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Antibacterial activity of natural-based toothpaste incorporated with nano-hydroxyapatite from fish bone against dental caries bacteria

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Abstract

Toothpaste formulation development has become important due to dental health problems. There has been a large array of improvements in the formulation including fluoride and microbeads (MBs) incorporation. However, the typical problems of the current toothpaste formulation are caused due to the fluoride and MBs toxicity issues on consumer health and aquatic animals. There is an urgent need to rectify this problem through the development of environmentally friendly hydroxyapatite (HAp) toothpaste using different natural components. This study was set out to determine the optimum gelling agent in toothpaste formulation and to investigate the effect of different combinations of the components in HAp-based toothpaste against *Streptococcus mutans*. The antibacterial activity of the developed toothpaste formulation was investigated using a well-diffusion method and the optimum concentration of the gelling agent (Arabic gum) was determined using a rheometer. One of the most significant findings in the study was the optimum concentration of the gelling agent was found at 55% and a combination of formulation with clove oil (CO) demonstrated the largest size of inhibition against *S. mutans* (CO = 24 mm, CO with formulation = 11 mm) compared to the other tested components. Findings from this study have suggested that the combination of formulation in HAp-based toothpaste with CO demonstrated the best bacterial inhibitory effect and the optimum gelling agent was at 55%. The overall finding is particularly important in the formulation development of HAp-based toothpaste, thus contributing to an increased supply of HAp in meeting its demand in the Halal market.

1. Introduction

There has been a growing recognition that more concern should be made to toothpaste formulation development as a result of dental health issues, particularly dental caries. Dental caries is a chronic infectious illness that affects tooth hard tissue, which has influenced toothpaste formulation (Takahashi and Nyvad, 2011; Cheng *et al*., 2017). The deposition of acidic metabolites such as glucose leads to a pH decline underneath the critical pH, at which tooth hard-tissue demineralization begins (Takahashi and Nyvad, 2011; Cheng *et al*., 2017). Recently, there has been a resurgence of interest in fluoride toothpaste, which has been discovered as an extremely effective caries preventative agent with numerous benefits (Marinho *et al*., 2013; Marinho *et al*., 2015; Marinho *et al*., 2016; Fontana, 2016; Bijle *et al*., 2018; Walsh *et al*., 2019). Preventive treatments for caries are mostly performed by decreasing bacterial acid production or by altering the mineralization imbalance (Buzalaf *et al*., 2011; Bijle *et al*., 2018).

Fluoride is widely accepted as a functional anti-caries agent in the dental industry (Hardwick *et al*., 2000; Loveren, 2001; Koo, 2008; Featherstone, 2009; Cheng *et al*., 2017). It is rapidly becoming a critical component of tooth treatment as an efficient preventative measure against dental caries. Caries prevention is mostly achieved by brushing with fluoride toothpaste, but the efficacy varies depending on the dosage (Walsh *et al*., 2019). Despite its safety and effectiveness, fluoride toothpaste has some significant disadvantages. Fluoride toothpaste's widespread use may result in inadvertent ingestion during toothbrushing, resulting in dental fluorosis (Zohoori *et al*., 2012). Fluoride usage

