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Anti-Inflammatory, Antimicrobial and Stability Studies of Poly-Herbal Mouthwashes against *Streptococcus mutans*

Ezemokwe Ikenna Chiedozie, Onyegbule Felix Ahamefule and Anwachaepe Amarachukwu Ukamaka

Abstract
Methanol extracts of *Moringa oleifera* stem, twigs of *Breynia nivosa, Azadirachta indica,* and *Psidium guajava* were evaluated for antibacterial activity against *Streptococcus mutans*. Qualitative and quantitative phytochemical components of each plant extract were determined. Three batches of mouthwash were formulated based on the multiples of minimum inhibitory concentrations of each plant extracts. The batches were evaluated for antibacterial and anti-inflammatory activities, which were compared with those of a standard marketed mouthwash (Minty Brett ®) and anti-inflammatory agent (Volterene®). The stability of the batches was also determined. The methanol extract of each plant presented appreciable antibacterial activity (with minimum inhibitory concentrations of 25.12 mg/ml, 6.31 mg/ml, 39.8 mg/ml and 12.5 mg/ml for *M. oleifera*, *B. nivosa*, *A. indica*, and *P. guajava* respectively) against *S. mutans*. Tannins, saponins, triterpenoids, steroids, alkaloids and cardiac glycosides were present in all the plant extracts. Proteins and *phlobatannins* were present in *B. nivosa*. Batch 2 of the mouthwash preparation demonstrated a better antibacterial activity with inhibition zone diameter of 14.2 mm and average kill time of 25 min, while batch 3 had the best anti-inflammatory activity (65.1% inhibition). The batches stored on the shelf and in the refrigerator were stable during 3 months of observation. Defined combination of different herbal extracts lead to synergy, better activity and stability. The poly-herbal mouthwash formulation demonstrated better antibacterial and anti-inflammatory activities than standard marketed mouthwash (Minty Brett®).

Keywords: Mouthwash, Anti-Bacterial, *Streptococcus mutans,* Stability Studies, Poly-Herbal

1. Introduction
In Nigeria, consistent increase in the burden of oral diseases such as caries and periodontal diseases has been reported [1, 2]. The use of mouthwash in the prevention oral diseases and maintenance of oral hygiene dates as far back as 2700 BC [3]. Standard marketed mouthwashes containing synthetic and semi synthetic active agents have merits as well as several demerits such as high cost, oral cancer, like staining of the teeth and irritation during, use, high degree of alcohol content and so on [4]. Therefore the need for an affordable natural/herbal alternative with few demerits for the maintenance of oral hygiene is required especially in patients with special health care concern.

Over 750 species of bacteria inhabit the oral cavity, *Streptococcus mutant* is the major cause of the most common dental diseases such as caries and periodontitis [5]. One of the important virulence properties of *S. mutans* is their ability to form biofilms known as dental plaque on tooth surfaces [6]. Biofilm cells have been shown to be up to 1,000-fold more tolerant of antibiotics, and this confers a great resistance to the organism [7]. If left unchecked, dental plaque can progress to dental caries and periodontal disease. Therefore inhibition of the biofilm formation will increase the sensitivity of the organism to antimicrobial agents, hence prevent the development of dental disease.

Naturally occurring antimicrobial herbs can be used individually or in combination in the formulation of mouthwash and also in addition will be cheap, highly biocompatible, and posses extra activity such as anti-inflammatory, wound healing property, antioxidant property and so on [8]. *Psidium guajava* [9, 10], *Moringa oleifera* [11], *Azadirachta indica* [12] and *Breynia nivosa* [13] has demonstrated varied degree of activity against *Streptococcus mutans* and also are used as chewing stick/powder in different part of Nigeria.

2. Materials and Method
2.1 Plants
Stems of *Moringa oleifera* and twigs of *Breynia nivosa, Azadirachta indica, and Psidium guajava*.

2.2 Test Organisms
Four strains of *Streptococcus mutans* isolated from extracted teeth of dental caries patients at the School of Dentistry Trans-Ekulu, Enugu State.

2.3 Chemicals and solvents
Nutrient agar (Oxoid Limited, England), nutrient broth (Oxoid Limited, England), Mueller Hinton agar (Oxoid Limited, England), Columbia blood agar base (Oxoid Limited, England). dimethyl sulfoxide (DMSO), MacFarland turbidity Standard (prepared from barium chloride, sulfuric acid and distilled water), methanol (Sigma Aldrich, Germany), Tween 80, Fehlings solution (A & B), ferric chloride (Sigma Aldrich, Germany), *Streptococcus* selective supplement (Oxoid Limited, England), concentrated sulphuric acid (Sigma Aldrich, Germany), distilled water, nitric acid (Sigma Aldrich, Germany)

2.4 Plant harvesting
The plant parts *Psidium guajava* twig, *Moringa oleifera* stem, *Azadirachta indica* twig and *Breynia nivosa* twig were collected in November, 2014 from Aniocha Local Government in Anambra state. They were identified by a plant taxonomist in the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria. The samples were air-dried under a shade and milled using a mechanical grinder.

