**Evaluation of Biological Activity of Mastic Extracts Based on Chemotherapeutic Indices**

RYUICHIRO SUZUKI¹, HIROSHI SAKAGAMI², SHIGERU AMANO², KUNIHIKO FUKUCHI³, KATSUYOSHI SUNAGA¹, TAISEI KANAMOTO³, SHIGEMI TERAKUBO⁴, HIDEKI NAKASHIMA⁴, YOSHIKI SHIRAKI¹, MINEKO TOMOMURA², YOSHIKO MASUDA², SATOSHI YOKOZE², AKITO TOMOMURA², HIROFUMI WATANABE³, MASAKI OKAWARA³ and YOSHIHARU MATAHIRA⁵

¹Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, Sakado, Japan; ²Meikai University School of Dentistry, Sakado, Japan; ³Graduate School, Showa University, Tokyo, Japan; ⁴St. Marianna University School of Medicine, Kawasaki, Japan; ⁵Sunsho Pharmaceutical Co., Ltd., Fuji, Japan

**Abstract.** Background: Most previous mastic investigators have not considered its potent cytotoxicity that may significantly affect the interpretation of obtained data. In the present study, we re-evaluated several biological activities of mastic extracts, based on chemotherapeutic indexes. Materials and Methods: Pulverized mastic gum was extracted with n-hexane and then with ethyl acetate or independently with methanol or n-butanol. Tumor specificity (TS) of the extracts was determined by their cytotoxicity against human malignant and non-malignant cells. Antibacterial activity was determined by their cytotoxicity against bacteria and normal oral cells. Antiviral activity was determined by their protection of viral infection and cytotoxic activity. Cytochrome P450 (CYP) 3A4 activity was measured by β-hydroxylation of testosterone. Results: Ethyl acetate extract showed slightly higher tumor specificity (TS=2.6) and one order higher antibacterial activity (selectivity index (SI)=0.813) than other extracts (TS=1.4-2.5; SI=0.030-0.063). All extracts showed no anti-human immunodeficiency virus (HIV) activity, but some anti-herpes simplex virus (HSV) activity, which was masked by potent cytotoxicity. They showed strong inhibitory activity against CYP3A4. Conclusion: Ethyl acetate extraction following the removal of cytotoxic and CYP3A4 inhibitory substances by n-hexane can enhance antitumor and antibacterial activity of mastic.

Mastic is the extract of sap from *Pistacia lentiscus*, grown only in the Chios island of Greece. Due to its unique shape and diverse efficacy, mastic has been called “the tear drop of Christ”. Mastic extracts have been reported to show antitumor activity (including the enhancement of anticancer drugs’ action and induction of apoptosis via oxidative stress) (1, 2), antioxidant activity (that correlated with phenolic and flavonoid contents) (3-8), antibacterial activity (9-11), modulating activity of drug-metabolizing enzymes (12, 13) and antiviral activity (14-17). However, most previous investigators did not describe the chemotherapeutic index of these activities (that is the ratio of the biological activity to cytotoxicity), even though there is a cautionary note that mastic extracts showed potent cytotoxicity (18).

In the present study, we separated the crude mastic extract into 5 different fractions using organic solvents and, then, re-examined the biological activity of unfractionated and fractioned extracts, based on chemotherapeutic indexes.

**Materials and Methods**

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI 1640 medium, doxorubicin, azidothymidine (AZT), 2′,3′-dideoxyctydine (ddC) (Sigma-Aldrich Inc., St. Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chemical Ind., Ltd., Osaka, Japan), curdlan sulfate (CRDS) (79 kDa; Ajinomoto Co. Inc., Tokyo, Japan). Culture plastic dishes and plates (96-well) were
Fractionation of mastic by organic solvent extraction. Pulverized mastic gum (5 g) was infused with n-hexane (50 ml) at room temperature for 24 h to give n-hexane soluble fraction. Subsequently, the residue was re-extracted with ethyl acetate (50 ml) by the same way to afford ethyl acetate soluble portion. Quite separate from this, pulverized mastic gum (5 and 10 g) had been prepared and was infused with methanol (macerated) and n-butanol for 24 h at room temperature, independently. Furthermore, pulverized mastic gum (10 g) was extracted with methanol in reflux to prepare methanolic extract. The organic solvent of each soluble portion was evaporated under reduced pressure affording corresponding extract (n-hexane extract, 2.1 g; ethyl acetate extract, 2.4 g; methanol extract (macerated), 3.6 g; methanol extract (refluxed), 6.4 g; and n-butanol extract, 9.0 g).

