Antioxidant and antibacterial activity of seven predominant terpenoids

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\textbf{ABSTRACT}

This study aimed to determine the efficacy of seven predominant wine terpenoids (i.e. \(\alpha\)-pinene, limonene, myrcene, geraniol, linalool, nerol, and terpineol) against foodborne pathogenic bacteria, as well as to observe their antioxidant activities. Antibacterial activities were observed against foodborne pathogenic bacteria. MIC\textsubscript{50} and MBC values for Escherichia coli, Salmonella enterica, and Staphylococcus aureus were in the ranges of 0.420–1.598 mg/mL and 0.673–3.432 mg/mL, respectively. The terpenoid \(\alpha\)-pinene showed the strongest DPPH free radical scavenging (IC\textsubscript{50} value = 12.57 ± 0.18 mg/mL) and the highest reducing power (213.7 ± 5.27 \(\mu\)g/mL of L-ascorbic acid equivalents). However, the DPPH free radical scavenging of the terpenoids was found to be lower than that of butylated hydroxytoluene, which is known to be a strong reducing agent. The seven predominant terpenoids in wines that were identified in this study could be new potential sources of natural antibacterial and antioxidant agents for use in the food industry.

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\textbf{Introduction}

A wide variety of aromatic substances are found in wines; the volatile composition is one of the most important parameters responsible for determining wine quality and consumer acceptance.\cite{1,2} Thus, studies have identified hundreds of species, such as terpenoids, aliphatic compounds, that are present in wine. Terpenoids are responsible for a typical aroma in wine, and they can, therefore, be used analytically for varietal characterization. Some popular wine products, such as Agudelo, Chardonnay, Gewurztraminer, Macabeo, Muscat, and Riesling, have characteristic terpenoids.\cite{3} The flavor threshold of nerol and terpineol is three to four times higher than that of linalool.\cite{4} Linalool has a flavor threshold of 25.2 \(\mu\)g/L.\cite{5} Pangzhen Zhang et al.\cite{6} reported five monoterpenoids, 24 sesquiterpenes, and four norisoprenoids were detected in grape samples. The terpenoids have also application to cancer treatment, prevention, cardioprotection, endocrine toxicity/ reproductive dysfunction, food supplement, traditional medicine, immunology, inflammation, menopausal syndromes, and neuroprotection.\cite{7} Foodborne pathogenic bacteria and oxidation in foods not only result in food deterioration and shelf life reduction but also lead to disease and economic losses. Currently, these two factors are considered major problems in the food industry and human nutrition. Many studies have demonstrated the antibacterial and antioxidant activities of wines\cite{8–10}, especially owing to essential oils or aromatic compounds found in them. Essential oils and their main components possess a wide spectrum of biological activity, which may be of great importance in several fields such as food chemistry, pharmacology, as well as pharmaceutics.\cite{11} The main advantage of essential oils is that they can be used in any...
foods and are generally recognized as safe (GRAS). However, the antibacterial activity of terpenoids in wines against foodborne pathogenic bacteria is yet to be studied, in addition to their potential antioxidant properties. Previous literature has only explored these properties in some essential oils. The terpenoids – α-pinene, limonene, myrcene, geraniol, linalool, nerol, and terpineol – have been identified as the seven most predominant terpenoids in wines.

Synthetic food additives generate a negative perception in consumers. Therefore, food manufacturers search for safer natural alternatives such as plant essential oils. The objective of this study was to determine the antibacterial activity of these seven predominant wine terpenoids. These activities were tested against typical foodborne pathogenic bacteria (*Escherichia coli*, *Salmonella enterica*, and *Staphylococcus aureus*). DPPH free radical and reducing power assays were used to evaluate the antioxidant activities of the generated mutants.

**Materials and methods**

**Samples**

Myrcene, limonene, geraniol, linalool, and nerol were purchased from Tokyo Chemical Industry (Tokyo, JAPAN); α-pinene was purchased from Sigma-Aldrich (St. Louis, MO, USA), and terpineol from ACROS Organics (Pittsburgh, PA, USA).

