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A Monoterpenoid Isolated from Citrus Aurantifolia Peel and Its Potential as An Antibacterial for The Inhibition and Eradication of Streptococcus Mutans Biofilm

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ABSTRACT

Background: Dental caries, which becomes a serious problem caused by its harmful effects on human health and can impair quality of life. This is a chronic infectious disease with multifactorial etiological factors due to the accumulation of biofilms on the tooth surface. Citrus aurantifolia is one of the plants which have antimicrobial activity. It originates from Southeast Asia, has been used as natural remedies for dyspepsia, antiseptic, antiviral, antifungal, anthelmintic, astringent, diuretic, insect repellent, constipation, appetite stimulant, and lower cholesterol levels. In this study, the isolation of one of the active compounds from the peel of aurantifolia orange will be carried out and testing of the active biological components that play a role in the formation of Streptococcus mutans UA 159 biofilms.

Methods: Citrus aurantifolia peel was extracted with methanol and fractioned by ethyl acetate, n-hexane, and water. The most active extract was purified by a combinational technic of chromatography provided the pure compound. UV, NMR, IR, and MS spectrometry were used to characterizes active biological structures. The MBIC and MBEC values are determined by adding an isolated compound to the S. mutans UA 159 biofilms.

Results: The isolation results from the skin of C. aurantifolia obtained a compound that has been determined as a derivative of monoterpenoid with the formula C10H16O2 and obtained an MBEC value of 34.75% for induction time of 30 minutes.

Conclusion: The antibacterial agent obtained from the monoterpenoid extracted from C. aurantifolia peel especially for the prevention of biofilm formation has the potential to be further developed.

Keywords: Streptococcus mutans biofilm, Monoterpenoid, Citrus aurantifolia peel

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INTRODUCTION

Severe untreated dental caries is common in preschool children in many countries and is associated with a negative impact on the quality of life of young children and their families.[1,2] Causative relationships between dental caries and Streptococcus mutans have been established for many years. Streptococcus mutans as the main causative agent has developed multiple mechanisms to integrate into the plaque (dental biofilm) to colonize tooth surfaces.[3]

Of all the oral streptococci, Streptococcus mutans is the most cariogenic bacteria, capable of colonizing the surface of the teeth, and producing many intracellular and extracellular polysaccharides. The initial stage of oral biofilm formation which further causes caries lesions to be the responsibility of these microorganisms because they are very aciduric and acidogenic, and metabolize some salivary glycoproteins.[4] The virulence factor of S. mutans is usually caused by adhesion attachment to cell surface proteins, acid tolerance, acid production, glucosyltransferase (GTF) production which plays a role in the production of glucans from sucrose, and also intracellular polysaccharides, which will then form a strong and cariogenic biofilm layer.[5–10]

Many plants have been curing human ailments for a long time due to their known properties, particularly their secondary metabolites, which have recently been shown to act as antimicrobial agents.[11,12] In recent years, phytochemical studies for its antibacterial activity have received a lot of attention and research has shown that antibacterial activity can be obtained from phytochemical content in various ways, such as suppression of virulence factors (inhibition of enzyme and toxin activity, as well as the formation of bacterial biofilms) and damage to bacterial membranes.[11,13]

The uses of natural ingredients, especially medicinal plants, have increased along with the high price of medicines and the phenomenon of resistance from chemical drugs. Medicinal plants can be used as an alternative herbal medicine for microorganisms that are resistant to chemical drugs.[13] In recent years, knowledge about the antibacterial activity of phytochemicals and bioactive compounds has grown increase very widely and many are known about the mechanisms of these antimicrobial actions. Generally, phytochemicals cause suppression of some virulence factors and damage to bacterial membrane.[11]

Citrus plants are included in the Rutaceae family which includes fruits such as lime as a source of multiple nutrients that are promising and beneficial to humans. The large amount of lime peels produced as a result of processing citrus by-products is generally disposed of as waste in the environment, even though this residue has the potential to be a rich source of phenolic compounds and dietary fiber. This compound acts as a potential nutraceutical source. Nutritional dietary supplements can be obtained from these wastes, because of their low cost and easy availability. To produce new nutraceuticals, the utilization of bioactive rich lime residues can provide an efficient, low cost, and environmentally friendly platform. Lime peel has been widely studied but research on the antibacterial activity of lime peel on the formation of S. mutans UA 159 biofilms has never been done.[14,15]

