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ORIGINAL ARTICLE

Inhibitory Effects of Fermented Soybean Tempeh on the Anti-Adhesive Properties of *Actinomyces viscosus* and Plaque Growth *in vitro*

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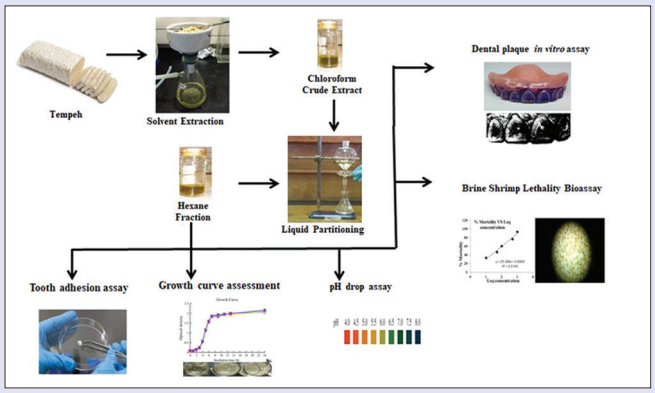
ABSTRACT

Background: Tempeh, the Southeast Asian traditional food, has garnered great attention for its antibacterial property against Gram-positive and Gram-negative pathogens, and antidiarrheal effect. We have previously reported the potentiality of tempeh hexane fraction (HXF) in ceasing *Actinomyces viscosus* biofilm formation *in vitro*. **Objective:** Here, we investigated the efficacy of tempeh HXF on other cariogenic virulence traits of *A. viscosus* such as adhesive properties, acid production, and plaque growth. **Materials and Methods:** Anti adhesion of HXF was assessed based on its effects on the number of cells adhering to the surface of tooth in sucrose-dependent (SD) and sucrose-independent (SI) medium. The potential of HXF to inhibit the capability of *A. viscosus* to generate acids was investigated by pH drop assay. The HXF at different concentrations were used to determine the LC_{50} based on brine shrimp lethality assay. Finally, the prospect of HXF as an inhibitor of plaque formation was investigated using artificial saliva-coated denture as an *in vitro* batch model. **Results:** HXF significantly decreased colony-forming unit of SD (1.07 log reduction) and SI (0.56 log reduction)-mediated adsorption of bacterial cells onto the tooth surface over 4- and 12-h incubation, respectively. Acid production was reduced after treated with HXF in a dose-dependent manner. Finally, a substantial reduction in plaque coverage area >55% was found on the HXF treated-denture. **Conclusion:** The anti-biofilm effect of HXF was associated with the suppression of *A. viscosus* adhesion to tooth surfaces and reduction in acid production. Furthermore, *in vitro* anti-plaque potential of HXF was demonstrated.

Key words: *Actinomyces viscosus*, anti-adhesion, anti-plaque, fermented food, periodontal diseases, tempeh

SUMMARY

- Tempeh's active fraction distrupted *Actinomyces viscosus* biofilm formation by inhibiting the adhesive ability and acid production without vanquishing its population
- Cytotoxic activity was observed for the active fraction.



Abbreviations used: CFUs: Colony-forming units; HXF: Hexane fraction; LC_{50}: Median lethal concentration; SD: Sucrose-dependent; SI: Sucrose-independent; OD: Optical density.

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INTRODUCTION

Formation of dental plaque biofilm is a biological process associated with the attachment, detachment, and proliferation of oral bacteria on the tooth surface. The dental biofilm is formedthrough adhesion of bacteria to a pellicle coating on the oral surface.[1] One of the significant bacterial adhesion mechanisms associated with dental biofilm formation is polysaccharide-mediated adhesion.[2] *Actinomyces viscosus* is regarded as early colonizers of tooth surfaces. *A. viscosus* produce fructosyltransferase (FTF), which synthesizes extracellular fructans through sucrose metabolism. These fructans function as short-term storage reservoirs in the plaque[3] and may also play a role in bacterial adhesion to the tooth surface. Studies on dental plaque have demonstrated the roles of sugars in the pathogenicity of dental biofilm. *A. viscosus* have a higher affinity toward sugars, as it was found that treatment with fructanase before the adhesion of *A. viscosus* resulted in a significant reduction in adhesion of this organism.[4] *A. viscosus* can also adhere to teeth through interactions with salivary proteins.[5]

Tempeh is fermented soybean with edible mold, *Rhizopus oligosporus*, which originated from Indonesia and is now, widely produced by cottage industry in Malaysia. Tempeh exhibits many bioactivities, such as antibacterial,[6-9] antidiarrheal,[8] antihyperlipidemic,[10] and anti-oxidant activities.[11-15] Nevertheless, the anti-cariogenic activity of tempeh against *A. viscosus* has not been studied in details. Recognition of the anti-adhesive role of tempeh against an enterotoxigenic *Escherichia*

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coli (ETEC) to intestinal epithelium cells of pigs[8] and human origin[16] has stimulated our interest on tempeh's potentiality as a protectant against dental biofilm.

