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Analgesic and Anti-inflammatory Activity of Resina Pini

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Abstract – In this study, we investigated the potential of Resina Pini (RP) for anti-inflammatory and analgesic agents to treat inflammatory diseases such as gingivitis and periodontitis. Crude RP (RP1), recrystallized RP (RP2), and Ramus Mori Albae-treated RP (RP3), plus their respective water extracts (RP1-WE, RP2-WE and RP3-WE) were prepared for *in vitro* and *in vivo* tests. We couldn't find any signs of heavy metals pollution in all the RP samples. RP2-WE exhibited the highest viability of human gingival fibroblasts (HGF) and the strongest scavenging activity on superoxide anion. RP1, RP2 and RP3, RP2 showed potent scavenging activity on DPPH free radical. RP2-WE displayed a stronger inhibition on hyaluronidase (HAase) activity and RP3 also displayed potent HAase inhibition. RP2-WE, RP3-WE, RP3 and RP2 were reduced admirably the production of PGE_2 in HGF. In addition, RP2-WE and RP3-WE exhibited potent inhibitory activities on arachidonic acid-induced ear edema in mouse. Moreover, RP-2 prevented completely acetic acid-induced writhing by 100.0% and RP1, RP3, RP1-WE and RP2-WE also exhibited excellent protective activities against writhing. While aminopyrine, the positive control, showed 76.9% analgesic effect at the same dose. Taken together, these results suggest that recrystallized aqueous extract of Resina Pini could be a promising drug for the treatment of periodontal diseases.
Keyword – Resina Pini, analgesic activity, anti-inflammatory, hyaluronidase, PGE_2, cytotoxicity

Introduction

Periodontal diseases, the most common form of oral disease, are a group of inflammatory and localized microbial-induced infections involving the supporting tissues of the teeth, the gingiva, periodontal ligament, and alveolar bone. Consisting of two major stages, gingivitis and periodontitis, periodontitis often starts with inflammatory lesions of the gingiva and, if left untreated, can result in tooth loss (Schwach-Abdellaoui, *et al*., 2002). The characteristic symptoms of periodontal diseases include swelling, redness, bleeding, heat, pain, pus around the teeth, tooth loss, and chronic bad breath. While good oral care products can improve the gingival conditions, effective therapeutic agents are needed to cure the inflammation, arrest the progression of the periodontal destruction, and restore gingival-periodontal health, plus the prognosis is quite predictable for gingivitis and chronic periodontitis.

Many kinds of oral care agent are currently used to reduce the level of supragingival plaque and arrest the development of periodontal disease. However, only a few agents are free from undesirable side-effects (Scheie, 1989), and there is no ideal agent for periodontal disease. The adverse reactions associated with the use of existing agents have stimulated research on alternative agents, and the recent use of natural products has proved to be a successful strategy for the discovery of effective plaque-control agents and/or new treatments, resulting in the investigation of a number of natural extracts (Harvey, 2000; Bacca, *et al*., 1997).

Resina Pini (RP), a resinous exudation obtained from *Pinus densiflra* (Pinacezae) (Yun, 1997), has traditionally been used to control infection, inflammation, and pain related to dental caries and periodontal disease (Yuk, 1989), RP could be an effective alternative to conventional therapeutic agents for periodontal tissue inflammation and breakdown. Although RP has been used for a long time, there are only a few reports on its therapeutic efficacy. Thus, to evaluate the anti-plaque and anti-gingivitis activity of RP, the current authors previously investigated the effect of RP in supra-gingival plaque control to prevent periodontal tissue inflammation and breakdown. There has been report on the inhibitory effect of RP water extracts against release of arachidonic acid from the cell membrane (Cho and Suk, 2002). We have demonstrated previously that RP significantly inhibits the growth and glucosyltransferase activity of *Streptococcus mutans*-associated plaque and gingivitis. These findings

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present evidence that RP water extracts can play an important role in supragingival plaque control, which prevents periodontal infection and inflammation (Seo, *et al*., 2005). In this study, the anti-inflammatory activity, analgesic efficacy and safety of RP samples were evaluated *in vitro* and *in vivo*.

