for *Eriobotrya japonica* (EJ) and *Callistemon citrinus* (CC) respectively [12]. *Eriobotrya japonica* showed no side effects in male and female rats [13], thus is considered a safe traditional medicine for clinical application. Another study also showed that *Callistemon citrinus* is well-tolerated and safe for use [14]. Even though the two plants are reported to be individually effective against *Streptococcus mutans,* there is no data in the literature showing additive, synergistic or potentiative effects from the combinations of the two plant extracts.

Streptococcus mutans is a gram-positive bacteria most implicated in the progression of dental caries [15]. It uses multiple mechanisms to colonize the tooth surface and form bacterial plaque biofilm which serves as a physical barrier to limit penetration of antimicrobial agents into the deep layers of biofilm [16-17]. The bacteria also produce organic acids through various carbohydrate metabolism processes that dissolve tooth enamel and dentine over time leading to the development of dental caries [18-19]. In advanced states, dental caries can affect the pulp of the tooth and destroy tooth structure leaving only root fragments that can lead to ulcerations and abscesses [20].

Dental caries is also one of the most prevalent and consequential oral diseases globally [21]. In Uganda, the overall caries prevalence was highest in adults than in children in randomly selected urban areas [22]. Available interventions to address the challenge of this condition include artificial sealants which protect the tooth surface from the acidic environment, antibacterial mouth rinses, and fluorides [23]. However, when cavitated carious lesions develop, restorative treatment is required for caries management [24]. In addition, antibiotics such as metronidazole, β-lactam antibiotics, tetracyclines may be used but their efficacy has decreased due to anti-microbial resistance [25]. Also, some of the ingredients used in the formulation of oral care products such as fluorides [26] and alcohol [27] have been associated with some negative effects such as dental fluorosis, burning sensation, dryness of the mouth, bad odor, and oral cancer. Overall, the costs incurred in the management of dental caries are high especially for people living in low-resource countries such as Uganda, thus the need for cheaper alternatives from nature [28].

The current work therefore aimed at understanding *in vitro* antimicrobial interactions between the combinations of the two plant hydro-ethanolic leaf extracts against *Streptococcus mutans* to support

their polyherbal usage for dental caries management.

MATERIALS AND METHODS

Materials

The standard strain of *Streptococcus mutans* ATCC 25175 (cariogenic bacteria) was obtained from the Microbiology Laboratory, College of Veterinary medicine, Makerere university. Ciprofloxacin powder was obtained from Kampala Pharmaceutical Industries (Uganda), Resazurin (Invitrogen™, USA), Mueller–Hinton broth (BD Difco™), Ethanol (Sigma-Aldrich, USA). All other reagents and solvents used in this study were of analytical grade.

Plant Collection and Authentication

The leaves of *Eriobotrya japonica* and *Callistemon citrinus* were collected near Mbarara University of Science and Technology at GPS coordinates -0.61723342 (latitude), 30.65666333 (longitude) for *Callistemon citrinus* and -0.61670506, 30.65656174 for *Eriobotrya japonica*. Herbarium specimens for both plants were prepared and submitted for botanical authentication to a taxonomist at the Department of Biology, Faculty of Science, Mbarara University of Science and Technology. Voucher numbers Healy Onen 001 and Healy Onen 002 for EJ and CC respectively were issued.

Preparation of Plant Extracts

Fresh leaves of both plants were dried in a well-aerated room under shade at room temperature and later pulverized to a coarse powder stored in amber bottles ready for extraction. The coarse powder (300 g) for each plant material was placed in a 2.5 L amber bottle and macerated using 60 % ethanol (1500 mL) for 3 days with constant agitation. The marc was filtered using a muslin cloth and later with a filter paper (Whatman No. 5). The filtrate was concentrated using a rotarod evaporator (IKA RV10, USA) at 40 ℃ and finally to a dry extract using an electric oven at 50 ℃. The extract was weighed and the percentage yield was calculated according to the formula,

$$\text{Percentage yield} = \frac{\text{Amount of dry extract recovered (g)}}{\text{Amount of plant sample (g)}} \times 100 \quad \text{…….. Eqn. 1}$$

The extracts were then packed in amber glass bottles and stored at -4 °C for subsequent activities.

Phytochemical Screening

The two extracts were subjected to qualitative phytochemical tests for alkaloids, flavonoids, saponins, tannins, and essential oils [29].

