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Editorial Board Members

Original Article

185 - 188 **Bacterial polysaccharides inhibit sucrose-induced hyperglycemia in silkworms.**
Masaki Ishii, Yasuhiko Matsumoto, Kazuhisa Sekimizu

189 - 196 **Green synthesis and inhibitory effects against oral pathogens of silver nanoparticles mediated by rice extracts.**
Temsiri Suwan, Sakornrat Khongkhunthian, Siriporn Okonogi

197 - 205 ***Caesalpinia sappan*: A promising natural source of antimicrobial agent for inhibition of cariogenic bacteria.**
Rinrampai Puttipan, Sunee Chansakaow, Sakornrat Khongkhunthian, Siriporn Okonogi

206 - 213 **Effect of rice variety and modification on antioxidant and anti-inflammatory activities.**
Siriporn Okonogi, Adchareeya Kaewpinta, Taepin Junmahasathien, Songwut Yotsawimonwat

214 - 223 **Fungal-derived xenobiotic exhibits antibacterial and antibiofilm activity against *Staphylococcus aureus*.**
Siddhartha Kumar, Arvind Kumar, Manisha Kaushal, Prince Kumar, Kasturi Mukhopadhyay, Antresh Kumar

224 - 232 **Differences in how bronchial asthma patients transmit experience about adverse reactions and usability of inhaled steroids to others: A qualitative focus-group study.**
Fuki Kurimoto, Satoko Hori, Yasufumi Sawada

233 - 238 **Constipation in the elderly in a Japanese long-term medical facility: An ultrasonographic investigation.**
Koichi Yabunaka, Gojiro Nakagami, Keiko Tabata, Junko Sugama, Masaru Matsumoto, Yoshifumi Kido, Terumi Iuchi, Hiromi Sanada

239 - 247 **Whole body vibration exercise in the management of cancer therapy-related morbidities: A systematic review.**
Patrícia Lopes-Souza, Carla Fontoura Dionello, Danúbia da Cunha Sá-Caputo, Eloá Moreira-Marconi, Eric Heleno Freire Ferreira Frederico, Renata Marques Marchon, Anke Bergmann, Trentham Furness, Mario Bernardo-Filho

248 - 253 **Criteria for the selection of switch OTC drugs based on patient benefits, efficacy, and safety [II]: Comparing the physicochemical and pharmaceutical properties of brand-name and switch OTC terbinafine hydrochloride cream.**

Motoari Takata, Yuko Wada, Yurika Iwasawa, Miyuki Kumazawa, Ken-ichi Shimokawa, Fumiyoshi Ishii

CONTENTS (*Continued*)

Case Report

254 - 258 **Atypical cases of filariasis from a non-endemic area.**

Parul Kodan, Nitin Gupta, Ankita Baidya, Ayan Basu, Abdul Razik, Ankit Mittal, Nishant Verma, Bijay Ranjan Mirdha, Sandeep Mathur, Madhu Rajeshwari, Neeraj Nischal, Manish Soneja, Naveet Wig

Erratum

E1 **Erratum for "Anesthetic activity of plant essential oils on *Cyprinus carpio* (koi carp)" by Khumpirapang N *et al*. (*Drug Discoveries & Therapeutics*. 2018; 12(1):21-30.).**

Guide for Authors

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Original Article

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Bacterial polysaccharides inhibit sucrose-induced hyperglycemia in silkworms

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Summary **Diabetes and obesity result from sucrose-induced hyperglycemia. Prevention of hyperglycemia contributes to inhibit the onset of these life-related diseases. Here we show that polysaccharides obtained from soil bacteria inhibit sucrose-induced hyperglycemia in an *in vivo* silkworm evaluation system. Ethanol precipitates of extracellular polysaccharides were prepared from viscous bacterial colonies. Among 24 samples obtained from different bacterial species, oral administration of 6 samples from *Rhizobium altiplani*, *Cupriavidus* sp., *Paenibacillus polymyxa*, *Pantoea eucalypti*, *Variovorax boronicumulans*, and *Xanthomonas cynarae* suppressed sucrose-induced hyperglycemia in silkworm insect larvae. The *R. altiplani* fraction treated further with DNase I, RNase A, and proteinase K, followed by phenol extraction also exhibited suppressive activity. Our results suggest that silkworms provide an efficient screening system of bacterial polysaccharides that inhibit sucrose-induced hyperglycemia.**

Keywords: Bacteria, polysaccharide, silkworm, sucrose-induced hyperglycemia

1. Introduction

Transient and rapid postprandial increases in blood glucose levels, referred to as a blood glucose spike, are a potential risk factor for diabetes. Suppression of the blood glucose spike is expected to be useful for preventing the onset of diabetes. Generally, blood glucose levels and their suppression by drugs are evaluated in mammalian animal models. We previously reported that an increase in blood glucose levels after glucose intake could also be evaluated in silkworms, an alternative model animal (*1*-*3*). Not only glucose, but also sucrose intake increases silkworm blood glucose levels (*4*). Glucose level increases in the silkworm hemolymph after sucrose intake are suppressed by acarbose and voglibose, α-glucosidase inhibitors that are used clinically for human diabetes patients (*4*). We propose that the silkworm evaluation system is useful for screening substances that suppress sucrose-induced hyperglycemia.

Bacterial polysaccharides have various structures and biologic activities (*5*-*7*). We recently reported a bacterial polysaccharide with high innate immunity-stimulating activity in a silkworm evaluation system (*8*). Bacterial polysaccharides can be obtained in large quantities at low cost. We therefore propose the use of bacterial polysaccharide libraries for screening seeds of medicines and supplements for human health. In this paper, we describe the collection of polysaccharide-producing bacteria and preliminary screening of polysaccharides that suppress sucrose-induced hyperglycemia in a silkworm model system.

2. Materials and Methods

2.1. *Collection of polysaccharide-producing bacteria*

Bacteria isolated from soil and plants that formed viscous colonies on agar plates were collected. The bacteria grown on the plates (10 cm) were recovered with a spreader and 15 ml of saline, and the cells were removed by centrifugation (8,000 rpm, 5 min). Ethanol (final concentration: 67%) was added to the centrifuge

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supernatant, and fibrous precipitates were collected by centrifugation. Bacterial 16S rRNA was sequenced and homology searches were performed to determine the bacterial species using the EZBiocloud database.

The crude polysaccharide fractions were treated enzymatically as follows. DNase I (1,000 U/mL; Promega) and RNase A (10 μg/mL; NIPPON GENE CO., LTD.) were added to the ethanol precipitate, dissolved in water, and incubated overnight at 37°C, and then further incubated overnight at 37°C with protease K (100 μg/mL). Phenol: chloroform: isoamyl alcohol (50:49:1) was added to the fraction, and the samples were vigorously shaken, followed by the addition of two volumes of ethanol to the upper layer fraction. The precipitates were then collected by centrifugation.

Table 1. Homology search of 16S rRNA sequences of bacteria producing extracellular polysaccharides

Species	Strain	Identities (%)
Bacillus megaterium	129-19	99
Cupriavidus sp.	No.48	97
Curtobacterium plantarum	126-8	99
Enterobacter kobei	126-4b	97
Enterobacter tabaci	118-13A	97
Escherichia coli	118-18	98
Ewingella americana	221-5-2	99
Gluconobacter cerinus	221-2-2	99
Kosakonia sp.	118-14	98
Lelliottia amnigena	112-13-1	99
Novosphingobium panipatense	208-110	98
Paenarthrobacter nicotinovorans	208-57	99
Paenibacillus lupini	110-24	99
Paenibacillus polymyxa	126-6-1A	99
Pantoea eucalypti	118-5-1	98
Pantoea sp.	126-2	99
Pantoea vagans	126-1	99
Paraburkholderia insulsa	118-3-2	99
Paracoccus aestuariivivens	126-5b	98
Pseudomonas nitroreducens	No.24	98
Pseudomonas palleroniana	118-25A	99
Rhizobium altiplani	No.26	99
Variovorax boronicumulans	110-14	99
Xanthomonas cynarae	No.4	100

Bacterial species of 24 strains isolated in this study are listed. Bacterial species exhibiting the highest sequence homology are presented. When the species of the highest homologous bacterial strain could not be specified, only the names of the genus are shown.

2.2. *Sucrose tolerance test of silkworms*

Silkworms (Hu Yo x Tsukuba Ne, Ehime Sericulture Incorporated Company, Ehime, Japan) were reared as described previously (*9,10*). The silkworm sucrose tolerance test was conducted according to the previously reported method (*4*). Briefly, sucrose (10%) and test samples were mixed with silkworm artificial diet. Sucrose diet with or without polysaccharide samples was fed to 5th-instar larva of silkworms for 1 h, the silkworm hemolymph was collected, and glucose concentrations were measured with a glucometer (Accu-Chek, Roche).

3. Results

We collected bacteria that formed viscous colonies on YME agar plates. The isolated bacteria comprised 19 genera (Table 1). Ethanol precipitates of crude polysaccharides (see Materials and Methods) were mixed with silkworm diet containing 10% sucrose and fed to the silkworms. After 1 h, the blood glucose levels of silkworms were measured. Among the 24 polysaccharide fractions tested, 6 samples from *Rhizobium altiplani*, *Cupriavidus* sp., *Paenibacillus polymyxa*, *Pantoea eucalypti*, *Variovorax boronicumulans*, and *Xanthomonas cynarae* exhibited suppressive effects on the increase in the blood sugar level of silkworms (Table 2). The differences in the glucose level between controls without bacterial samples and those with bacterial polysaccharides were statistically significant.

The crude polysaccharide fraction from *R. altiplani* was treated with DNase I, RNase A, and proteinase K, and further extracted with phenol. The treatment had little effect on the sugar content, whereas the amounts of DNA and protein were greatly reduced to 1/20 and 1/28, respectively (Table 3). This phenol-extracted fraction also exhibited suppressive activity against sucrose-induced hyperglycemia (Figure 1).

4. Discussion

The findings of the present study demonstrated that bacterial polysaccharides from *R. altiplani*

Table 2. Bacterial polysaccharides that suppress sucrose-induced hyperglycemia by oral administration

Species	Strain	Sample weight (mg/g diet)	Blood glucose (mg/dL)		*p* value
			Exp. 1	Exp. 2	
No bacteria (control)		-	320 ± 64	366 ± 47	
Cupriavidus sp.	No.48	55	N.D.	207 ± 91	0.001
Paenibacillus polymyxa	126-6-1A	12	210 ± 27	N.D.	0.004
Pantoea eucalypti	118-5-1	9.4	226 ± 60	N.D.	0.02
Rhizobium altiplani	No.26	60	121 ± 22	N.D.	0.00003
Variovorax boronicumulans	110-14	21	253 ± 29	N.D.	0.05
Xanthomonas cynarae	No.4	25	N.D.	252 ± 37	0.0004

Ethanol precipitation fraction was mixed with 1 g of 10% sucrose-containing artificial diet. The mixture was fed to silkworms for 1 h, and the glucose levels in the silkworm hemolymph were determined. Statistically significant differences between control and testing groups were evaluated using Student's *t*-test. N.D., not determined. $n = 5$-10.

Table 3. Comparison of the amounts of DNA and protein in a crude polysaccharide fraction and an enzyme-treated fraction prepared from *Rhizobium altiplani*

Fraction	Sugar (mg/g)	DNA (mg/g)	Protein (mg/g)
Crude polysaccharide	490	10	56
Enzyme-treated sample	520	0.45	2.1

Crude polysaccharide was incubated with DNase I(1,000 U/mL) and RNaseA (10 µg/mL) 24 h at 37˚C. Then, proteinase K (100 µg/mL) was added to the samples and incubated 24 h at 37˚C. Phenol/chloroform/isoamyl alcohol was added and vigorously mixed, followed by centrifugation. The upper layer fraction was collected and mixed with ethanol (final concentration: 67%). Fibrous precipitates were collected by centrifugation. The amounts of sugars, DNA, and proteins in the crude polysaccharide fraction and the enzyme-treated fraction were measured by the phenol-sulfuric acid method, the fluorescent-based Qubit assay, and Bradford assay, respectively.

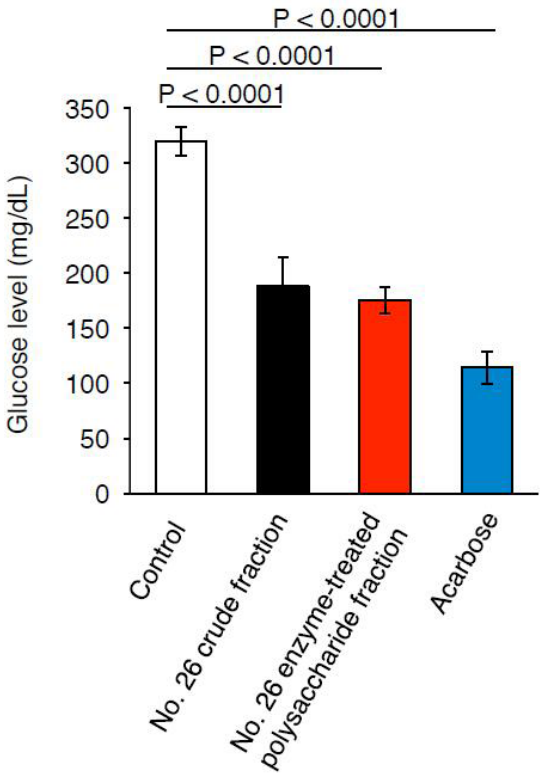

Figure 1. Effect of enzyme-treated polysaccharide fraction on sucrose-induced hyperglycemia. Crude fraction (50 mg/g diet) prepared from *R. altiplani* No. 26, enzyme-treated polysaccharide fraction (46 mg/g diet), and acarbose (40 mg/g diet) were mixed with 10% sucrose containing diet, respectively. Diets with the polysaccharide fraction were orally administered to silkworms for 1 h. The glucose levels in silkworm hemolymph were determined. Acarbose was used as a control according to a previous report (*4*). Statistically significant differences between control and testing groups were evaluated using Student's *t*-test.

markedly suppressed sucrose-induced hyperglycemia in silkworms. We propose that the polysaccharides screened using the silkworm model are promising candidates for healthy foods and medicines to prevent the onset and exacerbation of diabetes. The silkworm system is superior to mammalian systems in terms of cost and ethical issues (*11-13*). Therefore, the silkworm system is expected to be useful for screening bacterial polysaccharides that inhibit increases in blood glucose levels in humans. Bacteria secreting polysaccharides can be easily obtained as viscous colonies on agar plates. Polysaccharides secreted from bacteria have various structures depending on the bacterial species (*6,7*). Furthermore, industrial mass production of bacterial polysaccharides is possible. Based on these properties, it is expected that the library of bacterial polysaccharides will be useful for screening compounds with physiologic activities, such as agents with blood sugar lowering effects.

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Original Article

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Green synthesis and inhibitory effects against oral pathogens of silver nanoparticles mediated by rice extracts

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Summary **Rice is staple food for people in many countries for centuries. It is therefore considered as safe and environmental friendly material for pharmaceutical formulations. In the present study, aqueous extracts of three different parts of rice grain; rice bran (RB), rice husk (RH), and rice germ (RG) were compared for their use as reducing agents in synthesis of silver nanoparticles (AgNPs). AgNPs from those three different parts of rice, RB-AgNPs, RH-AgNPs, and RG-AgNPs, respectively showed different reducing activity, which the highest capacity was RB. RG-AgNPs and RB-AgNPs showed the maximum absorption of AgNPs at 440 nm whereas that of RH-AgNPs was at 480 nm. FTIR spectra of all AgNPs indicated the presence of different functional groups from rice attached to the nanoparticles and these groups prevented the particle agglomeration. Size analysis using dynamic light scattering revealed that RB-AgNPs was the smallest particles (346.4 ± 36.8 nm) and possessed the highest negative zeta potential. Antimicrobial test showed that the AgNPs obtained from green synthesis mediated by rice extracts have great antimicrobial activity against *Streptococcus mutans*, the severe oral pathogenic bacteria causing dental caries. These results suggest that aqueous extracts of RB, RH, and RG have potential to be used as reducing agents in synthesis of silver nanoparticles.**

Keywords: AgNPs, rice grain, reducing agent, antimicrobial, *Streptococcus mutans*

1. Introduction

Nanoparticles of certain metals, such as titanium, zinc, magnesium, gold, copper, and silver have been developed for various fields. Among them, silver nanoparticles (AgNPs) have gained interest for commercialization applications since they have considerably versatile properties, such as a variable surface area to volume ratio, which is very useful for many biomedical and technological applications. They have been used extensively in electronic industry and as excellent catalyst. In medical applications, many reports demonstrate their biological activities, such as anticancer (*1*), antioxidant (*2*), and antimicrobial activities (*3*). AgNPs have been used as antibacterial agent for many kinds of applications including home appliances and water treatment (*4*). The biological activity of metal nanoparticles is closely related to their size. The smaller size usually gives the higher activity. Thus, control the size and size distribution of these nanoparticles is an important issue. Generally, specific control of shape, size, and size distribution is often achieved by varying the synthesis methods, reducing agents, and stabilizers (*5*). Recently, the ability of AgNPs on inhibition of certain viruses, such as human immunodeficiency virus 1 (HIV-1) has been reported. AgNPs showed a half maximal inhibitory concentration against the virus of 11.2 ± 0.6 μg/mL ($p < 0.0001$) with no significant toxicity against normal cells (*6*). Silver has long been historical used since ancient times and it has been demonstrated that, in low concentrations, silver is nontoxic to human cells (*7*).

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In former time, AgNPs were generally synthesized by chemical reaction based on the reduction of silver nitrate ($AgNO_3$) by chemical reducing agent (*8*). In global efforts to reduce generated hazardous chemical waste, the use of chemicals is decreased in the synthesis protocols and green or biological synthesis of AgNPs has been increased. The biological methods are using eco-friendly resources, such as plant (*9*), algae (*10*), bacteria (*11*), and fungi (*12*). The extracts from many plants have been reported to act as reducing agent in the green synthesis of AgNPs (*9,13,14*). Synthesis of AgNPs using microorganisms is readily scalable and of course eco-friendly, however, production of microorganisms is more expensive than the production of plant extracts (*15*).

Rice (*Oryza sativa* L.) is a cereal plant in family Poaceae. It is the predominant dietary energy source for many countries in the world. Rice is low in fat and high in starchy carbohydrate, packed full of vitamins and minerals. Dietary minerals and trace elements play a significant role in maintenance of optimal health (*16*). Rice grain has a hard cover called rice husk (RH) to protect the kernel inside. After RH is removed, the remaining product contains the inside endosperm and the outside rice bran (RB) and rice germ (RG). Many parts of rice grain contain high amount of compositions having antioxidant activity and high reducing property (*17,18*). RB and RG are commercial available for health care consumers. The commercial rice-milling process separates RH from the kernel inside because this part of rice grain is inedible and used in non-food applications.

The aim of present study is to synthesize AgNPs by eco-friendly method using RB, RH, and RG extracts as reducing agents. The reducing property of the extracts was confirmed using ferric reducing antioxidant power (FRAP) assay. For AgNPs synthesizing, the extracts were reacted with $AgNO_3$ as a precursor in a certain condition. The obtained AgNPs were characterized and investigated for antimicrobial activity against oral pathogens.

2. Materials and Methods

2.1. *Materials*

RB, RH, and RG of Jasmine rice was purchased from the local producer in Chiang Mai, Thailand. 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) was purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA). $AgNO_3$ was supplied by RCI Lab-scan Co., Ltd. (Bangkok, Thailand). Potassium bromide (KBr) for infrared spectroscopy purchased from Fisher Scientific (Loughborough, UK). Tryptic soy agar (TSA) and broth (TSB) were supplied by Difco™ (Balti-more, Maryland, USA). Brain heart infusion agar (BHI-A) and broth (BHI-B) were purchased from Becton, Dickinson and Company (Franklin Lakes, New Jersey, USA). Sabouraud dextrose agar (SDA) and broth (SDB) were purchased from BBL™ (Baltimore, Maryland, USA). All other chemicals and solvents were of analytical reagent grade or the highest grade available.

2.2. *Microbial strains*

Two aerobic bacterial strains, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 which represent for Gram positive and Gram negative bacteria, respectively, and one facultative Gram-positive bacteria, *Streptococcus mutans* DMST 18777 were used as the oral pathogenic bacteria. *Candida albicans* ATCC 10231 was used as the oral pathogenic fungi.

2.3. *Preparation of rice extracts*

The obtained RB, RH, and RG powders were sieved through a 14-mesh sieve in order to remove the large particles. The sieved samples were pulverized and 2 g of RB, RH, and RG powder samples were dispersed in deionized water to obtain 2% aqueous dispersions. The dispersions were macerated with continuous stirring at room temperature for 24 h. Subsequently, they were filtered through Whatman No.1 filter paper and the filtrates were stored in the refrigerator at 4°C for further use.

2.4. *FRAP assay*

Reducing property of the rice extracts was determined using FRAP assay previously described (*19*) with some modification. The FRAP reagent was freshly prepared by mixing 2.5 mL of 10 mM TPTZ solution in 40 mM HCl with 2.5 mL of 20 mM FeCl3 and 25 mL of 0.3 M acetate buffer, pH 3.6. An amount of 20 µL rice extracts were mixed with 180 µL of FRAP reagent in 96 well plate. Then, they were incubated for 10 min at room temperature and the absorbance of was determined at 595 nm using a microplate reader (Bio-Rad, Model 680, Hercules, California, USA). All data were run in triplicate. The reducing power of the samples was evaluated by calculating the amount of Fe^{+2} produced by the rice extract samples using the calibration curved of $FeSO_4$.

2.5. *Synthesis of AgNPs*

The synthesis of AgNPs was done by the following procedure. The rice extract solution was filled into the 125 mL Erlenmeyer flasks and heated until 75°C. An exact amount of $AgNO_3$ solution (0.1 M) was added drop wise until the volume ratio of the rice solution to $AgNO_3$ solution was 100:1. The mixture was kept at 75°C under continuous stirring for 60 min. The obtained mixture was added thrice with deionized water and subjected to centrifugation (Heraeus™

Megafuge™ 40 Centrifuge Series, ThermoFisher Scientific, Waltham, Massachusettes, USA) at 8,000 rpm for 15 min to remove any traces of un-utilized phyto-constituents. After removing the liquid phase, the AgNPs obtained were kept in the refrigerator for further study. The AgNPs obtained from RB, RH, and RG were named as RB-AgNPs, RH-AgNPs, and RG-AgNPs, respectively.

2.6. *Characterization of AgNPs*

The obtained AgNPs were characterized using UV-vis and Fourier transform infrared (FTIR) spectroscopy measurement. Their particle size and zeta potential were measured using dynamic light scattering (DLS).

2.6.1. *Visualization and UV-vis spectroscopy*

The outer color appearance of the obtained AgNPs was early observed by visualization. The obtained particles were confirmed by UV-vis spectra using UV 2450 double-beam sspectrophotometer (Shimadzu-2450, Kyoto, Japan). The rice-AgNPs samples were diluted to 100 folds with deionized water before subjecting to this investigation. The optical property of the AgNPs solution was observed in the wavelength range of 300-700 nm. The UV-vis absorbance spectra were recorded at room temperature.

2.6.2. *FTIR*

In this experiment, the lyophilized RB-AgNPs, RH-AgNPs, and RG-AgNPs were used. The obtained AgNPs in the powder form were characterized using FTIR in order to investigate the possible role of the phyto-constituents presented in the rice extracts on the surface modification of the synthesized AgNPs. The KBr disc of the lyophilized AgNPs were prepared. The IR spectra in the range of 4,000-400 cm^{-1} of the samples were recorded using a Nicolet Nexus 470 FT-IR (Minneapolis, Minnesota, USA) in the diffuse reflectance mode at room temperature at a resolution of 4 cm^{-1}. The spectra were collected against a KBr disc background at room temperature. The instrument was maintained with the automatic dehumidifier to diminish water vapor interference.

2.6.3. *DLS*

The obtained AgNPs were investigated for their particle size and size distribution as well as zeta potential using DLS (Malvern Zeta sizer Nano-ZS, Malvern Instruments, Worcestershire, UK) at 25°C. Each sample was diluted to 100 folds with deionized water before investigation. The disposable plastic cuvettes and folded capillary zeta cell were used in the measurement. The measurement was done in triplicate of three independent AgNPs batches.

2.7. *Antimicrobial activity of AgNPs*

The antimicrobial activity of the obtained RB-AgNPs, RH-AgNPs, and RG-AgNPs against oral pathogenic microorganisms; *S. aureus*, *E. coli*, *S. mutans*, and *C. albicans* was tested based on Kirby-Bauer method (*20*). The aerobic and facultative bacteria were grown in TSA and BHI-A, respectively at 37°C for 24 h. The bacterial strains were diluted in TSB and BHI-B, respectively to a final density of 1.5×10^6 colony forming unit (CFU)/mL. *C. albicans* were cultured in SDA at 37°C for 36-48 h. The fungal suspension was prepared to a final concentration of $1\text{-}2 \times 10^5$ CFU/mL in SDB. The density of the microbial suspension was adjusted with 0.5 McFarland constant by observing the OD at 600 nm under a UV-vis spectrophotometer. The bacterial and fungal suspensions were swabbed on the surface of their corresponding agars. Freshly prepared lyophilized RB-AgNPs, RH-AgNPs, and RG-AgNPs suspensions (40 µL) were added onto the 6 mm-diameter filter paper discs which would be put onto the surface of the seeded agar plates. The discs filled with deionized water and 0.1 M $AgNO_3$ solution at the same amount (40 µL) were used as negative controls. The plates were incubated at 37°C for 24 h. The antimicrobial activity of the samples was evaluated by determining the diameter of the clear zone of inhibition around the paper discs. All samples were done in triplicate.

2.8. *Statistical analysis*

Descriptive statistics for continuous variables were calculated and reported as a mean ± standard deviation. Data were analyzed using a One-way analysis of variance and Duncan's multiple range test using Statistic a software version 17 (SPSS Inc., Chicago, Illinois, USA). The values were presented as means ± standard deviation which a *p*-value less than 0.05 was considered as significant difference.

3. Results

3.1. *Reducing property of rice extracts*

In the present study, the reducing property of three different parts of rice grain, RB, RH, and RG were compared. The values of FRAP assay showed wide variation among the samples (Figure 1). The significantly highest ($p < 0.05$) ferric reduction ability was obtained from RB extract (524.0 ± 7.68 µmol Fe^{2+}/g sample), followed by RH and RG with the reducing values of 247.1 ± 8.49 and 152.1 ± 4.24 µmol Fe^{2+}/g sample, respectively. Our results show that RG also possessed the reducing property, even less than RB and RH, respectively.

3.2. *Synthesis and characterization of AgNPs*

Reduction of silver ion to produce AgNPs during exposure to the rice extracts could be easily detected by color change. The color of RB, RH and RG extract solutions was pale yellow and the color of the precursor $AgNO_3$ was colorless. However, when AgNPs were formed, the color of the solution began to change. After complete reduction, the color of the system were yellow-brown as presented in Figure 2.

The intensity of the resultant color was observed during 0-60 min. It was found that the color change of the reaction mixtures of all rice extracts and $AgNO_3$ was gradually appeared as pale yellow-brown around 10-30 min of reaction, depending on the type of the rice extracts. It was noted that the color change in the formation of RB-AgNPs was observed within 10 min, significantly faster than that in the systems of RH-AgNPs (25 min) and RG-AgNPs (30 min). After 60 min, all systems turned to intense yellow-brown color, indicating the complete formation was occurred.

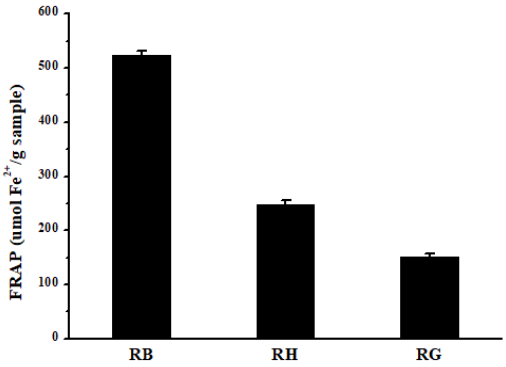

Figure 1. Reducing activities of RB, RH, and RG.

The UV-vis spectra of RB-AgNPs and RG-AgNPs showed the maximum absorption at 440 nm, whereas that of RH-AgNPs appeared at 480 nm, as shown in Figure 3. The absorption of the precursor $AgNO_3$ was at 216 nm, however the UV-vis spectra of all AgNPs did not exhibit the absorption at this wavelength, indicating that there was no trace $AgNO_3$ left in the obtained AgNPs systems.

After the synthesis of AgNPs, the dispersions containing nanoparticles were centrifuged to separate AgNPs from other rice compositions in the solutions. The FTIR of RB-AgNPs, RH-AgNPs, and RG-AgNPs are shown in Figure 4. The results showed the peaks at 965, 1,160, 1,445-1,453, 1,591-1,599, and 2,980 cm^{-1}.

Analysis using DLS reveals that the size of the AgNPs obtained from different part of rice grain is different. RB-AgNPs showed average diameter of 346.4 ± 36.8 nm whereas RH-AgNPs and RG-AgNPs showed similar particle size of 587.3 ± 49.6 and 510.9 ± 84.4 nm, respectively. Particle size distribution expressed as polydispersity index (PdI) of RB-AgNPs, RH-AgNPs, and RG-AgNPs were similar with the PdI values of 0.271, 0.260, and 0.266, respectively. The zeta potential of all AgNPs obtained was negative values of 32 ± 2.6,

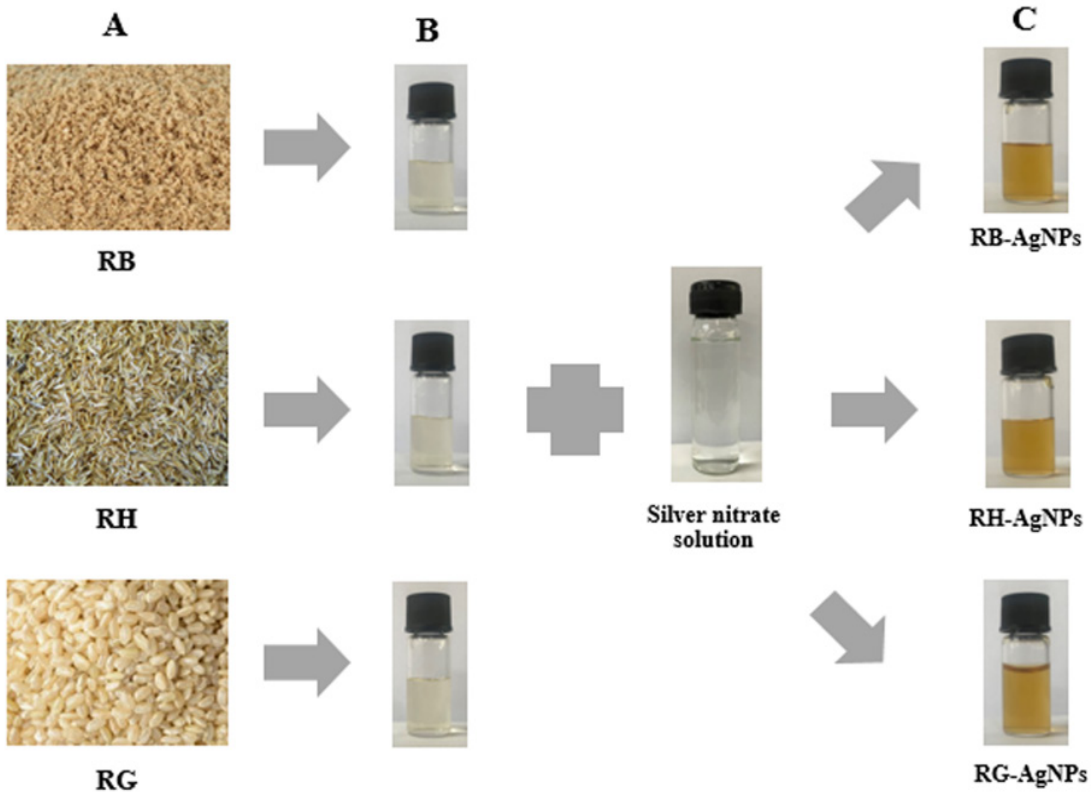

Figure 2. Outer appearance of three parts of rice grains (A), rice extracts (B), and the obtained AgNPs (C).

