

KASTURI TOBACCO LEAF FLAVONOIDS AS BIOACTIVE COMPOUNDS IN PERIODONTAL ANTIBACTERIAL MUCOADHESIVE GEL

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ABSTRACT

Objective: The tobacco leaves are a rich source of many biologically active substances. Flavonoids are one of the bioactive substances contained in tobacco leaves with a wide range of pharmacological properties. This study has been aimed to formulate the antibacterial periodontal gel containing the flavonoids fraction of Kasturi tobacco (*Nicotiana tabacum*) leaves.

Methods: Fraction flavonoids obtained from methanolic extracts of tobacco leaves, which have been purified from nicotine. Different concentrations of flavonoids fraction (0, 25, 0.5, 1, 2, and 4 mg/ml) were prepared with Carbopol-974P. The strength of flavonoid gel adhesion in *ex vivo* mucosa was tested by the falling liquid film method. Evaluation of *in vitro* antibacterial agent was followed by the disk diffusion method.

Results: The flavonoid gels have better adhesion parameters (time, strength, and distance) than gel bases. The antimicrobial by the zone of inhibition studies proved that flavonoids periodontal gels with concentrations of 4 mg/ml are highly active against *Porphyromonas gingivalis* with the zone of inhibition >10 mm which was higher than quercetin gel formulations (control groups).

Conclusion: Gel formulation containing the flavonoids fraction of Kasturi tobacco (*N. tabacum*) leaves could be a good candidate for periodontal gel with good mucoadhesive gel and antibacterial agent.

Keywords: Antibacterial periodontal gel, Flavonoid, Periodontitis, Tobacco leaves.

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INTRODUCTION

Gingivitis and periodontitis are the most common oral diseases which its initial stage is characterized by inflammation of the gingival tissue called gingivitis [1], further periodontitis is characterized by inflammation of the supporting tissues of the teeth, progressive damage to the periodontal ligament, and alveolar bone together with the formation of periodontal pockets and gingival recession. The immune response to the interaction of microorganisms and inflammatory cells in the tissues around the teeth contributes to the development of this disease. The enzymes and toxins released by periodontal pathogenic bacteria exaggerate the destruction of the periodontium [2]. Anaerobic Gram-negative bacteria involved in the development of periodontal disease are *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Actinobacillus actinomycetemcomitans* [3]. The bacteria colonization in the periodontal pocket constructs a firmly attached biofilm on the teeth and gingiva surface which the film helps bacteria fight antibiotics. Bacteria can be interconnected in biofilms, and this action helps gene transmission that results in resistance to antibiotics and the growth of resistant microorganism species [4].

In most cases, the important treatment modality is by controlling inflammation through dental plaque and calculus removal to reduce pathogenic microorganisms [5], but this procedure is not able to inhibit bacterial growth by systemic antibiotic therapy. As the etiologies of periodontal disease correlate to various types of bacterial infection, there is no ideal and effective antibiotic for all pathogens, and the improper different antibiotics uses can lead to medical side effects [6]. The short-term efficacy and inconsistency of antiseptic mouthwash and also the side effects of systemic antibiotics encourage the use of a therapeutic system locally. The use of minocycline gel and metronidazole gel in the

subgingival area is the development of drug delivery systems [7]. This system has several advantages including reducing prescription drugs, increasing drug concentration in the target tissue, reducing drug side effects, and lowering the dosage frequency [8]. Natural products such as flavonoids are capable to prevent the development of infections. Flavonoids are safe and cost-effective with neglectable side effects. Local drug delivery of natural products to the periodontal pocket is an additional therapy other than dental root planing and scaling for the treatment of periodontal disease [9].

With the increasing prevalence of antibiotic-resistant bacterial infections, the use of natural ingredients, such as flavonoids, is increasingly attracting potential utility as antibiotic replacement therapy or adjuvant therapy. Flavonoids have been known to possess antibacterial potency against various pathogenic microorganisms [10]. Therefore, the development of flavonoids as herbal mucoadhesive active compounds is very necessary and important to improve the success of periodontal disease treatment. The bioactive fraction used in this development of mucoadhesive gel is flavonoids from Kasturi tobacco leaf wastes in which our preliminary research on the crude extract showed antimicrobial activity. Nicotine constituent removal was the most important part in producing a satisfactory antimicrobial assay [11]. The preliminary research data encourage us to further carry out extraction and fractionation of nicotine-free flavonoids from the waste of Kasturi tobacco leaves.