Experimental

Chemicals – Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, fetal bovine serum (FBS), phosphate buffered saline (PBS) and 3-(4, 5-dimethylthiszol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Gibco Laboratories (Gaithersburg, MD, U.S.A.). Calcium ionophore A_{23187}, Arachidonic acid (AA), 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.).

Plant material and extraction – RP was acquired from traditional drug market in Daegu, Korea. The crude RP (unprocessed RP, RP1) was powdered and filtrated through gauze at 90 °C. After coagulation in cold distilled water, the recrystallized RP (RP2) was obtained and air-dried. To prepare the Ramus Mori Albae-treated RP (RP3), the mixture of RP1 : Ramus Mori Albae 20 : 3 (w/w) was boiled with distilled water (500mL) and filtered and recrystallized after cooling in the same way described above. The powdered RP 1, RP2 and RP3 were extracted with distilled water. 20g each of powdered plant materials was boiled with distilled water (500 mL) in rotary vacuum evaporator for 5 h at 90 °C. After cooling, the mixture was filtrated with Whatman 2 filter, and centrifuged at 4,000 rpm for 25 min. The supernatant was concentrated under vacuum below 60 °C to furnish a water extract solid. The yield of water extracts of RP1, RP2 and RP3 (RP1-WE, RP2-WE and RP3-WE) were 1.4, 0.7 and 2.2% w/w, respectively. The dried powder of plant material was dissolved in 10% DMSO (v/v) and filtered through a Millipore filter (0.22 μm, Gelman Sciences Inc., Ann Arbor, U.S.A.) prior to use.

Animals – ICR mice (approximately 30 g) of either sex were obtained from Korea Experimental Animal Center (Seoul, Korea). They were kept in departmental animal house on well cross-ventilated room at 23 ± 1 °C, 50 ~ 60% relative humidity and 12h/12h light/dark cycle for one week before and during the experiments. Animals were provided with standard pellet diet and water *ad libitum*.

Atomic absorption spectrophotometric analysis – The contents of heavy metal in unprocessed and processed RP were measured with a flame atomic absorption spectrophotometer (Pye Uunicam 929 AAS, Cambridge, U.K.). A sample with a mass of 5 g was weighed and placed into a 100 mL beaker. and 20 mL of concentrated H_2SO_4 was added and this was boiled gently for 30 min on a hot-plate. The porcelain crucible was cooled and 50 mL triple-distilled water, 25 mL concentrated ammonium oxalate were added. The mixture was heated to near dryness. The corners and walls of the beaker were washed with about 10 ml triple-distilled water, and the solution was again heated until dense white fumes developed. The beaker was cooled and then the ash was dissolved in 1 mL conc. H_2SO_4, 1 mL conc. HNO_3 and diluted with triple-distilled water up to a volume of 25 mL. The solution was analyzed by means of atomic absorption spectrophotometer. All samples were run in triplicate. A blank determination using the same procedure was performed. Atomic absorption spectrophotometer with deuterium background correction at 213.9 nm for Zn, 285.2 nm for Mg, 422.7 nm for Ca, 279.5 nm for Mn, 670.8 nm for Li, 248.3 nm for Fe, 193.7 nm for As, 357.9 nm for Cr, 253.7 nm for Hg, 228.8 nm for Cd, 232.0 nm for Ni and 240.7 nm for Co, 217.0 nm for Pb, and 324.8 nm for Cu were used, respectively. Detection limits for each element were 0.015 μg/L for Mn, 0.075 μg/L for Cr, 2.2×10^{-4} μg/L for Cd, 0.009 μg/L for Cu, 0.009 μg/L for Co, 0.038 μg/L for Zn, 0.085 μg/L for Fe, 0.0001 μg/L for As, 0.009 μg/L for Hg and 0.0048 μg/L for Pb, respectively.

Phytochemical analysis – A preliminary phytochemical analysis was carried out on the water and ethanol extracts of RP1, RP2 and RP3 using reported method (Farnsworth, 1966). For this analysis, the following reagents and chemicals were used: Molish for glycosides and carbohydrates, Fehling reagent for reducing sugar; Biuret reagent for protein and peptides; Mayer reagent for alkaloids; alcoholic picric acid for essential oils; 5% alcoholic $AgNO_3$ for acidic compounds; methanol, 10% $FeCl_3$-ethanol for phenolic compound, Flavonoid and Tannin. In addition, steroids, terpenoids and saponin were tested using the Leibermman-Burchard method.