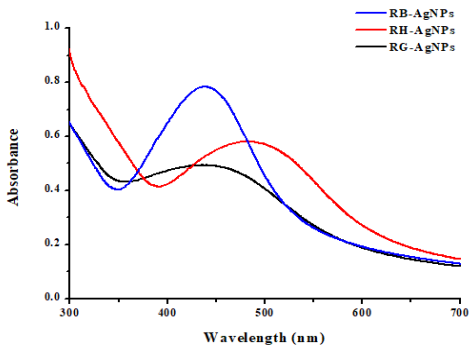

Figure 3. UV-vis spectra of the obtained AgNPs.

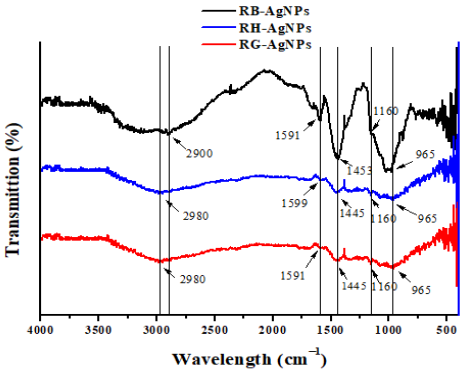

Figure 4. FTIR of the obtained AgNPs.

24.5 ± 3.1, 18.6 ± 2.4 mV for RB-AgNPs, RH- AgNPs, and RG-AgNPs, respectively.

3.3. *Antimicrobial activity*

The synthesized AgNPs were investigated for their antimicrobial activity against oral pathogenic bacteria and fungi. The results of our study showed that all AgNPs obtained possessed antimicrobial activity against all tested oral pathogens as shown in Figure 5. The inhibition potential of different AgNPs was similar, particularly on the inhibition of the facultative *S. mutans* and the pathogenic fungi *C. albicans*. Among the four tested pathogens, the inhibition zones of RB-AgNPs, RH-AgNPs, and RG-AgNPs against *S. mutans* were the largest diameters of 17.7 ± 0.6, 17.5 ± 0.5 and 17.7 ± 0.6 mm, respectively, indicating the highest potential antibacterial activity of the obtained AgNPs against this strain. RB-AgNPs showed similar activity against the aerobic *S. aureus* and *E. coli* with the inhibition zones of 14.3 ± 0.3 and 14.5 ± 1.8 mm, respectively. RH-AgNPs exhibited stronger inhibition against these strains with the inhibition zones of 12.7 ± 0.6 and 14.7 ± 0.3 mm, respectively. In contrast, RG-AgNPs exhibited stronger inhibition against both strains with the inhibition zones of 14.3 ± 0.5 and 13.3 ± 0.6, mm, respectively. All AgNPs exhibited the same potential inhibition of *C. albicans* with the inhibition zones of 11.2 ± 2.1, 11.5 ± 0.5, and 11.3 ± 2.1 mm for

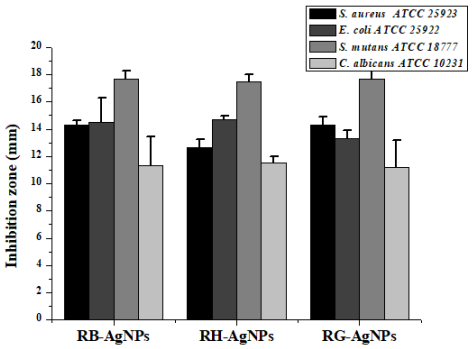

Figure 5. Inhibition zone diameters of the obtained AgNPs against four oral pathogens.

RB-AgNPs, RH-AgNPs, and RG-AgNPs, respectively. The negative controls show no inhibition zone against both bacterial strains and *C. albicans*.

4. Discussion

Phytochemicals, including phenolics and flavonoids, in rice have been reported. Oryzanol and other important compounds, such as tocols (topherols and tocotrienols) and phytosterols are the important bioactives in rice grain due to their antioxidant properties (*21*). The content of these compounds is different in different rice varieties (*22*). Moreover, different part of rice grain yields different type and amount of these compounds (*23,24*). RB extracts from many rice varieties contain high content of total phenolic and total flavonoid (*25*). These compounds are considered to play the important role on reducing property in RB. RH also contains high total phenolic content that can be an effective source of natural antioxidants (*26*). RG contains high amount of alpha tocopherol, small amount of gamma tocotrienol and alpha tocotrienol (*27*). These compounds are considered to support the reducing property of RG.

The present study reveals that rice extracts from different part of rice grain can act as a reducing agent in green synthesis of AgNPs. To detect AgNPs formation during the process of synthesis, color change can be observed. This visual signature was the easiest way for basic characterization of AgNPs. The intensity of the resultant color is dependent on the concentration of the reactants (*28*). The reaction time was also have an important role on the resultant color intensity (*29*). Therefore, in the current study, the reactant concentration and the reaction time were fixed. The spectra of AgNPs can be attributed to the surface plasmon resonance due to collective oscillation of surface electrons (*30*). The UV-vis absorption spectra of AgNPs generally appear in the range of approximately 350-600 nm, depending on the type of reducing agent. For example the gelatin-AgNPs showed their absorption at 400-450 nm (*31*), whereas those obtained from the extracts of *Plectranthus amboinicus* leaf and *Lantana camara* berry were at 428 nm (*32*) and in the range of

390-520 nm (*33*), respectively. The obtained AgNPs in the current study were confirmed by UV-vis spectra. Our results showed good agreement with the previous studies on the UV absorption range.

FTIR study was carried out in order to determine the possibility of the residual bio-reducing functional groups of the rice extracts involved in reduction process and their possible unique interactions with the surface of AgNPs. The organic functional groups like OH or C=O interacted to the surface of AgNPs can be detected by FTIR (*15*). The IR peaks of the obtained AgNPs in the current study indicate that many functional groups are involved. The peaks at 965 and 1160 cm^{-1} are considered to be the stretching vibrations of C–OCH_3, C–H stretching of alkenes and C–O stretching aromatic side chain of proteins (*34*). The peaks in 1,445-1,453 cm^{-1} are relevant to the N–O stretching of nitro groups (*35*). The peaks located at 1,591-1,599 cm^{-1} are assigned to C=O stretching vibrations of amides characteristic of rice proteins. The broad peaks at 2,980 cm^{-1} are assigned as –OH stretching in alcohols and phenolic compounds with strong hydrogen bonds (*36*). The presence of these peaks confirmed that the obtained AgNPs were covered by certain rice phytochemicals, including flavonoids and phenols, with functional groups, such as ketone, aldehyde, and carboxylic acid. The presence of these groups on the surface of AgNPs is considered to support the stability of the nanoparticles. They can prevent the pairing and agglomerating of the nanoparticles. If the amount of these compounds is high in the rice extracts, they can cover wide surface area of the obtained AgNPs. This can cause the size of the synthesized AgNPs to be extremely small since high agglomeration cannot occur.

The size of the nanoparticles obtained from DLS is hydrodynamic size which is slightly bigger than that measured by a transmission electron microscope due to the hydrodynamic radius (*37*). However, the size of the obtained nanoparticles can be compared by using the same equipment. In this study, the size of all AgNPs obtained was measured using DLS which their zeta potential could also be detected. The results reveal that among the three kinds of AgNPs, the smallest size and the highest negative value of zeta potential were obtained from RB-AgNPs. This was considered to be influenced by the high phytochemical content existed in the RB extract.

The oral cavity is the hub of an extremely diverse microflora consisting of about 500 species of microorganisms (*38*). The oral pathogenic bacteria (*S. aureus*, *E. coli*, and *S. mutans*) and fungi (*C. albicans*) used in the current study are the most common microorganisms found in oral cavity. The overgrowth of these microorganisms, particularly *S. mutans* and *C. albicans* can cause severe diseases in oral cavity. *S. mutans* is the major cause of dental caries (*39*). While *C. albicans* is the major cause of oral candidiasis (*40*), which the symptoms include pain, oral discomfort, and loss of taste (*41*). Several mechanisms of action on antibacterial activity of AgNPs have been proposed, such as the ability of AgNPs to attach bacterial cell wall and cause structure change in cell membrane, the ability to damage and make porous in the cell membrane resulted from free radicals of AgNPs, and the ability of silver ion that can be released to the inner cell and destroy several function in the cell (*42,43*). The mechanism of action on antifungal activity of AgNPs against *C. albicans* was previously proposed that AgNPs have high potential to disrupt cell membrane and arrest the cell cycle at the G2/M phase of *C. albicans* (*44*). The current study demonstrates that rice can be used as natural reducing agent to synthesize AgNPs. We explore the different reducing potential and advantages of many parts of rice grains in green synthesis of AgNPs without the use of any chemical stabilize and reducer. We also demonstrate the potential of the synthesized AgNPs on many important oral pathogens including aerobic bacteria, facultative bacteria, and fungi. The AgNPs obtained can inhibit all tested microorganisms especially *S. mutans* which is the most important oral pathogenic bacteria causing dental carries and oral infections. Among the three parts of rice grains, RB is the most effective and suitable part for the synthesis of AgNPs.

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Original Article

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Caesalpinia sappan: A promising natural source of antimicrobial agent for inhibition of cariogenic bacteria

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Summary **From the previous findings, the ethanolic fractionated extract of *Caesalpinia sappan* (F-EtOH) has high activity against *Streptococcus mutans*, the most severe cariogenic bacteria. The present study was aimed to isolate and identify the active compound of F-EtOH and compare its inhibitory activity against the biofilm of *S. mutans* as well as the cytotoxicity to oral fibroblast cells with F-EtOH. Compound isolation was done by column chromatography. The active compound was identified using liquid chromatography-mass spectrometry with electrospray ionization and nuclear magnetic resonance spectroscopy. It was found that the major compound of F-EtOH is brazilin. F-EtOH and brazilin were compared for inhibitory potential on the biofilms of three strains of *S. mutans*. The results exhibited that both F-EtOH and brazilin had potential on inhibiting biofilm formation and eradicating the preformed biofilms and their activity was dose dependent. F-EtOH showed significantly less toxic to normal periodontal ligament fibroblast than brazilin. At low concentration of 1- and 2-MBC, F-EtOH showed higher effective than brazilin. The results of our study suggest that the antibacterial activity of F-EtOH is according to the synergistic effects of the existing compounds including brazilin in F-EtOH.**

Keywords: Sappan wood, brazilin, oral pathogens, antibiofilm, cytotoxicity

1. Introduction

Many *Streptococcus* spp. are normal flora microorganisms in oral cavity but some insidious species are found to be oral pathogenic stains. *Streptococcus mutans* is considered to be one of the severe cariogenic bacteria leading to dental caries (*1,2*). They can early colonized on hard tooth surfaces and the epithelial tissues to form a biofilm or known as a dental plaque, which later contains multiple bacterial species (*3*). These biofilms can produce acid that destroys the tooth's enamel layer which leads to periodontitis and dental carries (*4*). Although, the oral pathogens may be controlled by meticulous mechanical oral hygiene but they cannot be completely exterminated from oral cavity. Controlling oral microorganisms and keeping dental plaque at levels compatible with oral health are important. Therefore, many oral health care products are formulated to contain antiplaque or antiseptic agents to achieve good oral health.

Using plant extracts instead of chemical synthetic agents in treatment of certain bacterial infections are of increasing interest for green environment. Various potential plant extracts have been reported on antimicrobial activity against oral pathogens (*5*). Nowadays, only a few of them are considered to be used as ingredients in dental products whereas many of them are not, according to their limited properties. For example curcumin, the high effective secondary metabolite compound from turmeric has been reported to have strong ability against biofilm formation of *S. mutans* (*6*). However, curcumin is not used as ingredient in toothpaste or mouthwash because of its low aqueous solubility and rapid degradation, as well as its yellowish color that affects the physical characteristics of the products (*7*). Therefore, searching for other potential plants is still needed.

Caesalpinia sappan or sappan wood is the plant

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commonly found in many Asian countries. In Thailand, it has been used as traditional medicine since the ancient time to promote blood circulation. The heartwood part of *C. sappan* can produce natural red dye that can be used as coloring agent in food, beverage, and cosmetics. For biological properties, it has been reported to have antioxidant (*8,9*), anti-inflammation (*10*), anti-rheumatoid arthritis (*11*) and antimicrobial activity (*12-14*). Many phenolic compounds, such as xanthone, coumarin, chalcones, flavones, and homoisoflavonoids have been found from *C. sappan* (*15*). Brazilin [(6a S-cis)-7, 11b-dihydrobenz[b]indeno[1,2-d]pyran-3,6a,9,10(6H)-tetrol] is reported as one of the major constituents present in the heartwood part of *C. sappan* (*16*). The color of brazilin can be changed from amber to red in pH ≥ 7 (*17,18*). The intensity of red color is depends on the amount of brazilin. The extract of *C. sappan* heartwood has been reported to have activity against several kinds of bacteria including *S. mutans* (*19,20*). While brazilin has also been reported to have inhibition activity against many oral pathogens (*14*). From the literature review, there is still lack of deep detail of *C. sappan* extracts against oral pathogens, particularly on inhibition of the biofilms of such severe pathogens. Our previous study presented that the ethanolic fractionated extract of *C. sappan* (F-EtOH) showed strong antibacterial activity on these bacteria (*20*). The current study was aimed to identify the active compound of F-EtOH and compare the antibiofilm activity and cytotoxicity between F-EtOH and its isolated active compound. The minimum bactericidal concentration (MBC) of both F-EtOH and the isolated active compound was determined before antibiofilm activity investigation.

2. Materials and Methods

2.1. *Chemicals and reagents*

Hexane, ethyl acetate, ethanol, methanol, chloroform, and dimethyl sulfoxide (DMSO) of analytical grade and HPLC grade were from RCI Labscan (Bangkok, Thailand). Difco™ Brain heart infusion (BHI) broth and agar were from Bacton, Dickinson and Company (Sparks, Maryland, USA). Human blood for blood agar preparation was supported by Maharaj Nakorn Chiang Mai Hospital (Chiang Mai, Thailand). Dulbecco's modified eagle medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS), and antibiotic-antimycotic solution (AA) were from Gibco™, Life Technologies (Grand Island, New York, USA). Thiazolyl blue tetrazolium bromide was from Sigma-Aldrich (St. Louis, Missouri, USA). The other chemicals and solvents were of the highest grade available.

2.2. *Plant materials and fractionated extracts preparation*

The heartwood of *C. sappan* was collected from the local area in Chiang Mai Province of Thailand and identified by the botanist in the botanical herbarium of Faculty of Pharmacy, Chiang Mai University to obtain the reference voucher numbers (002276). F-EtOH was prepared according to the previous report (*20*). Briefly, the dried powder of *C. sappan* heartwood was subjected to fractionated extraction by maceration method using 3 different solvents; hexane, ethyl acetate, and ethanol, respectively. The filtrate from ethanol extraction was subjected to a rotary evaporator, EYELA rotary evaporation N-1000 (Tokyo, Japan) for removing the solvent and to obtain F-EtOH.

2.3. *Identification of the major compound*

Liquid chromatography–mass spectrometry (LC-MS) with electrospray ionizationc (ESI), Flexar SQ300 MS (Single Quad) (PerkinElmer, Waltham, Massachusetts, USA) was used to determine the molecular mass of the compound. The maximum absorption was determined using UV-2450 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan) with spectrum mode in wavelength range of 200-700 nm. Nuclear magnetic resonance (NMR) spectra of the isolated compound were obtained from Bruker 400 MHz NMR spectrometer, UltraShieldTM (Billerica, Massachusetts, USA). ^{1}H NMR was operated at 400 MHz and ^{13}C NMR was operated at 100 MHz. CD_3OD was used as a solvent.

As brazilin was previously reported to be the major compound in *C. sappan* (*20*), for quantitative determination of the major compound of F-EtOH, the pure brazilin was dissolved in methanol at the final concentration ranges of 0.5-500 µg/mL and used for preparing calibration curve. HPLC analysis was carried out using HPLC Shimadzu CLASS-VP™ model (Kyoto, Japan) and the reversed phase Eurospher 100, *i.d*. 4 mm, C18 column, Knauer (Berlin, Germany). F-EtOH and brazilin were dissolved in methanol to proper concentrations before injection. Aqueous solution of 1% v/v acetic acid in DI water (A) was mixed with methanol (B) at a volume ratio of 3:1. This solution was used as the mobile phase with the flow rate of 1 mL/min, injection volume of 10 µL, running time of 30 min, and detected at 280 nm. Running system was performed at room temperature. A standard curve of brazilin was constructed. The amount of brazilin in F-EtOH was calculated from the linear equation of $y = 8710.9x + 435.18$ ($r^2 = 0.9998$).

2.4. *Bacterial strains culture conditions*

Three strains of *S. mutans* including *S. mutans* DMST9567, *S. mutans* DMST18777, and S. *mutans* DMST41283 were cultured and incubated under anaerobic condition at 37°C in 5% CO_2 using anaerobic chamber BACTRONII-2, SHEL LAB® (Cornelius, Oregon, USA). Blood agar plates were prepared from

5% human blood in BHI agar.

2.5. *MBC determination and antibiofilm susceptibility*

The MBC of the test samples was determined according to the method previously described (*20*). Briefly, the diluted samples were added into the suspension of 1 × 10^6 CFU/mL of the tested bacterial strains in the 96-well plates and incubated at 37°C in 5% CO_2 anaerobic chamber for 24 h. Subsequently, the cultures were streaked on blood agar plates and 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.

In antibiofilm susceptibility test, the antibiofilm formation and eradication of the preformed biofilms were investigated. For antibiofilm formation, the samples were prepared to have the final concentrations of 1-, 2-, and 4-fold MBC. The sample solutions of 100 µL were transferred to 96-well plates followed by adding 50 µL of BHI broth and 50 µL of the culture suspensions (1 × 10^6 CFU/mL). Chlorhexidine at 0.12% (CHX) was used as a positive control. The plates were incubated in anaerobic chamber for 24 h. After incubation, nonadherent planktonic cells were removed and the wells were gently rinsed with 200 µL of phosphate-buffered saline (PBS). The adherent biomass was stained with 200 µL of 0.1% (w/v) crystal violet at room temperature for 30 min. The solutions were removed, and the wells were rinsed 3 times with 200 µL of PBS. Then, 100 µL of 30% (v/v) acetic acid was added to dissolve the crystal violet stains and measured at 595 nm using a microtiter plate reader Model 680, BIO RAD (Tokyo, Japan). The percentage of biofilm formation was calculated by the following equation; *% biofilm formation = As × 100/Ac*. Whereas *Ac* is the absorbance of the control culture (untreated cell) and *As* is the absorbance of the culture treated with the sample. The lower percentage of biofilm formation indicates the higher inhibitory activity of the test samples.

For antibiofilm activity that the preformed biofilms were eradicated, 50 µL of the culture suspensions (1 × 10^6 CFU/mL) and 150 µL of BHI broth were transferred to 96-well plates and incubated in an anaerobic chamber for 24 h. After incubation, nonadherent planktonic cells were removed and the wells were gently rinsed with 200 µL of PBS. Next, 100 µL of BHI broth was added in each well and the adherent biomass was treated with 100 µL of the test sample at the concentrations described above. The plates were further incubated in an anaerobic chamber for 24 h. After incubation, the nonadherent planktonic cells were removed by gently rinsing the wells with 200 µL of PBS. The viability biomass was stained with 200 µL of 0.1% w/v crystal violet at room temperature for 30 min. The solutions were removed, and the wells were rinsed 3 times with 200 µL of PBS. Then, 100 µL of 30% v/v acetic acid was added to dissolve crystal violet stains and measured at 595 nm using a microtiter plate reader. The biofilm eradication activity of the samples was evaluated from the percentage biofilm left which was calculated by the following equation; *% preformed biofilm = As × 100/Ac*. Whereas *As* is the absorbance of the culture treated with the samples and *Ac* is the absorbance of the control culture (untreated cells). The lower percentage found indicates the higher eradication activity of the test samples.

2.6. *Cytotoxicity*

The cytotoxicity of F-EtOH and its major component against normal cells was evaluated. Periodontal ligament (PDL) fibroblast cells were collected from the healthy human subjects. This experiment was under ethical clearance No. 02/2015, approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University, Thailand. Cell viability was determined by MTT assay. The PDL cells were cultured in completed DMEM (supplemented with 10% v/v FBS and 1% v/v AA) and incubated in humidified atmosphere, 5% CO_2 at 37°C. For the test, the cell suspension at a density of 1 × 10^4 cells/well was cultured in 96-well plates and then incubated under the same condition for 24 h. After that, the medium was removed and replaced with 100 µL of completed DMEM and 100 µL of the samples (final concentration ranged from 3.9-2,000 µg/mL in 0.4% v/v DMSO). The plates were further incubated for 24 h. Then, 100 µL of the medium was removed from each well and 100 µL of MTT solution (0.5 mg/mL in PBS) was added and further incubated for 4 h. Next, the medium was removed, and the formed formazan crystals were dissolved by mixing with 100 µL of DMSO for 10 min. The absorbance was measured at 540 nm and 690 nm as a reference wavelength using a microtiter plate reader. The cell viability was compared with the untreated culture or vehicle control culture. The percentage of cell viability was calculated using the following equation; *% cell viability = OD × 100/* OD_0. Whereas *OD* is the optical density of the well containing cells treated with the samples and OD_0 is the optical density of the well containing cells treated with 0.4% v/v DMSO (a negative control). The higher percentage of cell viability indicates the lower cytotoxicity of the samples.

2.7. *Statistical analysis*

The results of all experiments were conducted in triplicate and 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. *Sappan wood extract and the major compound*

F-EtOH obtained had the same outer appearance as the previous report (*20*). Isolation of F-EtOH using column chromatography yielded many fractions but only fraction-6 (F6) was identify since our previous work found that F6 has the highest activity. It was found that F6 was a relatively pure compound. The HPLC chromatograms of F-EtOH and F6 were compared as shown in Figure 1. It is found that F6 demonstrated a major single peak at a retention time of 6.83 min (Figure 1A). Meanwhile, F-EtOH demonstrated 2 obvious peaks and 2 tiny peaks exhibited at obviously distinct retention times (Figure 1B). The peak of F-EtOH at the same retention time as F6 is the largest one. The results of identification of the single compound of F6 using ^{1}H NMR spectra and ^{13}C-NMR spectra are as follows and shown in Figure 2.

^{1}H NMR data (400 MHz, CD_3OD): δ 2.77 (1H, d, J = 15.6 Hz, H-7), 3.02 (1H, d, J = 15.6 Hz, H-7), 3.69 (1H, d, J = 11.2 Hz, H-6), 3.92 (1H, d, J = 11.2 Hz, H-6), 3.96 (1H, s, H-12), 6.29 (1H, d, J = 2.4 Hz, H-4), 6.46 (1H, dd, J = 10.5, 2.4 Hz, H-2), 6.60 (1H, s, H-11), 6.70 (1H, s, H-8), 7.19 (1H, d, J = 8.1 Hz, H-1). ^{13}C NMR data (100 MHz, CD_3OD): δ 43.0 (C-7), 51.2 (C-12), 71.0 (C-6), 78.2 (C-6a), 104.4 (C-4), 110.1 (C-2), 112.6 (C-11), 113.0 (C-8), 115.7 (C-1a), 131.5 (C-7a), 132.4 (C-1), 137.6 (C-11a), 145.5 (C-10), 145.8 (C-9), 155.9 (C-3), 158.0 (C-4a).

The UV-visible spectrum of F6 showed maximum absorption at 224.5 and 289 nm. The mass spectrum of the compound showed a molecular weight at *m/z* 286. Using the NMR spectra as well as UV absorption and mass spectrum to compare with the data reported previously (*21,22*), we considered that the isolated compound of F6 was identical to brazilin ($C_{16}H_{14}O_5$), which the chemical structure is shown in Figure 3.

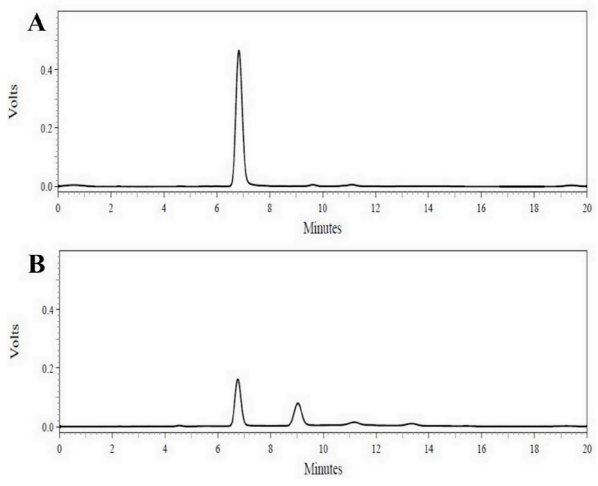

Figure 1. HPLC chromatograms of F6 (A) and F-EtOH (B) detected at 280 nm.

The physical appearance of F6 is orange red powder. An amber color solution was observed when it was solubilized in ethanol, methanol, DMSO or in the solutions of pH less than 7. The color of the solution changed to red or pink when the pH was increased to 7 or higher. These physical characteristics as well as the identified NMR spectra confirmed that the pure compound of F6 was brazilin (*17,21,22*). The physical appearance of F-EtOH was red brown powder. HPLC analysis indicated that the content of brazilin in F-EtOH was 325.14 ± 25.91 µg/mg of F-EtOH. It was also

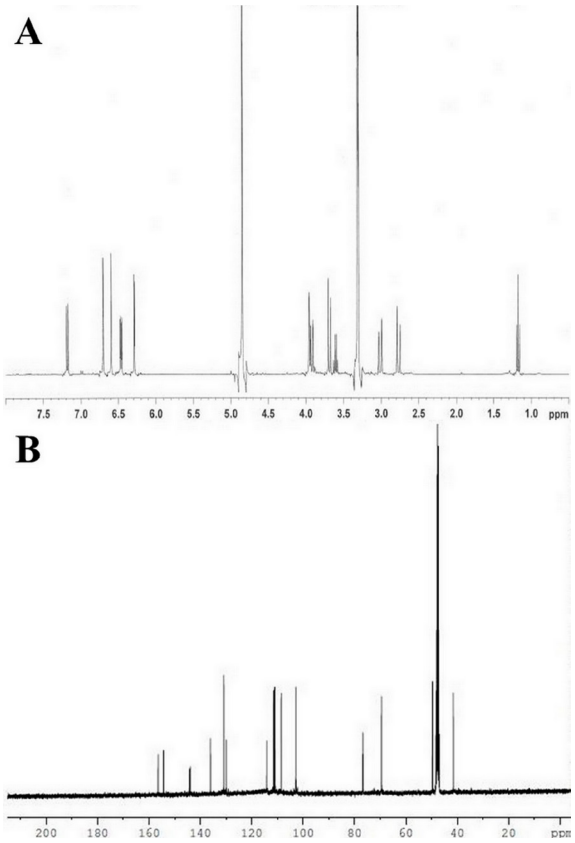

Figure 2. Nuclear magnetic resonance (NMR) spectra of brazilin from *C. sappan*, ^{1}H NMR (A) and ^{13}C NMR (B).

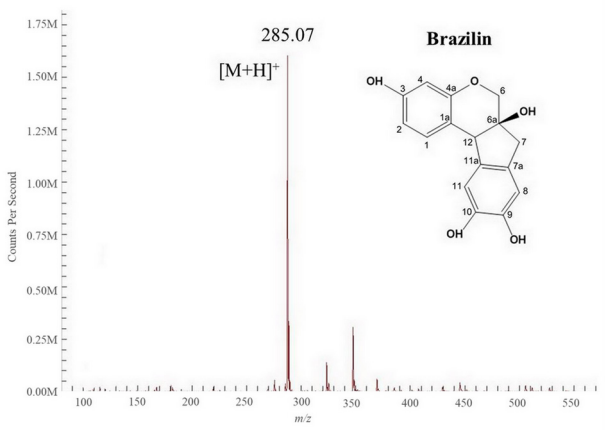

Figure 3. Fragmentation pattern from LC-MS by ESI technique (negative ion mode) of brazilin (*m/z* 286) isolated from F-EtOH.

found that brazilin played a role on the color of F-EtOH solution.

3.2. *MBC and antibiofilm susceptibility*

The MBC of F-EtOH and brazilin against the three strains of *S. mutans* was found to be the same value of 125 µg/mL. To evaluate the potential of F-EtOH as an antibiofilm agent, we examined the inhibition of biofilm formation and the ability to eradicate the preformed biofilms. We also compared these activities of F-EtOH with its major compound brazilin. The results are shown in Figure 4. It was found that all *S. mutans* strains were able to form biofilms completely (100%) after treated with the negative control but significant reduction after treated with F-EtOH and brazilin. Both F-EtOH and brazilin demonstrated dose dependent curves. Biofilm formation of all tested strains of *S. mutans* could be inhibited by F-EtOH and brazilin. After contacting with F-EtOH at 1-fold MBC, only 6.97 ± 0.79, 8.97 ± 1.60, and 9.17 ± 0.97% of biofilm of *S. mutans* DMST9567, *S. mutans* DMST18777, and *S. mutans* DMST41283 could be detected, respectively. After contacting with brazilin at the same concentration, 9.55 ± 1.08, 10.79 ± 0.33, and 14.29 ± 2.32% of biofilm of the three strains, respectively, could be detected. At the higher concentrations as 2-fold MBC, only 2.56 ± 0.21, 4.20 ± 0.13, and 5.25 ± 0.97% of the biofilm of the three strains, respectively, could be detected after contacting with F-EtOH. While, after contacting with brazilin, 9.55± 1.08, 5.59 ± 1.68, and 5.08 ± 1.17%, respectively, could be found. At 4-fold MBC, the biofilm formation of the three strains was almost completely inhibited and not significantly different from CHX. At this concentration, both F-EtOH and brazilin could inhibit more than 85% biofilm formation of all strains. At lower concentrations as 1- and 2-fold MBC, F-EtOH showed significantly higher effective on inhibition of biofilm formation of the three strains than brazilin. The results indicated that F-EtOH and brazilin even at low concentration as 1-fold MBC was sufficient to inhibit biofilm formation of all three strains of *S. mutans*.

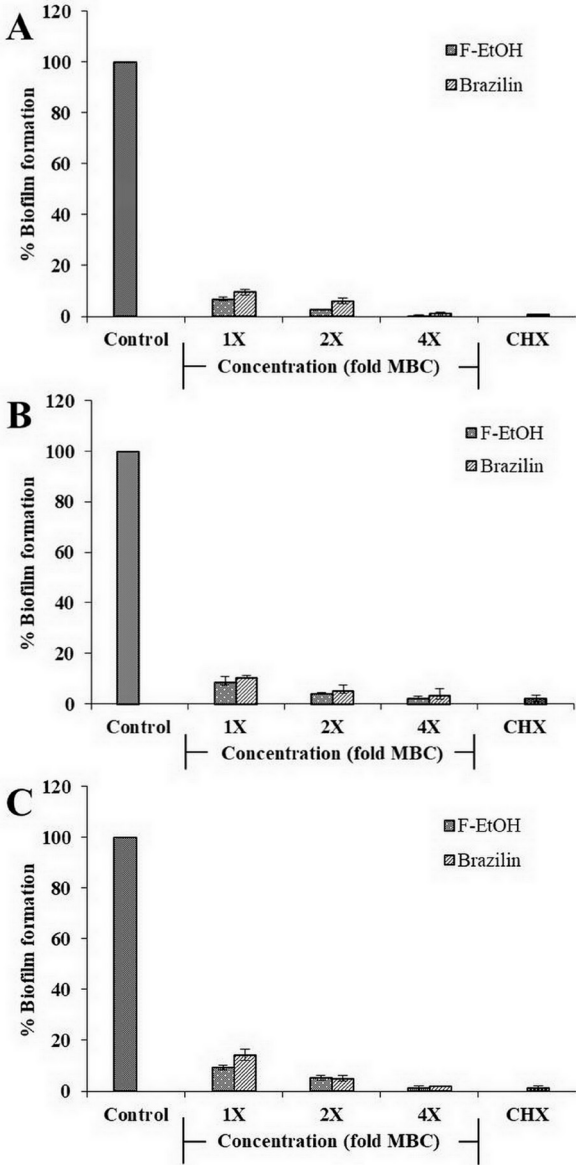

Figure 4. Biofilm formations of *S. mutans* DMST9567 (A), *S. mutans* DMST18777 (B), and *S. mutans* DMST41283 (C) after treating with F-EtOH, brazilin, and CHX in comparison with the untreated control.

