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Improved systemic delivery of insulin by condensed drug loading in a dimpled suppository

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Summary **The development of peptide therapeutics owing to the advances in biotechnology has overcome some unmet medical needs; however, the route of administration is still limited to injections. Systemic delivery of insulin *via* an enteral route remains a great challenge due to its instability and low mucosal permeability. In this study, we investigated the effect of drug condensation in a suppository on the efficacy of insulin after rectal administration. Suppositories with dimples are prepared by a mold method using a hard fat (Suppocire® AM). Insulin or fluorescein isothiocyanate-dextran (molecular weight: 3,000-5,000) (FD4) as a model of a hydrophilic macromolecule was loaded in the dimples, and sealed with other lipids with different melting points. The *in vitro* release test showed that the time to 50% drug release depends on the melting point of the lipid for sealing but not on the number of dimples. The suppositories with one-, or three-dimple containing insulin and caprylocaproyl macrogol-8 glyceride (Labrasol®) were administered to rats at 0.5 U/head. The reduction in plasma glucose level was more significant for the one-dimple-type suppository than for the three-dimple-type although the one-dimple-type suppository contained less amount of Labrasol by one-third compared to the three-dimple-type. These results suggest that condensation of an insulin dose in a limited surface area of a suppository improves systemic availability *via* the rectal route with a reduced amount of an absorption enhancer.**

Keywords: Dimple type suppository, insulin, peptide, rectal delivery, Labrasol

1. Introduction

The recent progress in biotechnology has overcome some of the problems related to an unestablished field of therapeutics (*1*). The novel highly functional peptide therapeutics, such as agents for cancer, diabetes mellitus, cardiovascular diseases, and rheumatoid arthritis, are clinically accessible and were found to have a favorable clinical effect (*2-4*). Although these peptides drastically improve the symptoms, their administration method is limited to injections, which lead to patient inconvenience due to pain, risks of infection, and the need for continuous ambulant use. In addition, polypeptides are scarcely absorbed in the gastrointestinal tract due to their high susceptibility to the digestive and mucosal enzymes (*5-7*) in addition to their poor membrane permeability (*8*). To overcome these drawbacks of peptide therapeutics, alternative routes of administration have been investigated by many researchers. Nasal (*9*) and pulmonary (*10,11*) routes are the representative alternatives. The bioavailability of insulin *via* pulmonary route is 10-46% (*12*). Exubera®, the inhalation formulation of insulin, was marketed, although the product is withdrawn.

Oral route is the most attractive route of administration but tends to produce significant variability in absorption rate and absolute availability because of various factors, *e.g.*, gastric emptying and gut motility, digestive enzymes, pH, luminal contents, among others. Rectal route is another alternative route of administration for peptides (*13,14*). Although some proteolytic enzymes exist in the rectal mucosa (*15*), the peptides administered rectally are less degraded than those ingested orally, owing to the presence of few secretory digestive enzymes in the rectum. In addition, it has been reported that the proteolytic enzyme activities of the rectal epithelial cells are lower than those of rat nose, lung,

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and Caco-2 cells (*15*).

Besides the investigation of alternative routes, improvement of intestinal absorption of polypeptide drugs has also been investigated by many researchers, such as by suppressing enzymatic degradation *via* co-administration of protease inhibitors (*16*) and enhancing the penetration across the mucosal epithelium through use of an absorption enhancer (*17-19*). There are two distinctive approaches to improve the intestinal absorption of peptides: reducing pH at the limited site of drug administration, where the activities of peptidases are suppressed (*20,21*) and using concentration gradient as a driving force by dosing with a highly concentrated drug at the limited site (*22,23*). In the case of drug absorption through passive diffusion, the rate of absorption essentially depends on the concentration in the lumen when the blood drug concentration is low enough, *i.e.* a sink condition can be assumed.

In the present study, to improve the enteral delivery for poorly absorbable drugs such as peptides, we designed a suppository with dimples where a drug is located only in a limited area and released at a higher rate when administered in the rectum (Figure 1). Insulin, was chosen as a model peptide drug and the effect of drug condensation in a dimple of a suppository on rectal delivery was evaluated in terms of plasm glucose level. Labrasol, which is composed of polyethylene glycol (PEG) esters, a small glyceride fraction, and free PEG, is a non-ionic water dispersible surfactant. We selected Labrasol as a nontoxic absorption enhancer for insulin, which is not only not irritable to the intestinal mucosa but also does not interact with other lipoid pharmaceutical additives such as hard fat.

Sinko *et al*. reported that a bolus intestinal administration of salmon calcitonin as a higher concentration solution provided higher bioavailability than the continuous administration of a lower concentration solution (*20*). We assumed that a rapid drug release, *i.e*. a rapid melting of the sealing material of the dimples, is required for improving the rectal absorption of insulin. In addition to the selection of the sealing material suitable for the rapid release, setup of the suitable evaluation method is also important. To evaluate *in vitro* release from a suppository, basket or paddle method (*24*), dialysis membrane method (*24,25*), and flow-through method (*26*) are used commonly. However, very few of the *in vitro* tests for suppositories are for the fast release-type suppository. For the fast release-type suppository, the drug release rate through a semipermeable membrane in the dialysis membrane method is not negligible in comparison with the release rate from a suppository. The European Pharmacopoeia suggests that *in vitro* release test should be adopted as the property of suppository, although it introduces flow-through method for the *in vitro* release test of suppository (*24*). In this study, we used the system illustrated in Figure 2. By placing a suppository fixed by mesh with the dimple side up, the drug phase composed of low-gravity oil can float in medium as soon as the sealing melts. Thereafter, the drug in the dimple can be released. To select an appropriate sealing material for rapid release, we examined drug release from suppositories using the above described method with fluorescein isothiocyanate-dextran (molecular weight of 3,000-5,000 (FD4)) as a marker.

The goal of this study was to clarify if a dimple-type suppository is useful for improving rectal insulin delivery and to suggest the feasibility of the dimple-type suppository for the rectal absorption of biomedicines including peptides as well as oligonucleotides.

2. Materials and Methods

2.1. *Materials*

Fluorescein isothiocyanate-dextran with a molecular weight of 3,000-5,000 (FD4) was purchased from Sigma-Aldrich Co., Ltd. (USA). Bovine insulin (from bovine pancreas, 27 USP-U/mg) was purchased from Sigma-Aldrich (St. Louis, USA). Suppocire® AM pastilles and Labrasol® were donated by Gattefossé (Lyon, France). Soybean oil was purchased from Wako Pure Chemicals (Osaka, Japan). A glucose test kit (Glucose C-test Wako) was purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents were of reagent grade.

2.2. *Preparation of dimple-type suppository*

Suppocire AM pastilles molten at 60°C were cast into an aluminum template (0.4 mL) and solidified at 4°C to form the molded sample (ϕ: ca.4 mm × height: ca.30 mm). One, three, or five dimples (diameter: 2.5-3.0 mm)

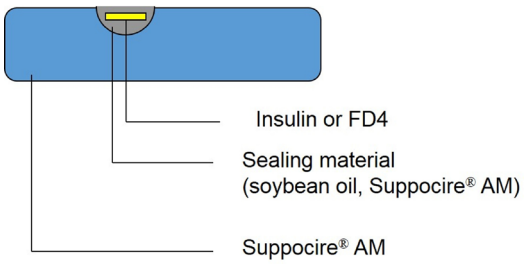

Figure 1. Illustration of a dimple type suppository.

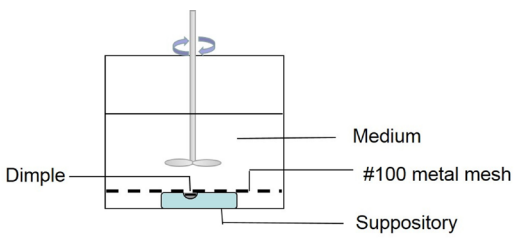

Figure 2. Scheme of the apparatus for the dissolution test.

were created on the lateral face of sample; 5 μL of 100°C glycerin was poured onto the lateral face to melt the surface and vacuum off the molten surface.

2.3. *Drug loading to the dimples of suppository*

Fifty percent (w/v) of Labrasol aqueous solution in which insulin or FD4 was dissolved was used as a loading solution. The solution was loaded onto the dimples of a suppository *via* the following procedure; 2 μL of a molten sealing material (soybean oil, a mixture of Suppocire AM pastilles and soybean oil (10:14, w/w) or Suppocire AM alone was loaded onto each dimple and solidified, following which 2.5 μL of the loading solution was poured into each dimple and allowed to dry in a desiccator for a couple of hours, and each dimple was covered with 2.5 μL of the sealing material. The final amount of insulin and FD4 was 0.5 IU and 100 μg in a suppository, respectively. Each dimple has 1 mg of Laborasol. The obtained suppositories were stored at −20ºC until use.

2.4. *In vitro release test*

In vitro release test was performed using the apparatus illustrated in Figure 2. Namely, a suppository was placed into 9.6 mM phosphate buffered saline (pH7.2; 20 mL) with the dimple side up at 37°C and covered by #100 metal mesh. The medium was stirred by a propeller mixer (ϕ 20 mm) at 100 rpm and kept at 37°C. Five-hundred microliters of the medium was collected at 30, 45, 60, 90, 120, 180, and 300 sec and fresh phosphate buffered saline (500 μL) was added. After all the samples were collected, the residual drug was extracted. Briefly, rest of the medium and the residual suppository were collected in a test tube and methylene chloride (5 mL) was added. The mixture was shaken at 100 rpm for 30 min and centrifuged at 2,000 rpm to collect the aqueous layer. The concentration of FD4 in the samples and the aqueous layer was determined using fluorescent spectrometry using the hybrid multi-mode microplate reader (Synergy H4, BioTek Instruments, Winooski, USA). The test was performed in triplicate. The time to 50% drug release (D_{50}) was calculated from the fitting curve of quadratic approximation on each dissolution profile.

2.5. *Animal experiments*

All animal experiments were performed in line with the Guidelines for Animal Experiment at Osaka Otani University. Male Wistar rats weighing 185-230 g were allowed to fast with free access to water for 16 h and anesthetized by an intraperitoneal administration of 50 mg/kg sodium pentobarbital. The jugular vein was exteriorized by surgery, and 50 μL blood was collected as blank. A suppository was administered into the rectum and the anus was immediately tied tightly to prevent leakage of the meltage. Fifty microliters of blood sample was collected from the jugular vein at the predetermined times. The blood samples were immediately heparinized to separate the plasma by centrifugation (12,000 rpm for 5 min). The glucose concentration in plasma was measured by the glucose test kit.

2.6. *Statistical analysis*

Experiments were replicated at least thrice. The differences between means for two groups were statistically analyzed using Student's *t*-test. *P* values < 0.05 indicated significant difference.

3. Results

3.1. *In vitro drug release from the dimple suppository*

The influence of sealing materials on the drug release from a dimple suppository was examined using FD4 as a maker. As shown in Figure 3a, the suppositories containing the mixture of Suppocire AM and soybean oil (10:14) (B type) showed the smallest D_{50} of 44.8 ± 8.8 sec (mean ± S.E.), while those containing soybean oil alone (A type) and Suppocire AM alone (C type) had D_{50} values of 103.5 ± 7.6 sec and 61.6 ± 20.0 sec, respectively. The drug release was proven to be independent of the number of dimples (Figure 3b).

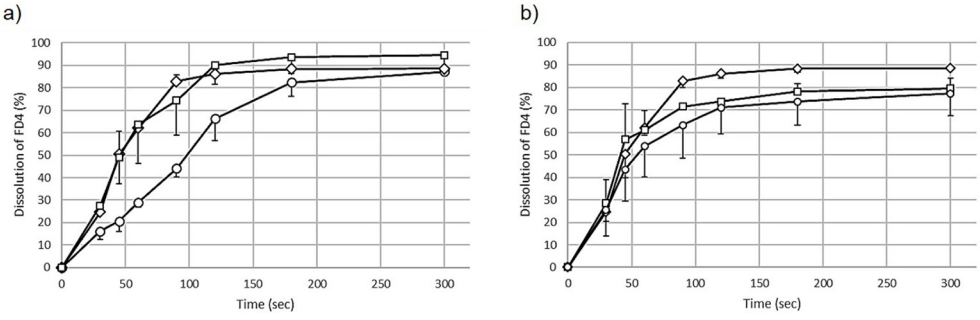

Figure 3. *In vitro* release from suppositories. a) Influence of the sealing material on drug release; ○: soybean oil (A type), ◊: the mixture of Suppocire® AM and soybean oil (= 10:14) (B type), □: Suppocire® AM (C type). **b)** Influence of the number of dimples on drug release, ◊: one-dimple, □: three-dimple, ○: five-dimple. FD4 1 mg/suppository. Labrasol 1 mg/dimple. Data represent mean ± S.E. (*n* = 3 batches).

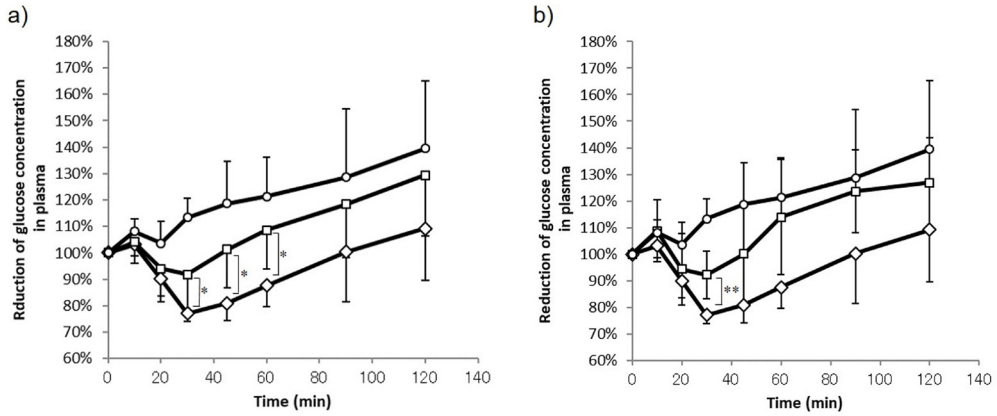

Figure 4. Plasma glucose concentration after the rectal administration of suppository. a) Effect of Labrasol on the reduction of glucose (◊: 0.5 U insulin and 1 mg LabrasolTM, □: 0.5 U insulin, ○: placebo). **b)** Influence of the number of dimples on the reduction of glucose (◊: one-dimple, □: three-dimples) or placebo (○). Insulin: 0.5 U/suppository. Labrasol^{TM}: 1 mg/dimple. Data represent mean ± S.D. (n = 6). The symbols * and ** represent $p < 0.05$ and $p < 0.01$, respectively.

The release from one-, three-, and five-dimple B-type suppositories was approximately 80-90% after 90 sec with D_{50} values of 44.8 ± 8.8 sec, 48.3 ± 9.3 sec, and 64.0 ± 27.4 sec (the mean ± S.E.), respectively. Consequently, no significant differences in drug release were detected among the suppositories with different number of dimples.

3.2. *Effect of Labrasol on the rectal absorption of insulin from the dimple suppository*

The plasma glucose level in rats after the administration of insulin-loaded one-dimple B-type suppository is shown in Figure 4a. The maximum reduction of plasma glucose level was observed at 30 min, with 22.9 ± 3.2% and 8.2 ± 5.8% (mean ± S.D. as the percentage to the initial level) reduction with and without Labrasol, respectively. The difference was statistically significant from 30 min to 60 min ($p < 0.05$).

3.3. *Effect of the number of dimples on the rectal absorption of insulin*

One- or three-dimple B-type suppositories loaded with insulin and Labrasol were administered to rats. The time profiles of plasma glucose level are shown in Figure 4b. Insulin (0.5 IU) and Labrasol (1 mg)-loaded one-dimple suppository reduced plasma glucose concentration up to 22.9 ± 3.2% (mean ± S.D.), while the three-dimple suppository in which insulin (0.5 IU) and Labrasol (3 mg) were divided into three dimples reduced plasma glucose level up to 7.8 ± 8.9% (mean ± S.D.). There was a statically significant difference in the reduction of plasma glucose level at 30 min between one- and three-dimple suppositories ($p < 0.01$).

4. Discussion

The absorption rate for a drug *via* passive transport depends on the drug concentration in the intestinal lumen. Therefore, we assumed that a rapid drug release, *i.e.* a rapid melting of the sealing material of the dimples, is required for improving the rectal absorption of insulin from the dimple suppository. First, we designed the fast release-type suppository. The drug release from the dimple-type suppository we used in this study was studied using the *in vitro* release test; the suppository with Suppocire AM alone and with the mixture of Suppocire AM-soybean oil as the sealing material, showed a more rapid release than that with soybean oil alone. FD 4 was observed to be mixed easily with soybean oil, a sealing material in a dimple, but not with the other sealing materials (data not shown). The suppository with soybean oil alone in a dimple provided a slower release of FD4 owing to the entrapment of FD4 into soybean oil, which retarded its release. With respect to variability among batches, the suppositories in which dimples were sealed with soybean oil alone and with the mixture of Suppocire AM-soybean oil showed less release variability than that with Suppocire AM alone. The melting points of Suppocire AM and soybean are around 37°C and −9°C, respectively. The 10:14 mixture of Suppocire AM and soybean oil was designed to be in solid state at 25°C and to melt at 32°C within 5 sec. It is speculated that the two sealing materials with lower melting points melt quickly with the least variability at 37°C, which leads to their sealing off within less time regardless of batches. Thus, in terms of rapid release and less variability, we selected the mixture of Suppocire AM-soybean oil as a sealing material for insulin-loaded suppository.

In this study, we used Labrasol as an absorption enhancer and showed that Labrasol is effective for the improvement of rectal insulin delivery. With respect to the mechanism of the enhancement by Labrasol, suppression of drug efflux due to P-gp (*27*), interaction with the lipid bilayer of the epithelial cell (*28*), and opening of the tight junction (*29*) are reported. However,

the suppression of drug efflux appears to contribute the least to the absorption enhancement of insulin because most of the substrates for P-gp are hydrophobic compounds.

Next, we evaluated the influence of number of dimples on rectal insulin delivery in the presence of Labrasol (Figure 4b). The results suggested that the localization of insulin in a suppository can improve the rectal absorption of insulin, although there seems to be little difference in the drug release rate between one- and three-dimple suppositories (Figure 3b). The insulin concentration in a dimple of one-dimple suppository is three times higher than that of three-dimple suppository. The loaded insulin in a dimple is covered by the hard fat except for at the sealing site. Therefore, the release of insulin from the dimple suppository is limited only towards the rectal mucosa without dilution by the luminal bulk fluid because insulin covered by the suppository base is restricted to diffuse to the lumen. The prevention of dilution of insulin by the luminal fluid or contents provides a high concentration of drug between the epithelium and a suppository. The concentration gradient between the epithelial surface and blood is the driving force for the drug to permeate across the mucosa. It is reported that dosing with a highly concentrated drug at the limited site leads to the improvement of the intestinal absorption of caffeine (*22*) *via* concentration gradient as a driving force. This could explain the improvement of the rectal delivery of insulin by one-dimple suppository.

The higher concentration may also induce the saturation of enzymatic degradation. It is reported that dosing with a highly concentrated drug at the limited site leads to saturation of enzymatic degradation to improve the intestinal absorption of erythropoietin (*23*). Among the enzymes that decompose insulin, insulin-degrading enzymes have been well investigated and it was revealed that decomposition of insulin by the enzymes played an important role in the intestinal absorption of insulin (*30-32*). It is also reported that the enzyme is located in the cytosol of rat small intestine mucosal cells, human colon mucosal cells, and Caco2 cells. The Km of the cytosolic insulin degrading activity (78 nM) is comparable to that of the enzyme (*31*). The insulin degrading activity in mucus appears to be more important for the enzymatic barrier of the rectum than that of the small intestine because of less contribution of secretory digestive enzymes in the rectum. In terms of accessibility of enzymes to insulin, the amount of the enzymes that can access insulin in the one-dimple suppository is three times lower than that for the three-dimple suppository because only one-third of the membrane area is in contact with the drug layer. Therefore, the loading of insulin per unit of enzyme for one-dimple suppository is 9 times larger than that of three-dimple suppository. If permeability through the mucosal membrane facing each dimple, which contains the same amount of Labrasol, is equally facilitated, one-dimple suppository would show. a greater reduction in plasma glucose level due to saturation of the enzymatic degradation of insulin in the intestine. For avoiding the enzymatic barrier, however, the effect of Labrasol on enzymatic activity must be considered. It is reported that Labrasol inhibits intestinal UDP-glucuronyl transferase (*33*). Although the effect of Labrasol on the activities of insulin degrading enzymes in the intestine is not clear, the condensed Labrasol in a dimple may inhibit them. If this is the case, we should estimate to what extent the enzyme inhibition by Labrasol and the saturation of enzymatic degradation by a high concentration of insulin contribute to the promotion of rectal insulin delivery.

For the absorption of insulin, the association of insulin is an important factor. Insulin exists as a monomer, dimer, trimer, or hexamer (*34*). In terms of the molecule association, addition of surfactants is one of the determinants. Shao and his co-workers report that sodium dodecyl sulfate, hexadecyl trimethylammonium bromide, and sodium glycolate dissociate porcine-zinc insulin hexamers into monomer (*35*). They also report that these surfactants enhance the bioavailability of intestinal absorption (*36*). Therefore, it is important to determine the status of insulin in the dimple of suppository in the presence of Labrasol because it appears to be an important factor for the rectal absorption of insulin at a high concentration.

We demonstrated that condensing insulin in a limited area of a suppository is a promising strategy for the enhancement of its rectal absorption. With respect to absorption enhancers, their effects generally depend more on the concentration than on the total amount of dose. When the absorption area is limited, the total dose of an absorption enhancer required and consequently, a risk of possible adverse effect can be remarkably reduced. On the other hand, this strategy not only applies to the rectal absorption of polypeptides but also can be expanded to other biomedicines such as oligonucleotides, which are poorly permeable across the mucosa and unstable against enzymes in the intestine. We demonstrated that our rectal siRNA delivery technique using lipid nanoparticles provided therapeutic gene silencing in the liver through its delivery *via* the lymphatic route (*37,38*). Feasibility of our dimple-type suppository to rectal oligonucleotide delivery is now under investigation.

5. Conclusion

The present study demonstrates that a dimple-type suppository can promote the rectal delivery of insulin by condensing it in a dimple with an absorption enhancer. This technique may lead to a new enteral delivery system for biomedicines such as peptides and oligonucleotides that are poorly permeable and unstable in the intestine.

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Both triazolyl ester of ketorolac (15K) and YM155 inhibit the embryonic angiogenesis *in ovo* (fertilized eggs) *via* their common PAK1-survivin/VEGF signaling pathway

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Summary **15 K is 1,2, 3-triazolyl ester of ketorolac, an old pain-killer, that blocks PAK1 by its R-form and inhibits COX-2 by its S-form. Mainly due to a robust increase in cell-permeability, 15K is over 500 times more potent than ketorolac in both anti-cancer and anti-PAK1 activities in cell culture with IC_{50} around 24 nM. However, 15K has no anti-AKT activity. Angiogenesis requires at least the kinase PAK1, and perhaps the kinase AKT as well, and is essential for a robust growth of solid tumors. Thus, in this study, we examined the potential antiangiogenic activity of 15K both *in ovo* and cell culture, prior to its *in vivo* (xenograft) anti-cancer activity test. The IC_{50} of 15K against the embryonic angiogenesis *in ovo* in CAM (chorioallantoic membrane) assay is around 1 nmol/egg. Surprizingly, however, 15K failed to inhibit the tube formation of HUVECs (human umbilical vein endothelial cells) in cell culture even at high as 150 μM. In an attempt to solve this mystery, we tested both *in ovo* as well as HUVECs-based anti-angiogenic activity of a potent survivin-suppressor called YM155, which blocks PAK1, in addition to AKT. YM155 is slightly more potent than 15K in CAM assay with IC_{50} around 0.5 nmol/egg, and apparenty inhibits the tube formation of HUVECs with IC_{50} around 18 nM. According to a few previous findings with the direct PAK1-inhibitor frondoside A (FRA), the tube formation of HUVECs depends solely on PAK1. Thus, the failure of 15K to affect their tube formation is most likely due to their drug (15K)-resistance. Furthermore, unlike FRA, YM155 killed HUVECs with IC_{50} around 18 nM, clearly indicating that AKT is essential for survival of HUVECs, instead of their tube formation.**

Keywords: PAK1, angiogenesis, HUVECs, ketorolac ester (15K), YM155, survivin suppressor

1. Introduction

PAK1 (RAC/CDC42-activated kinase 1) is the major oncogenic/ageing/melanogenic kinase. It is responsible for a wide variety of diseases/disorders such as cancers, neurofibromatosis (NF), Alzheimer's disease (AD), diabetes (type 2), hypertension, a variety of infectious and inflammatory diseases, epilepsy, schizophrenia, depression, autism, and obesity (*1*). In addition, PAK1 shortens the healthy lifespan of *C. elgans* (*2*), and is essential for PDGF/EGF-dependent melanogenesis as well (*3*), suggesting that PAK1-blockers could be elixirs (longevity-promoters) and skin-whitening cosmetics. Thus, the potential market value of natural or synthetic PAK1-blockers would be huge, and pharmaceutical giants such as Pfizer, Roche, Novartis and Astrazeneca recently started developing potent PAK1-blockers.

However, major problem of these synthetic PAK1-blockers for clinical application is their poor water-solubility and cell-permeability. Thus, through the copper-catalyzed "Click Chemistry" (CC) which was originally introduced by Barry Sharpless (2001 Nobel-laureate) and his colleagues in 2001 (*4*), we recently managed to robustly boost both anti-cancer activity and cell-permeability of several COOH-bearing PAK1-

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blockers without any loss of their water-solubility (*5*). Briefly, any COOH-bearing compounds could be esterized with water-soluble 1,2,3-triazolyl alcohol in a high yield through the CC. Among these 1,2,3-triazolyl esters, 15K, 1,2,3-triazolyl ester of an old pain-killer called ketorolac sold by Roche, is so far the most potent, inhibiting the PAK1/COX-2-dependent growth of A549 lung cancer cells with IC_{50} around 24 nM, and the growth of B16F10 melanoma cells with IC_{50} around 6 nM (*5*). In addition, by the CC, the anti-PAK1 activity of ketorolac in cell culture was boosted over 500 fold, and the anti-COX-2 activity *in vitro* was boosted 20 fold, respectively (*5*).

Angiogenesis, which is essential for the robust growth of all solid tumors, depends on PAK1 (*6*). It has been known that the oncogenic RAS-PAK1-RAF-MEK-ERK signalling pathway leads to activation of VEGF (vascular endothelial growth factor) gene which is essential for angiogenesis (*7*). Prior to its test *in vivo* (xenograft) anti-cancer test, we wonder if ketorolac or its very potent derivative (15K) is anti-angiogenic. However, so far there is no positive report on the anti-angiogenic activity of Ketorolac *per se* in cell culture or *in vivo*. Here in this study, we have confirmed the potent anti-angiogenic activity of 15K *in ovo* by chorioallantoic membrane (CAM) assay (*8*), although it has no effect on the tube formation (or survival) of HUVECs in cell culture. We wonder why.

