

Effects of the tooth-coating solution and its components on the proliferation and production of inflammatory cytokines in mitogen-activated human peripheral blood mononuclear cells

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ABSTRACT

The tooth-coating solution has been developed as a cosmetic. The tooth-coating solution is composed of rosin, copal and shellac which are suggested to have anti-inflammatory efficacies. The pharmacological efficacies of the tooth-coating solution and its components on the proliferation of T cell mitogen-stimulated peripheral blood mononuclear cells (PBMCs) derived from healthy subjects were evaluated. The effects of the components on production of seven kinds of Th1/Th2/Th17 cytokines from the stimulated PBMCs were also measured. The tooth-coating solution and its ingredients attenuated the proliferation of T cell mitogen-stimulated PBMCs. Rosin, copal and shellac significantly decreased the concentration of IL-6 in the culture supernatant of the stimulated PBMCs ($p < 0.05$). The tooth-coating solution, copal and shellac also decreased the concentration of TNF- α in the supernatant of the stimulated PBMCs ($p < 0.05$). From these observations, we suggest that the tooth-coating solution has potency to prevent inflammation by attenuation of T cell activation and subsequent production of inflammatory cytokines, which may be beneficial for suppression of periodontal disease and stomatitis.

KEYWORDS: peripheral-blood mononuclear cells

(PBMCs), inflammatory cytokine, tooth-coating solution, rosin, copal, shellac.

INTRODUCTION

Tooth-coating solution has been currently developed as a cosmetic for keeping the teeth white and clean. The tooth-coating solution is mainly composed of rosin, copal and shellac. The main component of rosin is abietic acid, which has been reported to have anti-inflammatory effects [1]. Abietic acid is known to inhibit endotoxin lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines such as IL-1 β , TNF- α , and IL-6 from RAW264.7 macrophages [1].

One of the most popular oral cosmetics is dentifrice, a mouth rinse and tooth whitening product. Dentifrice which contains shellac has the efficacy to prevent adherence of *Streptococcus mutans* to the enamel surface of human teeth. Shellac has also been used for the film coating of medical tablet and foods [2]. Thus, shellac is generally used as a constituent of several pharmaceutical, agriculture, confectionary and food products [3]. Copal varnish is known to serve as the standard cavity liner used under amalgam restorations to control microleakage of fluid at the amalgam/tooth interface [4].

Since the tooth-coating solution may have anti-inflammatory efficacy, its components may also have efficacies to suppress inflammatory immune systems. However, these regents have never been evaluated for their efficacies to suppress human PBMCs and

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the production of inflammatory cytokines from the activated PBMCs.

Therefore, in the present study, the tooth-coating solution and its components were examined for their suppressive efficacies on proliferation of T cell mitogen-activated PBMCs obtained from healthy subjects, and on the production of Th1/Th2/Th17 cytokines including IL (Interleukin)-2, IL-4, IL-6, IL-10, TNF- α , IFN- γ and IL-17 from the activated PBMCs.

MATERIALS AND METHODS

Reagents

The tooth-coating solution and its major components rosin, copal and shellac were provided by Hanic White Labo Co., Ltd. (Tokyo, Japan). Ficoll-Paque was obtained from Amersham Pharmacia Biotech Inc. (Buckinghamshire, UK). Concanavalin A was obtained from J-OIL MILLS, Inc. (Tokyo Japan). Tetrazolium salt of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640 medium and fetal bovine serum were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Lymphocyte separation solution was purchased from Nacalai Tesuque, Inc. (Kyoto, Japan). BD Cytometric Bead Array Human Th1/Th2/Th17 cytokine kits were from BD (San Jose, CA, USA). All other reagents were of the highest quality available from commercial vendors.

Subjects

The present study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The study was approved by the Ethical Committee of Tokyo University of Pharmacy and Life Sciences and written informed consent was obtained from all healthy subjects included in the study. 20 mL of heparinized venous blood was taken from 11 healthy subjects (3 males and 8 females). The mean (standard deviation [SD]) age of healthy subjects was 24.5 [7.2] years.

Isolation of PBMCs

The isolation and culture of PBMCs were carried out according to the method we described previously [5-7]. In brief, 5 mL of heparinized blood was loaded on to 4 mL of lymphocyte separation solution Ficoll-Paque, and centrifuged at 900 g for 20 min

at room temperature. The buffy coat containing PBMCs was taken and rinsed 3 times with RPMI-1640 medium containing 10% fetal bovine serum. PBMCs including lymphocytes were suspended in RPMI-1640 medium containing 10% fetal bovine serum to a cell density of 1×10^6 cells/mL.

PBMC culture and evaluation of tooth-coating solution and its component reagents

The cell suspension was placed into each well of a 96-well flat-bottomed microplate. Saline containing concanavalin A as a T cell mitogen was added to each well to a final concentration of 5.0 μ g/mL. The tooth-coating solution was dissolved in ethanol to give final concentrations of 0.0001, 0.001, 0.01, 0.1, and 1 %. Rosin, copal, and shellac were also dissolved in ethanol to give final concentrations of 0.1, 1, 10, 100, and 1000 μ g/mL. The same volume of each vehicle solution was added to the control wells. The plate was then incubated for four days in an atmosphere containing 5% CO₂ at 37 °C.