For the ability to eradicate the preformed biofilm, the results are shown in Figure 5. A dose dependent reduction in cell viability of *S. mutans* was observed. Treating with 1-fold MBC of F-EtOH and brazilin was not much effective on eradication of the preformed biofilm of *S. mutans* DMST41283. More than 50% (52.07 ± 2.65 and 59.59 ± 1.84) of viable cells could be found after treating with F-EtOH and brazilin, respectively. However, at this concentration, 38.54 ± 1.50 and 46.52 ± 0.77% of viable cells of DMST9567 and *S. mutans* DMST18777, respectively were detected after contacting with F-EtOH. Whereas, after contacting with brazilin at this concentration, higher amount of *S. mutans* DMST9567 (49.42 ± 0.91) and *S. mutans* DMST18777 (54.79 ± 3.11%) could be detected. Increasing the extract concentration, a higher reduction in cell viability of the three strains was observed. Treatment with 4-fold MBC of F-EtOH, only 2.34 ± 1.63, 2.34 ± 0.47, and 5.49 ± 1.13% of viable cells of *S. mutans* DMST9567, *S. mutans* DMST18777, and *S. mutans* DMST41283 could be detected, respectively. At the same concentration of brazilin, 2.46 ± 0.82, and 3.79 ± 1.04, and 9.86 ± 2.62% of viable cell could be detected, respectively. This viability reduction indicated that more than 90% of the pathogens could be killed. From these results, it is shown that F-EtOH presented the significantly higher effective than brazilin. F-EtOH and brazilin at concentration of 4-fold MBC could killed *S. mutans* DMST9567 and *S. mutans* DMST18777 as much as CHX. Meanwhile, the reduction of viable *S. mutans* DMST41283 from F-EtOH and brazilin was lower than CHX. It was

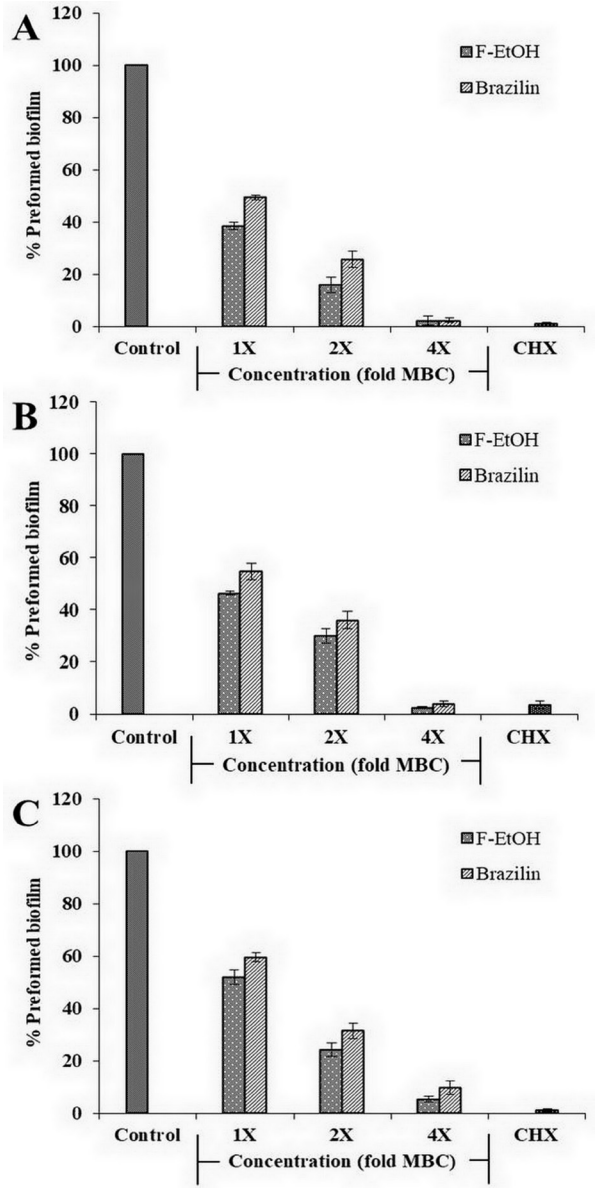

Figure 5. Percentage of preformed biofilms of *S. mutans* DMST9567 (A), *S. mutans* DMST18777 (B), and *S. mutans* DMST41283 (C) after treating with F-EtOH, brazilin, and CHX in comparison with the untreated control.

considered that the biofilm communication of this strain might be tolerant to F-EtOH and brazilin.

3.4. *Cytotoxicity*

The cytotoxicity of F-EtOH and brazilin on normal PDL cells of healthy volunteers was evaluated by MTT assay. DMSO at the final concentration of 0.4% v/v and its series of 2-fold dilution were also tested as it was used as a solvent for the test samples. The results showed that 85.01 ± 4.34% of cell viability could be detected when DMSO at 0.4% v/v was used indicating that DMSO at this concentration was not toxic to the cells. Comparison between F-EtOH and brazilin, the results are shown in Figure 6. Both F-EtOH and brazilin demonstrated the dose-response curves, however it was

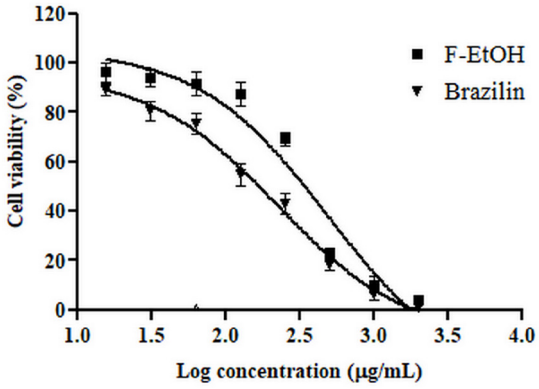

Figure 6. Viability of PDL cells after treating with F-EtOH (■) and Brazilin (▼).

obviously different in levels of toxicity. Cell viability higher than 80% demonstrates safety to normal cell. Monitoring at log concentration of 2.1 which referred to the concentration of 125 µg/mL, cell viability after treating with F-EtOH was 87.36 ± 4.83% whereas that treating with brazilin at the same concentration was obviously reduced to 54.73 ± 4.23%. Decreasing the concentration to 31.25 µg/mL which eqivalent to log 1.49, cell viability after treating with brazilin was 80.59 ± 3.77%. Therefore, brazilin at this concentration is safety to PDL.

4. Discussion

The oral cavity is the hub of an extremely diverse microflora consisting of about 500 species of microorganisms. The surface of mucosal membrane, teeth and tongue as well as gingival pocket can be the suitable habitats for microbial growth. These areas therefore can promote the formation of distinct oral microflora communities. Oral biofilm found on a tooth surface and define as a dental plaque is the important microbial community and identified by the initial adhesion of the early colonizers. The dental plaques commonly found in oral cavity are supragingival, subgingival, buccal mucosa, and tongue coating plaques. The metabolic activities of these normal flora communities can change the properties of the oral environment and to a suitable condition for promoting the growth of oral pathogens. The over growth of these oral pathogens can lead to several oral diseases such as dental caries, gingivitis, and periodontitis (*23,24*). Oral streptococci especially *S. mutans* is significantly role lead to dental caries and gingivitis (*2*). CHX is widely used in oral health care products for the prevention and treatment of oral diseases due to these pathogenic bacteria. However, CHX is cytotoxic to human periodontal cells, inhibits protein synthesis, affects mitochondrial activity, and thus, has adverse effects on vital tissues (*25*). Therefore, it is a need to find new agents that could be used as biological alternatives in management of these oral diseases.

Many bioactive compounds from various plants

have been reported their antimicrobial efficacy on a wide range of microorganisms including oral pathogens. Among phenolic compounds, tannins and the members of flavonoid group, especially flavan-3-ols and flavonols possessed broad spectrum, high antimicrobial activity and show synergism effect with antibiotics. Moreover, they are able to suppress the factors influence to microbial virulence, such as inhibition of biofilm formation, reduction of host ligands adhesion, and neutralization of bacterial toxins (*26*). Although, the specific mode of action of phenolic compounds is not completely understood (*27*). According to the numerous of report, phenolic compound and aromatics are disrupted at the cytoplasmic membrane of microbial cells by changing their structure and function influence to cell contents leak out and the microorganisms die (*28*).

In Thailand, it is well familiar that the most natural health care products contain the extracts of green tea. Its bioactive constituents are catechins and derivatives; epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC) have the strong oral protection by against oral pathogens such as streptococci and lactobacilli (*29,30*). *C. sappan* has been reported its antimicrobial activity against oral pathogens including gram positive and negative strains lead to oral diseases as dental caries and periodontitis. However, research related to this activity is still needed according to the lack of important detail such as antibiofilm activity which is directly related with severe dental diseases, particularly dental caries, gingivitis, and periodontitis. The current study provides the intensive data from our previous works (*20*) which reported the antibacterial activity of F-EtOH which is the ethanolic fractionated extracts of *C. sappan* possesses significantly higher antimicrobial activity against *S. mutans* and *S. intermedius* than the other extracts. In the present study, we therefore worked deeply on F-EtOH to identify the major active compound of this fractionated extract and compare their antibiofilm activities. The inhibitory activity test was performed against three strains of *S. mutans* because this pathogen is the most severe cariogenic bacteria that can cause dental carries and other important oral disease like periodontitis. Isolation of the major compound from F-EtOH was done by using silica gel column chromatography. The obtained isolated compound was confirmed to be brazilin by comparing the obtained ^{1}H NMR and ^{13}C NMR spectra as well as the supporting data of MS and UV with the data previously reported in the literatures (*11,15,21,22*). Considering the HPLC chromatogram of F-EtOH, it confirmed that F-EtOH contained not only brazilin but also one obvious minor peak and the other two tiny peaks. The calculation based on the HPLC chromatograms of F-EtOH and brazilin resulted that F-EtOH contained brazilin of approximately 300 µg/mg extract. According to the eradication of the preformed biofilm, the result clearly showed that the percentage of preformed biofilms of all strains after treating with F-EtOH at the concentration of 125 µg/mL were significantly less than after treating with brazilin at the same concentration indicating that F-EtOH had stronger inhibitory activity than brazilin. Using the above calculation, it is indicated that F-EtOH at the concentration of 125 µg/mL contains brazilin only 37.5 µg/mL. This result emphasizes that F-EtOH which consists brazilin at approximately 3 folds less than the pure brazilin has stronger activity than the isolated brazilin. The antibacterial potential of F-EtOH, therefore, is considered to be due to the synergistic effect of brazilin and the other minor compounds existed in the extract.

Biofilm formation of *S. mutans* is a severe etiological factor for dental caries. In the current study, we used two methods to investigate the antibiofilm activity of the test samples. One is the test on inhibition of biofilm formation and the other is the inhibitory activity test on eradication of the preformed biofilms of the test pathogenic bacteria. Both methods are different in the time that the samples are exposed to the test bacterial strains. For antibiofilm formation, the bacterial strains were mixed together with the samples at the first time before forming biofilms. This method indicates the ability of the sample to prevent the adsorption or adhesion of microorganisms to surfaces that is the initial stage of biofilm formation by killing the bacterial strains (*31*). Regarding to the inhibitory activity on eradication of the preformed biofilms, the bacterial strains were incubated for 24 h, that their biofilms were completely formed, before mixing with the test samples. F-EtOH showed the higher potential than brazilin on inhibition of biofilms of all strains by both methods. Our study demonstrated that the antibiofilm activity of F-EtOH and brazilin was dose dependent. At the low concentrations, such as at 1- and 2-fold MBC corresponding to 125 and 250 µg/mL, respectively, different potential of inhibition between F-EtOH and brazilin was obviously detected. F-EtOH showed significantly higher effective than brazilin. The concentration of 125 µg/mL was sufficient to inhibit the biofilms from *S. mutans*. Meanwhile, at the high concentration (4-fold MBC) corresponding to 500 µg/mL, brazilin and F-EtOH showed similar inhibitory power to almost complete inhibition (about 98%), and same as the positive control CHX.

The cytotoxicity test on normal cell is necessary for the candidates to be used in human. PDL is the connective tissue consists of fibroblasts cells in the periodontal area (*32,33*). These PDL cells were used in the present study to test for the toxicity of F-EtOH and brazilin to human normal cells. Our results show that PDL could tolerate to F-EtOH higher than brazilin. F-EtOH did not induce nonspecific toxicity to PDL cells when the concentration of 125 µg/mL was used, whereas brazilin at this concentration showed severe

toxic to the cells. This result confirmed the significant difference on higher safety of F-EtOH than brazilin.

In conclusion, this study reveals that the extract of *C. sappan* heartwood prepared from ethanolic fractionated extract F-EtOH and its isolated major compound brazilin possess the effective inhibition against three strains of cariogenic bacteria (*S. mutans*). Their inhibitory activity on pathogenic biofilms is along with the inhibition of biofilm formation and the eradication of the preformed biofilms of these pathogens. The antibacterial potential of F-EtOH at its MBC and 2-fold MBC is stronger than its major compound brazilin and it has significantly less toxicity to normal cells than brazilin. F-EtOH at 125 μg/mL is nontoxic to human normal cells. The antibacterial activity of F-EtOH is the results of synergism of the existed compounds including brazilin. From these results, F-EtOH of *C. sappan* is a promising natural antibacterial candidate suitable for further study on development of oral health care product for treatment and prevention of oral infection and related disorders like dental carries.

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Effect of rice variety and modification on antioxidant and anti-inflammatory activities

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Summary **The effects of variety and modification of rice on its antioxidant and anti-inflammatory activities were investigated. White rice varieties; Jasmine (JM) and Saohai (SH), and pigmented rice varieties; Doisket (DS) and Homnil (HN) were used. The modified rice samples were obtained from chemical modification using etherification reaction. The activities of the modified rice samples were compared with the ethanol extracts of the raw rice at the same rice concentration. Antioxidant activity was measured by the free radical scavenging activity tests and ferric reducing power assay. Results indicated that the ethanol extracts of raw rice had higher antioxidant activity than the modified rice. Among the raw rice tested, the pigmented rice showed higher antioxidant activity than white rice. Trolox equivalent antioxidant capacity values from free radical scavenging activity test were revealed that 50% ethanol extracts of HN and DS possessed the highest antioxidant activity. Ferric reducing power assay showed that 50% ethanol extracts of DS had the highest antioxidant activity. The anti-inflammatory activity was evaluated *in vitro* using a lipopolysaccharide-stimulated RAW264.7 macrophage cell model with enzyme-linked immunosorbent assay. Absolute ethanol extracts of HN reduced interleukin-6 secretion whereas that of DS suppressed interleukin-6 and tumor necrosis factor -α secretion. These results indicate that variety of rice, chemical modification, and extracting solvent were the factors that play an important role on antioxidant and anti-inflammatory activity. This study supports the potential use of the pigmented rice, especially DS, as a promising choice of a natural source because of its antioxidant and anti-inflammatory activities.**

Keywords: White rice, pigmented rice, modified rice, antioxidant, anti-inflammatory

1. Introduction

Oxidative stress is the resulting from the accumulation of reactive oxygen species and it can induce inflammatory cells to produce inflammatory mediators, such as cytokines and chemokines (*1*). Pro-inflammatory cytokines, such as tumor necrosis factor (TNF) -α, interferon-γ, interleukin (IL) -1β, IL-6, and IL-18, play an important role in signal transduction cascades during the progression of inflammation (*2*). Oxidative stress and inflammation increase the risk of various chronic diseases such as heart disease, cancer, diabetes (*3*), and Alzheimer's disease (*4*). There is a need for alternative treatment such as natural remedies due to conventional treatments show several side effects. Moreover, several studies have demonstrated the importance of diet in the control of chronic diseases. It has been reported that fruits, legumes, and vegetables, as well as grains consumption (*5*), have been associated with reduced the risk of chronic disease development (*6*). This could be attributed to the presence of natural bioactive compounds in these foods (*7*).

Rice (*Oryza sativa* L.) is one of the most important cereal grains and the economic agriculture in Asia. It

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is the principal staple food for more than half of the world's population. Based on the color of rice grains, rice can be categorized into two groups, non-pigmented rice and pigmented rice. Non-pigmented or white rice grains are white or pale yellow whereas pigmented rice grains show specific color, such as red, purple, and black. Rice grain is a rich source of many bioactive compounds including phenolics, flavonoids, and sterol derivatives (*8*). Phenolic compounds such as ferulic acid, p-coumaric and diferulate are presented in rice grains (*9*). Those compounds directly scavenge some reactive species as chain breaking antioxidants and suppress lipid peroxidation recycling other antioxidants, such as tocopherol (*10*). Some phenolic compounds may bind pro-oxidant metals, such as iron and copper, preventing the formation of free radicals from these pro-oxidants while simultaneously maintaining their capacity to scavenge free radicals. Previous studies indicated that phenolics might also suppress gene expression for pro-inflammatory factors (*11*).

Recent studies have reported that rice exhibit the potential to reduce risk of disease due to their antioxidant and anti-inflammatory activities. Especially in the pigmented rice, it has been reported that the pigmented rice contains natural anthocyanin compounds, such as cyanidin 3-glucoside and peonidin 3-glucoside (*12*), which possess antioxidant and anti-inflammatory activities (*13*). Those compounds have the potential to reduce the risk of chronic disease (*14*) and help to prevent cellular damage from oxidative stress.

Modification of rice starch has been shown to access various valuable features to the rice. Modified rice is widely used in pharmaceutical field because of their less toxicity and biodegradable properties. It can be used as gelling agents (*15,16*), flocculants, thickeners, stabilizers, fillers, binders, and disintegrants (*17*). Our previous studies reported that the modified rice obtained from chemical modification by etherification method could improve some property of rice (*15*) and it can be feasible to be useful in pharmaceutical preparation (*16,18*).

Although the antioxidant and anti-inflammatory activities of rice have been previously investigated (*19,20*), however, there is limited information available about local rice varieties that were chosen for these studies. Moreover, less investigation on the effect of chemical modification method to antioxidant and anti-inflammatory activity of modified rice was found. The aim of the present study is to investigate the effects of rice variety and rice modification on their antioxidant and anti-inflammatory properties.

2. Materials and Methods

2.1. *Materials*

Thai white rice grain varieties; Jasmine (JM) and Saohai (SH), and Thai pigmented rice grain varieties; Doisket (DS) and Homnil (HN), were obtained from a different rice cultivation area of Chiang Mai province, Thailand. Monochloroacetic acid, silver nitrate, Trolox, potassium persulfate, 2,'-azinobis-(3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt (ABTS), 2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ), thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), hydrochloric acid (HCl), and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine were obtained from Life Technologies (Carlsbad, CA, USA). Macrophage RAW264.7 cells were purchased from an American type culture collection. Enzyme linked immunosorbent assay (ELISA) kits were purchased from eBioscience (San Diego, CA, USA). Methanol, ethanol and glacial acetic acid were from RCI Lab-scan Co., Ltd. (Bangkok, Thailand). All other chemicals and solvents were of analytical grade or the highest grade available.

2.2. *Sample preparation*

2.2.1. *Preparation of raw rice and rice extracts*

Raw rice powder was prepared by wet milling method (*21*). Each rice sample was accurately weighed and extracted with absolute ethanol (Abs-EtOH) and 50% EtOH (50-EtOH), at a ratio of rice powder to a solvent of 1:10 w/v. The mixtures were stirred at 750 rpm for 24 h at room temperature. The mixtures were filtered through Whatman No. 1 filter paper. The filtrate was subjected to a rotary evaporator (EYELA N-1000, NY, USA) to remove the solvent. The obtained extracts of Abs-EtOH from JM (JM-Abs-EtOH), SH (SH-Abs-EtOH), DS (DS-Abs-EtOH), and HN (HN-Abs-EtOH) and those of 50-EtOH of JM (JM-50-EtOH), SH (SH-50-EtOH), DS (DS-50-EtOH), and HN (HN-50-EtOH) were kept at 4°C for further studies.

2.2.2. *Preparation of modified rice.*

Raw rice powder was modified using etherification method previously described by Okonogi *et al*. (*15*) with some modification. Briefly, 50% (w/w) sodium hydroxide aqueous solution was mixed with methanol at a ratio of 1:4 (w/w) in a 100-mL three necked round-bottom flask. The raw rice powder was added and stirred at room temperature until homogenous slurry was obtained. After that, proper amount of monochloroacetic acid was added. The temperature of the mixture was controlled at 50 ± 1°C for 3 h with continuous stirring. At the end of the reaction, the mixture was neutralized to pH 7.0. The solid phase was collected and washed by 80% (w/w) methanol until the filtrate testing for chloride by using silver nitrate test

was negative. The obtained slurry was dried at 50°C for 48 h. The obtained dried solid was pulverized and passed through the 80-mesh sieve. After passing the sieve, the obtained modified rice samples of JM (JM-M), SH (SH-M), DS (DS-M) and HN (HN-M) were kept in a desiccator for further use.

2.3. *Sample preparation for antioxidant and anti-inflammatory activity tests*

The samples used in antioxidant and anti-inflammatory activity tests were prepared as following. Exact amount of 4 g of raw rice powder was extracted using Abs-EtOH and 50-EtOH as extracting solvent. The solvents of the extract solution were evaporated. The yield of the extracts was recorded. The obtained semisolid mass extracts from Abs-EtOH and 50-EtOH were diluted with Abs-EtOH and 50-EtOH, respectively, to obtain the clear sample solutions of 40 mL. Meanwhile, exact amount of 4 g of modified rice was used without extraction. The modified rice powder samples were dissolved in water to obtain the clear sample solutions and the volume was adjusted with water to 40 mL. The sample solutions of rice extracts and modified rice solutions were further diluted with their respectively vehicles, *e.g*. Abs-EtOH and 50-EtOH for the rice extracts and water for the modified rice to obtain the 1,000-fold dilutions. This dilution of each sample was used for determination of antioxidant activity and anti-inflammatory activity test.

2.4. *Determination of antioxidant activity*

2.4.1. *Free-radical scavenging activity on ABTS*

ABTS assay described previously by Saeio *et al*. (*22*) was used in this experiment. Briefly, free radical ABTS was generated by reacting ABTS solution with potassium persulfate. The mixture was prevented from light and left to stand at room temperature for 12 h. Then, the mixture was diluted with Abs-EtOH to obtain the absorbance of approximately 0.7 units at 750 nm. The exact amount of 20 µL solution of each rice dilution was mixed with 180 µL ABTS free radical solution. The mixture was left to stand for 5 min at room temperature then the absorbance at 750 nm was recorded using microplate reader (Biorad 680, Hercules, CA, USA). Trolox was used to construct a standard curve. The antioxidant activity is expressed as Trolox equivalent antioxidant capacity (TEAC) in millimolar concentration of Trolox which antioxidant capacity is equivalent to 1 mg of the test sample.

2.4.2. *Free-radical scavenging activity on DPPH*

DPPH assay described previously by Okonogi *et al*. (*23*) was used in this experiment. Briefly, the solution of DPPH free radicals was prepared by dissolving the free radicals in Abs-EtOH to a concentration of 100 µM. The exact amount of 20 µL solution of each rice dilution was mixed with 180 µL DPPH free radical solution. The mixture was protected from light and left to stand for 30 min at room temperature. The amount of DPPH remaining in each period of stand was determined at 520 nm using the microplate reader. Trolox was used to construct a standard curve. The antioxidant activity is expressed as TEAC in millimolar concentration of Trolox which antioxidant capacity is equivalent to 1 mg of the test sample.

2.4.3. *Ferric reducing antioxidant power (FRAP) assay*

FRAP assay was determined according to a procedure described previously by Tachakittirungrod *et al*. (*24*). Briefly, the FRAP reagent was prepared by mixing TPTZ solution with $FeCl_3$ solution. The exact amount of 20 µL solution of each rice dilution was mixed with 180 µL FRAP reagent. After 5 min of mixing, the absorbance was taken at 595 nm using the microplate reader. The standard curve was constructed using $FeSO_4$ solution. The antioxidant compound will reduce the ferric ion into ferrous ion; the later reacts with TPTZ to form a blue complex which increases the absorption. The reducing power was expressed as equivalent concentration (EC). This parameter was defined as the concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$.

2.5. *Determination of anti-inflammatory activity*

RAW 264.7 macrophages were cultured in DMEM media containing 10% FBS, 4 mM L-glutamine, and 100 units-penicillin-streptomycin. The cells were seeded with a density of 2×10^6 cells per well into a 24 well plate and incubated at 37°C for 24h. Subsequently, the exact amount of 1 µL solution of each rice dilution was added to each well and the plates were incubated at 37°C for 3h. Then, the macrophages were activated by adding 1 µg/mL of LPS and the plate was further incubated at 37°C for 24 h. The positive control was the plates treated with LPS without the solution of rice dilution. The negative control was the plates treated without neither LPS nor the solution of rice dilution. The 500 µL supernatant of each well was removed and kept in −20°C for the ELISA assay.

MTT assay was used to determine cell viability. The 100 µL solution of 5 mg/mL MTT was added into the plates and incubated at 37°C for 2 h. After that, the supernatant was removed and the cells were lysed with 500 µL of lysis buffer (10% SDS in 0.01N HCl).The absorbance was recorded at 570 nm with reference at 690 nm using microplate reader.

Pro-inflammatory cytokines; IL-6 and TNF-α, in the cell supernatants were quantified using ELISA

kit according to the manufacturer's instructions. The absorbance was recorded at 450 nm with a reference at 570 nm using the microplate reader. The variation from cell density was reduced by using MTT values for normalization. The amount of cytokines of the positive control was defined as 100%. The results of the samples were calculated as a percent of this value. The inflammation assay was repeated in triplicate on independent days. The influence of the test compounds on cytokine secretion was defined as significant if the level of the positive control was changed by at least 25%.

2.6. *Statistical analysis*

Descriptive statistics for continuous variables were calculated and reported as a mean ± standard deviation (SD). Data were analyzed using a one-way analysis of variance (ANOVA) and Duncan's multiple range test ($p < 0.05$) using SPSS statistic software version 22.

3. Results

3.1. *Rice samples*

The raw rice powders of white rice grains (JM and SH) and pigmented rice grains (DS and HN) appeared different color. JM powder was white whereas SH powder was yellowish white. The color of both pigmented rice powder was purple. After extraction with Abs-EtOH and 50-EtOH, all rice extracts were clear viscous liquid. The color of white rice extracts was light brown-yellow for SH and light yellow for JM. The color of the pigmented rice extracts obtained from Abs-EtOH was dark purple whereas that obtained from 50-EtOH was different. The color of HN-50-EtOH was dark purple-green whereas that of DS-50-EtOH was dark red-purple.

The yield of all rice extracts ranged from 0.96% to 6.18 % (w/w), as shown in Table 1. Among the rice extracts obtained from Abs-EtOH, DS gave the highest yield of 6.18% whereas JM gave the lowest yield of 0.96%. Among the rice extracts obtained from 50-EtOH, DS also gave the highest yield of 3.91% whereas JM gave the lowest yield of 0.72%.

Table 1. Yield of rice extracts

Samples	Yield (%)*
SH-Abs-EtOH	1.46 ± 0.04
SH-50-EtOH	0.99 ± 0.01
JM-Abs-EtOH	0.96 ± 0.01
JM-50-EtOH	0.72 ± 0.01
HN-Abs-EtOH	2.26 ± 0.03
HN-50-EtOH	3.43 ± 0.02
DS-Abs-EtOH	6.18 ± 0.02
DS-50-EtOH	3.93 ± 0.03

*yield (%) = dry weight of extracts/raw weight of sample × 100%.

After subjecting to the chemical modification, the color of rice powder was changed to off-white. The obtained modified rice powders showed high ability to dissolve in water. Ethanol extraction was also carried out with the obtained modified rice samples using the same manner as the extraction of the raw rice. It was found that no yield of extract was obtained from all modified rice samples.

3.2. *Antioxidant activities of rice samples*

3.2.1. *ABTS radical scavenging activity*

The results from ABTS assay are presented in Figure 1. Raw rice extracts showed higher antioxidant activity than their modified rice ($p < 0.05$). Among the raw rice extracts, the samples extracted by 50-EtOH gave higher activity than those extracted by Abs-EtOH. HN-50-EtOH and DS-50-EtOH possessed the highest free radical scavenging property with TEAC values of 1.18 ± 0.17 mM/mg and 1.07 ± 0.07 mM/mg followed by SH-50-EtOH and JM-50-EtOH, respectively. The rice samples extracted by Abs-EtOH showed lower TEAC values than those extracted by 50-EtOH. Among the rice samples extracted by Abs-EtOH samples, HN-Abs-EtOH exhibited the highest free radical scavenging property with TEAC values of 0.62 ± 0.12 mM/mg followed by DS-Abs-EtOH and JM-Abs-EtOH, respectively. The lowest TEAC was found in SH-Abs-EtOH.

3.2.2. *DPPH radical scavenging activity*

The results of DPPH radical scavenging assay are shown in Figure 2. The raw rice extracts showed higher antioxidant activity than their modified rice ($p < 0.05$). Among the raw rice extracts, DS-50-EtOH and HN-50-EtOH showed the highest scavenging activity with TEAC values of 1.06 ± 0.07 mM/mg and 1.05 ± 0.02

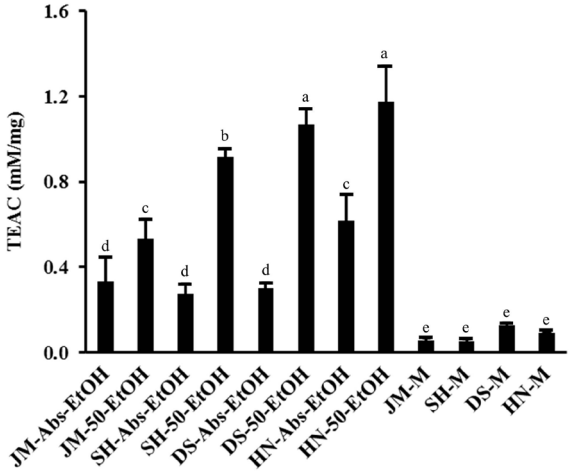

Figure 1. TEAC values of rice samples from ABTS assay. Different letters indicate significant differences ($p < 0.05$).

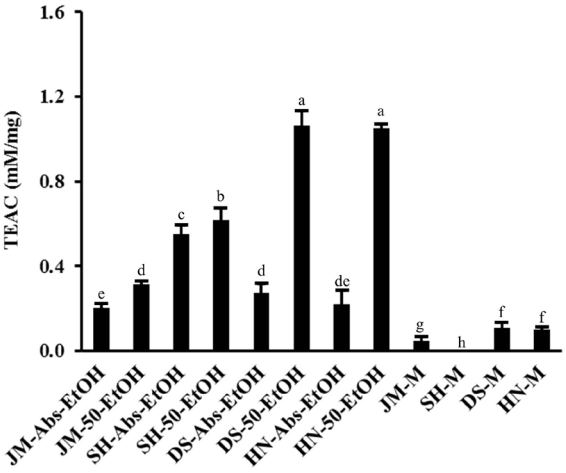

Figure 2. TEAC values of rice samples from DPPH assay. Different letters indicate significant differences ($p < 0.05$).

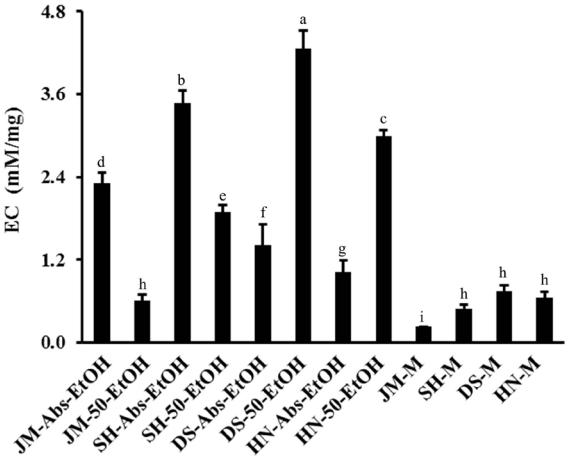

Figure 3. EC values of rice samples. Different letters indicate significant differences ($p < 0.05$).

mM/mg followed by SH-Abs-EtOH, SH-50-EtOH, DS-Abs-EtOH, HN-Abs-EtOH, and JM-Abs-EtOH, respectively.

3.2.3. *FRAP*

FRAP results are shown in Figure 3. The ferric reducing ability of raw rice extracts was significantly higher than their modified rice ($p < 0.05$). Among the raw rice extracts, significant differences were also observed among varieties of rice. DS-50-EtOH showed the highest EC with values of 4.26 ± 0.26 mM/mg followed by SH-Abs-EtOH, HN-50-EtOH, JM-Abs-EtOH, SH-50-EtOH, DS-Abs-EtOH, HN-Abs-EtOH, and JM-50-EtOH, respectively.