Gingival fibroblast culture – Primary cultures of HGF were prepared by the method of explant culture. Briefly, pieces of HGF were obtained from two patients between 7 and 8 yr of age with no clinical signs of periodontal disease undergoing surgical removal of the third molars. Minced pieces of the tissue were explanted to 35 cm^2 tissue culture dishes containing 10 mL of DMEM containing 10% FBS, 100 units/mL of Penicillin and 100 μg/mL of Streptomycin sulfate. The fibroblasts were

obtained by trypsination of the primary outgrowth of cells. The HGF were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C. The media were changed twice weekly. For subculture, the cells were washed twice with PBS, treated with 0.05% Tripsin/0.02% EDTA for 5 min, and transferred to 75 cm^2 culture flasks. The cells used for the experiments proliferated in logarithmic phase between the 8th and 15th passages.

MTT assay – *In vitro* safety test of RP on HGF was checked using MTT assay (Rubinstein, *et al.,* 1990). Cells were seeded in the growth medium (180 μL) into 96-well plates (2×10^4 cells / well) and incubated at 37 °C, 5% CO_2. A test sample was dissolved in 10% DMSO (v/v) and adjusted to final sample concentrations ranging from 20 μg/mL to 300 μg/mL by diluting with the growth medium. After standing for 2 h, 20 μL of various samples were added to each well and incubated for 48 hr. The same volume of 10% DMSO was added to the control group well. 48 h later, 50 μL MTT was also added to the each well (final concentration, 2 mg/mL) and incubated for another 4 hr. After 4 h, the plate was centrifuged for 5 min at 3000 rpm and then the medium was removed. The resulting formazan crystals were dissolved with 150 μL DMSO. The optical density (O.D.) was measured at 540 nm using ELISA plate reader (BioTEK Instruments, Winooski, U.S.A.). The IC_{50} value was defined as the concentration of sample to reduce at 50% of absorbance relative to the vehicle-treated control. The fractional absorbance was calculated by the following formula:

$$\% \text{ cell viability} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control well}} \times 100$$

Lethality – D_{50} values of RP1 were determined in mice (Lorke, 1983). The resin was administered orally to groups of 7 mice in different doses up to 2000 mg/kg and animals were observed up to 7 days at time intervals for mortality.

DPPH radical scavenging activity – DPPH radical scavenging activity was determined using the modified Tomohiro's method as follows (Hatano, *et al*., 1989). To 16 mg DPPH/100 mL ethanol, a sample solution (control: distilled water 100 μL) and 0.1 M sodium acetate buffer (pH 5.6) was added with the final sample volume of 2 mL. Then the solution with a testing sample was shaken vigorously and kept for 30 minutes at room temperature. The absorbance of the sample was measured by a spectrophotometer at 517 nm against a blank of ethanol without DPPH. A blank sample without a testing sample was also measured under the same condition. All tests were run in triplicate. Inhibition percentage was calculated according to the formula:

$$\% \text{ Inhibition} = [(A_B - A_A) / A_B] \times 100$$

Where A_B is the absorbance of the blank sample and A_A is the absorbance of the tested sample after 30 minutes.

Superoxide anion assay – Superoxide anion scavenging activity was measured spectorphotometrically (Marklund and Marklund, 1974). RP sample (30 μL) was added into 150 μl of 50 mM Tris-HCl buffer solution (pH 8.0). Then, 30 μl of 7.2 mM pyrogallol solution was added into the mixture, and absorbance at 450 nm was measured. A blank sample without RP sample was also subjected to the same experiment. Superoxide anion scavenging activity of the RP sample was expressed in the extent of a reduction in absorbance.