Lime (Citrus aurantifolia) is one of the plants which have antimicrobial activity.[16,17] Citrus peel consists of the outside (flavedo) and the inside (albedo). The outer skin is rich in essential oils and carotenoids, while the inner skin is rich in phenols and pectins. There are much functional substance is found in Citrus peel such as essential oils (0.6-1%), phenols (0.67-19.62 g/100 g db), vitamin C (0.109-1.150 g/100g db) and fibers (6.30-42.13g/100g db).[18]

Initial cell adhesion to solid surfaces followed by surface adhesion by self-produced extracellular polymeric substances (EPS) is a multi-stage process of biofilm growth.[19] Generally, passive transport of bacteria by flowing saliva to the surface of the teeth. Changes in the oral environment can be initiated by the presence of pioneering microorganisms through the production and secretion of metabolic products, where these products can stimulate mutually beneficial microbiota-biofilm interactions. The process of forming biofilms is complex and goes through a very rapidly developing process with the following stages: the binding of bacteria to a solid surface, the formation of an exopolysaccharide matrix, the occurrence of irreversible bonds between bacteria and the surface, the process of maturation structure, disintegration and dispersion of an organized structure, and finally the new habitat formation occurs. Oral microbiome analysis can be determined by the genotypic characteristics of the host and its metabolic phenotype, thus enabling all factors that play a role in maintaining the homeostasis of the host microbiota to be understood. The contribution of genetic maps including the host (microbiota) of the environment and detection of biofactors that show a predisposition to disease could guide new diagnostic methods that refer to the individual.[20]

Oral biofilms are one of the most complex polymicrobial communities discovered in nature as conglomerations of previous research regarding the isolation of compounds contained in the peel of aurantifolia orange, it shows that this compound is thermally stable in the evaporation surface-associated microorganisms, which can cause diseases on ecological and dysbiosis principles. Biofilm structure modulation, microenvironments, spatial organization, and the development of microbial communities are strongly influenced by saliva and diet of host. The opportunistic pathogens such as S. mutans can be triggered with sugar-rich diets to create an acidic microenvironment that is protected by an EPS rich matrix.[21,22] This investigation aimed to evaluate the antimicrobial activity of monoterpenoid isolated from C. aurantifolia peel as antibacterial against S. mutans biofilm.

METHODS

Chemicals and reagents

The column chromatography (Merck®, Darmstadt, Germany) using Kiesel G60 silica gel resin and the Octadecyl-bonded silica (ODS) was a Li Chroprep RP-18 (Merck®). TLC analysis was carried out using Kiesel G 60 F_{254} and RP-18 F_{254S} (Merck®). Deuterated solvents (methanol, ethyl acetate, *n*-hexane) were from Merck Co. Ltd. and Sigma Aldrich Co. Ltd. (St. Louis, MO, USA).

Plant material collection and determination

Lime fruit (Citrus aurantifolia (Christm.) was collected from a lime plantation in the village of Sukawana, Kecamatan Kertajati, Majalengka, Indonesia. The specimen was determined by Mr. Joko Kusmoro (Botanist) at the Laboratory of Plant Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences Universitas Padjadjaran, Bandung, Indonesia. The plant part used were peels and collected from their natural environment then stored at room temperature.

Test Organism

In this study, Streptococcus mutans UA 159 was used. Brain Heart Infusion broth and Muller Hinton agar as a medium, paper disc 6 mm, chlorhexidine was as positive control (from Merck Co. Ltd. and Sigma Aldrich), and anaerobic jar (for anaerobic condition) for antibacterial assay.

Instruments

To characterize the chemical structure of compound using several spectrometers. NMR spectra recorded on a 500 MHz FT-NMR spectrometer was performed using a JNM-*ECA 500* (JEOL) *instrument* from Japan, IR spectra were obtained from a Perkin Elmer Spectrum One FT-IR spectrometer (Buckinghamshire, England), Ultraviolet spectrum was recorded on UV-Vis spectrophotometer Shimizu (Japan), and Mass spectra were obtained from an ES-MS spectrometer (UPLC MS/MS TQD type, Waters). To evaluate antibacterial activity using micropipette biolab, microplate 96 well (Nest, America), Laminar airflow, autoclave machine HVE-50 Hirayama, incubator Memmert, jar and microplate reader EZ 400 Biochrom Diagnostic Automation Inc.