More interestingly, we recently found that YM155, a potent survivin-suppressor, also blocks PAK1 (in addition to another oncogenic/angiogenic kinase AKT) in cell culture (*9*). This is not a great surprise because it has been known that PAK1-deficient mice express far less survivin, clearly indicating that PAK1 is essential for survivin expression (*10*). Interestingly, however, YM155 blocks AKT by inhibiting epithelial growth factor (EGF) receptor (*11*). Thus, in an attempt to solve the mystery behind the inability of 15K to block the tube formation of HUVECs, we examined if YM155 affects the *in ovo* anti-angiogenesis as well as both tube formation and viability of HUVECs in cell culture (*8,12*), as their viability might depend on AKT, instead of PAK1.

2. Materials and Methods

2.1. *Chemicals and reagents*

15K, 1,2,3-triazolyl ester of ketorolac (see Figure 1A), was synthesized from ketorolac by Click Chemitsry as described previously (*5*). YM155 (see Figure 1B) was purchased from Adooq Bioscience (Irvine, CA, USA). Medium MCDB-104 was a product of Nihon Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Moregate (Brisbane, Australia). Cellgen was obtained from Koken (Tokyo, Japan). EGF was purchased from BD Biosciences

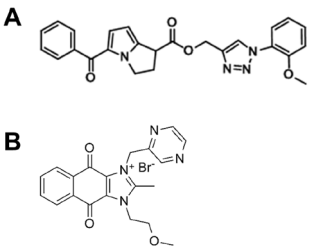

Figure 1. Chemical structure of 15K (A) and YM155 (B).

(Bedford, MA). Human basic fibroblast growth factor (FGF, recombinant) was purchased from Austral Biologicals (San Ramon, CA, USA). Medium 199 and all other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Fertilized chicken eggs were obtained from Pulmuone Farm (Danyang, Korea). Fat emulsion (20%) was from DongKook Pharmaceutical Co., Ltd. (Seoul, Korea).

2.2. *Cell culture*

HUVECs were grown in HUVEC growth medium (MCDB-104 medium supplemented with 10 ng/mL EGF, 100 µg/mL heparin, 100 ng/mL endothelial cell growth factor and 10% FBS) as previously described (*8*). Cell culture was carried out at 37°C under a humidified 95-5% (v/v) mixture of air and CO_2. The cells were seeded on plates coated with 0.1% gelatin and allowed to grow to subconfluence before experimental treatments.

2.3. *CAM angiogenesis assay*

The CAM assay was performed as previously described (*12,13*). In brief, fertilized chicken eggs were kept in a humidified incubator at 37°C. After 4 days of incubation, approximately 4 mL of albumen was aspirated and further incubated. Onto 5-day-old fertilized chicken embryos in the shells, 10 µL aliquots of samples (0.2, 1, 5, and 20 nmol/egg) or retinoic acid (5 nmol/egg), as a positive control mixed in 1% methylcellulose, were applied in 2 mm silicon rings placed on the surface of the growing CAM. After 2 days of incubation, an appropriate volume of a 20% fat emulsion was injected into the CAM to visualize the blood vessels. At least 15 eggs were used for each condition, and experiments were repeated 5 times. The % inhibition of angiogenesis by 15K or YM155 was calculated as a suppression ratio of new vessels within the area encircled by a white ring, compared with the control (non-treated).

2.4. *HUVECs tube formation assay*

Capillary tube-like structures formed by HUVECs were prepared as previously described with slight modifications (*8,12*). Briefly, HUVECs (6.0×10^4 cells/

cm^2) were seeded between two layers of collagen gel and then incubated in MCDB-104 medium with 0.5% FBS supplemented with 10 ng/mL of basic fibroblast growth factor, 8 nM phorbol 12-myristate 13-acetate, and 25 µg/mL ascorbic acid. They were treated with various concentrations of either 15K (50 and 150 µM), YM155 (1, 5, 10, 25, and 50 nM) or CAPE (50 µM) for up to 24 h. The resulting web-like capillary structure was viewed with a microscope under 200 x magnification and captured with a Leica-DFC295 digital camera (Leica, Wetzlar, Germany). Based on these photographs, the tube formation was quantified by determining the pixel number of tubes in each image using the NIH Image program.

2.5. *HUVECs viability during YM155 treatment*

The cell viability of HUVECs was determined by the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide) method as previously described (*3,5*). In brief, HUVECs (1.0×10^4 cells/cm^2) were seeded and then treated as above with YM155 at indicated concentrations (1-50 nM) for 24 h. Their viability was determined with MTT reagent which is converted to a pigment by a mitocondrial reductase in living cells. The absorbance (OD) of this pigment was measured at 490 nm, using a microplate absorbance reader (iMark™, Bio-Rad Laboratories, CA, USA).

2.6. *Statistical analysis*

Results are expressed as mean ± SD (standard deviation) from five independent experiments. A value of $p < 0.05$ was considered statistically significant. Data were evaluated statistically using one-way analysis of variance (ANOVA) followed by Holm-Sidak method and using Student *t*-test for analysis between the control and treatments (15K and YM155).

3. Results

3.1. *Suppression of embryonic angiogenesis in ovo by 15K and YM155*

To evaluate the effect of 15K (for chemical structure, see Figure 1A) and YM155 (for chemical structure, see Figure 1B) on the angiogenesis, we first used a CAM assay, an increasingly popular *in ovo* model for studying angiogenesis which was adapted by Folkman in an early 1970s (*14*). In CAM assay, anti-angiogenic activities of either 15K or YM155 were judged on 7th day after 2 days treatment, and retinoic acid (RA, 5 nmol/egg) was used as a positive (anti-angiogenic) control (*13,15*). As shown in Figure 2, 15K significantly inhibited the new blood vessel growth of chick embryos in a dose-dependent manner (0.2-20 nmol/egg). The IC_{50} of 15K against the embryonic angio-genesis was around 1 nmol/egg. At 5 nmol/egg, 15K and RA showed the basically same level of anti-angiogenic effect, inhibiting the angiogenesis by around 65%.

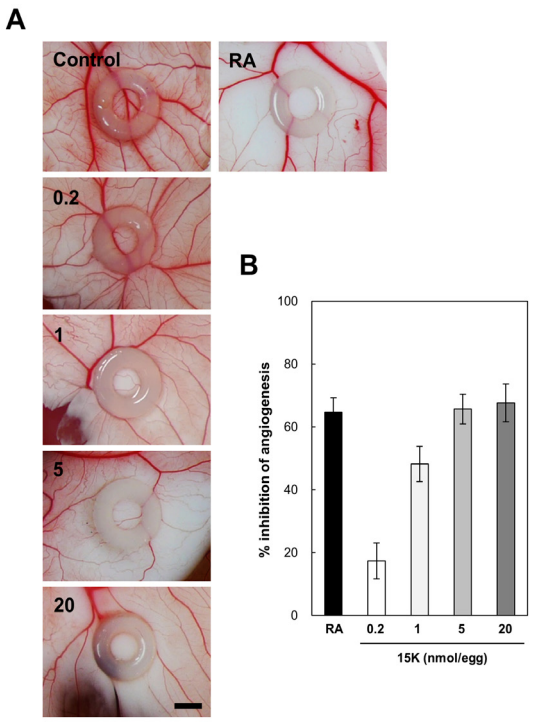

Figure 2. Anti-angiogenic activity of 15K *in ovo*. A. *in ovo* angiogenesis patterns: the control (non-treated) embryo angiogenesis, treated with either 15K (0.2-20 nmol/egg) or retinoic acid (RA, 5 nmol/egg). **B**. Quantification (% inhibition of angiogenesis), based on the new vessel formation within the area encircled by the white ring (compared with the control). 15K significantly inhibited the angiogenesis in CAM in a dose-dependent manner with IC_{50} around 1 nmol/egg. Scale bar = 2 mm. Values are expressed as means ± SD ($n = 5$).

As shown in Figure 3, YM155 also showed a strong anti-angiogenic activity *in ovo* in a dose-dependent manner, with IC_{50} around 0.5 nmol/egg, suggesting that YM155 appears to be even more potent than 15K under the CAM conditions. Interestingly, IC_{50} of YM155 against PAK1 in cell culture is around 500 nM, being 10 times higher than that of 15K (around 50 nM), and it is also true with anti-productivity (reducing brood size) and elixir (life-extending) activity in *C. elegans* (*9*). These observations strongly suggest, if not proven as yet, that the *in ovo* anti-angiogenesis of YM155 is not only due to its anti-PAK1 activity, but also due to its anti-AKT activity (or a third unknown activity). Is there any evidence for AKT-dependency of *in ovo* angiogenesis? Recently it has been suggested that AKT is involved in the COX-2-dependent *in ovo* angiogenesis (*16*).

3.2. *Effect of 15K and YM155 on HUVEC tube formation*

We next investigated the effect of 15K and YM155 on the angiogenesis *in vitro* (cell culture) using a tube formation model of HUVECs, in comparison with

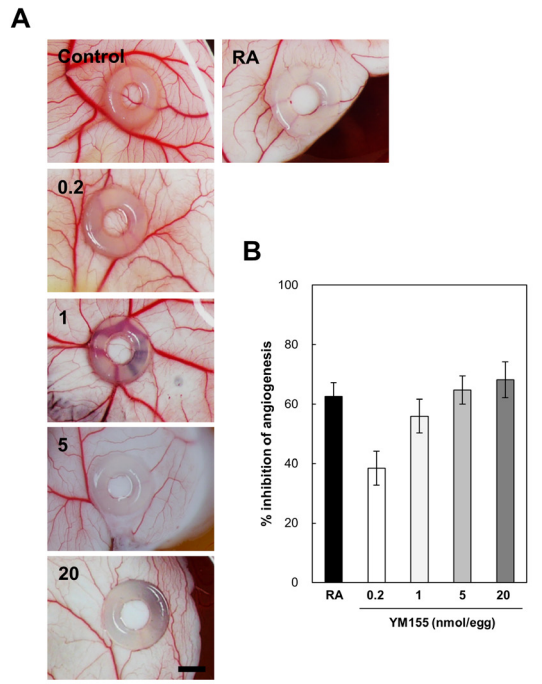

Figure 3. Anti-angiogenic activity of YM155 *in ovo*. A. *in ovo* angiogenesis patterns: the control (non-treated) embryo angiogenesis, treated with either YM155 (0.2-20 nmol/egg) or Retinoic acid (RA, 5 nmol/egg). **B**. Quantification (% inhibition of angiogenesis). YM155 also inhibited the *in ovo* angiogenesis in CAM in a dose-dependent manner with IC_{50} around 0.5 nmol/egg. Scale bar = 2 mm. Values are expressed as means ± SD (n = 5).

caffeic acid phenethyl ester (CAPE), an anti-angiogenic PAK1-blocker from propolis. During normal tube formation, the endothelial cells formed a network of capillary-like tubes, which were composed of multiple cells by gathering together and adhering to each other.

As shown in Figure 4D, YM155 clearly inhibited their elongation with IC_{50} around 18 nM. CAPE at 50 μM also completely inhibited their elongation, with around 50% cell death (see Figure 4B). To our big surprise, however, 15K, even at 150 μM caused little effect on tube formation (or survival) of these endothelial cells (see Figure 4B).

The IC_{50} of 15K and CAPE against the PAK1-dependent growth of A549 lung cancer cells for 72 h are 24 nM and 10 μM, respectively, indicating that 15K is over 400 fold more potent than CAPE, if both are equally allowed to penetrate through plasma membranes of their target cells. Thus, in theory, 15 K should have inhibited their tube formation for 24 h with IC_{50} around 120 nM, if 15K is allowed to penetrate as effectively as CAPE through HUVECs. In short, it is most likely that 15K fails to penetrate through the plasma membranes of HUVECs.

Is there any evidence for AKT-dependency of HUVEC tube formation? Both AKT and ERK (down stream of PAK1) signaling pathways have been reported to be involved in the HUVEC angiogenesis (*17*). Furthermore, the observed IC_{50} of YM155 (18 nM) aginst HUVEC tube formation is not far from the IC_{50} against the oncogenic EGFR-PI-3 kinase-AKT/ERK signalig pathway in pancreatic cancer cells (*11*). In addition, it should be worth noting that CAPE, which clearly inhibits the tube formation of HUVECs, is known to block AKT, in addition to MAPK/ERK, down-stream of PAK1 (*18*), as does YM155.

3.3. *Cell death of HUVECs during YM155 treatment*

CAPE at 50 μM caused around 50% death of HUVECs. We observed a significant % of their death during YM155 treatment as well. Thus, in an attempt to distinguish between cell death and *bona fide* inhibition of tube formation *per se*, we have quantified their YM155-caused death (see Figure 5), an an attempt to re-calculated the latter (net inhibition of tube formation), according to the formula as discussed below. Up to 10 nM, YM155 inhibited the *bona fide* tube formation by 20-30% (presumably due to its anti-PAK1 activity), but killed HUVECs by its anti-AKT activity with IC_{50} around 18 nM.

4. Discussion

First of all, these *in ovo* observations altogether have proven that both 15K and YM155 exert their potent anti-angiogenic activity by blocking their common oncogenic/angiogenic PAK1-survivin signaling pathway, as PAK1 is essential for survivin expression (*10*), and the potent survivin-suppressor YM155 indeed blocks PAK1 as well as AKT in cancer cells (*9,11*). To the best of our knowledge, both 15K and YM155 are the most potent anti-angiogenic PAK1-blockers so far. Moreover, since we recently found that 15K extends the healthy lifespan of *C. elegans* by 30% at 50 nM (*15*) as does PAK1-deficiency (*1,2*), it is most likely that 15K is also able to suppress effectively the angiogenesis-dependent growth of a variety of solid tumors such as pancreatic and colon cancers *in vivo* (for instance, human cancer xenografts in mice) without any side effect, as does YM155. Currently we are testing the effect of 15K on the growth, metastasis and angiogenesis of human cancer xenografts in mice.

In addition, it should be worth warning that the simple HUVECs cell culture system turns out to be a rather imperfect model to screen for some potent anti-angiogenic compounds such as 15K and retinoic acid which work *in ovo*, but clearly fail in this mono-cell culture, most likely due to their faillure to penetrate through plasma membrane of HUVECs. Ultimately siRNA-based AKT/PAK1 silencing approach should determine whether the tube formation of HUVECs depends on AKT or PAK1.

However, it should be worth proposing the following (far less expensive and quicker) alternative approach: a highly cell-permeable potent anti-cancer

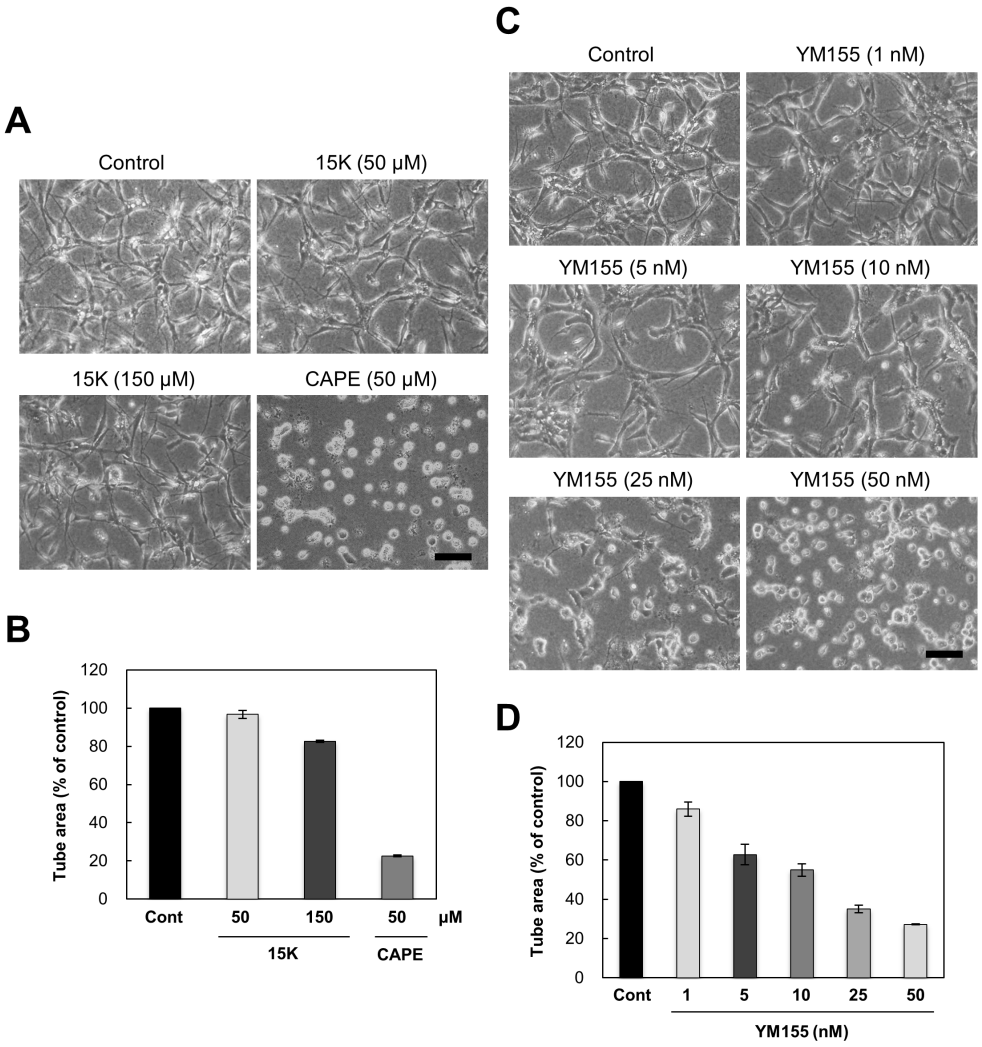

Figure 4. YM155 (C, D) inhibits the tube formation of HUVECs, but not 15K (A, B). HUVECs were sandwiched between two layers of collagen gels and induced to form blood vessel-like tubes. **Left**: HUVEC were treated with 15K at indicated concentrations (0, 50 and 150 μM). **The quantification (B)**: % tube area was measured by the length of tube-like structures containing connected cells. 15K failed to inhibit tube formation of HUVECs. **Right**: HUVECs were treated with YM155 at indicated concentrations (0, 1, 5, 10, 25, and 50 nM). **The quatification (D)**: YM155 apparently inhibits the tube formation in a dose-dependent manner, with IC50 around 18 nM. Each experiment was repeated three times and representative data are shown. Scale bar = 100 μm.

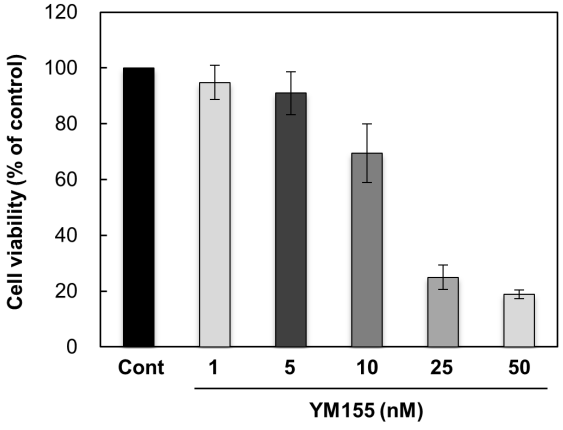

Figure 5. Cell viability of HUVECs during YM155 treatment. HUVECs were treated with YM155 at the indicated concentrations for 24 h, and their viability was measured by MTT method. YM155 reduced their viability in a dose-dependent manner with IC_{50} around 18 nM. Each experiment was repeated three times.

agent called frondoside A (FRA), a sulphated saponin, from a sea cucumber directly inhibits PAK1 with IC_{50} around 1 μM, and AKT with IC_{50} around 60 μM (*19*). If FRA fails to inhibit the tube formation around at 1 μM, but works around at 60 μM, it would be crystal clear that (unlike *in ovo* angiogenesis) the tube formation of HUVECs is AKT-dependent, and PAK1-independent, as is the survival of all normal cells. To a big surprise, however, the outcome is clearly opposite: their tube formation requires PAK1, while their survival requires AKT (see Figure 6). FRA has been found to inhibit the tube formation of HUVECs in cell culture by more than 80% at 0.5 μM without any cytotoxicity, proving that its anti-PAK1 activity alone is sufficient for its anti-angiogenic action against HUVECs (*20*). Thus, we shall conclude that the potent PAK1-blocker 15K fails to interfere with the tube formation of HUVEC simply by its failure in penetrating through this cell line, just like

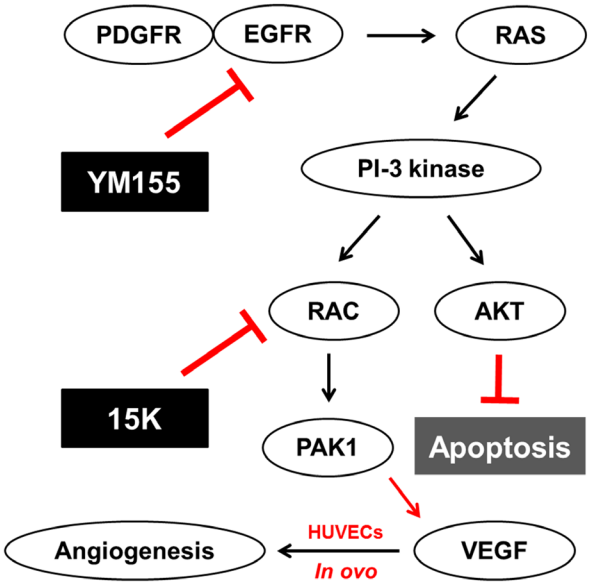

Figure 6. Mechanism underlying the anti-angiogenic action of 15K and YM155 *in ovo* and cell culture. 15K blocks PAK1 by inhibiting RAC directly, and inhibiting COX-2 directly, down stream of PAK1. YM155 blocks both PAK1 and AKT by down-regulating EGFR, and eventually suppressing survivin and COX-2 genes. EGFR-RAS-PI3 kinase-PAK1-MEK-ERK signalling pathway eventually activates VEGF gene. Thus, if *in ovo* angiogenesis depends on either PAK1 or AKT (or both), both 15K and YM155 could inhibit the embryonic angiogenesis. So far it is most likely that tube formation of HUVECS depends solely on PAK1 (*19,20*), while their survival depends mainly on AKT (*21*). Thus, the most likely reason why YM155 causes their death and inhibits their tube formation, but 15K fails is that HUVECs simply rejects 15K.

a few other PAK1-dependent cancer cell lines such as EMT6 (breast) and LIM-1899 (colon) (*5*).

In additon, we should point out another pitfall of HUVECs-based tube formation system. Many normal cells including HUVECs require AKT, but not PAK1, for their survival (*1,20,21*). Thus, any compounds with anti-AKT activity are expected to kill HUVECs at critical concentrations. In fact CAPE at 50 μM killed around 50% of HUVECs. Since the dead HUVECS would no longer form tubes, and the apparent reduction of tube formation by CAPE at this concentration is around 80%, the net inhibition of tube formation should be corrected to only 30% of total cells. In other words, the anti-PAK1 activity of CAPE inhibited the 60% of the remaining (50%) cells. Likewise, the apparent % reduction in tube formation (a) by YM155, which also kills HUVECS by its anti-AKT activity (see Figure 5), should be corrected by the following formula where their % death is b: (a – b)/(100 – b) × 100%. Thus, if a = b, in fact there is no net inhibition of tube formation *per se*. According to the above formula, up to 10 nM, the *bona fide* inhibition of tube formation by YM155 reached 20-30%, but at 25 nM (and higher concentrations), the cell death by its anti-AKT activity dominated.

Unfortunately, many scientists tend failing to distinguish between their death and *bona fide* inhibiton of tube formation, drawing an apparently conflicting (or incorrect) conclusion on PAK1/AKT-dependency of angiogensis in general.

In conclusion, due to the MDR (multi-drug resistance) of HUVECs, their tube formation assay appears to be a less reliable model for angiogenesis, compared with *in ovo* CAM assay. Furthermore, just like the case of CAPE and YM155, the apparent inhibition of tube formation is often caused by cell death (apoptosis), rather than the inhibition of cell migration and attachment *per se*. Despite of these pitfalls, as long as test compounds could permeate HUVECs without cell death as does FRA (*20*), the tube formation could provide an alternative cell culture screening system for PAK1-specific blockers, as is the PDGF-dependent melanogenesis without cell death in B16F10 melanoma cell line (*3*).

Lastly, 15K appears to be a far safer cancer therapeutic than YM155, mainly because it causes no harm on normal cells such as HUVECs which are clearly killed by YM155.

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Potential of *Piper betle* extracts on inhibition of oral pathogens

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Summary **In the present study, antimicrobial activity of *Piper betle* crude ethanol extract against 4 strains of oral pathogens; *Candida albicans* DMST 8684, *C. albicans* DMST 5815, *Streptococcus gordonii* DMST 38731 and *Streptococcus mutans* DMST 18777 was compared with other medicinal plants. *P. betle* showed the strongest antimicrobial activity against all tested strains. Fractionated extracts of *P. betle* using hexane, ethyl acetate, and ethanol, respectively, were subjected to antimicrobial assay. The result revealed that the fractionated extract from ethyl acetate (F-EtOAc) possessed the strongest antimicrobial activity against all tested strains. Its inhibition zones against those pathogens were 23.00 ± 0.00, 24.33 ± 0.58, 12.50 ± 0.70 and 11.00 ± 0.00 mm, respectively and its minimum inhibitory concentrations were 0.50, 1.00, 0.50 and 1.00 mg/mL, respectively. Interestingly, the minimum concentration to completely kill those pathogens was the same for all strains and found to be 2.00 mg/mL. Killing kinetic study revealed that the activity of F-EtOAc was dose dependent. HPLC chromatograms of *P. betle* extracts were compared with its antimicrobial activity. An obvious peak at a retention time of 4.11 min was found to be a major component of F-EtOAc whereas it was a minor compound in the other extracts. This peak was considered to be an active compound of *P. betle* as it was consistent with the antimicrobial activity of F-EtOAc, the most potential extract against the tested pathogens. It is suggested that F-EtOAc is a promising extract of *P. betle* for inhibition of oral pathogens. Separation and structure elucidation of the active compound of this extract will be further investigated.**

Keywords: *Piper betle*, oral pathogen, extract, antibacterial activity, antifungal activity

1. Introduction

Infectious disease occur in oral cavity is one of the serious dental problems. It causes impairment and functional limits of the infected organs and disability of the associated organs in oral cavity. According to the lesion and type of pathogens, oral infectious disease can be divided into many types such as oral candidiasis, gingivitis, periodontal disease, and dental caries. *Candida albicans* is a major cause of oral candidiasis (*1*). The oral streptococci bacteria such as *Streptococcus sanguinis*, *Streptococcus gordonii*, *Streptococcus intermedius*, and *Streptococcus oralis* enriching in oral biofilm are the major cause of gingivitis (*2,3*). Whereas many studies confirm that *Streptococcus mutans* is the most severe bacteria in oral cavity and is a major cause of dental caries (*4,5*).