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has increased toxicity and the evolution of fluoride-resistant *Streptococcus mutans* and other oral bacterial species (Liao *et al*., 2017; Bijle *et al*., 2018). Excess fluoride administration can cause dental fluorosis, a persistent discoloration of the tooth enamel (Carwile *et al*., 2020). Intentional intake of a high amount of sodium fluoride has been claimed to result in severe bleeding and cardiac failure (Bridwell *et al*., 2019). In addition, synthetic microbeads (MBs) with diameters ranging from 5 mm to 1 mm have become extremely prevalent in toothpaste formulations (King *et al*., 2017; Nam and Park, 2020). MBs used in facial cleansers and toothpaste pose a huge public health issue and are hazardous to marine, agricultural, and freshwater habitats (Ding *et al*., 2020). Due to their non-biodegradability and durability in the marine ecosystem, these compounds are frequently settled in marine creatures (Nam and Park, 2020). MBs may be chronically hazardous to organisms due to their polymer constituents, characteristics, and tiny size (Sun *et al*., 2019; Ding *et al*., 2020). It tends to absorb endocrine-disrupting compounds (EDCs), medicines, and personal care products in aqueous conditions (Carr *et al*., 2016). Previous studies have proven the detrimental effects of MBs on several marine creatures, including the growth of medaka (Chisada *et al*., 2019), planktonic crustaceans (Gambardella *et al*., 2017), floating freshwater plants (Kalčíková *et al*., 2017), and chronic toxicity on freshwater zebra mussels (Magni *et al*., 2018). As a consequence, various substitute compounds, such as theobromine (Nakamoto *et al*., 2016; Premnath *et al*., 2019; Golfeshan *et al*., 2021), caffeine (Golfeshan *et al*., 2021), plant extract (Annisa *et al*., 2022) and hydroxyapatite (Sharma *et al*., 2015) are being identified as promising active ingredients in the protection of hypersensitivity and dental cavities. Preliminary research suggests that using hydroxyapatite (HAp) in toothpaste can minimize dental caries-causing dentine hypersensitivity (Sharma *et al*., 2015).

HAp is one of the most widely researched biomaterials in medicine and dentistry due to its biocompatibility (Balhuc *et al*., 2021). It has been employed as an additive material to strengthen currently utilized dental materials, particularly in preventative applications (Balhuc *et al*., 2021). HAp can enhance crystallite formation and proliferation, resulting in enamel remineralization (Oubenyahya, 2021). The use of HAp is justified since it would obliterate the open dentinal tubules and merge in with them, lessening the pain of dentine hypersensitivity (Vano *et al*., 2018). Over the last decade, substantial research has been undertaken on the integration of HAp into toothpaste formulations due to its potential beneficial effect (Esteves-Oliveira *et al*., 2016; Hiller *et al*., 2018; Vano *et al*., 2018; Amaechi *et al*., 2019; Bossù *et al*., 2019; Jumanca *et al*., 2019; Meyer and Enax, 2019; Hasan *et al*., 2020; Ionescu *et al*., 2020; Körner *et al*., 2020; Rifada *et al*., 2020; Sarembe *et al*., 2020; Steinert *et al*., 2020; Hasan *et al*., 2021a, 2021b; O'Hagan-Wong *et al*., 2021). Although the majority of research implies that toothpaste containing HAp has the potential to prevent dental caries, lesions, and microbial viability, this topic is still disputed.

There has been little consensus on the effectiveness of HAp as a fluoride replacement, signalling the need for additional research, particularly on the role of HAp against the leading cause of dental caries. It is well-known that the *S. mutans* species is the most prevalent strain related to teeth problems and diseases (Nachu *et al*., 2022). However, there seems to be a lack of prior research on the effect of HAp toothpaste on dental *S. mutans* bacteria. There is certainly a significant need to investigate the relevance of HAp-based toothpaste as a fluoride-free alternative for dental caries prevention. Other than that, the synthesis of HAp from fishery by-products can significantly contribute to better management of such waste. It is well-known that such by-products such as the skin, bone and internal organs contain high protein and biopolymers that can be utilized into high-value added products (Zhou *et al*., 2006; Karim and Bhat, 2009; Mohtar *et al*., 2010; Boutinguiza *et al*., 2012; Fan *et al*., 2017; Chew *et al*., 2020; Hasan *et al*., 2020; Hasan *et al*., 2021a, 2021b; Alves *et al*., 2022; Dermawan *et al*., 2022; Derkach *et al*., 2022; Nie *et al*., 2022; Sockalingam *et al*., 2022). Therefore, the primary aim of this study was to evaluate the optimal gelling agent for toothpaste formulation and to assess the antimicrobial activity of various component combinations in HAp-based toothpaste against *S. mutans*. This study is an ideal opportunity to explore the combined effect of HAp and a few selected essential oils in toothpaste formulations against the aforementioned bacteria associated with dental caries.