The rationale of the current study is based on our previous findings that reveal the potentiality of tempeh hexane fraction (HXF) as a biofilm inhibitor to *A. viscosus*.[17] The aims of this study were three aspects as follows: (1) to investigate the anti-cariogenic effects of tempeh against *A. viscosus* surface adhesion activity and acid production; (2) to evaluate the cytotoxicity of tempeh by using brine shrimp lethality assay; and (3) to determine the anti-plaque effect of tempeh by using an acrylic denture model.

MATERIALS AND METHODS

Tempeh extraction and fractionation

The commercial tempeh was purchased from a local cottage industry producer in Malaysia. The tempeh blocks were lyophilized and ground to a fine powder. The sample was sequentially extracted with n-hexane, dichloromethane, and chloroform as previously reported.[17] The organic extracts were concentrated by using a Rotary Vacuum Evaporator (Büchi, Switzerland) at 40°C followed by oven-drying to constant weight. Water extract was lyophilized.

Chloroform extract suspended in water was solvent-partitioned with an equal volume of hexane using a separatory funnel. The organic fraction was concentrated *in vacuo* with rotary evaporation and oven-dried.

HXF was solubilized in dimethyl sulfoxide (DMSO) (3%) before use. The preliminary study indicated that 3% DMSO is innocuous both to bacterial growth and biofilm formation.[17]

Bacterial strain and culture conditions

The bacterial strain used in this study was *A. viscosus* from the American Type Culture Collection 43146. This strain was cultured in brain heart infusion broth (BHI; Merck, Germany) under an anaerobic condition in a pack-rectangular anaerobic jar (Mitsubishi Gas Chemical Co. Inc., Japan) at 37°C. An overnight culture was inoculated into fresh BHI to allow the bacteria to reach the exponential growth phase with a turbidity of 0.5 McFarland.

Assay of bacterial adherence on the acrylic tooth

The effect of HXF against bacterial adherence on the acrylic tooth was determined by counting the number of colony-forming units (CFUs) based on method modified from Barbieri *et al.*[18] Sterile tooth was introduced into a test tube containing 0.9 mL of the BHI medium with or without 5% (w/v) sucrose and autoclaved at 121°C for 15 min. Standardized cell suspension of 2.1×10^9 cells/mL inoculum of 0.1 mL with and without active fraction at sub-inhibitory concentration were then added to the tube. Assay was carried out at 37°C for different incubation period (4 and 12 h), with three repetition for each analyzed period. After each preexposure period, the tooth samples were rinsed thrice with distilled water to remove weakly adhered cells. Following sonication for 10 s and vortex, the dislodged *A. viscosus* was used to create three decimal dilutions in phosphate buffer saline (10^{-1}, 10^{-2}, and 10^{-3}), which were then plated on Muller-Hinton (MH) agar plate. Following incubation at 37°C for 24–48 h, the CFUs formed was enumerated.

Growth curve assessment

The dynamics of *A. viscosus* growth in the presence of HXF was monitored over 24 h. Planktonic growth curves were obtained by placing 100 µL of HXF (3, 1.5, and 0.75 mg/mL) in BHI into a 96-well microtiter plate. The wells were inoculated with 100 µL of an overnight *A. viscosus* culture in triplicate. An equivalent amount of DMSO, which used to dissolve extract, was employed as vehicle control and medium-containing extract without inoculum was employed as a blank. Plates were incubated at 37°C and the optical density (OD) at 600 nm was recorded at every 1-h interval up to 24 h. The viable cell count was obtained by spreading 100 µL of culture on MH agar plates after desired dilutions. The plates were incubated for 48 h at 37°C and cells were counted as CFUs.

pH drop assay

Effect of HXF on acid production by *A. viscosus* was measured, as described elsewhere.[19] Five ml of BHI broth containing 5% (w/v) of sucrose and HXF (ranging from 1 to 3 mg/mL) were inoculated with 100 µL of overnight cultures of *A. viscosus* to obtain a final inoculum of 1.5×10^4 CFU/mL and incubated at 37°C for 24 h. The pH of the bacterial culture was assessed at the onset and after incubation. All determinations were performed in triplicates using growth controls.