Dilution of Plant Extracts and Standard Drug

The dry extracts from both plants were mixed in five different combinations of *Callistemon citrinus* (CC): *Eriobotrya japonica* (CJ) – 1:0, 3:1, 1:1, 1:3 and 0:1. Each proportion was dissolved in dimethyl sulfoxide (DMSO, 10 %) to produce a concentration of 0.1 g/ml. This was diluted to obtain a stock solution of a concentration of 80 mg/mL using sterile water. The ciprofloxacin (standard drug), was dissolved in sterile water to obtain a concentration of 0.02 mg/mL.

Preparation of Resazurin (0.015 %)

The resazurin powder (0.015 g) was dissolved in sterile water and made to 100 mL using the same solvent. The solution was vortexed, sterilized by filtration (0.22 µm filter), and stored at 4 °C for a maximum of two weeks before use [30].

Preparation of Standard Inoculum of *Streptococcus mutans* (ATCC 25175)

The bacterium was sub-cultured on blood agar and incubated at 37 °C for 24 hours [29]. A cotton swap was used to inoculate the colonies in sterile normal saline (0.85 %) tube to obtain a McFarland standard streptococcus suspension of 0.5 at OD_{600}, which is equivalent to 1.5×10^8 CFU/mL [31]. Approximately 5 $x10^5$ CFU/mL was then prepared by carrying 1:100 Mueller–Hinton broth dilution. The inoculum prepared was dispensed into the 96-well microtiter plates in less than 15 min.

Determination of the Minimum Inhibitory Concentration (MIC)

This was done by broth dilution assay as described previously [32], with modifications. 10-fold serial dilutions were carried out to obtain final concentrations in the range of 20000 µg/mL to 39.0625 µg/mL for extract combinations and 5 µg/mL to 0.009765625 µg/mL for the standard drug. DMSO (2.5 % v/v) was used as a negative control. The plates were covered and then incubated in an oven at 37 °C for 24 hours. 0.015 % resazurin (30 µL) was added to all wells and further incubated for 4 hours for the observation of any microbial growth. The lowest concentration of the antimicrobial sample which showed no visible microbial growth was scored as the minimum inhibitory concentration (MIC) value [30].

Determination of the Minimum Bactericidal Concentration (MBC)

20µl of the contents from the wells before the MIC value were drawn and inoculated on fresh blood agar and later incubated in an oven at 37 °C for 24 h. The minimum concentration with no colony growth was taken as the minimum bactericidal concentration (MBC) [33].

Determination of Antimicrobial Interactions

Fractional inhibitory/bactericidal concentration indices (FICI/FBCI) were used as reported previously [34-45] to determine the presence or absence of antimicrobial interactions as shown below:

$$FICI = \frac{\text{MIC of extract A in combination with extract B}}{\text{MIC of extract A alone}} + \frac{\text{MIC of extract B in combination with extract A}}{\text{MIC of extract B alone}} \quad \text{..... Eqn. 2.}$$

$$FBCI = \frac{\text{MBC of extract A in combination with extract B}}{\text{MBC of extract A alone}} + \frac{\text{MBC of extract B in combination with extract A}}{\text{MBC of extract B alone}} \quad \text{.....Eqn. 3}$$

The index was calculated for every value of MIC and MBC obtained in triplicates and presented in Mean ± SEM.

Data Analysis

The data were summarized into Mean ± SEM and presented in suitable tables and graphs. Statistical tools i.e. one-way ANOVA and Tukey's Multiple Comparison Test were used for data analysis. The differences were considered statistically significant at $P \leq 0.05$. GraphPad Prism (version 8.0.2) software was used to perform the analysis. Checkerboard analysis utilizing pharmacological mathematical model – Fractional Inhibitory/ Bactericidal Concentration Index, was used for determination of synergistic, antagonistic and other *in vitro* antimicrobial interactions.

RESULTS

Eriobotrya japonica (EJ) had a higher percentage yield than *Callistemon citrinus* (Table 1). Both plant extracts contained a similar profile of phytochemical groups (Table 2), showing the presence of flavonoids, saponins, and tannins, and the absence of alkaloids and volatile oils. However, *Callistemon citrinus* extract showed higher intensity of flavonoids than *Eriobotrya japonica*.