2. Materials and methods

2.1 Preparation of raw materials and extraction of hydroxyapatite

Fish bones were collected from the local fish processing industries and were processed according to Hasan *et al*. (2020). They were then washed thoroughly and boiled to remove excess meat. They were then dried in an oven for 24 hrs. The dried bones were then heated in a furnace (Carbolite ELF 11/14B/301, UK) at temperatures ranging from 600 to 1000°C, with a heating rate of 10°C/min for 5 hrs and it was cooled isothermally for 3 hrs. The calcined bones were milled using the ball-mill (Retsch PM 100, Germany). The extraction was carried out based on the method of Boutinguiza *et al*.

(2012).

2.2 Preparation of toothpaste formulation

The preparation of toothpaste formulations was formulated based on the method of Ogboji *et al*. (2017). The abrasive agent and binder were weighed using fine balance before the binder was premixed with water at room temperature with continuous mixing until the jelly structure gradually formed. The gel was mixed with an abrasive agent with continuous stirring. The formed gel was mixed with oil components such as peppermint, clove, and neem oils at 40°C with continuous stirring to form an emulgel. This step is crucial step to ensure a homogeneous emulgel. The emulgel was next mixed with glycerine and olive oil and the homogeneous paste was deaerated and stored at 4°C for 24 hrs. The ingredients used for the formulation of the toothpaste are outlined in Table 1.

Table 1. List of ingredients used in the formulation of toothpaste in this study and their respective functions.

Components	Function	Percentage (%)
Arabic gum	Binder/emulsifier	55.0
HAp	Abrasive/active	3.0
Glycerine	Humectant	3.0
Olive oil	surfactant	1.5
Peppermint oil	Flavouring	1.5
Clove/Neem oils	Antibacterial	5.0
Water	Medium	31.0

2.3 Rheological analysis

The rheometer was used to measure the rheological properties of toothpaste formulations. All toothpaste formulations were kept at 25°C before they were analyzed using a rheometer (Hybrid Discovery HR-2, USA) with spindle no. 34 at speeds ranging from 10 to 100 rpm. The device was initially analyzed using a blank sample before the analysis as a control. A small amount of toothpaste sample was mounted on a sample disc and pressed with constant pressure. Experimental data was automatically recorded via a computer connected to a rheometer through Rheocalc software. Each experiment was carried out three times.

2.4 Evaluation of antibacterial

2.4.1 Preparation of medium culture

The nutrient agar (NA) and tryptone soy broth (TSB) were used for the subculturing medium of bacteria in this study. The agar and broth mediums were prepared by dissolving 28 g of NA powder and 30 g of TSB in 1 L of distilled water. Both of the mediums were then proceeded to the sterilization process using an autoclave at 121°C for 45 mins. The sterile NA solution was poured directly onto Petri dishes with an average of 25 mm thickness and a centrifuge tube for TSB. The poured agar solution was then left for 20 mins in the laminar flow for the solidification process. The Bunsen burner was used throughout the process to avoid any cross-contamination. The culture mediums were then kept at 4° C until further used.

2.4.2 Preparation of bacteria culture

The cross-streaking method was used in this study for the sub-culturing process of bacteria. The freeze-dried bacteria were purchased from a supplier and sub-cultured three times to obtain a pure culture. The process was carried out in a biological safety cabinet (BSC) and sprayed with 70% ethanol before the process. The inoculation loop was burned and a few colonies of single species of bacteria were inoculated and streaked onto the NA. The cultures were cultivated at 37°C for 40 to 48 hrs. The pure culture bacteria were then stored at -80°C in glycerol stock with a ratio of 1:1 until further use.