Oral candidiasis, gingivitis, and dental caries can be treated and prevented by using denture cleanser and mouthwash, respectively. Previous studies presented that chemical agents such as alkaline peroxide, alkaline hypochlorite, chlorhexidine, and disinfectant in denture cleanser and mouthwash can reduce dental plaque and the number of oral pathogens, significantly (*6,7*). However, long use of these agents can cause some defects in oral cavity such as certain adverse effects (*8*), increasing of tooth staining, and calculus formation

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(*9*). Moreover, changing oral taste can be easily found including bleaching of acrylic resin which is a denture base material (*8,9*).

Research in activity of plant extracts have been increasing interested for utilizing as alternatives and replacing the use of those chemical compounds for treatment or prevention of infection of oral cavity (*10*). Many plant extracts show high potential on antibacterial activity for these purposes. For example, the ethanol crude extract from aerial part of *Andrographis paniculata* shows antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Vibrio alginolyticus*, *Shigella sonnei*, *Salmonella typhimurium*, *Vibrio cholera*, *Shigella boydii*, and *Vibrio alginolyteus* (*11*). The ethyl acetate extracts of *Momordica charantia* leaves and *Sesbania grandiflora* bark have the inhibitory activity against *S. aureus*, *E. coli*, and *Bacillus cereus* (*12,13*) whereas the extract of *Phyllanthus emblica* that can inhibit *Bacillus subtilis*, *Bacillus cereus*, *Salmonella typhi*, *Salmonella paratyphi* and *S. aureus* (*14*). It is noted that most plant extracts possess the activity against both Gram positive and Gram negative bacteria. However, some plant extracts exhibit the inhibitory activity against only Gram positive such as the extracts of *Psidium guajava* leaves that has the inhibitory activity against *S. aureus* and *B. cereus* but cannot inhibit the growth of *E. coli* and *Salmonella enteritidis* (15).

Piper betle is a medicinal plant in family Piperaceae. Many studies have explored its biological properties such as antimicrobial, radioprotective, antioxidant, anti-inflammatory and immunomodulatory activities (*16-19*). For the antimicrobial activity, *P. betle* have been reported to inhibit the growth of various kinds of bacteria such as Gram positive *S. aureus* and *Enterococcus faecalis* as well as Gram negative *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Klebsiella pneumoniae* (*18,20*). However, the research on activity of *P. betle* extracts against oral pathogens including both fungi and bacteria which are the major cause of oral candidiasis, gingivitis, and dental caries is still less. It is therefore interesting to investigate the activity of this plant against these oral pathogens to find an alternative option for the treatment of oral infectious disease or using as an active extract in oral hygiene products.

2. Materials and Methods

2.1. *Chemicals*

Ethanol, ethyl acetate, and hexane were from RCI Labscan (Bangkok, Thailand). Dimethyl sulphoxide (DMSO) was from Merck (Darmstadt, Germany). Brain heart infusion broth (BHI), brain, heart infusion agar (BHA), sabouraud dextrose broth (SDB) and sabouraud dextrose agar (SDA) were purchased from Difco (Maryland, USA). Human blood was supported by Maharaj Nakorn Chiang Mai Hospital (Chiang Mai, Thailand). Tystatin (Nystatin) was from T.O. Pharma Co., LTD (Bangkok, Thailand). Other chemicals and solvents are of the highest grade available.

2.2. *Plant Materials*

P. betle and other five medicinal plants including *A. paniculata*, *M. charantia*, *P. emblica*, *S. grandiflora* and *P. guajava* were collected from the northern area of Thailand. The voucher specimens of these plants were deposited in the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand. The reference number and part used of these plants were presented in Table 1. The fresh medicinal plants were dried in a hot air oven at 50°C for 24-48 h. The dried medicinal plants were ground into fine powder.

2.3. *Preparation of plant extracts*

The dried powder samples of all plants were macerated in ethanol for 24 h. Then, the mixture was filtered through Whatman No. 1 filter paper. The dried macerated plant residue was re-macerated in ethanol and filtered again. The obtained filtrates were gathered and subjected to a rotary vacuum evaporator in order to remove the solvent. The crude ethanol extract (CE) obtained was kept in the refrigerator until used.

The fractionated extracts of *P. betle* were prepared using organic solvents with different polarity. Hexane was the first solvent used to macerate the plant material. Then, the dried macerated plant residue was further extracted with ethyl acetate, and ethanol, respectively. The hexane fractionated extract (F-Hexane), ethyl acetate fractionated extract (F-EtOAc) and ethanol fractionated extract (F-EtOH) obtained after solvent evaporation were kept in the refrigerator until used.

2.4. *Oral pathogenic strains and growth conditions*

The reference strains of oral pathogens used in this study were *C. albicans* DMST 8684, *C. albicans* DMST 5815, *S. gordonii* DMST 38731 and *S. mutants* DMST 18777. *C. albicans* DMST 8684 and *C. albicans* DMST 5815 were cultured in SDB at 37°C under aerobic condition for 24-48 h whereas *S. gordonii* DMST 38731 and *S. mutans* DMST 18777 were cultured in BHI at 37°C under anaerobic condition (5% H_2, 5% CO_2 and 90% N_2) for 24-48 h. The suspension of these pathogenic strains was prepared and the concentration was adjusted using a McFarland densitometer (DEN-1 Biosan, Riga, Latvia) to the turbidity of 0.5 McFarland standard.

2.5. *Comparing antimicrobial activity of P.betle CE with other plants*

Stock solutions of CE of each plant were prepared by

dissolving in DMSO to a concentration of 100 mg/mL. The antimicrobial test was performed using the disk diffusion method. An exact amount of 20 µL stock solution of CE was added on a paper disk (CE-disk). The suspension of pathogenic strains after adjusting to 0.5 McFarland standard was swabbed over the entire surface of SDA for *Candida spp*. and 5% human blood in BHA (bBHA) for bacterial strains. The CE-disks were placed on the surface of each cultured medium. The plates were inverted and incubated in an aerobic condition for Candida strains and in anaerobic condition for bacterial strains at 37°C for 16-18 h. After incubation, the diameter of the clear zone indicating complete inhibition was measured. Nystatin suspension at a concentration of 100,000 unit/mL (20 mg/mL) and 1.2 mg/mL chlorhexidine solution (CHX) were used as positive controls for antifungal and antibacterial determinations, respectively. DMSO was used as a negative control.

2.6. *Antimicrobial activity of P. betle fractionated extracts in comparison with its CE*

This experiment was performed using broth dilution method. Stock solutions of *P. betle* extracts including CE and the fractionated extracts; F-Hexane, F-EtOAc, and F-EtOH were prepared by dissolving in DMSO to have a final concentration of 32 mg/mL. Two fold serial dilutions of the stock solution were prepared until the lowest concentration obtained was 0.03 mg/mL were prepared in a 96-well plate using SDB or BHI media as a diluent for Candida strains or bacterial strains, respectively. The suspension of each pathogen was adjusted to have final microorganism concentrations of 1×10^4 and 1×10^6 cfu/mL for Candida strains and bacteria strains, respectively. The plates were inverted and incubated in aerobic condition for Candida strains and in anaerobic condition for bacterial strains at 37°C for 16-18 h. Minimum inhibitory concentration (MIC) of the extracts that inhibited the visual growth of the microorganism in this step was recorded. All dilutions were subsequently streaked on the entire surface of SDA for Candida strains and bBHA for bacterial strains and further incubated in the same conditions as in the determination of MIC. After incubation, the minimum concentrations of the extracts that showed complete inhibition of Candida strains and bacterial strains were determined as minimum fungicidal concentration (MFC) and minimum bactericidal concentration (MBC), respectively.

2.7. *Killing kinetic study*

The study was performed according to the method previously described by Okonogi *et al*. (*19*). F-EtOAc of *P. betle* was used in this experiment as it showed the highest antifungal and antibacterial activities against all tested pathogens. F-EtOAc was firstly dissolved in DMSO to have a concentration of 100 mg/mL. Then the extract solution was further diluted with SDB or BHI, a diluent for Candida strains or bacterial strains, respectively to obtain concentrations of 1-fold, 2-fold, and 4-fold MFC and MBC for Candida strains and bacterial strains, respectively. Suspensions of the pathogenic strains having microorganism concentration of 10^4-10^6 cfu/mL were added to the extract solution in the 96-well plates. The cultures were then incubated in the same conditions as in the determination of MIC for 24 h. Viable counts were determined at the time intervals of 0, 1, 2, 4, 6, 12 and 24 h by plating 20 µL of known dilutions of the culture samples on the entire surface of SDA and bBHA for Candida strains and bacterial strains, respectively. The SDA and bBHA plates were subsequently incubated for up to 24-48 h in the suitable condition of each strain. The plates with 30 to 300 colonies were used for cfu counts. Log cfu/mL was plotted against time for construction of the killing kinetic curves. Nystatin and CHX were used as a positive control for kinetic study of antifungal and antibacterial activities, respectively. All assays were analyzed in triplicate.

2.8. *High performance liquid chromatography (HPLC) analysis*

HPLC analysis of *P. betle* extracts including CE, F-Hexane, F-EtOAc, and F-EtOH was performed using a Hypersil ODS column (4.6 i.d. × 250 mm) with an Agilent 1100 HPLC system (Massachusetts, USA). The exact weight of 1 mg extracts were dissolved in 1 mL ethanol (HPLC grade) and filtered through a 0.22 µm filter membrane before injection with an injection volume of 20 µL. The HPLC mobile phase consisting of methanol (A) and water (B) at a volume ratio of 7:3 was isocratic pumped at a flow rate of 0.70 mL/min for 20 min and detected by the UV/VIS detector at a wavelength of 280 nm.

2.8. *Statistical analysis*

All experiments were done in triplicate and the results are expressed as mean ± SD. Statistical analysis was done by using one-way ANOVA and *p*-value at a level of 95% confidence limit.

3. Results

3.1. *Preparation of plant extracts*

The outer appearance of *P. betle* CE was dark green fluidized mass with specific intense odor whereas that of *P. emblica*, *S. grandiflora*, and *P. guajava* were solid mass and easily to be ground into powder. The CE of *A. paniculata* and *M. charantia* appeared as viscous mass. It was noted that the color of CE extracted from the leaves was dark green whereas that extracted from

the barks was rusty brown. The yield of CE obtained from each plant was presented in Table 1. It was found that *P. emblica* gave the highest yield followed by *A. paniculata* and *P. betle*. Fractionated extraction of *P. betle* resulted the fractionated extracts with different yield. The yield of F-EtOAc was the highest of 8.8% w/w whereas that of F-Hexane and F-EtOH were 8.2 and 4.2% w/w, respectively.

3.2. *Comparing antimicrobial activity of P. betle CE with other plants*

The results as shown in Table 2 demonstrated that CE of *P. betle* possessed the strongest antimicrobial activity against both antifungal and antibacterial activity against the tested pathogens with the inhibition zones of 22.30 ± 2.10 and 17.30 ± 0.67 mm for *C. albicans* DMST 8684 and *C. albicans* DMST 5815, respectively and 7.80 ± 0.30 and 7.10 ± 0.00 mm for *S. gordonii* DMST 38731 and *S. mutans* DMST 18777, respectively. The CE of *M. charantia* and *P. guajava* showed inhibition to *C. albicans* DMST 5815 and *S. gordonii* DMST 38731 whereas the CE of *P. emblica* and *A. paniculata* showed only antifungal activity. In addition, it was found that the CE of *S. grandiflora* showed no activity against the tested oral pathogens. In comparison with the control, Nystatin showed the inhibitory effect to both *C. albicans* DMST 8684 and *C. albicans* DMST 5815 and CHX showed the strong effect to both *S. gordonii* DMST 38731 and *S. mutans* DMST 18777. From these results, *P. betle* was considered to be suitable for further study.

Table 1. Detail of plants used and the yield of CE

Plant samples	Voucher specimen	Used part	Yield (%)
A. paniculata	004046	Leaf	24.6
M. charantia	023225	Leaf	6.6
P. emblica	008895	Leaf	40.1
S. grandiflora	023176	Bark	8.9
P. guajava	008610	Leaf	19.5
P. betle	008612	Leaf	19.2

3.3. *Antimicrobial activity of P. betle fractionated extracts in comparison with its CE*

The results as shown in Table 3 demonstrated that different fractionated extracts of *P. betle* possessed antimicrobial activity in different levels. F-Hexane possessed the inhibition zone of 21.00 ± 1.40 and 20.67 ± 0.58 mm, against both *C. albicans* DMST 8684 and *C. albicans* DMST 5815 whereas only *S. gordonii* DMST 38731 could be inhibited with the inhibition zone of 8.00 ± 0.00 mm. F-EtOAc possessed obviously clear inhibition zone of 23.00 ± 0.00 and 24.33 ± 0.58 mm against *C. albicans* DMST 8684 and *C. albicans* DMST

Table 2. Inhibition zone of CE against oral pathogens

CE of plant samples and controls	Inhibition zone (mm)			
	C. albicans DMST 8684	*C. albicans* DMST 5815	*S. gordonii* DMST 38731	*S. mutans* DMST 18777
A. paniculata	NZ	10.00 ± 0.00	NZ	NZ
M. charantia	NZ	10.33 ± 0.58	7.7±0.70	NZ
P. emblica	10.70 ± 0.80	NZ	NZ	NZ
S. grandiflora	NZ	NZ	NZ	NZ
P. guajava	NZ	12.00 ± 0.00	8.10 ± 0.0	NZ
P. betle	22.30 ± 2.10	17.30 ± 0.67	7.80 ± 0.30	7.10 ± 0.00
Nystatin	19.67 ± 0.58	17.00 ± 0.00	-	-
CHX	-	-	8.00 ± 0.00	15.00 ± 0.00
DMSO	NZ	NZ	NZ	NZ

NZ: no inhibition zone. Data were represented as mean ± SD.

Table 3. Inhibition zone of *P. betle* extracts against oral pathogens

P. betle extracts and controls	Inhibition zone (mm)			
	C. albicans DMST 8684	*C. albicans* DMST 5815	*S. gordonii* DMST 38731	*S. mutans* DMST 18777
F-Hexane	21.00 ± 1.40	20.67 ± 0.58	8.00 ± 0.00	NZ
F-EtOAc	23.00 ± 0.00	24.33 ± 0.58	12.50 ± 0.70	11.00 ± 0.00
F-EtOH	NZ	NZ	NZ	NZ
CE	21.00 ± 0.71	20.00 ± 0.00	11.30 ± 0.40	10.67 ± 0.58
Nystatin	19.67 ± 0.58	17.00 ± 0.00	-	-
CHX	-	-	8.00 ± 0.00	15.00 ± 0.00
DMSO	NZ	NZ	NZ	NZ

NZ: no inhibition zone. Data were represented as mean ± SD.

Table 4. MIC, MFC and MBC (mg/mL) of *P. betle* extracts

P. betle extracts and controls	*C. albicans* DMST 8684		*C. albicans* DMST 5815		*S. gordonii* DMST 38731		*S. mutans* DMST 18777	
	MIC	MFC	MIC	MFC	MIC	MBC	MIC	MBC
F-Hexane	1.00	2.00	1.00	4.00	1.00	2.00	2.00	2.00
F-EtOAc	0.50	2.00	1.00	2.00	0.50	2.00	1.00	2.00
CE	1.00	2.00	1.00	2.00	1.00	4.00	2.00	8.00
Nystatin	6×10^{-4}	2.4×10^{-3}	3.1×10^{-3}	9.8×10^{-3}	-	-	-	-
CHX	-	-	-	-	$< 3 \times 10^{-4}$	$< 3 \times 10^{-4}$	$< 3 \times 10^{-4}$	6×10^{-4}

5815 and of 12.50 ± 0.70 and 11.00 ± 0.00 mm against both *S. gordonii* DMST 38731 and *S. mutans* DMST 18777, respectively. F-EtOH showed no inhibitory effect to all tested pathogens. In comparison with CE, it was found that CE also possessed the inhibitory activity against both tested fungi and bacteria. However, the inhibition zones of CE against all tested oral pathogens were significantly less than F-EtOAc.

Determination of MIC, MFC and MBC of *P. betle* extracts was performed in order to confirm the results of inhibition zone. F-EtOH was not subjected to this experiment as it showed no inhibition zone. The results were shown in Table 4. F-EtOAc showed the highest inhibitory activity against all tested oral pathogens. Antifungal activity of CE was almost similar to F-EtOAc, however, antibacterial activity was significantly lower. It was found that the MBC values of F-EtOAc against *S. gordonii* DMST 38731 and *S. mutans* DMST 18777 were 2 times and 4 times, respectively, less than CE indicating that F-EtOAc was significantly higher effective than CE. F-Hexane showed minor antimicrobial activity against the oral pathogens. Nystatin, a positive control for antifungal activity, showed an activity against *C. albicans* DMST 8684 and *C. albicans* DMST 5815 with MIC values of 6.1×10^{-4} and 3.1×10^{-4} mg/mL and MFC values of 2.4×10^{-3} and 9.8×10^{-3} mg/mL, respectively. CHX, a positive control for antibacterial activity, showed an activity against *S. gordonii* DMST 38731 and *S. mutans* DMST 18777 with the same MIC value of $< 3 \times 10^{-4}$ mg/mL and MBC values of $< 3 \times 10^{-4}$ and 6×10^{-4} mg/mL, respectively.

3.5. *Killing kinetic study*

As F-EtOAc showed the highest inhibitory activity against all tested oral pathogenic strains, it was selected for this experiment. The killing kinetic of F-EtOAc against *C. albicans* DMST 8684 was dose dependent as shown in Figure 1. It was found that at concentration of 1-fold MFC (2 mg/mL) and 2-fold MFC (4 mg/mL), the efficiency of F-EtOAc to completely kill the microorganism could be done within 1 h. However, strong killing efficiency was obviously seen when the concentration of F-EtOAc was increased to 4-fold MFC (8 mg/mL). At this concentration, F-EtOAc could

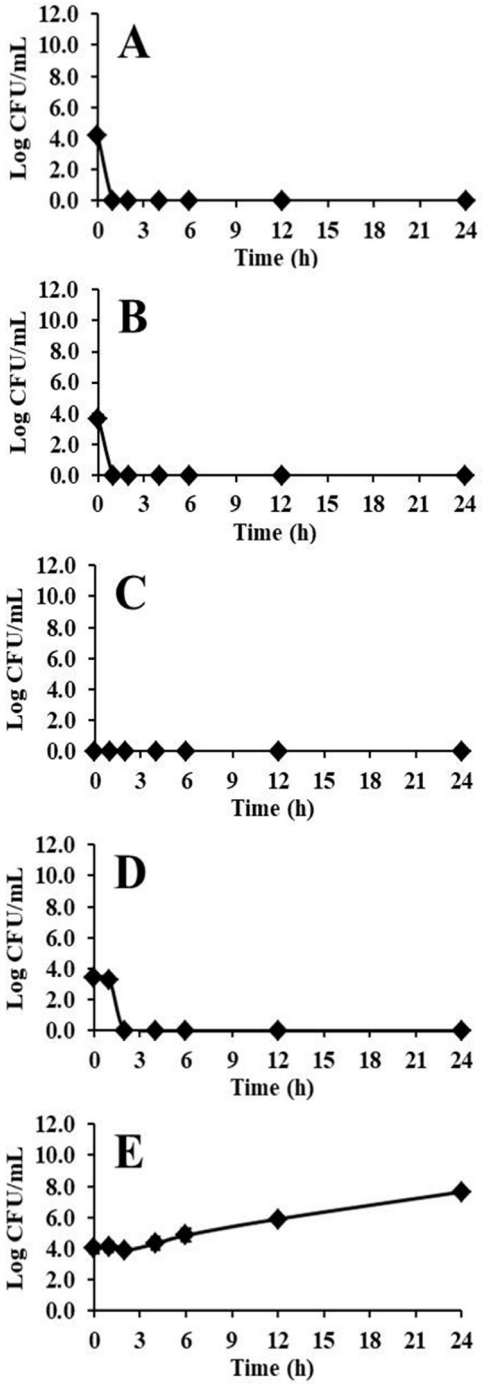

Figure 1. Killing kinetics of F-EtOAc at the concentration of 1-fold MFC (A), 2-fold MFC (B), 4-fold MFC (C) in comparison with nystatin (D) and DMSO (E) (n = 3) against *C. albicans* DMST 8684.

completely kill the microorganism suddenly once after the microorganism exposed to the extract. Nystatin could completely kill the pathogen within 2 h. It was obviously seen that the killing rate of F-EtOAc against *C. albicans* DMST 8684 was significantly faster than nystatin.

The killing kinetic of F-EtOAc against *S. gordonii* DMST 38731 was dose dependent as shown in Figure 2. At concentration of 1-fold MBC (2 mg/mL), F-EtOAc could completely kill the pathogenic bacteria within 4 h. However, increasing concentration of the extract to 2-fold MBC (4 mg/mL) and 4-fold MBC (8 mg/mL), higher killing efficiency was obviously seen. The extract at these concentrations could completely kill the bacteria within 2 and 1 h, respectively. CHX, a positive control for antibacterial activity, could completely kill this pathogenic bacterial strain within 4 h. It was obviously seen that the killing rate of F-EtOAc against *S. gordonii* DMST 38731 was significantly faster than CHX.

The killing kinetic of F-EtOAc against *S. mutans* DMST 18777 was also dose dependent as shown in Figure 3. It was found that at a low concentration of 1-fold MBC (2 mg/mL), F-EtOAc could completely kill all pathogenic bacteria. However, after comparison with the bacterial growth curve in the negative

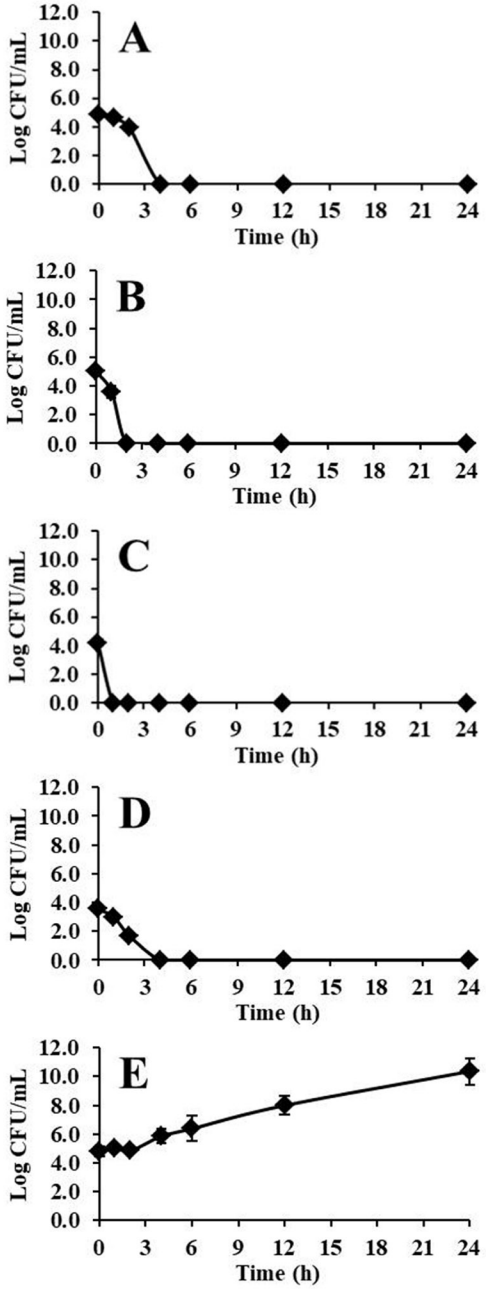

Figure 2. Killing kinetics of F-EtOAc at the concentration of 1-fold MBC (A), 2-fold MBC (B), 4-fold MBC (C) in comparison with nystatin (D) and DMSO (E) (*n* = 3) against *S. gordonii* DMST 38731.

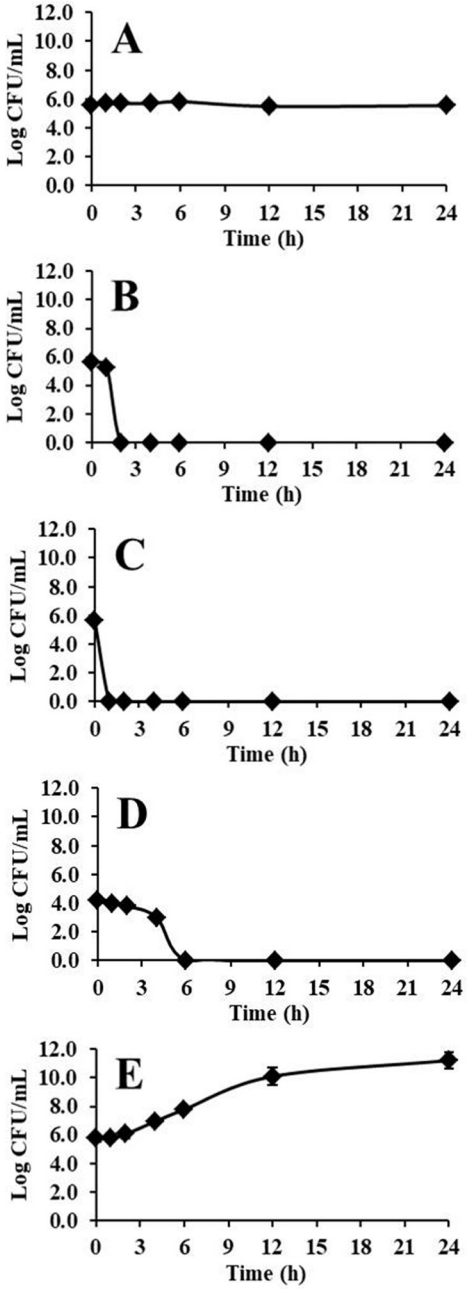

Figure 3. Killing kinetics of F-EtOAc at the concentration of 1-fold MBC (A), 2-fold MBC (B), 4-fold MBC (C) in comparison with nystatin (D) and DMSO (E) (*n* = 3) against *S. mutans* DMST 18777.