Kasturi tobacco leaf waste is a tobacco leaf located in the lower stem, brownish-yellow in color, damaged or perforated leaf shape, and has a low selling value; hence, it is often discarded and destroyed by burning. Disposal of this waste will cause serious problems because

tobacco leaf waste contains highly toxic nicotine. Thus, efforts need to be made to use controlled tobacco leaf waste to avoid harmful effects on the environment and make Kasturi tobacco leaf waste as a source of flavonoids that can be used as a basis for biopharmaceutical development to treat periodontal disease.

METHODS

Fractionation of flavonoids from the waste of Kasturi tobacco leaf

Kasturi tobacco leaf waste was collected from tobacco plantations in Pakusari, Jember, Indonesia. Tobacco leaves are dried at room temperature for 2 days before further drying in an oven for 24 h at 40°C. To dried tobacco leaf powder (50 g), methanol (400 mL) was added and the mixture was then stirred with an orbital shaker at 150 rpm for 24 h. The solution was obtained through filtration which the supernatant was volume reduced (200 mL) through vacuum evaporation. To the methanol extract (200 mL), hexane (20 mL) was added before stirring with a magnetic stir for 10 min to form two layers. The top layer was removed, while the bottom layer is washed again with hexane (20 mL). This process is repeated 15 times. All the upper layers were evaporated with a rotary evaporator until a thick extract (20 mL). A portion of the thick extract (10 mL), hydrogen chloride (10%, 20 mL), distilled water (10 mL), and ethyl acetate (20 mL) was added and the mixture was stirred under a magnetic stir for 10 min to form two layers. The top ethyl acetate layer was separated and collected. This fractionation process was repeated 3 times. The top layer is a solution rich in flavonoids, while the lower layer is a solution rich in nicotine. The top layer that is

rich in flavonoids is taken and tested for the nicotine content with the Dragendorff test to make sure there are no nicotine traces in the layer. The result of fractionation of flavonoids (the top layer formed) is taken and evaporated. The alkaloid removal followed a standard procedure by Docheva et al. with a modification [12,13].

Flavonoid mucoadhesive gel formulation

Flavonoids with a series concentrations of 0.25, 0.5, 1, 2, and 4 mg/mL were dispersed in dimethyl sulfoxide 1% along with a gel base consisting of Carbopol-974P, triethanolamine, and aquades (2:5:100). This mixture is slowly stirred to homogenize and forms a thick gel mass. The gel is poured into a petri dish and left overnight for stabilization. Metronidazole (2%) and quercetin (50 µM) gels were used as the control.

Ex vivo flavonoid gel adhesion

The strength of the flavonoid gel adhesion was performed through the falling liquid film method. In this study, the rat gastric mucous layer was used as a model to determine the strength of gel adhesion. The gastric mucosa of rats was cleaned with 0.9% sodium chloride and was cut (1.5 cm × 3 cm). The rat's gastric mucosa is placed between two glass plates and the top of which was a hole with a 2 cm diameter. Flavonoid gel (200 mg) was attached to the steel plate as a buffer. The steel plate was then attached to the texture analyzer tool pipe. The texture analyzer was managed using a computer with stable microsystem activation. The steel plate will slowly drop into the glass