2.5 Extraction of plant materials
2 kg of each pulverised plant parts were extracted using soxhlet extraction method with 1 Litre of methanol each as the solvent. The methanol extracts of each plant were concentrated using rotary evaporator (RE300UK) at 40 ^{o}C. The concentrated crude methanol extract obtained was kept in sample bottles and stored in a refrigerator at 4^{o} until it was required.

2.6 Test organisms
Four strains of *Streptococcus mutans* were used in this study. These were clinical isolates from dental swabs collected from extracted tooth of patients with dental caries at School of Dentistry Trans-Ekulu Enugu State. The isolates were maintained in Columbia blood agar base (supplemented with 5% sheep blood).

2.7 Phytochemical Screening of plant extracts
In the phytochemical analysis of the extracts of stem of *Moringa oleifera*, twigs of *Breynia nivosa, Azadirachta indica* and *Psidium guajava* were screened qualitatively for alkaloids, tannins, saponins, flavonoids, steroids, cardiac glycosides, and terpenoids were carried. Also a quantitative estimation of alkaloids, tannins and saponins were carried out using standard methods reported by Trease and Evans [14].

2.8 Isolation and identification of microorganisms
Dental swabs obtained from fresh extracted tooth patients with dental caries at School of Dentistry, Trans-Ekulu, Enugu State, Nigeria were inoculated in sterile test tubes containing Nutrient broth. After overnight incubation at 37 ^{o}C, samples were streaked onto Petri plates containing blood agar to which *Streptococcus* selective supplement has been added. Plates were then incubated anaerobically in a candle jar at 37 ^{o}C for 24 h. After incubation, plates were observed for colonies with alpha-hemolysis. Suspected *S. mutans* colonies were maintained in Columbia blood agar base (supplemented with 5% sheep blood). Identification for *Streptococcus mutans* was confirmed by observation of cultural, morphological and microscopic characteristics, Gram staining, and biochemical testing procedures like mannitol and sorbitol fermentation and catalase test.

2.9 Determination of minimum inhibitory concentration (MIC) of the crude extracts on test isolates
The MIC of the plant extracts on the test isolates was determined by the agar dilution method [15]. The stock solution (1000 mg/ml) was further diluted in a 2-fold serial dilution to obtain the following concentrations: 500, 250, 125, 62.5, 31.25, and 15.625 mg/ml. Agar plates were prepared by pouring 9 ml of molten double strength MHA into sterile Petri plates containing 1 ml of the various dilutions of the extract making the final plate concentrations to become 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 mg/ml. The test isolates which were grown overnight in broth were adjusted to McFarland 0.5 standard and streaked onto the surface of the agar plates containing dilutions of the extract. The plates were then incubated in anaerobic candle jar at 37 ^{o}C for 24 h after which all plates were observed for growth. The minimum dilution concentration of the extracts completely inhibiting the growth of each organism were taken as the MIC.

2.10 Formulation of poly-herbal mouthwashes
The poly-herbal mouthwash formulations were prepared based on the MIC results obtained for each extract and the method with little modifications was adopted from [9, 10]. Three batches of mouthwashes were formulated (Table 1) with 1.5 x MIC (Batch 1), 2 x MIC (Batch 2) and 1.5 x MIC (Batch 3) for the respective plant extract. A pilot study on the formulations was carried out to determine the best formula for the product.

Table 1: Formula for different batches of Mouthwash

Ingredients	Batch 1	Batch 2	Batch 3
B. nivosa	0.48 g	0.631 g	0.631 g
A. indica	3 g	4 g	4 g
M. oleifera	1.95 g	2.6 g	2.6 g
P. guajava	0.94 g	1.25 g	1.25 g
Chloroform water	6 ml	6 ml	-
Ethanol	-	-	6 ml
Peppermint emulsion	1.25 ml	1.25 ml	1.25 ml
Tween 80	3.5 ml	3.5ml	3.5 ml
Distilled water to	50 ml	50 ml	50 ml

2.11 Antibacterial evaluation of the poly-herbal mouthwash formulations
The antibacterial activity of the formulation against the test isolates was determined by the agar well diffusion method. Dilutions of 1:0, 1:1, 1:2, 1:4, 1:6, 1:8 of the various formulations and control (Minty Brett®) were prepared. A volume of 20 ml of molten Mueller Hinton Agar (MHA) was poured into sterile Petri dishes (90 mm) and allowed to set. Standardized concentrations (McFarland 0.5) of overnight cultures of test isolates were swabbed aseptically on the agar and holes (6 mm) were made in the agar plates using a sterile metal cork-borer. Also 20µl of the various dilutions of the formulations and control were put in each hole under aseptic condition. Gentamicin (10 µg/ml) was used as positive control, while DMSO was used as the negative control. The

plates were then incubated anaerobically in a candle jar at 37 ºC for 24 h and the inhibition zones diameters (IZDs) were measured and recorded. The size of the cork borer (6 mm) was deducted from the values recorded for the IZDs to get the actual diameter. This procedure was conducted in triplicate and the mean IZDs calculated and recorded.

2.12 Determination of Kill Time

The kill time of the formulations and control was carried out according to standard methods [8]. A volume of 0.1 ml of $1x10^6$ cfu/ml suspension of the test organisms was added to test tubes containing 5 ml of the formulations and control (Minty Brett ®). These were kept for 1 h. At time intervals of 0, 10, 20, 30, 40, 50, and 60 min, a volume of 0.1 ml of the mixture was aseptically withdrawn and sub cultured on fresh nutrient agar plates and incubated anaerobically in a candle jar at 37 ºC for 24 h. After incubation, the plates were observed for the presence or absence of growth.