Ciprofloxacin showed the lowest MIC and MBC while EJ: CC (1:0) had the highest MIC and MBC values (Figures 1 and 2 respectively). The MIC and MBC for the standard drug were similar to those of *Callistemon citrinus* (CC: EJ = 1:0) but significantly different from ones of *Eriobotrya japonica* (CC: EJ = 0:1) at $P \leq 0.05$. Increasing the quantity of *Eriobotrya japonica* in the proportions increased the MIC/MBC values whereas increasing the quantity of *Callistemon citrinus* in the proportions decreased the MIC/MBC values. The FICI/FBCI of the different extract proportions ranged from 1.5 to 3.917 as shown in Table 3.

DISCUSSION

The percentage yields of both plants were higher than those obtained in previous studies for *Callistemon citrinus* [30] and *Eriobotrya japonica* [36], This could be attributed to the different methods of extraction and solvent systems i.e. soxhlet extraction compared to our cold maceration and 5 % ethanol versus 60 % ethanol used in the current study. Geographical variations may also explain the differences in the above-stated values of percentage yield. The phytochemical profiles reported in our study were comparable to other studies [37], for *Callistemon citrinus* and [38], for *Eriobotrya japonica* where ethanol was also used for extraction. In comparison to the fruit 50 % ethanolic extract of *Callistemon citrinus*, [39] reported from India, the presence of alkaloids, glycosides, flavonoids, and carbohydrates. The phytochemical groups absent in this Asian study included tannins, proteins, terpenes, saponins, and steroids. Contrary to our findings, crude alkaloids were isolated from leaves of CC in Zimbabwe using a 10 % ammonia solution and an undeclared percentage of ethanol [40]. The absence of volatile oils according to our findings may also be attributed to the method of extraction since earlier studies [41], already documented antimicrobial activities of essential oils distilled from leaves of *C. citrinus* against *streptococcus mutans* with twenty-eight (28) compounds being identified from this oil of the plant.

The MIC and MBC findings indicate that the *Callistemon citrinus* was more efficacious than *Eriobotrya japonica* and its quantity in the proportions affected values accordingly. No combination level showed MIC or MBC lower than those of CC alone. The promising activity of *Callistemon citrinus* independent of EJ could be due to the higher quantity of flavonoids. However, our MIC value for CC was higher than the one reported for fruit extract of the same plant [42] – 31.2 and 3.9 µg/ml for ethanolic extract and n-hexane fraction respectively. In the same study

[42], the leaf extract of *C. citrinus* also gave better activity (MIC = 62.5 µg/ml for both the crude ethanolic and n-hexane fractions). This difference could be due to variations in the geographical locations since the previous study was done in Korea. A recent study on the antimicrobial activity of CC against *Streptococcus mutans* in Uganda reported a better zone of inhibition for a petroleum ether extract compared to the ethanol one [43]. Piceatannol (3,3',4',5-tetrahydroxystilbene) compound has also been isolated from CC fruit and tested against *Streptococcus mutans* giving MIC of 15.6 µg/mL [44]. But the presence of this compound in the leaves has not been ascertained though it was reported in the stem bark of *Callistemon lanceolatus* [45].

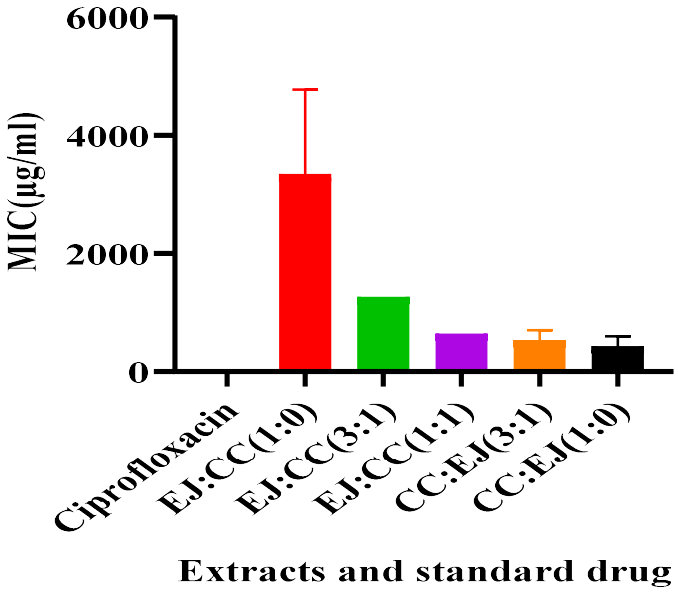

Figure 1: MIC of different extract proportions and standard drug. Each bar is a mean from triplicate measurements and its corresponding SEM.