3.3. *Anti-inflammatory activity of rice samples*

All rice extracts and the modified rice at the concentration used did not have a significant cytotoxic effect towards macrophages as determined using MTT assay. Pro-inflammatory cytokines IL-6 and TNF-α were detected using commercial ELISA. The results of anti-inflammatory activity were shown in Figure 4. All pro-inflammatory cytokines exhibited significantly higher production in the positive control than the negative control. From the results, the anti-inflammatory activity of the raw rice extracts was significantly higher than their modified forms. Moreover, the activity of the rice extracts obtained from Abs-EtOH was significantly higher than those obtained from 50-EtOH. Furthermore, the pigmented rice extracts showed higher activity than the white rice extracts. The secretion of IL-6 was significantly reduced by 33.24 ± 2.75% after adding DS-Abs-EtOH and by 60.96 ± 7.27% after adding HN-Abs-EtOH. The secretion of TNF-α was also significantly reduced by 56.48 ± 0.10% after adding DS-Abs-EtOH and by 82.80 ± 0.97% after adding HN-Abs-EtOH. From these results, it can be concluded that DS-Abs-EtOH has

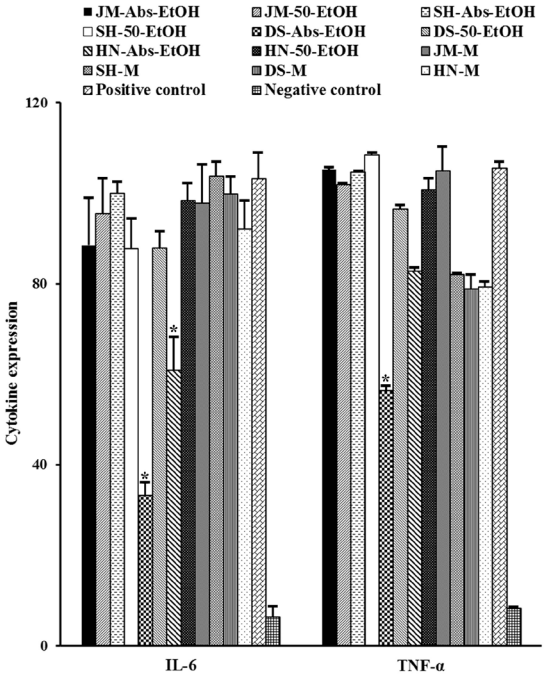

Figure 4. Effects of rice samples on IL-6 and TNF-α expression in LPS-stimulated macrophages (*the inhibitory activity of rice sample on cytokine secretion was significantly reduced at least 25% from a positive control).

the highest anti-inflammatory activity.

4. Discussion

The amount and type of compounds existing in each rice variety is different (*25*). The bioactivities of rice grains are hypothesized that it is according to the constituents existing in them. In this study, four different rice varieties including white rice, JM and SH, as well as pigmented rice, DS and HN, were investigated for their antioxidant and anti-inflammatory activities in comparison with the respective rice after subjecting to the chemical modification. The modified rice used

in the present study was obtained from etherification. Under this chemical reaction, raw rice starch can be modified into carboxymethyl starch (*15*) which can easily dissolve in water. Raw rice powder was found to be water insoluble, therefore extraction with organic solvent was used to collect essential compounds from raw rice samples. Abs-EtOH, containing 99.9% ethanol, was select as a suitable solvent because of its less polarity than water and less toxicity than other organic solvents (*26*). Water-cosolvent systems have been used for extraction of various plant compounds as an alternative to the extraction processes (*27*). Therefore 50-EtOH, containing 50% ethanol, was also selected to use in this study. The obtained rice extracts possessed different color due to rice variety and type of extracting solvent system. Different yield of extracts was found even from the same rice variety, depending on the solubility in the used extracting solvents and amount of the beneficial compounds in each rice grain variety. Extraction of the modified rice using the same manner and same extracting solvent showed that no any extract could be obtained. This might be due to the loss of certain ingredients at the final step of rice modification that the residual reagents and the unwanted products were completely washed out using ethanol. All solutes in the modified rice samples that could be dissolved in ethanol were removed in this step. Therefore, we decided to use the whole part of modified rice in the activity comparison test. To be comparable, the amount of the modified rice sample used was the same as that of the raw rice that gave the extract concentration used in the test.

Many reactions and mechanisms are involved in antioxidant processes. The reactions of antioxidant to decrease free radical are direct and indirect processes. The direct processes are *via* free radical scavenging and metal ion chelating activities. The indirect processes are *via* inhibiting the activity of free radical generating enzymes and enhancing the activity of intracellular antioxidant enzymes (*28*). To demonstrate antioxidant activity by a free radical scavenging mechanism, ABTS and DPPH assay were applied. The principle of ABTS assay is to monitor the decay of the radical-cation of ABTS resulting from the oxidation of ABTS. Radical-cation of ABTS is soluble in both aqueous and organic solvents. This assay is not affected by ionic strength and can be done at different pH levels, therefore it was commonly used (*29*). Another method for detection of antioxidant activity based on free radical scavenging mechanism is DPPH assay. This assay is according to the color changes of the DPPH free radicals. The purple radical DPPH solution was converted to the yellow non-radical DPPH by the antioxidant having electron donating activity. These two methods can determine the free radical scavenging activity of the test samples directly to indicate the antioxidant activity of the samples. These ABTS and DPPH assays were selected to use for antioxidant activity testing in the present study. Moreover, due to the different mechanism of antioxidant action, FRAP assay was used for determination of reducing activity. This method was applied to measure antioxidant activity by determination of the total reducing capacity of the compound based on the ability to reduce Fe^{3+} into Fe^{2+}. Two mechanisms complement one another and give useful information of antioxidant activity (*30*).

From the results of ABTS assay, the highest potential of ABTS free radical scavenging was DS-50-EtOH and HN-50-OH. This result is confirmed by the result from DPPH assay that DS-50-EtOH and HN-50-EtOH also showed the highest potential of DPPH free radical scavenging. The result from FRAP assay reveals that the highest reducing power was obtained from DS-50-EtOH. These results demonstrated that 50-EtOH is the better extracting solvent than Abs-EtOH. This was due to the ability of solvent to dissolve antioxidant compounds in rice grains. Moreover, the results indicated that the pigmented rice grains possess antioxidant activity with respect to the mechanisms of free radical scavenging and reducing activity. Previous studies demonstrated that pigmented rice exhibited higher antioxidant activity than white rice (*31*). This is according to the existing anthocyanins and proanthocyanins or condensed tannins which are the most prevalent phenolic compounds found in the pigmented rice (*19*). Those compounds have ability to donate hydrogen and act as reducing agents (*32*). Rice bioactive compounds such as anthocyanin are water soluble and the existing phenolic compounds are in the soluble and insoluble forms (*30,33*) Therefore, extracting solvent systems also play an important role on the antioxidant activity of rice due to the amount and type of the compounds that can be extracted.

Besides the results of bioactive analysis and antioxidant activity, the *in vitro* anti-inflammatory effect of rice was evaluated with macrophages cells. The effects of white rice and pigmented rice on IL-6 and TNF-α expression were examined for their potential anti-inflammatory activities. From our results, the pigmented rice has a potent anti-inflammatory action. The extracts of DS and HN obtained from Abs-EtOH achieved the inhibition against IL-6. The anti-inflammatory activity of the test samples is accounted when the pro-inflammatory cytokines, such as IL-6 and TNF-α were significantly reduced by at least 25%. In the present study, DS-Abs-EtOH significantly suppressed IL-6 and TNF-α secretion, indicating that DS-Abs-EtOH extract has high potential as an anti-inflammatory agent. To the best of our knowledge, this is the first study which reports the significantly high anti-inflammatory effects of DS, an important pigmented rice variety of Thailand. In previous report from other groups, the Abs-EtOH extracts of two varieties of HN grown in Phayao province showed low anti-inflammatory activity against IL-6, TNF-α, and

nuclear factor-kappa B. Those results contrast to our results that HN-Abs-EtOH in the present study exhibits high inhibition against IL-6. This difference might be due to the variation of different cultivation area that the yield of active compounds obtained is different amount (*13*).

Different polarity of extracting solvents can affect the bioactive composition of the obtained extracts. Previous report from other groups shows that the water extracts of *Chaenomeles sinensis* have higher antioxidant activity whereas the ethanolic extracts have higher anti-inflammatory activity (*34*). Another report demonstrates that the polar fractions of *Suaeda asparagoides* have higher antioxidant activity than the non-polar fractions whereas the non-polar fractions have higher anti-inflammatory activity than the polar fractions (*35*). In the present study, the higher polar rice extracts from 50-EtOH showed higher antioxidant activity than the lower polar extracts from Abs-EtOH, while Abs-EtOH extracts showed higher anti-inflammatory activity than 50-EtOH extracts. These results are in line with the previous reports and that was according to the different bioactive compounds in the extracts.

The present results from antioxidant and anti-inflammatory studies clearly indicate that modification of rice structure has an effect to reduce antioxidant and anti-inflammatory property of rice. It is hypothesized that some bioactive compounds were degraded during the chemical modification process. In our process of rice modification, rice powder was treated with acid, base, and high temperatures. Previous studies reported that phenolic compounds and anthocyanins are labile to heat treatment and unstable under the alkaline pH. These factors induced the degradation and resulting in color change of bioactive compounds (*34*). Moreover, as mentioned above, the moodified rice was washed several times with ethanol at the final step of rice modification. This step also caused the loss of many active ingredients that could be dissolved in the ethanol. This was confirmed by visual observation, that the color of the modified rice was different from the raw rice indicating that some active compounds were lost. In addition, high viscosity of the systems containing modified rice might retard the reaction of the test. According to these factors, DS-M and HN-M showed less activities of antioxidant and anti-inflammatory than their respective raw rice. Our results confirm that after rice modification, the antioxidant and anti-inflammatory activities can be obtained from only pigmented rice variety. From the results of MTT assay, cytotoxicity to the macrophage cells was not found in all rice samples indicating the safety of the samples.

In conclusion, difference in rice varieties leads to the difference antioxidant and anti-inflammatory potential. Chemical modification of rice causes significant reduction on the antioxidant and anti-inflammatory activity of rice. The pigmented rice possesses better antioxidant and anti-inflammatory activities than the white rice. In addition, the results suggest that DS is a promising source of antioxidant and anti-inflammatory compounds.

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Original Article

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Fungal-derived xenobiotic exhibits antibacterial and antibiofilm activity against *Staphylococcus aureus*

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Summary ***Staphylococcus aureus*** **is an opportunistic pathogen, responsible for superficial and invasive infections both in nosocomial and community-acquired settings. The incidences of infection have become more problematic attributable to emerging drug resistance and biofilm formation. These challenges suggest the need for new antimicrobial agents against *S. aureus*. In present work, we purified a fungal xenobiotic (FI3) which elicits a potent antimicrobial activity against a list of tested microbes including methicillin sensitive (MSSA) and methicillin resistance (MRSA) *S. aureus*. The cell growth of MSSA and MRSA were completely ceased with the 1× minimum inhibitory concentration (MIC); 32 µg/mL and 128 µg/mL, respectively. The cell viability severely decreased within 90 min, due to disturbance of membrane homeostasis. This bactericidal effect was enhanced at lower pH (pH 4) with a speculation to retain positive charge. The FI3 potently disrupts biofilm adherence at 64 µg/mL and found to be a safe with no toxic effect on mammalian tissue. FI3 also leads to increase the potency of tested antibiotics. Taken together, we established that FI3 has a potent antimicrobial activity against tested microbes and safer to human tissue. It may be proven a leading molecule for the treatment of bacterial infections.**

Keywords: Staphylococcal infections, antibacterial, antibiofilm, fungal xenobiotic, Staphylococcus aureus

1. Introduction

Staphylococcus aureus is a dangerous human pathogen which causes a diverse range of superficial and invasive diseases such as acute skin and tissue lesions to severe necrotizing pneumonia, osteomyelitis endocarditis, septicemia, and catheter-associated bacteremia (*1-4*). This notorious bacterium continues to parade higher morbidity, mortality, and a significant financial burden to the public. Individuals at high risk for *S. aureus* infection include patients with surgical, organ transplantation, indwelling catheters, ventilator-assisted respiration, diabetics, tracheal intubation, late-stage renal disease, immunosuppressive or cancer therapy and individuals with low-birth weight neonates, and hospital residents (*5-8*). In spite of high rate of infections, continuous and rapidly emerging drug resistance against available different antibiotics has made it difficult to treat (*9*). Secretion of virulence factors and formation of biofilm in *S. aureus* are the two hallmark determinants that majorly contribute to therapeutic failure. A multitude of virulence factors including enterotoxins, pore-forming toxins (α-hemolysin, Panton-Valentine leukocidin) is considered to be the key factors essential for the establishment of infection (*10*). In contrast, formation of multicellular biofilm over on cell boundary acts as a major obstacle for drugs to enter inside and that makes them 100 folds more resistant to antibiotics, antimicrobials, and immune defense (*11,12*). Many *Staphylococcus* species showed an increased resistance to available drugs (*13,14*). Consequently, the prognosis of such infections is problematic and has now

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become an alarming issue both in developed as well as developing countries.

The drug resistance condition is developed by frequent and indiscriminate uses of antibiotics. More than 11,000 people died from drug resistance *S. aureus* related infections in the U.S. (CDC report 2013) and now the situations have reached to an epidemic proportion with a serious health concern (*15*). In India, the incidence of drug resistant *S. aureus* infections has endemic nature and it increased steadily up to 54% both in hospital-acquired (HA) and community-acquired (CA) infections (*16-18*). There is a major deficit of effective and sustainable treatment against *S. aureus*. Hence, there is an urgent need for finding new antibacterial and novel approaches against both planktonic and sessile bacteria.

Since a long time, the discovery of new active compounds from both natural and synthetic sources has been explored and still gaining much attention (*19,20*). Fungi have been used as a natural source of traditional medicine and variety of drugs has been derived and still, the research on fungi is of a great interest. Antibiotics penicillin, streptomycin, immunosuppressant cyclosporine, anti-hypercholesterolemic compactin, and lovastatin are such examples. In fact, fungal secondary metabolites have a vital ecological role; they act as a weapon of defense and competitor to protect themselves against parasites and predators. The endophytic fungi, habitat to colonize on plants, represent a storehouse of the diverse array of bioactive secondary metabolites which can prove to be a miracle and combat plethora of pathogens including resistant microorganisms. With this background, the objective of this study is to evaluate the antimicrobial and anti-biofilm activity of the endophytic fungal xenobiotic (FI3) against methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). We showed that FI3 leads to eradicate *S. aureus* infection load speedily by targeting cell membrane and biofilm adherence of Staphylococcal strain without showing any detrimental effect to *E.coli* and human tissue.

2. Materials and Methods

2.1. *Bacterial and fungal strains and culture media*

The methicillin-sensitive *S. aureus* (MSSA); ATCC29213 and methicillin-resistant *S. aureus* (MRSA); ATCC33591, *Pseudomonas aeruginosa* MTCC1034, *Bacillus subtilis* MTCC441 and, *Escherichia coli* strains were used in this study. MSSA was received as a gift by Prof. Kasturi Mukhopadhyay, JNU, New Delhi, India and *P. aeruginosa* MTCC1034, *B. subtilis* MTCC441, and *E. coli* were purchased from MTCC, Chandigarh, India. These strains were used for analysis of drug susceptibility assay. Drug susceptibility test was also performed using MRSA. *S. aureus* strains were cultured in Muller-Hinton Broth/Agar (MHB/MHA). Tryptic Soya Agar/Broth (TSA/TSB) was used for biofilm study. *P. aeruginosa* MTCC1034, *B. subtilis* MTCC441 and *E. coli* were cultured in LB (Luria-Bertani) broth. The fungal isolates (FI) were isolated from the bark and dried tissue of the plant from the Sanjay Gandhi Zoological Park (SGZP), Patna, Bihar and also received from the National Culture Collection of Pathogenic Fungi (NCCPF), PGIMER, Chandigarh, India. All media were purchased from the Hi-media laboratory.

2.2. *Purification of fungal xenobiotic (FI3)*

The fungal isolates maintained on YEPD agar plate (seed culture) was inoculated in YEPD medium and allowed to incubate on shaking condition for six days at 30ºC. Cells were pelleted by centrifugation at 12,000× g for 15 min and the culture supernatant was filtered through Whatman filter No 1. The cell-free supernatant was extracted as described by Samuel *et al*. (*21*). The supernatant was gently agitated for 30 min after addition of equal volume of chloroform. This step was repeated thrice by adding fresh Chloroform. Fractions recovered from chloroform extraction were evaporated in the rotatory evaporator, dried under vacuum, weighed and dissolved in phosphate buffer saline (PBS) for purification. The sample was loaded on C-18 column using a gradient flow rate with 1 mL/min of acetonitrile (A) and water (B) mobile solvents. The chromatogram was run as A5% + B95% (0 min); A15% + B85% (0-5 min); A95% + B5% (5-25 min); A15% + B85% (25-40 min). The prominent peak with high intensities were collected in a sterile glass vial for further processing and antimicrobial analysis. Peak No. 6 showed postive results was collected and termed this xenobiotic as FI3.

2.3. *Susceptibility assay*

The antimicrobial susceptibility of FI3 was determined by two different methods mentioned here under.

2.3.1. *Spot assay*

FI3 was initially screened for antimicrobial activity against *S. aureus* by spot assay described elsewhere (*22,23*) with minor modifications. For this, both MSSA and MRSA cells were cultured in Muller-Hinton broth (MHB) till the cell density (Abs 600 nm) reaches 0.5. It was achieved at the mid-exponential phase of the cell growth (data not shown). One OD_{600nm} of *S. aureus* cell density was calculated which equals to 2×10^8 cells/mL (*24*). The cell pellet was resuspended in PBS to maintain 0.1 OD for spotting. 2×10^6 (0.01 OD_{600nm}) MSSA or MRSA cells were uniformly spread over the surface of the Muller-Hinton agar (MHA) plate and FI3 was put on plate for antimicrobial screening for

calculation of zone of inhibition. 5 µL of ten folds serial dilutions were spotted on MHA plate in the absence and presence of FI3 and other tested drugs. The difference in *S. aureus* cell growth was observed after incubation for 24 h at 37°C.

2.3.2. *Minimum inhibitory concentration (MIC)*

MIC was determined by broth microdilution method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (*25*). The assay was done in three independent experiments. Briefly, different dilutions of FI3 and other tested drugs *viz*. Tet (tetracycline), Genta (gentamycin), and Van (vancomycin) were prepared in 96 wells plate by addition of final volume 100 µL of MHB medium in each well of the plate. Subsequently, it was serially diluted in 1:2 ratios. After successful dilutions of tested compounds and drugs, 2×10^6 cells of mid-exponential phase *S. aureus* (MSSA and MRSA), *P. aeruginosa*, *B. subtilis* and *E. coli* were added in each well. MIC value was calculated by observing the optical density (OD_{600nm}) after 24 h of plate incubation at 37°C. 1× MIC was defined as the concentration of tested compound (FI3) that enables to restrict cell growth 100% as compared to the cell growth control. The FI3 showing 100% cell growth inhibition was considered as the 1× MIC.

2.4. *Cell viability assay*

To determine the antimicrobial activity of FI3, 2×10^6 MSSA cells of mid exponential phase were taken as described elsewhere (*26*). The cells were incubated with varying concentration of FI3 *i.e*. 16 µg/mL, 32 µg/mL, 64 µg/mL and 128 µg/mL, those correspond to 0.5× MIC, 1× MIC, 2× MIC and 4× MIC, respectively. The mixture of tube content was incubated at 37°C at 120 rpm. After each 30 min of interval up to 90 min, 10 µL of tube content was taken and serially diluted up to 100 times in PBS to reduce FI3 and then spread out on MHA plate at 37°C. Viable colonies were calculated after overnight incubation.

2.5. *Biofilm attachment assay and inhibition of biofilm formation*

The effect of FI3 on biofilm inhibition of MSSA was tested as described elsewhere (*27-29*). Briefly, the overnight culture of *S. aureus* was diluted up to 2×10^5 cells/mL in TSB. This diluted cell suspension with 5 mM glucose was seeded in to 24-well polystyrene plate in addition to introducing different concentrations of FI3. The assay was done in three independent experiments. In biofilm attachment assay, sterile glass slide (10 × 10 mm) was put in the wells. The plates were incubated at 37°C for 48 h and 3 h for inhibition of biofilm formation and biofilm attachment assay, respectively. After incubation, both slides and wells were washed with 250 µL of PBS to remove planktonic bacteria and air dried. The cells were then fixed with 200 µL of methanol for 15 min and plates were allowed to dry. The plate wells or glass slides were stained with 200 µL of 0.1% (v/v) crystal violet for 5 min. Excess stain was gently rinsed off and plates were air-dried. After staining, the attachment of biofilm was observed under the inverted microscope. The stained wells were re-solubilized in 200 µL of 95% (v/v) ethanol and cell concentration was measured at OD_{595nm} for biofilm inhibition analysis respectively (*30*). The FI3 untreated *S. aureus* cells were considered as a positive control in both experiments.

2.6. *Haemolysis assay*

To test the hemolytic activity FI3, chicken blood and human red blood cells (RBCs) were taken as reported elsewhere (*31,32*). Briefly, fresh human blood was collected and layered on histopaque-1077. The RBCs cells were recovered from the pellet by centrifugation at 400× g for 30 min. The pellet was washed with 0.9% (w/v) NaCl for three times and finally resuspended in 0.9% NaCl. Then, various concentrations (16-256 µg/mL) of FI3 were added in 95 µL of the RBCs cells suspension and it was then incubated at 37°C with gentle mixing for 3 h. After incubation, the supernatant was recovered by centrifugation as above. The cell lysis was quantified at 415 nm. As a positive control, 1% triton X-100 was added in RBCs suspension for measuring the complete lysis and in the negative control, RBCs suspension was incubated only in the presence of PBS. Haemolysis was calculated as: Percentage haemolysis = $[(A - A0)/(A100 - A0)] \times 100$. Where: A, represents absorbance of the FI3 treated sample at 415 nm and A0 and A100 represent zero percent and 100% lysis determined in PBS and 1% Triton X-100, respectively.

2.7. *Cytoplasmic membrane permeability assay*

To investigate the membrane permeabilizing property of the FI3 against *S. aureus* cell membrane, calcein release assay was performed using calcein acetoxymethyl ester (calcein-AM) as described elsewhere with minor modifications (*33*). Calcein-AM is a non-fluorescent, lipid-soluble, cell membrane permeable dye. Upon hydrolysis by cytoplasmic esterase, it breaks into non-permeable, fluorescent calcein with an excitation (Ext) and emission (Em) 494 nm and 527 nm, respectively. Briefly, *S. aureus* cells were harvested to mid-exponential phase and diluted to maintain 2×10^4 cells/mL in PBS, supplemented with 5 mM glucose. Cells were then incubated with 2 µM calcein-AM for 1h for maximal uptake of dye followed by addition of 1× MIC concentration (32 µg/mL) of FI3 and then

fluorescence was monitored at different time intervals (up to 90 min). Gramicidin B (35 µg/mL) was used as a positive control. During analysis, 1 mL of the sample was withdrawn at 15 min intervals and supernatants were recovered by centrifugation for fluorescence measurement using Spectrofluorometer (Perkin Elmer, LS 55).

2.8. *Propidium iodide (PI) uptake assay*

PI is a non-permeable, cell membrane, fluorescent dye that having excitation (Ext) and emission (Em) wavelength 544 nm and 620 nm, respectively. When the bacterial cell membrane is rendered permeable, PI enters and interacts with cellular DNA and fluorescence can be detected by spectrofluorometer. This assay was done according to the method described by Madhuri *et al*. (*33*). For this, *S. aureus* cells were harvested to mid-exponential phase and cells turbidity were diluted to maintain 2×10^4 cells/mL in PBS buffer. 2 mM PI was supplemented in the cell culture and incubated at 37°C for 10 min for dye equilibration. Cells were then exposed to 1× MIC of FI3 (32 µg/mL) at 37°C for different time periods up to 120 min. Gramicidin B (35 µg/mL) was used as a positive control since it kills the bacteria through membrane disruption (*34*). Fluorescence was measured at every 30 min intervals for 3 h using Spectrofluorometer (Perkin Elmer, LS 55).

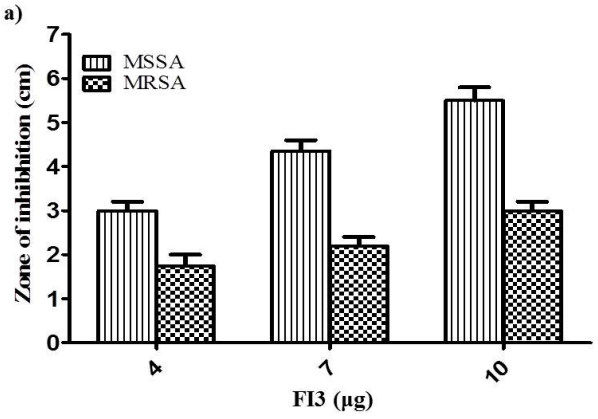
2.9. *Statistical analyses*

Statistical analyses were assessed using GraphPad prism 6.0 (Graph Pad Software, La Jolla, CA). *p* values were calculated *via* Student *t*-test. $p < 0.05$ was deemed significant.

3. Results

3.1. *Susceptibility profile of FI3 against both methicillin sensitive and resistant S. aureus*

The antimicrobial activity of fungal isolates was tested by spot assay for initial screenings, one such fungal isolate (FI) was identified as *Aspergillus nidulans* based on 18S rDNA sequence analysis (data not shown). The antimicrobial activity of different solvent extracts of *A. nidulans* was tested. The chloroform extract showed the highest antimicrobial activity among the all tested solvents. The solvent extract was then powdered and dissolved in PBS, filter through the 0.22 µm filter. The antimicrobial xenobiotic (FI3) was purified by reverse phase chromatography using C-18 column (Figure 1S, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=28*). Figure 1a



b)

Strains	1X MIC
MSSA (ATCC29213)	32µg/mL
MRSA (ATCC33591)	128µg/mL
P. aeruginosa MTCC1034	128µg/mL
B. subtilis MTCC441	256µg/mL
E.coli	ND*

*ND= not detectable

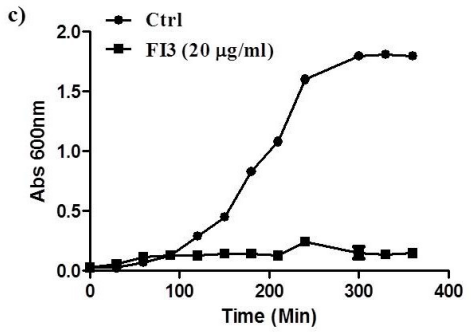


Figure 1. Susceptible profile of FI3 against *S. aureus*. Six days aged fungal culture broth extracted with equal volume Chloroform. Chloroform extract (FI3) was then evaporated and it was dissolved in PBS **(a)**. FI3 was spotted at the indicated amounts (4 µg, 7 µg, and 10 µg) on MSSA and MRSA suspended in MHA plate. Plate incubated at 37°C for 24 h and zone of inhibition was calculated. **(b)** Determination of MIC of FI3 against MSSA, MRSA, ***P. aeruginosa B. subtilis*** and ***E. coli*** bacterial strains. The 1× MIC) was measured that can be defined as the concentration of FI3 at which 100% of cell growth was inhibited. **(c)** Effect of FI3 (20 µg/mL) on MSSA growth.

illustrates that cell growth of both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistance *S. aureus* (MRSA) was inhibited in the presence of varying concentration of FI3. These results clearly demonstrate that FI3 is effective against both MSSA and MRSA. The antimicrobial activity of FI3 was further assessed against a panel of Gram+ve and Gram−ve strains using microdilution assay. Figure 1b demonstrates that FI3 completely inhibits the cell growth of the tested bacteria strains including resistant MRSA. The growth of MSSA and MRSA were completely arrested (1× MIC) at 32 µg/mL and 128 µg/mL, FI3 respectively. FI3 also retains its MIC values for *P. aeruginosa* MTCC1034 and *B. subtilis* MTCC441, 128 µg/mL and 256 µg/mL, respectively. On the contrary, FI3 has no appreciable inhibition of *E. coli* growth and was found to be insensitive even at higher concentration. Strikingly, MSSA growth was not recovered and remain suppressed when growth curve was performed in the presence of more than 0.5× MIC (16 µg/mL) concentration of FI3 (Figure 1c). These findings revealed that the FI3 effectively inhibits the growth of *S. aureus* (MSSA and MRSA), *P. aeruginosa* and *B. subtilis* without affecting the growth of *E. coli.*

3.2. *Combined effect of FI3 with topical antimicrobials*

Combination of two or more antimicrobials together has been explored as an alternative strategy for the treatment of systemic *S. aureus* infections. In order to test the combinatorial effects of FI3 together with tested topical antibiotics on *S. aureus* susceptibilities, broth microdilution and spot assays were performed. Figure 2a illustrates the drug susceptibility profile of three different antibiotics named as Tet, Genta, and Van in combination with FI3. The MIC of these individual antibiotics was measured as 1 µg/mL, 1 µg/mL, and 2 µg/mL, respectively (Figure 2a). The drug susceptibility of Tet, Genta, and Van was further enhanced when cells were grown with 8 µg/mL FI3 along with tested antibiotics. The MIC values of tested antibiotics; Tet, Genta, and Van in combination with FI3 were now calculated as 62.5 ng/mL, 125 ng/mL, and 250 ng/mL, respectively. Spot assay further confirmed that the supplementation of FI3 together with tested antibiotics were noticeably more susceptible to Tet, Genta, and Van than the individual effects of the tested antibiotics (Figure 2b).

3.3. *Bactericidal activity of FI3*

To assess the bactericidal activity of FI3, mid-exponential phase cells were treated with different concentration of FI3 *i.e.* 0.5× MIC (16 µg/mL), 1× MIC (32 µg/mL), 2× MIC (64 µg/mL) and 4× MIC (128 µg/mL) for 30 min, 60 min, and 90 min of action time. Figure 3a illustrates that the FI3 treatment shows time and concentration-dependent of cell killing. For example, at 0.5× MIC concentration, 19%, 24%, and 53% cells were killed within 30 min, 60 min, 90 min, respectively whereas at 1× MIC, 38%, 67% and 96% cells were killed in 30 min, 60 min and 90 min, respectively. The cells were found to be completely eliminated with no significant viable cells (± 2.5%)

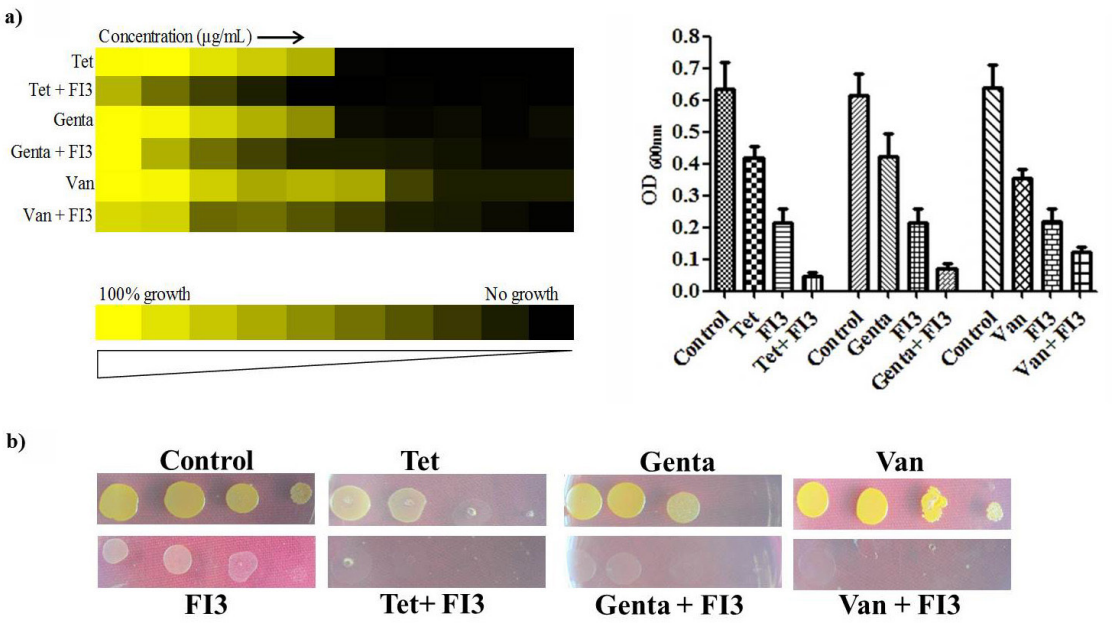


Figure 2. Combinatorial effect of FI3 with tropical antimicrobials. (a) Broth dilution assay to determine MIC value of tested antimicrobial drugs Tet (tetracycline), Genta (gentamycin), and Van (vancomycin) in absence and presence of FI3. Data was quantitatively showed with color (see the color bar in lower panel), where each shade of color represents relative optical densities of the cell and as bar graphs (see right panel). **(b)** Spot assays of *S. aureus* in presence of Tet, Genta, and Van (62.5 ng/mL, 62.5 ng/mL, and 125 ng/mL) alone and with FI3 (8 µg/mL).