**Chemicals**

L-Ascorbic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (St. Louis, MO, USA), potassium hexacyanoferrate and butylated hydroxyanisole (BHA) were from Acros Organics (Pittsburgh, PA, USA), iron(III) chloride from Showa (Saitama, JAPAN), and trichloroacetic acid (TCA) from Alfa Aesar (Ward hill, MA, USA).

**Antibacterial activity**

**Microbial strains**

*Escherichia coli*, *Salmonella enterica*, and *Staphylococcus aureus* were used in all experiments. These strains represent foodborne pathogenic bacteria. These strains were supplied by the author’s laboratory, National Kaosiung University Science and Technology. Strains were stored at −80°C through cryopreservation. For preparation of the inoculum, strains were cultured in tryptone soya broth (TSB) (Oxoid, Basingstoke, Hampshire, England) overnight. All microbial cultures were subcultured before the assay and were later diluted with phosphate buffer saline (pH 7.4) to obtain a microbial suspension of 1.00 × 10^6 colony-forming units (CFUs)/mL for further experiments.

**Minimum inhibitory concentration (MIC_{50}) assay**

The minimum inhibitory concentration (MIC_{50}) was determined using the agar dilution method described in a previous report with slight modifications. Samples were diluted with sterilized 10% Tween-80 to a final concentration of 8 mg/mL to 0.4 mg/mL. Each dilution was inoculated with 100-µl aliquots of bacterial culture with a concentration of approximately 1.00 × 10^6 CFU/mL. This was done for each of the strains in triplicate, followed by incubation at 37°C for 16 h at 125 rpm. Sterilized 10% (v/v) Tween-80 without the inoculum was used as the growth control, while the medium without the inoculum was used as the blank control (negative). The growth in each test group was compared with that of the growth control. The MIC_{50} was defined as the lowest concentration of test samples with a growth reduction greater than or equal to 50%, compared with the growth control.
**Minimum bactericidal concentration (MBC) assay**

MBC was defined as the lowest concentration of the samples wherein no visible turbidity or colonies were observed. In order to determine the MBC, the solution for concentration being tested was spread on sterile PCA (Oxoid, Basingstoke, Hampshire, England) plates and incubated at 37°C for 24 h. This was done for all three bacterial strains in triplicate.

**Time-kill curve assay**

The time-kill curve assay was conducted based on methods used in the previous research with slight modifications. Experiments were performed using 25 mL of TSB (Oxoid, Basingstoke, Hampshire, England) broth and inoculated with the sub-cultured sample to obtain a solution with approximately 1.00 × 10⁶ CFU/mL as the final concentration. Time-kill curve assay was conducted for each bacterial strain using the predetermined concentrations of MIC.

Samples for viable count determination were taken during the experiment at the 0, 1, 2, 4, 8, 12, 16, and 24 h during the experiment. Each sample was serially diluted and pour plating was used to prepare the plates. The number of CFUs per mL were determined following an incubation at 37°C for 24 h. Samples were compared with growth control (un-inoculated sterilized 10% (v/v) Tween-80) and the time-kill curves were generated using the log of CFU/mL over time. This was done for all three bacterial strains in triplicate.

**Antioxidant activity**

**2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity**

The free radical scavenging activity was determined using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method. The hydrogen atom or electron donation ability of the sample was measured using a methanol solution of 2,2-Diphenyl-1-picrylhydrazyl (DPPH). A 20-µL aliquot of different sample concentrations (6.25–50 mg/mL) was mixed with 180 µL of DPPH solution (180 µM) in methanol in a 96-well plate.

DPPH solution was used as the blank sample, and BHA was used as positive control. After 40 min of incubation in the dark at room temperature, the samples were observed using a 96-well plate reader (HT 340 Elisa reader, Bio Tec) to monitor the decrease in absorbance at 517 nm. This was done for all three bacterial strains in triplicate. The free radical scavenging activity was expressed as the percentage inhibition of DPPH and was calculated using the following formula:

\[
\text{Inhibition percentage (\%)} = \left(\frac{(A_0 - A_A)}{A_0}\right) \times 100
\]

where \(A_0\) and \(A_A\) are the absorbance values of the blank and test samples, respectively. The absorbance of the samples was measured at 517 nm against a blank and IC₅₀ values were calculated using linear regression analysis. IC₅₀ value was defined as concentration required for 50% inhibition, as compared to the control.