MTT assay

After 4 days of incubation of the culture, 10 μ L of 5 mg/mL MTT solution dissolved in saline was added to each well, and then the cultures were re-incubated under 5% CO₂ at 37 °C for 4-5 hours [6, 7]. The plates were centrifuged at 375 g for 5 min to precipitate the cells and the formazan produced by the growing cells. Aliquots of the supernatant were removed from each well, and dimethylsulfoxide was added followed by shaking of the plate on a microshaker for 10 min to dissolve the formazan crystals. The absorbance was read with a microplate reader at 550 nm. Dose-response curves were plotted, and the concentration that inhibited cell growth by 50% (IC₅₀) of the reagent was calculated.

Cytokine analyses

Concentrations of seven cytokines IL-2, IL-4, IL-6, IL-10, IFN- γ , TNF- α and IL-17 in the medium of the activated PBMCs cultured for four days in the presence or absence of the reagents were measured [5, 6]. The culture supernatants of PBMCs incubated for 96 h in the presence of 5.0 μ g/mL concanavalin A and the serial concentrations of the tooth-coating solution and its components were stored at -80 °C until the measurement of the cytokine concentrations. The concentrations of

these cytokines in the PBMC culture supernatants were measured with bead-array procedures using a Human Th1/Th2/Th17 Cytokine Kit, followed by flow cytometry, according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Differences in the IC_{50} values of the tooth-coating solution and the components rosin, copal and shellac on the proliferation of the activated PBMCs of healthy subjects were analyzed by Dunnet's multiple comparison test. Dunnet's multiple comparison test was also used for the statistical analysis of the differences in the cytokine concentrations between the supernatants of PBMCs treated with the tooth-coating solution or its components and those of PBMCs cultured in the absence of the reagents. These data analyses were performed using the PASW statistics software program (version 18.0,

SPSS Japan Inc., an IBM company), Graphpad prism 7 for windows (version 7.05 Graph pad software Inc.) and EXCEL 2016 (Microsoft).

RESULTS

We first examined the pharmacological efficacies of the tooth-coating solution rosin, copal and shellac on the *in vitro* proliferation of concanavalin A-stimulated PBMCs of healthy subjects (Figure 1A-D). The tooth-coating solution dose dependently suppressed the PBMC proliferation (Figure 1A). Rosin, copal and shellac also dose dependently inhibited the PBMC proliferation (Figure 1B-D). Individual suppressive potencies exhibited by the IC_{50} values of copal widely deviated between the subjects of PBMC donors, whereas the deviation of the IC_{50} values of rosin and shellac were relatively small (Figure 2). The median (range) of the IC_{50} values of these agents is presented in Table 1.