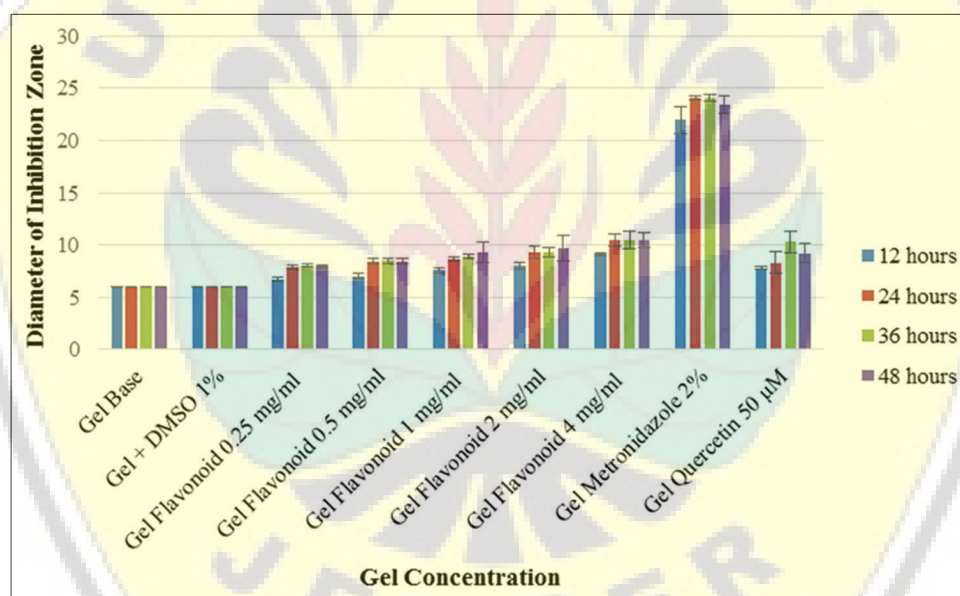


Fig. 1: *Porphyromonas gingivalis* inhibition zone in incubation 12, 24, 36, and 48 h in all control and treatment groups. Significance values in all groups at each time of exposure showed a value of p<0.05

Table 1: Diameter of *Porphyromonas gingivalis* growth inhibition zone

Groups	Diameter of inhibition zone (mm)				P
	12 h	24 h	36 h	48 h	
Gel base	6±0.00	6±0.00	6±0.00	6±0.00	-
Gel+DMSO 1%	6±0.00	6±0.00	6±0.00	6±0.00	-
Gel flavonoid 0.25 mg/ml	6.71±0.17	7.87±0.16	8.07±0.09	7.98±0.15	0.000
Gel flavonoid 0.5 mg/ml	7.05±0.32	8.44±0.266	8.50±0.29	8.47±0.24	0.000
Gel flavonoid 1 mg/ml	7.58±0.29	8.70±0.20	8.93±0.15	9.30±0.98	0.009
Gel flavonoid 2 mg/ml	8.05±0.33	9.35±0.61	9.33±0.49	9.75±1.24	0.078
Gel flavonoid 4 mg/ml	9.13±0.16	10.46±0.55	10.51±0.84	10.49±0.71	0.045
Gel metronidazole 2%	21.96±1.31	24.12±0.17	24.04±0.33	23.47±0.85	0.022
Gel quercetin 50 µM	7.79±0.14	8.31±1.03	10.29±1.03	9.24±0.90	0.019

DMSO: Dimethyl sulfoxide

Table 2: The flavonoid gels mucoadhesion parameters

Groups	Time (s)	Force (g)	Distance (mm)
Gel quercetin 50 µM	17.290	3.15	2.993
Gel flavonoid 4 mg/ml	17.135	2.78	3.146
Gel flavonoid 2 mg/ml	17.415	3.13	2.525
Gel flavonoid 1 mg/ml	17.500	4.75	3.233
Gel flavonoid 0.5 mg/ml	18.255	5.24	4.090
Gel flavonoid 0.25 mg/ml	16.970	3.53	2.830
Gel+DMSO 1%	19.545	5.03	4.233
Gel base	16.505	1.34	2.641

DMSO: Dimethyl sulfoxide

plate hole and touch the rat's gastric mucosa, then after a few seconds, the steel plate will rise quickly.

In vitro antibacterial activity of flavonoid gel against *P. gingivalis*

The antibacterial effect of gel formulations on *P. gingivalis* was evaluated using the disk diffusion method. *P. gingivalis* ATTC 33277 was cultured in medium blood agar by adding Vitamin K and kanamycin. Culture media were incubated for 72 h under anaerobic conditions at 37°C ± 2°C. After bacterial growth, the bacterial suspension was prepared in 0.9% saline and the turbidity was adjusted to a standard of 0.5 McFarland (equivalent to 10⁶ CFU/ml) and was then aseptically transferred to the culture medium. Sterile paper discs with a diameter of 5 mm were then smeared with flavonoid gel concentrations of 0.25, 0.5, 1, 2, and 4 mg/ml and were placed on the surface of the plates containing the culture medium within the same distance. The plates were then incubated at 37°C ± 2°C for 48 h under anaerobic conditions. After this period, the diameter of the resistance zone around the disc was measured in millimeters and the average diameter was recorded. The test is performed as triplicates. Sterile paper discs with base gel were used as negative controls, while metronidazole gel concentrations of 2% and quercetin gel 50 µM were used as positive controls.