**Reducing power assay**

A sample volume of 1 mL was mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1% potassium ferricyanide. This mixture was then incubated at 50°C for 20 min. This was then subjected to centrifugation at 3000 rpm for 10 min. A volume of 1 mL trichloroacetic acid solution (10%) was then added to the mixture followed by centrifugation again at 3000 rpm for 10 min. A volume of 100 µL supernatant was mixed with 100 µL of distilled water and 100 µL of ferric chloride (0.1%), and the absorbance was measured at 700 nm. This was done for all three bacterial strains in triplicate. L-Ascorbic acid was used as a positive control was used to generate a linear regression analysis for the calibration curve. The results of reducing power were expressed in micrograms of L-ascorbic acid equivalents per milliliter (µg/mL).
Statistical analysis

Data were expressed as mean ± standard deviation of three independent replicates. Statistical analysis was performed using the software SPSS for Windows 14.0. All data were analyzed using one-way analysis of variance (ANOVA). Post hoc analysis of group differences was performed by Tukey’s multiple comparison tests. The criterion for significance was set at p < 0.05.

Results and discussion

Antibacterial activity

Comparative antibacterial activity results of the seven predominant terpenoids in wines are shown in Tables 1 and 2. The three test microorganisms that were used to represent foodborne bacteria included one Gram-positive bacterium, *Staphylococcus aureus*, and two Gram-negative bacteria, *Escherichia coli* and *Salmonella enterica*. MIC\(_{50}\) values ranging from 0.420 to 0.747 mg/mL were obtained for the seven terpenoids against *Staphylococcus aureus*, 0.421–0.747 mg/mL for *Escherichia coli*, and 0.420–1.598 mg/mL for *Salmonella enterica*, as shown in Table 1. For all seven terpenoids, the concentration of 3.432 mg/mL was determined to be the MBC, as shown in Table 2. At a concentration of 0.421 mg/mL, limonene showed the strongest antibacterial activity among the three terpenoids without a hydroxyl group – α-pinene, limonene, and myrcene. These results are similar to those reported previously.\[^{22}\] Nerol and geraniol exhibited similar antibacterial activity among the four terpenoids with a hydroxyl group – geraniol, linalool, nerol, and terpineol. The effects of bioactivity could be due to the diastereomeric structure.\[^{23,24}\]

| Compounds | MIC(mg/mL) | *Escherichia coli* | *Salmonella enterica* | *Staphylococcus aureus* |
|-----------|------------|--------------------|-----------------------|------------------------|
| α-Pinene  | 0.421\(^aE\) | 0.421\(^aE\)      | 0.421\(^aC\)          |
| Limonene  | 0.686\(^aB\) | 0.686\(^aB\)      | 0.420\(^bC\)          |
| Myrcene   | 0.635\(^aD\) | 1.598\(^aA\)      | 0.635\(^aD\)          |
| Geraniol  | 0.445\(^aC\) | 0.445\(^aC\)      | 0.445\(^aC\)          |
| Linalool  | 0.686\(^aB\) | 0.420\(^aE\)      | 0.420\(^aC\)          |
| Nerol     | 0.441\(^aC\) | 0.441\(^aC\)      | 0.441\(^aC\)          |
| Terpineol | 0.747\(^aA\) | 0.462\(^aC\)      | 0.747\(^aA\)          |

Values within the row followed by the different small letters are significantly different (p < 0.05) by Tukey’s multiple range test.