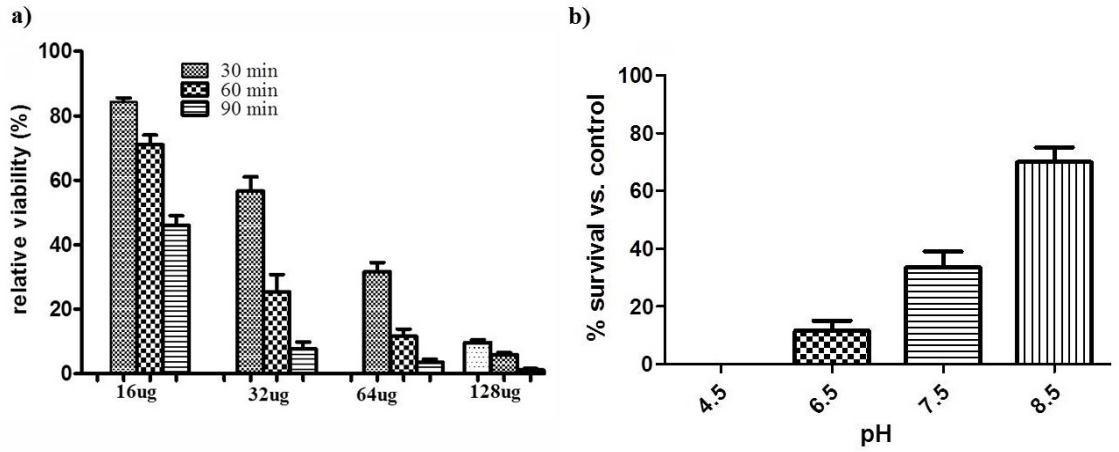


Figure 3. Antimicrobial activity of FI3. (a) Cell killing assay to determine the antimicrobial activity of FI3. Mid-exponential phase *S. aureus* cells were treated with 0.5×, 1×, 2× and 4× MIC *i.e.* 16 µg/mL, 32 µg/mL, 64 µg/mL, and 128 µg/mL, respectively. Cells were incubated up to 90 min at 30 min intervals. **(b)** *S. aureus* cells were grown till mid-log phase and cells were exposed to 16 µg/mL (0.5× MIC), a sub-inhibitory concentration of FI3 for 2 h at different pH.

within 90 min exposure of 1× MIC or at higher concentrations. These results ensure that the FI3 has a potent antimicrobial activity.

To access the effect of pH on cell susceptibility of *S. aureus* in response to FI3 treatment (0.5× MIC), cell viability assay was performed at varying pH ranging from 4.5 to 8.5. Figure 3b illustrates the impact of pH on the antimicrobial activity of FI3. Cells viability at individual pH was measured in presence or absence of FI3. Without FI3 treatment at different pH was considered as control (Ctrl). The relative cell viability (FI3/Ctrl) was analyzed at different tested pH for monitoring pH dependent effect of FI3. The data suggested that antimicrobial activity of FI3 is pH dependent and more susceptible at lower pH. The increased susceptibility at lower pH is expected to be increased a net positive charge on the molecule. This gain in the positive charge on FI3 molecule tends to interact to the negative charged cell membrane more firmly.

3.4. *Membrane permeability effect of FI3*

The staphylococcal cell permeabilization effect of FI3 was tested by quantification of preloaded calcein leakage *via* spectrofluorimetric analysis. Gramicidin B; a known pore-forming peptide was used as a positive control. For this, we performed cell permeability analysis by measuring calcein258 AM hydrolysis. Calcein AM is a cell-permeable, non-fluorescent dye which converts into a green fluorescent calcein after acetoxymethyl (AM) ester hydrolysis by intracellular esterase. When cells membrane becomes more permeable, more amount of calcein AM dye enters the cell and get hydrolyzed into membrane-impermeable green fluorescent calcein dye. Dead cells with compromised cell membrane do not retain calcein fluorescence inside the cell and release outside the cell membrane that can be measured at 515nm. Similar to the positive control, cells treated with 0.5× MIC FI3 (16 µg/mL) increased calcein fluorescence that indicates enhanced cell permeability. The cell permeability was further corroborated when cells were spotted with cell-disrupting chaotropic agent SDS and by PI uptake assay. Figure 4B illustrates that cells treated with SDS became hyper-sensitive in presence of FI3 and PI was 45% up taken upon treatment of 0.5× MIC FI3 as compared to positive control (Figure 4c).

3.5. *FI3 potentially clears up matured biofilm*

To consider the potential staphylocidal activity of FI3, we also considered the possibility that FI3 would also be effective against matured biofilm. *S. aureus* cells were grown in biofilm forming medium with the subsequent increasing order of FI3 concentration and biofilm mass was measured after 2 h of treatment. More than 90% biofilm formation was inhibited at 64 µg/mL (2× MIC) concentration (Figure 5a). FI3 showed a significant inhibitory activity against *S. aureus* biofilm formation at 48 h (data not shown). The biofilm inhibitory effect of FI3 was further confirmed by microscopic analysis. Cells treated with 1× MIC (32 µg/mL) and 2× MIC (64 µg/mL) concentration of FI3 reduce the biofilm cell aggregation (cleared up) approximately 40% and 89%, respectively as compared to untreated (control) (Figure 5b).

3.6. *Haemolytic effect of FI3*

The antimicrobial and antibiofilm activity of FI3 against both planktonic as well as surface-attached cells prompted us to further find out any toxic effect of FI3 on human tissue. This was achieved by using human as well as chicken blood tissues. For this, RBC cells were treated with subsequently increasing concentration of FI3. 0.9% NaCl and 1% Triton X-100 were taken as a negative and positive control respectively. The FI3 did not show any haemolytic effect on both human as well as chicken blood tissues as compared to tested control. This study suggests that FI3 is safer with low hemolytic

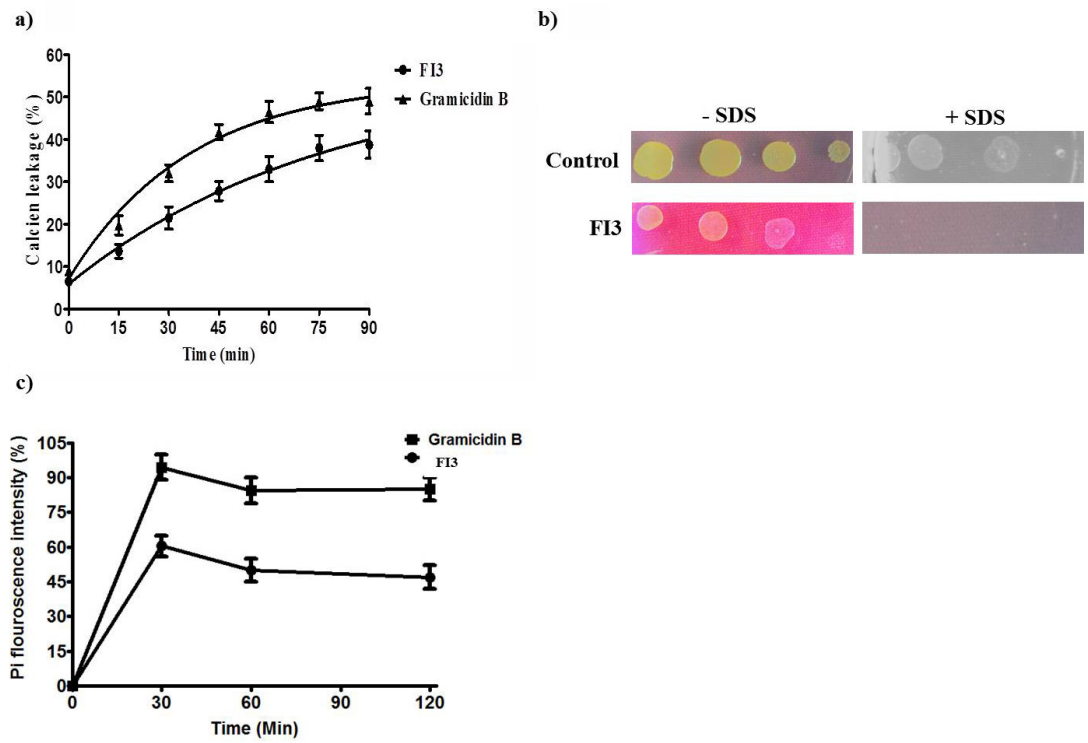


Figure 4. Cell membrane permeabilization activity of FI3 against *S. aureus*. (a) To assess the FI3 effect on membrane permeabilization, mid-log phase cells treated with 32 µg/mL (1× MIC) FI3 up to 90 min at 15 min intervals. 35 µg/mL gramicidin was used as a positive control. **(b)** Spot assay of *S. aureus* in the absence (negative control) and presence of 16 µg/mL (0.5× MIC), a sub-inhibitory concentration of FI3 and cell perturbation agent SDS at 0.03%. **(c)** PI uptake assay performs to determine cell deprivation in presence of 16 µg/mL, FI3 and 35 µg/mL, gramicidin B (positive control).

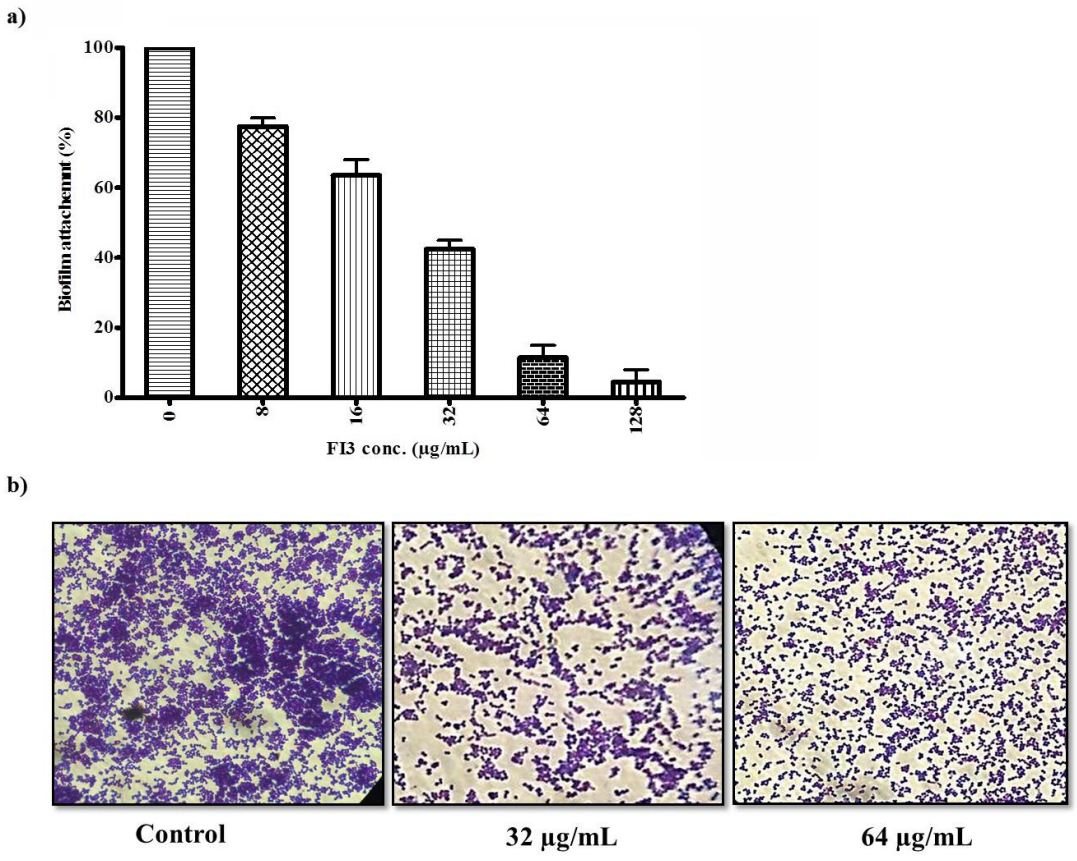


Figure 5. Anti-biofilm activity of FI3. The 2×10^5 cells/mL *S. aureus* cells suspended in the TSB-glucose medium, were seeded in pre- kept glass slide, in 24 well polystyrene plate and added different concentrations of FI3 (in triplicate) as indicated. The plate was incubated at 37°C for **(a)** 4 h for biofilm attachment analysis and **(b)** 48h for inhibition of biofilm formation.

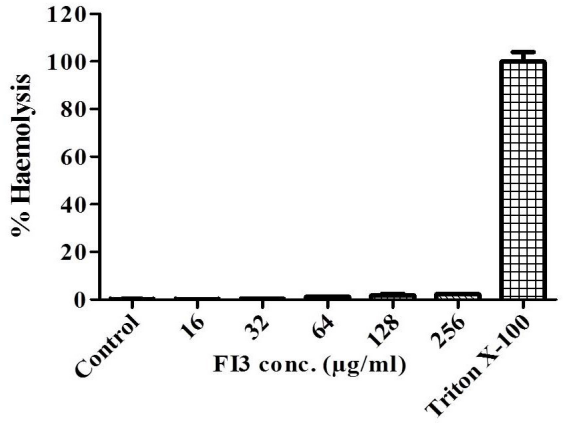


Figure 6. Hemolytic effect of FI3 on mammalian cells. FI3 toxicity was measured against human blood RBCs by measuring hemolytic activity as described elsewhere (Chopra *et al*., 2015). Triton X-100 as a positive control and NaCl (0.9%) as a negative control.

activity to the human as well chicken tissue as depicted in Figure 6.

4. Discussion

The prevalence of methicillin resistance *S. aureus* (MRSA) globally cause a major risk in the treatment of hospital-acquired (HA) and community-acquired (CA) infections (*35*) and become endemic in India (*15*). Rapidly emerging such threats and clinical complications in Staphylococcal infections demand the discovery of novel antibacterial candidates against both planktonic and drug resistant sessile bacteria. Since a long time, the discovery of new active compounds from natural sources has been explored and still gaining much attention. Natural products isolated from fungi have been represented one of the most successful source for the treatment of such infectious diseases. Indian geographical diversity is favorable to fungi which covers one-third of fungal diversity of the World. More than 75% of the total fungal species remain undiscovered and need to be explored against a plethora of pathogens especially multidrug-resistant microorganisms. The objective of the present study was to examine antibacterial properties of *A. nidulans* fungal xenobiotic in different micro-environmental conditions. The purified fungal xenobiotic (FI3) showed a strong antibacterial activity against different bacteria such as *P. aeruginosa* MTCC1034, *B. subtilis* MTCC441, MSSA and MRSA strains with a MIC 128 µg/mL, 256 µg/mL, 32 µg/mL and 128 µg/mL, respectively (Figure 1c). Similar antibacterial effect of natural products extract was earlier reported (*19,36,37*). Exploration of combined effects of two or more antimicrobials together can be an alternative strategy for the treatment of life-threatening multi-drug resistant *S. aureus* infections. The clinical impacts of such strategy are not only to synergize the bacteriostatic or bactericidal activities but also suppress the multi-drug resistance (MDR) problem in systemic *S. aureus* infections. Interestingly, FI3 was capable to synergize the antimicrobial effect up to 15 folds when it used in combination with Tet, Genta, and Van as compared to individual effects of the tested antibiotics (Figure 3). In addition, FI3 also has a strong antimicrobial activity that effect was observed dose-dependent. When *S. aureus* cells exposed with 16 µg/mL (0.5× MIC) FI3, 19%, 24%, and 53% cells were killed within 30 min, 60 min, 90 min, respectively whereas at 1× MIC (32 µg/mL), 38%, 67%, and 96% cells were killed in 30 min, 60 min and 90 min, respectively. FI3 completely eliminated with no significant viable *S. aureus* cells within 90 min exposure. The antimicrobial activity of FI3 was maximum at pH 4 compared to other tested pH (Figure 3b). The increased susceptibility at lower pH is expected to be increased a net positive charge on the molecule which promotes interaction with negatively charged cell surface thereby showed higher bactericidal activity. Similar results were observed when α-MSH (α-melanocyte stimulating hormone) peptide was tested against *S. aureus* at varying pH (*33*). The antimicrobial effect of FI3 was due to cell membrane disruptions which correlated by enhancement of cell permeability. The cell membrane permeability increased by 33% when cells were incubated for 90 min exposed with 0.5× MIC FI3. Such membrane disruption effect was further corroborated by cells spotted with cell-disrupting chaotropic agent SDS and PI uptake assay. In presence of SDS, FI3 made cells hyper-sensitive similar to the study reported by Pal *et al*. (*38*) and 45% higher PI was taken up upon treatment of 0.5× MIC FI3 as compared to positive control.

Formation of biofilm over the cell surface of *S. aureus* makes it difficult for antibiotics and immune cells to attack. Biofilm forming cells elicit several folds higher drug-resistant and adhered to implanted medical devices and damaged tissue and are one of the major attributes of acute and chronic infections (*39,40*). In this context, it is interesting to note that FI3 effectively inhibited biofilm formation of *S. aureus* and almost 89% biofilm was cleared up by the treatment of 64 µg/mL (2× MIC) FI3 (Figure 5a). A similar antibiofilm effect was also reported by Bakkiyaraj *et al*. (*19*). In contrary, ebselen that strongly shows antibacterial activity against *S. aureus* is strongly inhibited biofilm formation at 16× MIC concentration (*41*). It has also been remarkably noted further that FI3 showed low hemolytic effect on human blood tissues and safe to mammalian cells (Figure 6). Moreover, FI3 has a good bactericidal activity against both *S. aureus* strains with little difference in susceptibility to MSSA and MRSA strains. This high bactericidal activity of FI3 against both MSSA and MRSA strains may have tremendous therapeutic consequence.

Taken together, our results demonstrate that the purified FI3 of fungal *A. nidulans* shows a potent

bactericidal and anti-biofilm activity against tested microbes including *S. aureus*. FI3 rapidly ceases the growth of both the sensitive (MSSA) and drug-resistant (MRSA) *S. aureus*, without affecting *E. coli* growth. The growth of MSSA was rapidly ceased at 32 µg/mL (1× MIC) within 90 min. In spite of antimicrobial activity, biofilm formation of *S. aureus* was also remarkably inhibited at 64 µg/mL, without showing any hemolytic effect on human blood cells. Both bactericidal, antibiofilm activity of FI3 can be used for the treatment of bacterial infections and may be proven a leading molecule.

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Original Article

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Differences in how bronchial asthma patients transmit experience about adverse reactions and usability of inhaled steroids to others: A qualitative focus-group study

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Summary **Patients' experience of adverse reactions (ADR) and usability of drugs is important for proper use and post-marketing development of drugs, but it remains unclear whether and how patients are transmitting such information to others. The aim of this study was to explore differences in the ways in which bronchial asthma (BA) patients transmit experience of ADR to inhaled corticosteroids and usability of inhalers to others, including the reasons for these differences. A qualitative study involving focus-group interviews was conducted. Participants were fifteen Japanese BA patients treated with inhaled steroids who belonged to an association for BA patients. Data were analyzed using the constant comparative method. Almost all participants reported behavioral differences concerning ADR and usability. Participants actively expressed their doubts and anxieties about ADR to members of a patients' association and their attending physician. In contrast, information about patients' needs, including opinions and questions about the usability of steroids inhalers and anxieties regarding potential ADR to prolonged use of inhaled steroids, was shared only with members within the association and not disseminated outside, with some participants even choosing to keep it personal. Underlying this behavior was a mindset of perceiving efficacy and ADR to be more important than usability, and thinking "it is useless to inform anyone." In conclusion, behavioral differences of how BA patients transmit experience about ADR and usability was obvious, because benefit to inform usability was not perceived. It is necessary to make patients aware that transmitting their experience and comments about drugs is beneficial.**

Keywords: Inhaled steroids, dissemination of information, usability of inhalers, adverse reactions

1. Introduction

Post-marketing information about efficacy and safety, as well as feedback about the usability of drugs are important not only to improve compliance, but also to facilitate proper use of drugs and post-marketing development (Ikuyaku, in Japanese; the phrase meaning fostering drugs). In recent years, systems to collect information regarding adverse drug reactions directly from patients have been investigated in many countries (*1,2*). However, it seems that such information from patients is not collected enough, and patients are less conscious that information transmitted by them will lead to proper use of drugs or effective post-marketing development (*3*). In addition, it is not clear whether patients actively transmit information about the usability of drug preparations and their preferences related to drugs. Therefore, in order to encourage the proper use of drugs and to enable effective post-marketing development, it is important to ascertain whether and how patients are transmitting such information, and to understand the background of patients' perceptions about such actions.

With respect to treatment of bronchial asthma,

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Table 1. Characteristics of the participants

Group	Sex	Age group (years)	Asthma history	Asthma condition	History of inhaled steroid	Current inhaled steroid	History of current Inhaled steroid usage
A	F	60–69	40 years	Less than once a week	20 years	Symbicort®	8 months
A	F	60–69	11 years and 6 months	Less than once a week	11 years 6 months	Symbicort®	1 year 3 months
A	F	50–59	20 years and 9 months	Less than once a week	18 years	Symbicort®	1 year
A	F	70–79	35 years	Less than once a week	about 20 years	Alvesco®	3 years
B	M	40–49	30 years	Less than once a week	13 years	Pulmicort®	13 years
B	M	70–79	25 years 2 months	Not every day but more than once a week	10 years	Symbicort®60	9 months
B	F	60–69	about 20 years	Less than once a week	About 20 years	Advair®	3 months
B	F	50–59	31 years 9 months	Less than once a week	More than 10 years	Symbicort® Alvesco®	9 months
B	M	60–69	6 years 8 months	Less than once a week	6 years 8 months	Symbicort®	8 months
B	M	60–69	35 years 5 months	Less than once a week	20 years	Symbicort®	1 year 10 months
B	M	30–39	35 years	Less than once a week	6 years	Advair®100	6 years
C	M	80–89	37 years	Less than once a week	unknown	Advair®250	2 years
C	F	60–69	23 years 4 months	Less than once a week	18 years 2 months	QVAR®100	3 years
C	F	60–69	28 years	Less than once a week	18 years	Alvesco®200	10 years
C	F	30–39	7 years	Not every day but more than once a week	7 years	Advair®500	5 years 6 months

F, female; M, male.

treatment based on inhaled steroids is generally the first-line choice (*4*). In order to control asthma, continued treatment is essential, and the patient's medication compliance affects the efficacy of the treatment (*5*). Since inhaled steroids are administered using special devices, not only efficacy and safety, but also patients' preferences regarding the inhaler's operability, portability, and preferred usability are important factors influencing medication compliance (*6,7*).

Patients' associations, which are formalized non-profit patient-interest organizations, promote self-help (*8*), through group activities such as exchanges between members, regular events, and medical lectures, providing a sense of mental security, as well as information about diseases and drugs. In addition, the associations enable patients to face the disease through exchange, sharing, and accumulation of "experience-based knowledge" that only patients can engage in (*8,9*). Although the members of patients' associations may have a greater tendency to actively seek information regarding drugs as compared to patients who do not belong to any association, their role in information dissemination about drugs is not clear.

The purpose of this study was to explore differences in the ways in which bronchial asthma patients transmit experience of adverse reactions to inhaled corticosteroids and usability of inhalers to others, including the reasons for these differences. Since there have been few previous studies in this area, we adopted focus-group interview (FGI) methodology, which is an exploratory qualitative technique, for the present investigation.

2. Methods

2.1. *Design*

In this study, the FGI was adopted as a qualitative technique, because interactive discussion among multiple participants has the potential to develop greater insights and to clarify perspectives that cannot necessarily be derived from one-on-one interviews (*10*). To validate the results, FGIs were conducted with three groups, a follow-up questionnaire was conducted after the FGIs to confirm the participants' remarks, and the results were discussed and confirmed with other researchers who had participated in the interviews.

2.2.*Subjects*

Members of two associations for bronchial asthma patients in the Kanto district (the megalopolis of Tokyo and its suburbs), who were undergoing treatment with inhaled steroids, were invited to participate in the interviews by post and e-mail. Fifteen patients responded (Table 1). A financial incentive was provided to the participants.

2.3. *Data collection*

Focus groups were conducted in September 2011. The total duration of one interview was approximately 1.5 hrs, and the interviews were conducted in community centers or community meeting rooms. The groups were moderated by an academic staff member and an undergraduate student who were research team members (Hori and Kurimoto) and were both women. The moderators were not acquainted with the participants and were familiar with this research theme. An independent note taker was present. The basic FGI guide used in this study was prepared based on common guidelines for conducting focus group research (*10,11*). This guide consisted of an introduction, stimulus questions, probes, and a conclusion. The interview was conducted

Table 2. Topic guide used for the focus groups

1) Remarks on doubts and anxieties regarding adverse reactions to inhaled drugs
   Do you consult someone when you have doubts or anxieties about adverse reactions to inhaled drugs? or Do you not consult anyone?
   – *Do you use the internet?*
   – *Do you make inquiries to companies?*
   – *Do you ask the physician or the pharmacist about drugs based on your doubts and anxieties?*
   – *Is there any advantage in being a member of a patient association when you have doubts and anxieties?*
   – *Or, do you do nothing and leave it as is?*

2) Thoughts driving behavior related to doubts and anxieties regarding adverse reactions to inhaled drugs
   Why do you consult the person?
   - *Why do you not consult persons around you?*
   - *When you consult someone, why did you decide to talk to that person? Why did you not select someone else?*

3) Remarks about worries about usability
   Do you consult someone when you have trouble understanding the method of operation or perceive that usability is poor?
   or Do you not consult anyone?
   – *Do you use the internet?*
   – *Do you make inquiries to companies?*
   – *Do you ask the physician or the pharmacist about drugs based on your worries?*
   – *Is there any advantage of being a member of a patients' association when you have trouble understanding the method of operation or perceive that usability is poor?*
   – *Or, do you do nothing and leave it as is?*

4) Thoughts driving the behavior related to usability
   Why do you consult the person?
   - *Why do you not consult persons around you?*
   - *When you consulted someone, why did you decide to talk to that person? Why did you not select someone else?*

5) The reasons behind the differences in behavior
   5-1. Comparing the persons you consult when you have worries about adverse reactions and usability, is there any difference?
   5-2. Why do you consult different persons?

using an interview guide consisting of five open-ended questions, as shown in Table 2. The survey items for the interview were behavior when the patients had doubts or anxieties after experiencing adverse reactions due to inhaled steroids or felt that the usability of the inhalers was poor, and their reasons for these feelings. Information regarding differences in behavior and the corresponding reasons was also collected. If the topics shown in italics in Table 2 did not emerge during FGIs, these areas were probed by the moderators. A video camera and digital voice recorder were installed in the room used for the interview to record the proceedings, after participants' consent had been obtained. Each interview was conducted by one interviewer and one sub-interviewer, with an observer watching and recording the proceedings. The subjects were divided into groups of four to seven members.

In order to make it easier for the participants to recall their own experiences about inhaled steroid drugs, placebo samples of steroid inhalers were placed on the table, and were available for practice.

2.4. *Data analysis*

The interviews were recorded with permission of the participants, and then transcribed verbatim and used along with observation data. Transcripts were independently analyzed by the first and second authors using the constant comparative method. This method combines inductive category coding with simultaneous comparison of all units of meaning obtained. First, open-coding was used to develop categories based on commonly recurring themes, and each new unit of meaning was compared with all other units and subsequently grouped with similar units of meaning. Categories were continuously refined until saturation of themes and subthemes was reached after three focus groups. Saturation of themes was determined when the range of ideas was identified and no new information was being obtained.

The focus groups and analysis were conducted in Japanese. The statements in italics were then translated into English with the aim of capturing the meaning of the statements, rather than literal translation. Regarding remarks related to each code, a four-stage weighting was adopted based on the method proposed by the previous literature (*12*). In the weighting scheme, A was assigned when "similar remarks were heard from three groups", B was assigned when "similar remarks were heard from several members from two groups", C was assigned when "similar remarks were heard from one member each, from two groups, or several members belonging to one group", and D was assigned when "similar remarks were heard from only one member from one group". Letters in parenthesis in the Results section and in Tables 3-5 show the results of weighting. In addition, numbers given in the Results section correspond with the sub-themes shown in Tables 3-5.

Table 3. Behaviors of participants when they had doubts or anxieties after experiencing adverse reactions of inhaled steroids, or when they felt that the usability of the inhaler was unsatisfactory

Main Themes	Sub-themes
Behaviors taken	*Consulting*
#1. when they had doubts or anxieties after experiencing adverse reactions to inhaled steroids	*Members of a patients' association (#1-1, A) (#2-1, B)*
	An advising physician of a patients' association (#1-2, C)
	An attending physician (#1-3, A) (#2-2, B)
	A pharmacist (#1-4, D) (#2-3, C)
#2. when they felt that the usability of the inhaler was unsatisfactory	*A nurse (#1-5, D) (#2-4, D)*
	Getting information from the bulletin of a patients' association (#1-6, A)
	Attempting to deal with their doubts and anxieties themselves without consultation (#1-7, B) (#2-5, C)
	Gathering information from the internet (#1-8, B)
	Devising measures for lessening adverse reactions on their own (#1-9, B) (#2-6, B)

Numbers and letters in parenthesis represent the sub-theme numbers and the results of weighting, respectively.

Table 4. Perceptions and reasons to consult someone or not to do

Main Themes	Subthemes
#3. Perceptions and reasons to consult members or a advising physician of a patients' association	*Tremendous reliance on an advising physician (#3-1, C)*
	Past experience of success (#3-2, B)
	Achieving peace of mind (#3-3, C)
	Understanding of suffering (#3-4, B)
	Atmosphere encouraging free speech (#3-5, C)
	Not being able to communicate with asthma patients in their hospital (#3-6, C)
	No understanding of asthma from general public (#3-7, C)
#4. Perceptions and reasons to consult medical staff	*Possessing specialized knowledge (#4-1, A)*
	Providing appropriate information on asthma (#4-2, C)
	Encouragement from medical staff (#4-3, A)
	Specified to an attending physician;
	Positive response from the attending physician (#4-4, C)
	Good relationship with the attending physician (#4-5, C)
	Specified to a pharmacist;
	A similar footing between physician and pharmacist (#4-6, D)
#5. Perceptions and reasons not to consult medical staff	*Patient's pride in possessing correct information (#5-1, C)*
	Related to attending physician;
	Distrust of attending physician (#5-2, A)
	Hesitation regarding communicating with the attending physician (#5-3, A)
	Related to pharmacist;
	Perception of lack of specialized knowledge (#5-4, A)
	Poor response from a pharmacist (#5-5, D)
	Insufficient information-sharing by a pharmacist (#5-6, C)

Numbers and letters in parenthesis represent the sub-theme numbers and the results of weighting, respectively.

Table 5. Perceptions and reasons not to disseminate information about adverse reactions or poor usability

Main Themes	Subthemes
#7. Perceptions and reasons not to disseminate information about adverse reactions	*Is this really an adverse reaction? (#7-1, C)*
	Already having been informed about adverse reactions (#7-2, C)
	Occurrence of adverse reactions cannot be avoided (#7-3, C)
#8. Perceptions and reasons not to disseminate information about poor usability	*Should be solved by oneself (#8-1, B)*
	Useless to talk about it (#8-2, C)
	Preference for efficacy over usefulness (#8-3, B)
	Adverse reactions more important than usability (#8-4, C)
	Not sure whether the information will be forwarded by medical staff to pharmaceutical companies (#8-5, B)
	Questioning whether an attending physician would take any action (#8-6, C)
	Usability is acceptable (#8-7, C)
	Usability has been improved (#8-8, B)

Numbers and letters in parenthesis represent the sub-theme numbers and the results of weighting, respectively.

