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Yoshihiro Inoue
Showa Pharmaceutical University, Machida, 194-8543 Tokyo, Japan

Takahisa Nakane
Showa Pharmaceutical University, Machida, 194-8543 Tokyo, Japan

Yoko Arai
Showa Pharmaceutical University, Machida, 194-8543 Tokyo, Japan

Corresponding Author:
Yoshihiro Inoue
Showa Pharmaceutical University, Machida, 194-8543 Tokyo, Japan

A novel screening method for ingredients of essential oil to identify antibacterial compounds based on growth curve shape

Yoshihiro Inoue, Takahisa Nakane and Yoko Arai

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Abstract
We established a novel screening method to identify antibacterial compounds and used the method to screen various triterpenoids, which are difficult to assay using existing methods. The method involves comparison of the shape of bacterial growth curves based on changes in culture turbidity. Growth curves were grouped using multiple classification analysis. The antibacterial compounds tested belonged to a different chemical group than the controls. Six and four of the triterpenoids screened using the method suppressed the growth of *Staphylococcus aureus* FDA209P and *Escherichia coli.* W3110, respectively. Two triterpenoids exhibited bactericidal activity against *S. aureus*, and the others exhibited bacteriostatic activity.

Keywords: Terpenoids, antibacterial activity, screening method

Introduction
Novel medicines are needed in all medical fields. This need is particularly pressing for infectious diseases, as pathogens exhibiting resistance to antibiotics continue to emerge, and innovative medical treatments such as an organ transplantation involve the use of immunosuppressants, which place patients at increased risk of infection [1, 2]. The risk of acquiring an infectious disease continues to increase. As such, efforts to identify or develop novel antibiotics have continued, often targeting natural products using standard methods such as observation of inhibitory zones on agar media. However, such approaches have limitations, including the subjectivity of the observer in making visual judgments, the inability to characterize effects on growth during incubation based on analysis of results at only one time point, and the low assay reproducibility for hydrophobic compounds. We have addressed these issues in our research in an effort to develop a method for high-throughput screening of antibacterial compounds. An important aspect of our method development strategy was to ensure objective determinations regarding changes in bacterial growth over time.
The screening method we developed was applied to the identification of triterpenoid antibacterial compounds. The triterpenes examined in this study are natural products derived from various plants and contain many substances. Some triterpenes reportedly exert pharmacologic properties, such as carcinostatic activity [3], but the nature of other properties remains unclear. Therefore, triterpenes are very attractive compounds for research, as they are expected to exhibit novel useful activities. The objective of the present study was to identify antibacterial seed compounds using the highly sensitive screening method that we developed.

Experimental
Growth Curves
Staphylococcus aureus FDA209P as a gram-positive microorganism and *Escherichia coli* W3110 as a gram-negative microorganism were used as test microorganisms. Brain-heart infusion (BHI) and Mueller-Hinton (MH) (Becton Dickinson and Company, Franklin Lakes, NJ, USA) media were used for the cultivation of *S. aureus* and *E. coli*, respectively.
Stock solutions of triterpenes were prepared in dimethyl sulfoxide at a concentration of 500 μg/mL. An aliquot of 100 μL of growth medium at 2-fold concentration was added to each well of a 96-well microplate, and then 90 μL of bacterial suspension was added to each well. Next, 10 μL of triterpene stock solution was added, and the suspension was mixed well by pipetting. The microplate was sealed with a transparent sanitized sheet, and bacterial growth was followed by monitoring the change in turbidity after intermittent vortexing. Bacterial growth was monitored over time as the increase in optical density at 690 nm (OD_{690}) using a

microplate reader (iMF reader, Dai Nippon Pharmacy, Osaka, JAPAN). Change in turbidity over time was then recorded to prepare growth curves.

Grouping of Growth Curves

Growth curves illustrating change in turbidity over time were grouped based on similarity by multiple classification analysis using the statistical software R (R Development Core Team) [4]. The degree of similarity was illustrated using a tree diagram (*i.e.*, dendrogram) and colored chart showing changes in turbidity (*i.e.*, heatmap).

Plant Material

Fronds and roots were collected from various types of pteridophytes and dandelions. Voucher specimens were deposited in the Herbarium of Showa Pharmaceutical University, Tokyo, Japan.

Extraction and separation of triterpenoids were carried out as follows: fresh fronds and roots were extracted with n-hexane three times to gibe extract [5]. The insoluble materials were filtered off. The extract was refluxed with benzene for 1 h. The insoluble materials were filtered off, and the filtrate was evaporated to dryness to afford an extremely viscous residue, which was then chromatographed on silica gel.