2.13 Anti-inflammatory Studies

Xylene induced ear oedema [16] was adopted. Overnight fasted mice were divided into six groups (n=5). The groups and the treatment were as follows:

Group 1 = Volterene cream® (positive control)
Group 2 = Minty Brett®
Group 3 = Batch 1
Group 4 = Batch 2
Group 5 = Batch 3
Group 6 = no treatment

A 1 ml volume of the mouthwash (treatment) was applied topically to the inner surface of the right ear of the mice in different groups. The left ear was considered to be the control. One hour afterwards (after the treatment above), 30 µl xylene was applied to the inner surface of the right ear to induce oedema and was left for another 30 min. The animals were sacrificed and with the aid of a cork borer with diameter of 7 mm, a circular section of both ears was removed and weighed. The percentage inhibition of ear oedema was calculated relatively to the left ear that was without xylene using the mathematical relationship below.

Percentage (%) inhibition = (1 - et/ec) x 100

Where
et = weight of edema of treated ear
ec = weight of edema of untreated ear

2.14 Stability studies

2.14.1 Determination of stability of the formulation using surface tension

The study was carried out using drop volume method [17]. A pipette was used to measure the volume of a drop of the preparation by taking the average of 5 drops. The weight of the drops was measured using an analytical weighing balance. The surface tension of the batches was determined over the period of 13 weeks using the equation below,

$$\gamma \text{ (surface tension)} = \frac{mg}{2\pi rf}$$

Where,
m =weight
g = acceleration due to gravity
f = correction factor
2π = constant

2.14.2 pH studies

Well calibrated pH meter was used to determine the pH of different batches of the mouthwash formulations kept on shelf and in refrigerator (12 ºC) respectively. The results were documented and compared over 13 weeks. The study was replicated in triplicate.

2.14.3 Colorimetric studies

The absorbance of the three batches kept on the shelf and in refrigerator (12 ºC) respectively were determined and compared over 13 weeks using colorimeter at 600 nm. A 1:2 fold dilution of the samples were used to determine the absorbance.

2.15 Statistical Analysis

Using SPSS (version 20) One-way ANOVA was used to test for differences. Charts and graphs were also used to compare the batches stored in the shelf and refrigerator. P-values ≤ 0.05 indicates a significant difference.

3. Result

3.1 Plant extraction

The methanol extract of the plant materials produced a percentage weight yield of *M. oleifera* extract was 4.8% w/w, *B. nivosa* extract 7.5% w/w, *A. indica* extract 5.7% w/w, and *P. guajava* extract 5.3% w/w.

3.2 Qualitative and quantitative studies

Table 2 shows the result of the phytochemical analysis of crude extract of the plant samples. The result revealed the presence of tannin, saponin, steroid, alkaloids, triterpenoids, and cardiac glycosides in all the plant samples. There was absence of carbohydrate, and reducing sugars in the entire plant sample with varied presence of proteins, flavonoids and phlobatannins. The quantity of tannins, saponins and alkaloids in each plant crude extract was determined and presented in Table 3.

Table 2: Qualitative phytochemical constituents of *M. oleifera* stem, *B. nivosa* twig, *A. indica* twig, and *P. guajava* twig.

Phytochemicals	*B. nivosa*	*P. guajava*	*A. indica*	*M. oleifera*
Tannin	++	++	+	+
Saponin	++	++	+	+++
Phlobatannins	+	-	-	-
Flavonoid	+	+	+	-
Steroid	+	+	+	+
Alkaloids	+	++	+	+
Cardiac glycosides	++	+	+	+
Carbohydrate	-	-	-	-
Protein	+	-	-	-
Reducing sugar	-	-	-	-
Triterpenoids	+	+	+	+

Key: - absent
+ Present in small quantity
++ Present in moderately high quantity
+++ Present in high quantity

Table 3: Quantitative phytochemical composition of *M. oleifera* stem, *B. nivosa* twig, *A. indica* twig and *P. guajava* twig

Constituent	Percentage (% w/w)			
	M. oleifera	*B. nivosa*	*A. indica*	*P. guajava*
Alkaloid	0.31 ± 0.24	0.20 ± 0.32	0.21± 0.11	0.82± 0.38
Tannin	0.40 ± 0.73	0.92 ± 0.43	0.36 ± 0.24	0.79 ± 0.51
Saponin	0.74 ± 0.83	0.63 ± 0.37	0.17 ± 0.28	0.57 ± 0.81

Data represent the mean ± standard deviation of triplicate readings

3.3 Preliminary antibacterial sensitivity study

The result of the preliminary antibacterial sensitivity study (Table 4) shows that *S. mutans* is susceptible to different dilutions of the crude methanol extract of each plant sample. Also the minimum inhibitory concentration of the plant extracts on *S. mutans* was determined and recorded in Table 5.