2.5. *Conduct and analysis of the questionnaire survey*

We conducted three questionnaire surveys for participants of the FGIs at the timings shown below. 1) Before conducting the FGIs, a self-report mail form pre-survey was conducted to gather information about the participants' sex, age group, asthma history, current asthma status (frequency of attacks), inhaled

steroid usage history, and current inhaled steroid usage. The survey form was collected on the day the FGI was held. 2) On the same day, after completing the FGI, a questionnaire survey on the operability of steroid inhalers was conducted. 3) After analyzing the interview results, in order to evaluate the accuracy and validity of the results, a self-reporting survey form was developed and a mail survey was conducted. The collected survey forms were aggregated and analyzed.

2.6. *Ethics*

Ethical approval for the study was obtained from the Ethics Committee of Graduate School of Pharmaceutical Sciences, the University of Tokyo. The authors confirm all patient/personal identifiers have been removed so that the patient/person(s) described are not identifiable and cannot be identified through the details of the story.

3. Results

Three FGIs were conducted with 15 participants (6 males, 9 females), who were members of asthma patients' associations. The age of the participants was between 30 and 89 years. Participants' characteristics were summarized in Table 1.

3.1. *Behavior of participants*

The behavior of the participants when they had doubts or anxieties after experiencing adverse reactions to inhaled steroids, or when they felt that the usability of the inhaler was unsatisfactory could be categorized into "consulting members of a patients' association or an advising physician," "consulting medical staff," "not consulting anyone," and "doing nothing because there was no doubt or anxiety" (Table 3).

3.1.1. *Behavior of participants when they had doubts or anxieties after experiencing adverse reactions of inhaled steroids*

With regard to the behavior of the participants when they had doubts or anxieties after experiencing adverse reactions of inhaled steroids, most participants mentioned that they firstly consulted members of a patients' association (A) (#1-1). They also put into practice various measures for lessening adverse reactions (B) that they had learned from other members, *via* mutual exchange of information. Some participants also collected information about adverse reactions from an advising physician of the patients' association (C) (#1-2). Even though most of the participants consulted their attending physicians (A) (#1-3), some of them also commented that since one needs to muster enough courage to consult the attending physician after encountering a possible adverse reaction, they would first wish to confirm that the symptom is in fact an adverse reaction (C) by getting information from members or the bulletin of the patients' association (A) (#1-1, #1-6), and would then report it to the attending physician (C). A few participants said that they consulted a pharmacist (D) (#1-4) or a nurse (D) (#1-5) regarding their doubts and anxieties about adverse reactions. In contrast, some participants mentioned that they did not consult a pharmacist.

Some participants said that they attempted to resolve their doubts and anxieties themselves without any consultation (B) (#1-7). In order to ascertain whether the symptom was an adverse reaction, they gathered information from the internet (B) (#1-8), and to lessen adverse reactions (B), they devised measures on their own (#1-9). Some of the participants mentioned that they did nothing because they had no doubt or anxiety (C).

3.1.2. *Behavior of participants when they felt that the usability of the inhaler was unsatisfactory*

With regard to the behavior of the participants when they felt that the usability of the inhaler was unsatisfactory, some participants mentioned that they first consulted members of a patients' association (B) (#2-1), and put into practice various measures for improving inhaler usability (B). Many of them also mentioned in this context that they did not consult an attending physician (B) or a pharmacist (D). In contrast, there were some participants who informed an attending physician (B) (#2-2), a pharmacist (C) (#2-3) or a nurse (D) (#2-4).

Some participants said that they attempted to overcome their doubts and anxieties themselves without consultation (C) (#2-5), and devised measures on their own to lessen poor usability (B) (#2-6). Some of the participants mentioned that they did nothing because they have no doubt or anxiety (B).

3.2. *Perceptions and reasons underlying behaviors*

The following sections describe the perceptions and reasoning underlying the above behaviors (Tables 4-5).

3.2.1. *Perceptions and reasons to consult members of a patients' association or an advising physician*

One of the patients' associations selected in this study was a branch of a nationwide association with several advising physicians. The participants had tremendous reliance on one physician who was involved in editing the association's bulletin (C) (#3-1). The factors behind this were obtaining the latest information from the bulletin or in exchange meetings (C) and obtaining a specific response to consultation by e-mail or phone (D), which made the advising physician an invaluable partner for consultation and information collection (A).

With respect to the reasons for consulting a member of the patients' association, the responses were

Table 6. Information dissemination by participants only within the patients' association

■Anxiety about the adverse reactions/risks regarding inhaled steroids in the future
"I am worried about what will happen a few years from now" (Participant 5)
"Not sure how the adverse reactions will really emerge after prolonged use. Currently, I do have physical strength to cope with them, but, with time, these will accumulate, and as I grow older and lose physical strength, I am always concerned about what might happen then." (Participant 7)
■Measures taken by the patients to alleviate adverse reactions
"I try to create a thin layer of milk within my mouth before I inhale, and it has always worked for me" (Participant 1)
■Specific opinions about poor usability and measures taken by the patients
Regarding ambiguity in gauging the remaining amount of the drug
"I always write the date, from the day I actually start inhaling" (Participant 1)
"I write here <on the actual inhaler>, then I write on the calendar, and I also write here" (Participant 14)
Regarding difficulty in operation of the inhaler
"That is why I write with a color pen myself. First write it once on the right, then go towards the left and using red ink pen when I write here <on the rotation clip of the inhaler>" (Participant 2)

psychological factors, such as achieving peace of mind (B) (#3-2), compassion for the suffering of patients afflicted with the same disease (C) (#3-3), or simply an atmosphere encouraging open dialog (B) (#3-4). Some of the participants also mentioned that they had experience having their doubts or anxieties resolved by advice from the members of the patients' association (C) (#3-5). Some of the participants also mentioned apparently paradoxical reasons, such as not being able to communicate with asthma patients in a hospital where they were visiting (C) (#3-6), and the general public has no understanding of asthma (C) (#3-7).

There were discussions about their concerns regarding possible adverse reactions in the future (C). Table 6 summarizes the information dissemination based on the remarks regarding exchanges between patients' association members only.

3.2.2. *Perceptions and reasons to consult medical staff, or not*

The participants expected that the persons they consulted would have expertise about asthma, and thus they thought that medical staff having such knowledge would be suitable for consultation (A) (#4-1). At the same time, they also expected that medical staff would provide appropriate information on asthma to patients (C) (#4-2). Some participants said that the reason behind not consulting medical staff was pride that the patient possessed the correct information (C) (#5-1).

Other reasons to consult the attending physician were encouragement from the attending physician (A) (#4-3), positive response from the attending physician (C) (#4-4), or a good relationship with the attending physician (C) (#4-5). In contrast, some participants said that the main reason behind not consulting an attending physician was distrust (A) (#5-2) or hesitation (A) (#5-3). Some of the reasons for distrust were responding with blunt answers when consulted regarding adverse reactions or requests of drugs (B), not being satisfied with diagnosis or instructions (B), insufficient sharing of information or lack of expertise (B), and lack of consensus among medical staff (B). Moreover, not having any understanding of the pain and suffering of the patient (B), and difficulty in communicating (C) were also mentioned as factors causing distrust. With respect to hesitation, the reasons were limitations of consultation time (B), experience of being offended by the attending physician in the past (C), and the perception of avoiding a situation where consulting may result in being considered a troublesome patient (C).

The participants mentioned that the reasons to consult pharmacists were on a similar footing between physicians and pharmacists (D) (#4-6), and encouragement from the pharmacists (C) (#4-3). With respect to not consulting a pharmacist, the reasons given were the negative perception of pharmacists not having expertise about asthma (A) (#5-4), providing insufficient information (D) (#5-5), or lack of response to questions (C) (#5-6). Because of these factors, some participants said that they had rejected advice from pharmacists (C).

One participant mentioned that the reason to consult nurses was encouragement from a nurse (D) (#4-3).

3.2.3. *Perceptions and reasons not to disseminate information about adverse reactions*

As a reason not to disseminate information about adverse reactions, the participants mentioned that they did not know whether it was really an adverse reaction or not (C) (#7-1). There were also some participants who had no doubt or anxiety when an adverse reaction occurred, resulted from situations such as already having been informed by the patients' association or the attending physician about adverse reactions (C) (#7-2), or the perception that the occurrence of adverse reactions cannot be avoided (C) (#7-3).

3.2.4. *Perceptions and reasons not to disseminate information about poor usability of inhalers*

In all the focus groups, participants expressed opinions about usability (A), such as difficulty in inhaling and knowing how much drug remained in an inhaler. The reasons for not disseminating information about usability even under such conditions were that one

should solve usability issues oneself (B) (#8-1), and that it was useless to communicate with others (C) (#8-2). Moreover, based on the experience of improvements in symptoms as a result of inhaling steroids (C), and experience and anxiety concerning adverse reactions (A), the perception was that efficacy (B) (#8-3) and adverse reactions (C) (#8-4) were more important than usability. On the other hand, even if an opinion was expressed, participants questioned whether medical staff would notify the drug manufacturers (B) (#8-5), and even if poor usability was reported, they questioned whether the attending physician would take any action (C) (#8-6).

Some of the participants mentioned that they do nothing because they have no doubt or anxiety (C), because the usability is acceptable (C) (#8-7), or because the usability has been improved (B) (#8-8). Some of the participants with a long history of asthma even favorably evaluated usability as much better than before (C) as a result of successful research and development by pharmaceutical companies.

Regarding voluntary reports to drug manufacturers, neither the patient as an individual, nor the patients' association as an organization took any initiative (B). Some of the participants felt that it would be nice to have measures whereby the patients themselves could report poor usability (C). During the FGI, some of the participants mentioned that they had come to realize that it was desirable to exchange opinions about inadequacies in inhaler usability either as an individual or through the patients' association as an organization (C).

3.3. *Results of the follow-up questionnaire*

Responses were obtained from all 15 participants. The validity of the analysis was supported by the results of the follow-up.

Among the participants, five reported that their thoughts and behavior had changed after participating in the FGI. Regarding specific changes, participants mentioned the following points.

"It was important for the patients to engage more actively in information exchanges regarding opinions and questions."

"I noted that it was acceptable to disseminate information to pharmacists and pharmaceutical companies, and things such as inferior usability could likely be improved by reporting them as a summary of patients' association members' opinions. Previously, we used to rely only on the attending physician, bulletin, and members of the patients' association."

"When I told the pharmacist that I marked the direction of rotation of the inhaler with a color marker on the clip, she gave me some stickers to place on the clips. I also mentioned about the ambiguity in display of the remaining number of doses. This was significant for me because, based on the experience of the interview, I was able to take a different action."

4. Discussion

The participants in the study placed tremendous reliance on the advising physician of the patients' association, and in some situations, even preferred the advising physician to their own attending physician for consultation. Sharing the latest information and dealing with the patient with a caring attitude were mentioned as factors promoting dissemination of information to the advising physician, in accordance with patients' perceptions of desirable traits in a physician (*13*).

For participants who belonged to a patients' association, the members of the patients' association were regarded as peers with the same disease, whom they could safely consult about doubts and anxieties, and exchange information based on their own experience of the disease or knowledge gained in patients' association meetings. These are well known characteristics common to patients' associations and SHGs (*14*).

While many of the participants chose to consult the attending physician when they had doubts or anxiety after experiencing adverse reactions, distrust and hesitation about the attending physician were barriers to information dissemination for some of the patients. Since dissatisfaction with the information and treatment provided by the physician and the attitude of the physician may lead to distrust (*15*), this is likely to be a barrier to communication between the patient and the physician. Moreover, the observations that patients tend to refrain from expressing their opinion when the physician is very busy or because they do not want to be seen as troublesome patients are in agreement with the findings of previous studies (*16,17*). With respect to pharmacists, the general perceptions of an insufficient volume of information sharing and lack of expertise about asthma acted as barriers to communicating their opinion.

Most of participants in the FGIs actively disseminated information when they had doubts and anxieties after experiencing adverse reactions. However, they did not actively discuss with medical staff any anxieties regarding adverse reactions in the future resulting from prolonged use of inhaled steroids. With respect to poor usability, while information was shared among the members of the association, there was neither any active dissemination of information to medical staff nor any engagement with pharmaceutical companies. In the first place, the participants did not even perceive that it was acceptable to disseminate such information to medical staff or pharmaceutical companies. The factors influencing such behavior were the perception of efficacy being more important, and the mindset that "it was useless to inform someone." However, as a result of participating in the FGI, some of the participants realized that "it was acceptable to actively disseminate

information about usability either as an individual or *via* the patients' association as an organization," and some of them actually practiced it after the FGI.

Although medical staff should ideally capture patients' needs in the field and transmit them to the pharmaceutical companies, in practice that may be difficult to implement considering circumstances such as the busyness of the medical staff and the fact that communication between patients and medical staff is not always smooth. In view of this, a more useful approach may be to implement measures through which the patient's needs can be directly obtained from the patients. Some of the participants evaluated the opportunity to express their opinion in an FGI as highly useful.

Limitations of the study are as follows: This study was conducted with participants who were members of asthma patients' associations. The information dissemination behavior or mindset of asthma patients who belong to different types of patients' associations, or who do not belong to any patients' association, may be different.

With respect to doubts and anxieties after experiencing adverse reactions, the main barriers to dissemination of information by participants were distrust and hesitation about communicating with medical staff. Distrust was reported to stem from insufficient information availability from medical staff and lack of good communication capability. In order to resolve these issues, further education of medical staff is required. Previous studies have indicated that even for questions about the validity of the treatment proposed by the physician, which are difficult for a patient to ask, appropriate words from the physician can encourage patients to ask questions (*18,19*), and appropriate education of physicians led to better communication between patients and physicians, as well as an improvement of patients' condition (*20*). According to a structured review, communication between patients and medical staff regarding drugs, guiding patients, and encouraging them to ask questions to pharmacists can be effective measures to encourage patients to express their opinion (*15*). Moreover, according to the previous survey (*21*), medication compliance improved for patients who read educational materials on communication with the physician during checkups, as compared to that of patients who did not read such materials. This suggests that, in addition to measures for medical staff, simultaneously educating patients is likely to be a useful strategy. With respect to the medical staff being very busy, which is one reason for the hesitation of patients to communicate with physicians or nurses, there may be no easy solution, but one possibility would be to provide patients with opportunities to disseminate information to the attending physician and the primary care pharmacist by utilizing settings such as patients' associations, which are different from usual checkups or guidance about medication in hospitals. In fact, some hospitals have set up patients' associations within the hospitals; these hold monthly meetings in which the physicians also take part to facilitate communication with patients.

In conclusion, behavioral differences in how bronchial asthma patients transmit experience about the adverse reactions and usability of inhaled corticosteroids were obvious. Information about the patients' needs, including opinions and questions about the usability of steroids inhalers, was shared only with members within the association and not disseminated outside, with some participants even choosing to keep it personal. Additionally, many patients believed that it was not acceptable to disseminate such information to the outside. Underlying this behavior was a mindset of perceiving efficacy and adverse reactions to be more important than usability, and thinking that "it is useless to inform someone."

The present qualitative study suggests that measures should be implemented to make patients aware that transmitting their needs, experience and comments about drugs is beneficial. In addition, new platforms are needed within which the patients themselves can freely transmit their opinions about improving drugs and delivery devices. Above all, such implementations would also improve awareness that patients are subjectively involved in their pharmacotherapy.

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Original Article

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Constipation in the elderly in a Japanese long-term medical facility: An ultrasonographic investigation

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Summary **This study aims to assess the fecal retention in elderly patients using colonic ultrasonography (US) in Japanese long-term care facility and determine the correlation between nutrition management methods and the fecal retention by US. This cross-sectional, single-center study was conducted in a long-term care facility in Japan. Patients with chronic constipation fulfilled the Rome III criteria for the diagnosis of functional constipation. US was performed on constipation patients with 4-day fecal retention before starting the standard management of constipation. After patients had defecated, nurses checked the outside of feces using King's Stool Chart and Bristol Stool Chart. All of 32 patients underwent the management of suppository laxative, the daily life independence level in grade C. In all cases, the King's Stool Chart did not detect > 200 g of fecal matter; the Bristol Stool Chart revealed type 5-7 in 56.2% of patients. The total parenteral nutrition and tube feeding did not completely detect type 1-2 in 0%. While the fecal retention groups comprised 15.6%, the non-fecal retention groups comprised 84.4%. The total parenteral nutrition did not completely detect the fecal retention in 0%. In the non-fecal retention groups, the King's Stool Chart indicated < 100 g in 81.8%, and the Bristol Stool Chart indicated type 5-7 in 100%. In conclusion, fecal properties of elderly constipation patients with the long-term parenteral nutrition should be assessed in follow-up examination by US, which is possible for personalized medical care by US, to avoid the administered regular management of constipation.**

Keywords: Elderly patient, management of constipation, nutrition, ultrasonography

1. Introduction

Chronic constipation is a frequent problem of elderly people, with its symptoms reported in up to 50% of patients in long-term care (LTC) facilities (*1-3*). However, no consensus exists on the definition of constipation regarding what the elderly perceive as constipation and what physicians traditionally consider constipation (*4*). As Japan has become a super-aging society with an increasing number of older adults with cerebrovascular diseases and dementia, it could be considered that the number of older adults who could not complain of subjective symptoms or who have difficulty with communication is increasing.

Chronic constipation is attributed to various factors and can result in complications, such as impaction, even perforation and death, when left untreated or not adequately treated (*5-8*). Hence, the prevention of

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chronic constipation by the nursing staff is imperative, and when it does occur, efforts should be focused on initiating an appropriate treatment to manage the condition. Several methods can prevent and manage constipation, including changes in diet and lifestyle, as well as drug therapy options. However, Japan witnesses the highest prevalence of inappropriate medication in patients with chronic constipation (*9*) because it tends to provide excessive management of constipation to prevent fecal impaction (*10*).

Hence, the precise assessment of the fecal retention in the colon and rectum is crucial. Although typically recommended diagnostic tests for constipation include plain abdominal radiography, barium enema, colonoscopy, defecography, abdominal computed tomography, and magnetic resonance imaging (*11-13*), these procedures might provide inadequate information. Moreover, various procedures (*e.g.*, plain abdominal radiography, barium enemas, defecography, and computed tomography) are unsuitable for follow-up testing because of inherent problems related to radiation exposure. For instance, while barium enema and defecography require the use of contrast medium, colonoscopy is often poorly tolerated by patients, and magnetic resonance imaging and defecography are expensive and lack standardization.

In contrast, transabdominal sonography has been extensively applied in the clinical practice because of its low cost, safety, speed, and nonionizing radiation (*14,15*). Recently, several studies have reported using a pelvic sonography technique to diagnose constipation by measuring the rectal diameter in children; sonography images reveal a fecal mass in the rectum as a crescent-shaped acoustic shadow (*16-18*). Previously, several studies have proposed the use of colorectal ultrasonography (US) as the first-line clinical imaging and initial diagnostic technique in the colon (*19,20*). Furthermore, US can be used concomitantly to assess the fecal retention in adults along with a physical follow-up examination to assess constipation (*21,22*).

However, little information is available on the sonographic visualization of constipation among Japanese elderly patients in an LTC facility because the elderly and adults have different meal form, gastrointestinal function and rectal sensitivity (*23*). Hence, this study aims to assess the fecal retention in elderly patients by US and determine the correlation between nutrition management methods as meal form and fecal retention by the colonic US.

2. Patients and Methods

2.1. *Patients*

This cross-sectional, noninterventional, single-center study was conducted in a Japanese LTC medical facility (Sengi Hospital, Ishikawa, Japan) between March and April 2016. We enrolled patients with chronic constipation if they aged, at least, 65 years and fulfilled the Rome III criteria for the diagnosis of functional constipation. In contrast, we excluded patients with a history of abdominal surgery, Irritable bowel syndrome, organic disease, and colon gas because of the difficulty in viewing the inside of the colon because of the gas. This study protocol was approved by the Research Ethics Committee of the Graduate School of Medicine and Faculty of Medicine at the University of Tokyo (Tokyo, Japan; approval number, 10789). In addition, we obtained written informed consent from all patients or their families. Notably, all participants were free to retract their consent at any time and were encouraged to report any pain or discomfort during the colonic US examination.

2.2. *Ultrasound technique*

We first assessed patients with constipation by US imaging before starting the standard management of constipation without the 4-day defecation. Soon after, nurses administered regular management of constipation (*e.g.*, laxative, enema, and stool extraction) every day to patients until defecation. After patients defecated, nurses checked the outside of feces using the King's Stool Chart and Bristol Stool Chart. We scanned the colorectum of all patients using our systematic scanning method (*20,21*), and the resulting images were performed at the center of the ascending colon, transverse colon, descending colon, rectum, and up to the portion just beyond the left iliopsoas muscle of the sigmoid colon to easily identify all cases by transverse and longitudinal sonographic scans (Figure 1). The sonographic examinations lasted for approximately 10 min, which were performed by a certified sonographer with 30 years of experience. We used the US system (Noblus; Hitachi Aloka Medical Ltd., Tokyo, Japan) with a curved-array (5 MHz) probe.

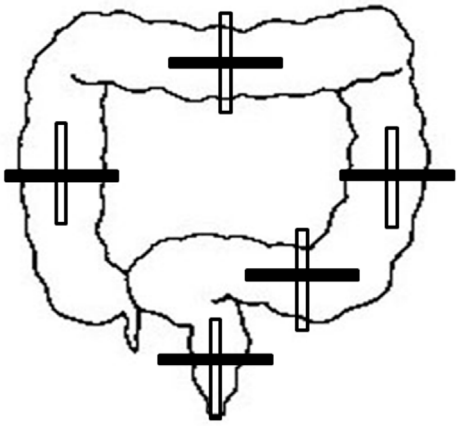

Figure 1. Sonographic scans were performed at the center of the ascending colon, transverse colon, descending colon, rectum, and up to the portion just beyond the left psoas muscle of the sigmoid colon by transverse (closed bar) and longitudinal (open bar) sonographic scans.

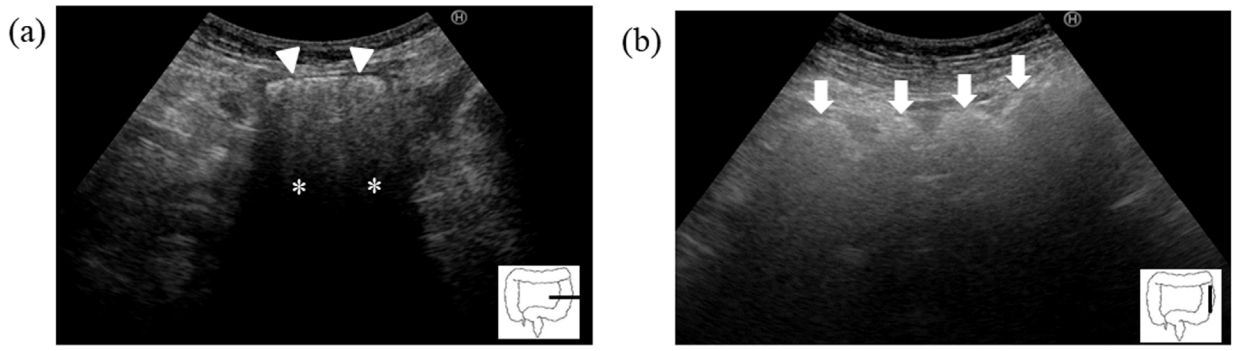

Figure 2. The presence of the fecal retention. US images of an 88-year-old female who intakes oral food. (a) A transverse scan showing a high echogenicity by the brightness of the colon wall (arrowheads) and acoustic shadow behind the descending colon (asterisks). **(b)** A longitudinal scan showing a clear crescent-shaped high echogenicity by the brightness of the colon wall with haustrations (arrows).

For US imaging, we used the focal range of 4 cm and the image depth of 6-8 cm. Furthermore, we used echo gain and dynamic range to determine the appropriate range to display.

2.3. *Data analysis*

We defined the US images before management of constipation as the fecal retention (fecal retention/non-fecal retention). The fecal retention groups suggested high echogenicity by the brightness of the colon wall with posterior echoes (acoustic shadows) by a transverse scan and visualized haustrations by a longitudinal scan (*22*); these findings were detected in any of the five sites (*i.e*., ascending, transverse, descending, sigmoid colon, and rectum; Figure 2). All US images were classified visually as the fecal retention or non-fecal retention, and two independent certified sonographers reviewed the US images to ensure the inter-rater reliability. Furthermore, an expert sonographer assessed the data derived from the US images. Of note, all images were evaluated under blinded conditions. We assessed the correlation between the visual evaluation (fecal retention/non-fecal retention) using Cohen's kappa statistic to reach a consensus between the two certified sonographers. In this study, the correlations between the fecal retention and other variables, nutrition management method [total parenteral nutrition (TPN), gastric fistula tube, tube feeding (TF), oral foods – also includes the use of TPN or gastric fistula tube in combination], amount of the King's Stool Chart and quality of the Bristol Stool Chart and bowel movement frequency were analyzed using the Fisher's exact test. We set the statistical significance level at < 0.05. Statistical analyses were conducted using IBM SPSS Statistics 21.0 for Microsoft Windows (IBM Corp., Armonk, NY).

3. Results

Table 1 summarizes patients' characteristics. Of 41 eligible patients, 8 patients were excluded because they did not defecate while nurses were providing defecation care, and 1 patient was excluded because of insufficient image quality. Thus, the final analysis comprised 32 patients (8 males and 24 females; mean age, 87.2 years; range, 74-106 years). All patients were receiving the management of constipation with laxative suppositories (bisacodyl), the daily life independence level in grade C. Table 2 presents the correlation between nutrition management methods and bowel movements. In all patients, the King's Stool Chart did not detect > 200 g of fecal matter, and the Bristol Stool Chart revealed type 5-7 (diarrhea) in 56.2% (18 of 32 patients). In addition, TPN and TF did not completely detect type 1-2 (constipation) in 0% (0 of 17 patients); TPN tended to delay the defecation. We observed a significant difference between the nutrition management method and the Bristol Stool Chart ($p = 0.01$). Table 3 summarizes US findings of the fecal retention (fecal retention/non-fecal retention). While the fecal retention groups comprised 15.6% (5 of 32) of patients, the non-fecal retention groups comprised 84.4% (27 of 32) of patients. In addition, nutrition management methods of the fecal retention groups, TPN, did not completely detect the fecal retention in 0% (0 of 15) of patients. For the non-fecal retention groups, the following three

Table 1. Participants' characteristics ($N = 32$)

Items	Mean ± SD, Min–Max or *n* (%)
Age (years)	87.7 ± 8.1, 74 – 106
Women	24 (75)
The level of independence	
Grade C (bed ridden)	32 (100)
Main disease	
Cerebrovascular disease	11 (34.4)
Fracture femoris	5 (15.6)
Lung disease	5 (15.6)
Diabetes	4 (12.5)
Cancer	2 (6.3)
Parkinson's disease	1 (3.1)
Others	4 (12.5)
Defecation care	
Suppository Laxative (Bisacodyl)	32 (100)

Table 2. The correlation between nutrition management methods and bowel movements ($N = 32$)

Items	King's Stool Chart				Bristol Stool Chart				Bowel movement frequency (day)					
	< 100 g $n = 22$	100 – < 200 g $n = 10$	> 200 g $n = 0$	$P^{1)}$	Type 1–2 (Constipation) $n = 5$	Type 3–4 (Easier to pass) $n = 9$	Type 5–7 (Diarrhea) $n = 18$	$P^{1)}$	4	5	6	7	8	$P^{1)}$
Total parenteral nutrition ($n = 15$)	13 (86.7)	2 (13.3)	0	0.111	0	3 (20.0)	12 (80.0)	0.01*	4 (26.7)	0	1 (6.7)	7 (46.7)	3 (20.0)	0.069
Gastric fistula tube ($n = 6$)	4 (66.7)	2 (33.3)	0		1 (16.7)	3 (50.0)	2 (33.3)		5 (83.3)	0	1 (16.7)	0	0	
Tube Feeding ($n = 2$)	0	2 (100.0)	0		0	1 (50.0)	1 (50.0)		2 (100.0)	0	0	0	0	
Oral foods ($n = 9$)	5 (55.6)	4 (44.4)	0		4 (44.5)	2 (22.2)	3 (33.3)		7 (77.8)	2 (22.2)	0	0	0	

Data were shown as n (%). [1]Fisher's exact test, $^*P < 0.05$.

results were obtained: the King's Stool Chart indicated under < 100 g in 81.8% (18 of 22) patients; the Bristol Stool Chart indicated type 5-7 (diarrhea) in 100% patients; and bowel movement frequency was 5-8 days in 100% of patients.

We observed a significant difference between the nutrition management method and the Bristol Stool Chart ($p = 0.01$). A significant difference was also observed in nutrition management methods between the fecal retention and non-fecal retention groups ($p = 0.041$). In addition, we observed a significant difference in the Bristol Stool Chart between the fecal retention and non-fecal retention groups ($p = 0.002$). Based on the Kendall W test, the results obtained by two independent sonographers (A and B) at 0.83 and 0.84, respectively, exhibited a significant correlation with each other.

4. Discussion

This study investigated elderly patients with constipation by colonic US imaging in LTC facilities. Consequently, US images categorized constipation as fecal retention and non-fecal retention. In the fecal retention groups, the fecal retention was < 15.6% (5 of 32 patients) before management of constipation. Several factors are accountable for the fecal retention in the colon in elderly patients with constipation.

Perhaps, the low detection rate in this study could be attributed to the nutrition management methods. Reportedly, parenteral nutrition of this condition is widely used in hospitalized patients, especially elderly, who are unable to eat to aid in patients' ability to recover from illness (*24*). The most common adverse effect of such treatment is diarrhea, which is reported in 68% of intensive care unit patients (*25*) and 96% of patients with dysphagia (*26*). In this study, we compared nutrition management methods with US finding in elderly patients with constipation. All TPN were not completely detected in the fecal retention groups of US finding; even the oral foods groups did not detect fecal retention in 66.4% of patients by US. Perhaps, the dietary intake of elderly patients was not sufficient for the fecal retention for 4 days.

Another probable reason for the low detection rate in this study could be that nurses only checked the outside of feces using the King's Stool Chart and Bristol Stool Chart. While the King's Stool Chart tends to detect < 200 g of defecated feces, the Bristol Stool Chart tends to report diarrhea in the non-fecal retention groups. This study reports a markedly high prevalence and use of medications to manage elderly constipation in LTC settings (*27*). The most frequently used solution to prevent or treat constipation was laxative medications. Despite considering laxatives as the solution, it was impossible to manage the balance between constipation and diarrhea because diarrhea is the common side

Table 3. The correlation between clinical features and fecal retention by evaluation on ultrasonography

Items	Fecal retention (n = 5), n (%)	Non-fecal retention (n = 27), n (%)	P[1)]
Nutrition management methods			0.041*
Total parenteral nutrition	0 (0)	15 (100)	
Gastric fistula tube	1 (16.7)	5 (83.3)	
Tube Feeding	1 (50.0)	1 (50.0)	
Oral foods	3(33.3)	6 (66.4)	
King's Stool Chart			1.000
< 100 g	4 (18.2)	18 (81.8)	
100-200 g	1 (10.0)	9 (90.0)	
> 200 g	0 (0)	0 (0)	
Bristol Stool Chart			0.002*
Type 1-2 (Constipation)	3 (60.0)	2 (40.0)	
Type 3-4 (Easier to pass)	2 (22.2)	7 (77.8)	
Type 5-7 (Diarrhea)	0 (0)	18 (100.0)	
Bowel movement frequency (day)			0.517
4	5 (27.8)	13 (72.2)	
5	0 (0)	2 (100)	
6	0 (0)	2 (100)	
7	0 (0)	7 (100)	
8	0 (0)	3 (100)	

[1)]Fisher's exact test. *P < 0.05.

effects of laxative use, and some patients do respond to laxative treatment with diarrhea (*28,29*). In addition, the repeated use of laxative medications might weaken the efficacy of laxatives and result in anorectal burning. In particular, the first-line treatments for constipation in bedridden patients should be not laxative overdoses and need a long-term administration to keep the normal bowel movement (*30*). Perhaps, intractable diarrhea might be the major indication for TPN.

In this study, nurses administered regular management of constipation (laxative suppository) in patients without the 4-day defecation in an LTC facility because the healthcare provider attempt to avoid severe chronic constipation in complications like fecal impaction or idiopathic perforation of the colon. Thus, it is a tendency for the uniformity and excessive management of constipation. This study suggests that a follow-up examination by the colonic US can locate the fecal retention and assess it in the colon, supposedly proposing the most optimum management of constipation, which facilitates in selecting laxatives or enemas to treat constipation.

This study has some apparent limitations. First, constipation could not be assessed adequately on US images alone. No single test can adequately assess the pathophysiology of constipation because of the physiological phenomenon of the fecal retention in the colon. Hence, these patients must undergo a comprehensive diagnostic evaluation based on their clinical condition and other examination findings. Second, an additional consideration is the dependence of the efficacy of US on operators' skill and technique.