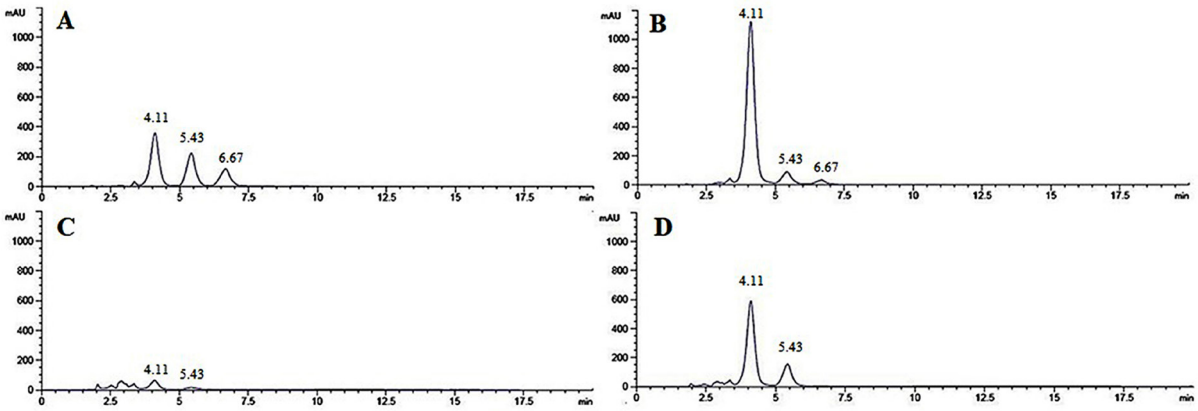

Figure 4. HPLC chromatograms of *P. betle* extracts; F-Hexane (A), F-EtOAc (B), F-EtOH (C) and CE (D).

control, it could be clearly seen that the extract exactly showed some inhibition to the growth of the pathogen. Increasing concentration of the extract to 2-fold MBC (4 mg/mL) and 4-fold MBC (8 mg/mL), higher killing efficiency was obviously seen. The extract at 4 mg/mL could completely kill the bacteria within 2 h whereas at 8 mg/mL of F-EtOAc, the pathogen was completely killed within 1 h. CHX, a positive control for antibacterial activity, could completely kill this pathogenic bacterial strain within 6 h. It was obviously seen that the killing rate of F-EtOAc against *S. mutans* DMST 18777 was significantly faster than CHX.

3.6. *HPLC analysis of P. betle extracts*

HPLC chromatograms of *P. betle* extracts including F-Hexane, F-EtOAc, F-EtOH and CE were demonstrated in Figure 4. F-Hexane and F-EtOAc contained 3 major compounds at the same retention time of 4.11, 5.43 and 6.67 min but different quantity. The compound of F-EtOAc at a retention time of 4.11 min was obviously seen as a major ingredient. Whereas, F-EtOH and CE contained 2 compounds that were presented in the same retention time of 4.11 and 5.43 min.

4. Discussion

It has been reported worldwide of the side effects of chemical antiseptic compounds used in oral hygiene products (*8*). Meanwhile, researches have explored many biological activities of plants extracts for treatment of various diseases (*13,16,22*). For inhibition of oral pathogens, many plant extracts have been reported to have the potential on this purpose, for example, ethyl acetate extract of *Camellia sinensis* leaves was reported to decrease the incidence of dental caries (*23*) whereas water extract of *Vaccinium macrocarpon* fruits showed an inhibitory effect against protease enzyme of *Porphyromonas gingivalis* (*24*). Moreover, the extract from *Salvadora persica* stem can inhibit many oral pathogenic bacteria, such as *S. mutans*, *Lactobacillus acidophilus*, *Aggregatibacter actinomycetemcomitans*, and *P. gingivalis* (*25*). Present study was done on *P. betle*, a plant widely grown in Southeast Asian countries. The leaves of this plant have been widely consumed by local people as a mouth freshener. There are some reports on its antibacterial activity against many types of bacteria (*18,20*) but none of them are oral pathogens. The current study reports on inhibitory effects of *P. betle* extracts against four strains of oral pathogens including two strains of oral pathogenic fungi; *C. albicans* DMST 8684 and *C. albicans* DMST 5815 and two strains of oral pathogenic bacteria; *S. gordonii* DMST 38731 and *S. mutants* DMST 18777. These pathogenic microorganisms are the major cause of oral candidiasis, gingivitis and dental caries, respectively (*2,26,27*). The antibacterial and antifungal activities were investigated by two methods which are disk diffusion method and broth dulution method following the standard protocol of the National Committee for Clinical Laboratory Standards (NCCLS) (*28,29*) The antimicrobial activity of CE of *P. betle* against these pathogens was firstly compared with the CE of other five plants which have been reported to have antimicrobial activity. The results indicate that even the tested plant extracts showed antibacterial activity, but not all of them can inhibit the oral pathogens. CE of *P. betle* was found to be the most effective against all tested strains with inhibition zones of 22.30 ± 2.10 and 17.30 ± 0.67 mm for *C. albicans* DMST 8684 and *C. albicans* DMST 5815, respectively and 7.80 ± 0.30 and 7.10 ± 0.00 mm for *S. gordonii* DMST 38731 and *S. mutans* DMST 18777, respectively. However, the activity against these oral pathogens of the CE is significantly lower than the fractionated extract from ethyl acetate (F-EtOAc). F-EtOAc showed the highest effective with the inhibition zones of 23.00 ± 0.00 and 24.33 ± 0.58 mm against *C. albicans* DMST 8684 and *C. albicans* DMST 5815 and of 12.50 ± 0.70 and 11.00 ± 0.00 mm against *S. gordonii* DMST 38731 and *S. mutans* DMST 18777, respectively.

The inhibition zone can roughly indicate the

inhibitory effects of the tested extracts but MIC, MBC, or MFC values present deeper and more proper information particulary for comparative effects of the extracts. The results confirm that F-EtOAc is the most effective extract against the oral pathogenic bacteria and fungi. The MBC values of F-EtOAc against *S. gordonii* and *S. mutans* show that F-EtOAc is 2 times and 4 times, respectively, higher effective than CE. In addition, antimicrobials are usually regarded as bactericidal or fungicidal if the MBC/MIC or MFC/MIC ratio is ≤ 4 and bacteriostatic or fungistatic if > 4 (*30*). The ratios obtained for all the test organisms were above 4 which indicated that F-EtOAc was bactericidal and fungicidal actions against the tested oral microorganisms.

The bactericidal and fungicidal actions of F-EtOAc were confirmed by the killing kinetic study. Bactericidal and fungicidal agents can completely inhibit the growth or multiplication of pathogenic microorganisms (*31*). From the killing kinetic patterns of F-EtOAc, it is indicated that F-EtOAc exhibits bactericidal and fungicidal actions. The pharmacological actions of some antimicrobial agents are dose dependent and some are time dependent (*21,32*). The effective antimicrobial activity of F-EtOAc from the killing kinetic patterns is considered to be dose and time dependent.

Many previous reports have shown that *P. betle* contains extensive biologically active compounds such as eugenol, allylpyrocatechol, chavibetol, chavibetol acetate, caryophyllene, hydroxychavicol, which are related to its activities (*33-35*). The HPLC condition used in the current study was modified from the previous studies of Ferreres *et al*. that the major chemical compounds in *P. betle* were reported (*33*). Considering the HPLC patterns of *P. betle* extracts, it is considered that the antimicrobial activity of the extracts against the tested oral pathogens are likely according to the compound at a retention time of 4.11 min. This compound was found to be the most abundant of F-EtOAc and extremely higher amount than other extracts. This compound therefore is considered to be an active compound of *P. betle* for antimicrobial actions against the oral pathogens.

In conclusion, *P. betle* is a medicinal plant that possessed the strong potential action against oral pathogens causing candidiasis, gingivitis and dental caries. The evaluation of antimicrobial activity of fractionated extracts of *P. betle* confirms that the major bioactive compound of *P. betle* extract is a moderate polar compound similar to ethyl acetate. *P. betle* extract is the promising natural source of antimicrobial compounds against oral pathogens. Purification and structure elucidation of the active compounds as well as clinical trials are challenges for further studies.

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Effects of *Caesalpinia sappan* on pathogenic bacteria causing dental caries and gingivitis

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Summary **The present study explores antimicrobial activities of *Caesalpinia sappan* extracts against three strains of oral pathogenic bacteria; *Streptococcus mutans* DMST9567 (*Smu9*), *Streptococcus mutans* DMST41283 (*Smu4*), and *Streptococcus intermedius* DMST42700 (*Si*). Ethanol crude extract of *C. sappan* (Cs-EtOH) was firstly compared to that of other medicinal plants using disc diffusion method. Cs-EtOH showed significantly higher effective inhibition against all tested strains than other extracts and 0.12% chlorhexidine with the inhibition zone of 17.5 ± 0.5, 18.5 ± 0.0, and 17.0 ± 0.0 mm against *Smu9*, *Smu4*, and *Si*, respectively. Three fractionated extracts of *C. sappan* using hexane, ethyl acetate, and ethanol, respectively, were further investigated. The fractionated extract from ethanol (F-EtOH) presented the strongest activities with the minimum bactericidal concentration (MBC) of 125-250 μg/mL. Killing kinetics of F-EtOH was depended on the bacterial species and the concentration of F-EtOH. Two-fold MBC of F-EtOH could kill all tested strains within 12 h whereas its 4-fold MBC showed killing effect against *Si* within 6 h. Separation of F-EtOH by column chromatography using chloroform/methanol mixture as an eluent yielded 11 fractions (F1-F11). The fingerprints of these fractions by high-performance liquid chromatography at 280 nm revealed that F-EtOH consisted of at least 5 compounds. F6 possessed the significantly highest antimicrobial activity among 11 fractions, however less than F-EtOH. It is considered that F-EtOH is the promising extract of *C. sappan* for inhibiting oral pathogenic bacteria and appropriate as natural antiseptic for further develop of oral hygiene products.**

Keywords: Oral pathogens, antimicrobial activity, extracts, medicinal plants, *Ceasalpinia sappan*

1. Introduction

Dental caries, a scientific term for tooth decay, is the localized destruction of a susceptible tooth surface whereas gingivitis is one of the periodontal diseases which is characterized by bleeding, plaque formation and gum inflammation (*1*). Both dental caries and gingivitis play an impressive impact on the human health and welfare. These health problems and the consequent cost of dental treatment leads to costing billions of dollars each year (*2*). Oral microorganisms are one of the most important factors responsible for dental carries and gingivitis (*3*). The most insidious bacteria are oral pathogenic strains particularly *Streptococcus spp*. Although this genus is the normal flora microorganisms in oral cavity, some species such as *Streptococcus mutans* and *Streptococcus intermedius* are the pathogenic bacteria. *S. mutans* is considered to be the most cariogenic bacteria initiate to dental caries whereas *S. intermedius* displays the significant role to generate gingivitis (*4,5*). They can early colonized on tooth surface (*6*) and form organized microorgamism communication as biofilm or dental plaque (*7*). They can also produce acid that destroys the tooth's enamel layer. Many evidences show that elimination of these

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pathogenic bacteria and microbial dental plaque biofilm can prevent gingivitis and dental caries (*8,9*). However, these oral pathogens cannot be easily eliminated completely from oral cavity with common daily sanitation. Antiseptics appear to be the efficient tools for this prevention, however the prolong use of these agents has led to emergence of multidrug-resistant pathogens (*10*).

Recently, much attention has been given to natural products with health-promoting benefits. Novel therapeutic products from plants have been extensively reported (*11-13*). Plants produce various active molecules that are able to fight bacterial infections successfully (*14,15*). Many potential plant extracts have been reported on their antimicrobial activity against oral pathogens (*16,17*). Moreover, the synergistic interactions of secondary plant metabolites with antibiotics or certain bioactive compounds were reported (*18,19*).

Caesalpinia sappan is a member in family Leguminosae. The heartwood of *C. sappan* has been used as coloring agent in many products of food, beverage, and cosmetics. Importantly, *C. sappan* has long been used as traditional medicine in treatment of many kinds of infectious diseases. Some phenolic compounds existing in this plant have been isolated as well as the finding on their pharmacological activities such as antioxidant (*20*) and anti-inflammation (*21*). For antimicrobial activity, it has been reported that *C. sappan* extracts can inhibit the growth of several aerobic and facultative bacteria such as *Salmonella ebony*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus subtilis*, *Salmonella faecalis*, *Enterobacter aerogenes*, *Pseudomonas aerogenosa*, and *Staphylococcus aureus* (*22*). Moreover, the extract showed antifungal activity against *Aspergillus niger* and *Candida albicans* (*23*). However, up to our best knowledge, there is no scientific evaluation of *C. sappan* extracts on inhibition of oral pathogenic bacteria such as *S. mutans* and *S. intermedius* that are the major cause of dental carries and gingivitis, respectively. This study was designed to elucidate their potential effects on those pathogens.

2. Materials and Methods

2.1. *Chemicals*

Chloroform, *n*-hexane, ethyl acetate, ethanol, methanol, and dimethyl sulfoxide (DMSO) were from RCI Labscan Ltd. (Bangkok, Thailand). Difco™ Brain heart infusion (BHI) broth and agar were from Bacton, Dickinson and Company (Maryland, USA). Silica gel 60 (0.040-0.063 mm) for column chromatography (230-400 mesh ASTM) was from Merck KGaA (Darmstadt, Germany). The other chemicals and solvents were of the highest grade available.

2.2. *Plant materials and preparation of ethanol crude extracts*

The fresh samples of *C. sappan* and other medicinal plants presented in Table 1 were collected from the local area in Chiang Mai Province, Thailand. All plants were identified by the botanist in the botanical herbarium of Faculty of Pharmacy, Chiang Mai University to obtain the reference numbers. The plant materials were dried and pulverized into fine powder before macerating in 95% ethanol (24 h × 3 at room temperature). After maceration, the mixtures were filtered and the solvent was removed from the filtrates to obtain the ethanol crude extracts. The obtained ethanol crude extract of *C. sappan* (Cs-EtOH) and those of other plants were kept in the refrigerator until use.

2.3. *Fractionated extracts preparation*

The dried powder of *C. sappan* was subjected to fractionated extraction previously described (*24*) using 3 different solvents; *n*-hexane, ethyl acetate, and ethanol in orderly to obtain the fractionated extracts namely F-Hexane, F-EtOAc, and F-EtOH, respectively.

2.4. *Bacteria strains and culture*

Three strains of Gram-positive facultative anaerobic pathogenic bacteria including *S. mutans* DMST9567 (*Smu9*), *S. mutans* DMST41283 (*Smu4*), and *S. intermedius* DMST42700 (*Si*) were cultured and incubated under anaerobic condition at 37°C in 5% CO_2. Blood agar plates were prepared from 5% human blood in BHI agar.

2.5. *Comparing antimicrobial activity of ethanol crude extracts*

The antibacterial activity of Cs-EtOH in comparison with the ethanol crude extracts of other medicinal plants, which their antibacterial activity has been previously reported, was investigated using disc diffusion method. Before testing, the amount of the cultures was adjusted by adding BHI broth until the turbidity was equivalent to 0.5 McFarland standard which referred to 1×10^8 CFU/mL. Each blood agar plates were streaked with bacterial stock suspension. The extracts were dissolved in DMSO to a final concentration of 200 mg/mL before adding onto the sterile paper discs of 5 mm in diameter, and then put them on the top layer of the blood agar plates. Chlorhexidine (CHX) 0.12% solution and DMSO were used as positive and negative controls, respectively. The plates were incubated at 37°C in 5% CO_2 anaerobic chamber for 24 h. The antimicrobial activity was evaluated by measuring the diameter of the inhibition zone.

Table 1. Inhibition zone of ethanol crude extracts of various medicinal plants against oral pathogenic bacteria

Plant species	Reference No.	Diameter of inhibition zone (mm)		
		Smu9	*Smu4*	*Si*
Acacia catechu	009208	NZ	NZ	NZ
Caesalpinia sappan	002276	17.5 ± 0.5	18.5 ± 0.0	17.0 ± 0.0
Cajanus cajan	023173	10.0 ± 0.0	11.0 ± 0.0	15.5 ± 0.0
Cassia alata	023179	10.7 ± 0.6	NZ	NZ
Phyllanthus amarus	006540	NZ	14.7 ± 0.3	15.0 ± 0.0
Piper sarmentosum	006283	NZ	NZ	NZ
Psidium guajava	008610	NZ	7 ± 0.0	7.3 ± 0.3
Sesbania grandiflora	023176	NZ	NZ	NZ
Syzygium aromaticum	023230	10.0 ± 0.0	10.3 ± 0.3	10.3 ± 0.6
Syzygium cumini	023177	NZ	NZ	8.8 ± 0.3
0.12% CHX		16 ± 0.0	15 ± 0.0	10.0 ± 0.0

NZ = No zone.

2.5. *Antimicrobial susceptibility of C. sappan extracts*

The antimicrobial susceptibility of F-Hexane, F-EtOAc, F-EtOH, and Cs-EtOH was evaluated by comparing the value of minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the extracts against the tested pathogenic strains using broth microdilution method. The sample stock solutions (in 25% DMSO) were diluted to the serial concentrations of 7.8-2,000 µg/mL and added into the suspension of 1×10^6 CFU/mL of the bacterial strains in the 96-well plates. The plates were then incubated at 37°C in 5% CO_2 anaerobic chamber for 24 h. The lowest concentration that inhibited the bacteria was considered as the MIC of the extract. The cultures were further investigated for MBC by streaking on blood agar plates. The plates were incubated at 37°C in 5% CO_2 anaerobic chamber for 24 h. The lowest concentration in the plates that bacterial growth could not be visible was considered as the MBC.

2.6. *Separation of F-EtOH and fingerprint preparation*

F-EtOH was separated using column chromatography. Silica gel 60 was used as the stationary phase and the mixture of chloroform/methanol at the ratios of 15:1, 10:1, and 5:1 was used as the mobile phase. Thin layer chromatography (TLC) was used for characterized the spots of the collected fractions. The fractions having similar TLC pattern were pooled and concentrated by rotary evaporator. The yield of each fraction was recorded. The fingerprint of all fractions were prepared by means of high performance liquid chromatography (HPLC) using HPLC Shimadzu CLASS-VP™ model (Kyoto, Japan) and the reversed phase Eurospher 100, i.d. 4 mm, C18 column, Knauer (Berlin, Germany). The system was conducted with isocratic at room temperature. The mixture of 1 % v/v acetic acid in DI water (A) and methanol (B) at A:B ratio of 75:25 was used as the mobile phase. HPLC procedure was operated with the mobile flow rate of 1 mL/min, injection volume of 10 µL, running time of 30 min, and monitored with UV/VIS detector at 280 nm.

2.7. *Killing kinetics of F-EtOH*

The suspensions of the tested bacterial strains (1×10^6 CFU/mL) were prepared in BHI broth in 96-well plates. F-EtOH was diluted with BHI broth and added into the bacterial suspension and gently mixed to have the final concentrations of 2 and 4 folds of its MBC. The plates were incubated at 37°C in 5% CO_2 anaerobic chamber for 24 h. The surviving bacteria were monitored at 0, 1, 2, 4, 6, 12, and 24 h by collecting the culture at each time point and cultured on the blood agar plates. The number of viable bacterial colonies was counted. CHX 0.12% solution was used as a positive control and the BHI broth was used as a negative control. Killing kinetic profiles were generated between log colonies of the survival bacteria and time.

2.8. *Statistical analysis*

The experiments were conducted in triplicate. The results were expressed as mean ± SD and statistically analyzed *via* SPSS statistic 17.0 software. ANOVA and Turkey's Multiple test have been determined the significant at $p < 0.05$.

3. Results

3.1. *Antimicrobial activity of crude ethanol extracts*

As shown in Table 1, among various ethanol crude extracts of the tested plants, Cs-EtOH showed the highest potential against the tested oral pathogens with the widest inhibition zone against *Smu9*, *Smu4*, and *Si* of 17.5 ± 0.5, 18.5 ± 0.0, and 17.0 ± 0.0 mm, respectively. Its inhibition activity was significantly higher than a positive control, CHX solution, which showed the inhibition zone of only 16 ± 0.0, 15 ± 0.0, and 10.0 ± 0.0 mm, respectively. Followed Cs-EtOH was the extract of flowers of *Syzygium aromaticum* which showed the

Table 2. MIC/MBC of the fractionated and ethanol crude extracts of *C. sappan* against oral pathogenic bacteria

Oral pathogenic bacteria	MIC/MBC (µg/mL)							
	F-Hexane		F-EtOAC		F-EtOH		Cs-EtOHs	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Smu9	> 2,000	> 2,000	125	250	62.5	125	125	250
Smu4	> 2,000	> 2,000	250	500	125	250	250	500
Si	> 2,000	> 2,000	125	250	125	125	125	250

Table 3. Yield and MIC/MBC of the fractions of F-EtOH separated by column chromatography

Fraction	Yield (% w/w)	MIC/MBC (µg/mL)					
		Smu9		*Smu4*		*Si*	
		MIC	MBC	MIC	MBC	MIC	MBC
F1	1.0	> 2,000	> 2,000	> 2,000	> 2,000	> 2,000	> 2,000
F2	1.2	> 2,000	> 2,000	> 2,000	> 2,000	> 2,000	> 2,000
F3	3.0	> 2,000	> 2,000	> 2,000	> 2,000	> 2,000	> 2,000
F4	2.0	> 2,000	> 2,000	> 2,000	> 2,000	> 2,000	> 2,000
F5	1.4	1,000	> 2,000	1,000	> 2,000	500	1,000
F6	8.6	125	500	125	500	125	250
F7	14.6	125	500	125	1,000	125	500
F8	19.0	125	1,000	250	2,000	250	1,000
F9	17.2	250	2,000	250	2,000	250	1,000
F10	3.4	500	2,000	250	2,000	250	2,000
F11	3.6	500	2,000	250	2,000	250	2,000

inhibition zone against *Smu9*, *Smu4*, and *Si* of 10.0 ± 0.0, 10.3 ± 0.3, and 10.3 ± 0.6 mm, respectively. The extract from the aerial part of *Phyllanthus amarus* showed the inhibition zone against *Smu4*, and *Si* wider than that of *S. aromaticum*, but it could not inhibit *Smu9*. The extracts from some common medicinal plants such as *Cassia alata* and *Psidium guajava* showed slight inhibition and against only one strain whereas the extracts from some plants such as *Piper sarmentosum* and *Sesbania grandiflora* showed no activity against the tested oral pathogens.

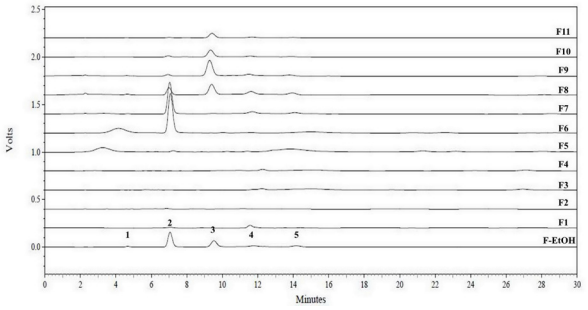

Figure 1. HPLC fingerprints of F-EtOH in comparison with its fractions obtained from the separation by column chromatography and detected at 280 nm.

3.2. *Antimicrobial susceptibility of C. sappan extracts*

Antimicrobial susceptibility against the 3 strains of oral pathogenic bacteria; *Smu9*, *Smu*, and *Si*, was expressed as their MIC and MBC values. The results are showed in Table 2. It was found that among Cs-EtOH and the three fractionated extracts of *C. sappan*, F-Hexane presented MIC and MBC values higher than 2,000 µg/mL for all strains. Meanwhile, F-EtOAc, F-EtOH, and Cs-EtOH revealed lower MIC and MBC values. It was clearly seen that F-EtOH possessed the lowest MIC of 62.5 and 125 µg/mL against *Smu9* and *Smu4*, respectively indicating its highest antibacterial activity. The results on MBC values also confirmed that this fractionated extract of *C. sappan* possessed the highest antibacterial activity against the tested oral pathogenic bacteria as it showed the least MBC values. Therefore, F-EtOH was selected for further studies on killing kinetics patterns against those pathogens.

3.3. *Separation of F-EtOH and fingerprint preparation*

Separation of F-EtOH by column chromatography yielded 11 fractions (F1-F11). After removing solvents, the yield of each fraction was recorded. It was found that the yield of each fraction was different as shown in Table 3. It was found that approximately 25% of the extract was loss during separating process since a summation of the yield of all fractions was only 75%. The yields of F6-F9 were relatively higher than the others. Optimal HPLC conditions previously reported for *C. sappan* extracts (*25*) were used to prepare HPLC fingerprints of the extracts.

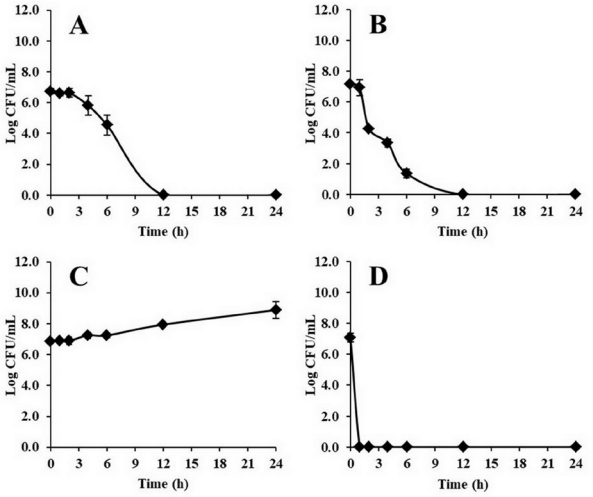

Figure 2. Killing kinetics of F-EtOH at the concentrations of 2-fold MBC (A) and 4-fold MBC (B) in comparison with negative control (C) and positive control (D) against *S. mutans* DMST9567.

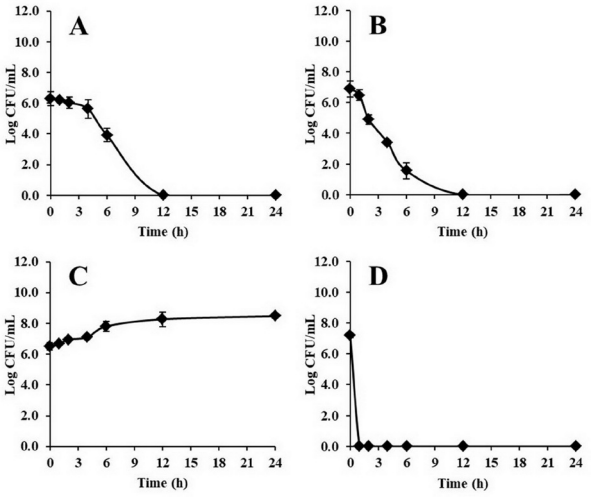

Figure 3. Killing kinetics of F-EtOH at the concentrations of 2-fold MBC (A) and 4-fold MBC (B) in comparison with negative control (C) and positive control (D) against *S. mutans* DMST41283.

The HPLC fingerprints of 11 fractions isolated from F-EtOH in comparison with that of F-EtOH were shown in Figure 1. The results revealed that F-EtOH consisted of at least 5 compounds which exhibited at different retention times. Peak 2 is the largest one followed by peaks 3, 5, 4, and 1, respectively. Comparing the HPLC fingerprints of 11 fractions, F1-F4 did not exhibit any peak at the same retention times as peaks 1-5 of F-EtOH. F6 revealed the highest pure fraction that contained mostly peak 2 and the very less of one more peak at the position prior to peak 2.