Assay for cytotoxic activity. Human squamous cell carcinoma cell lines (Ca9-22, derived from gingiva and HSC-2, HSC-3, HSC-4 derived from tongue, all purchased from Riken Cell Bank, Tsukuba, Japan, and human normal oral cells [gingival fibroblast (HGF), periodontal ligament fibroblast (HPLF) and pulp cell (HPC)], established from the first premolar tooth extracted from a 12-year-old girl (19), were inoculated at 2.5×10⁴ cells/0.1 ml in a 96-microwell plate. After 48 h, the medium was replaced with 0.1 ml of fresh medium containing different concentrations of each sample. Cells were incubated further for 48 h and the relative viable cell number was then determined by the MTT method (20). The relative viable cell number was determined by the absorbance of the cell lysate at 562 nm, using a microplate reader (Infinite F50R, TECAN, Kawasaki, Kanagawa, Japan). Control cells were treated with the same amounts of DMSO and the cell damage induced by DMSO was subtracted from that induced by test agents. The concentration of compound that reduced the viable cell number by 50% (CC₅₀) was determined from the dose-response curve and the mean value of CC₅₀ for each cell type was calculated from triplicate assays.

Tumor specificity index (SI) was calculated using the following equation: SI = mean CC₅₀ against normal cells / mean CC₅₀ against tumor cells (D/B) in Table 1). Since Ca9-22 cells, as well as HGF cells, were derived from gingival tissue (21), the relative sensitivity of these cells was also compared ((C/A) in Table 1).

Assay for antibacterial activity. Streptococcus mutans ATCC 25175, Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923 (all from American Type Culture Collection (ATCC), Manassas, VA, USA) were grown in Brain Heart Infusion (BHI) medium under aerobic conditions. Porphyromonas gingivalis 381 and Fusobacterium nucleatum ATCC 31647 were grown in Gifu Anaerobic Medium (GAM) containing 5 μg/ml hemin and 1 μg/ml menadione under anaerobic conditions with mixed gas of nitrogen (83%), hydrogen (7%) and CO₂ (10%). Aerobic and anaerobic menadione under anaerobic conditions with mixed gas of nitrogen medium under aerobic conditions. Porphyromonas gingivalis 381 of bacterial growth (IC₅₀) was determined. The selectivity index containing serially diluted mastic fractions or vehicle (DMSO), with (SI) was determined by the ratio of IC₅₀ to CC₅₀ against human normal oral cells (D in Table I).

Measurement of CYP3A4 activity. CYP3A4 activity was measured by β-hydroxylation of testosterone in human recombinant CYP3A4 (Cypex Ltd., Dundee, UK). The reaction mixture, containing 200 mM potassium phosphate buffer (pH 7.4), NADPH regenerating system (1.3 mM NADPH, 1.3 mM glucose-6-phosphate, 0.2 U/ml glucose-6-phosphate dehydrogenase and 3.3 mM MgCl₂) and the human recombinant CYP3A4 (16.5 pmol/ml) along with 0, 1, 3, 11, 33 and 100 μg/ml of mastic (dissolved in DMSO), was pre-incubated at 37°C for 5 min. The reaction was started by the addition of 300 μM testosterone substrates. The final volume of the reaction mixture was 250 μl with a final DMSO concentration of 2%. The reaction was stopped by the addition of 500 μl ethyl acetate after 15 min. After centrifugation (15,000 x g for 5 min), 400 μl of supernatant was collected, dried and re-suspended in 100 μl of methanol. Analyses of the metabolites were performed by high-performance liquid chromatography (HPLC) (JASCO PU2089, AS2057, UV2075 ChromNAV equipped with a TSK gel ODS-120A, 4.6 mm ID×25 cm, 5 μm column (TOSOH, Tokyo, Japan). The mobile phase consisted of 70% methanol and 30% water. The metabolites were separated using an isocratic method at a flow rate of 1.0 ml/min. Quantification of the metabolites was performed by comparing the HPLC peak area at 254 nm to that of 11α-progesterone, the internal standard. The retention times for β-hydroxytestosterone and 11α-progesterone were approximately 5.0 and 6.7 min, respectively. The concentration that inhibited the CYP3A4 activity by 50% (IC₅₀) was determined from the dose-response curve.
Statistical treatment. Experimental values are expressed as the mean±standard deviation (SD). Statistical analysis was performed by using Student’s t-test. A p-value <0.05 was considered to be significant.