Data analysis

Quantitative data are presented in mean ± SD, compared with ANOVA and followed by *post hoc* test. Significance values were determined as $p < 0.05$. Data were analyzed by SPSS software.

RESULTS

Antibacterial test of tobacco leaf flavonoid Kasturi gel on *P. gingivalis* bacteria showed its ability to inhibit bacterial growth in a dosage-dependent manner. The average diameter of the inhibition zone is shown in Table 1 and Fig. 1

The average diameter of the highest inhibition zone of the experiment groups (Table 1 and Fig. 1) is indicated by the flavonoid gel 4 mg/ml at all exposure times and the lowest inhibition zone indicated by the flavonoid gel 0.25 mg/ml at all times of exposure. However, the average diameter of the flavonoid gel inhibition zone was 4 mg/ml lower than the 2% metronidazole gel. Statistical analysis showed that exposure time significantly affected the diameter of the inhibitory zone in the treatment group ($p < 0.05$).

The mucoadhesive test was carried out to determine the strength of the flavonoid gel of Kasturi tobacco leaf that can attach to mucous tissue in which the experiment indicated the flavonoid gels to have a better adhesion parameters (time, strength, and distance) than the gel base (Table 2).

DISCUSSION

Exploration of natural materials with biological activity has been part of development of a non-surgical chemotherapeutic treatment strategy in periodontal disease management such as the use of therapeutic agents of flavonoid on local delivery vehicles. The efficacy of antimicrobial agents applied locally in the treatment of periodontitis depends on adequate subgingival delivery of the agent,

achieving sufficient contact time between the antimicrobial agent and target microorganisms, and achieving an effective concentration of antimicrobial agents. The local delivery devices provide a potential in therapy improvement where conventional treatment cannot be applied [14].

Flavonoids have been known as a source for antibacterial agents against various pathogenic microorganisms [10]. This study indicated that tobacco leaf flavonoids gel tends to work well as an antibacterial against *P. gingivalis* at 24-h incubation. The antibacterial mechanism of flavonoids is related to inhibition of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism [15].

Mucoadhesion is the main property for the treatment of periodontal disease. Mucoadhesive drug delivery systems have been introduced as new dosage forms due to their ability to remain in the mucous membranes and release their drug content slowly. The data of this study indicate that the gels have better adhesion parameters (time, strength, and distance) than gel bases. The potential use of the mucoadhesive system as a drug carrier lies in the extension of residence time at the absorption site, thus allowing intensive contact with the epithelial barrier [16]. Thus, the use of bioadhesive molecules aims to maintain preparations at worksites and direct drugs to specific locations or tissues [17]. Therefore, bioadhesive systems that control drug release can improve treatment and maintain effective drug concentrations at the worksite [18].

CONCLUSION

Based on *in vitro* antibacterial and mucoadhesion studies, the flavonoid gel 4 mg/ml was selected as the best formulation. Flavonoid gel 4 mg/ml showed a satisfactory mucoadhesion property and produced significant growth inhibition zone against *P. gingivalis*. The polymer concentration in the gel base can be used as a controlling drug release rate and for increasing adhesive strength, contact time with the mucosa, and residence time in periodontal pockets. Overall, the gel formulation containing the flavonoids fraction of Kasturi tobacco (*Nicotiana tabacum*) leaves a good candidate for periodontal gel with good mucoadhesive gel and antibacterial agent.

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AUTHORS' CONTRIBUTIONS

Conceptualization, B.K.; methodology, B.K., E.P., and A.S.N.; investigation, B.K., E.P., Q.N.F., M.R., and D.A.A.; data curation, Q.N.F., M.R., and D.A.A.; resources, B.K., E.P., Q.N.F., M.R., and D.A.A.; formal analysis and data interpretation, B.K. and E.P.; original draft preparation, B.K., E.P., Q.N.F., M.R., and D.A.A.; and review and editing, B.K. and A.S.N.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest and all authors participated actively in work. All authors read and approved final version of manuscript for publication.

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