Values within the column followed by the different capital letters are significantly different (p < 0.05) by Tukey’s multiple range test.

| Compounds | MBC(mg/mL) | *Escherichia coli* | *Salmonella enterica* | *Staphylococcus aureus* |
|-----------|------------|--------------------|-----------------------|------------------------|
| α-Pinene  | 3.432\(^aA\) | 1.716\(^bB\)      | 1.716\(^bD\)          |
| Limonene  | 1.683\(^aF\) | 0.673\(^bF\)      | 1.682\(^aE\)          |
| Myrcene   | 3.176\(^aB\) | 3.176\(^aA\)      | 3.176\(^aA\)          |
| Geraniol  | 1.778\(^aD\) | 0.711\(^bD\)      | 1.778\(^aC\)          |
| Linalool  | 1.716\(^aE\) | 0.686\(^bE\)      | 1.716\(^aD\)          |
| Nerol     | 1.762\(^aD\) | 0.705\(^bD\)      | 1.762\(^aC\)          |
| Terpineol | 1.868\(^aC\) | 0.747\(^aC\)      | 1.868\(^bB\)          |

Values within the row followed by the different small letters are significantly different (p < 0.05) by Tukey’s multiple range test.

Values within the column followed by the different capital letters are significantly different (p < 0.05) by Tukey’s multiple range test.
of MIC\textsubscript{50} and MBC values, Gram-positive bacteria were observed to be slightly more sensitive than Gram-negative bacteria, possibly due to the hydrophilic cell wall structure of Gram-negative bacteria.\cite{25} The cell wall of Gram-negative bacteria is constituted mainly by a lipo-polysaccharide, which blocks the penetration of hydrophobic components of terpenoids and for this reason, Gram-positive bacteria are found to be more sensitive to the seven terpenoids.\cite{26}

Furthermore, the time-kill curve assay was used to determine the antibacterial activity at the predetermined MIC\textsubscript{50} values. The time-kill dynamic process of seven predominant terpenoids in wines against all three bacterial samples is presented in Figure 1. At the time points of 16 h to 24 h, the antibacterial activity of all seven predominant terpenoids was shown to reach a plateau, while the

Figure 1. Time.kill curve of seven predominant terpenoids in wines against foodborne pathogenic bacteria (Escherichia coli, Salmonella enterica and Staphylococcus aureus) at concentrations of MIC\textsubscript{50}. The time.kill curve of used for α-pinene (a), limonene (b), myrcene (c), geraniol (d), linalool (e) nerol (f), terpineol (g) and control (h).
growth control showed a steep increase in antibiotic activity. Therefore, we found that the seven predominant terpenoids in wines exhibited effective antibacterial activity against all three bacterial samples at their predetermined MIC\textsubscript{50} after 16 h.

**Antioxidant activity**

Increases in free radicals in the body have been implicated in damaging human life.\textsuperscript{[27,28]} Antioxidant properties are important in counteracting the deleterious effects of free radicals in foods and biological systems.\textsuperscript{[29]} For this reason, the antioxidant activity of seven predominant terpenoids in wines was determined using two methods, the determination of DPPH free radical scavenging activity and the reducing power assay.

The DPPH free radical is characterized as a stable free radical, which has been widely accepted as a method for estimating the free radical scavenging activity of antioxidant.\textsuperscript{[30]} Both are spectrophotometric methods for determining antioxidant activity, based on electron transfer reactions, which visually relies on the reduction of a colored oxidant, commonly used to investigate the in vitro antioxidant capacity of foods and biological samples.\textsuperscript{[31]} The results obtained from these methods present an excellent correlation.

The results of the assessment of the antioxidant activity by DPPH free radical scavenging activity are shown in Figure 2. In this study, the antioxidant activity of seven predominantly terpenoids in wines were expressed as IC\textsubscript{50}, wherein a low IC\textsubscript{50} value indicates that the sample is an effective DPPH scavenger.

The DPPH free radical scavenging activity measures the ability of the sample to donate hydrogen to the DPPH radical, resulting in bleaching of the stable DPPH free radical from the purple color of DPPH cation to the yellow-colored diphenylpricyhydrizinze. The greater the bleaching action observed, the higher the antioxidant activity, which is reflected in a lower IC\textsubscript{50}.\textsuperscript{[32]}

All seven terpenoids were determined to be effective DPPH scavengers with \(\alpha\)-pinene showing the strongest DPPH free radical scavenging effect at 12.57 ± 0.18 mg/mL. This was followed by limonene at 13.35 ± 0.26 mg/mL and nerol at 26.08 ± 2.28 mg/mL. Myrcene (40.83 ± 1.68 mg/mL) showed the lowest scavenging activity. The determined IC\textsubscript{50} values were in the order BHT < \(\alpha\)-pinene < limonene < nerol < terpinol < geraniol < linalool < myrcene.

