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Stability and Antibacterial Property of Polyherbal Mouthwash Formulated Using Local Ingredients

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Abstract. The oral cavity is a home to more than 500 bacterial species. Although some of the oral bacteria are harmless, there are certain species that may cause oral plaques, bad breath, and mouth disease. Thus, maintaining a good oral hygiene is essential for a healthy mouth and body. The present study aimed to formulate a polyherbal mouthwash that may have antibacterial properties. Mouthwash formulations were prepared containing varying percentages of herbal extracts, with each formulation stored at 12 °C and 25 °C. Over the course of 12 weeks, the appearance and pH of the formulated mouthwash were measured. The mouthwash formulations maintained good homogeneity and color when stored at 25 °C, displaying lower pH level ranging between 3.71 and 4.85. Although the mouthwash stored at 12 °C maintained good homogeneity, a change in color in the formulation was evident and a more unstable pH readings were recorded. Antibacterial assay showed that mouthwash formulations stored at 25 °C had better inhibitory activity compared to those stored at 12 °C. Furthermore, mouthwash formulation containing (30% v/v) aleppo oak extract as the major ingredient conferred the greatest inhibition zone diameter (IZD = 10-18 mm) against salivary bacteria compared to formulations with (30% v/v) clove and (30% v/v) turmeric extracts as major ingredients. The best polyherbal mouthwash formulation in terms of inhibiting bacterial growth followed the 3:1:2 ratio for aleppo oak extract, clove extract, and turmeric, respectively. Therefore, the polyherbal mouthwash formulated in this study has the potential to be optimized and commercialized to antagonize growth of pathogenic oral bacteria.

Key words: Antibacterial; Polyherbal; Mouthwash; Aleppo Oak; Clove; Turmeric

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INTRODUCTION

The oral cavity is a reservoir of many microorganisms in the mouth. The mouth provides a wet environment and with food leftovers it becomes a hotspot for bacteria and fungi to grow and thrive. In fact, the oral cavity is a home to more than 500 bacterial species. Oral bacteria aggregate forming biofilms that adhere to the hard and soft surfaces of the oral cavity (Jiao et al., 2019). Although some of these oral bacteria are harmless, there are certain species that may cause oral plaques, bad breath, and mouth diseases (Kilian et al., 2016). Mouth bacteria have also been linked to cardiovascular diseases, osteoporosis, and diabetes. For instance, *Streptococcus mutans*, *Porphyromonas gingivalis* and *Lactobacillus acidophilus* are considered to be the main causes of dental caries and other mouth disease (Mahyari et al., 2016). Thus, protecting the teeth and mouth generally from microbes and maintaining oral hygiene are essential for a healthy mouth and body (Anderson et al., 2019). Brushing teeth two to three times a day is highly recommended as it cleans the teeth, kills unwanted microbes, and removes dental plaques and food leftovers (Hayasaki et al., 2014). Furthermore, flossing the teeth is also important to remove interdental plaque (Lin et al., 2020; Toyama et al., 2019), as well as the food that is stuck between the teeth, which cannot be removed by toothbrush (Alali et al., 2018). Using mouthwash is also highly recommended to kill bacteria in spots where the toothbrush cannot reach (Mitha et al., 2016).

A mouthwash is an aqueous solution for gargling which is often used for its refreshing and antiseptic properties and efficacy to control dental plaques (Amador-Medina et al., 2019). In fact, many pathologic oral cavity conditions require mouthwash as a type of medication (Javali et al., 2020). Despite its effectiveness, regular commercialized mouthwash tends to include alcoholic substances and other chemicals which dry up the oral cavity and eventually promote bacterial growth. Some of these chemicals may also cause unwanted allergic reactions and even increase the risk of oral cancer (Ustrell-Borràs et al., 2020). Other adverse effects may include staining,

alteration of taste, and mucosal desquamation (Tartaglia et al., 2019).

There has been an increasing evidence contraindicating the use of alcoholic mouthwash, like those containing cetylpyridinium chloride (CPC) (Chowdhury et al., 2013). There have been tremendous efforts towards replacing the commonly used mouthwash with safer products made of natural ingredients that can provide similar or even better oral protection but with minimal adverse effects. Several mouthwash companies are trying to reduce the amount of harmful chemicals in their mouthwash ingredients by replacing them with more natural components inspired by homemade recipes. The present study sought to formulate a polyherbal mouthwash that can be optimized and commercialized in controlling pathogenic oral bacteria. With its all-natural ingredients, a mouthwash formulation that is safer and user friendly can be used as an alternative for commercially available alcoholic mouthwash.