Extraction of Citrus aurantifolia peel

Collected C. aurantifolia (50 kg) were washed thoroughly under water and squeezed, then disposed of water and seeds. The peel (27.4 kg) was extracted in 50 l methanol under continuous shaking for 24 hours. The extract was filtered through a 22 µm paper filter, then the filtrate was evaporated to dryness using a rotatory evaporator at 40°C. Based on

process at a temperature of 40°C and will not damage its constituent components. Prevention of decomposition of the compounds in the extract can be carried out at a

temperature of 40 ° C. Methanol was used in the extraction process because it can extract non-polar, semi-polar, and polar compound. The methanol extract (0.5 kg) was partitioned by *n*-hexane, ethyl acetate, and water to get *n*-hexane, ethyl acetate and water extract were 2 g, 51 g, and 440 g, respectively. All extracts were tested antibacterial activity to determine the most active extract.

Separation and purification of the ethyl acetate extract of Citrus aurantifolia peel

Separating concentrated n-hexane extract from the skin of C. aurantifolia and producing 21 fractions can be done using liquid column chromatography using stationary G60 silica gel (70-230 mesh) and 5% (v / v) n-hexane-ethyl acetate as eluent.

Thin layer chromatography (TLC) with stationary silica gel phase G60 F254 can be used to analyze the content of each fraction, to determine the suitable solvent composition for the next purification process. Liquid column chromatography can then be used to purify fraction 13 (1 g) with a stationary G60 (70–230 mesh) an octadecyl silane (ODS) stationary phase and 2.5% (v/v) *n*-hexane-ethyl acetate as the eluent, then produced 21 fractions. Furthermore, TLC with stationary phase silica gel (G60 F254) was used to analyze the fraction of the 2nd column chromatography stage. Fraction 13-8 has a single spot on TLC chromatogram silica gel and ODS (Silica gel RP-18) indicating this fraction is pure compound. Wight of this fraction is 8.3 mg (compound 1). Meanwhile, there is the same pattern of fraction 13-9 to 13-12 so they are combined to further purify. Fraction 13-9 to 13.12 (23.3 mg) was purified by isocratic column chromatography using Silica G60 F254 as the stationary phase and *n*-hexane-ethyl acetate (9:1, v/v) as the eluent, resulting in 20 fractions. Fraction analysis of the 3rd column chromatography stage can be carried out using TLC with the ODS stationary phase which shows the presence of one compound in the last four fractions. This pure compound (compound 2) has Rf of 0.45 in TLC of silica gel with *n*-hexane-ethyl acetate (7:3, v/v) as a mobile phase.

Structural determination of compound

Spectrum analysis of 1H NMR, 13C NMR, DEPT 135 °, HMQC, 1H-1H COZY and HMBC can be used to determine the chemical structure of the compounds obtained on a 500 MHz FT-NMR spectrometer was performed using a JNM-*ECA 500* (JEOL) instrument from Japan. Delta ™ NMR processing and control software, copyright 1990–2004 by JEOL USA, Inc. Version: 4.3.2 [Windows_NT] Network port = 6422 used in this study. Determination of the IR spectrum of compounds using the FT-IR Perkin Elmer Spectrum One spectrometer (Buckinghamshire, UK).

The compound was measured using NMR (nuclear magnetic resonance) spectrophotometer to determine the number and type of protons and to determine the number, type and breakdown of carbon signals that depend on the number of bound protons (metin, methylene, methyl, and quaternary carbon). The compound is a white crystalline soluble in methanol. Based on 13C- and 1H-NMR spectrum data, the compound has 10 carbon atoms, 16 hydrogen atoms and 2 oxygen atoms. The results of this study found that the most active compounds isolated from *C. aurantifolia* peel was determined to be a monoterpenoid derivative with the formula $C_{10}H_{16}O_2$.