Triterpenoids were assigned by melting point, ^{1}H- and ^{13}C-NMR spectra, and mass spectrometry (MS). Melting points were measured on a Yanagimoto micro melting point apparatus without correction. ^{1}H- and ^{13}C-NMR spectra were acquired at 500 and 125 MHz, respectively, in $CDCl_3$ solution with tetramethylsilane as an internal standard. MS was performed (direct inlet) at 30 eV, and the relative intensities of peaks were reported with reference to the most intense peak above *m/z* 100.

Optical rotations were analyzed in $CHCl_3$ solution (C=0.1-0.3) at 22-24 °C.

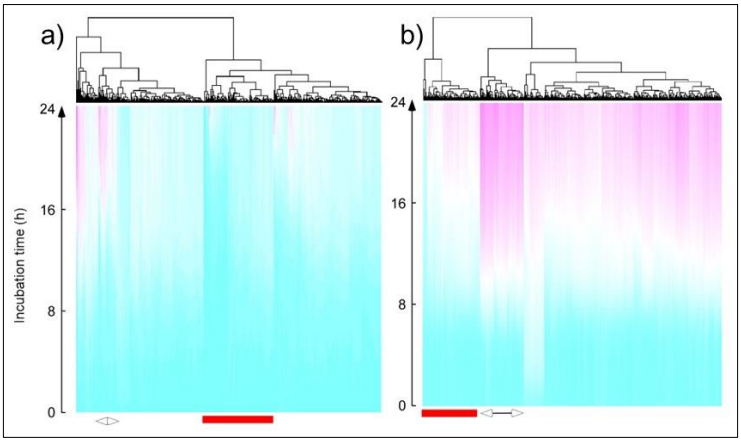
Time-Kill Assay

Aliquots of growth medium (100 µL) concentrated 2-fold, bacterial suspension (90 µL), and terpene solution (10 µL) were mixed in a 2000-µL microtube, and the suspension was incubated at 37 °C with inversion. Samples of 10 µL were withdrawn from the suspension at 0, 1, 3, and 5 h, diluted, and then spread on an agar medium plate. The plates were incubated at 37 °C for 20 h, after which the colonies appearing on the agar medium were counted. Time-Kill assays were also carried out with Davis minimum medium instead of 2-fold concentrated growth medium.

Results and Discussion

Screening methods to identify antibacterial agents have typically involved monitoring bacterial viability based on turbidity of liquid cultures or measurement of growth inhibition zones on solid media. These methods use only a single determination time point (*i.e.*, 20 h) and thus do not provide information regarding the status of bacteria during incubation [6, 7]. Therefore, it is not possible to differentiate reagents that enhance growth from reagents that slow growth when the turbidity of the suspension is measured at a single time point, for example, after reaching an optical density of 1.0 at 20 h. Growth curves, by contrast, provide an indication of the viable cell count or turbidity of the bacterial suspension over time, thereby reflecting the state of the bacterial cells in response to various factors. Growth curves prepared under conditions that neither accelerate nor inhibit growth are similar to curves for cultures with no reagent added to the medium (*i.e.*, control condition). When a growth curve differs from that of the control, this indicates that the cells were affected by the factor that differed between the two conditions. We generated growth curves showing the change in the turbidity of bacterial suspensions containing triterpenoids and classified the curves based on shape. A total of 150 different triterpenoids were examined.

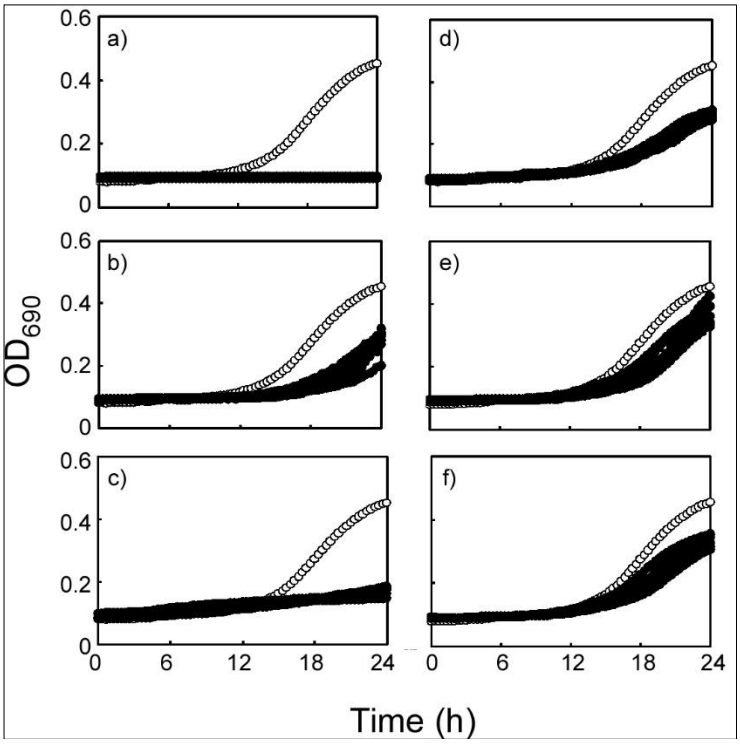
The results of the classification analyses are shown in Figures 1 a) and b). Each figure consists of two parts, an upper panel showing a dendrogram and a lower part showing a heatmap. The heatmaps indicate the change in turbidity visually, with blue corresponding to low turbidity and red corresponding to high turbidity. Nine combinations consisting of one triterpene and one bacterial species were examined. Three trials of nine combinations were carried out in the same 96-well plate, and the examination was repeated three times. Curve classification was carried out objectively using the statistical software R. Growth curves exhibiting high similarity clustered as a group, and those exhibiting low similarity were located distant from this group. When curves for more than six trials of one triterpenoid and one bacteria formed a group far from the group of curves for the control condition, the triterpenoid was deemed to have affected bacterial growth. The locations of the growth curves of the control condition and the inhibitory state are shown by arrows and red bars, respectively.