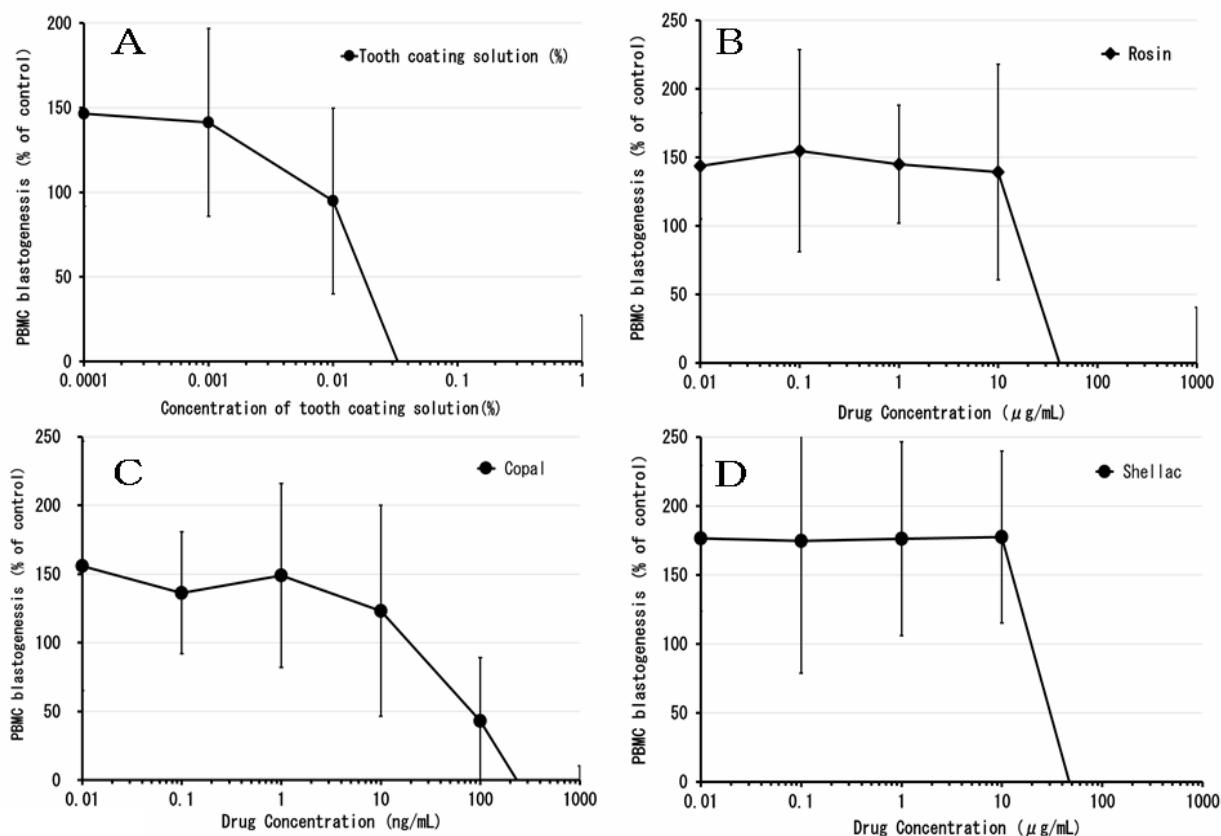


Figure 1. Effects of the tooth-coating solution (A), rosin (B), copal (C) and shellac (D) on the concanavalin A-stimulated proliferation of PBMCs obtained from 11 healthy subjects.

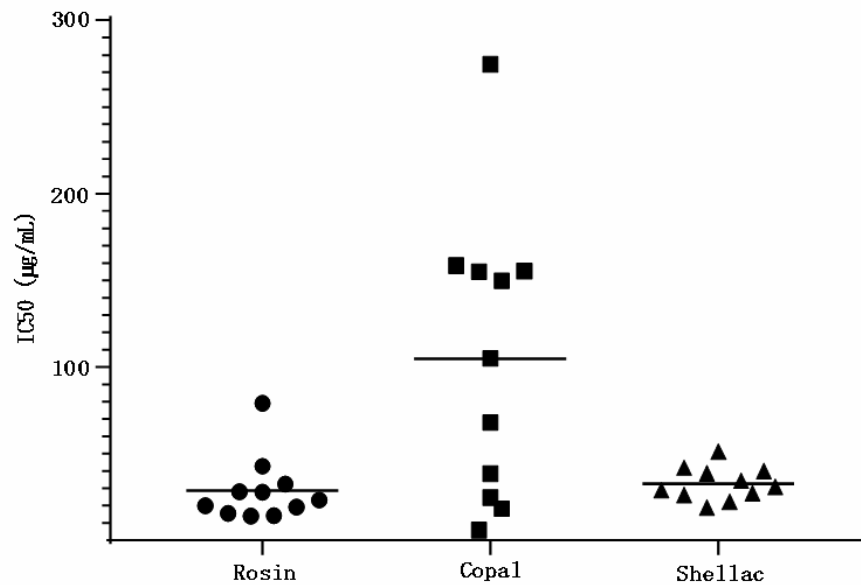


Figure 2. Individual variations in the IC₅₀ values of rosin, copal and shellac due to the concanavalin-A stimulated proliferation of PBMCs obtained from 11 healthy subjects.

Table 1. Individual IC₅₀ variation in the tooth-coating solution composed of rosin, copal and shellac.

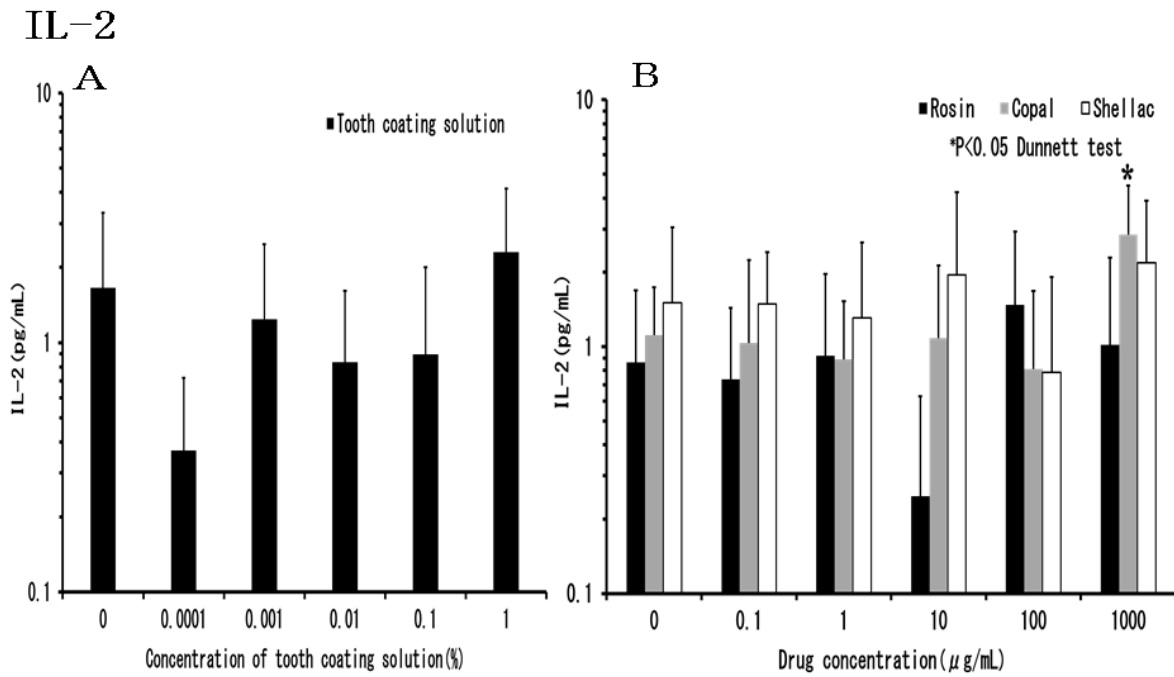
Reagents	Mean(SD)	Median	Minimum	Maximum
Tooth-coating solution (%)	0.02 (0.01)	0.02	0.001	0.037
Rosin (µg/mL)	28.7 (18.8)	23.2	14.0	78.8
Copal (µg/mL)	104.8 (82.6)	105.0	5.9	274.4
Shellac(µg/mL)	32.8 (9.5)	30.9	18.9	51.1