Brine shrimp toxicity bioassay

The assay was carried out according to the principle and protocol previously described by Meyer *et al.*[20] with slight modifications. Eggs of brine shrimp (*Artemia salina*) were purchased from local aquatic shop and mixed with artificial seawater (prepared by dissolving 38 g sea salt per liter of water). The eggs were allowed to hatch at room temperature for 48–72 h. Phototropic nauplii were collected from the lighted side with Pasteur pipette. To determine if the HXF was cytotoxic, 1–5 mg/mL were tested, in triplicates. Ten brine shrimp larvae were then added to each vial containing the aforementioned fraction. After incubating for 24 h at room temperature, the number of survivor of nauplii was counted.

Lethality was calculated from the mean survival of larvae in treated tubes and that of control. Mean percentage of mortality was plotted against the logarithm of concentrations. The concentration killing 50% of the larvae (LC_{50}) was calculated from the linear equation by taking the antilogarithm. Potassium dichromate was used as positive control.

In vitro primary phase of dental plaque model

This experiment was conducted to test the inhibitory effect of HXF on plaque formation. The sterile denture was preconditioned with artificial saliva to enable formation of the acquired pellicle which was prepared by a method described by Macknight-Hane and Whitford.[21] Meanwhile, bacterial cells were harvested by centrifugation (5000 rpm, 5°C, 10 min), washed twice with distilled water and re-suspended in artificial saliva. The OD was adjusted to 0.46 at 600 nm which corresponds to a microbial concentration of 3.65×10^8 cells/mL.[22] The denture was exposed to bacterial culture with and without HXF (3 mg/mL) for 12 h at 37°C followed by washing in sterile distilled water. The inoculated denture incubated in artificial saliva for extended period of 120 h.[23]

A digital photograph of the denture was taken after the accumulated plaque on the anterior aspect of the upper teeth was disclosed by applying 1% (w/v) methylene blue solution for approximately 10 s followed by a thorough rinse with sterile distilled water to remove excess disclosing agent.[24] The images of the disclosed plaque were taken with a DSLR camera (Canon EOS 70D, Canon Inc., Ōta, Tokyo, Japan) at a fixed focal length, and hence, the distance to the object was maintained constant (17 cm). All image-processing procedures were written by using the ImageJ software package (National Institutes of Health, Montgomery, MD). Using the "Freehand tool" on the setting, the irregular shape of the front four teeth was outlined. Each image was converted to monochrome to calculate the area fraction of plaque regions.

Data analysis

The comparison between different experimental groups was determined using one-way ANOVA tailed by a *post hoc* Tukey's test. All the

experiments were performed in triplicate and the results were expressed as means ± standard error of replicates. Differences were accepted as statistically significant at $P < 0.05$ or less.

RESULTS

Contribution of sucrose to bacterial adherence to acrylic tooth

Results of the adhesion of *A. viscosus* to tooth surfaces in the presence and absence of 1% (w/v) sucrose are presented in Figure 1. In this study, an acrylic tooth was used as substratum in order to mimic the hard surface of the natural tooth. Adherence of the tested bacteria to tooth in the absence of sucrose was significantly lowered ($P < 0.05$) compared to their adherence with sucrose.

Effect on sucrose-independent and sucrose-dependent adherence to acrylic tooth

Anti-adherence activity of HXF was assessed based on its effects on the number of cells adhering to the tooth surface. An acrylic tooth was used as substratum to mimic the hard surface of the natural tooth. The results illustrated that the total number of cells adhering to the HXF-treated tooth samples were found to be much less [Figure 2] compared with their respective controls. HXF significantly showed inhibitory adhesive activity by 0.56 and 0.75 log reduction in the absence of sucrose, over 4- and 12-h of exposure. Adherence of *A. viscosus* after treated with (3 mg/mL) HXF in the presence of sucrose was inhibited by 1.07 and 1.3 log reduction when exposed for 4 and 12 h, respectively. Significantly, less bacterial cells were observed to be adhering to the tooth surface when 0.2% chlorhexidine was used instead of the extract ($P < 0.05$).