2.4.3 Antibacterial assay

2.4.3.1 Preparation of turbidity standard

A few colonies of bacteria on the surface of nutrient agar (NA) were inoculated and suspended into sterile and tryptone soy broth (TSB) and mixed well using a vortex for homogeneous suspension. The bacterial suspension was measured optically using an Ultraviolet-Visible Spectrophotometer (UV-Vis) at 550 nm wavelength. It was then diluted several times until reached the reading of 0.125 of optical density (OD) which is equivalent to the 0.5 McFarland turbidity standards.

2.4.3.2 Well diffusion

A toothpaste solution was prepared by mixing 3 g of toothpaste in 3 mL of distilled water (1:1 dilution). An amount of 0.2 mL of the mixture was incorporated into a nutrient agar (NA) plate using a micropipette and left for 1 hr. A sterile cork borer with a 6 mm diameter was used to form wells before the sample solutions were introduced on the surface of the agar. Gentamycin was used as the positive control while the formulation without oil was for the negative control. All of the plates were cultivated for 40 to 48 hrs at 37°C. The inhibition zone was then determined after the cultivation period.

2.5 Statistical analysis

One-way Analysis of Variance (ANOVA) and multiple comparisons (Post hoc test) were applied for all analyses and experiments in this study. A significant level was applied at $\alpha = 0.05$. The analyses were conducted using SPSS for Windows (Version 23.0).

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3. Results and discussion

3.1 Rheological properties of the toothpaste formulation

The rheological property of the toothpaste formulation is important as it may influence the character of the formulation system. Determination of the rheological behaviour of three toothpaste formulations with various concentrations of Arabic gum (AG) was carried out using the rheometer. By using spindle no. 34 at a temperature of 25°C and rotational speed ranging from 10 to 100 rpm, the viscosity profiles of the three formulations of toothpaste samples were determined. Based on the results obtained, the results revealed that the difference in the viscosity profile of toothpaste samples was influenced by the combination of all ingredients and the concentration of the gelling agent. The viscosity analysis was conducted to determine the optimum concentration of gelling agent that was compatible to be applied in toothpaste formulation as a comparable concentration with standard toothpaste. Figure 1 shows a comparison of the viscosity of toothpaste formulation samples compared with standard toothpaste (ST) for clove and neem formulations.

The analysis of data shows that all of the toothpaste samples disobeyed the Newtonian interaction between viscosity, shear rate, and shear stress. This phenomenon can be considered as non-Newtonian fluids due to the decreasing value of viscosity over shear rate. The result demonstrated that C30 and N30 showed the lowest viscosity value compared to the others. This phenomenon could be due to poor network structure (Liu *et al.*, 2015). Meanwhile, C60 and N60 demonstrated the highest viscosity value and can be considered the most viscous and stable formulations. However, C55 has a viscosity value which was comparable to ST. Different concentrations and different components in toothpaste formulations could be significant contributors to the changes in the rheological behaviour of those formulations (Cai *et al*., 2017). According to Liu *et al*. (2015), different types of commercial toothpaste have different viscosity values; Darlie (3.4 kPa/s), Colgate (4.2 kPa/s), and Ynby (4.8 kPa/s). They added a point that the viscosity values of the three toothpaste formulation samples were significantly decreased as the shear rate increased which indicated that all samples follow shear-thinning behaviour. According to the Newtonian rule, a liquid can be considered Newtonian if its viscosity varies linearly with the shear rate (Ahuja and Potanin, 2018). This indicated that the viscosity of all toothpaste samples in the present study followed a non-Newtonian behaviour which had pseudoplastic properties, aligned with the theoretical viscosity behaviour of toothpaste and other such gelling agents (Carboxy-methyl cellulose and xanthan gum) (Barros *et al.,* 2022). This situation could be due to the aggregation of molecules in the sample which started to be destroyed and deformed when the molecules moved on the spindle surface. Based on the rheological properties, the optimum concentration of the AG was found to be 55% and it was used in the formulation for further analysis.