Then, as shown in Figures 3-9, we analyzed the concentrations of seven cytokines secreted into the culture medium of PBMCs stimulated by concanavalin A in the presence of the tooth-coating solution (A), rosin, copal and shellac (B). In this study, the concentrations of Th1/Th2/Th17 cytokines IL-2 (Figure 3), IL-4 (Figure 4), IL-6 (Figure 5), IL-10 (Figure 6), TNF- α (Figure 7), IFN- γ (Figure 8) and IL-17 (Figure 9) were measured using a Human Th1/Th2/Th17 Cytokine Kit, followed by flow cytometry.

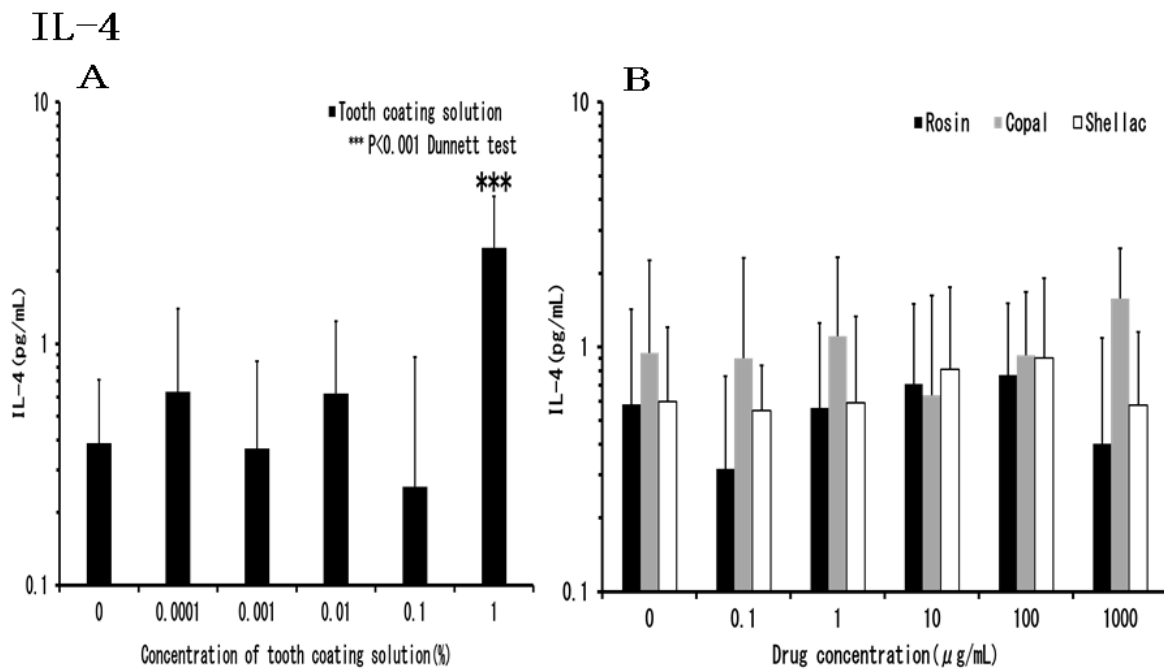
The tooth-coating solution significantly decreased the concentrations of IL-10 at 0.01, 0.1 and 1% ($p < 0.05$) (Figure 6A), TNF- α at 0.1 and 1% ($p < 0.05$) (Figure 7A), and IFN- γ at 0.1 and 1% ($p < 0.01$) (Figure 8A). Whereas, the tooth-coating solution significantly increased IL-4 concentration at 1%. ($p < 0.001$) (Figure 4A).