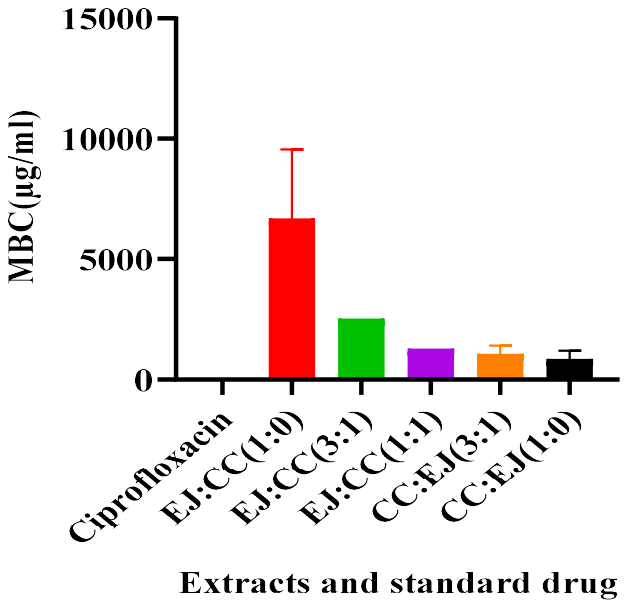

Figure 2: MBC of different extract proportions and standard drug. Each bar is a mean from triplicate measurements and its corresponding SEM.

Table 1: Percentage Yield of the Hydro-ethanolic Extracts

Plant	Yield (%)
EJ	20.05
CC	15.45

Key**:** EJ = *Eriobotrya japonica*; CC = *Callistemon citrinus*

Table 2: Phytochemical Screening

Phytochemical group	CC	EJ
Alkaloids	-	-
Flavonoids	++	+
Saponins	+	+
Tannins	+	+
Volatile oils	-	-

Key: - = absent; + = present; ++ present in higher quantities; CC = *Callistemon citrinus*; EJ = *Eriobotrya japonica*

Table 3: FICI and FBCI of the Different Extract Proportions

Extract proportions	FICI (Mean±SEM)	FBCI (Mean±SEM)
EJ: CC (3:1)	3.917±0.8457	3.750±1.465
EJ: CC (1:1)	1.958±0.4229	1.875±0.649
CC: EJ (3:1)	1.583±0.4583	1.500±0.649

Key: FICI **=** Fractional inhibitory concentration index; FBCI = Fractional bactericidal concentration index; CC = *Callistemon citrinus*; EJ = *Eriobotrya japonica*

The MIC findings of *Eriobotrya japonica* (EJ) in our study (3,333 µg/mL) are lower than those reported previously (6,250 µg/ml for an aqueous extract) [46]. Though the communities in Uganda are using the plant for the management of dental caries symptoms including pains, its local medicinal value may be due to the excellent antinociceptive activity reported previously [47], since it exhibited lower antimicrobial activity against *Strep. mutans* according to our findings. Conversely, the plant may be utilizing other mechanisms such as inhibition of the biofilm formation as reported for other plants [48] as opposed to direct antibiotic activity against the cariogenic bacteria. But this will need to be confirmed in a separate study.

FICI/FBCI ≤0.5 shows synergy; 0.5 < FICI/FBCI ≤1 is indicative of additivity; 1 < FICI/FBCI ≤4 means absence of interaction (indifference); and FICI/FBCI >4 indicates antagonism [35]. In the present study, the FICI/FBCI values lay in the 1 < FICI/FBCI ≤ 4 range, meaning none of these *in vitro* antimicrobial interactions were observed between the two plant extracts.

CONCLUSION

A combination of *Calistermon citrinus* and *Erybotrya japonica* hydro-ethanolic leaf extracts may not have any synergistic, additive, or potentiative *in vitro* antimicrobial activity against *Streptococcus mutans*. The *Calistermon citrinus* leaf extract is more efficacious against the cariogenic bacteria when used singly than its mixture with *Erybotrya japonica* leaves extract.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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