*Brief communication*

# Antibacterial abietane-type diterpenoid, taxodone from *Metasequoia glyptostroboides* Miki ex Hu

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In an attempt to isolate bioactive constituents, ethyl acetate cone extract of *Metasequoia glyptostroboides* was subjected to a column chromatographic analysis that resulted in isolation of an abietane-type diterpenoid, taxodone. Its structure was elucidated by spectroscopic means. Further, taxodone showed potential antibacterial effect as diameters of zones of inhibition against foodborne pathogenic bacteria, such as *Listeria monocytogenes* ATCC 19166, *Salmonella typhimurium* KCTC 2515, *S. enteritidis* KCTC 2021, *Escherichia coli* ATCC 8739, *E. coli* O157:H7 ATCC 43888, *Enterobacter aerogenes* KCTC 2190, *Staphylococcus aureus* ATCC 6538 and *S. aureus* KCTC 1916, were found in the range of 9.4 to 14.2 mm. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of taxodone against the employed bacterial pathogens were found in the range of 250 to 1000 and 250 to <2000 $\mu$g/ml, respectively. Also the compound had a strong antibacterial effect on the viable counts of the tested bacteria. These findings indicate that the bioactive compound taxodone present in *M. glyptostroboides* could be used as an antibacterial agent in food industry to inhibit the growth of certain important foodborne pathogens.

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## 1. Introduction

Plants contain a great number of secondary metabolites, many of which display biological activity as "natural products" with a role in plant defense against bacteria, fungi and other microorganisms. There has been a growing interest in new and effective antibacterial substances from natural sources like plants to reduce cases of bacterial diseases. In many cases, antibacterial compounds from plants, herbs and spices in low concentrations have been used effectively in the food industry. Biologically active secondary metabolites of spices, herbs and medicinal plants, due to their diverse range of antibacterial activities, are becoming increasingly important in food industry as potential food preservatives to control foodborne pathogenic bacteria. Several terpenoid compounds have been isolated from many plant species and reported to have various biological activities (Gao and Han 1997; Singh and Singh 2003).

Foodborne pathogenic bacteria cause several foodborne diseases (Tauxe 2002). The indiscriminate and excessive use of a wide range of chemical bactericides has led to extended environmental pollution and the production of resistant pathogen populations. During the last few years, research on plant-based antibacterial agents has produced a diverse range of products with novel modes of action, which are expected to have a significant impact on the control of foodborne diseases in coming decade. Therefore, the demand for plant based antimicrobials as novel bactericide sources for controlling foodborne pathogens is rapidly increasing (Ulubelen *et al*. 2000; Kubo *et al*. 2004; Giang *et al*. 2006).

Some synthetic chemicals have been used to control microbial growth and reduce the incidence of foodborne

**Keywords.** Antibacterial activity; diterpenoid; foodborne pathogens; *Metasequoia glyptostroboides*; taxodone

Abbreviations used: DMSO, dimethylsulphoxide; KFDA, Korea Food and Drug Administration; LB, Luria–Bertani; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration

illness. Although these synthetic preservatives are effective, they might be detrimental to human health. Consumers are concerned about the safety of food products that contain artificial preservatives. Recently, the ban of chemical additives by some consumers has driven the food industry and food research towards the search for natural antibacterial compounds from plant origin. Many natural compounds (e.g. terpenoids) found in dietary plants possess antibacterial activities against foodborne pathogens (Dellar *et al*. 1996; Singh and Singh 2003; Aiyelaagbe *et al*. 2007).

*Metasequoia glyptostroboides* Miki ex Hu is a deciduous conifer of the redwood family of Cupressaceae. It is only living species in the genus *Metasequoia* propagated and distributed in many parts of eastern Asia and North America as well as Europe. Previously we reported various biological properties of organic extracts and essential oils from *M. glyptostroboides* such as antibacterial (Bajpai *et al*. 2007a; Bajpai and Kang 2009b), antioxidant/antibacterial (Bajpai *et al*. 2009a), antidermatophytic (Bajpai *et al*. 2009c) and antifungal (Bajpai *et al*. 2007b) activities. However, there is no report available in the literature on the biologically active constituents of *M. glyptostroboides* and their antibacterial properties against foodborne pathogenic bacteria.