3.4. *Antimicrobial susceptibility of F-EtOH fractions*

Serial dilutions of 11 fractions separated from F-EtOH were prepared and determined for MIC and MBC. The results as shown in Table 3 revealed that F1-F4 had no

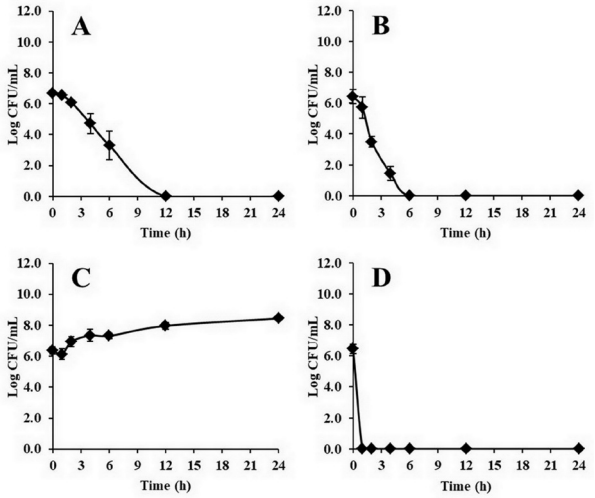

Figure 4. Killing kinetics of F-EtOH at the concentrations of 2-fold MBC (A) and 4-fold MBC (B) in comparison with negative control (C) and positive control (D) against *S. intermedius*.

antibacterial activity against all tested bacterial strains. Interestingly, F5-F11 that one of their HPLC peaks was at the same retention time as peak 2 (of F-EtOH) had antibacterial activity against all tested oral pathogenic strains. It was noted that F8-F11 that HPLC peaks were the same retention time as peaks 3, 4, and 5 were also able to inhibit the bacterial strains. Among 11 fractions, F6 was the most effective fraction, however, its ability was less than F-EtOH. It was considered that the compound at peak 2 was the major active antibacterial agent of F-EtOH. The high antibacterial activity of F-EtOH was considered to be due to the synergism of this active compound and the minor constituents existing in this fractionated extract. F-EtOH was therefore selected for further study.

3.5. *Killing kinetics of F-EtOH*

The killing kinetic profiles of F-EtOH against both strains of *S. mutans*, *Smu9* and *Smu4*, were shown in Figures 2 and 3, respectively. It was found that the killing kinetic profiles of 2-fold MBC and 4-fold MBC against these strains during the first 3-6 h were slightly different. F-EtOH of 4-fold MBC showed higher potential than that of 2-fold MBC. However, the bacteria were completely killed by both concentrations within 12 h. The killing kinetic profiles of F-EtOH against *S. intermedius* were shown in Figure 4. It was found that the bacterial inhibition activity of F-EtOH against this strain was significantly higher than against *S. mutans* and it was clearly dose dependent. *Si* could be completely killed within 12 h by 2-fold MBC whereas it was only 6 h that the pathogen was completely killed by 4-fold MBC.

4. Discussion

Currently, there is a trend to use the active ingredient

from natural sources such as plants. Many plants that used to be the ingredients in the traditional medicinal remedies for oral health care have been researched. The secondary metabolite compounds of plants have been reported as bioactives for several activities including antimicrobial activity against many pathogens and many of them are purposed to use instead of chemicals (*26*). *C. sappan* has been extensively studied and reported on its bioactivities such as antioxidant, anti-inflammatory, and antimicrobial activities. Previous study reported the antimicrobial activities of *C. sappan* extract obtained from water extraction of its wood (*27*). It is found that the main active compounds from its aqueous extraction is brazilin (*28,29*). Unfortunately, this compound is unstable. Therefore, it is less possible to use this pure compound for clinical applications. For some plants, the whole extracts show higher benefits than their pure compounds regarding to the synergistic effects on stability and activity (*15,18,19,30*). However, for aqueous extract, it is difficult to remove water from the aqueous solution of the extract by common rotary evaporator. Ethanol is easier to remove than water. Ethanol has been reported to be safe and effective for extraction of many bioactive compounds, especially phenolic compounds and volatile oils (*31,32*). Therefore, ethanol was chosen as extracting solvent for preparation of the crude extract of *C. sappan* and the other plants in the present study. Although, extensive researches have widely reported on biological activities of *C. sappan* (*21-23,28,29,33,34*), the researches on inhibition of oral pathogens are still limited. The current article provides more scientific data of *C. sappan* extracts on the oral pathogenic bacteria. The results show many interesting issues. It is found that not all of the extracts, which were previously reported to have inhibitory activity against some other bacterial species, could inhibit the oral pathogenic bacteria. Among the crude extracts from 10 medicinal plants, there are only 4 plants that possess the antibacterial activity against all tested strains of oral pathogens. Among them, Cs-EtOH is the most effective extract on inhibition of all tested strains of oral pathogenic bacteria. More interestingly, when the fractionated extracts of *C. sappan* from three different extracting solvents, *n*-hexane, ethyl acetate, and ethanol are compared, it is found that only F-EtOH has the highest potential on inhibition of those tested pathogens. This result indicated that the polarity of the active ingredients of *C. sappan* are relatively high. Our result was in accordance with the previous report which presented that the most active extract of *C. sappan* on antimicrobial activity against *Salmonella ebony*, *Klebsiella pneumonia*, *Escherichia coli*, and *Bacillus subtilis* was found in the polar solvent fraction (*22*). It has also been reported that *C. sappan* contains many active ingredients which mostly are phenolic compounds that can be categorized into several structural groups such as brazilin, chalcone, protosappanin, and homoisoflavonoids (*21*). Brazilin was reported to have strong antimicrobial activity (*35*). Recently, it has been reported that brazilein which is the oxidizing form of brazilin could be easily occurred during extraction or storage, and separation from brazilin is quite difficult (*36*). Therefore, it might be possible that either brazilin or brazilein or both of them possessed high potential for the antibacterial activity against the test oral pathogens in the present study.

In addition, after separation of F-EtOH by column chromatography, F-EtOH demonstrates its activity significantly higher than its purified fractions. This result is considered to be due to the synergistic effect of the major active compounds and the other minor compounds inside the extracts.

Kinetic inhibitory activity against oral pathogenic bacteria of F-EtOH reveals its high effects on killing the tested bacteria. F-EtOH can completely kill the tested oral pathogenic bacteria within 6-12 h. The killing activity is depended on the type of the pathogenic species and the dose of F-EtOH. It is concluded that F-EtOH is the most effective extract of *C. sappan* and it is worth for further study in animal or clinical trial.

Acknowledgements

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Original Article

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In vitro oral epithelium cytotoxicity and *in vivo* inflammatory inducing effects of anesthetic rice gel

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Summary ***In vitro*** **cytotoxicity of lidocaine hydrochloride (LH) and prilocaine hydrochloride (PH) to oral epithelial cells, isolated from tissue specimens of healthy volunteers, were evaluated. Cell vitality after treating with 1-20% anesthetic solutions for 5 and 30 min was investigated using F-actin and 4',6-diamidino-2-phenylindole staining technique and observed by fluorescence microscopy. Vitality rate of more than 90% was found in all anesthetic groups at both durations whereas no survived cell was found in a positive control group (sodium dodecyl sulfate). Lactate dehydrogenase (LDH) assay was performed to confirm the safety of both anesthetic solutions. Cell culture medium after treating with LH or PH for 5 and 30 min were collected and analyzed using commercial kits. The results showed no significant difference between the test groups and negative control group (untreated culture) with low LDH levels.** ***In vivo*** **inflammatory inducing effect of 5, 10, 20% LH or PH loaded rice gels was investigated in healthy volunteers. Tumor necrosis factor alpha (TNF-α) in gingival cervicular fluid was determined by ELISA technique. It was found that the expression of TNF-α was not different from the baseline. The expression of this inflammatory mediator caused by the commercial gel was higher than those of both anesthetic rice gels. It might be due to the effects of other excipients in the formulation of the commercial product. It is concluded that LH or PH possess no cytotoxicity to oral epithelium and the developed rice gel base and LH and PH rice gels do not induce inflammatory effect to oral tissues.**

Keywords: Local anesthetic gel, lidocaine, prilocaine, epithelial cell, gingival tissue

1. Introduction

Local anesthetics are used in clinical setting for pain management during minor interventional treatments and postoperative care. Currently, they are extensively used in several procedures of dental treatment to eliminate pain (*1,2*). However, the drugs have possibility to produce various toxic effects in many tissues because of their chemical structures (*3*). Previous studies identified rates of incidence for systemic local anesthetic toxicity associated with various clinical forms of regional anesthesia (*4*). Local anesthetics can be classified into two groups based on the chemical moiety of amides [–NH–CO–] and esters [–O–CO–]. In general, local anesthetics exhibit their activity mainly by binding to specific receptor sites on the sodium (Na^+) channels innerves and blocking the movement of ions through these pores. Both chemical and pharmacologic properties of individual local anesthetics determine their clinical properties (*5*). Lidocaine and prilocaine belong to amino amide class of local anesthetics (*6*). Lidocaine was created in 1943 (*7*). Since it is non-addictive and well tolerated by most patients, lidocaine quickly became the standard by which we currently compare all anesthetic medications. By the mid-1960s, other local anesthetics including prilocaine, mepivacaine, and

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bupivacaine were available. Prilocaine has subsequently been widely reported in dental treatment. The formulation contained 4% prilocaine has been used as a primary buccal infiltration (*8,9*). It was reported that the effect of lidocain and prilocaine as primary buccal infiltration was similar to each other when compared with articaine (*8,10*).

Local anesthetics including lidocaine applied topically have been reported to use for surgical anaesthesia with reduced cardiotoxicity and central nervous system toxicity (*11,12*). However, many studies have shown their cytotoxicity towards several cultured cells (*13,14*). Cytotoxicity of local anesthetic including lidocaine and prilocaine has been reported on mesenchymal stem cells and osteoblastic cells as well as human oral and tumor cells (*15-18*). Even though these local anesthetics have been long used in several complications, there is inadequate information about cytotoxic activity of these agents particularly for dental applications that the use of local anesthetics has been increased. Inflammation inducing effects of these anesthetics after clinical applications to gingival tissues have also not been well reported. Therefore, these need to be studied in detail.

2. Materials and Methods

2.1. *Materials*

Lidocaine hydrochloride (LH) and prilocaine hydrochloride (PH), BPC pharmaceutical grade, were obtained from Gufic Bioscince Ltd. (Mumbai, India). Sodium hydroxide and glacial acetic acid were from RCI Lab-scan Co., Ltd. (Bangkok, Thailand). ELISA kit (Biolegend®, Cat. No. 430203) was purchased from Biolegend Co., Ltd. (Tokyo, Japan). Alexa Fluor® 488-conjugated phalloidin was from Invitrogen (Carlsbad, CA). 4',6-Diamidino-2-phenylindole (DAPI) was from Biotium, Inc. (Hayward, CA), Commercial anesthetic gel (5% LH) was from Septodont Ltd. (Kent ME16 0JZ, UK). HEPES-buffered saline (fungizone), trypsin-EDTA, and Dulbecco's modified Eagle medium (DMEM) were from GIBCO-BRL, Life Technologies (Grand Island, NY). Fetal bovine serum was from Gemini (Calabasas, Calif). Protease enzyme (thermolysin) was from Sigma Chemical Co. (St. Louis, MO). Serum-free keratinocyte growth medium (KGM) was from Lonza (Walkersville, MD, USA). Trypsin-EDTA was of Invitrogen™ (Grand Island, NY, USA). The culture dishes were of Nunc™ (Roskilde, Denmark). All other chemicals and solvents were of AR grade or the highest grade available unless otherwise stated.

2.2. *Preparation of anesthetic solutions and rice gels*

Aqueous solutions of LH and PH at concentrations of 1, 5, 10, and 20% were prepared as followed. Exact amount of 1, 5, 10, and 20 g of LH or PH was dissolved in distilled water and the volume was adjusted to 100 mL. Rice gel base was prepared according to the method previously described (*19*). Exact amount of LH or PH was incorporated into certain amount of the prepared rice gel base using small amount of glycerin as wetting agent. Subsequently, the gel base was added until the total weight of the mixture was 100 g to obtain 5, 10, and 20% of drug in each riced gel formulation. The mixture was well triturated until the homogenous anesthetic transparent gel was obtained.

2.3. *Tissue collection and epithelial cell culture*

This experiment protocol (No. 22/2555) was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University. Gingival tissue was obtained from 10 healthy human subjects (18-20 years old age), who were patients undergoing impacted teeth surgical removal at Faculty of Dentistry, Chiang Mai University. Informed consent was obtained. Tissue samples that appeared to be severely traumatized were excluded. Normal non-inflamed gingival tissues were collected and washed twice with cold HEPES-buffered saline containing 1% penicillin and 10 mg/mL streptomycin and 25 µg/mL amphotericin B, and subsequently cut into pieces, approximately 2 × 2 mm, and placed in the culture dishes, containing 0.5 mg/mL thermolysin and 1.125 mM Ca^{2+} at 4°C for 90 min. After that, the epithelial sheet was separated from the connective tissue. The cells were isolated from the epithelial sheet by trypsinization with trypsin-EDTA, and cultured in serum-free KGM, while the connective tissues were placed on a 60 mm culture dish, which contained DMEM, supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin (Invitrogen™) until the cells spread from the tissue and their number was further expanded. After the cells reached 80% confluence, they were washed twice, trypsinized and then transferred to new culture flasks.

2.4. *Cell vitality visualization*

Epithelial cell vitality after treating with the anesthetic solutions (1-20% LH or PH) for 5 and 30 min was evaluated by visualization after staining. The cells were seeded in 96-well culture plates with sufficient density of cell for optimal well coverage and then incubated in a humidified atmosphere of 5% CO_2 at 37°C. After treating with the anesthetic solutions with different concentrations and durations, the cells were fixed with para-formaldehyde and stained with Alexa Fluor® 488-conjugated phalloidin for F-actin visualization and DAPI for nuclei staining (*20*). Sodium dodecyl sulfate (SDS) was used as a positive control and the untreated culture was used as a negative control. The viable cells were visualized and counted under fluorescence microscopy (Olympus BH2-RFC, Tokyo, Japan) with

standard filter blocks for violet (355-425 nm), blue (450-490 nm), and green (515-560 nm) excitation light (*21,22*). Photograph was taken using Kodak Tri-X-pan 400, Provia Fujichrome 400, Ilford 400 and Kodak Ektachrome 400 films.

2.5. *Lactate dehydrogenase (LDH) assay*

Cytotoxicity induction was also assessed by LDH leakage into the culture medium. After exposure to the anesthetic solutions, the culture medium was centrifuged at 3,000 rpm for 5 min to separate the cells. Subsequently, the LDH in the cell free supernatant was determined using a commercially available kit from Sigma Diagnostics (LD50). The assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD. The formation of NADH from the above reaction results in a change in absorbance at 340 nm. Aliquots of media and warm reagent were mixed in a 96-well plate (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and absorbance was recorded using a microplate spectrophotometer system (Spectramax 190 UV-Vis Microplate Reader, Molecular Devices, Sunnyvale, California, USA). Results were analyzed with the Soft max pro software (version 2.2.1) and are presented as percentage of control values. Cadmium chloride was shown not to interfere with the determination of LDH within a range from 0 to 2,000 μM.

2.6. *Cytokines assay using ELISA*

To investigate the inflammatory inducing effect of the anesthetics, tumor necrosis factor alpha (TNF-α) of the gingival crevicular fluid was determined using ELISA technique, modified from the previous method (*23*). The experiment protocol was approved by the Human Experiment Committee of the Faculty of Dentistry, Chiang Mai University (Process No. 26/2556). Exact amount of 0.2 mL of rice gels containing 5, 10, and 20% LH or PH as well as positive and negative controls were applied to the gingiva of 10 healthy volunteers (25-60 years old age) at the gingival margin of 8 teeth separately per person. Rice gel base was used as a negative control and the commercial gel containing 5% LH was used as a positive control. After contact time of 2 min, the gel was removed from gingiva and washed with normal saline solution. Sterile paper strips (2 mm × 10 mm) were inserted to the gingival sulcus of the selected sites (Figure 1) to collect the gingival crevicular fluid at time intervals of 0, 3, 6, and 24 h. Determination of TNF-α was performed using ELISA kit (Biolegend®, Tokyo, Japan, Cat. No. 430203). ELISA results were recorded using microplate reader (Model series UV 900 Hdi, USA) at wavelengths of 450 and 550 nm. Calculation of the relative absorbance units and the TNF-α concentration for each sample from the standard curve of TNF-α standard (0-500 pg/mL), was performed as described in the instruction manual. Each sample was measured in duplicate and concentrations were derived from the standard curve. Percentage of relative amount of TNF-α expression causing by the test samples was calculated based on the control.

2.7. *Statistical analysis*

Statistical evaluation of LDH and TNF-α release experiment was performed by one-way ANOVA (Bonferroni test). Data were presented as mean ± SD. The value of $p < 0.05$ was considered to indicate significant differences.

3. Results

3.1. *Epithelial cell culture*

After epithelial cells from gingival tissue was isolated and cultured for 2 days in appropriated conditions, the morphology of polygonal or squamous shape was noticed. By using KGM, the cells could be subcultured. Cells cultured at the second to the fourth passages were used throughout this study.

3.2. *Cell vitality after contacting with the anesthetic solutions*

After treating with 1, 5, 10, and 20% anesthetic solutions for 5 and 30 min, high survival rates of epithelial cells were found as shown in Figure 2. It was noted that at longer contact time, the highest concentration of both anesthetic did not inhibit epithelial cell growth. Staining with DAPI and F-actin showed clearly that the cells could survive after 30 min exposure with solutions of either LH or PH whereas no cell was seen after contacting with SDS as presented in Figure 3.

3.3. *Cytotoxicity by LDH assay*

Detecting cytotoxicity induced by LH or PH solutions was assessed by LDH leakage from ruptured cells into the culture medium. As shown in Figure 4, LDH levels were not significantly increased after exposure to anesthetic solution for both contact durations of 5 and 30 min. However, the amount of LDH leakage found in the highest concentration of both anesthetic solutions were about 20%, similar to the negative control group.

3.4. *TNF-α release*

The amount of TNF-α in the gingival crevicular fluid of the volunteers after 2-min contact with LH and PH rice gels in comparison with the rice gel base and

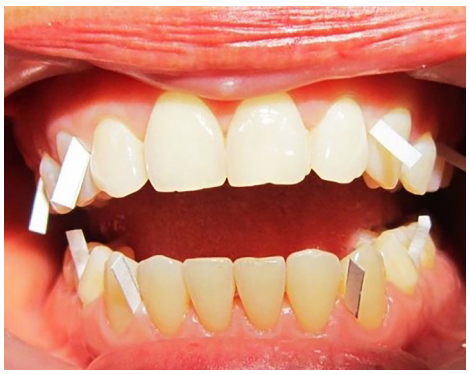

Figure 1. Collection of gingival crevicular fluid.

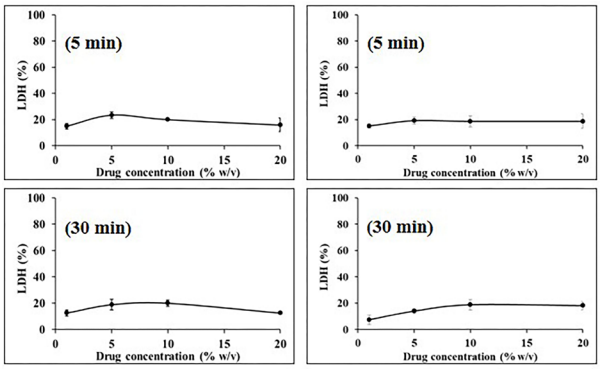

Figure 2. Survival rate of gingival epithelial cells after exposure to LH solutions (left) and PH solutions (right) for 5 min and 30 min.

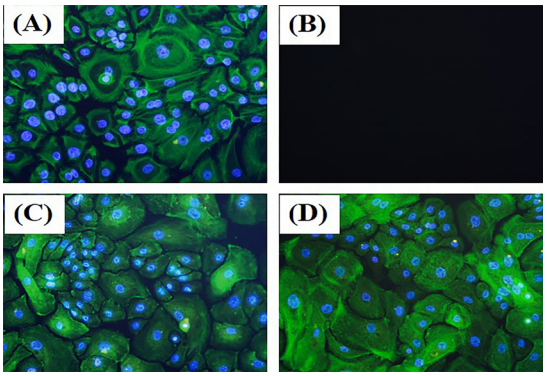

Figure 3. Gingival epithelial cells of the untreated group (A) and after exposure to 20% SDS solution (B), 20% LH solution (C), and 20% PH solution (D) at contact time of 30 min.

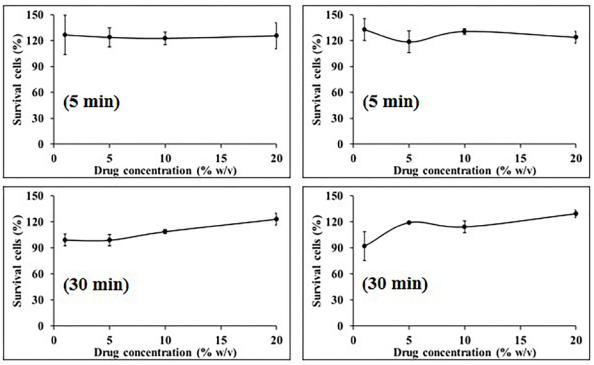

Figure 4. LDH leakage in cell culture medium after exposure to LH solutions (left) and PH solutions (right) for 5 min and 30 min.

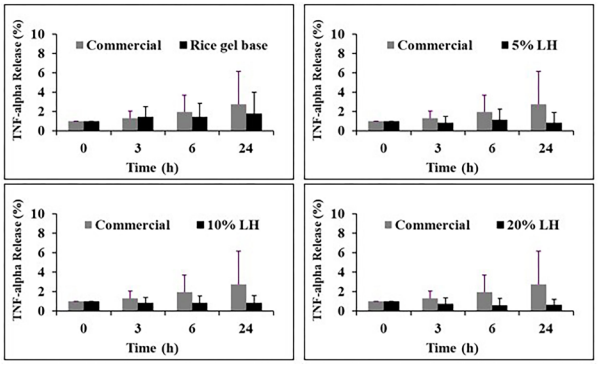

Figure 5. Expression of TNF-α after exposure to rice gel base, rice gels containing LH at 5%, 10%, and 20% in comparison with commercial product.

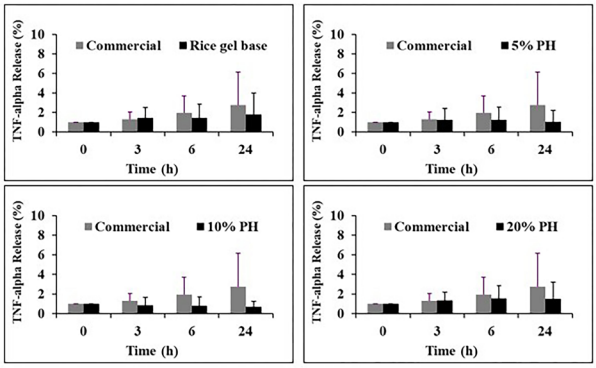

Figure 6. Expression of TNF-α after exposure to rice gel base, rice gels containing PH at 5%, 10%, and 20% in comparison with commercial product.

the commercial gel was shown in Figures 5 and 6, respectively. At the period of 0-3 h after removing gels, the rice gels containing either LH or PH and the commercial gel showed similar levels of TNF-α to the rice gel base which was about 1-2%. However, after 3 h, there was some difference occurred. The rice gel base and the PH gels for all test concentrations showed similar results with the steady TNF-α release but the commercial gel caused significantly slightly increase of TNF-α particularly at 24 h. Interestingly, LH gels showed gradually decrease of TNF-α along with the time and concentration. At 24 h, the gels with 10 and 20% LH showed significantly lower amount of TNF-α than at 0 h.

4. Discussion

Today, local anesthetics have been widely used in dental treatments for relive of pain caused by the treatment procedure. Among several local anesthetics,

lidocaine and prilocaine were of the most commonly used drugs due to their anesthetic fast onset and suitable duration (*24*). In addition, they have been widely studied and reported on relative low side effects than the others in the same group based on chemical structure (*25,26*). The use of lidocaine and prilocaine gel for intrapocket anesthesia demonstrates the high potency for pain relive in scaling and root planing procedure (*27*). However, there was not well investigated on the toxicity and inflammatory inducing effects of lidocaine and prilocaine to gingival tissue which might be occurred during dental procedures. In the present study, these effects of both drugs were investigated using LH and PH as drug models in order to confirm the safety of lidocaine and prilocaine during being exposure to gingival epithelial cells. In the study, the epithelial cells were isolated from healthy human subjects with wide range of age. It was found that age of the subjects providing tissue samples did not appear to affect the success rate in the culture which was in good agreement with the previous report (*28*). After contacting with various concentrations of LH and PH solutions with different contact times, it is shown that the cells could be survived indicating that both drugs are fully safe. In fluorescent images of the epithelial cells staining with DAPI and F-actin, the nucleus and cytoplasm, respectively, of the cells were obviously seen confirming the cells could be survived after contact with lidocaine and prilocaine. For investigation of cytotoxicity using LDH assay, high LDH release indicates high amount of cell death (*29*). The results of LDH assay revealed that lidocaine and prilocaine at 5-20% had no toxic effect to the cells.

Inflammation in gingival tissue can lead to severe dental diseases. Oral inflammation can be occurred due to several factors including noxious stimuli, oral bacteria, and certain drugs (*30*). Mild inflammation has been reported when the cream containing both anesthetics was inserted subcutaneously to rats (*31*). For side effect comparison between the two anesthetics, prilocaine has been reported to increase higher level of methemoglobin than lidocaine (*32*). Many mediators including TNF-α are released from the host cells during inflammatory process. TNF-α is a cell signaling cytokine involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. Therefore, determination of this cytokine can indicate the severity of inflammation. To investigate the inflammatory inducing effects of lidocaine and prilocaine, we applied the anesthetics in the form of gel formulation using modified rice as gelling agent in order to enhance the adhesion of the anesthetics to the gingival tissue along with the test period. The results indicated clearly that rice gel base as well as lidocaine and prilocaine rice gels did not induce inflammatory effects in volunteers. Therefore, we considered that lidocain and prilocain have no effect on inflammatory induction. The commercial lidocaine gel at the same drug concentration showed higher inflammatory inducing effect to the gingival tissues. It is considered that this effect might be due to the other excipients in the products.

In conclusion, the results of the present study demonstrate that both lidocaine and prilocaine are safe to use in oral cavity even in high concentration of 20% and prolong period of application. Rice gel base and rice gels containing these anesthetics also show no inflammatory inducing effect to the gingivitis.