HAase activity assay – HAase activity was spectrophotometerically determined by measuring the amount of N-acetylglucosamine formed from sodium hyaluronate (Tung, *et al*., 1994). 50 μL of bovine HAase (7,900 units/ml) dissolved in 0.1 M acetate buffer (pH 3.5) was mixed with 100 μL of sample and then incubated at 37 °C for 20 min. The control group was treated with DMSO. The reaction mixture was added with 100 μL of 12.5 mM $CaCl_2$ and then incubated at 37 °C for 20 min. This Ca^{2+}-activated HAase was treated with 250 μL of 0.12% hyaluronic acid-K in 0.1 M acetate buffer (pH 3.5) and then incubated at 37 °C for 40 min. 100 μL of 0.4N-NaOH and 0.4 M potassium borate were added and then incubated in a boiling water bath for 3 min. After cooled to the room temperature, 3 mL of DMAB (4 g of p-dimethylaminobenzaldehyde dissolved in 350 mL of 100 % acetic acid and 50 mL of 10 N-hydrochloric acid) was added and incubated at 37 °C for 20 min. Optical density at 585 nm of the reaction mixture was measured by using a spectrophotometer (Pharmacia Biotec, Cambridge, U.K.).

Analysis of PGE_2 production – Gingival fibroblasts were seeded at 1×10^4 cells per well in flat-bottomed 96-well plates and treated with RP samples or aspirin (250 μg/mL) for 24 h. 1 μM Ca-ionophore A_{23187} were then added to the culture medium, and incubated at 37 °C for 24 h. The medium was collected in a microfuge tube and then centrifuged at 3000 rpm for 5 min. The amount of PGE_2 released into the culture medium was determined using a PGE_2 Enzyme-Immuno Assay kit (Amersham Biosciences, Piscataway, U.S.A.).

AA-induced ear edema bioassay – The acute anti-inflammatory activity was determined by AA-induced ear edema assay in mice of either sex following the slightly modified method (Kim, *et al*., 1993) of the original

procedure of Tonnelli, *et al*. (1965). Test sample was topically applied to the inner and the outer surface of ear. Thirty minutes later, 2.5% AA in acetone (25 μL/ear) was applied and the thickness increased was measured 1 hr after the AA treatment using a digital thickness gauge (Mitutoyo co., Kawasaki, Japan). Percent inhibition of ear edema was calculated compared to the control group treated with vehicle and AA only.

Acetic acid-induced writhing test – Analgesic activity of sample was studied by reduction of the acetic acid induced writhing in the mice. Thirty minutes after the administration of sample (200 mg/kg, p.o.) or the reference standard, the animals received 0.1 mL/10g acetic acid (1%, i.p.). The number of abdominal contractions and stretching with a jerk of the hind limb were counted for 10 min after administering acetic acid and then inhibition percent was calculated (Silva, *et al*., 2003, Koster, *et al*., 1959).

Statistics – Each data were expressed as mean ± SD. Group means were compared by using a one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. Statistical significances were considered significant at $p < 0.05$.

Results and Discussion

Atomic absorption analysis – Metals like Zn, Cu, Ca, Fe, Mg and Mn, which are required for metabolic activity in organisms, lies in the narrow spectrum between their essentiality and toxicity. Others heavy metals like Cd, Hg, Cr, As and Pb *etc*. may exhibit extreme toxicity even at low levels thus necessitating basal monitoring of heavy metal contents. Therefore, the purpose of this analysis was to determine the potential for heavy metal contamination of RP. The other aim was to compare the concentrations of heavy metals in the crude and processed resins. The results of the quality assurance studies are shown in Table 1. Ca concentrations in samples were relatively high ranged from 1.98 to 8.42 mg/L. The highest concentration of Ca was found in RP3. Level of Zn in samples ranged from 2.40 to 5.19 mg/L. The highest concentrations were found in RP1. The concentrations of Pb, Mn, Co, Cu, Cd and Cr were extremely low in the tested RP samples. Happily, toxicants like As, Hg and Ni were not found in samples. Consequently, we couldn't find any signs of heavy metals pollution in RP samples.