METHODS

Preparation of polyherbal mouthwash

Three different formulations of polyherbal mouthwash were developed (Table 1). The mouthwash formula made use of three main herbal ingredients: aleppo oak, clove, and turmeric. Three minor non-herbal ingredients were also included, such as the peppermint oil, sweetener, sodium benzoate, and salt. The minor components were used for preservation and for improving the taste. In order to test the antibacterial activity of the mouthwash herbs, different percentages of the herbal extracts were prepared. Meanwhile, the concentrations of the minor ingredients were fixed for all three mouthwashes. A similar amount of water was also added until the desired volume for the mouthwash was achieved.

For the formulation, the mouthwash herbal ingredients were ground to obtain their powder form. Ten grams of each aleppo oak, clove, and turmeric powder were separately soaked into 100 mL of distilled water and incubated at 37 °C for 48 hours (Mahyari et al., 2016; Paula et al., 2014). After incubation, the herbal extracts were filtered. The extracts were then boiled separately and left to cool. Ten grams of each solid minor ingredients (sweetener, salt, and sodium benzoate) were added separately into 100 mL of distilled water. After the ingredient extracts cool down, the major and minor ingredients were mixed following the formulation in Table 1. Peppermint oil, unlike other ingredients, was directly added to each of the formulation as it was in aqueous form. Finally, 30 mL of distilled water was added to constitute a 100 mL total volume of the mouthwash formulations.

Table 1. Three different formulations of polyherbal mouthwash

Ingredients	F1 (mL)	F2 (mL)	F3 (mL)
Aleppo oak extract (10g/100mL)	30	20	10
Clove extract (10g/100mL)	10	30	20
Turmeric extract (10g/100mL)	20	10	30
Water	30	30	30
Peppermint oil (100%)	0.8	0.8	0.8
Sweetener solution (10g/100mL)	3	3	3
Salt solution (10g/100mL)	6	6	6
Sodium benzoate solution(10g/100mL)	0.2	0.2	0.2
Total volume	100 mL	100 mL	100 mL

Stability test

Different mouthwash formulations were subjected to stability test prior to antibacterial testing. Stability test aims to ensure that the mouthwash formulations are usable and can maintain the same characteristics in the long term, before undergoing antibacterial assay. Physical stability test included recording the visual appearance, physical separation, and homogeneity of the formulated mouthwash. In addition, pH stability was also monitored using a well-calibrated pH meter. To investigate the changes and variability in the pH readings, the mean and the standard deviation for the pH readings were calculated. Different mouthwash formulations were then kept on the shelf (25 °C) and in the refrigerator (12 °C). The results were recorded and compared over the course of twelve weeks (Chiedozie et al., 2016).

Antibacterial assay

The antibacterial assay made use of saliva samples, rather than a readily available bacterial sample, in order to obtain a more realistic result that would simulate the effect of the mouthwash in a real-life scenario. Serial dilution was performed on the saliva sample to test the effect of the mouthwash on different concentrations of saliva. The serial dilution aimed

to form diluted samples with concentrations of 10^{-1},10^{-2} and 10^{-3}. The stock saliva sample (100 µL), as well as the three diluted samples, were separately spread on top of nutrient agar plates. In total, eight petri plates were prepared; each of the two plates were spread with a similar amount of the saliva sample.

The petri plates with saliva were incubated for 24 hours at 37 °C. Each petri plate was divided with a marker into three different sections. Each section was assigned for a different formulation of the mouthwash that was prepared previously. To apply the mouthwash onto the agar plates, a small filter paper with a diameter of 0.7 cm was soaked into the mouthwash and aseptically overlaid on top of the agar medium. To avoid contamination, the following procedures were performed under the laminar flow hood. Following treatment, the petri dishes were incubated for 24 hours at 37 °C. The inhibition zone diameter (IZD) was then measured the following day to estimate the bacterial inhibition capacity of the formulated mouthwash.

RESULTS AND DISCUSSIONS

Physical and color stability analysis

Three different mouthwash formulations were prepared. Each formulation was then split in half and incubated at two different temperatures: in the refrigerator at 12 °C and at room temperature at around 25 °C. Two different temperatures were chosen to determine the optimum storage conditions for the mouthwash formulation in which they were able to maintain their activity for the longest time possible. The same protocol was adapted by previous studies (Chiedozie et al., 2016; de Oliveira Marreiro et al., 2014). The visual appearance, phase separation, and homogeneity of each formulation were monitored by ocular examination for the course of twelve weeks.