Acknowledgements

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Original Article

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Protective effects of *Phaseolus vulgaris* lectin against viral infection in *Drosophila*

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Summary **Phytohemagglutinin (PHA) isolated from the family of *Phaseolus vulgaris* beans is a promising agent against viral infection; however, it has not yet been demonstrated *in vivo*. We herein investigated this issue using *Drosophila* as a host. Adult flies were fed lectin approximately 12 h before they were subjected to a systemic viral infection. After a fatal infection with *Drosophila* C virus, death was delayed and survival was longer in flies fed PHA-P, a mixture of L4, L3E1, and L2E2, than in control unfed flies. We then examined PHA-L4, anticipating subunit L as the active form, and confirmed the protective effects of this lectin at markedly lower concentrations than PHA-P. In both experiments, lectin feeding reduced the viral load prior to the onset of fly death. Furthermore, we found a dramatic increase in the levels of the mRNAs of phagocytosis receptors in flies after feeding with PHA-L4 while a change in the levels of the mRNAs of antimicrobial peptides was marginal. We concluded that *P. vulgaris* PHA protects *Drosophila* against viral infection by augmenting the level of host immunity.**

Keywords: Antiviral effect, innate immunity, plant lectin

1. Introduction

Lectins are carbohydrate-binding proteins that have been found in a number of organisms, including microorganisms, plants, and animals (*1*). Lectins exist in intracellular and extracellular compartments and bind mono- and oligosaccharides with specificity in certain sugar residues. When lectins act intracellularly, lectin-tagged cellular components may change their functions and/or localization. In contrast, plasma membrane-bound or extracellular lectins serve either as ligands or receptors as well as bridging molecules to link cells (*1*). When extracellular lectins function as ligands, they stimulate receptors at the surface of target cells in order to activate signal transduction pathways for the induction of a number of biological phenomena.

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Phytohemagglutinin (PHA) in the seeds of the red kidney bean *Phaseolus vulgaris* consists of a tetramer of subunits E and L, and five different compositions of PHA have been identified, i.e., E4, E3L1, E2L2, E1L3, and L4 (*2*). All these *P. vulgaris* PHAs possess an agglutination activity against red blood cells as well as a mitogen activity against lymphocytes, and subunits E and L appear to be responsible for the former and latter activities, respectively (*3*). *P. vulgaris* PHA has the potential as an agent against viral infection. Lectins isolated from the family of *P. vulgaris* beans, such as extra-long autumn purple beans, French beans, and Anasazi beans, exert inhibitory effects on the reverse transcriptase of human immunodeficiency virus *in vitro* (*2,4*). However, the antiviral activity of *P. vulgaris* PHA has not yet been demonstrated *in vivo*.

Insects have provided researchers with animal models suitable for the study of human diseases (*5*). Evidence for the application of insect models for the development of medicines to treat human diseases has been accumulated (*6-8*). The fruit fly *Drosophila melanogaster* has been widely used in research to elucidate the underlying

mechanisms of self-defense systems (*9,10*). In addition to the availability of robust genetic approaches, the small size of *Drosophila* allows us to utilize a large number of flies in each experiment, which in many cases results in accurate and reliable data. Despite size differences, the organization of the genome is very similar between humans and *Drosophila*, and a number of *Drosophila* models of human diseases exist including infectious diseases, diabetes mellitus, and cancer (*11-14*). Furthermore, similarities have been identified in antiviral mechanisms between humans and *Drosophila* (*15-18*). Therefore, we herein used *Drosophila* as a host for viral infection in order to elucidate whether *P. vulgaris* PHA exhibits antiviral activity *in vivo*.

2. Materials and Methods

2.1. *Materials*

The fly line w^{1118} obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, Indiana, U.S.A.) was maintained with standard cornmeal/agar medium at 25°C and used throughout this study. *P. vulgaris* lectin was extracted from red kidney beans, purified to homogeneity by affinity chromatography, and sub-types were separated by ion-exchange column chromatography, according to established procedures (*3*) with slight modifications. A mixture of L4, L3E1, and L2E2, which we operationally named PHA-P (J-Oil Mills code number J113), and purified L4 (PHA-L4) (J-Oil Mills code number J112) were individually tested for their antiviral activities. The amount of proteins was measured by the Bradford method using the Bio-Rad Protein Assay with bovine serum albumin as a standard protein. PHA-L4 and purified E4 (PHA-E4) labeled with fluorescence isothiocyanate (FITC), J-Oil Mills code numbers J512 and J511, respectively, were used to locate the lectin in the digestive tracts of flies.

2.2. *Lectin feeding of flies*

Males of *Drosophila* adults, 3-7 days after eclosion, were collected in vials (30-35 flies per vial) in which filter paper (Whatman 3MM Chr) was placed at the bottom and kept for 3 h with no food or water for starvation. A total of 0.3 mL of water containing or not containing *P. vulgaris* PHA at various concentrations was added to the vials, and flies were given free access to water absorbed by filter paper for 19 h. Flies were then maintained with regular food for 12 h for recovery from starvation before the abdominal infection with *Drosophila* C virus (DCV).

2.3. *Preparation of DCV, infection, and viral titer assays*

DCV, a natural pathogen of *Drosophila*, is a non-enveloped positive-strand RNA virus assigned to the Dicistroviridae genus *Criparivus*. In order to prepare DCV stocks, cultures of S2 cells, a *Drosophila* cell line established from embryonic hemocytes, were incubated with DCV, and culture media were collected, subjected to the assessment of viral titers with S2 cells as a host, and stored at –80°C until used, as described previously (*19*). Adult flies, with and without lectin feeding, were infected with DCV (2,750 or 27,500 50% tissue culture-infective dose ($TCID_{50}$)) by the abdominal injection of the viral suspension using a nitrogen gas-aided microinjector and maintained in vials containing regular fly food, as described previously (*19*). An assay for fly survivorship and the assessment of the viral load in flies were conducted as described previously (*19*).

2.4. *Messenger RNA analysis of antimicrobial peptides*

Males of *Drosophila* adults (approximately 15 flies), with and without lectin feeding, were suspended in TRIzol reagent (Invitrogen) in a plastic microtube and homogenized using a plastic pestle on ice. After centrifugation, RNA in the supernatants was precipitated with isopropanol and dissolved with water. Extracted total RNA was subjected to reverse transcription using a random hexamer as a primer to generate cDNA. Sequences corresponding to those of antimicrobial peptide mRNAs were amplified from the cDNA by PCR using primers specific to attacin-A, defensin, diptericin, drosomycin, and metchnikowin. The levels of the mRNAs of Draper and integrin αPS3-βv, engulfment receptors responsible for the phagocytic removal of apoptotic cells in *Drosophila* (*18*), were similarly determined. A sequence corresponding to the mRNA of the ribosomal protein rp49 was also amplified as an unchanged internal control. We first determined the ratio of two cDNA preparations, obtained from RNA of flies fed lectin or water, that gives a similar level of the signal derived from rp49 mRNA. Then, PCR amplification of the sequences corresponding to the mRNAs of antimicrobial peptides and engulfment receptors was carried out using two cDNA preparations at the determined ratio. The concentrations of all cDNAs used in PCR were within a quantifiable range. The PCR products were separated by polyacrylamide gel electrophoresis followed by staining with ethidium bromide. The intensity of each PCR product was digitized and shown relative to that obtained with RNA of water-fed flies. The nucleotide sequences of the PCR primers were: 5'-CCCGGAGTGAAGGATG-3' (forward) and 5'-GTTGCTGTGCGTCAAG-3' (reverse) for the mRNA of attacin-A (*20*); 5'-GTTCTTCGTTCTCGTGG-3' (forward) and 5'-CTTTGAACCCCTTGGC-3' (reverse) for the mRNA of defensin (*20*); 5'-GCTGCGCAATCGCTTCTACT-3' (forward) and 5'-TGGTGGAGTGGGCTTCATG-3' (reverse) for the mRNA of diptericin (*20*);

5'-CGTGAGAACCTTTTCCAATATGATG-3' (forward) and 5'-TCCCAGGACCACCAGCAT-3' (reverse) for the mRNA of drosomycin (*20*); 5'-AACTTAATCTTGGAGCGA-3' (forward) and 5'-CGGTCTTGGTTGGTTAG-3' (reverse) for the mRNA of metchnikowin (*20*); 5'-CGGAATTCTCTGCCGCAC GGGTTACATAG-3' (forward) and 5'-CCGCTCGAGC CGGCTCGAATTTTCGCTT-3' (reverse) for the mRNA of Draper (*21*); 5'-AGATACCTACTCCTGGGCTT-3' (forward) and 5'-TCCGCATTTGGAGCTCCATT-3' (reverse) for the mRNA of integrin αPS3 (*21*); 5'-AAGCCAACTCTACCCATGATT-3' (forward) and 5'-GTGGGACAGTTGCAATAGGT-3' (reverse) for the mRNA of integrin βv (*21*); and 5'-GACGCTTCAAGGGACAGTATCTG-3' (forward) and 5'-AAACGCGGTTCTGCATGAG-3' (reverse) for the mRNA of rp49 (*20*).

2.5. *Statistical analysis*

All data were collected from at least three independent experiments except for those presented as Figures 3B and 4B. The numerical data were statistically analyzed by the Log-rank test (the Kaplan-Meier method) for a survivorship assay or the two-tailed Student's *t*-test for other experiments. A p-value < 0.05 between two sets of experimental data was considered to be significant.

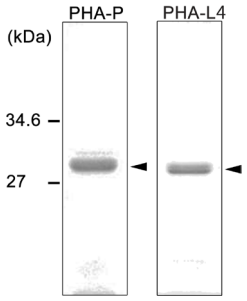

Figure 1. Purity of *P. vulgaris* PHA used in this study. Chromatographically prepared PHA-P (0.5 μg) and PHA-L4 (0.5 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis, and visualized by staining with Coomassie Brilliant Blue. The arrowheads point to the positions of lectin, and the positions and molecular masses of standard proteins are shown on the left. Subunits E and L possess a similar molecular mass of about 28 kDa.

3. Results

3.1. *Prolonged survival of DCV-infected flies after feeding with PHA-P*

Throughout this study, chromatographically purified *P. vulgaris* lectins were used (Figure 1). We first examined whether the procedures for feeding flies with lectin adopted in the present study allowed flies to take lectin into the digestive tract. Male adult flies were starved as described in the Materials and Methods and then fed FITC-labeled PHA-E4 or PHA-L4, which had been dissolved with water and adsorbed by filter paper placed in a vial. The digestive tracts of these flies were dissected out and examined under a fluorescence stereomicroscope. Fluorescence signals derived from FITC were clearly detected in a part of the midgut (*22*) of flies fed either isolectin (Figure 2), indicating the success of the oral administration of lectin in these procedures.

We then investigated the effects of PHA-P on the survival of flies after the abdominal infection with DCV. Adult flies were fed PHA-P, subjected to a fatal infection with DCV, and analyzed for survivorship. We found that flies pre-fed this lectin lived longer after the infection with DCV than unfed control flies (Figure 3A). PHA-P gave effective concentrations of 60-120 μg/mL during feeding (Figure 3B).

3.2. *Delay in the onset of fly death and prolongation in the survival time of DCV-infected flies after feeding with PHA-L4*

We performed similar experiments using PHA-L4, which consists of a tetramer of subunit L (see Figure 1). The administration of PHA-L4 extended the survival time of DCV-infected flies (Figure 4A), as did PHA-P, with effective concentrations of 1.2-6.0 μg/mL (Figure 4B), which were 20-50-fold lower than those of PHA-P. Furthermore, the pre-feeding of flies with PHA-L4 delayed death in flies (Figure 4A). The results obtained from repeated experiments revealed that PHA-L4-

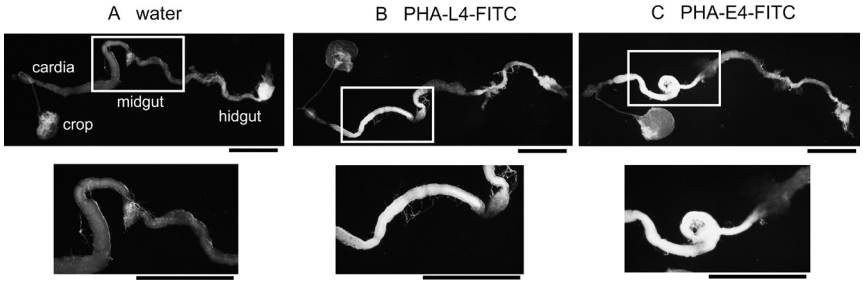

Figure 2. Existence of *P. vulgaris* PHA in the midgut of flies after feeding. Adult flies were fed FITC-labeled PHA-E4 (0.1 μg/mL), FITC-labeled PHA-L4 (0.1 μg/mL), or water alone as a control for 2 h. Digestive tracts were dissected out and examined by fluorescence microscopy. The bottom panels in each set of the data are magnified views of the squared area in the upper panels. Fluorescence signals derived from FITC are shown in white. The data represent one experiment of 4 replicates that showed a similar result. Scale bars = 200 μm.

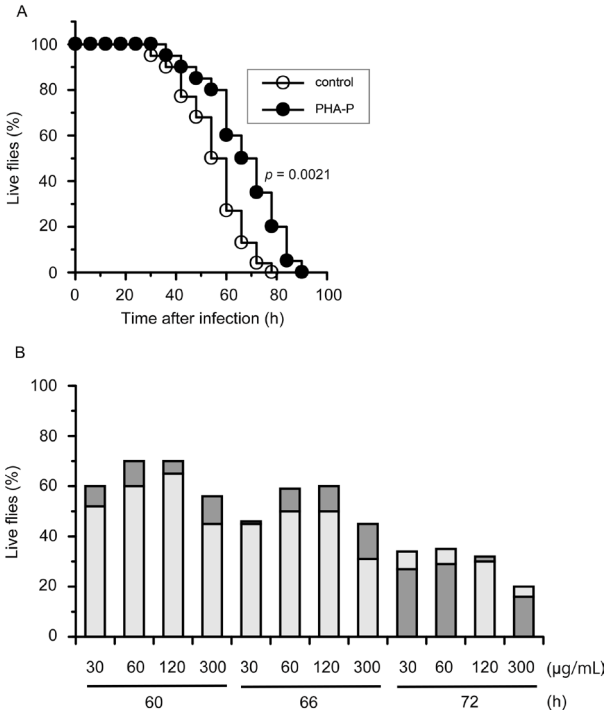

Figure 3. Effects of PHA-P on the survival of flies after DCV infection. (A) Adult flies were fed PHA-P (60 μg/mL) or water alone as a negative control for 19 h, abdominally infected with DCV at 27,500 $TCID_{50}$, and subjected to an assay for survivorship. Data obtained from one out of 3 independent experiments with similar results are presented. **(B)** Adult flies were fed PHA-P at various concentrations for 19 h, abdominally infected with DCV at 27,500 $TCID_{50}$, and examined for the ratio of live flies at the indicated time points. Data from two independent experiments are shown as white and gray bars.

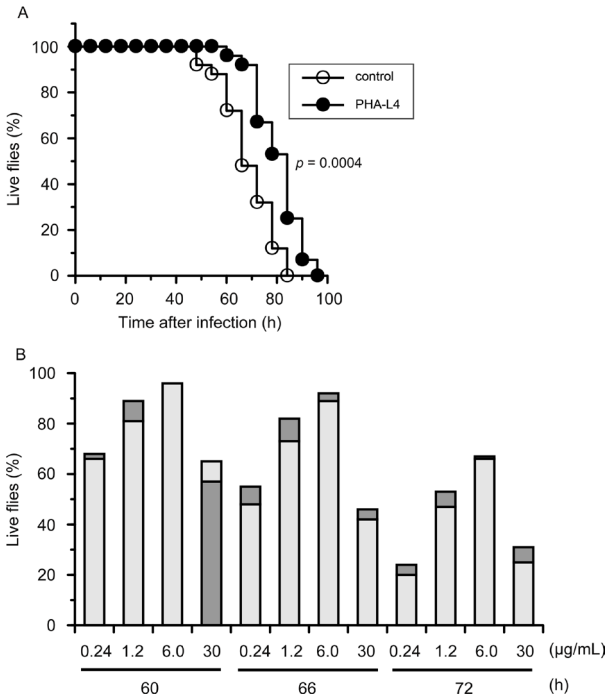

Figure 4. Effects of PHA-L4 on the survival of flies after DCV infection. (A) Adult flies were fed PHA-L4 (6 μg/mL) or water alone as a negative control for 19 h, abdominally infected with DCV at 27,500 $TCID_{50}$, and subjected to an assay for survivorship. Data obtained from one out of 5 independent experiments with similar results are presented. **(B)** Adult flies were fed PHA-L4 at various concentrations for 19 h, abdominally infected with DCV at 27,500 $TCID_{50}$, and examined for the ratio of live flies at the indicated time points. Data from two independent experiments are shown as white and gray bars.

Table 1. Timings of fly death

Items	2,750 $TCID_{50}$		27,500 $TCID_{50}$	
	Onset	Extinct	Onset	Extinct
Water-fed flies	42 - 48	90	42 - 54	78 - 90
PHA-L4-fed flies	48 - 66	96	54 - 60	84 - 102

Hours post-infection when flies start to die (onset) and all flies are dead (extinct) are shown as a summary of the data from three independent experiments with two different viral doses.

treated flies started to die approximately 10 h later and lived approximately 10 h longer than control flies (Table 1). In order to confirm these effects of PHA-L4, we carried out an infection experiment at a 10-fold lower dose of the viral burden. We found a similar protective effect against DCV infection in flies administered PHA-L4 as that observed in the experiment with a higher viral dose (Table 1).

3.3. *Inhibitory effects of PHA-L4 on the viral load in Drosophila*

We next investigated the level of the viral load in flies at various time points after the abdominal infection with DCV at two different doses. We found that the level markedly increased during the first 12 h, and then more gradually increased in control unfed flies at either viral dose (Figures 5A and 5B). The pre-administration of PHA-L4 to flies inhibited the growth of the virus, apparently in a different manner at the two viral doses used. In flies infected with DCV at a higher dose, lectin reduced the level of the viral load during 30-42 h post-infection, a period when the viral level reached its maximum (Figure 5A). In contrast, an increase in the level of the viral load was inhibited at early time points, 12-24 h post-infection, in flies administered a lower dose of the virus (Figure 5B). The inhibition of DCV growth by PHA-L4 appeared to occur well before the onset of fly death, irrespective of the initial level of the viral load (Table 1).

3.4. *Increases in the level of the mRNAs of immunity-related proteins in flies after feeding with PHA-L4*

Feeding with lectin might alter the antiviral status in flies. Both humoral (*23*) and cellular (*19*) immunity play roles in the protection of flies against viral infection, including that with DCV. Therefore, we compared the amounts of the mRNAs of antimicrobial peptides and

phagocytosis receptors in adult flies before and after lectin feeding by reverse transcription-mediated PCR. The mRNA levels of various antimicrobial peptides, the Toll pathway-induced attacin and diptericin as well as the Imd pathway-induced defensin, drosomycin, and metchnikowin (*10*), were determined, and we found that the mRNA levels of some antimicrobial peptides, such as diptericin and defensin, marginally increased in flies after feeding with PHA-L4 (Figure 6A). We then similarly analyzed the mRNAs of Draper and integrin αPS3-βν, engulfment receptors that activate two partly overlapping signaling pathways for the induction of phagocytosis of apoptotic cells by *Drosophila* hemocytes (*21*). The data indicated a dramatic increase in the levels of the mRNAs of both Draper and integrin βν in flies after lectin feeding (Figure 6B): signals derived from the mRNA of integrin αPS3 were not detected under the conditions adopted in this study (data not shown). These results suggest that *P. vulgaris* PHA enhances Toll and Imd pathways as well as the phagocytic activities of hemocytes in adult flies.

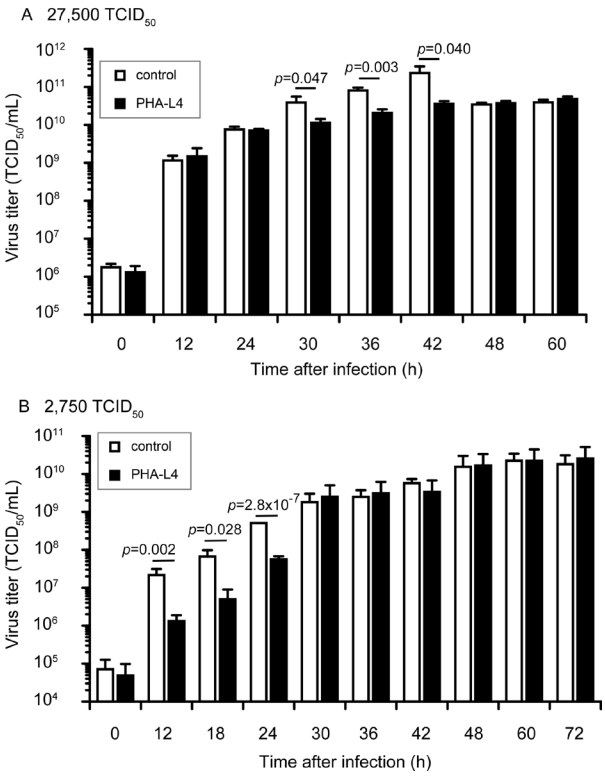

Figure 5. Effects of PHA-L4 on the viral load in adult flies. Adult flies were fed PHA-L4 (6 μg/mL) or left untreated as a negative control for 19 h, abdominally infected with DCV at 27,500 $TCID_{50}$ **(A)** or 2,750 $TCID_{50}$ **(B)**, and subjected to an assay for the viral load at the indicated time points. The means ± SD of data obtained from 3 independent experiments are shown. The data from two groups were statistically analyzed, and *p*-values are indicated when they were found to be significantly different.

4. Discussion

Pre-feeding with lectin isolated from *P. vulgaris* beans extended the survival period of *Drosophila* adults after an infection with DCV. This result indicates that *P. vulgaris* lectin exhibits antiviral activity in *Drosophila*. Based on the similarities in antiviral mechanisms between insects and humans, the present results confirmed that *P. vulgaris* PHA protects a host organism against viral infection.

Lectin-fed flies started to die later and survived longer than control unfed flies, and decreased the levels of the viral load. Antiviral actions accompanied by a decrease of the viral load suggest that the lectin treatment enhanced fly resistance, not tolerance against the virus (*24*). An analysis with fluorescence-labeled lectin indicated the presence of lectin in the midgut of the digestive tracts of flies. However, we speculate that lectin did not enter the body across the wall of the tract due to the peritrophic matrix, which serves as a barrier against microbial pathogens (*25*). Therefore, the antiviral effects of *P. vulgaris* PHA do not appear to occur through a direct interaction with the virus, but by indirectly altering host responses against viral infection. The antiviral defenses of *Drosophila* have been shown to rely on RNA interference, the Toll-Dorsal pathway, the JAK-STAT pathway, the phagocytosis of virus particles, and the phagocytosis of virus-infected cells (*16-19,26-28*). Therefore, humoral and cellular innate immune responses appear to participate in the protection of *Drosophila* against viral infection. We showed a dramatic increase in the levels of the mRNAs of phagocytosis receptors, which are responsible for the removal of cells undergoing apoptosis (*18*), whereas an increase of antimicrobial peptide mRNAs was only marginal. Taking our previous finding that the apoptosis-dependent phagocytosis of DCV-infected cells plays an important role in the antiviral actions of *Drosophila* immunity (*19*) into consideration, an increase in the phagocytic activity of immune cells could be a mechanistic interpretation of the antiviral effects of *P. vulgaris* lectin. Subunit L appears to be responsible for the antiviral actions of *P. vulgaris* lectin, as in the induction of lymphocyte growth (*3*). It is, thus, likely that PHA-L4 is a major *P. vulgaris* isolectin that influences the level of immunity in both vertebrate and invertebrate animals. Further investigation is required to clarify the mechanisms of actions of *P. vulgaris* PHA to protect animals from microbial infections.

In conclusion, *P. vulgaris* lectin, particularly PHA-L4, was shown to exhibit protective activity against DCV infection in *Drosophila* by augmenting the resistance of the host.

Acknowledgements

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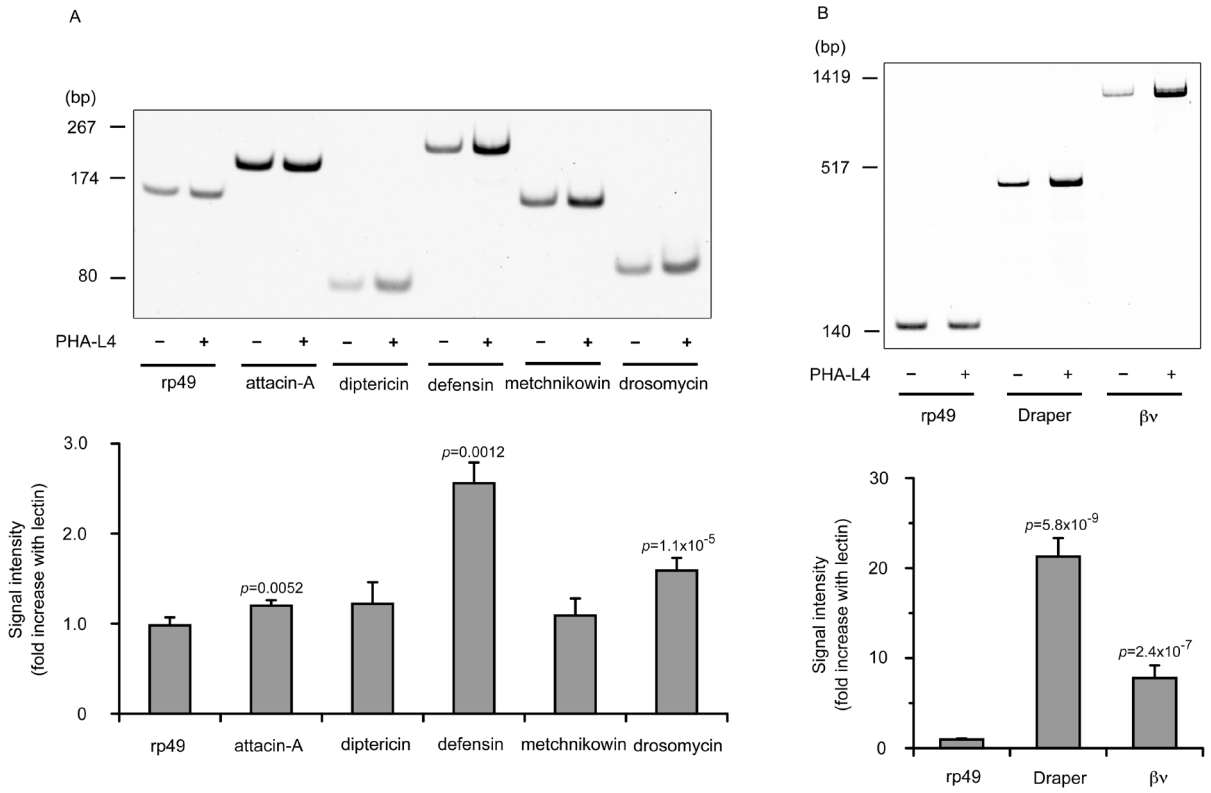

Figure 6. Effects of PHA-L4 on the levels of the mRNAs of antimicrobial peptides and phagocytosis receptors. Adult flies were fed PHA-L4 (6 μg/mL) or water alone for 19 h. Total RNA was extracted and analyzed by reverse transcription-mediated PCR for the mRNAs of the indicated antimicrobial peptides **(A)** and phagocytosis receptors **(B)**, together with that of rp49 as an unchanged internal control. Products in PCR were separated on a 6% polyacrylamide gel and visualized by the staining with ethidium bromide, and the intensities of the signals were digitized. Shown at the top are examples of ethidium bromide-stained gels with the positions and sizes, in base pairs (bp), of standard DNAs on the left. The intensities of the signals in four independent experiments were determined and shown relative to those obtained with RNA of water-fed flies, taken as 1, as means ± SD at the bottom. βν, integrin βν.