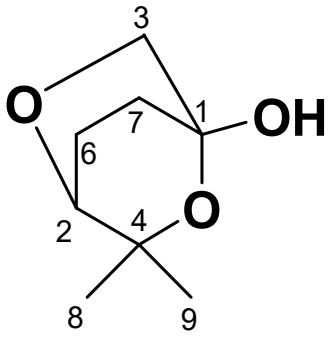

Figure 1. The structure of compounds of monoterpenoid ($C_{10}H_{16}O_2$)

Bacterial strain and inocula preparation

This study used Streptococcus mutans UA159 (ATCC® 700610 ™). [23] Initially Streptococcus mutans UA159 (ATCC® 700610 ™) was spread on BHA (Brain Heart Agar), then incubated for 48 hours, at 37 ° C, under facultative anaerobic conditions (5% CO2).

One inoculation loop of bacteria was grown in liquid BHA medium for 24 hours, at 37 ° C for inoculum preparation and adjusted for optical density (at 620 nm) using a Microplate reader (Biochrom EZ 400). Then, the bacterial suspension is diluted to contain about 108 CFU / mL or reach the McFarland standard of 0.5.[24]

Determination of the minimum inhibitory concentration (MIC)

According to the procedure described by Eloff[25], the monoterpenoid MIC of the skin of C. aurantifolia was determined using a series of broth micro solutions. Serial dilution of monoterpenoids was carried out in 96-well microplates until a concentration of 15-30000 ppm was obtained with BHI broth solvent. One hundred microliters of inoculum (bacterial suspension) was added to the well, then covered the plate using parafilm and incubated for 48 hours at 37 ° C. In the well samples, observations were made to determine the MIC value by observing the decrease in the first optical density (OD) value.

Each well solution was spread into the agar to obtain a minimum bactericidal concentration (MBC) value. After that, check which plates do not contain bacteria after incubating for 48 hours.

Analysis of the minimum biofilm eradication concentration (MBEC)

Biofilm eradication of S. mutans with *C. aurantifolia* peel based on monoterpenoid ability was analyzed using microdilution with a modified version of the method described by LaPlante.[26] Each well of the 96-hole micro-filled brain heart infusion (BHI) and 2% sucrose medium between 200 mL of bacterial culture with a cell density of 108 CFU / mL. Each well was washed using 300 µl of phosphate buffered saline (PBS) after incubation for 48 hours. A total of 200 µL of monoterpenoid compound was added to each well after the biofilm was formed and the samples were left to stand for 30 minutes. The negative control using wells containing bacteria, and as positive control using chlorhexidine 0.2%. The lowest monoterpenoid concentration capable of eradicating biofilms is expressed by the MBEC value. The culture and test compounds were removed after incubation for 30 minutes, then each well was washed twice with 300 µL PBS. After that, staining was done by adding safranin to 150 µL and incubated for 15 minutes. Perform three

rinses using 300 µL of PBS to remove excess safranin stains. The absorbance was measured using a microplate reader with a wavelength of 490 nm.

RESULTS

Thin-layer chromatography analysis

Compound **1**: Figure 1 shows the mobile phase resulting from the spot pattern of TLC fraction 17-20 through third column chromatography purification with n-hexane-ethyl acetate (7: 3, v / v). The fraction found to have a component with Rf = 0.69 was then given the name monoterpenoid and 9.9 mg of the compound was isolated. Compound **2**: Fraction 13-8 has a single spot on TLC chromatogram silica gel with *n*-hexane-ethyl acetat (7:3, v/v) of Rf 0.5 and TLC chromatogram ODS (Silca gel RP-18) with methanol-water (9:1, v/v) of Rf = 0.69 indicating this fraction is pure compound (Fig. 2). This compound named sesterterpenoid.

Inhibition of S. mutans biofilm formation by a monoterpenoid from C. aurantifolia peel

The MIC value of the isolated monoterpenoid towards S. mutans was 15000 ppm (1.5%).

Results of the ability of the monoterpenoid from C. aurantifolia peel to eradicate S. mutans biofilm

The MBEC value of the monoterpenoid from *C. aurantifolia* peel towards the S. mutans biofilm for the 30 min induction time was 34.75%, it's mean the monoterpenoid from *C. aurantifolia* peel was able to eradicate approximately 34.75% of the biofilms after 30 min of treatment.