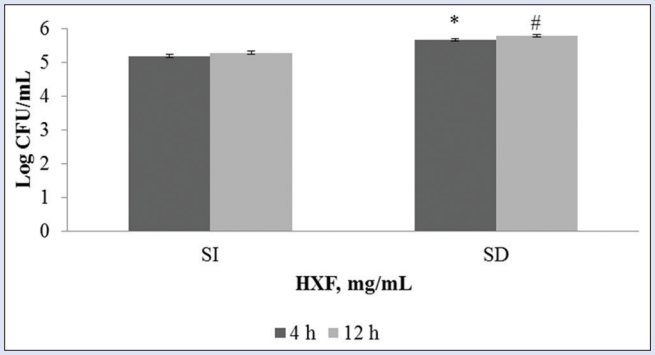

Figure 1: Influence of sucrose as a carbon source on *A. viscous* adherence assay in the absence (sucrose-independent) and presence of sucrose (sucrose-dependent). Asterisks (*) and hashes (#) indicate significant differences ($P < 0.05$) between sucrose-independent and sucrose-dependent. Data are expressed as mean ± standard error

Effect of hexane fraction on *Actinomyces viscosus* growth profile

Responses of *A. viscosus* when cultured in the BHI nutrient medium (growth control) with different concentrations of HXF are shown in Figure 3. No inhibitory activity could be attributed by the fraction, because no extension of the exponential phase was detected. Cultures containing the HXF attained slightly greater levels of growth than the control as measured by increased turbidity. This response is indicative of the presence of active substrates in the HXF which could be utilized by the cells. To confirm the activity, the cells were plated on MH agar plates after an overnight growth in the presence of the extract, with appropriate dilutions. The growth inhibitory activity was found to be non-bacteriostatic in nature.

Effect of hexane fraction on the acid production by *Actinomyces viscosus*

The ability of HXF to inhibit *A. viscosus* acid production in sucrose-rich liquid culture was investigated. In the control, the initial pH 7.11 was decreased to pH 4.77 after 24 h of incubation [Table 1]. HXF significantly change the pH of the culture from acidic level to weak acid in a concentration-dependent manner. Acid production by *A. viscosus* was

Table 1: Effect of hexane fraction on the acid production by *Actinomyces viscosus*

Treatment (mg/mL)	pH±SD	
	Onset	After 24 h
Growth control	7.11±0.14	4.77±0.12
HXF (1.0)	7.09±0.11	5.18±0.06*
HXF (2.0)	7.08±0.11	5.31±0.05*
HXF (3.0)	7.08±0.10	5.57±0.09*

*Significant differences ($P<0.05$) between 0 mg/mL (control) and each HXF treatment as determined by *t*-test. Data are expressed as mean±SE. SE: Standard error; SD: Sucrose-dependent; HXF: Hexane fraction

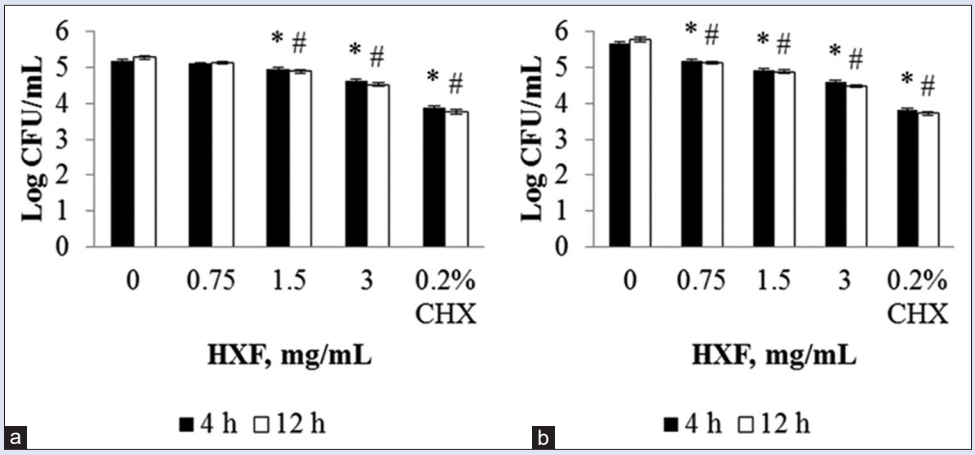

Figure 2: Inhibitory effect of hexane fraction in (a) sucrose-independent and (b) sucrose-dependent assays against *Actinomyces viscosus*. Asterisks (*) (for 4 h) and hashes (#) (for 12 h) indicate significant differences ($P < 0.05$) between 0 mg/mL hexane fraction (untreated control) and each treatment. Data are expressed as mean ± standard error

reduced after treated with HXF (3 mg/mL) as the pH values obtained was 5.57. However, the active components of this fraction did not show any apparent influence in pH value.