Table 4: Preliminary antibacterial sensitivity of methanol extract of *M. oleifera* stem, *B. nivosa* twig, *A. indica* twig and *P. guajava* twig

Test Isolates	Inhibition Zone Diameters (IZDs)(mm)							
	500mg/ml	250mg/ml	125mg/ml	62.5mg/ml	31.25mg/ml	15.625mg/ml	Positive Control	Negative Control
B. nivosa								
S. mutans1	11.7±0.57	9.67±0.57	7.67±0.57	5.67±0.57	4.0±0.0	1.67±0.0	24.0±0.0	+
S. mutans2	12.0±0.0	9.67±0.57	8.0±0.0	6.0±0.0	4.0±0.0	2.0±0.0	18.0±0.0	+
S. mutans3	13.7±0.57	12.0±0.0	10.0±0.0	8.0±0.0	6.0±0.0	4.0±0.0	22.0±0.0	+
S. mutans4	12.0±0.0	9.67±0.57	8.0±0.0	6.0±0.0	4.0±0.0	2.0±0.0	24.0±0.0	+
A. indica								
S. mutans1	7.67±0.577	6.0±0.0	4.0±0.0	2.0±0.0	1.0±0.0	+	24.0±0.0	+
S. mutans2	8.0±0.0	5.67±0.57	4.0±0.0	2.0±0.0	+	+	18.0±0.0	+
S. mutans3	6.33±0.577	4.0±0.0	2.0±0.0	+	+	+	22.0±0.0	+
S. mutans4	8.0±0.0	6.0±0.0	4.0±0.0	2.0±0.0	+	+	24.0±0.0	+
P. guajava								
S. mutans1	9.67±0.57	7.67±0.57	5.67±0.57	3.67±0.57	1.67±0.57	+	24±0	+
S. mutans2	12.0±0.0	10.0±0.0	8.0±0.0	6.0±0.0	4.0±0.0	2.0±0.0	18±0	+
S. mutans3	12.0±0.0	10.0±0.0.0	8.0±0.0	6.0±0.0	4.0±0.0	2.0±0.0	22±0	+
S. mutans4	12.0±0.0	10.0±0	8.0±0.0	6.0±0.0	4.0±0.0	2.0±0.0	24±0	+
M. oleifera								
S. mutans1	12.0±0.0	10.0±0.0	8.0±0.0	6.0±0.0	4.0±0.0	2.0±0.0	24±0.0	+
S. mutans2	8.0±0.0	6.0±0.0	4.0±0.0	2.0±0.0	+	+	18±0.0	+
S. mutans3	8.33±0.57	6.33±0.57	4.33±0.57	2.33±0.57	1.0±0.0	+	22±0.0	+
S. mutans4	8.0±0.0	6.0±0.0	4.0±0.0	2.0±0.0	+	+	24±0.0	+

Data represent the mean ± standard deviation of triplicate readings. +=presence of growth (no inhibition)

Table 5. Minimum inhibitory concentrations (MICs) of the extracts on test isolates

Test Organisms	MICs (mg/ml)			
	B. nivosa	***A. indica***	***P. guajava***	***M. oleifera***
S. mutans	6.31	39.8	12.5	25.12

3.4 Antibacterial evaluation of the herbal mouthwash formulations

Table 6 shows that the antibacterial potency of the formulations and control as batch 2 > minty Brett® > batch 3 >batch 1. The three formulations had varied activity against the four strains of *S. mutans* even at lower concentration ratio, while the activity of the positive control (Minty Brett®) discontinued after 1:1 dilution. The IZD of the stock formulation of batch 2 (14.2±18 mm) herbal mouthwash was higher than the IZDs of the stock of each plant extract (when not in combination) as shown in Table 4 (*B. nivosa* (12.35 mm), *P. guajava* (11.4 mm), *M. oleifera* (9.1 mm), *A. indica* (7.5 mm).

Table 6: Antibacterial evaluation of the herbal mouthwash formulations

	Inhibition Zone Diameters (IZDs)(mm) Produced by Different Dilutions of the Formulations						
		1:0	1:1	1:2	1:4	1:6	1:8
Batch 1	*S. mutans1*	4.67±0.57	2.33±0.57	+	+	+	+
	S. mutans2	4.67±0.57	2.0±0.0	+	+	+	+
	S. mutans3	5.67±0.57	2.67±0.57	+	+	+	+
	S. mutans4	6.67±0.57	3.67±0.57	1.33±0.57	+	+	+
	Average	5.42±0.96	2.67±0.22	0.33±00	+	+	+
Batch 2	*S. mutans1*	11.7±0.57	3.67±0.57	+	+	+	+
	S. mutans2	15.0±0.0	8.0±0.0	2.0±0.0	+	+	+
	S. mutans3	14.0±0.0	4.67±0.57	2.0±0.0	+	+	+
	S. mutans4	16.0±0.0	9.67±0.57	6.0±0.0	+	+	+
	Average	14.2±1.8	6.50±2.8	2.5±2.5	+	+	+
Batch 3	*S. mutans1*	4.33±0.57	1.67±0.57	+	+	+	+
	S. mutans2	7.33±0.57	5.0±0.0	2.33±0.57	+	+	+
	S. mutans3	10.0±0.0	5.67±0.57	2.0±0.0	+	+	+
	S. mutans4	7.67±0.57	6.0±0.0	2.67±0.57	+	+	+
	Average	7.33±2.3	4.59±2.0	1.75±1.2	+	+	+
Control (Minty Brett®)	*S. mutans1*	8.0±0.0	4.0±0.0	+	+	+	+
	S. mutans2	8.67±0.57	4.0±0.0	+	+	+	+
	S. mutans3	10.0±0.0	4.0±0.0	+	+	+	+
	S. mutans4	10.0±0.0	4.67±0.57	+	+	+	+
	Average	9.17± 1.0	4.17±0.33	+	+	+	+