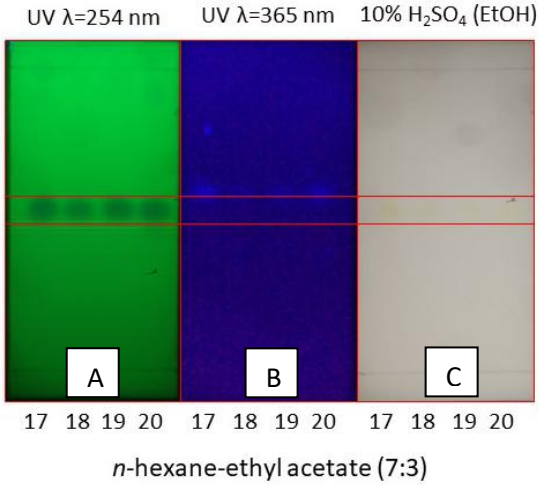

Figure 2. TLC chromatogram of fractions 17-20 using an ODS plate with n-hexane-ethyl acetate under UV light at λ 254 nm (a); UV light at λ 365 nm (b); and after spraying with 10% H2SO4 in ethanol to make the colour persist (c)

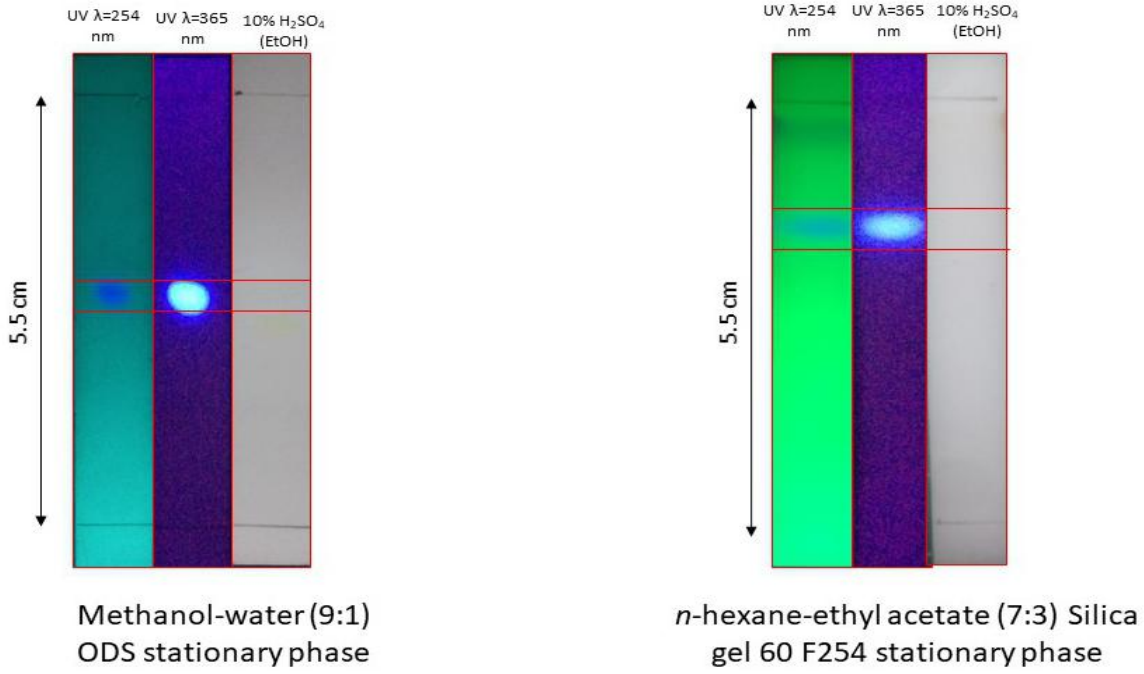

Figure 3. TLC chromatogram of fraction 13-8

DISCUSSION

In this study, the results of the NMR experiment showed that the first compound obtained was a monoterpenoid with a molecular formula of $C_{10}H_{16}O_2$ and the second compound was a sesterterpenoid. The MIC and MBC value of this compound towards S. mutans was 15000 and 30000 ppm, respectively. Biofilms contain exopolysaccharides so that it will be more difficult to penetrate, this will cause a higher concentration of compounds needed to inhibit the growth of a bacterial biofilm compared to that required for planktonic biofilms. An active compound from natural ingredients exerts a bactericidal and bacteriostatic effect shown by preventing the adhesion of bacteria to the surface of the skin and oral mucosa; decrease in pH; inhibition of glycolytic enzymes; decreased biofilm and plaque formation; and decreased cell surface hydrophobicity.[27] When the highest concentrations of the monoterpenoid from *C. aurantifolia* peel were tested for 30 min in this