Rosin significantly decreased the concentrations of IL-6 at 100 and 1000 µg/mL ($p < 0.01$) (Figure 5B), IL-10 at 10 µg/mL ($p < 0.01$) (Figure 6B), and IFN- γ at 1 µg/mL ($p < 0.001$), 10 µg/mL ($p < 0.05$), 100 and 1000 µg/mL ($p < 0.01$) (Figure 8B), compared with control. Similarly, copal significantly decreased the concentrations of IL-6 at 1000 µg/mL ($p < 0.05$) (Figure 5B), TNF- α at 1000 µg/mL ($p < 0.05$) (Figure 7B), and IFN- γ at 1 µg/mL ($p < 0.05$), 10, 100 and 1000 µg/mL ($p < 0.01$) (Figure 8B), while it significantly increased the concentration of IL-2 at 1000 µg/mL ($p < 0.05$) (Figure 3B).

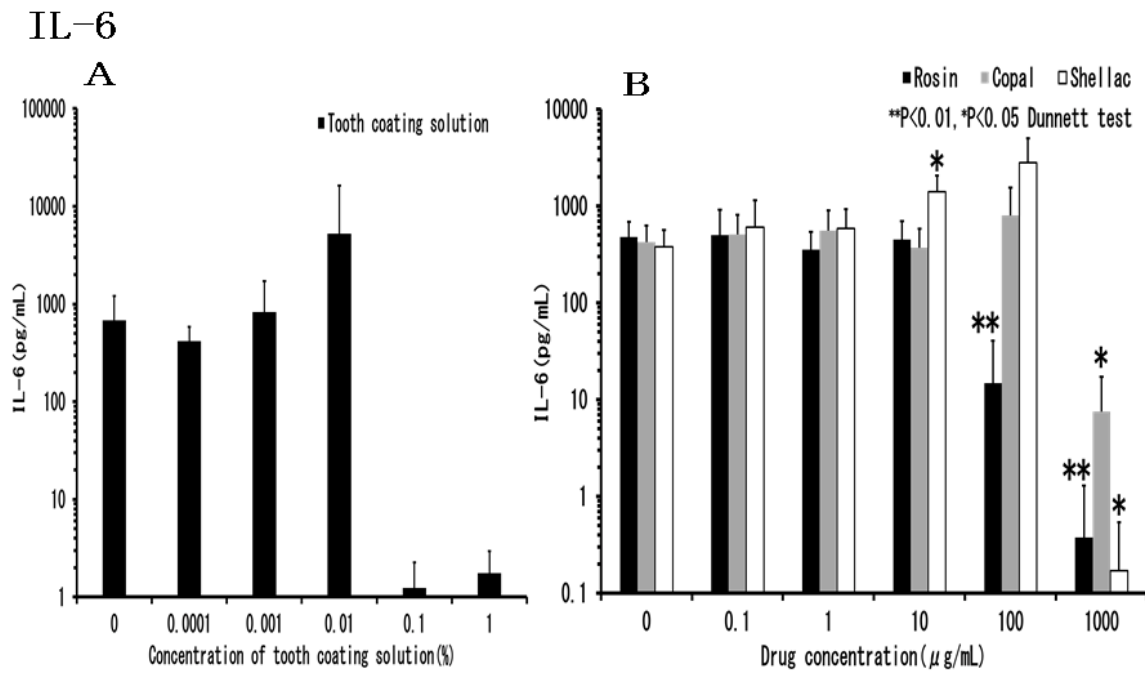
Shellac significantly decreased the concentrations of IL-6 at 10 and 1000 µg/mL ($p < 0.05$) (Figure 5B), IL-10 at 10, 100 and 1000 µg/mL ($p < 0.05$) (Figure 6B), TNF- α at 0.1 ($p < 0.01$), 100 and 1000 µg/mL ($p < 0.05$) (Figure 7B), and IFN- γ at



Figures 3. IL-2 concentrations in the supernatant of concanavalin A-stimulated PBMCs in the absence or presence of the tooth-coating solution (A), rosin, copal and shellac (B). IL-2 concentrations measured using bead-array procedures followed by flow cytometry. *p < 0.05 as compared to each control.



Figures 4. IL-4 concentrations in the supernatant of concanavalin A-stimulated PBMCs in the absence or presence of the tooth-coating solution (A), rosin, copal and shellac (B). IL-4 concentrations measured using bead-array procedures followed by flow cytometry. ***p < 0.001 as compared to each control.



Figures 5. IL-6 concentrations in the supernatant of concanavalin A-stimulated PBMCs in the absence or presence of the tooth-coating solution (A), rosin, copal and shellac (B). IL-6 concentrations measured using bead-array procedures followed by flow cytometry. * $p < 0.05$ and ** $p < 0.01$ as compared to each control.