3.2 Antibacterial properties of the toothpaste formulation

The antibacterial test was carried out to discover the bacterial inhibitory properties of the toothpaste formulation against gram-positive *S. mutans*. This species of bacteria was chosen due to the species' domination on teeth and it is usually related to mouth diseases such as gingivitis and periodontitis. The test was

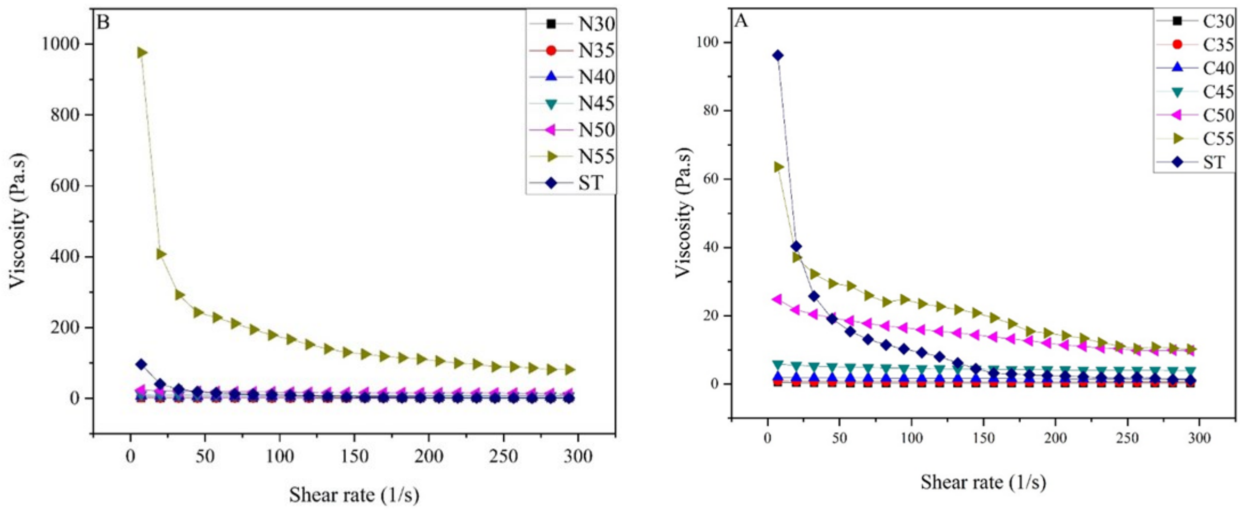

Figure 1. Comparison of the viscosity of toothpaste formulation with the addition of (A) clove and (B) neem and comparison with ST.
C30, C35, C40, C45, C50, and C55 represent toothpaste formulation with the addition of clove oil 30%, 35%, 40%, 45%, 50% and 55%, respectively.
N30, N35, N40, N45, N50, N55 represent toothpaste formulation with the addition of neem oil 30%, 35%, 40%, 45%, 50% and 55%, respectively.

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carried out on hydroxyapatite (HAp), clove oil (CO), neem oil (NO), HAp + formulation (HF), clove oil + formulation (CF), and neem oil + formulation (NF). The size of the inhibition zones is presented in Table 2.

The most interesting aspect of the result was that the CO formulation had the largest size of inhibition (21 mm) compared to the other tested components followed by the CF formulation (11 mm). In Table 2, there is a clear trend of the size of inhibition which decreased with the combination of the formulation. Further statistical tests revealed the significant difference in the inhibition zone demonstrated by the CO. However, no significant difference was observed in the size of inhibition for other samples. The most surprising aspect of the data is that the size of inhibition decreased with the combination of the formulation. The combination of different ingredients of toothpaste formulation resulted in the interaction of the components occurring, hence reducing the antibacterial effect.

Table 2. Size of inhibition zone of the samples against *S. mutans* species.