study, about 34.75% of the biofilm that has been formed can be destroyed.

This study has successfully demonstrated the antimicrobial effect of monoterpenoids from *C. aurantifolia* peel against S. mutans biofilms. Therefore the formation of biofilms (dental plaque) can be prevented by natural compounds that can be considered as potential molecules, through inhibition of the expression of glucosyltransferase activity in S. mutans.[28] This will directly affect the formation of cariogenic biofilms. [22,23] Antibiotics and other disinfectants can exhibit a pattern of high adaptive resistance to bacteria living in biofilms. The increasing number of adaptive antibiotic resistance globally is a barrier to the treatment of acute and chronic biofilm-related infections.[22,23,29]

The antibacterial properties of these natural compounds include secondary metabolites which are grouped based on their chemical types such as terpenes, steroids, alkaloids, polyketides, peptides, and aromatics.[30] The main components of plant volatiles and essential oils are terpenoids, which exhibit a wide range of biological activities including antimicrobial, antiviral, anticancer, antiparasitic, and antiallergic properties.[31]

Phytochemicals and essential oils (EO), both semi-synthetic and synthetic, are compounds that occur naturally and can be used as antimicrobial agents. The ability of natural secondary metabolites to have a molecular weight of 500 g/mol is likely to act as adjuvants for antimicrobials and exhibit synergy effects. Biotransformation processes such as microbial modification are an important alternative in the exploration of new antimicrobial agents. Natural products with low molecular weight such as terpene derivatives can be used as combination therapy of antimicrobial agents, because they have shown promising effects with the ability to eliminate the production of bacterial and fungal biofilms. Antimicrobial activity against susceptible and resistant pathogens has also been shown to be shared by terpenes and their derivatives, which are secondary metabolites commonly found in EO. Combination therapy between natural compounds and drugs can increase the action potential of the drug by responding to the function of the existing antimicrobial agents.[32]

The characteristics of the antimicrobial activity possessed by terpenoids derived from the five-carbon isoprene unit, so that the terpenoids can represent a large class of plant-produced compounds with multicyclic structures that differ from each other in the basic carbon framework and functional groups. In essential oils extracted from many plants can be found monoterpenes, this component is a class of terpenes consisting of two isoprene units. Over the past two decades, scientists have studied the antimicrobial activity of this compound, the growth of many bacteria can be inhibited by thymol and carvacrol (phenolic monoterpenoid) which has been investigated through several articles.[33]

CONCLUSIONS

The plant-derived phytochemicals represent a possible source of efficient, low cost, and environment-friendly platform for the production of safe antimicrobial agents. However, it is still necessary to confirm the selection of active and nontoxic antimicrobial phytochemicals, both in vitro and in vivo. The monoterpenoid extracted from *C. aurantifolia* peel with a molecular formula of $C_{10}H_{16}O_2$ has the potential especially to eradicate the formation of biofilms (about 34.75% of S. mutans biofilms) and be developed as antibacterial agents.

Abbreviations

ATCC: American type culture collection; CFU: Colony forming unit; COSY: Correlation spectroscopy; NMR: Nuclear magnetic resonance; TLC: Thin-layer chromatography; UPLC: Ultra-performance liquid chromatography; UV-VIS: Ultraviolet-visible

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Authors' contributions

JJ provided idea for this research and drafted this paper. SS, DK, and MHS helped to evaluate and edit this paper. All of the authors have approved this article.

Ethics approval and consent to participate

Ethical exemption from the research ethics committee Universitas Padjadjaran Bandung Indonesia no. 1315/UN6.KEP/EC/2018

Competing interests

The authors declare that they have no competing interests.

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