![Figure 2. IC\textsubscript{50} values for DPPH free radical scavenging activity of seven predominant terpenoids in wines. Results are the mean of three separate determinations ± SEM.](image)
The reducing power of antioxidants is an important indicator of potential antioxidant activity. The antioxidant effect exponentially increases as a function of the reducing power, indicating that the antioxidant properties are concomitant with the development of reducing power.\(^{[33]}\) In the reducing power assay, the presence of reductants (antioxidants) in the seven predominant wine terpenoids causes the reduction of the Fe\(^{3+}\) or ferricyanide complex to its ferrous form.\(^{[34]}\)

The reducing power activity of the seven predominant wine terpenoids was determined using L-ascorbic acid as a standard. Results were expressed as micrograms of L-ascorbic acid equivalents per milliliter (\(\mu\)g/mL). The reducing power of the seven predominant wine terpenoids is presented in Figure 3. Among them, \(\alpha\)-pinene showed the highest reducing power at 213.7 ± 5.27 \(\mu\)g/mL, while myrcene showed the lowest reducing power at 21.59 ± 1.14 \(\mu\)g/mL. The reducing power was in the order: \(\alpha\)-pinene (213.7 ± 5.27 \(\mu\)g/mL) > limonene (133.48 ± 6.22 \(\mu\)g/mL) > nerol (79.15 ± 3.75 \(\mu\)g/mL) > terpinol (73.92 ± 3.34 \(\mu\)g/mL) > geraniol (73.78 ± 3.94 \(\mu\)g/mL) > linalool (43.97 ± 1.09 \(\mu\)g/mL) > myrcene (21.59 ± 1.14 \(\mu\)g/mL).

The results of the reducing power test were shown to agree with the results of the DPPH free radical scavenging activity. Additionally, a similar correlation or relative activity was observed between the DPPH free radical scavenging activity and the reducing power assay, indicating that the test samples had comparable activities in two independent methods for the determination of antioxidant activity. The antioxidant in vivo effects of \(\alpha\)-pinene has previously been reported using the peroxidation assay.\(^{[35]}\)

In a separate study, the antioxidant activity of limonene has been demonstrated to have an important role in the ulcerative colitis (UC) rat model.\(^{[36]}\) Therefore, our results suggest that these seven predominant wine terpenoids have significant antioxidant activity. These results can be used as a reference for the food industry and nutrition in the future.

**Conclusion**

In the present study, antibacterial activity and antioxidant activity of seven predominant wine terpenoids were observed. \(\alpha\)-pinene was found to have the highest antioxidant activity, as determined through DPPH free radical activity and reducing power, while limonene was found to have the highest antibacterial activity against Gram-positive/Gram-negative foodborne pathogenic bacteria. The seven predominant wine terpenoids displayed effective antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enterica* at a predetermined MIC\(_{50}\) after the 16-h timepoint. In addition, the samples’ DPPH free radical scavenging effectiveness is of main interest.

![Figure 3](image-url). Reducing power assay of seven predominant terpenoids in wines. Results are the mean of three separate determinations ± SEM and expressed as ascorbic acid equivalents (\(\mu\)g/mL).
to the food industry as oxidation in foods is accepted to be a major factor in determining food quality. For this reason, the use of seven predominant wine terpenoids in foods will require the identification of bioactive compounds as well as the determination of their mechanism of action. These could potentially be a source for natural, raw materials for nutrient products, cosmetic products, and food additives. In conclusion, this study verified the antibacterial activity and antioxidant activity of seven predominant wine terpenoids, which can be used as a helpful reference for the food industry in the future. The authors declare no conflicts of interest.

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