In this study, we report for the first time an abietane-type diterpenoid, taxodone from the ethyl acetate cone extract of *Metasequoia glyptostroboides* and evaluated its efficacy as a potential antibacterial agent against some important foodborne pathogens.

## 2. Materials and methods

### 2.1 *General*

Melting points were recorded on a Gallenkamp apparatus. Optical rotations were measured using a JASCO DIP-1000 (Tokyo, Japan) automatic digital polarimeter. IR spectra (KBr) and UV spectra (MeOH) were obtained from Shimadzu DR-8001 IR and LKB 4053 UV spectrophotometer, respectively. 1D and 2D NMR spectra were measured in $CDCl_3$ on a Brucker ARX 250 MHz instrument with TMS as an internal standard. Purity of the compounds was checked by TLC and HPLC.

### 2.2 *Microorganisms*

Microorganisms as foodborne pathogenic bacteria such as *Listeria monocytogenes* ATCC 19166, *Salmonella typhimurium* KCTC 2515, *S. enteritidis* KCTC 2021, *Escherichia coli* ATCC 8739, *E. coli* O157:H7 ATCC 43888, *Enterobacter aerogenes* KCTC 2190, *Staphylococcus aureus* ATCC 6538 and *S. aureus* KCTC 1916 were used in this study. The bacterial pathogens were obtained from the Korea Food and Drug Administration (KFDA), and were maintained on Luria–Bertani (LB) agar medium at 4°C.

### 2.3 *Plant material*

The cones of *M. glyptostroboides* were collected from the local area of Pohang, Republic of Korea, in November and December 2008, and identified by the morphological features and the database present in the library at the Department of Biotechnology. A voucher specimen (DUB-0038) was deposited in the herbarium of College of Engineering, Department of Biotechnology, Daegu University, Republic of Korea.

### 2.4 *Extraction and isolation*

Dried cones of *M. glyptostroboides* (2 kg) were milled into powder and then extracted with ethyl acetate at room temperature for 12 days. The extract was evaporated under reduced pressure using a rotary evaporator (EYELA N1000). The dried ethyl acetate extract (7 g) was subjected to column chromatography over silica gel (230–400 mesh, Merck, Germany) and was eluted with hexane-ethyl acetate-methanol solvent system to give 20 fractions. Of the fractions obtained, the fraction-12 was further purified by preparative TLC over silica gel GF254 by using hexane-ethyl acetate (2:1) as a mobile phase to give one compound (96 mg).

### 2.5 *Taxodone*

The purified compound was crystallized from *n*-hexane-methanol to yield yellow round crystals (96 mg), mp 176–177°C; $^1$H NMR (pyridine-$d_5$, 250 MHz) $\delta$: 5.30 (1H, m, $W_{1/2}$ = 23), 3.45 (1H, d, $J$ = 2.5 Hz), 3.19 (1H, br s), 7.00 (1H, septet), 2.51 (1H, s, 11-OH), 8.78 (9H), 8.83 (3H), 8.90 (3H); $^{13}$C NMR (pyridine-$d_5$, 250 MHz) $\delta$: 181.6 (C-1), 149.3 (C-2), 143.3 (C-3), 141.3 (C-4), 135.7 (C-5), 130.3 (C-6), 126.2 (C-7), 77.5 (C-8), 77.0 (C-9), 76.4 (C-10), 69.9 (C-11), 57.8 (C-12), 43.1 (C-13), 40.6 (C-14), 36.6 (C-15), 34.1 (C-16), 26.6 (C-17), 21.3 (C-18), 20.7 (C-19), 18.7 (C-20).