Expectantly, the color of the mouthwash should be maintained throughout the experimental phase to ensure that the mouthwash formulations were acceptable. As shown (Figures 1 and 2), mouthwash formulations that were stored in the refrigerator (12 °C) rendered a light brown color throughout the experiment, whereas those kept at room temperature (25 °C) maintained a dark brown color.

As indicated in Table 2, the original color of the mouthwash was dark brown following preparation and prior to storage. The dark brown color is due to the influence of clove extract (Mahulette et al., 2020); the level of darkness of each formulation reflected the percent composition of the clove extract within the formulation. Interestingly, the formulations stored at 25 °C did not experience changes in color, unlike the formulations stored at 12 °C where there was a color shift from dark brown to light brown. The change in color might be attributed to the oxidation of the mouthwash ingredients. Although the herbs that were used in the mouthwash ingredients have natural antioxidants (Zhang et al., 2017), the low storage temperature might have disabled their antioxidant machinery leading to a change in coloration. Thus, lower temperature storage might affect the color stability of the mouthwash formulations. Phase separation in the mouthwash was not observed. Throughout the 12-week period, the mouthwash formulations remained well-merged and with similar consistency. The mouthwash formulations maintained the same homogenous state they originally had at the time of preparation (Table 3).

pH stability analysis

To observe any variation in the pH readings, pH stability was tested for the mouthwash formulations kept at 12 °C and 25 °C (Table 4). Using a calibrated pH meter, the pH values were measured in triplicates once a week for twelve weeks period. The mouthwash formulations stored at 25 °C showed a good pH stability. As shown in Figure 3, the mouthwash formulations had consistent pH values with no major shift in the pH curve and while maintaining a small standard deviation in the pH readings. In the contrary, the mouthwash formulations stored at 12 °C experienced significant drops and rises in the pH readings, thus the pH curve appeared erratic and unstable (Figure 4).

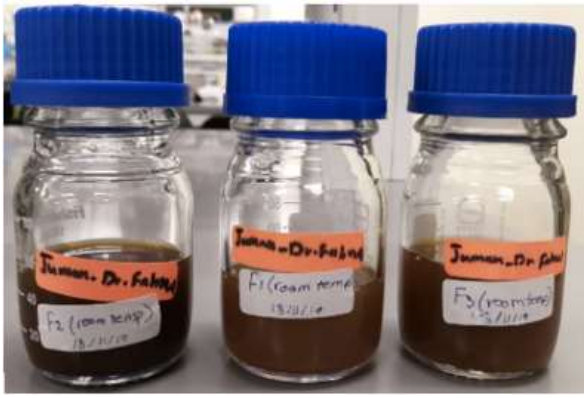

Figure 1. Mouthwash formulations stored at 25 °C

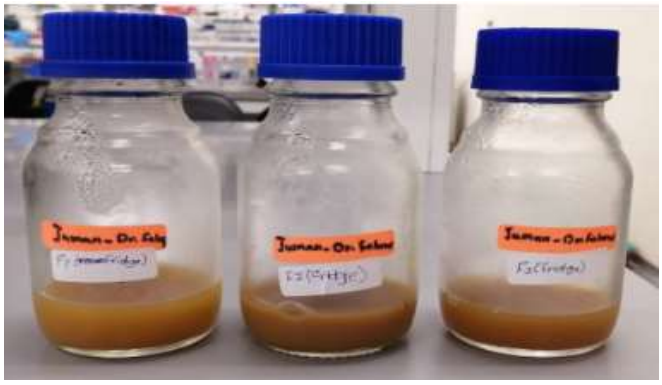

Figure 2. Mouthwash formulations stored at 12 °C

Table 2. The physical characteristics of different mouthwash formulations prior to incubation to different stoarge temperatures

Mouthwash Formulation	Evaluation Parameter	Observation
F1	Visual Appearance	Dark brown
	Phase Separation	Nil
	Homogeneity	Good
F2	Visual Appearance	Dark brown
	Phase Separation	Nil
	Homogeneity	Good
F3	Visual Appearance	Dark brown
	Phase Separation	Nil
	Homogeneity	Good

Table 3. The physical characteristics of different mouthwash formulations following exposure to different storage temperatures