In conclusion, this study demonstrates that US could evaluate the risk factors associated with elderly constipation in LTC facilities. The fecal properties of elderly patients with constipation and parenteral nutrition should be assessed and followed up by colonic US, personalized medical care by US (if possible), avoiding the administered regular management of constipation.

Acknowledgements

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Conflict of Interest

This study was a joint research program with FUJIFILM Corporation and was conducted under the sponsorship of this organization.

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Original Article

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Whole body vibration exercise in the management of cancer therapy-related morbidities: A systematic review

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Summary **The purpose of this systematic review was to investigate the effects of whole body vibration (WBV) exercise in the management of cancer therapy-related morbidities. The PubMED and PEDro databases were used to access publications published in English about the use of whole body vibration (WBV) exercises in cancer patients until February 22nd 2017. The studies included were classified according to the level of evidence (LE) by the National Health and Medical Research Council Hierarchy of evidence and the methodological quality (MQ) by the PEDro scale. The four included studies (2 of them with "high" LE-II and MQ) were performed in patients with different types of cancer (*i.e.* breast, lung, prostate, solid or hematological), treated with WBV exercise to counteract the cancer therapy-related morbidities. The variables evaluated were muscle activity, subjective rate of perceived exertion, exercise capacity, muscle strength, quality of life, resting urinary incontinence and severity of peripheral neuropathy. Although WBV exercise appears to be a potential treatment procedure of cancer therapy-related morbidities, further additional studies are required to determine specific and tailored protocols to be used in the different stages of the disease.**

Keywords: Whole body vibration, cancer, oncology, rehabilitation, exercise

1. Introduction

Cancer is a group of diseases that causes the growth of abnormal cells, which exceed their usual limits and can invade adjacent parts of the body and/or spread to other organs (*1*). Although it actually represents one of the main causes of morbidity and mortality worldwide, with approximately 14 million new cases in 2012, the amount of cancer survivors is progressively increasing due to advances in early detection and treatment (*2,3*).

Cancer development and its treatment are usually associated with fatigue (*4*) pain (*5*), anxiety or depression (*6*), and sleep disturbances (*7*) which determine a decrease of physical and psychological functions and negatively affect the patients' quality of life (QoL) (*8*).

One way to promote health, to diminish the

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physiologic and psychological effects of cancer and its treatment, to achieve the maximum possible physical, social and vocational well-being is to acquire or maintain a healthy lifestyle, entailing physical activity as a component of cancer rehabilitation (*9-11*). Physical activity is any movement of the body that involves the muscles action with energy expenditure above rest and the exercise is this physical activity with planning (*12*). According to the American Cancer Society, cancer survivors should exercise at least 150 minutes per week, including strength training exercises at least 2 days per week (*2*). The exercise can be beneficial to counteract cancer-related fatigue (*10,13-15*), improve overall quality of life, as well as relief of symptoms and side effects (*10*) of the disease and/or of the treatment. In this respect, recent studies have shown the positive effect of physical activity on breast (*16,17*), prostate (*18*), ovarian (*19*) and lung cancer (*20*).

Among the different exercise modalities, whole body vibration (WBV) exercise (*21*) has gained progressive popularity, being safe and well accepted by the patients. WBV exercises are generated by mechanical vibrations produced in oscillating/vibratory platforms (OVP) and can be transmitted to the body of the individual when is in contact with the OVP (*22-24*).

Several authors have already demonstrated the effects of WBV exercise with different protocols in improving muscle strength (*25,26*), bone formation (*25,27*), balance (*28*), flexibility (*29,30*), function ability (*26,27*), relief of pain (*31*) and fatigue (*32*). Moreover, investigations have shown the WBV exercise benefits in the rehabilitation programs of patients with chronic diseases, such as metabolic syndrome (*24*), chronic obstructive pulmonary disease (*33-35*), fibromyalgia syndrome (*36*), multiple sclerosis (*32,37*), rheumatoid arthritis (*27*) and cancer therapy-related morbidities (*39*).

Due to the importance of the exercise in several clinical conditions, the aim of this systematic review was to investigate the effects of WBV exercise in the management of morbidities related to the cancer therapy. This is the first systematic review to assess whether WBV exercises are safe and beneficial in the management of morbidities for cancer patients.

2. Materials and Methods

2.1. *Search strategy used to find the publications*

Two databases were accessed in the *Universidade do Estado do Rio de Janeiro* on February 22nd 2017 and two searches were performed. The keywords "whole body vibration" and "cancer", and "whole body vibration" and "oncology" were searched in the PubMed and PEDro databases for three reviewers independently. This systematic review adheres to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) Statement (*39*).

2.2. *Criteria to select the publications*

All the publications were screened following inclusion and exclusion criteria.

Inclusion criteria: In the search, all the publications found in the databases (PubMed and PEDro) were preliminarily considered to be included in this current review. To be included in this review, all studies had to investigate effects of WBV on cancer patients. A flowchart (Figure 1), based in the PRISMA analysis, was done to show the steps in the selection of the full papers analyzed in this review (*39*).

Exclusion criteria: Exclusion criteria allowed the elimination of unnecessary publications. Papers were excluded if they were: (*i*) published in a language other than English; and (*ii*) findings not related to cancer.

2.3. *Levels of evidence (LE) of the selected papers*

The National Health and Medical Research Council Hierarchy of evidence (NHMRC, 2003-2007) (*40*) were used to classify the included studies in this systematic review (Figure 2). Each article was assigned to one reviewer and cross-checked by a second reviewer and where there was disagreement a third party was consulted and the issue discussed until consensus was reached. Moreover, the methodological quality of these studies was determined by the PEDRo scale (*41*). In the PEDRo scale, each publication was evaluated according to: (a) eligibility criteria, (b) subjects were randomly allocated to groups, (c) concealed allocation, (d) the groups with baseline similarity, (e) blinding of the patients, (f) blinding of the therapists, (g) blinding of all assessors, (h) measures obtained from more than 85% of the subjects, (i) all subjects received the treatment or control condition or, at least one key outcome was analyzed by "intention to treat", (j) results of the groups with statistical comparisons and (h) point measures and measures of variability of outcome. Those publications with a score of seven or greater in the PEDro scale were considered of 'high' methodological quality, those with a score of five to six would be of 'fair' quality and a score of four or below were classified as 'poor' quality (*42*).

3. Results

The steps to select the full papers analyzed in this systematic review are shown in the flowchart (Figure 1). Of the twelve papers firstly screened, only four have reached the inclusion criteria.

The characteristics of the participants, the protocols used, the aims of the studies, the tools for evaluation and the outcomes of the selected articles and the level of evidence of the selected papers and the methodological quality are shown in the Table 1.

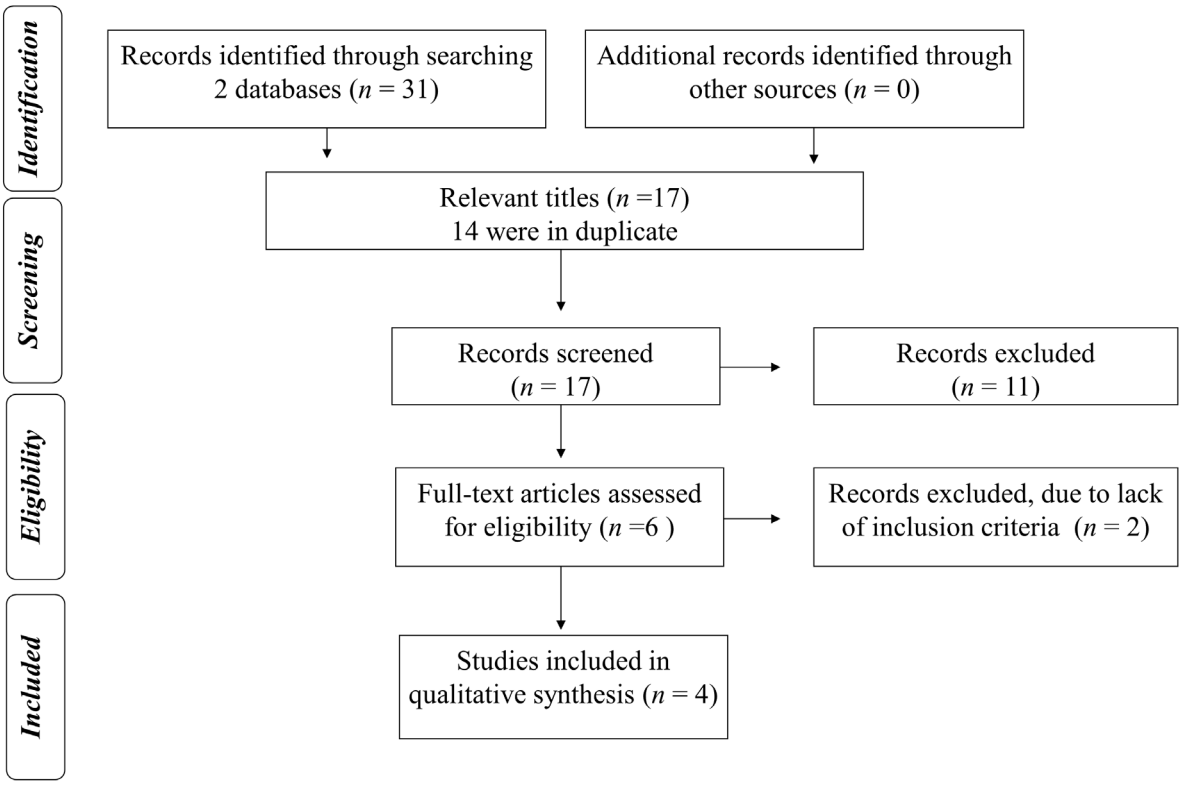
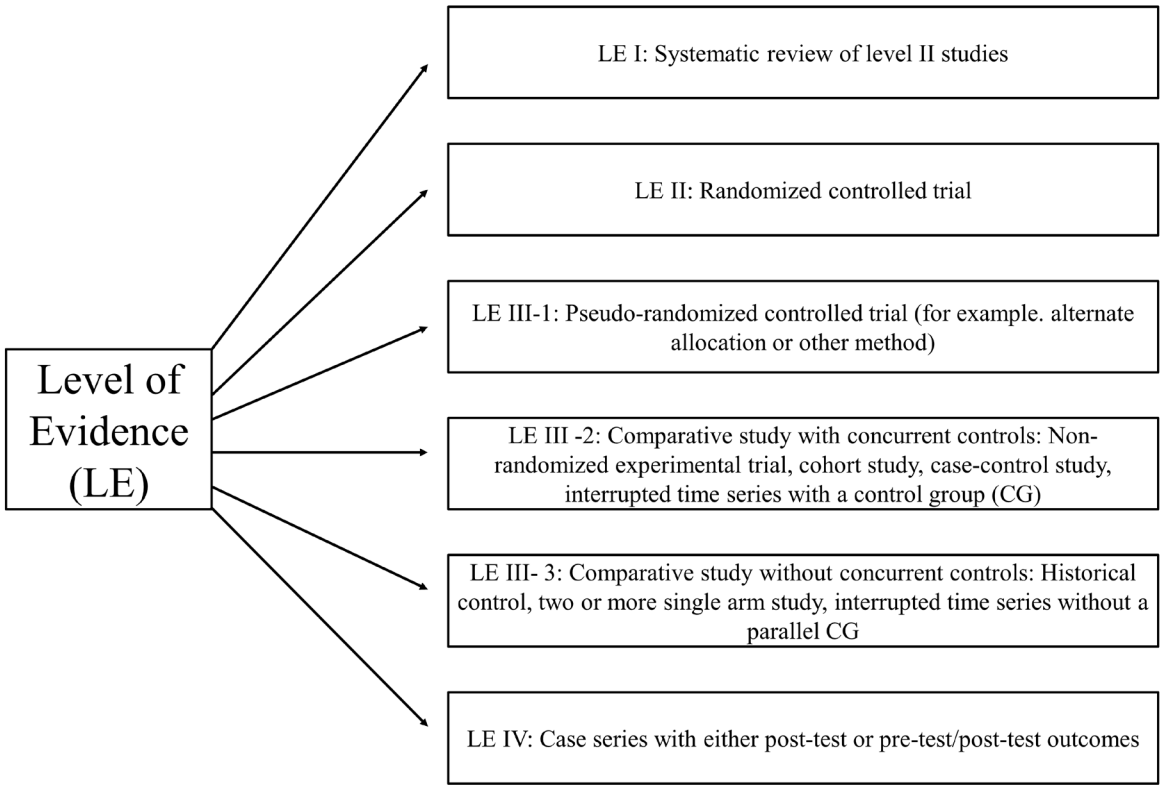


Figure 1. Flowchart indicating the steps to select the full papers analyzed in this review. A flowchart based in the PRISMA analysis, was done to show the steps in the selection of the full papers analyzed in this review.



CG – control group, LE – level of evidence

* Adapted from National Health and Medical Research Council (NHMRC), Q5 2003-2007.

Figure 2. Designation of levels of evidence according to the intervention research question. The National Health and Medical Research Council Hierarchy of evidence (NHMRC, 2003-2007) were used to classify the included studies in this systematic review.

Table 1. Characteristics of the participants, protocols used, aims of the studies, tools for evaluation, outcomes of the selected articles, level of evidence of the selected papers and methodological quality

Study	Type of cancer/ Treatment performed/ number of individuals	Morbidities of the treatment	WBV Intervention	Aim	Tools for evaluation	Outcome (s)	Level of evidence/ methodological quality
Van Ruymbeke *et al*., 2014	Breast cancer survivors/ all type of treatment. Breast cancer survivors (n = 20) and Control group-healthy woman (n = 20)	No comorbidity was reported	Two groups were undergoing vibration, the women were standing on a synchronous vibrating platform with a knee joint angle of 55°. Each condition: nonvibration condition (0Hz) and vibration condition at 20-30-40-50Hz vibration frequencies (amplitude, 4mm) lasting for 30 s with 2-min rest between conditions.	To analyze muscle activity and subjectively rate of perceived exertion	Surface EMG analysis of the RF, VM, VL, TA and GT muscles. The level of subjectively perceived exertion was rating on a combination of a VAS and revised Borg.	The muscle activation did not differ between breast cancer survivors and healthy controls. There was a significant frequency × muscle interaction effect. For the VAS scores no significant group × frequency interaction was found and no significant main effect for the factor group, in contrast the factor frequency was significant. The values of perceived exertion in both groups increased with increasing frequency.	III-1/ Fair
Salhi *et al*., 2015	Stages I-III lung cancer or mesothelioma/radical resection with or without a chemotherapy or radiotherapy CON group (n = 21), CRT group (n = 20), WBV group (n = 17)	Post-treatment QF was either equal or less than 70% of the predicted normal value or showed a decrease of at least 10% from the baseline value	CON group - patients were discouraged to improve their exercise tolerance. CRT group - 20 min of aerobic training on the bicycle and treadmill at 70% of the respective Wmax and speed plus resistance training on multigym equipment starting with three sets of eight repetitions for each exercise at 50% 1RM. WBV group - 20 min of aerobic training on the bicycle and treadmill at 70% of the respective Wmax and speed plus performed exercises on the synchronous vibration platform, three sets of 30 s for each exercise at 27 Hz. Patients trained three times a week for 12 weeks.	To assess the potential beneficial effect of rehabilitation	Exercise capacity with 6MWD and Wmax, muscle strength by the QF with isometric handheld dynamometer, and QoL with EORTC QLQ-C30	6MWD not increased in CON and in WBV group, and increased in CRT group. Wmax was significantly increased in both CRT and WBV group. QF significantly increased only in CRT group. For none of the groups, the score of EORTC QLQ-C30 Global changed significantly.	II/ High

Electromyography = EMG; Rectus Femoris = RF; Vastus medial = VM, Biceps femoris BF, Tibialis anterior = TA; Gastrocnemius = GT; Visual analogue scale = VAS; Control = CON; Conventional resistance training = CRT; Whole Body Vibration = WBV; Quadriceps force = QF; One-repetition-maximum = 1RM; Maximal workload (Wmax); 6-min walking distance = 6MWD; Quality of life = QoL; European Organisation for Research and Treatment of Cancer Quality of Life Cancer Questionnaire = EORTC QLQ-C30; Chemotherapy-induced polyneuropathy = CIPN; Functional Assessment of Cancer Therapy/ Gynecologic Oncology Group neurotoxicity subscale = FACT/GOG-NTX; Warm detection threshold = WDT.

Table 1. Characteristics of the participants, protocols used, aims of the studies, tools for evaluation, outcomes of the selected articles, level of evidence of the selected papers and methodological quality (continuted)

Study	Type of cancer/ Treatment performed/ number of individuals	Morbidities of the treatment	WBV Intervention	Aim	Tools for evaluation	Outcome (s)	Level of evidence/ methodological quality
Crevenna *et al*., 2016	Prostate cancer/radical prostatectomy/Case report	Urinary incontinence	WBV- 20-26 Hz, synchronous platform plus performing pelvic floor exercises, supine position on the device, 2 times a week for a period of 6 weeks	To treat the disabling and isolating symptom incontinence	Number pads/day	After intervention, the number of pads decreased from 5 pads/day to 1 safety pad/day.	IV/ Poor
Schönsteiner *et al*., 2017	Cancer patients- solid or hematological neoplasms /Chemotherapy/ Experimental group (n = 44), Standart group (n = 50)	CIPN grade II–III according to National Cancer Institute Common Toxicity Criteria	All patients received massage and passive mobilization in posture and transport layers for 30 min per side. Experimental group - training with WBV 9 Hz- 23Hz, alternate platform for 18 minutes alternating positions and frequencies. Standart group - Alternating training exercises with a focus on training of posture and transport movements were initiated including 21 separate exercises. patients were invited to practice the exercises at home on a daily basis and asked to document their efforts. In addition, all patients were motivated to walk as frequently and long as possible.	To evaluate the potential benefits of WBV to patients with CIPN	Severity of peripheral neuropathy was evaluated with chair-rising test, FACT/GOG-NTX, quality of life questionnaire (EORTC QLQ-C30), patellar tendon reflex and Achilles tendon reflex, quantitative evaluation of pallesthesia by using a Rydel-Seiffer tuning fork (C64), and the quantitative sensory testing followed a standardized protocol.	The time needed for the chair-rising test decreased significantly over time and that patients treated in experimental arm had a significantly higher reduction in the time needed for the chair-rising test compared to those treated in standard arm. FACT/ GOG-NTX categories "tingling" as well as "discomfort" in the feet were significantly EORTC QLQ C30 improved over time but again without differences between the study groups. The neurological reflex, Achilles and patellar tendon reflexes, the. difference did not reach statistical significance. Quantitative sensory testing before the intervention and after completion of the program revealed a significant reduction in the WDT in the experimental arm compared to the standard group.	II/ High

Electromyography = EMG; Rectus Femoris = RF; Vastus medial = VM, Biceps femoris BF, Tibialis anterior = TA; Gastrocnemius = GT; Visual analogue scale = VAS; Control = CON; Conventional resistance training = CRT; Whole Body Vibration = WBV; Quadriceps force = QF; One-repetition-maximum = 1RM; Maximal workload (Wmax); 6-min walking distance = 6MWD; Quality of life = QoL; European Organisation for Research and Treatment of Cancer Quality of Life Cancer Questionnaire = EORTC QLQ-C30; Chemotherapy-induced polyneuropathy = CIPN; Functional Assessment of Cancer Therapy/ Gynecologic Oncology Group neurotoxicity subscale = FACT/GOG-NTX; Warm detection threshold = WDT.

Table 2. Quality evaluation of the studies according to the PEdro scale

Author/year	Van Ruymbeke *et al*., 2014	Salhi *et al*., 2015	Crevenna *et al*., 2016	Schönsteiner *et al*., 2017
Eligibility criteria were specified*	No	Yes	No	Yes
Subjects were randomly allocated to groups	Yes	Yes	No	Yes
Allocation was concealed	No	Yes	No	Yes
The groups were similar at baseline regarding the most important prognostic indicators	Yes	Yes	No	Yes
There was blinding of all subjects	No	Yes	No	No
There was blinding of all therapists who administered the therapy	No	No	No	No
There was blinding of all assessors who measured at least one key outcome	No	No	No	Yes
Measures of at least one key outcome were obtained from more than 85 % of the subjects initially allocated to groups	Yes	Yes	Yes	Yes
All subjects for whom outcome measures were available received the treatment or control condition as allocated or data for at least one key outcome was analyzed by "intention to treat"	Yes	Yes	Yes	Yes
The results of between-group statistical comparisons are reported for at least one key outcome	Yes	Yes	No	Yes
The study provides both point measures and measures of variability for at least one key outcome	Yes	Yes	No	Yes
Total PEDro Scale	06	08	02	08

*It is not scored in the total score.

The level of evidence (NHMRC, 2003-2007) (*40*) of the two studies included in the current review (*38,43*) were considered LE II , one study (*44*) was considered LE III-1 and one study (*45*) was considered LE IV.

As far as the methodological quality evaluated following the PEDRo scale (Table 2) is concerned, two studies (*38,43*) were considered of "high" quality, one study (*44*) was considered of "fair" quality and one study (*45*) was considered of "poor" quality.

Individuals with different types of cancer, such as breast cancer (*44*) lung cancer or mesothelioma (*43*), prostate cancer (*45*) and solid or hematological neoplasms (*38*) were evaluated in these studies. The ages of the participants ranged from 18 up to 80 years old. Although different variables were investigated, the common aim of the all the selected papers was to evaluate the effects of the WBV exercise on cancer therapy-related morbidities.

Van Ruymbeke *et al*., 2014 (*44*) analyzed the muscle activity and subjectively rate of perceived exertion in breast cancer survivors and healthy women. The authors demonstrated that the muscle interaction effect and the values of perceived exertion significantly increased, being comparable in the two groups.

Salhi *et al*., 2015 (*43*) evaluated the effects of whole body vibration on exercise capacity, muscle strength and QoL in patients with stages I-III lung cancer or mesothelioma undergone to radical treatment. Although 6-min walking distance (6MWD), muscle strength and QoL did not significantly increase, maximal workload (Wmax) significantly increased ($p = 0.002$).

Crevenna *et al*., 2016 (*45*), have studied a patient suffering from severe urinary incontinence after radical prostatectomy due to prostate cancer. After whole body vibration therapy the patient regained continence, the urine loss almost stopped completely, the ability to work and to attend social and private activities increased.

Schönsteiner *et al*., 2017 (*38*) have evaluated the benefits of whole body vibration in chemotherapy-induced polyneuropathy. The authors reported a reduction of the time needed to complete the chair-rising test (CRT) in the individuals of the WBV group compared with individuals of a group with an intervention with other training exercises. In addition, a significant reduction in the quantitative sensory testing warm detection threshold (WDT) was found in the WBV group, and the categories "tingling" as well as "discomfort" in the feet of the program Functional Assessment of Cancer Therapy/ Gynecologic Oncology Group neurotoxicity subscale (FACT/GOG-NTX) were also significantly lower ($p < 0.001$, $p < 0.001$) in the WBV group. However, in the global status, functional, symptoms score and overall QoL, there was a significant improvement over time but no difference between the two groups.

The treated morbidities of the studies included on this review were functional exercise capacity reduction, fatigue, weakness, urinary incontinence and peripheral neuropathy. The WBV exercise frequencies ranged from 9 to 50 Hz, only one study was performed in one session, the others ranged from 12 to 36 sessions. The authors report no side effects during the interventions and among the studies that reported compliance, this ranged from 67 to 80%.

4. Discussion

The exercises have been used as one of the possibilities to rehabilitation for the morbidities caused for the treatment of the cancer survivors (*46,47*). This systematic review included articles in which WBV exercise was used to manage morbidities due to cancer treatment.

The WBV loads a mild cardiovascular exertion and its neural as well as muscular mechanisms may play a

role of fatigue (*48,49*). The frequency of mechanical vibration used to generate WBV exercise affects parts of the body through of which it is transmitted (*50*). The enhancement in muscle strength and power after vibration can be attributed to the increased muscle activity as a result of interaction of the mechanical vibration.

Authors have investigated the effects of frequency on the muscle activity (*51,52*). Herrero *et al*., 2011 (*51*) verified an increase of the electromyography (EMG) activity of the vastus lateral (VL) and vastus medial (VM) muscles in patients with spinal cord injury (SCI) exposed to WBV. Liao *et al*., 2016 (*52*) have also reported an increase of the vastus lateral and gastrocnemius (GS) muscle activity in patients with chronic stroke exposed to WBV. Similarly, to these findings, Van Ruymbeke *et al*., 2014 (*44*) showed a muscle interaction effect with frequency of the mechanical vibration with an increase of the muscle activation in individuals with breast cancer after therapy.

Salhi *et al*., 2015 (*43*) evaluated treated stages I-III lung cancer or mesothelioma performing aerobic training plus performed exercises on the synchronous vibration platform and found increase in maximal workload (Wmax) in these patients. In a study developed in the early phase after lung transplantation in which the patients remained in a static position on the platform and were exposed to WBV exercise, also revealed a significant improvement of the Wmax (*53*). It is relevant to consider that the Wmax might be a parameter to detect changes in aerobic endurance capacity (*54*) and it is suggested in this current revision that WBV exercise should be an important, safe and feasible intervention to improve aerobic capacity in individuals after cancer treatment.

The urinary incontinence is other morbidity that has been found after radical prostatectomy in prostate cancer patients (*55*). This dysfunction has noninvasive modalities considered first-line treatment during the first 6-12 months following prostatectomy and conservative modalities include pelvic floor muscle training (*56*). Pelvic muscle exercises are important to active retention strength of the striated muscles improving coordination of the contraction and relaxation process for better control and quality of muscle contraction (*57*). Crevenna *et al*., 2016 (*45*) showed a benefit of the additional use of high-intensity whole body vibration therapy in a patient suffering from severe post radical prostatectomy urinary incontinence. WBV exercise also showed the beneficial effects in patients without cancer as women with stress urinary incontinence (*58*).

The neuropathic disease is common in cancer survivors and may result from the infiltration of nerve tissue by the tumor, radiation treatment, chemotherapy, or cancer-related surgery, leading to symptoms such as pain and functional impairment (*59*). Schönsteiner *et al*., 2017 (*38*) have demonstrated the beneficial impact (symptoms relieve, physical fitness and sensory function) on chemotherapy-induced polyneuropathy (CIPN) of a program including massage, mobilization as well as physical exercises and WBV. Moreover, other studies have shown the positive effects (reduction of pain (*60*), enhanced muscles strength and balance (*61,62*) of WBV exercise on peripheral neuropathy in diabetic patients.

Several limitations of this review should be recognized. A relevant limitation is that we could not draw certain conclusions because there is still limited knowledge about WBV exercise on cancer patients. Moreover, it is difficult to prove the effects of whole body vibration on outcomes, because differences exist in multiple WBV parameters and morbidities among the studies. In addition, relevant studies published in other languages other than English may be missed; and there may be publication bias, due to the greater possibility of publication of studies with favorable intervention results.

Despite the limitations, it is important to consider that, in our knowledge, this is the first review about the use of suitable, non-invasive and simple procedure. Moreover, relevant findings are presented and demonstrated that the WBV exercise must be more understood and known to be used in the management of morbidities due to the cancer therapy.

In conclusion, the WBV exercise might be one modality of treatment to morbidities due cancer treatment for the benefits demonstrated, however other studies should be performed to determine the parameters and specific protocols that will be used to each morbidity.

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Original Article

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Criteria for the selection of switch OTC drugs based on patient benefits, efficacy, and safety [II]: Comparing the physicochemical and pharmaceutical properties of brand-name and switch OTC terbinafine hydrochloride cream

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Summary **The physicochemical properties (pH, yield value, and squeeze force) of a drug for dermatomycosis, a terbinafine hydrochloride-containing cream (a brand-name product), and 12 over-the-counter drugs (OTCs) were measured and compared to ascertain the characteristics of each product. The pH of the brand-name product, Lamisil, was 4.1, and that of the OTC products ranged from 4.2 to 7.6; Lamisil Plus (7.6) had a significantly higher pH. Moreover, the yield value for Lamisil, as an index of cream ductility, was 128 dyn/cm^2, and that for the OTC products ranged from 110 to 887 dyn/cm^2. In particular, the OTC products Damalin (887 dyn/cm^2), Barriact (512 dyn/cm^2), and Exiv Deep (663 dyn/cm^2) had a significantly higher yield value. In addition, the squeeze force was measured by attaching a HapLog® to the thumb and second finger. The squeeze force for Lamisil was 12.9 N, and that for the OTC products ranged from 1.8 to 14.6 N. The OTC product Bilumon (1.8 N) had a significantly lower squeeze force. These results indicated that there were marked differences in the pharmaceutical properties of brand-name and OTC products. External preparations are characterized by their feel during use. Based on the current results, the pharmaceutical characteristics of drugs resulted in differences in their feel during use, suggesting that products appropriate for individual patients can be recommended.**

Keywords: Cream, terbinafine hydrochloride, brand-name drug, OTC, HapLog®

1. Introduction

In Japan, the continued increase in national healthcare expenditures poses a serious social problem (*1*). One way to reduce medical expenditures is by promoting the use of over-the-counter (OTC) drugs in self-medication (*2*). Since January 1, 2017, if a person buys a "switch OTC" (an OTC drug switched from a prescription drug), the cost of the drug can be deducted from the person's income *via* an exemption for healthcare costs under the system for taxation of self-medication as part of an effort to maintain and promote health and disease prevention (*2*). This reflects the government's policy to promote self-medication and encourage a shift from drugs that require a prescription to OTC drugs that require no prescription ("Patients should be responsible for their own health and treat mild physical ailments themselves (*3*)") (*4*).

Pharmacists can contribute greatly to the promotion of self-medication by sufficiently understanding the equivalence of OTC drugs to brand-name drugs and their physicochemical characteristics and encouraging their use with appropriate evaluation. However, there are no clear criteria for the selection of OTC drugs, so drugs cannot be easily selected to suit individual patients.

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The current authors previously reported that various patient needs could be met by ascertaining the physicochemical and pharmaceutical properties of various dosage forms, most of which are external preparations that markedly differ in their feel during use: ointments, creams, lotions (*5-7*), ophthalmic liquids/solutions (*8*), nasal sprays (*9*), and tapes (*10,11*).

The aim of the current study was to compile information to help pharmacists select appropriate preparations according to patient needs by comparing and assessing pharmaceutical characteristics of a brand-name drug and OTC products. The brand-name and OTC drugs were creams containing terbinafine hydrochloride, which is a treatment for dermatomycosis.

2. Materials and Methods

2.1. *Materials*

Pharmaceutical creams containing terbinafine hydrochloride (1 brand-name "Lamisil® cream 1%" and 12 OTCs) were used in this study. The product name, abbreviated name, class, company name, lot number, and container of these products are listed in Table 1. In addition, OTC products were specifically those on the market (in drugstores, on the Internet, *etc*.) among all products registered with the Pharmaceutical and Medical Devices Agency (PMDA) (*12*).

2.2. *Measurement of pH*

The pH of each cream was measured using the HI 99161N pH meter (Hanna Instruments Japan Co. Ltd., Chiba, Japan) for dairy products and semi-solid foods. Measurement was performed 10 times at 24 ± 2°C, and the mean ± standard deviation (S.D.) was calculated.

2.3. *Measurement of ductility*

The spread diameter of each cream was measured using the 419 spreadmeter (Rigo Co., Ltd., Tokyo, Japan). Measurement was performed 8 times at room temperature (24 ± 2°C), and the mean value was calculated. The spread diameter was determined at 16 points between 5 and 1,800 seconds after the start of measurement. A semilog graph was prepared by plotting the spread diameter (cm) of each cream on the longitudinal axis and plotting the time (seconds) needed for the cream to spread on the transverse axis. The spread and viscosity of each cream were calculated from the slope and y-intercept (*13,14*). Moreover, the yield value was calculated using a previously reported formula (*15*).

2.4. *Measurement of squeeze force*

To assess the squeeze force with which a preparation was expelled from a container, a wearable tactile sensor (Haptic Skill Logger (HapLog®), Kato Tech Co., Ltd., Kyoto, Japan) was used to evaluate the sense of touch (*16,17*). Tactile sensors were attached to the right thumb and second finger, and the total force (thumb + second finger) required to squeeze 1 fingertip unit (FTU: volume of cream squeezed between the first joint and tip of the second finger) of cream while placing the center of the container between the thumb and second finger was regarded as the squeeze force (N). These sensors facilitate the simultaneous assessment of the finger contact force and sense of touch of the person wearing the sensor through the free sense of touch at the fingertip, but there are errors in the contact force due to individual differences in the method of attachment or size of the finger. This is why the values were corrected for each person. Measurement was performed 7 times per container of cream with the same lot number. In addition, measurement was performed for a total of 3 bottles if a product had different lot numbers. The mean of the respective means was then calculated to serve as the value for the product.