Phytochemical analysis – The main components of RP are rosine, including levopimaric acid, neoabietic acid, dextropimaric acid, and isodextropimaric acid. In addition, terpenoids, including α-pinene, β-pinene, myrcene, β-phellandrene, linalool, and linalyl acetate have also been isolated from RP (Song and Kim, 1994; Namba, 1980). Since topically applied RP will be incorporated in the saliva, the factors associated with water solubility were considered based on comparing the constituents in ethanol extracts and water extracts of RP1, RP2, and RP3. As shown in Table 2, the preliminary qualitative analysis showed that both ethanol and water extracts of RP1, RP2,

Table 1. Concentration of heavy metals by atomic absorption spectrometry

Elements	RP1	RP2	RP3
As	ND	ND [b)]	ND
Li	ND	ND	ND
Ca	1.98[a]	2.30	8.42
Cr	ND	0.01	0.03
Co	0.22	0.47	0.30
Cu	0.01	0.05	0.04
Cd	0.01	0.01	ND
Fe	0.92	1.47	2.58
Hg	ND	ND	ND
Mg	0.05	0.07	1.38
Mn	0.52	0.46	0.42
Ni	ND	ND	ND
Pb	0.33	0.45	ND
Zn	5.19	4.70	2.40

[a]Each sample was run in triplicate and the unit is mg/L. [b]ND : not detected.

Table 2. Preliminary phytochemical analysis of Resina Pini

	RP1		RP2		RP3	
	Water ex.	Ethanol ex.	Water ex.	Ethanol ex.	Water ex.	Ethanol ex
Alkaoids	–	–	–	–	–	–
Flavonoids	+	–	+	–	+	–
Reducing sugar	+	+	+	+	+	+
Glycosides and Carbohydrates	+	+	+	+	+	+
Phenolic compound and tannin	+	+	+	+	+	+
Protein and Peptides	+	–	+	+	+	+
Steroids, Terpenoids, Saponin	+	+	+	+	+	+
Essential oils	+	–	+	–	+	–
Acidic compounds	+	–	+	–	+	–

Table 3. Effect of various RP samples on HGF viability

Concentration (μg/mL)	Cell viability (%)					
	RP1	RP2	RP3	RP1-WE	RP2-WE	RP3-WE
20	91.6 ± 4.0[d]	93.4 ± 9.5[d]	92.2 ± 4.7[d]	92.6 ± 13.9[b]	98.1 ± 4.7[c]	94.3 ± 10.4[b]
50	78.1 ± 9.4**[, d]	89.3 ± 2.4[c, d]	89.0 ± 1.7**[, d]	90.8 ± 2.2[b]	94.8 ± 5.4[c]	91.4 ± 2.9[b]
100	63.1 ± 4.0**[, c]	79.3 ± 3.8**[, c]	74.5 ± 1.8**[, c]	88.6 ± 4.8[b]	90.0 ± 6.0[b, c]	87.4 ± 1.8[b]
200	45.5 ± 2.0**[, b]	64.1 ± 2.0**[, b]	57.1 ± 3.2**[, b]	75.0 ± 1.3**[, a, b]	82.1 ± 3.2**[, a, b]	82.2 ± 2.6**[, a, b]
300	28.8 ± 5.6**[, a]	47.4 ± 2.9**[, a]	37.2 ± 4.2**[, a]	63.2 ± 3.3**[, a]	74.0 ± 2.0**[, a]	70.8 ± 2.5**[, a]

HGF was treated for 48 h with RP samples at the indicated concentrations. The cell viability was determined by MTT assay. Each value represent as mean ± SD (n = 3). **$p < 0.01$, significantly different from vehicle control (PBS). [a-e] Values with different superscript letters in the same column indicate differences among the groups at $p < 0.05$ (ANOVA/Tukey's multiple comparison test).

and RP3 contained glycosides, carbohydrates, reducing sugar, tannin, proteins, peptides, terpenoids, steroids and saponins in common. They didn't have alkaloids equally and ethanol extract of RP1 did not possess even protein and peptides. On the basis of a comparison of water extracts and ethanol extracts of RP1, RP2 and RP3, it is likely that constituents such as flavonoids, phenolic compound, essential oils and acidic compounds exist in only their water extracts. This result gives an intimation of more components come from the water extract of RP samples. Further investigations will be necessary to obtain information about component profile for RP samples.