a) *S. aureus* FDA209P b) *E. coli* W3110
Concentration of triterpenoid: 25 µg/mL
Area indicated by arrows denotes growth curves of the no-reagent-added condition.
Area indicated by the red bar denotes growth curves indicating growth suppression.

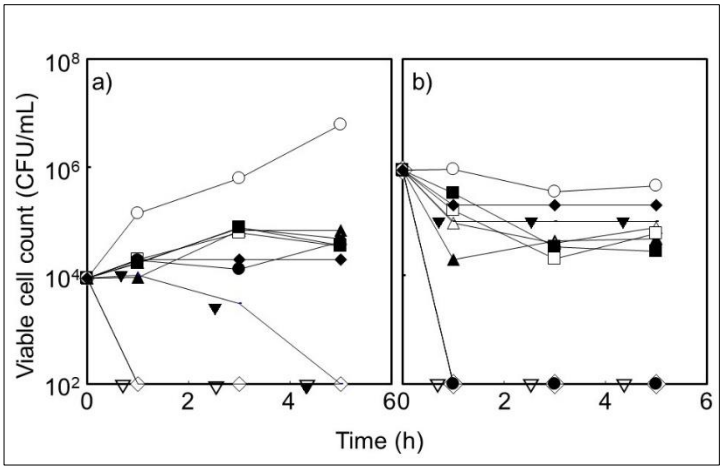
Fig 1: Heatmap and dendrogram of growth curves of in the presence of various triterpenoids

As shown in Figure 1a), six triterpenes clustered in the red bar area. The growth curves of *S. aureus* affected by these six triterpenoids are shown in Figure 2. Ursolic acid (a) and β↑onocerin (c) suppressed growth for 24 h, whereas glaucanol A (b) delayed growth for approximately 6 h, and other terpenes decreased the rate of growth even though the time of the start of exponential growth (increase in turbidity) was similar to the control (Figure 2). To more precisely characterize antibacterial activity, the change in viable cell number was measured using two different media (Figure 3). BHI medium is nutrient rich and enables *S. aureus* to grow. Davis minimum medium contains the minimum amount of nutrients necessary to keep bacteria alive but does not allow *S. aureus* to divide.



Concentration of triterpenoid: 25 μg/mL
a) ursolic acid, b) glaucanol A, c) sumaresinolic acid, d) glycol B, e) hydroxyhopane, f) β–onocerin

Fig 2: Growth curves of *S. aureus* FDA209P affected by various triterpenoids



a) BHI medium, b) Davis minimum medium
Concentration of triterpenoid: 25 μg/mL
open circle: control, open rhombus: ursolic acid, closed triangle: glycol B, open triangle: glaucanol A, open square: hydroxyhopane, closed square: sumaresinolic acid, closed circle: β–onocerin, inverted closed triangle: penicillin G, closed rhombus: tetracycline, inverted open triangle: mellitin