Storage Temperature	Evaluation Parameter	Observation (Months)		
		1	2	3
25°C	Visual Appearance	Dark brown	Dark brown	Dark brown
	Phase Separation	Nil	Nil	Nil
	Homogeneity	Good	Good	Good
12°C	Visual Appearance	Light brown	Light brown	Light brown
	Phase Separation	Nil	Nil	Nil
	Homogeneity	Good	Good	Good

Table 4. The mean and standard deviation of the pH readings of the mouthwash formulations

Storage	Mouthwash Formulation	Mean ($\bar{X}$)	Standard Deviation (σ)
25 °C	F1	3.79	0.018
	F2	3.80	0.029
	F3	4.11	0.025
12°C	F1	4.24	0.063
	F2	4.23	0.040
	F3	4.41	0.036

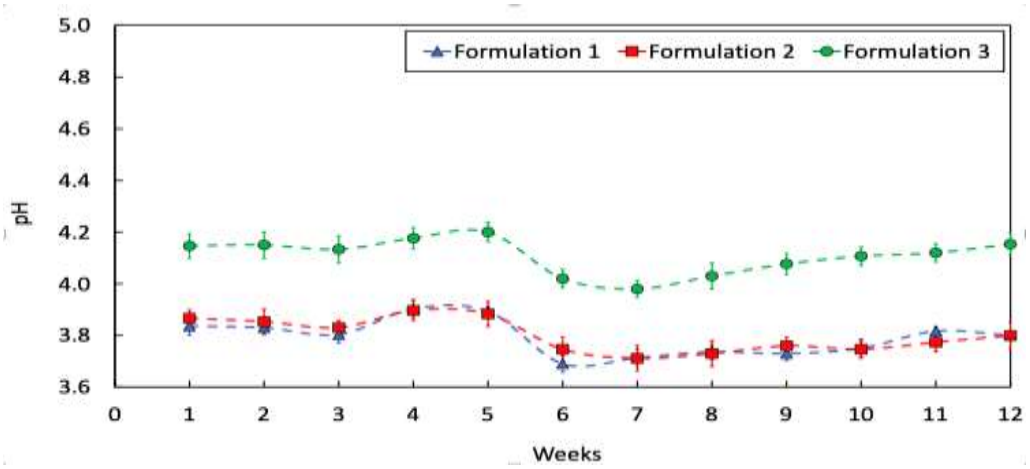

Figure 3. pH recorded over 12 weeks time for each mouthwash formulation stored at 25 °C. Readings were taken in triplicates ± standard deviation

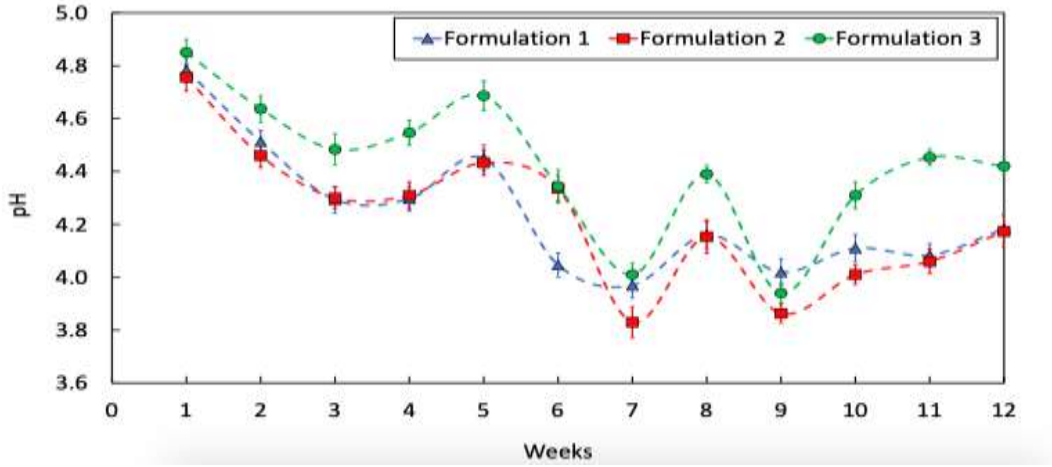

Figure 4. pH recorded over 12 weeks time for each mouthwash formulation stored at 12 °C. Reading were taken in triplicates ± standard deviation

Furthermore, the pH curves also showed that formulations stored at 25 °C generally had lower pH compared to those incubated at 12 °C. Temperature plays a significant role in pH measurements. Temperature affects the equilibrium of the reaction of water dissociating into hydrogen and hydroxide ions (Scerri, 2019). The dissociation reaction for water is represented in the following equation:

H_2O (l) ⇌ H^+ (aq) + OH^- (aq)

The dissociation of water is an endothermic process, and so the forward reaction tends to absorb heat. Increasing the water's temperature favors the forward reaction. As a result, more hydrogen and hydroxide ions are formed raising the value of Kw, the dissociation constant for water, and consequently lowering the pH (Bandura & Lvov, 2005).