Safety evaluation – *In vitro* safety test of RP was evaluated through the effects of the RP samples on the HGF viability. As shown in Table 3, the cell viability of the RP samples on HGF decreased in a concentration-dependent manner, ranging from 28.78 to 98.07% at a concentration of 20 - 300 μg/mL. The highest cell viability was exhibited by RP2-WE with the IC_{50} (the inhibitory concentration at 50%) estimated at 442.0 μg/ml, followed by RP3-WE with the second highest viability (IC_{50} = 396.8 μg/mL). The IC_{50} for RP1-WE and RP2 were estimated at 343.6 and 308.2 μg/mL, respectively. Meanwhile, RP1 exhibited the lowest cell viability on HGF with IC_{50} estimated at 211.6 μg/mL. Processing RP in various ways such as recrystallization and water extraction seems to reduce its toxicity towards the cultured cells and these results give the validity to the fact that processed RP has been invariably used in folk medicine for a long time.

In vivo safety evaluation of RP on HGF was measured by acute toxicity test. RP1 was chosen for the lethality test, as it exhibited the lowest IC_{50} value (211.57 μg/mL) for HGF viability. RP1 was orally administered to mice up to 2000 mg/kg body weight. During 14 days after administration, any significant toxic symptom including the death of the animal and change in body and main organ weight was not observed (results not shown). These implied that administered doses were essentially non-toxic and the minimal LD_{50} for RP was found to be greater than 2000 mg/kg.

Table 4. Free radical scavenging effects of RP samples and their inhibitory effects on HAase activity (IC_{50} values)

Treatment	Superoxide[c] (μg/mL)[f]	DPPH[d] (μg/mL)	HAase[e] (μg/mL)
RP1	676.7	1038.0	663.0
RP2	467.1	590.5	758.0
RP3	232.4	949.8	372.9
RP1-WE	620.9	NA	1284.5
RP2-WE	203.9	NA	286.4
RP3-WE	385.6	NA	745.4
Ascorbic acid[a]	NA[g]	157.5	NA
Aspirin[b]	NA	NA	313.0

[a,b]positive control
[c]Superoxide anion radical
[d]DPPH free radical
[e]hyaluronidasse
[f]The values are the means of triplicate data.
[g]NA: not applicable

***In vitro* antioxidant activity** – Oxygen reactive intermediates are generated under normal and/or pathogenic conditions for microbicidal activity, yet they can also be harmful to surrounding cells and matrix components at the inflammation site. So, the antioxidant activities of RP samples were evaluated by measuring their free radical scavenging effects on DPPH and superoxide anion. As shown in Table 4, RP2-WE had the strongest superoxide anion scavenging effect, demonstrated by the lowest IC_{50} value of 203.9 μg/mL compared to the others. RP3, RP3-WE and RP2 also showed a potent effect, as confirmed by the low IC_{50} values of 232.4, 385.6 and 467.1 μg/mL, respectively. RP1 and RP1-WE weakly inhibited superoxide anion formation, by IC_{50} values of 676.7 and 620.9 μg/mL, respectively. Water extracts of RPs previously demonstrated the concentration-dependent increase in electron-donating capacity to DPPH (Cho and Suk, 2002). Hence, in this reporter, the DPPH radical scavenging effects of the unprocessed and processed RP samples,

except for water extracts, were investigated. All of RP1, RP2 and RP3 showed DPPH free radical scavenging effect. Compared with this three samples, RP2 had the strongest effect with an IC_{50} value of 590.5 μg/mL, which was still weaker than that of ascorbic acid as the positive reference drug with IC_{50} of 157.5 μg/mL. RP1 and RP3 exhibited similar IC_{50} values, which were estimated at 1038.0 and 949.8 μg/mL, respectively. These data suggest that the process of recrystallization and water extraction increase free radical scavenging activity of RP. Antioxidant ability plays an important role in the defense mechanism against the harmful effects of the oxygen free radicals in cell. Therefore, above marked HGF viabilities of processed RPs might be due to their potent antioxidant activities.