Data represent the mean ± standard deviation of triplicate readings. +=presence of growth (no inhibition)

3.5 Anti-inflammatory activity of the mouthwash formulations

Then trend of anti-inflammatory property of the batches and the control are Batch 3> Volterene® > Minty brett® >batch 1>batch 2. It was observed in the chat (Figure 1) that batch 3 of the formulation has the highest percentage inhibition which is better than the positive controls. Using a one way ANOVA, there was a significant difference ($p<0.05$) in these anti-inflammatory activities compared with the positive control (volterene). Also in a multiple comparison analysis there was a significant difference ($p<0.05$) between the mean differences of all the batches and the negative control (no treatment) but no significant difference when the batches are compared with other treatments except negative control.

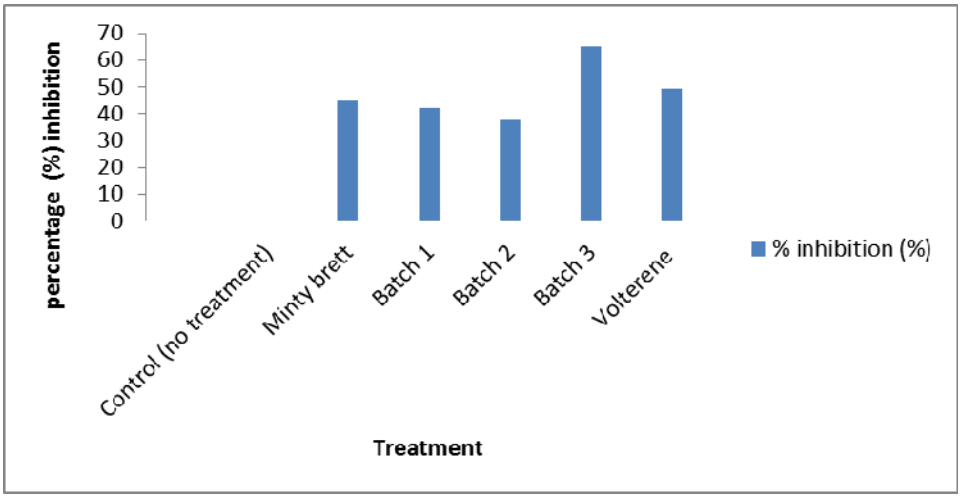

Fig 1: Anti-inflammatory activity of different mouthwash formulations

3.6 Stability studies

3.6.1 Surface tension analysis result

The surface tension analysis was carried out weekly on the batches of mouthwash formulations and the difference in range of value of the surface tension values for the shelf storage (Batch 1: 31.2- 34.3; Batch 2: 28.1- 41.1; Batch 3: 34.3 – 44.3) and refrigerated storage (Batch1: 31.2- 41.1; Batch 2: 28.5 – 41.1; Batch 3: 28.5 – 41.1) over 13 weeks were not significant ($P>0.05$) for all batches