### 2.6 *Antibacterial activity assay*

Standard agar diffusion method was used for antibacterial assay (Murray *et al*. 1995). Petri plates were prepared by pouring 20 ml of LB medium and allowed to solidify. Plates were dried, and 100 $\mu$l of standardized inoculum containing $10^7$ CFU/ml of bacterial suspension was poured and uniformly spread. The inoculum was allowed to dry for 5 min. A Whatman No. 1 sterile filter paper disc (6 mm diameter) was impregnated with 50 $\mu$g/disc of the compound

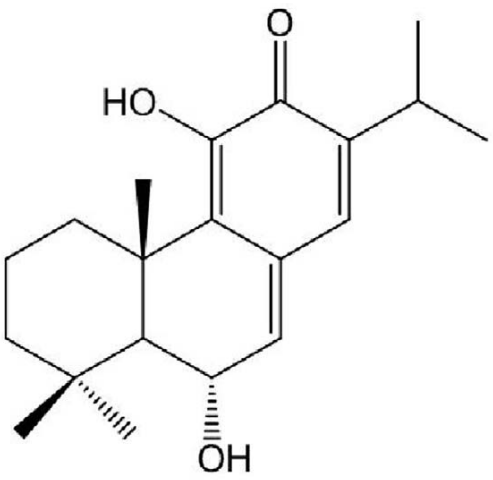


**Figure 1.** Chemical structure of taxodone isolated from the ethyl acetate cone extract of *Metasequoia glyptostroboides*.

taxodone. The compound was dissolved in 5% DMSO. Negative controls were prepared using the same solvent that was employed to dissolve the sample. A standard reference antibiotic, streptomycin (10 $\mu$g/disc, from Sigma-Aldrich Co., St. Louis, MO, USA), was used as the positive control for the tested bacteria. The plates were incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the diameter of the zones of inhibition against the tested bacteria. Each assay in this experiment was replicated three times.

### 2.7 *Determination of minimum inhibitory and bactericidal concentrations*

The minimum inhibitory concentration (MIC) of the compound was tested by a two-fold serial dilution method (Bajpai *et al*. 2009a). The test compound was first dissolved in dimethylsulphoxide (DMSO), incorporated into LB medium for bacterial pathogens to obtain a concentration of 4000 $\mu$g/ml and serially diluted to achieve 2000, 1000, 500, 250 125, 62.5, 31.25, 15.62 and 7.81 $\mu$g/ml. The final concentration of DMSO in the culture medium was maintained at 0.5% (v/v). A 10 $\mu$l standardized suspension of each tested organism (approximately $10^7$ CFU/ml) was transferred to each tube. The control tubes containing only bacterial suspensions were incubated at 37°C for 24 h. The lowest concentration of the compound, which did not show any growth of test organisms after macroscopic evaluation, was determined as MIC, and was expressed in $\mu$g/ml. Further, the concentrations showing complete inhibition of visual growth of bacterial pathogens were identified, and 50 $\mu$l of each culture broth was transferred on to the agar plates and incubated for specified time and temperature, as mentioned above. The complete absence of growth of bacterial colonies on the agar surface in the lowest concentration of sample was defined as the minimum bactericidal concentration (MBC). Each assay in this experiment was replicated three times.

### 2.8 *Effect of taxodone on viable count of the tested bacteria*

Active cultures for viable count assay were prepared in LB broth medium (Bajpai et al. 2009a). For each strain, 1 ml of active stock solution was transferred to 4 ml of LB broth. All treated cultures were kept at 37°C for 2 h. The cultures were then centrifuged at 10000 rpm for 10 min. The pellets were retained and re-suspended with 1 ml of phosphate-buffered saline. For viable counts, each of the tubes containing re-suspended bacterial suspension (approximately $10^7$ CFU/ml) of *L. monocytogens* ATCC 19166, *E. coli* ATCC 8739, *S. aureus* ATCC 6538 and *E. aerogenes* KCTC 2190 was inoculated with 250 $\mu$g/ml concentration of the compound in 10 ml LB broth and kept at 37°C. Samples for viable cell counts were taken out at 0, 20, 40, 60, 80, 100 and 140 min time intervals. The viable plate counts were monitored as follows: After incubation, 1 ml of the re-suspended culture was diluted in 9 ml buffer peptone water, thereby diluting it 10-fold. Also, 0.1 ml sample of each treatment was diluted and spread on the surface of LB agar. The colonies were counted after 24 h of incubation at 37°C. The controls were inoculated without the compound for each bacterial strain with the same experimental condition as mentioned above. Each assay in this experiment was replicated three times.