Inhibitory activity of HAase – HAase (hyaluronidase) is an endohexosaminidase that initiates the degradation of hyaluronic acid, which in turn inhibits the phagocytic ability of macrophages, one of the important reactions in inflammation (Forrester, *et al*., 1990). The effects of the RP samples on HAase activity are shown in Table 4, where all the tested samples reduced HAase activity. In particular, RP2-WE had a stronger inhibitory effect on HAase activity with IC_{50} of 286.4 μg/mL than aspirin used as the positive control with IC_{50} of 313.0 μg/mL. HAase exhibition of RP3 displayed similar to aspirin with IC_{50} values of 372.9 μg/mL. Meanwhile, RP1, RP2, and RP3-WE moderately inhibited HAase activity with IC_{50} values estimated at 663.0, 758.0 and 745.4 μg/mL, respectively. RP1-WE caused the weakest inhibitory effect, as demonstrated by the lowest IC_{50} value at 1284.5 μg/mL compared to the other samples. These results suggest that inhibitory effects of RP2-WE and RP3 against HAase activity may contribute to care and treat inflammatory disease like gingivitis and periodontitis.

Inhibitory activity of PGE_2 production –Prostaglandins are one of the pain neurotransmitters involved in the inflammatory reaction. In particular, PGE_2 plays a crucial role in the pathogenesis of periodontal disease (Saito, *et al*., 1990; Offenbacher, *et al*., 1986). As shown in Table 5, all the RP samples, except for RP1 and RP1-WE, exhibited a stronger inhibitory effect against 1 μM Ca-ionophore A_{23187}-stimulated PGE_2 production in HGF than that of aspirin. Among the tested samples, RP2-WE, RP3-WE, RP3 and RP2 were reduced admirably the production of PGE_2 by 66.5, 57.2, 54.9 and 50.7%, respectively, at a concentration of 250 μg/mL, when compared with aspirin, which was used as a positive control by 42.0 % under the same conditions. Meanwhile, RP1-WE caused a weak inhibition of PGE_2 production by 19.9% and RP1 didn't decrease PGE_2 production in HGF. Considering the important role of PGE_2 in the development of inflammation, these result implied that processed RP samples could be used to help control inflammation associated with periodontal disease, or aid in healing. Moreover, our data adduced grounds for traditional use of the processed RP since early times.

Table 5. Inhibitory effect of RP samples on PGE_2 production in HGF and their analgesic effect on acetic acid-induced writhing in mice

Treatment	PGE_2[1] (%)[3]	Analgesic effect[2] (%)[4]
RP1	<1**, a	96.6 ± 5.9**, c, d
RP2	50.7 ± 5.0c, d	100.0 ± 0.0**, d
RP3	54.9 ± 8.8c, d	82.2 ± 7.9b, c
RP1-WE	19.9 ± 9.5**, b	96.7 ± 2.2**, c, d
RP2-WE	66.5 ± 6.2**, d	93.4 ± 3.2*, c, d
RP3-WE	57.2 ± 2.1c, d	57.2 ± 7.4**, a
Aspirin	42.0 ± 5.8c	NA
Aminopyrine	NA[5]	76.9 ± 6.1b

[1]Gingival fibroblasts were seeded at a density of 1×10^4 cells/well and treated with samples or aspirin (250 μg/mL).
[2]RP sample and aminopyrine were tested at a dose of 200 mg/kg.
[3]Data (n = 4) are represented as mean ± SD. $^{**}p < 0.01$. Significantly different from the positive control group treated with aspirin. [a-d] Values with different alphabet in the same column indicate differences among the groups at $p < 0.05$ (ANOVA/Tukey's multiple comparison test).
[4]Data (n = 5) are represented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$. Significantly different from the positive control group treated with aminopyrine. [a-d] Values with different alphabet in the same column indicate differences among the groups at $p < 0.05$ (ANOVA/Tukey's multiple comparison test).
[5]NA: not applicable

Inhibitory activity of acute inflammation – The *in vivo* anti-inflammatory activities of the RP samples were evaluated using an acute inflammation model of mouse ear edema provoked by AA. Based on topical application, all of the RP samples exhibited the inhibitory effects on ear edema in mice by 47.2 ~ 100.0% at a dose of 250 and 500 μg/ear, as shown in Table 6. Especially, RP2-WE and RP3-WE had superior inhibitory effect by 83.0 - 100.0% at a dose of 250 and 500 μg/ear, respectively. Prednisolone, which was used as a reference steroidal anti-inflammatory drug, showed 88.8% inhibition at a dose of 200 μg/ear. The other samples, except for RP1, RP3 and RP1-WE, also inhibited potently ear edema similar to that of prednisolone at the tested dose. However, the inhibitory activities of RP1 (at a dose of 500 μg/ear), RP3 and RP1-WE (at a dose of 250 μg/ear) were significantly different from that of prednisolone. In agreement with the *in vitro* anti-inflammatory effect, these results clearly demonstrated that RP2-WE as well as RP3-WE possessed potent efficacy against acute inflammation similar to predni-