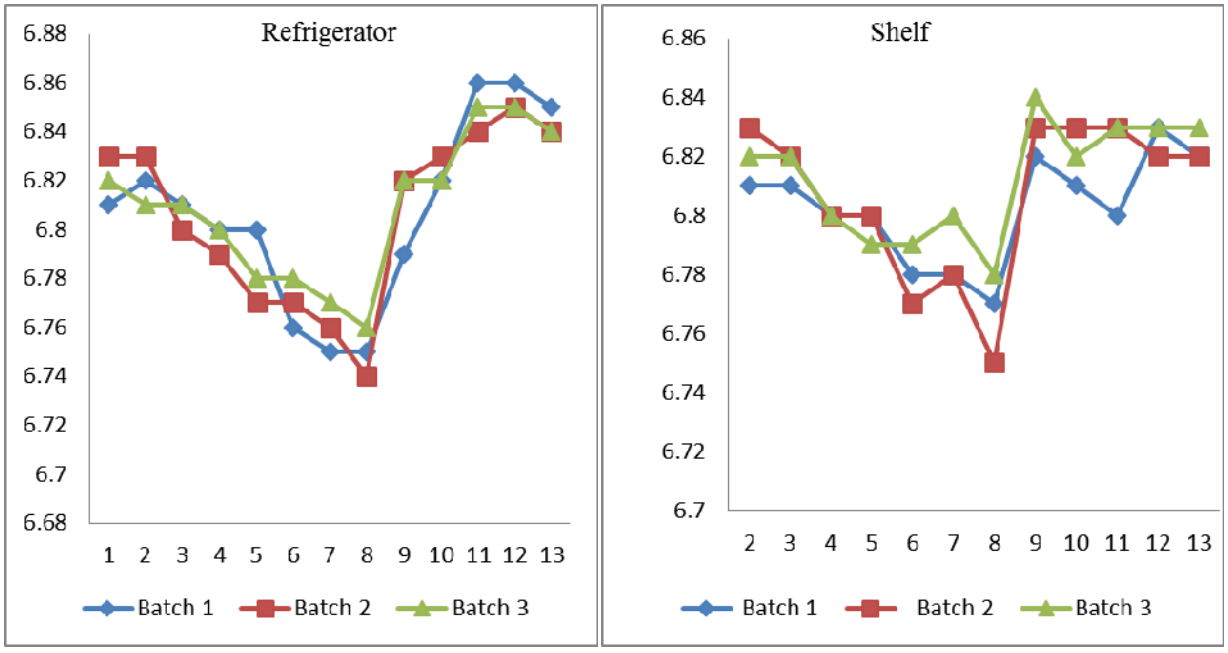

Fig 2: pH analysis charts of mouthwash formulation stored on the shelf and refrigerator

3.6.2 pH analysis result

From the table, it could be seen that the range of pH for shelf storage (Batch 1: 6.75- 6.82; Batch 2: 6.74- 6.85; Batch 3: 6.76 – 6.85) and refrigerator storage (Batch1: 6.77 - 6.83; Batch 2: 6.75 – 6.83; Batch 3: 6.78 – 6.83) over 13 weeks corresponds. The result of independent t-test on pH studies comparing refrigerator and shelf storage among the different batches revealed no significant difference in the mean score for all batches of the formulation ($p>0.005$).

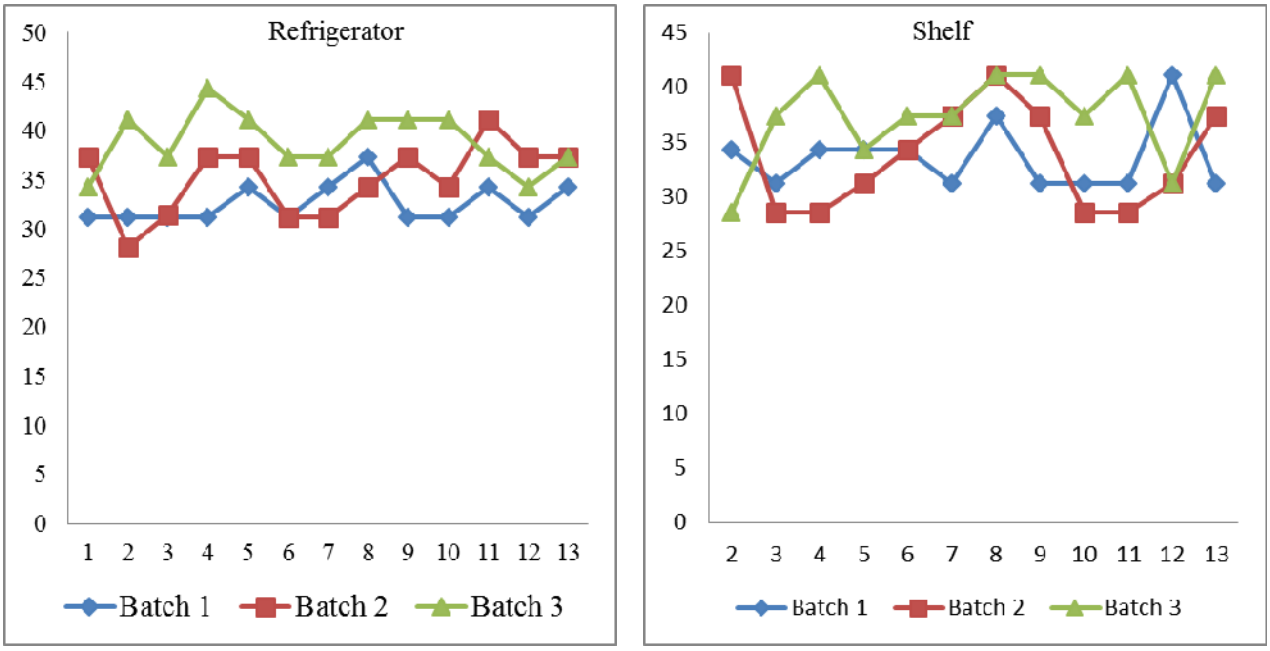

Fig 3: Surface tension analysis charts of mouthwash formulation stored on the shelf and refrigerator.

3.6.3 Colorimetric analysis result

From the table, it is seen that the upper and lower limit of the range of absorbance for shelf storage (Batch 1: 1.20-1.24; Batch 2: 1.21- 1.26; Batch 3: 0.8 – 0.9) and refrigerated storage (Batch1: 1.20 -1.23; Batch 2: 1.21 -1.24; Batch 3: 0.81 – 0.83) over 13 weeks also corresponds.