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Brief Report

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Digital PCR for determination of cytochrome P450 2D6 and sulfotransferase 1A1 gene copy number variations

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Summary ***CYP2D6*** **and** ***SULT1A1*** **occasionally show copy number variations (CNVs), with a larger number generally indicating greater enzymic activity. However, those variations are difficult to calculate using standard methods. With digital PCR, a recently introduced method for CNV analysis, DNA molecules are subjected to limited dilution and separated into nano-scale droplets prior to a PCR assay. Absolute quantitation of copy number can then be performed with high accuracy and sensitivity by determining the number of droplets showing an amplified signal for the target gene. This is the first report of analyses of** ***CYP2D6*** **and** ***SULT1A1*** **CNVs using a digital PCR method with blood sample from Japanese subject. Primers and probes were synthesized for the target and reference genes, and copy number calculation was performed using a QX200 Droplet Digital PCR System. Our results showed that the copy numbers in** ***CYP2D6*5 hetero*****, non-CNV, and** ***CYP2D6xN*** **subjects were 1, 2, and 3 to 4, respectively. In addition, in non-CNV and multiplication subjects, the number of copies for** ***SULT1A1*** **was 2 and 3 to 6, respectively. We found that the present digital PCR method was useful as well as accurate. In the future, a combined genotyping, allele distinction, and copy number calculation technique will be helpful for analysis of enzymic activity.**

Keywords: Cytochrome P450 2D6, sulfotransferase 1A1, copy number variation, digital PCR

1. Introduction

Cytochrome P450 2D6 (CYP2D6) metabolizes several different drugs, including timolol, propranolol, amitriptyline, propafenone, flecainide, and tamoxifen (*1-6*). The metabolic ratios of the probe drugs vary, thus patients can be classified into 4 different genotypes; poor metabolizer (PM), intermediate metabolizer (IM), extensive metabolizer (EM), and ultra-rapid metabolizer (UM) (*7*). Generally, when a PM or IM patient is administered a drug that is inactivated by CYP2D6, the blood concentration and risk of side effects will be increased as compared to EM patients. On the other hand, should a UM patient be administered such a drug, the concentration and therapeutic effect will be lower as compared to EM patients (*8-12*).

CYP2D6 has been reported to have over 100 gene polymorphisms (*13*). For example, *CYP2D6*4*, **5*, and **14* lack enzymic activity, while *CYP2D6*10* and **21* show decreased enzymic activity (*14-16*). Approximately 7% of Caucasians are classified as PM and considered to have *CYP2D6*4* or **5*, while the frequency of PM in the Japanese population is under 1%, and the main mutations are *CYP2D6*5* and **14* (*17-23*). In contrast, the frequency of IM is high in Japanese at 15%, which can be explained by *CYP2D6*10* (*24,25*). Since the frequencies of these 3 mutations in Japanese are high, analysis is important to elucidate CYP2D6 activity. In addition, *CYP2D6* occasionally shows copy number variations (CNVs), including gene deletion (*CYP2D6*5*), duplication, and multiplication (*CYP2D6xN*). *CYP2D6*1*, **2*, **4*, **9*, **10*, **17*, **35*, **36*, and **41* have been reported as 2-copy alleles (*CYP2D6x2*), among which *CYP2D6*2* is recognized to have 3 to 5 and 13 copies (*10,11,26-31*). A larger

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Table 1. Sequences of primers and probes used for digital and long PCR

Name	Sequence (5' → 3')
CYP2D6_Dup_F1 (*38*)	CTT CAC CTC CCT GCT GCA G
CYP2D6_Dup_R1 (*38*)	TCA CCA GGA AAG CAA AGA CA
CYP2D6_Dup_P1 (*38*)	FAM-CCG GCC CAG CCA CCA TGG-BHQ
SULT1A1_Dup_F1	AAA GGA TGT GGC AGT TTC CT
SULT1A1_Dup_R1	CAC ACT TTC CTT CCT CCC AT
SULT1A1_Dup_P1	FAM-CTC AGG GTG CAC CTT GGC CA-BHQ
RPPH1_F1	GGG AGG TGA GTT CCC AGA G
RPPH1_R1	CGT GAG TCT GTT CCA AGC TC
RPPH1_P1	HEX-CTG CCC AGT CTG ACC TCG CG-BHQ
P2×2f (*39*)	GCC ACC ATG GTG TCT TTG CTT TC
P2×2r (*39*)	ACC GGA TTC CAG CTG GGA AAT G
CYP13 (*40*)	ACC GGG CAC CTG TAC TCC TCA
CYP24 (*40*)	GCA TGA GCT AAG GCA CCC AGA C

FAM: 5-Carboxyfluorescein hydrate; HEX: 6-Carboxy-4,7,2',4',5',7'-hexachloro-fluorescein-3',6'-dipivaloate; BHQ: Black Hole Quencher™.

number of copies generally indicates greater enzymic activity, which induces UM.

The genetic polymorphisms of CYP2D6 are complex and widespread, thus precise estimation of phenotype using a gene test is difficult. Notably, "copy number" and "metabolism activity" are matters for CNV analysis. As for copy number, that is difficult to calculate with existing PCR methods as it is not possible to clearly determine the amount of change in enzymic activity. A more accurate method is needed, as conventional quantitative PCR assays are limited in regard to resolution and can at best distinguish a twofold difference in copy number of a particular gene in a DNA sample. Regarding metabolism activity, if the causal genes of PM (*e.g.*, *CYP2D6*4* and **36*) become duplicated (*e.g.*, *CYP2D6*4x2* and **36x2*), that also indicates development of PM (*27,30,31*). Similarly, we previously reported that if *CYP2D6*10* (cause of IM) is increased by 2 to 3 copies, there is not a significant difference (*29*), thus in the case of *CYP2D6xN*, the activity will be different depending on which allele has the mutation (*28*). For example, in a comparison of *CYP2D6*1x2/*10* and *CYP2D6*1/*10x2*, the former shows activity nearly to the level of EM, while the latter shows activity lower than EM.

Tamoxifen, a drug developed for treating breast cancer, is metabolized by CYP2D6, while its metabolite, 4-hydroxy tamoxifen, is metabolized by sulfotransferase 1A1 (SULT1A1) (*32,33*). In other words, CYP2D6 activates tamoxifen and SULT1A1 is involved in its inactivation. The major mutations of SULT1A1 are *SULT1A1*2* and **3*, whose frequencies are approximately 10% and under 1%, respectively, in Asian individuals (*34*). *SULT1A1*2* shows a lower level of activity than the wild type (*35*). Moreover, *SULT1A1* occasionally shows CNVs (1 or 3-6 copies), thus it is important to calculate the copy number as with *CYP2D6*. These are important, as such mutations cause individual differences in regard to the effects of tamoxifen in patients administered the drug (*36,37*).

Digital PCR, in which DNA molecules are subjected to limiting dilution and separated into nano-scale droplets prior to the assay, was recently introduced as a method for CNV analysis. With this technique, absolute quantitation of copy number can be performed with high accuracy and sensitivity by determining the number of droplets with an amplified signal for the target gene. In the present study, we calculated *CYP2D6* and *SULT1A1* copy numbers using a digital PCR method.

2. Materials and Methods

2.1. *Samples*

Blood samples from randomly selected unrelated Japanese subjects who participated in a previously reported study were obtained (*25,29,37*). The *CYP2D6* and *SULT1A1* genotypes were previously analyzed in these subjects. Approval for these studies were obtained from the local Institutional Review Board (*25,29*) and Chiba Institute of Science (*37*), separately. The sequences of the primers and probes used in the present study are shown in Table 1.

2.2. *Preprocessing for human genome DNA sample*

A single DNA molecule was separated to a droplet prior to performing digital PCR. When the target gene has multiple copies, they are closely connected on the same molecule, thus they behave as a single molecule and the target gene copy number is underestimated. Accordingly, preprocessing of the samples was performed using restriction enzymes (New England Biolabs Japan, Tokyo, Japan), with *Nde* I used for *CYP2D6* and *Pst* I used for *SULT1A1* (Figure 1). A 20-μL reaction mix containing 2 μL of 10x buffer, 4 IU of restriction enzymes, and 200 ng of genome DNA was utilized. Incubation was performed at 37°C for 120 minutes.

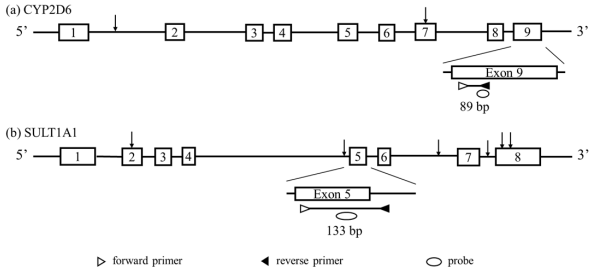

Figure 1. Strategy used to determine genomic DNA in human blood samples. We utilized PCR amplification followed by digestion of *Nde* I (*CYP2D6*) **(a)** and *Pst* I (*SULT1A1*) **(b)**. Multiple copies of the target gene might be closely connected on the same chromosome, thus they would behave as a single molecule and the copy number would be underestimated. Restriction enzymes were used to separate connected copies of the gene. Enzyme restriction sites are noted.

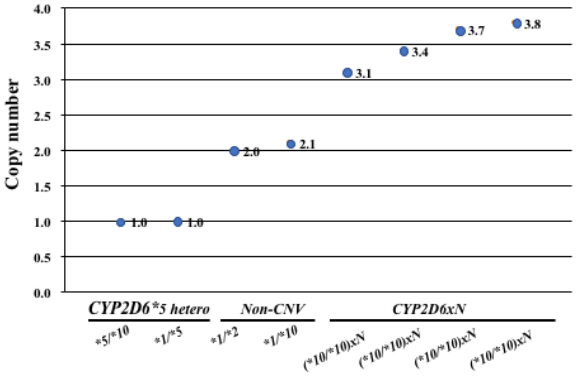

Figure 2. Determination of *CYP2D6* gene CNVs using digital PCR. The *x*-axis shows the sample genotype and category. Genotype was determined prior to digital PCR analysis. The *y*-axis shows the copy numbers for *CYP2D6*.

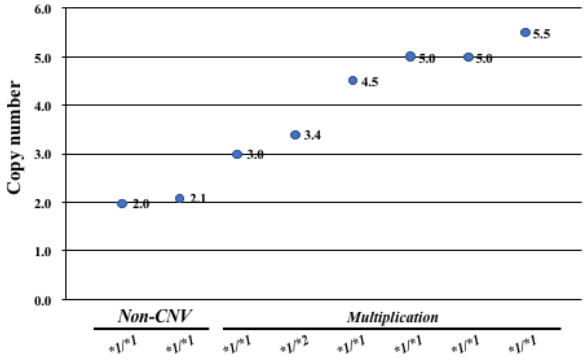

Figure 3. Determination of *SULT1A1* gene CNVs using digital PCR. The *x*-axis shows the sample genotype and category. Genotype was determined prior to performing digital PCR analysis. The *y*-axis shows the copy numbers for *SULT1A1*.

2.3. *Digital PCR for CYP2D6*

Digital PCR for *CYP2D6* was performed using specific primers and probes, as previously described by Qin J, *et al*. (*38*). Briefly, a 20-μL PCR mix containing 10 μL of 2× ddPCR supermix for probes, 0.625 μM each of primers for *CYP2D6* and the reference gene (*RPPH1*), 0.5 μM each of the probes for those, and 20 ng of genome DNA cut with *Nde* I was prepared (Figure 1). The PCR mix was partitioned into a discrete water-in-oil emulsion using a QX200 Droplet Generator (Bio-Rad Laboratories, Inc. Tokyo, Japan) prior to the PCR assay. The thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 minutes, followed by 45 cycles of denaturation at 94°C for 15 seconds, then annealing and extension at 60°C for 1 minute, with a final inactivation step performed at 98°C for 10 minutes. Following amplification, fluorescence of the samples was analyzed using a QX200 Droplet Reader (Bio-Rad Laboratories, Inc. Tokyo, Japan) to calculate the copy numbers.

2.4. *Digital PCR for SULT1A1*

We designed primers and probes specific for the *SULT1A1* gene. A 20-μL PCR mix containing 10 μL of 2× ddPCR supermix for probes, 0.9 μM each of primers for *SULT1A1* and *RPPH1*, 0.5 μM each of the probes for those, and 10 ng of genome DNA cut with *Pst* I was prepared (Figure 1). The PCR mix was partitioned into a discrete water-in-oil emulsion using a QX200 Droplet Generator prior to the PCR assay. Thermal cycling conditions consisted of an initial denaturation step at 95°C for 10 minutes, followed by 45 cycles of denaturation at 94°C for 15 seconds, then annealing and extension at 54°C for 1 minute, with a final inactivation step performed at 98°C for 10 minutes. Following amplification, fluorescence of the samples was analyzed using a QX200 Droplet Reader to calculate the copy numbers.

2.5. *Detection of CYP2D6xN and CYP2D6*5*

Detection of *CYP2D6xN* and *CYP2D6*5* was performed using long PCR testing, as previously described by Johansson I, *et al*. (*39*) and Steen VM, *et al*. (*40*), respectively.

3. Results and Discussion

For analysis of *CYP2D6*, we categorized the samples into 3 groups (*CYP2D6*5 hetero*, non-CNV, *CYP2D6xN*) based on known genotypes and compared those with the digital PCR data obtained in the present study. The copy numbers of those were 1, 2, and 3 to 4, respectively, thus there were no contradictions (Figure 2). However, the primers used in this study targeted exon 9 of *CYP2D6* and cannot be used to analyze copy numbers that have gene conversion to *CYP2D7* in exon 9 (*e.g. CYP2D6*36* or **36x2*). Thus, it is necessary to design primers for other regions in *CYP2D6*.

For analysis of *SULT1A1*, the samples were categorized into 2 groups (non-CNV, and multiplication) based on known genotypes, then compared using our

digital PCR findings. Their copy numbers were 2, and 3 to 6, respectively (Figure 3). In our previous study, we did not find samples with an *SULT1A1* gene deletion (*37*). In the present study, none of the samples had 0 or 1 copy, thus there were no contradictions. In that previous study, we were only able to obtain ambiguous copy number results, which were noted as "4 or more", while the present method allowed us to more clearly determine copy numbers.

To the best of our knowledge, this is the first report of analyses of *CYP2D6* and *SULT1A1* CNVs using a digital PCR method with clinical samples. Copy numbers are primarily presented as a single integer, though some are shown as an ambiguous number (*e.g.*, 3.4). We rounded off the numbers obtained and considered them as final copy numbers. We speculated that an imperfect reaction of the restriction enzymes was the primary reason of the ambiguous numbers, while use of clinical samples instead of cell lines may have also been related to that result.

CYP2D6 and SULT1A1 are involved in the main metabolism pathway of tamoxifen, with the former having effects on activation and the latter on inactivation. Accordingly, the effects and side effects of tamoxifen are increased in *CYP2D6xN* patients, whereas patients with *SULT1A1* multiplication will not see sufficient effects from the drug.

Several reports regarding the relationships between the genotypes of enzymes that metabolize tamoxifen and effects of the drug have been presented. Xu Y, *et al*. (*41*) found that the *CYP2D6*10* mutation had effects on tamoxifen efficacy in Chinese patients, while Gjerde J, *et al*. (*42*) conducted a study in Norway and reported that the *CYP2D6* genotype influenced conversion of tamoxifen to potent hydroxylated metabolites. On the other hand, investigations performed by Wegman P, *et al*. (*43*) in Sweden and Okishiro M, *et al*. (*44*) in Japan found no relationship of *CYP2D6* or *SULT1A1* genotypes with survival time. Similarly, Lum DWK, *et al*. (*45*) analyzed the *CYP2D6* genotype and tamoxifen response by meta-analysis, and found no association, while Motamedi S, *et al*. (*46*) performed a study in Iran, and reported that there was no significant relationship between *CYP2D6* copy number and tamoxifen resistance in their patients.

Estimation of response to tamoxifen given for breast cancer is extremely difficult because *CYP2D6* mutations include many variations and high complexity, and the drug is related to some genetic polymorphisms of enzymes such as SULT1A1. Few studies have performed analyses using a combination of multiple mutations, or combined genotyping and CNV analysis. We found that the present digital PCR method was able to calculate *CYP2D6* and *SULT1A1* copy numbers. In the future, a combined method for genotyping, distinguishing alleles, and calculating copy numbers will be helpful for estimating the effects and side effects of drugs such as tamoxifen.

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Brief Report

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Do scleroderma patients look young?: Evaluation by using facial imaging system

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Summary **These days various collagen supplements have widely been marketed. However, it has not been scientifically proved whether increasing collagen can actually prevent skin aging. Systemic sclerosis (SSc) is an autoimmune disease that is characterized by thickening of the skin caused by accumulation of collagen. In this study, we tried to evaluate facial skin characteristics and skin aging of SSc patients by using digital imaging system. As the result, the severity of wrinkles, texture and pores were significantly lower in SSc patients than control subjects. Among them, wrinkles showed better correlation with skin thickness score. Therefore, increased amount of collagen in scleroderma skin may directly affect wrinkles. In conclusion, attempt on collagen induction itself is reasonable and effective strategy in order to keep young appearance, although oral collagen supplementation may not directly reach to the skin.**

Keywords: Skin aging, collagen, wrinkles

1. Introduction

Nowadays, various collagen supplements have widely been marketed. However, it has not been scientifically proved whether increasing collagen can actually prevent skin aging. The evaluation of skin aging of patients with increased collagen deposition may answer this question. Systemic sclerosis (SSc) is a collagen disease mainly featured by fibrosis of the skin and various internal organs (*1*). Although the pathogenesis of fibrosis in SSc is still poorly understood, it may include inflammation, aberrant immune activation and endothelial cell injury, resulting in the activation of fibroblasts to increase the production of various collagens, mostly type I collagen (*2*).

To note, there is "folklore" that SSc patients look younger due to collagen deposition and the sclerosis of facial skin. Recent studies have indicated the usefulness of objective computer assessment of facial skin (*3,4*). Herein, we tried to evaluate facial skin characteristics and skin aging of SSc patients by using digital imaging system.

2. Methods

2.1. *Clinical assessment and patient material*

Twenty SSc patients (17 females and 3 males; mean age, 64.7 ± 7.7 years) who were hospitalized between March 2016 and April 2017 were enrolled in this study. Patients fulfilled the criteria proposed by the American College of Rheumatology and the European League Against Rheumatism (*5*). Modified Rodnan total skin thickness score (MRSS), semi-quantitative skin sclerosis assessment tool, was obtained at the first visit (*6*). Control data were also collected from the same number of age- and sex-matched control subjects (mean age, 63.1 ± 7.8 years). The control subjects consist of hospitalized patients with other diseases randomly chosen as indicated in Table 1. These patients did not have facial skin lesions.

2.2. *Photographing and facial skin analysis*

Photographing and facial skin analysis were conducted as objective computer assessments by VISIA®-

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Table 1. list of control subjects

Items	number
Dermatomyositis	5
Malignant melanoma	3
Squamous cell carcinoma/Bowen's disease	3
Basal cell carcinoma	2
Chronic skin ulcer	2
Chronic prurigo	1
Parapsoriasis	1
Neurofibromtosis type 1	1
Non-Langerhans cell histiocytoses	1
Lipoma	1
Total	20

Evolution (Canfield Imaging Systems, Fairfield, NJ). The eight skin characteristics (spots, wrinkles, texture, pores, UV spots, brown spots, red areas, and porphirins) were evaluated by percentiles that were calculated comparing with sex-, age-, and ethnicity-matched healthy subject data: higher percentile scores indicate less severity of each characteristic. Average percentile scores were obtained from two independent analysis.

2.3. *Statistical analysis*

The statistical analyses were carried out with Mann-

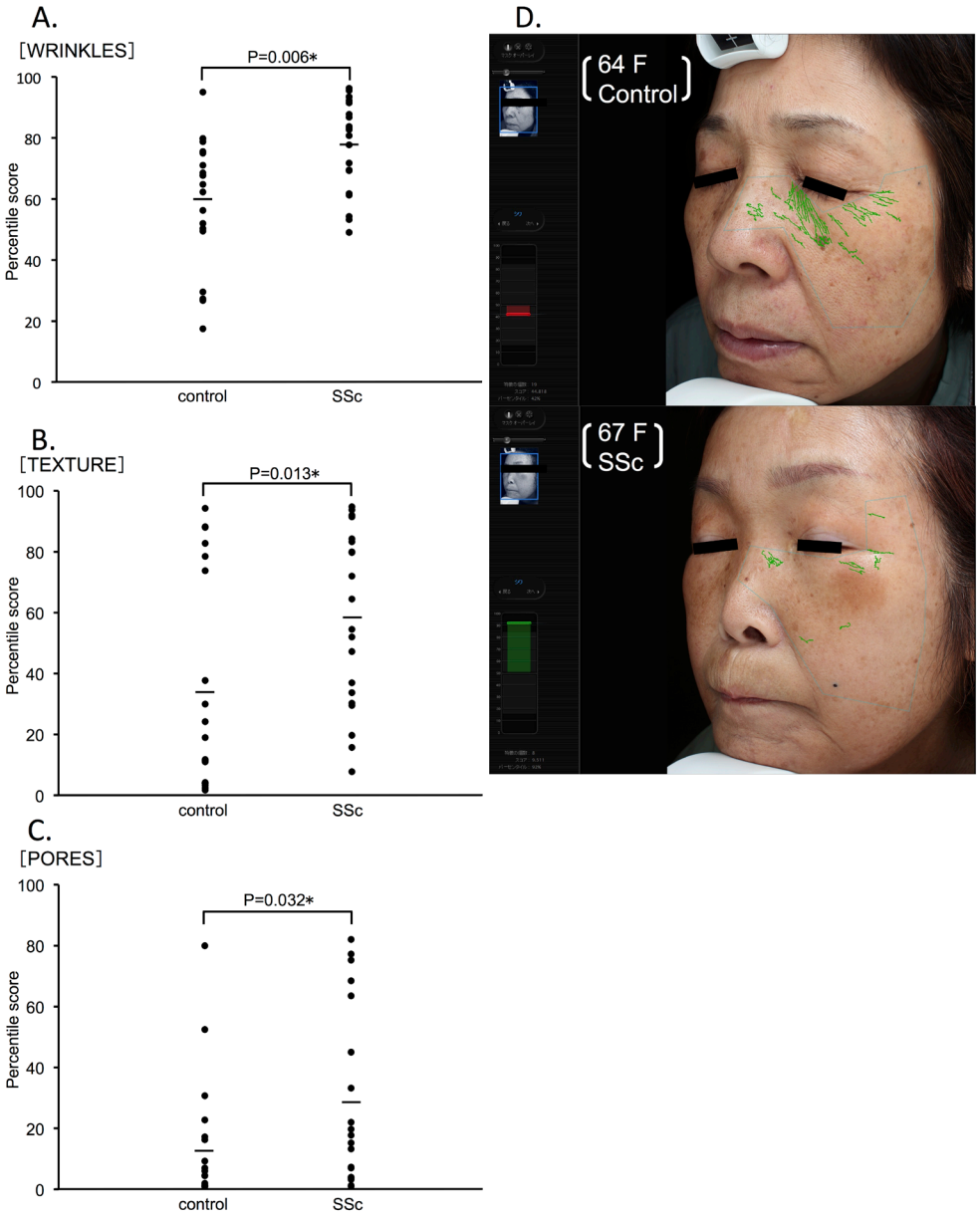

Figure 1. Objective computer assessments of three skin characteristics evaluated using VISIA®-Evolution. (A-C) The percentile scores of wrinkles **(A)**, texture **(B)**, and pores **(C)** in patients with systemic sclerosis (SSc) and in control subjects (Control) are plotted along the ordinate. Bars show their means. P-values are determined by Mann-Whitney *U*-test. **(D)** A representative photograph of comparing an SSc patient with age- and sex-matched control subject. Green lines on the face indicate detected wrinkles.

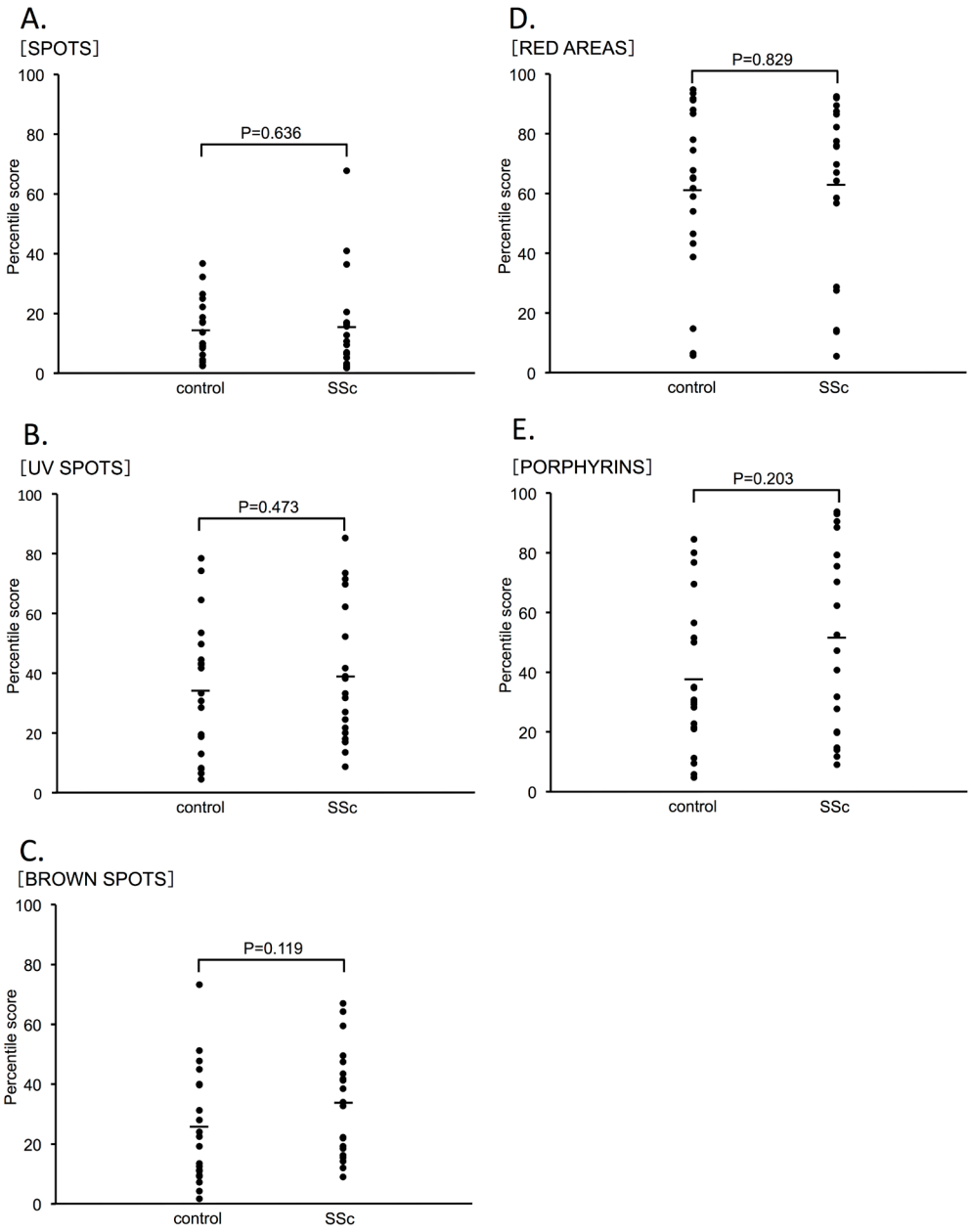

Figure 2. Objective computer assessments of five skin characteristics evaluated using VISIA®-Evolution. The percentile scores of spots **(A)**, UV spots **(B)**, brown spots **(C)**, red areas **(D)**, and porphyrins **(E)** in patients with systemic sclerosis (SSc) and in control subjects (Control) are plotted along the ordinate. Bars show their means.