The pH stability in the mouthwash might also be attributed to the minor preservative ingredients used, especially sodium benzoate. In a slightly acidic pH (around 4.5), sodium benzoate significantly reduces anaerobic fermentation of glucose and may inhibit the growth and survival of microorganisms within food products (Shahmohammadi et al., 2016). The active component of benzoate is an undissociated benzoic acid which is strongly lipophilic and can quickly enter the cell by interfering with the permeability of the cell membranes in many microorganisms (Liang et al., 2020). It is probable that sodium benzoate in this study does not only extend the shelf life of the mouthwash, but also contributes to its antibacterial effects. In the same manner, sodium chloride (NaCl) may also aid in the preservation and may provide antibacterial properties in the newly formulated mouthwash. NaCl is traditionally used as a gargling agent, especially when dealing with sore throat or pharyngitis attributed to viral or bacterial infections (Sallih & Bakar, 2019). Similarly, peppermint oil, a minor ingredient used in this study, may prevent food spoilage and provide antibacterial effects (Qu et al., 2020). Another ingredient that may contribute to the mouthwash stability is the sweetener. For this particular study, the sweetener stevia was used. Stevia has been proven to have no significant effect on plaque pH which makes it as an alternative sweetener in oral

preparations and confectionaries (Siraj et al., 2019). The addition of stevia extract may also improve the taste of the mouthwash.

Antibacterial analysis by disc diffusion method

Mouthwash formulations were applied to petri plates loaded with saliva in order to test their antibacterial efficacy. For mouthwash formulations stored at 25 °C, F1 with an IZD of 10 mm demonstrated better inhibitory potential as compared to F2 with IZD of only 8 mm. Meanwhile, F3 exhibited very poor inhibition. It was also noticeable that the antibacterial activity of the mouthwash formulations improved when using diluted saliva samples (Table 5). Similar assay was repeated for formulations kept at 12 °C. Unlike the formulations stored at 25 °C, those that were maintained at 12 °C rendered poor antibacterial properties (Table 6). All formulations showed limited inhibition when tested against undiluted saliva with F1 scoring the highest IZD of 6 mm and F3 scoring the lowest IZD of 2 mm. Unfortunately, no formulation inhibited bacterial growth found in diluted saliva samples.

Table 5. Inhibition zones diameter (IZD) for mouthwash formulations stored at 25 °C

Mouthwash	IZD in undiluted saliva	IDZ in	IDZ in	IDZ in
F1	10 ± 0.019	14 ± 0.023	16 ± 0.015	18 ± 0.019
F2	8 ± 0.023	10 ± 0.020	10 ± 0.012	12 ± 0.018
F3	4 ± 0.025	5 ± 0.042	6 ± 0.051	6 ± 0.037

*Mean ± standard deviation of triplicate sample

Table 6. Inhibition zone diameter (IZD) for mouthwash formulations stored at 12 °C

Mouthwash	IZD in undiluted saliva	IDZ in	IDZ in	IDZ in
F1	6 ± 0.016	0	0	0
F2	4 ± 0.024	0	0	0
F3	2 ± 0.022	0	0	0

*Mean ± standard deviation of triplicate sample

Basing from the IZD scores, F1 kept at room temperature (25 °C) had the best antibacterial activity, whereas F3 was the least effective. This may be due to the fact that F3 had higher pH levels compared to F1 and F2. Lower pH levels may inhibit bacterial growth which could explain the higher efficacy of F1 and F2 compared to F3. This may also be the reason why storage at 25 °C showed better inhibition results compared to storage at 12 °C. The lack of antibacterial activity of formulations stored at 12 °C may be due in part to their higher pH levels. In addition, a previous study had also noted that there are certain bioactive molecules within formulations that may be deactivated when exposed to lower temperatures (Shetty et al., 2016). Overall, mouthwash formulations stored at room temperature (25 °C) were more effective than those kept in the refrigerator (12 °C). Similar results were obtained by Shetty et al. (2016) in which the hot extract mouth rinse was more efficacious against oral pathogenic bacteria compared to the cold extract mouth rinse.