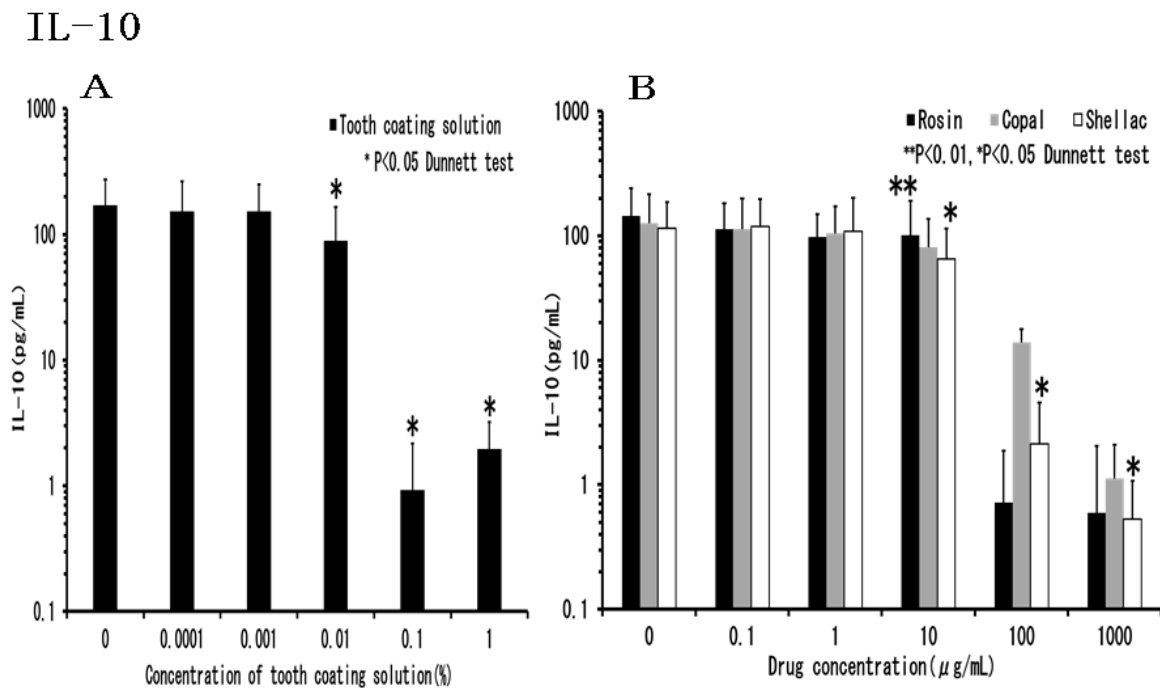


Figure 6. IL-10 concentrations in the supernatant of concanavalin A-stimulated PBMCs in the absence or presence of the tooth-coating solution (A), rosin, copal and shellac (B). IL-10 concentrations measured using bead-array procedures followed by flow cytometry. * $p < 0.05$ and ** $p < 0.01$ as compared to each control.