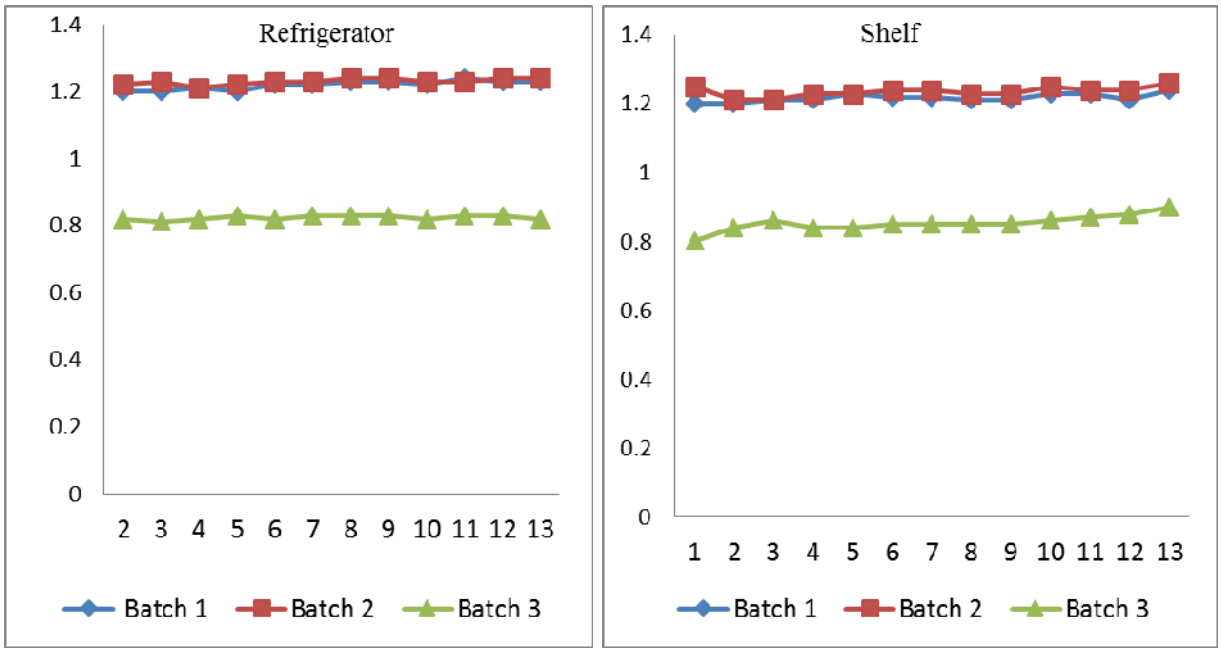

Fig 4: Absorbance charts of mouthwash formulations stored on the shelf and refrigerator

4. Discussion

The presence of certain Phytochemicals has been demonstrated in different studies to be linked to the anti-bacterial and anti-inflammatory of different plant extract against *S. mutans* [14, 18-20]. A study reported that the presence of plant flavonoid Quercetin-3-O-alpha-l-arabinopyranoside (guaijaverin) demonstrated high potential antiplaque agent by inhibiting the growth of the *S. mutans* [21].

The result of preliminary antibacterial sensitivity study reveals that all the extracts has activity (measure as inhibition zone diameter (IZD)) against the four isolates of *S. mutans*. This could be due to inhibition of its resistant mechanism (Biofilm) conferred by its physiological and metabolic makeup.

Batch 2 has the same concentration of plant extracts as batch 3 formulation, therefore the lower activity of demonstrated by batch 3 could have been reduced by the presence of ethanol in the formulation which may alter the bioavailability/activity of the formulation. The improved activity (measured as IZD) of the formulation as compared with the activity of the individual plant samples could be due to the combination of the different bioactive secondary metabolite in each plant

which conferred a synergistic/additional effect on the formulation. The findings of this study agree with other findings in the study of herbal mouthwash formulation. In a study, Rosemary mouthwash was found as a potent antimicrobial than chlorhexidine mouthrinse [22] using zone of inhibition against *Streptococcus mutans* to evaluate. Another study on herbal mouthwash comparing the activity of mono-herbal and ploy-herbal mouthwash also demonstrated synergy in activity and greater activity of the poly-herbal mouthwash compared to the mono-herbal preparations [10].

Batch 3 of the formulation demonstrated the highest percentage inhibition when compared with the positive controls. This is in agreement with the observation by Goral (2007) that moderate ethanol use is associated with lower production of mediators of inflammation and has anti-inflammatory effect. Ethanol exposure can result in functional alterations of macrophages and other cells of the innate immune system [23].

The stability study results shows that the formulations were stable both in shelf and refrigerator over the period of study. Change in the stability of a pharmaceutical product can lead to a drift in the physicochemical and pharmacokinetic properties of the product such as concentration, formation of new compounds, drug penetration, bioavailability etc and this may entirely change the action of the pharmaceutical product. In this study, the result of surface tension, pH and absorbance recorded revealed a negligible variation in of the stability of the formulations in both storage conditions and this implies that the product will maintain its basic activity and integrity over a long period of time. Consistency in the absorbance of the formulations in both storage condition shows consistency in the concentration of the formulation according to Beer-Lambert law.

5. Conclusion

The study shows that a calculated combination of herbal extracts which originally possess activities (example antibacterial and anti-inflammatory) will lead to synergy and improved activity better than that produced when the plants are used individually. It also revealed that herbal mouthwash can serve as a good compliment/substitution to standard marketed mouthwash in maintaining oral hygiene and prevention of oral diseases (dental caries and dental inflammation) caused as a result of infection by *S. mutans*.