Samples	Size of inhibition (mm)
Clove oil	21 ± 3.06^a
Neem oil	11 ± 3.61^b
HAp	10 ± 1.53^b
Clove with formulation	11 ± 2.65^b
Neem with formulation	7 ± 2.52^b
HAp with formulation	9 ± 2.00^b
Gentamycin	28 ± 1.53^c

Values are presented as mean±SD of triplicates. Values with different superscripts are statistically significantly different ($p<0.05$).

Dental caries are the specific damage of susceptible dental hard tissues induced by acidic by-products of bacterial fermentation of dietary carbohydrates. These plaques can attract the presence of anaerobic bacteria such as *S. mutans* to surround the plaque area. These bacteria could carry anaerobic respiration by metabolizing the sugar from the plaque. Anaerobic respiration is necessary for their survival through the production of cellular energy in the form of adenosine triphosphate (ATP). However, the anaerobic glycolysis process of the bacteria could cause the release of lactic acid as a by-product. The demineralization of enamel, dentine, or cementum happens when the pH drops below a threshold range. Therefore, the present study was designed to determine the effect of a different combination of the components in HAp-based toothpaste against *S. mutans.*

The current study found that the inhibition size of the tested toothpaste formulation in this study ranged from 7 mm to 21 mm, with CO exhibiting the highest size of inhibition zone against *S. mutans*. This also accords with earlier findings from Yumas *et al.* (2022), which showed that the inhibition size against *S. mutans* of the tested toothpaste formulation containing different concentrations of unfermented cocoa powder ranged from 18 mm to 21 mm. One unanticipated finding was that the combination of the different components has resulted in the reduction of the antibacterial effect of the samples. This finding is consistent with that of Sunitha *et al.* (2015) who found that toothpaste containing different ingredient-based (neem = 16.66 mm, Vicco Vajradanti = 15.6 mm, Himalaya Herbal = 16.66 mm Colgate Herbal = 18 mm, Dabur Red = 17.33 mm, and Dabur Babool = 19 mm) demonstrates the different size of inhibition against *S. mutans*. This finding broadly supports the work of other studies in this area which suggested that different combinations of toothpaste ingredients had different antibacterial effects. A possible explanation for these results may be due to the interaction of the components, thus minimizing the antibacterial effect of the oil. The antibacterial effect of the CO was mainly contributed by the primary active compounds which consisted of eugenol and eugenol acetate. Cloves' chemical composition has been found to exhibit 81 to 84% eugenol, 3 to 4% ß-caryophyllene, and 81 to 86% eugenol (Sohilait, 2015).

The underlying mechanism is believed to be the penetration of the active compound into the cytoplasmic membrane which then inhibits the normal synthesis of deoxyribonucleic acid (DNA) and proteins that are required for bacterial growth. Essential oils (EO) can infiltrate bacterium cells, altering the regular functions of cells due to volatility and water solubility (Nair *et al.,* 2022). It destroys cell walls and membranes, next causing the loss of vital intracellular materials, which finally results in the apoptosis of bacteria cells (Xu *et al*., 2016). However, the combination of the components has ceased and reduced the penetration of the active compound to the bacteria cell. This result is suggestive of a link between the combination of different components in toothpaste formulation and the inhibition effect on bacteria which provides an insight into the toothpaste product research and development. However, the finding in this study is somehow limited to revealing the inhibitory effect of different concentrations of samples which indicates future studies on this matter are therefore recommended.

4. Conclusion

This study revealed that a combination of formulation with CO had the largest size of inhibition against *S. mutans* compared to the other tested

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components. This finding suggests that the combination of formulation with CO in HAp toothpaste demonstrated the best bacterial inhibitory effect. This work contributes to the existing knowledge of toothpaste formulation development particularly HAp-based toothpaste products by providing a different angle of knowledge in which, a synergistic effect of HAp-based toothpaste with different EO had shown improved antibacterial properties. Overall, this finding implies that both single component and combination effects should be taken into account when developing a toothpaste formulation due to the different degrees of effectiveness.

Conflicts of interest

The authors have no financial or commercial conflicts of interest in this paper.

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