Results

Mastic components were separated by organic solvents into n-hexane extract, ethyl acetate extract prepared following n-hexane extraction, as well as with methanol extract and n-butanol extract, either macerated or refluxed, as described in Materials and Methods, and subjected to assay for various biological activities.

Tumor specificity. All extracts showed higher cytotoxicity against four human oral squamous cell carcinoma cell lines (Ca9-22, HSC-2, HSC-3, HSC-4) (CC50 ranged from 13.5 to 24.4 μg/ml) as compared with three human normal oral cells (HGF, HPLF, HPC) (ranged from 28.1 to 84.8 μg/ml), giving TS indexes from 1.4 to 2.6 (determined by the ratio of D/B) and from 1.3 to 2.3 (determined by the ratio of A/C) (Table I). Among them, ethyl acetate extract showed the highest TS values (TS=2.6), although its values were two-order lower than that of doxorubicin (TS=244.7), which was used as positive control.

Antibacterial activity. All extracts significantly (p<0.05) reduced the viable cell number of Streptococcus mutans in dose-dependent manners (Figure 1A). Ethyl acetate extract showed approximately eight or nine times higher antibacterial activity (IC50=104 μg/ml), as compared with other fractions (831-936 μg/ml). It should be noted that ethyl acetate extract at 1,000 μg/ml completely eliminated the bacteria, while treatment with unfractionated sample retained 27% of the bacteria viable (Figure 1A). When IC50 value was divided by mean CC50 value against three human normal oral cells, SI values could be obtained (Table II). Ethyl acetate extract showed the highest SI value of antibacterial activity (0.813), followed by unfractionated (0.587) > methanol (reflux) (0.063) > methanol (coolly immersed) (0.050) > n-butanol (0.034) > n-hexane extractable fractions (0.030) (Table II).

The most sensitive bacterial strain was Porphyromonas gingivalis (IC50=32.7 μg/ml), followed by Staphylococcus mutans (IC50=104 μg/ml), Staphylococcus aureus (IC50=609.4 μg/ml), Fusobacterium nucleatum (IC50=630 μg/ml), and Escherichia coli (IC50=1,000 μg/ml). Each point represents mean from three independent assays. *Significant reduction of viable bacterial number (p<0.05).
Fusobacterium nucleatum (IC$_{50}$=759.6 μg/ml) and Escherichia coli (IC$_{50}$=907.4 μg/ml) (Figure 1B).

**Antiviral activity.** When HTLV-I-bearing CD4-positive human T-cell line MT-4 cells were infected with HIV-1$_{IIIB}$ at a MOI of 0.01, viability was reduced to 18.8±1.9% (n=9) of mock-infected cells (calculated by the absorbance value of control mock- and HIV-infected cells). All mastic extracts did not prevent HIV-induced cytopathic effects (SI<1) (A-F), whereas three anti-HIV agents (AZT, ddC, CRDS) showed excellent anti-HIV activity (SI=5,624, 3,868, 7,142) (G-I) (Figure 2).

When Vero cells were infected with HSV-1 (MOI=0.01), viability was reduced to 12.5±7.6% (n=6) of mock-infected cells (Figure 3). All mastic extracts partially but significantly (p<0.05) reduced the HSV-induced cytopathic effects, recovering the cell viability up to 43.2±5.3% of mock-infected cells. Lower recovery of cell viability (less than...
50%) did not allow us to calculate the SI value. Higher concentrations of extracts reduced the anti-HSV activity, possibly due to the potent cytotoxicity of mastic (Figure 3).

**CYP3A4 inhibitory activity.** All mastic extracts were found to be potent inhibitors of CYP3A4, as judged from β-hydroxylation assay with testosterone (Figure 4). n-Hexane extract (B) exhibited the highest CYP3A4-inhibitory activity (IC₅₀=3.1 μg/ml), followed by methanol extract (macerated) (IC₅₀=4.1 μg/ml) (E), n-butanol extract (IC₅₀=12.1 μg/ml) (D), unfractionated sample (IC₅₀=14.3 μg/ml) (A), ethyl acetate extract (IC₅₀=14.8 μg/ml) (C) and, finally, methanol extract (refluxed) (IC₅₀=24.4 μg/ml) (F).