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7. References

1. Sunny AO, Chiwendu UN, Oral health trends and service utilization at a rural outreach dental clinic, Udo, Southern Nigeria. J Int Soc Prev Community Dent. 2015; 5(2):S118-S122.
2. Arigbede AO, Umanah AU, Ukegheson GE, Braimoh OB, Omitola OG, Akadiri OA. Prevalence of dental caries and oral hygiene status of a screened population in Port Harcourt, Rivers State, Nigeria. J Int Soc Prev Community Dent. 2015; 5(1):59-63
3. Shifman A, Orenbuch S, Rosenberg M. Bad breath--a major disability according to the Talmud. Isr Med Assoc J. 2002; 4(10):843-5.
4. Kishore KA, Bindu Priya S, Sravani C, Amrutha Sai K. Poornodaya S. Ravindra NR. Comparitative Evaluation of Antibacterial Efficacy of Herbal Extracts and Mouth WashesAgainst Subgingival Plaque Bacteria. An In-vitro Study. J. Dent. Her, 2014; 1(1).
5. Su-Mi C, Gi-Ug K, Jeong-Dan C. Synergistic antimicrobial activity of apigenin against oral Pathogens, Int. *Int*. J. *Eng. Res. Gen*. *Sci*, 2016; 2(1).
6. Krzyściak W, Jurczak A, Kościelniak D, Bystrowska B, Skalniak A. The virulence of *Streptococcus mutans* and the ability to form biofilms. Eur J Clin Microbiol Infect Dis; 2014 33(4):499-515.
7. Geraint BR, Mary PC, Kenneth DB. Enhancing the utility of existing antibiotics by targeting bacterial behaviour? British Journal of Pharmacology. Br J Pharmacol 2012; 165(4):845-857.
8. Hooda A, Rathee M, Singh J. *Chewing sticks in the era of toothbrush:* A Review. Int J of Fam Practe. 2009; 9(2):113-121.
9. Ravi K, Divyashree P. *Psidium guajava*: A review on its potential as an adjunct in treating periodontal disease *Pharmacogn Rev*. 2014; 8(16):96-100.
10. Zubaidah HA, Sameer S, Wan Nordini Hasnor Wan Ismail, Wan Himratul-Aznita Wan Harun and Fathilah A R. The effect of selected plant extract in the development of single-species Dental Biofilm. J Coll Physicians Surg Pak. 2014; 24(11):796-80.
11. Rao PK, Rao DB, kiran CR, Nadh MR, Madhavi Y, Rao TR. In vitro antibacterial activity of Moringa oleifera against dental plaque bacteria. J Pharm Res. 2004; 4(3):695.
12. Yerima MB, Jodi SM, Oyinbo K, Maishanu HM, Farouq AA, Junaidu AU, *et al*. Effect of neem extracts (Azadirachta indica) on bacteria isolated from adult mouth. Nigerian J Basic App Sc. 2012; (20):64-67.
13. Amadi ES, Oveka CA, Onveagba RA, Ugbogu OC, Okoli I. Antimicrobial screening of Breynia nivosus and Ageratum conzynoides against dental caries organisms. J Bio Scis. 2007; 7(2):354-358.
14. Trease E, Evans WC. Pharmacognosy William Charlses Evans as edited in 2009 16th ed.
15. Russell AD, Furr JR. The antibacterial activity of a new chloroxylenol preparation containing ethylenediamino tetracetic acid. J App Bacteriology. 1977; (43):253.
16. Junping K, Yun N, Wang N, Liang L, Zhi-Hong H. Analgesic and anti-inflammatory activities of total extract and individual fractions of Chinese medicinal plants. Polyrhachis lamellidens, Biol Pharmaceutical Bull. 2005; (28):176-180.
17. Boon-Beng L, Pogaku R, Eng-Seng C. New drop weight analysis for surface tension determination of liquids. *Colloids and Surfaces A:* Physicochem. Eng. Aspects. 2009; (332):112-120.
18. Goyal D, Sharma S, Mahmood A. Inhibition of dextransucrase activity in *Streptococcus mutans* by plant phenolics. Indian J Biochem Biophys. 2013; 50(1):48-53.
19. Teleb-contini BH, Salvador MJ, watanabe E, Ito DX, Oliveira DCR. Antimicrobial activity of flavonoids and steroids isolated from two chromolaena species. Brazilian J pharm sci. 2003; 39(4).
20. Jyothi A, Dennis TT. Antibacterial activity of medicinal

plant Cyclea peltata (Lam) Hooks & Thoms, Asian Pac J Trop Med, 2012; S280-S284.
21. Prabu GR, Gnanamani A, Sadulla S. Guaijaverin. A plant flavonoid as potential antiplaque agent against Streptococcus mutans. J Appl Microbiol. 2006; 101(2):487-95.
22. Dalirsani Z, Aghazadeh M, Amirchaghmaghi M, Pakfetrat A. In vitro comparison of the antimicrobial activity of ten herbal extracts against streptococcus mutans with chlorhexidine. J Appl sci. 2011; 11(5):878-882.
23. Goral J. Ethanol and inflammation (Anti-Inflammatory and Anti-Allergy agents in medicinal chemistry). Bentham Sci Pub. 2007; 6(7):264-270.