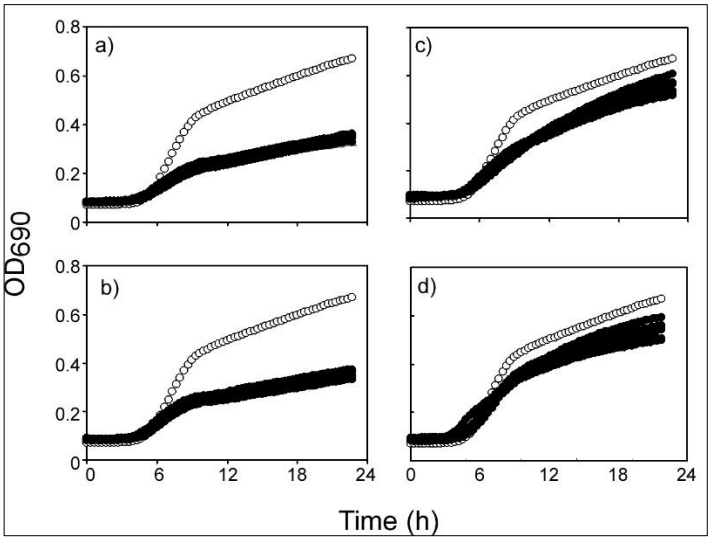
Fig 3: Time-Kill assays of triterpenoids against *S. aureus* FDA209P

The modes of action of various antibiotics are known and include inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis, and cell membrane disruption. Antibiotics are classified into two groups based on whether the mechanism is dependent on or independent of cell division. In medium that allowed cell division (Figure 3a), penicillin G exhibited bactericidal activity against *S. aureus* and decreased the viable cell count to zero within 1 h. However, penicillin G did not decrease the viable cell count of *S. aureus* in Davis minimum medium

(Figure 3b). In contrast, mellitin was able to disrupt the cell membrane independent of bacterial cell division (Figure 3a and b). The results shown in Figure 3 suggest that mellitin decreases the viable cell count of *S. aureus* independent of the status of the cells. By comparison, tetracycline exhibited bacteriostatic activity, with no marked change observed in the viable cell count.

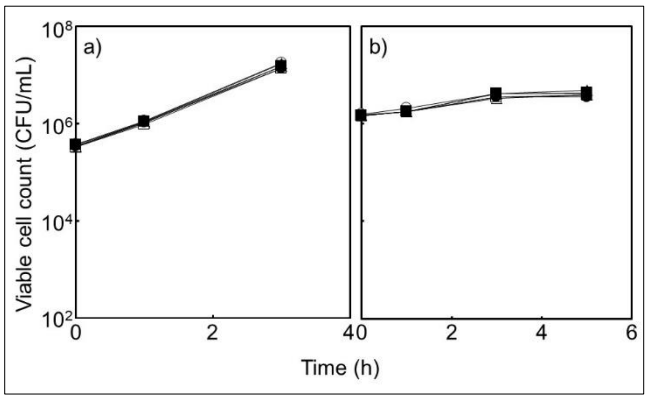
Ursolic acid decreased the viable cell count in both BHI medium and Davis minimum medium, similar to mellitin. Other triterpenes did not decrease the viable cell count (Figure 3a). The viable cell count in BHI medium was similar for all triterpenes. Ursolic acid decreased the viable cell count in Davis minimum medium, similar to mellitin. These results indicate that ursolic acid exhibits bactericidal activity against *S. aureus*. The growth curves for ursolic acid and sumaresinolic acid were similar (Figure 2a and c), but the change in viable cell count was different, perhaps due to a difference in the mode of action of these compounds.

In the screening against *E. coli*, the four triterpenes affected the shape of the growth curves. The growth curves for the four triterpenes are shown in Figure 4. None of the four triterpenes affected the timing of the start of exponential growth, but all four did decrease the rate of the growth and suppressed the maximum turbidity compared with the control. Figure 5 shows that none of the four triterpenes decreased the viable cell count in either medium. It is speculated that a change in the physical state of cells occurred, such as aggregation.



Concentration of triterpenoid: 25 μg/mL
a) glaucanol A, b) dryocrassol, c) 19-α-hydroxyisoadiantone, d) ketohanonanol

Fig 4: Growth curves of *E. coli* W3110 affected by various triterpenoids



a) MH medium, b) Davis minimum medium
Concentration of triterpenoid: 25 μg/mL
open circle: control, closed triangle: 19–α–hydroxyisoadiantone, open triangle: glaucanol A, open square: dryocrassol, closed square: ketohanonanol

Fig 5: Time-Kill assays of triterpenoids *E. coli* W3110

Ursolic acid and glaucanol A affected the growth of both *S. aureus* and *E. coli*, indicating that ursolic acid and glaucanol A affect both gram-positive and -negative bacteria. Common characteristics of the chemical structures of these compounds that are important for activity could facilitate the development of new broad-spectrum antibiotics.

These results are expected to play a key role in efforts to develop new antibiotics exhibiting high selective toxicity against *S. aureus*. The proposed screening method may accelerate this process.

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