Toxicity screening

The HXF was tested in a brine shrimp lethality assay to determine their cytotoxic activities. The result is reported as LC_{50} values, the concentrations required to kill 50% of a group of brine shrimps [Table 2]. The LC_{50} value for the percentage of mortality brine shrimp treated with this fraction as indicated by the regression equation (R^2 = 0.9748) was found to be 6.3 mg/mL. The resulting in LC_{50} value of <1 mg/mL is considered as significantly active[20] which suggests that the HXF was non-toxic against brine shrimp compared to positive control. Meanwhile, the untreated group using DMSO and seawater showed no mortality.

Effects of hexane fraction on plaque development *in vitro*

The potential of HXF as an anti-plaque was investigated by using sterile denture coated with artificial saliva as an *in vitro* model. No plaque was developed when denture were exposed to cultures of *A. viscosus* culture for overnight (Data not shown). The immersion of the saliva conditioned denture in *A. viscosus* culture for 4 h at 37°C, allowed visible plaque formation only after 120 h [Figure 4a]. When compared to the growth controls, HXF (3 mg/mL) reduced the formation of primary plaque growth of *A. viscosus* on the acrylic denture by 54.21% [Figure 4b and Table 3] without causing any discoloration on the teeth.

Table 2: Brine shrimp cytotoxicity of the hexane fraction, LC_{50} (mg/mL)

Treatment	LC_{50} (mg/mL)
HXF	6.30±2.3
Potassium dichromate	0.29±0.2

HXF: Hexane fraction

Table 3: Plaque coverage area (%) of control and hexane fraction treated denture calculated using the image J software

Treatment	Mean plaque formation (%)±SE
Growth control	100
HXF (3 mg/mL)	45.79±4.34*

*Significant differences (P<0.05) between growth control and HXF treatment as determined by *t*-test. Data are expressed as mean±SE. SE: Standard error; HXF: Hexane fraction

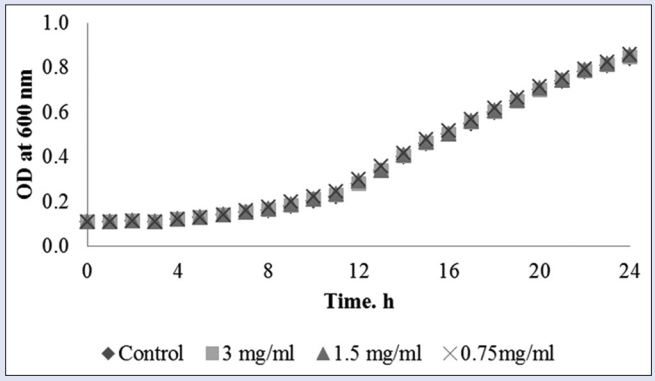

Figure 3: Growth response of *Actinomyces viscosus* when cultured for 24 h on brain heart infusion media-containing different concentrations of hexane fraction as compared with the growth control

DISCUSSION

The results discussed here further strengthen our previous reports on the anti-biofilm properties of HXF derived from tempeh Chloroform extract.[17] Sugar metabolism by the test consortium via enzyme FTF promotes adherence through the production of sticky fructans, whereas sucrose-independent (SI) attachment is mediated by nonspecific physicochemical forces, primarily relies on hydrogen bonding and hydrophobic interactions. In the absence of sucrose, *A. viscosus* can adhere through alternative adhesion mechanisms, but when sucrose is present, they may facilitate bacterial adhesion to form a dental biofilm.[4] The addition of sucrose in our study improved the adhesion of *A. viscosus* to the surface [Figure 1].

Tempeh showed higher anti-adherence activity against Gram-negative, an enterotoxic strain of *E. coli* to the intestinal epithelium cells of pigs[8] and humans.[16] In other study, soybean extracts in combination with milk fermentation reported to inhibit enterocyte adherence of Gram-positive bacteria, *Staphylococcus aureus* and *Listeria monocytogenes*. In the present study, treated *A. viscosus* culture containing HXF induced significant decreases in adherence under sucrose-dependent (SD) and SI conditions as compared to untreated control. This implies that the HXF can influence the SD adherence of the bacteria either by inhibit or reduce glucans synthesis. This inference is in accordance with an earlier report, wherein SD adherence depends on glucan production.[25]

The potential rationale of the reduction in SI adherence in the presence of HXF could be hypothesized due to the effect of oils from the soybean plant, which reduces the cell-surface hydrophobicity of the bacteria. Islam *et al.*[26] also stated that the presence of oils in plant extract could affect the hydrophobicity of bacteria. Recent studies on essential oils[27] and other oily substances such as guava and neem extracts[28] demonstrated that certain small hydrophobic compounds are known to penetrate easily to the cells generating pores in the outer membranes. The efficiency of HXF on *A. viscosus* in our study suggests the presence of such nonpolar molecules.