2.5. *Statistical analysis*

Values were compared using *Dunnett's* test (*18*). A *p*

Table 1. Creams used in this experiment

Product name	Abbreviated name	Class	Company	Lot number	Container
Lamisil® Cream 1%	Lamisil	Brand-name	Novartis Pharma K. K.	P0942	AT
Damalin Grande X	Damalin	OTC	Taisho Pharm. Co., Ltd.	024P1	AT
Barriact Hi Cream	Barriact	OTC	Zeria Pharm. Co., Ltd.	SZ01	AT
Salirabate EX Cream	Salirabate	OTC	Japan Medic Co., Ltd.	14013	AT
Mentholatum Exiv Cream E	Exiv	OTC	Rohto Pharm. Co., Ltd.	4E4	LT
Mentholatum Exiv Deep 10 Cream	Exiv Deep	OTC	Rohto Pharm. Co., Ltd.	4D3	PB
Terubine EX Cream	Terubine	OTC	Chugai Iyaku Seisan Co., Ltd.	4202	AT
Next Cream 24	Next	OTC	Shinsei Yakuhin Co., Ltd.	4T1	AT
Bilumon TF Cream EX	Bilumon	OTC	Shinshin Pharm. Co., Ltd.	E301	LT
Mailuzon TL Cream	Mailuzon	OTC	Fuji Pharma Co., Ltd.	30101	AT
Lamisil AT Cream	Lamisil AT	OTC	Novartis Pharma K. K.	P0121	AT
Lamisil Plus Cream	Lamisil Plus	OTC	Novartis Pharma K. K.	4B02	AT
Lamisil Cure Gel	Lamisil Gel	OTC	Novartis Pharma K. K.	4A01	AT

AT: Aluminum tube, LT: Laminated tube, PB: Plastic bottle.

value of 0.05 or 0.01 was considered significant.

3. Results

3.1. *Measurement of pH*

Previous studies have indicated that the pH influences the stability of drugs. The pH of each preparation used in this experiment was measured, and the results are shown in Figure 1. The pH of the brand-name drug, Lamisil (pH 4.1), and that of the OTC products ranged from 4.2 to 7.6 (Figure 1). In particular, the OTC products Exiv Deep (pH 6.9), Next (pH 6.9), Lamisil Plus (pH 7.6), and Lamisil Gel (pH 6.7) had a significantly higher pH.

3.2. *Measurement of ductility*

When a cream is applied to the skin, its feel depends on its ductility and viscosity. The spread diameter and time needed for each cream to spread were measured using a spreadmeter. In addition, a semilog graph (longitudinal axis: spread diameter, transverse axis: time needed to spread) was prepared to calculate the slope and y-intercept for each preparation. The results are shown in Figures 2 and 3. The slope for Lamisil was 0.21, whereas that for the OTC products ranged from 0.03 to 0.22 (Figure 2). In particular, the OTC products Damalin (0.04) and Barriact (0.03) had a significantly lower slope than that of Lamisil.

In contrast, the y-intercept for Lamisil was 3.11, whereas that for the OTC products ranged from 2.22 to 3.42, as shown in Figure 3. In particular, the OTC product Exiv Deep (2.22) had a significantly lower y-intercept than that of Lamisil, and Barriact (3.34) and Bilumon (3.42) had a higher y-intercept than that of Lamisil.

The yield value is known to be an index of cream ductility. As shown in Figure 4, the yield value for Lamisil was 128 dyn/cm^2, whereas that for the OTC products ranged from 110 to 887 dyn/cm^2. In particular, the OTC products Damalin (887 dyn/cm^2), Barriact (512 dyn/cm^2), and Exiv Deep (663 dyn/cm^2) had a significantly higher yield value.

3.3. *Measurement of the squeeze force*

The squeeze force is expressed as the force required to expel a dose of each preparation. The squeeze force was measured by attaching a HapLog® to the right thumb

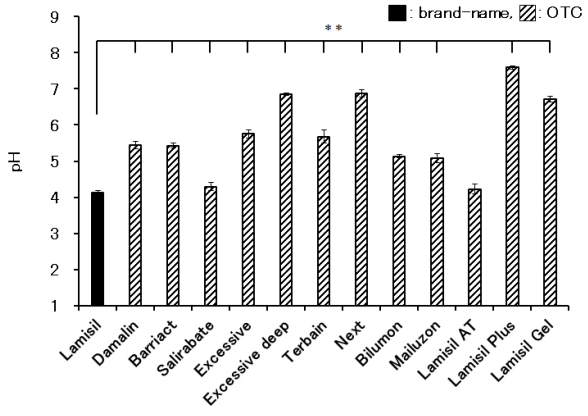

Figure 1. Measured pH of various preparations (*n* = 10). ***p* < 0.01 (*vs*. LLamisil cream, *Dunnett's* test)

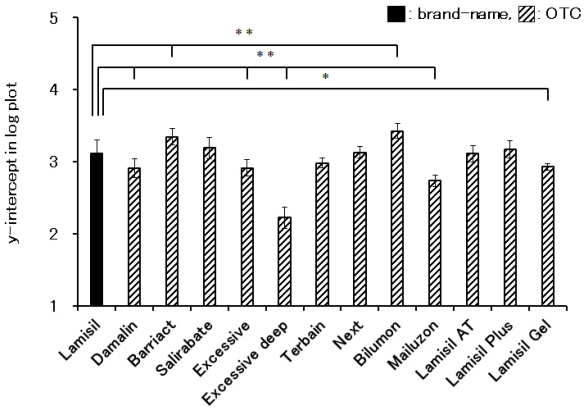

Figure 3. Y-intercept for various creams (*n* = 8). **p* < 0.05, ***p* < 0.01 (*vs*. Lamisil cream, *Dunnett's* test).

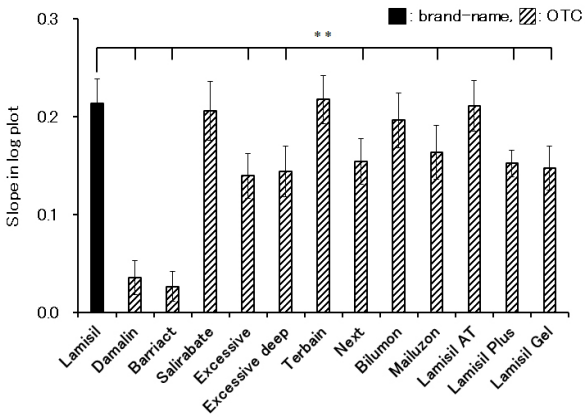

Figure 2. Measured slope for various creams (*n* = 8). ***p* < 0.01 (*vs*. Lamisil cream, *Dunnett's* test).

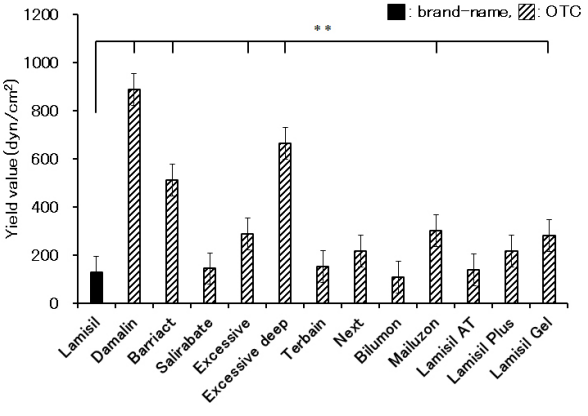

Figure 4. Yield value for various creams (*n* = 8). ***p* < 0.01 (*vs*. Lamisil cream, *Dunnett's* test).

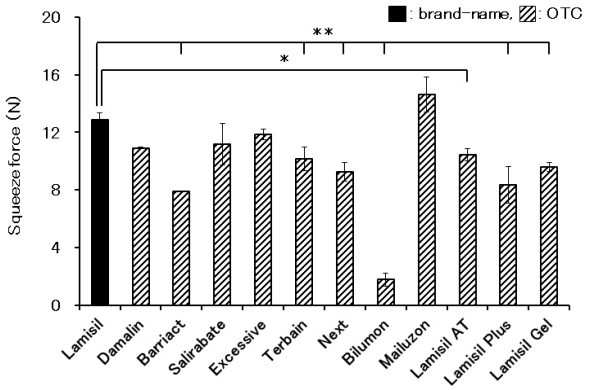

Figure 5. Squeeze force for various creams ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ (*vs*. Lamisil cream, *Dunnett's* test).

and second finger, as shown in Figure 5. The squeeze force for Lamisil was 12.9 N, whereas that for the OTC products ranged from 1.8 to 14.6 N. In particular, Bilumon (1.8 N) had a significantly lower squeeze force. However, Exiv Deep was in a plastic bottle, so its squeeze force could not be measured because it was not in an aluminum or laminated tube.

4. Discussion

External preparations are expected to have both desirable pharmaceutical and pharmacological properties as well as other appropriate physiological and psychorheological characteristics such as tactile feel, ductility, color, and smell (*19*). Since patients rub ointments and creams onto the affected area by hand, poorly ductile and highly viscous preparations are considered difficult to apply, and a study has that suggested the need to improve ointment bases for patient convenience and comfort (*20*).

In general, external preparations with a pH close to that of the surface of skin reportedly have less skin corrosivity and cause less irritation. The pH of the skin is neutral near the epidermis, mildly acidic in the stratum corneum, and 4.5-6.0 on the surface (*21*). This suggests that a pH of 4.5-6.0, which is the same as the pH of the surface of the skin, is optimal for external preparations. Since the use of external preparations itself may cause exacerbation of symptoms in patients with sensitive skin, preparations with a pH close to that of the surface of the skin need to be selected to avoid that risk.

In this study, Exiv Deep had a significantly higher pH than the brand-name drug Lamisil, followed by Next, Lamisil Plus, and Lamisil Gel (Figure 1). Since Exiv Deep contains 10% urea, which has keratolytic and keratin peeling action, and Next, Lamisil Plus, and Lamisil Gel contain 2% l-menthol, which can cause irritation, in addition to the active ingredient, caution is required when selling these products to patients with sensitive skin depending on the condition of the affected area (Table 2).

A spreadmeter was used to determine the ductility

Table 2. Composition of various creams

Product name	Anti-fungal Terbinafine hydro-chloride (%)	Anti-fungal 4-Iso propyl-3-methyl-phenol (%)	Anti-pruritic Crotamiton (%)	Anti-pruritic Lidocaine hydro-chloride (%)	Anti-pruritic Diphen-hydramine hydro-chloride (%)	anti-infla-mmatory Glycyr-rhetinic acid (%)	anti-infla-mmatory l-menthol (%)	Keratolysis Urea (%)
Lamisil	1.0							
Damalin	1.0	0.3		2.0		0.5		2.0
Barriact	1.0	0.3			0.5	0.5		
Salirabate	1.0	0.3			0.5	0.5		
Exiv	1.0			2.0	1.0	1.0		
Exiv Deep	1.0			2.0	1.0	1.0		10
Terubine	1.0	0.3		2.0	0.5	0.5		
Next	1.0		5.0			0.5	2.0	
Bilumon	1.0	0.3		2.0	0.5	0.5		
Mailuzon	1.0			2.0				
Lamisil AT	1.0					0.5	2.0	0.5
Lamisil Plus	1.0					0.5	2.0	
Lamisil Gel	1.0							

of various creams. A semilog graph was prepared with the diameter of the spread of the preparation (cm) along the vertical axis and the time that the cream needed to spread (seconds) along the horizontal axis. A linear formula was derived for the log approximation curve for the plot. Based on the brand-name drug (Lamisil), a higher "slope" is considered to indicate greater ductility, and a higher "y-intercept" is considered to indicate lower viscosity (Figures 2 and 3). Damalin and Barriact had a significantly lower "slope," and they were deemed to be poorly "ductile" (Figure 2). Exiv Deep had a significantly lower "y-intercept" than that of the brand-name drug Lamisil, indicating it has a high "viscosity" (Figure 3).

The "yield value," which is the minimum stress that induces a semisolid such as a cream or ointment to deform or flow, is an index of the feel during use, and a preparation with a low "yield value" is considered to be easy to spread with minor force. When the "yield value" for the brand-name drug Lamisil and OTC products was compared, Damalin, Barriact, and Exiv Deep had a significantly higher "yield value." Damalin and Exiv Deep presumably had a low yield value partly because they contain urea at a respective concentration of 2 and 10% in addition to the active ingredient (Table 2). In contrast, Salirabate, Terbain, Bilumon, and Lamisil AT had a "yield value" close to that of the brand-name drug, and these preparations are considered to spread even with little force (Figure 4).

Measurement of the force of finger contact would presumably be possible while maintaining the free feeling of contact of the fingertip by measuring the force exerted on the fingertip directly in contact with the container using HapLog® attached to the fingers. With this method, a dose could be expelled with a "squeeze force" similar to, or lower than, that needed to expel Lamisil; this was true for the OTC products other than Mailuzon (Figure 5). A dose of Bilumon could be expelled with a squeeze force one-seventh that of Lamisil presumably because Bilumon is contained in a laminated tube while the other products are contained in aluminum tubes (Table 1) (*16*).

In conclusion, pharmacists need to recommend OTC products that cater to diverse patient needs based on the results of this study, but some OTC products contain a skin irritant (l-menthol) or skin-softening ingredients (Irish moss chondrus crispus, urea) and may exacerbate symptoms (Table 2). Since the properties of OTC products are not always appropriate for all patients, pharmacists need to properly select a preparation based on its physicochemical properties depending on the individual patient's skin condition.

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Case Report

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Atypical cases of filariasis from a non-endemic area

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Summary **Filariasis can present in many different ways and pose significant dilemma to the clinician. We report four atypical cases of filariasis which presented as abdominal mass, cervical lymph node enlargement, fever in pregnancy and nosocomial febrile illness respectively. All the four cases were treated successfully with oral antifilarial agents. It is essential to be aware of such atypical presentations of filariasis so that prompt therapy can be initiated.**

Keywords: *Wuchereria bancrofti*, pregnancy, lymphadenopathy

1. Introduction

Filariasis is a neglected tropical disease which poses a significant burden in endemic areas. An estimated 68 million people are infected worldwide, with 40 million being symptomatic (*1,2*). India shares a heavy burden of the disease and despite having a successful national program, it is not rare to find people from endemic areas presenting with swollen limbs and scrotal swelling even today (*3*). The social, psychological and economic implications of this disease are enormous. Unfortunately, the disease is often missed in the early stages and when it manifests atypically. It can present in many different ways including fever, lymphangitis, cough or wheeze and limb or scrotal swelling. Since it may be incidentally detected in the blood or tissue aspirate, it is imperative for the laboratory technicians and clinicians to be aware of such findings. Timely diagnosis and appropriate management can significantly decrease the morbidity associated with the disease. We present four atypical cases of filariasis which created significant diagnostic dilemmas but were successfully managed.

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2. Case Report

2.1. *Case 1: Filariasis mimicking a malignant abdominal tumor*

A forty-eight-year old female, resident of Uttar Pradesh, presented with a lump in the right lumbar area, which developed over a year. Her blood investigations were normal. Magnetic Resonance Imaging (MRI) of the abdomen revealed a large midline, complex cystic mass of size 8.5 cm in the greater pelvis showing fluid-debris level as well as non-dependent membranous structures within (Figure 1A). The ovaries were seen separately from the mass. There was another complex cystic mass of size 5 cm in the right pararenal space of retroperitoneum, inferior to the right kidney (Figure 1B). Some of the loculi showed T2-hypointense debris. In addition, conglomerate tubular channels were seen along the bilateral external and internal iliac vessels, which raised the suspicion of dilated lymphatics (Figure 1C). The magnetic resonance lymphangiogram subsequently performed confirmed the lymphatic nature of these tubular channels. The cystic lesions in the pelvis and retroperitoneum also showed partial opacification with gadolinium, suggesting lymphatic communication and hence the origin of these cysts. Imaging differentials of multifocal macrocystic lymphatic malformation as well as of

cystic mesothelial and germ cell neoplasms were kept. Since imaging could not rule out malignancy, fine needle aspiration cytology (FNAC) of the pelvic mass was done, which revealed microfilaria (Figure 2). She was treated with oral diethycarbazine (DEC) for twenty-one days, subsequent to which the abdominal lump gradually decreased in size. The follow-up MRI performed at one year showed complete resolution of the pelvic and retroperitoneal lumps as well as the lymphatic dilatation (Figure 1D).

2.2. *Case 2: Microfilariae in the lymph node*

A eleven-year-old female, resident of Delhi, presented with on and off fever with chills for six months. On physical examination, she was found to have an enlarged right cervical lymph node measuring 4 × 2 cm. Her routine blood investigations were normal. Mantoux test was negative. Cytopathology of the lymph node aspirate revealed reactive changes with presence of microfilariae. She was treated with DEC and

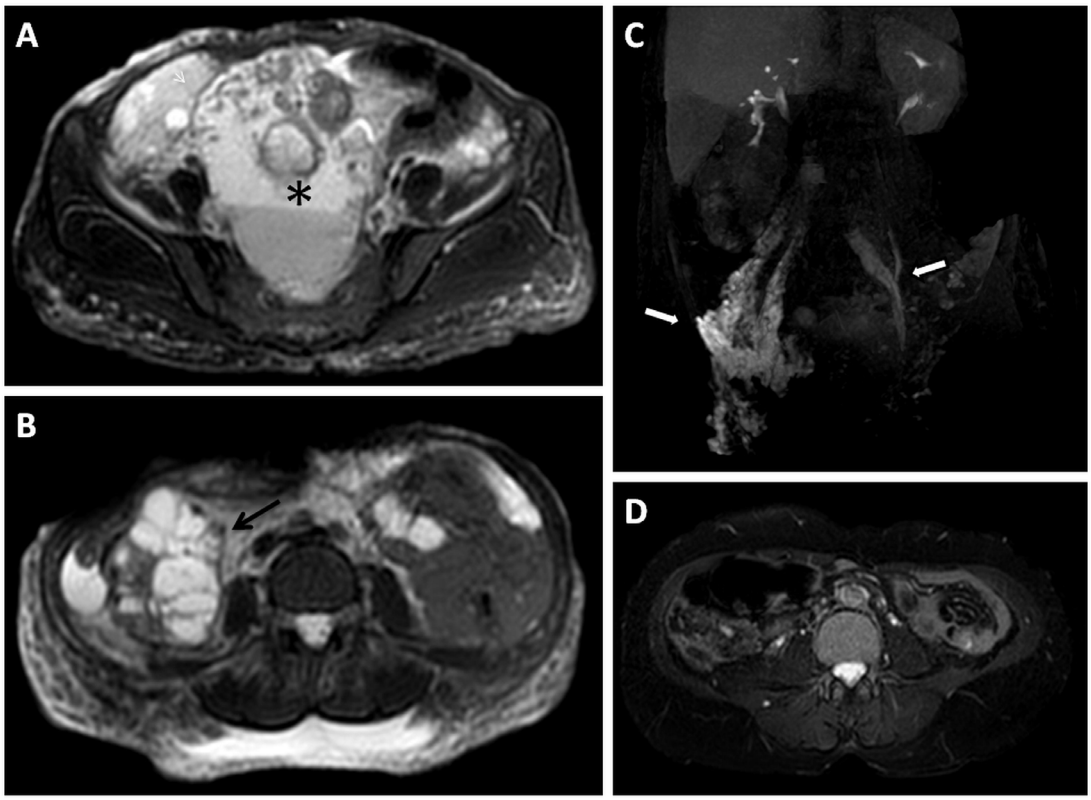

Figure 1. Fat suppressed T2-weighted MRI showing (A) large complex pelvic cystic mass (asterisk) with fluid-debris level as well as numerous T2-hypointense membranous contents, and **(B)** a complex multiseptate cyst (arrow) in the retroperitoneum on the right side. Some of the loculi show differential T2-hypointensity, consistent with debris. **(C)** Coronal maximum intensity projection images of MR lymphangiogram showing dilated lymphatics (open arrows) along the bilateral external and common iliac vessels. **(D)** Representative post-treatment T2-weighted MRI at the level of the second image showing complete resolution after one year of therapy.

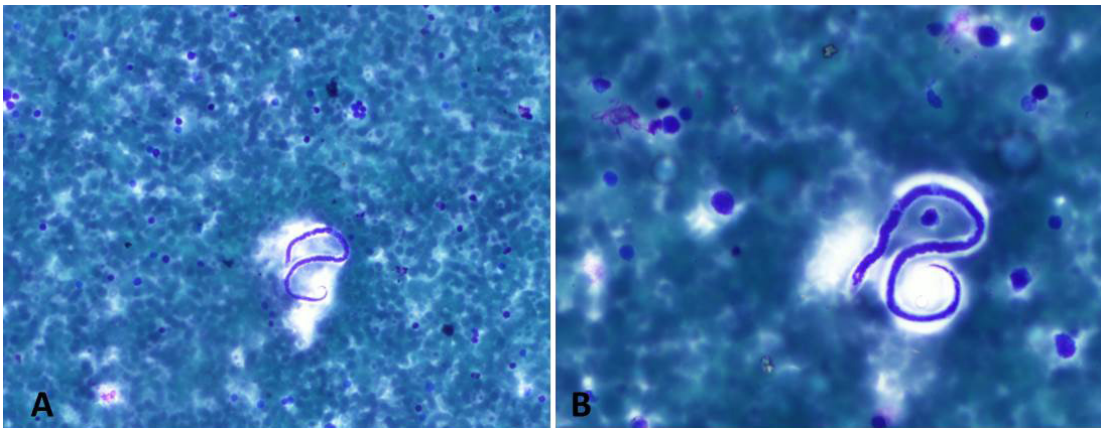

Figure 2. Fine needle aspiration smear from abdominal lump showing microfilaria in a background of blood and inflammatory cells (A). A higher power view of the microfilaria surrounded by a faintly staining sheath (B).

doxycycline, subsequent to which the fever resolved and did not recur at four months of follow. However, the lymph node swelling was persistent.

2.3. *Case 3: Detection of microfilariae in the screening peripheral smear of a pregnant patient with fever*

A 24-year-old pregnant female, resident of Delhi, presented in the second trimester of her pregnancy with fever, chills and joint pain lasting three days. The physical examination was unremarkable. Her routine peripheral blood smear showed microfilariae. Oral albendazole was initiated and the patient became afebrile on the second day of treatment. She remained asymptomatic at six months of follow-up and no microfilariae were seen in follow-up smear.

2.4. *Case 4: From the postoperative ward for fever*

A 55-year-old hypertensive male from Delhi presented acutely with altered sensorium and hemiparesis. Unenhanced computed tomography (CT) of the brain showed acute bleed in the left basal ganglion with midline shift and intraventricular extension. He underwent craniotomy with hematoma evacuation and frontal external ventricular drain (EVD) insertion. On the seventh postoperative day, he developed high-grade fever with chills. Physical examination revealed no focus of infection. He was started on broad-spectrum antibiotics, however there was no improvement in the fever. His blood counts, liver and kidney function tests, blood and urine cultures, cerebrospinal fluid examination, serum procalcitonin levels and chest radiograph were normal. Blood mount showed motile microfilariae (Figure 3), which were identified as *Wuchereria bancrofti* on giemsa staining (Figure 4). He was started on oral DEC and became afebrile on the second day of treatment.

3. Discussion

Filariasis is a major public health problem in the tropical world with both lymphatic and extralymphatic manifestations. A retrospective analysis of the records of our outpatient clinics and infectious disease consultations from 2017 revealed 20 patients of proven filariasis. Five patients showed atypical presentations, with the findings of one among them (massive splenomegaly with bone marrow aspirate showing microfilariae) having been already published (*4*).

Microfilariae have been detected incidentally in unsuspected swellings in many reports from across the world (*5-8*). In such cases, microfilariae may either be the direct etiologic agent for the swelling or may have gotten entrapped in an already existent swelling. Diagnosing extralymphatic filariasis remains a clinical challenge as it is often unsuspected and as it mimics non-filarial diseases. Our series describes four cases of filariasis which presented at unusual and rare sites, but were promptly diagnosed and managed.

The first case showed complex cystic masses in the pelvis and retroperitoneum, which usually raise the suspicion of macrocystic lymphatic malformation or malignancy. Since the ovaries were separately visualized, the possible differentials included primary cystic mesothelial and germ cell neoplasms. Because of the unusual morphology, filariasis was not kept as a differential until the cytology revealed otherwise. Timely diagnosis and prompt institution of antihelminthic therapy enabled complete recovery

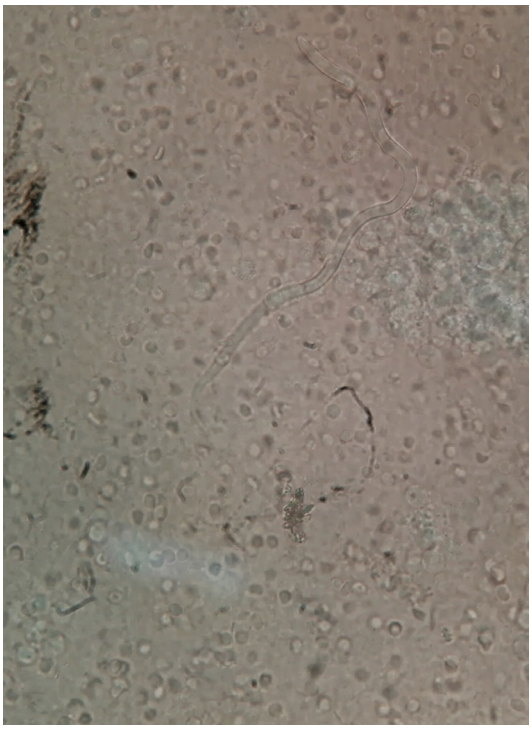

Figure 3. Wet mount of blood showing microfilaria.

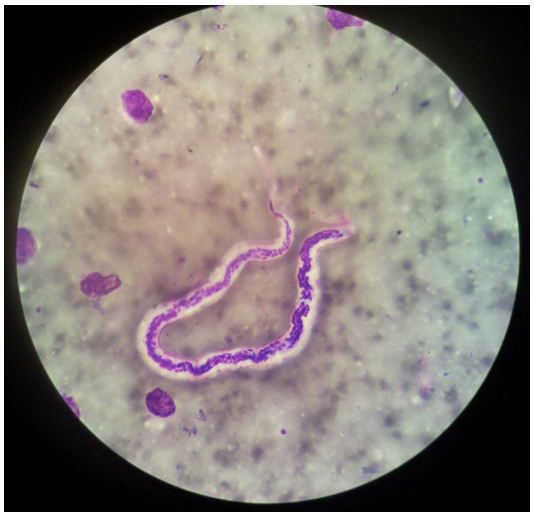

Figure 4. Peripheral blood smear (Giemsa stain) showing microfilaria of *Wuchereria bancrofti*.

without surgery. Only four reports of filariasis presenting as abdominal masses or cysts are available in the current literature, all of which are from the Indian subcontinent (*9-12*). Except for one, all showed large unilocular retroperitoneal cysts which were resected. Giri *et al*. (*12*) reported a complex solid-cystic retroperitoneal mass which, similar to our case, resolved completely on oral DEC therapy. Although the exact mechanism of the origin of abdominal cysts in filariasis is not known, possible hypothesis includes lymphatic obstruction resulting in rupture and extravasation of chyle as well as the involvement of ectopic lymphatic tissue (*9*). Although rare, keeping a high index of suspicion can result in timely diagnosis, which avoids surgery and significantly changes the course of the disease.

Many case series and reports have shown incidental detection of microfilaria in lymph node aspirates (*6-8*). Presence of microfilariae in lymph nodes may only be an incidental finding with no proven causal association. Cases of microfilariae in aspirates from malignant lymph nodes have also been reported, although the association with malignancy is controversial (*13-17*). It is important to be aware of this observation and so that the samples are carefully screened. Timely diagnosis can prevent future morbidity and complications.

There is no definitive evidence as to whether pregnancy, surgery or other forms of stress can activate the dormant worm and release more microfilariae into blood. No cases of filariasis presenting as nosocomial fever has been described in the literature. However, two among the four cases in our series presented for the first time in periods of stress (pregnancy and postoperative period respectively). Whether these presentations were incidental or had any possible relation to stress remains speculative. Another noteworthy finding in our case series was that none of the patients had eosinophilia. This highlights the fact that eosinophil counts may be normal in filariasis and the diagnosis should not be ruled out for the mere absence of eosinophilia.

There have been several reports of filariasis from non-endemic areas such as Delhi. Two among the four patients belonged to Delhi and had no history of travel to other endemic areas. Literature suggests that transmission of filariasis requires prolonged stay in endemic areas and hence is uncommon in travelers.

Detection of microfilaria or adult worm in the laboratory samples should prompt the physician to initiate treatment so as to avoid any long-term consequences of the disease. DEC is highly effective in eliminating microfilaria, but has only modest activity against the adult worm. Many recent studies support the synergistic effect of combining this drug with doxycycline. Doxycycline acts by targeting *Wolbachia*, which engages in endosymbiotic relationship with the filarial parasite and is important in maintaining the reproductive capacity of the worm (*18-20*). Albendazole and ivermectin have been used in combination as alternatives. Ivermectin and doxycycline should be avoided in pregnancy. In our series, all the patients responded well to treatment with oral anti-filarial drugs (DEC or albendazole). All patients tolerated the drug well and did not report any side effects or intolerance. All patients showed improvement in symptoms at follow up.

As we envision a world free of filariasis, the World Health Organization (WHO) has launched the Global Programme to Eliminate Lymphatic Filariasis (GPELF) by 2020. WHO recommends preventive chemotherapy with annual mass drug administration (MDA) of albendazole together with ivermectin or with DEC. Enhanced public health strategies, disease awareness, prompt diagnosis and treatment can help us in paving the path towards elimination.

In conclusion, these cases highlight the unusual and diagnostically challenging presentations of filariasis. It underscores the fact that timely diagnosis and prompt management can prevent severe complications and long-term sequelae. Each patient who gets diagnosed and treated moves us a step closer towards the vision of filariasis-free world.

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Erratum

DOI: 10.5582/ddt.2018.E1

It has come to the authors' attention that their article entitled "Anesthetic activity of plant essential oils on *Cyprinus carpio* (koi carp)" (*Drug Discoveries & Therapeutics. 2018; 12(1):21-30.*) included an error in Table 2. The corrected Table 2 is printed below.

Table 2. Chemical compounds existing in OBO, OCO, and OSO

Retention time (min)	Components	Percentage of chemical compound		
		OBO	OCO	OSO
4.08	α-Pinene	0.09	-	0.06
4.39	Camphene	-	-	0.06
4.93	Sabinene	0.09	-	-
5.03	β-Pinene	0.17	-	0.05
5.33	β-Myrcene	0.29	-	-
6.43	Limonene	0.19	-	-
6.60	1,8-Cineole	3.54	-	-
7.02	1,3,6-Octatriene	2.37	-	-
8.35	Terpinolene	0.16	-	-
8.79	Linalool	0.53	3.63	0.94
10.42	L-Camphor	1.49	-	-
11.23	Borneol	0.57	-	1.73
11.32	α Terpineol	-	0.60	-
13.04	Methyl chavicol	**78.12**	-	-
14.74	Z-Citral (Neral)	-	**37.04**	-
15.38	Geraniol	-	0.79	-
16.11	E-Citral (Geranial)	-	**41.01**	-
18.66	α-Cubebene	-	-	0.16
19.23	Eugenol	-	-	11.56
19.76	α-Copaene	-	0.17	2.39
20.55	β-Elemene	0.70	-	9.67
21.43	Methyl eugenol	0.44	-	**46.18**
21.81	β-Caryophyllene	-	2.47	8.99
22.21	Trans-α-Bergamotene	3.02	-	-
22.79	β-Selinene	0.34	0.75	2.06
23.08	β-Farnesene	-	0.39	0.07
23.90	Germacrene-D	1.36	1.42	7.35
24.10	Trans-β-Farnesene	0.23	0.69	-
25.10	β-Bisaboloene	-	0.12	-
25.66	β-Sesquiphellandrene	0.25	-	-
26.48	Cis-α-Bisabolene	-	3.01	-
30.08	δ-Cadinene	2.79	-	-
Total		96.74	92.09	91.27

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