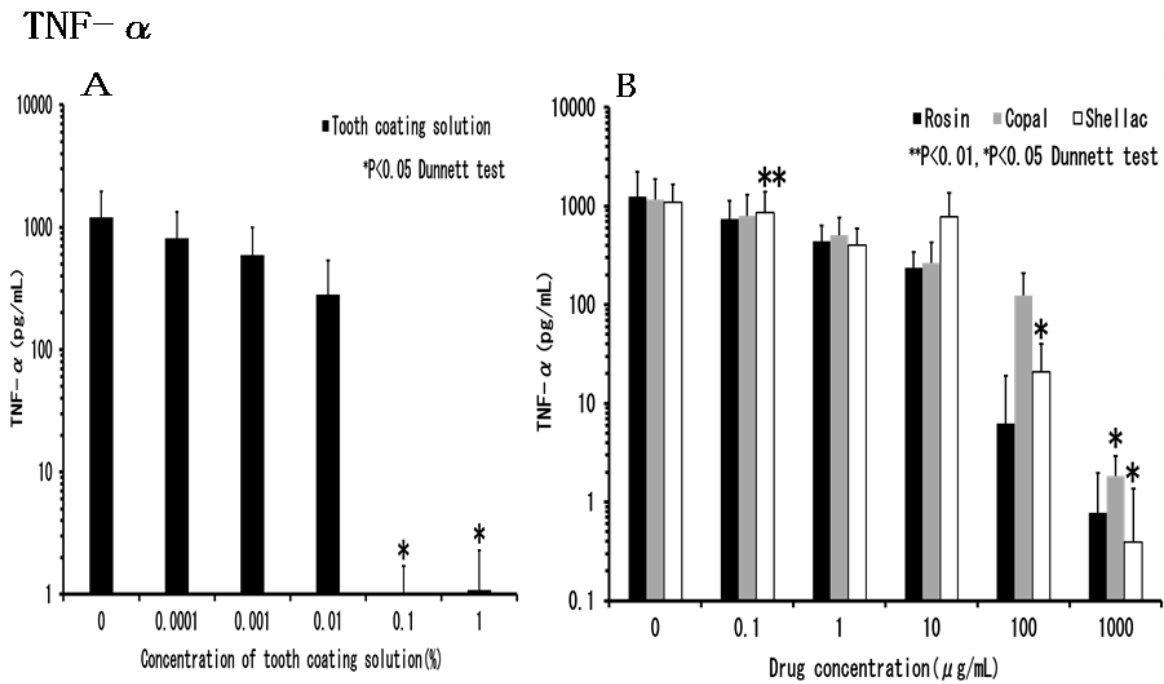


Figure 7. TNF- α concentrations in the supernatant of concanavalin A-stimulated PBMCs in the absence or presence of the tooth-coating solution (A), rosin, copal and shellac (B). TNF- α concentrations measured using bead-array procedures followed by flow cytometry. *p < 0.05 and **p < 0.01 as compared to each control.

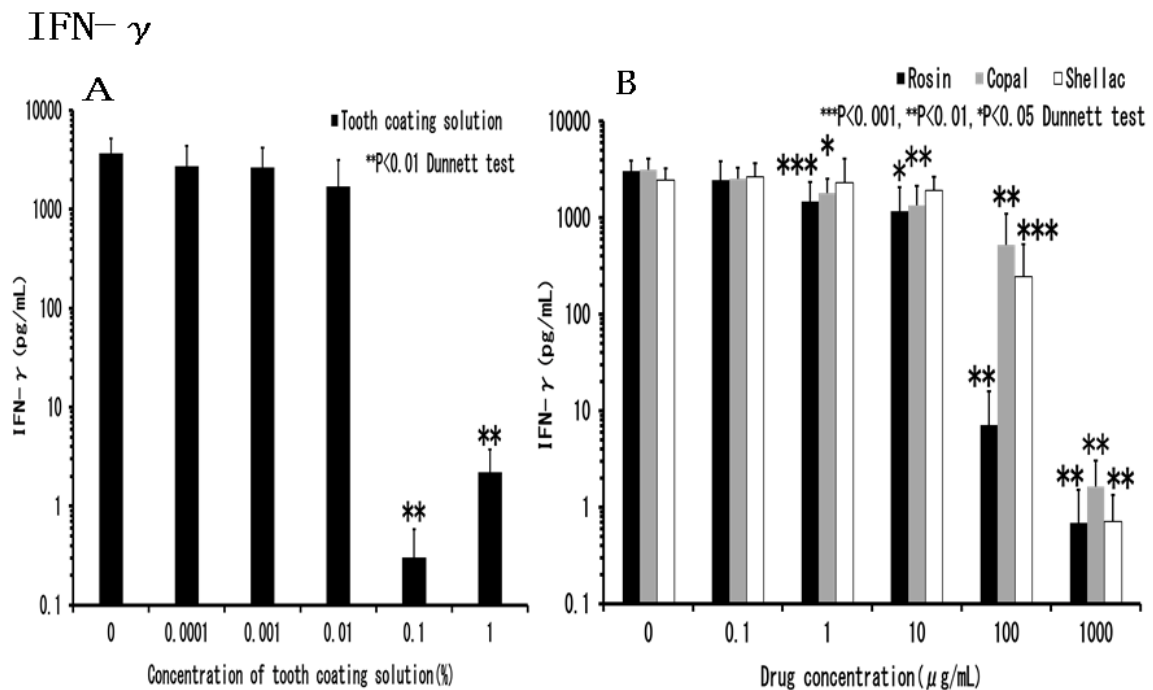


Figure 8. IFN- γ concentrations in the supernatant of concanavalin A-stimulated PBMCs in the absence or presence of the tooth-coating solution (A), rosin, copal and shellac (B). IFN- γ concentrations measured using bead-array procedures followed by flow cytometry. *p < 0.05, **p < 0.01 and ***p < 0.001 as compared to each control.