An ideal anti-biofilm agent is should not hinder the basic metabolic activity of the organism in order to avoid instigation of antibiotic resistance.[29] The growth curves [Figure 4] of the growth control and the HXF treated cells gave a typical sigmoidal pattern. There was inconspicuous in the exponential phase between these curves, which clearly indicates that growth of bacteria was not inhibited by the treatment. The ability of tempeh to disengage *A. viscosus* from an intact biofilm, without affecting their viability, may prove clinically advantageous since selective pressure and overgrowth emergence of resistant bacteria would be avoided. The previous study has showed that tempeh had no growth inhibitory effect on ETEC but exhibited strong anti-adherence activity against ETEC to intestinal epithelial cells which is caused by an interaction between *E. coli* and soybean compounds.[16]

The metabolic system of biofilm-forming bacteria generates an acidogenic niche, triggering progressive changes in the mineral layer of the dental surfaces.[30] The cariostatic effect can be attained by reducing

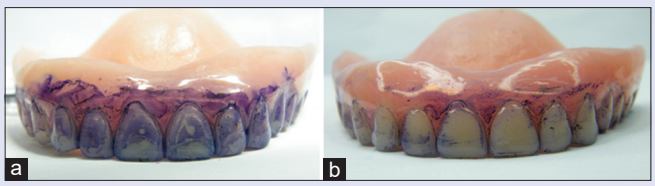

Figure 4: Plaque formation on acrylic tooth after disclosed by applying 1% methylene blue solution. (a) Growth control (b) hexane fraction application (3 mg/mL)

bacterial acidogenesis.[31] Therefore, the alteration of pH is used as an indicator to determine the effect of anti-cariogenic agents. Hence, the potential of HXF to inhibit the capability of *A. viscosus* to generate acids in the presence of sucrose as carbon source was investigated. The results obtained showed notable reduction in pH drop by growth control, suggesting the acid-forming ability of *A. viscosus*. The study further revealed that HXF was found to be associated with a gradual decrease in acid production in a dose-dependent manner.

Although tempeh is conventionally consumed as an edible food, it is still pertinent to confirm that HXF has minimal cytotoxicity, considering its potential application as an oral care ingredient. To this end, the brine shrimp lethality bioassay, a preliminary screening tool which has been widely used to assess the *in vitro* cytotoxicity of numerous natural products, was carried out.[32] Studies have demonstrated a positive correlation between the brine shrimp lethality and oral lethality test in mice in medicinal plant research.[33] The LC_{50} value of HXF was recorded 21-fold higher than the positive control, indicating it is non-toxic.

In the final part of this study, the potential of HXF as an inhibitor of plaque formation was investigated by using sterile denture coated with artificial saliva as an *in vitro* batch model. The selection of incubation duration for plaque growth by monospecies specific was a challenge. The formation of mature plaque *in vitro* was developed after 120 h. This finding agrees with earlier studies showed the development of a biofilm forming bacteria within 48 h, and after 120 h, the plaque covered nearly 80% of the bovine teeth using an artificial mouth.[23] The obtained results demonstrated that HXF at 3 mg/mL reduced plaque growth by >55%. Notably, the HXF application did not cause any discoloration on the teeth, unlike CHX. Thus, this may encourage its use for cosmetic reasons in addition to its high antiplaque activity.

CONCLUSION

This study demonstrated for the first time that, HXF derived from soybean tempeh can protect against the adhesion, acid production, and plaque growth of *A. viscosus* to the saliva-coated tooth surface *in vitro*. This effect is not a result of bactericidal activity, but of an interaction between the extracts and the bacteria, resulting in a loss of adhesion capability of the *A. viscosus* to the tooth surface. More intensive research on HXF or tempeh in general, is warranted in future to characterize its potency and safety *in vivo*, besides unraveling its chemical composition and modes of action in greater details.

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Conflicts of interest

There are no conflicts of interest.

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