**Discussion**

The present study demonstrated, for the first time, that ethyl acetate extract of mastic prepared after n-hexane washing showed slightly higher antitumor (TS=2.6) (Table I) and antibacterial activity (SI=0.813) (Table II) compared to unfractionated mastic (TS=2.0, SI=0.587) (Table II). This may be due to the removal of cytotoxic substances by rinsing with n-hexane (Tables I and II). However, antitumor activity of all mastic extracts is much lower than that of anticancer drugs (TS=4-2,961) (25). It has recently been reported that the cytotoxic effect of antineoplastic drugs (cisplatin, 5-fluorouracil and etoposide) against FTC-133 thyroid cancer cells was enhanced by essential oils from the aerial parts (leaves, twigs and berries) of *Pistacia lentiscus* (1). It, thus, remains to be investigated whether ethyl acetate extract of mastic and antitumor agents show such synergistic effect in the tumor cells.

It is surprising that mastic extracts show very potent CYP3A4 inhibitory activity. CYP3A4 is the most abundant CYP family (26) and, therefore, inhibition of CYP3A4 by mastic should increase the pharmacological action (in a good way) or side-effects (in a bad way) of concomitantly administered drugs. Washing out these CYP3A4 inhibitory substances with n-hexane may reduce these pharmacological action or side-effects of combined drugs.

We also found that ethyl acetate extract of mastic selectively killed *Porphyromonas gingivalis*. This microorganism has been reported to dominate the biofilm community (27), colonize the microbial flora around dental implants (28), be present into the saliva and pooled subgingival plaque samples of aggressive periodontitis (29) and Fanconi's anemia patients (30). Toothpaste that contains of mastic is available in Japan. Ethyl acetate extract of

![Figure 3. Anti-HSV activity of mastic extracts.](image)
mastic, which has higher antibacterial activity than unfractionated mastic, may be appropriate for the treatment of periodontal diseases.

We found that mastic has some anti-HSV activity, but not anti-HIV activity, suggesting that the antiviral mechanism of mastic against these two viruses is different. Removal of cytotoxic substances may further enhance the anti-HSV activity, since anti-HSV activity seems to be masked by cytotoxic substances (judging from the overlap of loss of viability and that of anti-HSV activity; Figure 3). Further

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**Table I. Cytotoxicity and tumor specificity of mastic extracts.**

<table>
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<tr>
<th></th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (μg/ml)</th>
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<th>Human oral normal mesenchymal cells</th>
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<td></td>
<td>(A)</td>
<td>(B)</td>
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<td>Unfractionated</td>
<td></td>
<td>Ca9-22 22.4</td>
<td>HSC-2 26.9</td>
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<td>n-Hexane extract</td>
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<td>HSC-3 16.8</td>
<td>HSC-4 27.1</td>
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<td>n-BuOH extract</td>
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<td>HGF 43.9</td>
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<td>MeOH extract (Reflexed)</td>
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<td>DXR</td>
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<td>DXR 0.26</td>
<td>HGF 0.14</td>
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<td>DXR</td>
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<td>DXR 0.26</td>
<td>HGF 0.14</td>
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HGF, Human gingival fibroblast; HPC, human pulp cells; HPLF, human periodontal ligament fibroblast; CC<sub>50</sub>, 50% cytotoxic concentration; DXR, doxorubicin; TS, tumor specificity; EtOAc, ethyl acetate; BuOH, butanol; MeOH, methanol; SD, standard deviation. Oral squamous cell carcinoma cell lines: Ca9-22 (derived from gingival tissue), HSC-2, HSC-3 and HSC-4 (derived from tongue). Each value represents mean of triplicate assays.
Table II. Antibacterial activity of mastic extracts.

<table>
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<tr>
<th>Fractionation</th>
<th>Antibacterial activity IC₅₀ (µg/ml) (A)</th>
<th>Cytotoxicity against normal oral cells CC₅₀ (µg/ml) (D)</th>
<th>SI (D/A)</th>
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<td>n-Hexane</td>
<td>936</td>
<td>28.1</td>
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<td>EtOAc</td>
<td>104</td>
<td>84.8</td>
<td>0.813</td>
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<tr>
<td>n-BuOH</td>
<td>900</td>
<td>30.8</td>
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<td>MeOH (Macerated)</td>
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<td>MeOH (Reflected)</td>
<td>831</td>
<td>52.6</td>
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</table>

SI, Selectivity index; EtOAc, ethyl acetate; BuOH, butanol; MeOH, methanol; CC₅₀, 50% cytotoxic concentration; IC₅₀, half-maximal inhibitory concentration. Each value represents mean of triplicate assays.

studies are needed to elucidate the mechanism(s) of antiviral action and investigate the possible synergistic effect(s) of mastic with acyclovir.

Conflicts of Interest

This study was funded, in part, by Sunsho Pharmaceutical Co., Ltd. (Hiroshi Sakagami), causing, however, no prejudice against the impartiality of the research reported.

References