Table 6. Inhibitory effect of RP samples on AA-induced mouse ear edema

Treatment (μg/ear)	% Inhibition
RP1 250	$79.7 \pm 20.1^{b, c, d}$
RP1 500	$48.1 \pm 12.8^{**, a}$
RP2 250	$69.7 \pm 18.2^{a, b, c}$
RP2 500	100.0 ± 0.0^{d}
RP3 250	$59.9 \pm 13.9^{a, b}$
RP3 500	$81.6 \pm 2.8^{b, c, d}$
RP1-WE 250	$47.2 \pm 19.6^{**, a}$
RP1-WE 500	$83.4 \pm 2.5^{b, c, d}$
RP2-WE 250	$83.0 \pm 9.2^{b, c, d}$
RP2-WE 500	100.0 ± 0.0^{d}
RP3-WE 250	$98.7 \pm 12.9^{c, d}$
RP3-WE 500	100.0 ± 0.0^{d}
Prednisolone	$88.8 \pm 13.1^{b, c, d}$

RP samples or prednisolone was applied topically to the ears of mice. Data represent mean ± SD (n = 10). $^{**}p < 0.01$. Significantly different from the positive control group treated with prednisolone. $^{a-d}$ Values with different superscript letters indicate differences among the groups at $p < 0.05$ (ANOVA/Tukey's multiple comparison test). RP1, crude RP; RP2, recrystallized RP; RP3, Ramus Mori Albae-treated RP; RP1-WE, water extract of RP1; RP2-WE, water extract of RP2; RP3-WE, water extract of RP3.

solone. Based on previous findings for *in vitro* tests, it is thought that anti-inflammatory activities of RP samples might be largely due to inhibition of PGE_2 production. Accordingly, our data support the speculation that processed RP will be useful in the immediate care and treatment of periodontal disease, inhibiting inflammation of and damage to periodontal tissues surrounding the teeth.

Analgesic activity by writhing test – The analgesic efficacy of RP samples was investigated, as pain is another diverse symptom of inflammation. The writhing test is widely used to measure the degree of pain relief, and an intraperitoneal injection of acetic acid known to induce a prostaglandin-mediated rapid onset of pain, including constriction of the abdomen, turning of the trunk (twist), and extension of the hind legs (Mogil, *et al*., 1996). As presented in Table 5, all the RP samples tested, with exception of RP3-WE, exhibited stronger analgesic activities against the acetic acid-induced writhing responses of mice than aminopyrine. Among the RP samples, RP-2 prevented perfectly the writhing by 100.0% at the oral dose of 200 mg/kg, while aminopyrine, the positive control, showed 76.9% analgesic effect under the same condition. RP1, RP3, RP1-WE and RP2-WE also exhibited excellent inhibition against the acetic acid-induced writhing by 96.6, 82.2, 96.7 and 93.4%, respectively at the dose of 200 mg/kg. But RP3-WE exposed less inhibition by 57.2% than that of aminopyrine. Based on *in vitro* findings, analgesic activities of RP samples on writhing response are likely to be attributable to their inhibitory activities against PGE_2 production, mainly. Consequently, these results suggest that oral administration of RP samples can be used to block and control the pain associated with dental caries and periodontal diseases, since these efficacies of RP samples against chemical (acetic acid injection) painful stimulus are characteristic of peripheral analgesic properties like aminopyrine.

Conclusion

Among tested RP samples, RP2-WE exhibited the most effective inhibition against inflammation and pain as well as excellent safety *in vitro and in vivo,* and its efficacy was found to be the superior or equivalent to that of NSAIDs (Aspirin, Aminopyrine) and other medicine, prednisolone. These results provides evidence clearly suggesting that RP2-WE can be safely applied to treat and control the progression of inflammation and damage of periodontal tissues. Consequently, recrystallized aqueous extract of Resina Pini may be a promising agent for periodontal diseases.

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