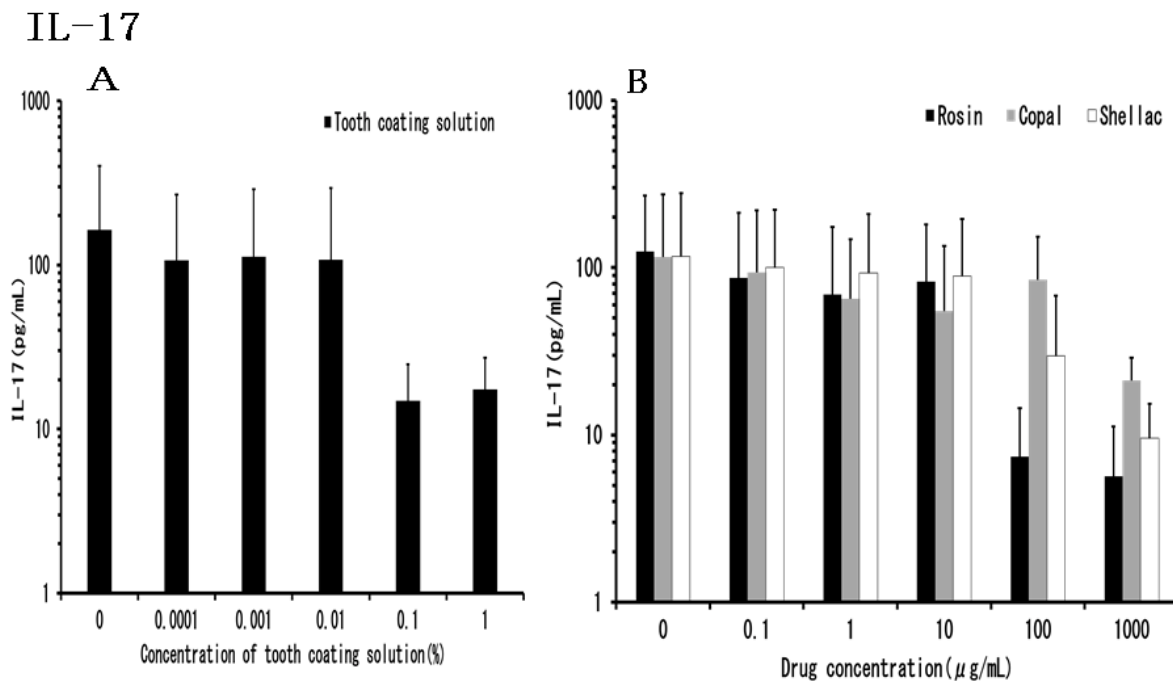


Figure 9. IL-17 concentrations in the supernatant of concanavalin A-stimulated PBMCs in the absence or presence of the tooth-coating solution (A), rosin, copal and shellac (B). IL-17 concentrations measured using bead-array procedures followed by flow cytometry. No significant difference was observed between the PBMC cultures incubated with and without the reagents.

100 µg/mL ($p < 0.001$) and 1000 µg/mL ($p < 0.01$) (Figure 8B). Thus, the secretions of IL-6, IL-10, TNF- α and IFN- γ were almost commonly suppressed by the tooth-coating solution, rosin, copal and shellac.

DISCUSSION

Periodontal diseases are chronic infectious inflammatory disorders characterized by the destruction of tooth-supporting structure [8]. The chronic inflammatory conditions of the periodontium involve interactions between bacterial products, numerous cell populations and inflammatory mediators. Several proinflammatory cytokines including IL-1, IL-6, IL-12, IL-17, IL-18, IL-21, TNF- α and IFN- γ have been demonstrated to be involved in the pathogenesis of periodontitis [9]. Furthermore, serum levels of TNF- α , IL-6, IL-17 and IL-23 were reported to be significantly higher in rats in the apical periodontitis group than in rats in the control group [10].

In this study, we evaluated potencies of the tooth-coating solution and these components, rosin,

copal and shellac to attenuate proliferation and inflammatory cytokine secretion in T cell-mitogen-stimulated PBMCs of healthy subjects. It is the first time, to the best of our knowledge, that the suppressive potencies of the tooth-coating solution and its components on the activated human PBMCs are evaluated. The data showed that the tooth-coating solution and its main ingredients rosin, copal and shellac dose dependently suppressed the proliferation of the T cell mitogen-activated PBMCs *in vitro*. Furthermore, IL-6, IL-10, TNF- α and IFN- γ levels were also significantly attenuated by the treatment of PBMCs with the tooth-coating solution and its ingredients. IL-6, IL-17, TNF- α and IFN- γ have been categorized as pro-inflammatory cytokines in gingival tissue [9]. Therefore, the present observations suggest that the tooth-coating solution and its ingredients are able to decrease the amounts of pro-inflammatory cytokines in gingival tissue of patients with periodontal diseases. IL-10 has been categorized as an anti-inflammatory cytokine [9]. However, in our study, the tooth-coating solution and the ingredients also

decreased the IL-10 level. Garlet reported that anti-inflammatory cytokine IL-10 is associated with the attenuation of periodontal tissue distribution and do not compromise the control of periodontopathogens [8]. Periodontal disease is the leading cause of tooth loss among adults [11]. The tooth-coating solution has been widely used as a teeth cosmetic in Japan. The present data suggest that the tooth-coating solution and its ingredients may have benefits to attenuate gingival inflammation by decreasing the production of pro-inflammatory cytokines from the activated T cells. Rheumatoid arthritis and chronic periodontitis are the most common chronic inflammatory diseases with remarkable pathological and clinical similarities [12].

CONCLUSION

These observations also give a possibility that the tooth-coating solution may have efficacies for the treatment of rheumatoid arthritis. The tooth-coating solution has been widely used as a teeth cosmetic in Japan. In addition to the cosmetic use, the tooth-coating solution and the ingredients rosin, copal and shellac may have benefits to attenuate gingival inflammation by decreasing production of pro-inflammatory cytokines from the activated T cells.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest to disclose.

ABBREVIATIONS

IL - Interleukin
PBMCs - Peripheral blood mononuclear cells

LPS - Endotoxin Lipopolysaccharide
MTT - Tetrazolium salt of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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