Whitney *U*-test in the comparison of medians. Correlations were assessed using Pearson's correlation coefficient. All analyses were performed with Statcel4 software (OMS, Saitama, Japan): $p < 0.05$ was considered significant.

3. Results and Discussion

When we compared the percentile scores of SSc patients to control subjects, the scores of wrinkles, texture and pores were significantly higher in SSc patients than control subjects (Figure 1). This indicated that SSc patients have fewer wrinkles/pores and more fine-textured skin, which objectively supported the notion that SSc patients look young. On the other hand, there were no statistically significant differences in the scores of other characteristics between SSc and control subjects (Figure 2): for example, although telangiectasia is also common feature of SSc skin (*7*), the scores of red areas were not significantly different between the two groups.

Next, we examined the correlation of MRSS with percentile scores of the three characteristics (wrinkles, texture, and pores) in SSc patients. Among them, wrinkles showed better correlation with MRSS (R = 0.234) than the others (Figure 3). Therefore, increased amount of collagen in SSc skin may directly affect wrinkles. Histopathologically, SSc skin shows increased and thickened collagen bundles in the dermis as well

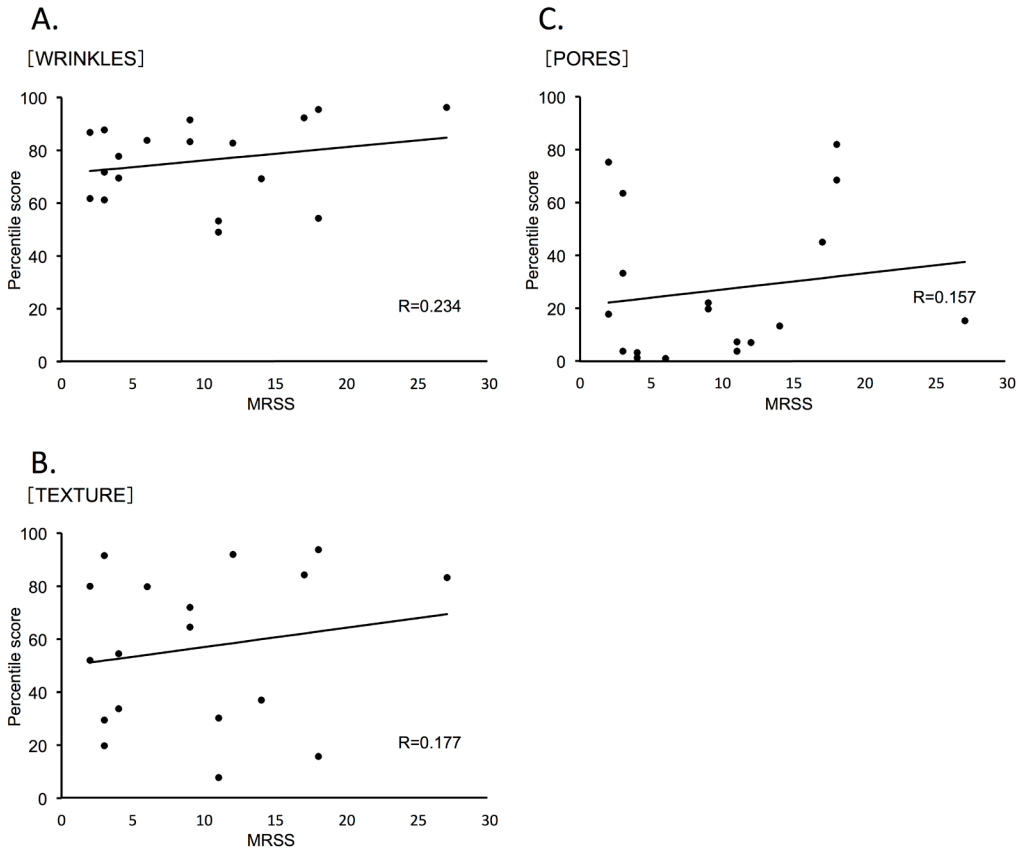

Figure 3. Correlation of MRSS with percentile scores of three characteristics (wrinkles, texture and pores) in SSc patients. Correlations of MRSS with percentile scores of wrinkles **(A)**, texture **(B)** or pores **(C)** were assessed by Pearson's correlation coefficient.

as subsequent atrophic epidermis and appendages. The atrophic epidermis and appendages may result in improved texture or pores, respectively.

As described above, the percentile scores derived from VISIA system were based on the huge database of healthy individuals. A limitation of this study is that control subjects consisted of patients with various diseases, because they were randomly chosen from hospitalized patients. This is a pilot study with a small number of patients and controls, and larger studies with increased and organized controls (*e.g.* 20 patients for each disease listed in Table 1) are needed in the future.

In conclusion, our result supported the notion that SSc patients look young. Therefore, although oral collagen supplementation may not directly reach to the skin, attempt on collagen induction itself is reasonable and effective strategy in order to keep young appearance.

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Case Report

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Bullous dermatosis on legs of elderly: A new clinical entity?

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Summary **A lot of diseases occur on the skin of elderly persons. We report four elderly cases of bullous dermatosis that did not meet various differential diagnoses. Japanese, heart failure, atrophic skin and leg edema probably due to aging, as well as flaccid or tense bullae localized in legs were the common factors to our patients. Such conditions may be increased in coming aging society. Accordingly, it is worth regarding such symptom as the new clinical entity, which may comfort patients with similar condition and attract further attention.**

Keywords: Leg, bulla, elderly

1. Introduction

A lot of diseases occur on the skin of elderly persons (*1,2*). For example, when we see bullous formation in elderly patients, pemphigus, bullous pemphigoid or insect bite will be considered as differential diagnoses. Recently, we experienced four elderly patients with bullous skin changes on their legs. Because their manifestations did not meet various differential diagnoses, we suspect they may be a new clinical entity.

2. Case Report

2.1. *Case 1*

An 89-year-old Japanese female visited our hospital, for the treatment of tense or flaccid bullae of her legs that started to appear 4 months ago (Figure 1a). She had been diagnosed as having chronic heart failure and subsequent leg edema for a long time. The skin had become atrophic and fragile probably by aging. As laboratory findings, the index of an enzyme-linked immunosorbent assay (ELISA) with the recombinant protein of BP180 NC16a domain was 12.4 (normal range, < 9), whereas that with recombinant desmoglein 1 or 3 was negative (< 3.0 index). We suspected bullous pemphigoid, but a skin biopsy from the eruption revealed the formation of both subepidermal and intraepidermal bullae as well as subepidermal edema (Figures 1b and 1c). Acantholysis was not found. Infiltration of lymphocytes and eosinophils in the upper dermis were mild and slight, respectively.

Deposition of IgG or C3 along the basement membrane zone or keratinocyte cell surface was not found by direct immunofluorescence. Indirect immunofluorescence indicated that IgG antibodies against basement membrane zone or keratinocyte surface were absent in the patient's serum. Furthermore, the epidermal side of 1 mol/L NaCl-split skin did not react with the serum of the patient.

Taken together, autoimmune bullous diseases were denied. We also suppose the presence of stasis dermatitis, but ultrasonography of the leg veins did not detect valve failure or deep venous thrombus. She was treated by acrinol macrogol ointment as well as elastic stockings. Existing bullae epithelized after one week, and newborn of bullae stopped after about one year without further treatment. BP180 index reduced to be within normal limits spontaneously.

2.2. *Case 2*

A 97-year-old Japanese male had noticed skin fragility, pitting edema and tense bullae on both legs (Figure 2) two months before the first visit to our hospital. Autoimmune bullous diseases were suspected at the first visit. However, the indexes of ELISAs with the recombinant protein of BP180 NC16a domain or desmogleins were within normal limits (all values < 5 index). We did not perform skin biopsy or direct immunofluorescence because consent from the patient

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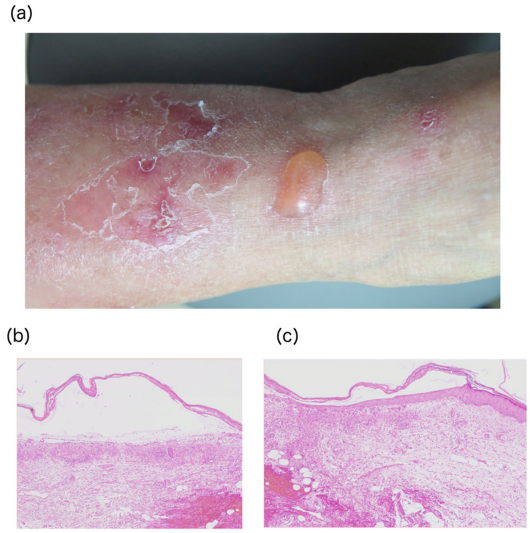

Figure 1. The clinical picture and histopathological findings of case 1. (a) Edema and tense bulla of right leg. Scars of previous bullae were also observed. **(b)** Histopathological subepidermal bulla. **(c)** Histopathological intraepidermal bulla formation.

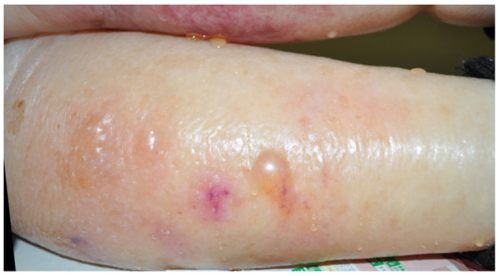

Figure 2. The clinical picture of case 2. Edema and multiple tense bullae of left leg.

could not be obtained. Indirect immunofluorescence was not performed due to the lack of patient serum. Chronic heart failure was found by heart function tests, and he was treated by acrinol macrogol ointment and by resting bed with legs up. The bullae were healed and the patient has not visited the hospital again.

2.3. *Case 3*

A 96-year-old Japanese female visited our hospital because of flaccid bulla suddenly appeared on her fragile skin of left leg (Figure 3). Skin biopsy and direct/indirect immunofluorescence were not performed because of her age. No remarkable abnormities were found in laboratory findings including ELISA indexes of BP180 NC16a domain, desmoglein 1, or desmoglein 3 (all values < 5 index), and the patients were also treated with acrinol macrogol ointment and resting. The bulla was epithelized within two weeks, and did not recurred to date.

2.4. *Case 4*

An 83-year-old Japanese male suffered from persistent

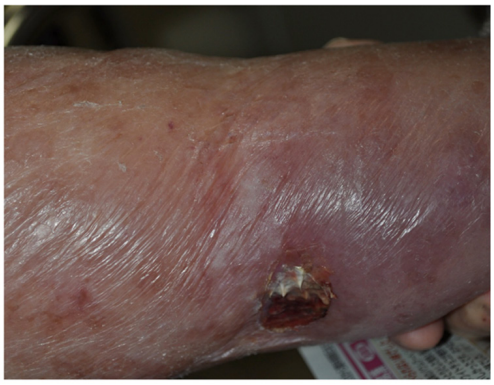

Figure 3. The clinical picture of case 3. Ruptured flaccid bullae of left leg.

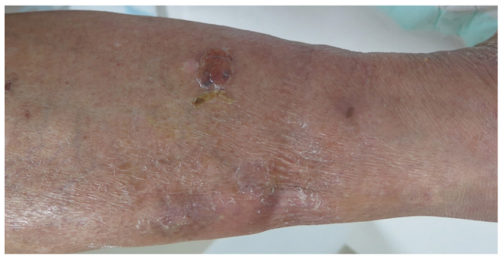

Figure 4. The clinical picture of case 4. Pitting edema (indicated by socks marks) and small flaccid bulla of right leg.

leg edema caused by heart failure. Then flaccid bullae started to appear repeatedly (Figure 4). ELISA indexes for BP180 NC16a domain, desmoglein 1 or desmoglein 3 were all negative (all values < 3 index). Although skin biopsy and direct/indirect immunofluorescence were not performed, the symptom was healed by acrinol macrogol ointment and resting. New born of bullae has stopped for more than half a year.

3. Discussion

We considered autoimmune bullous diseases, stasis dermatitis, diabetic bulla, insect bite, burn, and contact dermatitis as the differential diagnoses of the eruptions seen in our patients. Autoimmune bullous diseases were denied by the presence of both tense and flaccid bullae, negative direct/indirect immunofluorescence, and negative ELISA indexes for BP180 or desmogleins: Slightly increased BP180 index in case 1 may be the false positive. Furthermore, there were no past histories of diabetics, insect bites, heat source exposure, or contactant exposure in these patients. There have been no previous reports of bullous formation in patients with stasis dermatitis.

Elderly Japanese, heart failure, atrophic skin, and leg edema as well as flaccid or tense bullae localized in legs were the common features to our patients. Thus, the reported cases are characterized by bulla formation based on edema and fragility of the senile leg skin, and such condition will be increased in coming

aging society. As far as we searched, we could not find similar cases in databases such as Pubmed, and it should be worth regarding such symptom as the new clinical entity, which may comfort patients with similar conditions and attract further attention. Dermatologists may sometimes have seen similar conditions, but have not thought much about it.

As a limitation, biopsy or direct/indirect immunofluorescence could not performed in cases 2-4. To establish the disease concept, accumulation of patient number and additional examinations including skin biopsy or immunofluorescence are needed in the future.

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Case Report

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Breakthrough mucormycosis after voriconazole use in a case of invasive fungal rhinosinusitis due to *Curvularia lunata*

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Summary **Invasive fungal rhinosinusitis (FRS) is a potentially fatal illness requiring early diagnosis and aggressive treatment with surgery and antifungals. We report a case of chronic FRS in a recently diagnosed diabetic individual due to *Curvularia lunata*. Imaging revealed extension into the right orbit and right basifrontal lobe. This was further complicated by development of nosocomial mucormycosis which was attributed to voriconazole therapy. The patient responded well to debridement and amphotericin B based therapy. To our knowledge, there are no reported cases of invasive FRS due to *Curvularia lunata*. Also, breakthrough mucormycosis on voriconazole therapy is rarely seen in non-malignancy, non-transplant settings. The possibility of rare fungal infections (community and nosocomial) should be entertained in developing settings where fungal spores are ubiquitous.**

Keywords: Phaeohyphomycetes, Sino-orbital cerebral

1. Introduction

Rhinosinusitis is the inflammation of mucus membrane of nose and paranasal sinuses causing obstruction of the opening of the sinuses. Rhinosinusitis due to fungi can either be caused by the direct invasion of fungus into the nasal and paranasal sinus tissues or by allergic inflammatory response of the host to the fungus. The invasive fungal rhinosinusitis (FRS) can be further divided into acute (< 4 weeks) or chronic (> 12 weeks). Acute invasive FRS is a rapidly progressive infection of the immunocompromised host which commonly extends to involve orbit and brain. Mucormycetes and *Aspergillus* spp. are commonly implicated in acute FRS. Chronic FRS, on the other hand has a slow and indolent course and is usually limited to the sinuses and orbit and does not involve the brain. The common fungi implicated in chronic FRS is *Aspergillus* spp. Fungal rhinosinusitis is commonly reported from the Asian countries, more so from the Indian subcontinent. Invasive fungal sinusitis, particularly acute FRS, are potentially fatal, if not identified early and treated with aggressive surgery and systemic antifungals. We report an unusual case of chronic invasive FRS caused by *Curvularia lunata* which got complicated by development of superimposed acute invasive FRS due to mucormycosis.

2. Case Report

A 55 years old gentleman, known hypertensive (controlled) on irregular medication (Tablet amlodipine) presented with fever for three and half months, headache for three months and loss of vision in the right eye for one and half month. He was apparently asymptomatic three and half months back, when he started having continuous fever with evening rise in temperature and cough with mild expectoration. There was no associated history of breathlessness, haemoptysis, loss of appetite or loss of weight. Two weeks into the illness, patient started having right sided intermittent headache associated with redness and watering of right eye with right peri-orbital swelling. The patient was prescribed topical and oral antibiotics after which the eye symptoms resolved partially but the fever and headache persisted. Two months into the illness, he had sudden

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painless loss of complete vision in the right eye. On general examination, his core body temperature was 101 Fahrenheit, pulse was 110/minute and blood pressure was 150/94 mm of mercury. On ophthalmological examination, he had ptosis in the right eye and loss of perception of light in the right eye. The right optical disc showed complete pallor and optic atrophy. There was mild tenderness over the frontal and maxillary sinus. Rest of the general and systemic examination was normal. On initial laboratory investigations, haemogram, liver function tests and kidney function tests were within normal limits. His HbA1c was found to be 6.9%. Enzyme linked immunosorbent assay (ELISA) for human immunodeficiency virus (HIV) 1 and 2 was non-reactive. Chest X-ray showed areas of consolidation with air bronchogram in right upper and middle zone suggestive of active infection. The patient was started on empirical anti-tubercular therapy with isoniazid, rifampicin, pyrazinamide and ethambutol. Magnetic resonance imaging (MRI) of the brain, orbits and paranasal sinus revealed extensive sinusitis involving the bilateral maxillary, ethmoidal and frontal sinus with extension into the right orbit (sub periosteal abscess) and right basifrontal lobe in form of multiple ring enhancing lesions with perilesional oedema (Figure 1). The patient was started on intravenous amphotericin B with a suspicion of invasive fungal infection. Biopsy from the right maxillary sinus showed septate hyphae with acute angle branching on potassium hydroxide (KOH) mount (Figure 2). There was no granulomatous reaction on histopathology. The biopsy sample was inoculated in three tubes containing Sabourad dextrose agar (SDA), SDA with gentamycin and SDA with gentamycin and cycloheximide. All the three tubes were incubated at 37 degrees celsius. Four days after inoculation, a black mould was seen to be growing on the obverse with dark pigmentation on the reverse in the tube with SDA containing gentamycin and cycloheximide. The dematiaceous fungi was identified as *Curvularia lunata* based on the micro-morphological features (Figure 3). With a provisional diagnosis of cerebral phaeohyphomycosis, voriconazole was added to the existing regimen of amphotericin B. The contrast enhanced computed axial tomography (CECT) Chest showed areas of consolidation with ground glass opacities, multiple cavities and centrilobular nodules with tree in bud appearance. The features were consistent with both pulmonary tuberculosis and aspergillosis. Meanwhile, a broncho-alveolar lavage (BAL) was planned to confirm the diagnosis of tuberculosis. The BAL from the right upper lobe was positive for gene Xpert

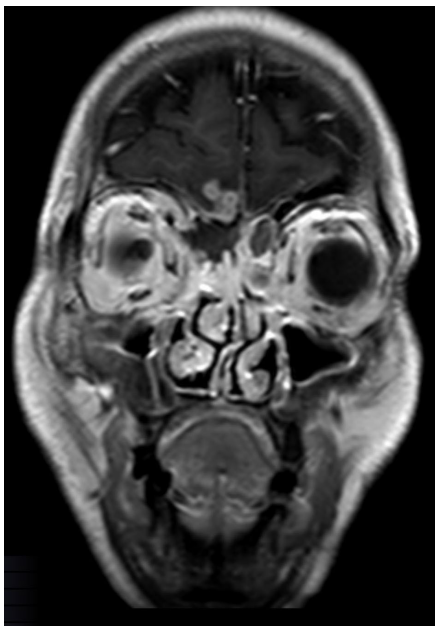

Figure 1. CEMRI showing extensive sinusitis involving the bilateral maxillary, ethmoidal and frontal sinus with extension into the right orbit (sub periosteal abscess) andextension into right basifrontal lobe in form of multiple ring enhancing lesions with perilesional oedema.

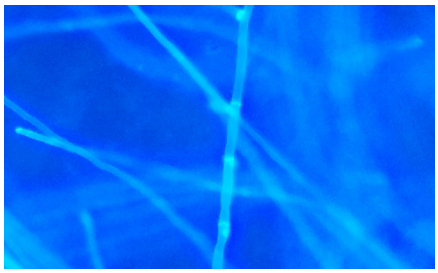

Figure 2. Biopsy from the right maxillary sinus showed septate hyphae with acute angle branching on Calcofluor-KOH mount.

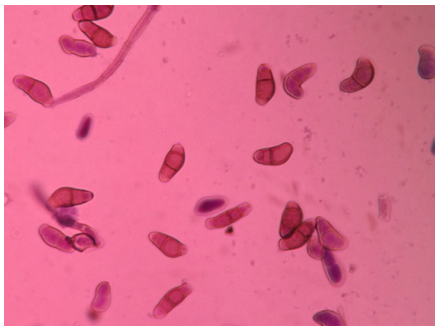

Figure 3. Lacto phenol cotton blue mount made from the growth on SDA tube shows micromorphological features suggestive of *Curvularia lunata*.

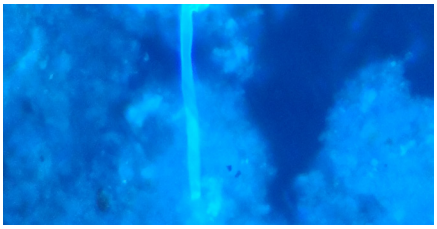

Figure 4. Calcuofluor KOH mount from the emergency debridement showed broad aseptate hyphae suggestive of invasive mucormycosis.

(cartridge based nucleic acid amplification test). Direct KOH mount and fungal culture were negative for BAL. Both serum and BAL galactomannan antigen (OD: – 0.7 and 1.54, respectively) were positive. Since *Curvularia lunata* does not give a positive galactomannan and is rarely associated with the current clinical scenario, it was considered as a contaminant. The diagnosis was revised to sino-orbital cerebral aspergillosis with possible pulmonary aspergillosis and tuberculosis with type II diabetes mellitus. In the hospital course, he showed improvement with respect to fever and headache and was planned for discharge on oral voriconazole. Therefore, his amphotericin was stopped after he had received 16 days of intravenous amphotericin B and 7 days of intravenous voriconazole therapy. After three days of isolated voriconazole therapy, he developed fever, swelling in the right eye and headache. With a suspicion of acute invasive FRS, emergency debridement was planned where the unhealthy hypertrophic mucosa from the maxillary sinus was removed. The KOH mount showed broad aseptate hyphae suggestive of invasive mucormycosis (Figure 4). Fungal cultures were negative. Voriconazole was immediately stopped and the patient was again started on intravenous amphotericin B. After initiation of amphotericin B, the symptoms improved again. The patient received 25 more days of intravenous amphotericin B after which a repeat MRI was done which showed partial resolution in the sinus and orbital lesions. The vision loss and partial ptosis, however did not improve. An ophthalmological consultation was sought and the vision loss and partial ptosis was attributed to optic nerve and oculomotor nerve involvement secondary to invasive fungal infection. None of the samples were positive for fungal culture except the previous sample which showed growth of *Curvularia lunata* in one of the tubes. Pan fungal polymerase chain reaction (PCR) assay was positive for both the samples but primers specific for mucormycetes was positive only in the second sample suggestive of acquisition of mucormycosis during the voriconazole therapy (*1*). Aspergillosis specific primers were however, negative for both the samples (*2*). Considering the stable status of the patient, he was discharged on oral posaconazole therapy. The patient was maintained well on anti-tubercular therapy and posaconazole on follow-up after three months of discharge.

3. Discussion

Chronic invasive FRS is commonly reported from patients who are mildly immunocompromised like the ones with diabetes mellitus or those who are on long term steroids. Our patient was diagnosed with type 2 diabetes mellitus at the time of admission based on his persistently elevated sugar levels and HbA1c reports. The patient was empirically started on broad spectrum amphotericin B at presentation. The initial biopsy showed septate hyphae which was later proven to be *Curvularia lunata*. Although, there has been some reports of *Curvularia lunata* being a cause of allergic FRS and cerebral abscess, there are no reports of it being associated with chronic invasive FRS (*3-6*). The treatment for infections due to *Curvularia lunata* are not standardized but most retrospective studies show a combination of amphotericin B and triazole to be the best choice (*7*). Therefore, voriconazole was added to our patient in addition to amphotericin B.

Sino-orbital-cerebral aspergillosis is a specific entity described mainly from the Indian sub-continent. It usually manifests with extra-ocular palsies and features of mass lesion in the brain. Imaging usually shows masses within the paranasal sinuses with extension into the orbit and cranial fossae (*8*). Owing to the positive galactomannan and the known fact that Aspergillus spp. is the most commonly implicated pathogen in chronic invasive FRS (*6,9*), the diagnosis was further revised. But, after the molecular results were available, in hindsight, the patient was likely to be suffering from *Curvularia lunata* infection and not invasive aspergillosis. The initial biopsy sample was positive by pan fungal PCR but negative when Aspergillus spp. specific primers were used. Also, the positive galactomannan could have been explained by the piperacillin-tazobactam, the patient was receiving at that time.

The patient showed resurgence in symptoms during the hospital stay. This debridement sample was positive by microscopy and PCR assay for mucormycetes. The diagnosis of newly acquired mucormycosis was based on the fact that in comparison to the second sample, the first sample was negative for mucormycetes by both microscopic and molecular methods. Though mucormycosis is commonly known as a community-acquired disease, nosocomial infections have been reported in recent years (*10*). The risk factors that would have increased the susceptibility of our patient to mucormycosis were voriconazole use, pre-existing sinusitis and diabetes mellitus. Voriconazole exposure is known to cause breakthrough infection with mucormycetes. It has been noted in experimental fly and mouse models that mucoraceous fungi become more virulent after voriconazole exposure (*11*). Most cases of mucormycosis in patients receiving voriconazole has been reported in transplant recipients and patients with haematological malignancy (*12*). Ours was a rare case of breakthrough mucormycosis in an individual who was otherwise immunocompetent, except for his diabetes.

It is imperative to understand, in the age where diabetes is so common, and in settings where fungal spore burden is very high, rare species will cause more and more disease. Also, in patients with risk factors for mucormycosis, even as rare as voriconazole use, intensive monitoring should be a rule, considering high mortality associated with the disease. The patient could

be saved from a potentially rare and intractable disease because of early suspicion, quick diagnosis and prompt initiation of treatment.

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