Some populations of the microbes in the tested petri plates seemed to be resistant to all the mouthwash formulations. Around 70% of the bacteria in the petri plates were inhibited by the formulated mouthwash, while the remaining 30% were resistant. Salivary microbes are generally part of the normal mouth flora that are harmless and non-pathogenic. As the saliva sample was obtained from individuals with good oral hygiene and no oral medical history, the saliva may contain benign oral microbiota (Ulloa et al., 2019). It was found that the oral microbiome contributes to the generation of nitric oxide (NO), an essential cardiovascular signaling molecule (Tribble et al., 2019). Such beneficial oral microbes are damaged by regular, chemical-based mouthwash formulations containing chlorhexidine (CHX) (Bescos et al., 2020). Hence, the results of the present study suggest that these mouthwash formulations do not affect the useful oral microbiome since they are formulated mainly of herbs.

A variety of natural herbs have been reported to have antimicrobial properties (Guerrero & Notarte, 2020). The F1 mouthwash, in particular, was noted to have superior antibacterial activity compared to F2 and F3. F1 is mainly composed of aleppo oak extract, as it formed 30% of the total mouthwash formulation. Aleppo oak (*Quercus infectoria*) was reported to have antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Candida albicans* (Tayel et al., 2013). Aleppo oak extract contains tannins and several flavonoids, such as naringin and rutin (Tayel et al., 2018). These components act on bacteria by

interfering with cell permeability and chelating essential metals which serve as co-factors in microbial metabolic pathways (Carvalho et al., 2018). In addition to aleppo oak, clove extract was another major component in F1, making up 10% of the total formulation volume. Clove (*Syzygium aromaticum*) is known for its antioxidant and antibacterial activities due to its high content of eugenol (Abdelkhalek et al., 2020). The broad-spectrum antibacterial properties of eugenol are attributed to their efficacy of reducing the hydrophobicity of bacteria, which is a key factor in cell attachment and formation of biofilms. Eugenol has the ability to breakdown the lipids in the bacterial cell membrane causing leakage of cellular components and eventually leading to cell lysis (Hadidi et al., 2020). The final main ingredient in the F1 mouthwash was the turmeric extract, which contributed to 20% of the total formulation volume. Turmeric (*Curcuma longa*) owes its unique color, flavor, and bioactivities to curcumin (Alsammarraie et al., 2018). Curcumin, in particular, is a good inhibitor of *Porphyromonas gingivalis* which is one of the key pathogens causing chronic periodontitis (Sha & Garib, 2019). Similar to tannins and eugenol, the antibacterial property of curcumin is attributed to its interference with the bacterial cell membrane permeability (Tyagi et al., 2015).

Generally, the main ingredients of the mouthwash formulations shared similar mechanism of action in inhibiting bacterial growth as all attack the cell membrane integrity and change the bacterial cell morphology which eventually lead to cell lysis. The antibacterial assay showed that F1 had the best inhibitory activity for both storage temperatures. F1 ingredients of aleppo oak extract, clove extract, and turmeric extract were made in the ratio of 3:1:2, respectively. This indicates that the herbal ingredient ratio of F1 was the most successful in inhibiting bacterial growth. Thus, increasing the concentration of the aleppo oak extract, being the main ingredient, may lead to better stability and antibacterial activity of F1.

Therefore, for the first time current study has achieved the synergistic effects of all ingredients (aleppo oak extract, clove extract and tumeric extract) inhibition against the selected bacterial species that is possible to be added in mouth wash formula. The advantage of this study is to give value added and benefits to consumers with all-natural contents which is human friendly if it is use as commercial product in the future.

CONCLUSION

The best polyherbal mouthwash formulation in terms of inhibiting bacterial growth followed the 3:1:2 ratio for aleppo oak extract, clove extract, and turmeric, respectively. With aleppo oak as the main ingredient, this suggests that aleppo oak is a better mouthwash component compared to clove and turmeric. Furthermore, the best mouthwash formulation was observed to be more stable when maintained at 25 °C. Given its stability and antibacterial properties, the polyherbal mouthwash formulated in this study has the potential to be optimized and commercialized for maintaining oral health.

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