## 3. Results and discussion

### 3.1 *Taxodone*

The ethyl acetate cone extract of *M. glyptostroboides* after column chromatography over silica gel yielded a pure compound which was obtained as yellow glass crystal with a specific melting point (mp 176–177°C). The $^1$H NMR data (pyridine-$d_5$, 250 MHz) showed the presence of two hydroxyl groups at 3.0 and 2.77. The quinonoid carbonyl, hydrogen bonded to the hydroxyl group at C-11 (doublet at 6.14 and 6.18), was also present. The NMR spectrum established the presence of the C-15 H, the C-11 enolic hydroxyl group, the C-14 H and the C-7 H. The one-proton broad signal was present at 5.30 ($W_{1/2}$ = 23 Hz), which was assigned to the C-6 $\beta$-axial methine proton. Irradiation at 5.31 resulted in collapse of a doublet at 3.45 (C-7 H), confirming that this olefinic proton was coupled to the C-6 methine proton. Also, the $^{13}$C NMR data displaying 20 carbon signals including one carbonly group strongly suggested this compound to be an abietane diterpenoid. The structure of this compound was determined to be taxodone by 1D and 2D NMR analysis,

and confirmed by comparing the physical and spectroscopic data with those in the literature (Kupchan *et al*. 1969). However, this is the first report on the isolation of taxodone from the cones of *M. glyptostroboides* and evaluation of its antibacterial activity against foodborne pathogenic bacteria.

### 3.2 *Antibacterial activity*

The antibacterial activity of the compound taxodone against the employed bacteria was qualitatively and quantitatively assessed by the presence or absence of inhibition zones. As shown in table 1, the compound taxodone (50 $\mu$g/disc) exhibited potent inhibitory effect against the tested bacterial pathogens. *L. monocytogens* ATCC 19166, *S. aureus* ATCC 6538 and *S. aureus* KCTC 1916 were found to be most inhibited by taxodone, with their respective diameter zones of inhibition of 14.2, 12.2 and 12.6 mm, whereas *S. typhimurium* KCTC 2515, *S. enteritidis* KCTC 2021, *E. coli* O157:H7 ATCC 43888, *E. coli* ATCC 8739 and *E. aerogenes* KCTC 2190 were inhibited moderately, with diameter of zones of inhibition of 10.2, 9.8, 9.4, 10.3 and 10.2 mm, respectively (table 1). In an earlier study conducted by Peres *et al.* (1997), it has been reported that terpenoid compounds isolated from *Croton uracurana* exhibited potential antibacterial effect against some representative foodborne pathogenic bacteria such as *Staphylococcus aureus* and *Salmonella typhimurium*. Four pentacyclic triterpenoids isolated from *Combretum imberbe* have been reported to exhibit antibacterial activity against *Staphylococcus aureus* (Katerere *et al*. 2003). In this study, the compound taxodone exhibited a higher antibacterial activity than that of standard streptomycin in regard to gram-positive bacteria; however, gram-negative bacteria were also inhibited to some extent by taxodone (table 1).

### 3.3 *Minimum inhibitory and minimum bactericidal concentrations*

The compound taxodone showed potent inhibitory effect in terms of MIC and MBC values against all the tested bacterial pathogens. As shown in table 2, the MIC and MBC values of taxodone against the tested bacterial pathogens were found in the range of 250 to 1000 and 250 to <2000 $\mu$g/ml, respectively. *L. monocytogenes* ATCC 19166 and *S. aureus* KCTC 1916 were found highly susceptible bacterial pathogens to the taxodone having MIC and MBC values of 250 and 250 $\mu$g/ml, respectively, for each bacterial pathogen. Taxodone also had potential antibacterial effect against the remaining bacterial pathogens tested. Similarly, Mathabe *et al.* (2008) reported the antibacterial activity of plant-based terpenoid compounds against a panel of foodborne pathogenic bacteria, showing their potential antibacterial effect.

### 3.4 *Effect of taxodone on viable counts*

Further study was carried out to evaluate the effect of the compound taxodone on the viable count of some Gram-negative and Gram-positive bacteria such as *L. mono-cytogens* ATCC 19166, *E. coli* ATCC 8739,

**Table 1.** Antibacterial activity of taxodone against foodborne pathogens isolated from *M. glyptostroboides*

| | [a]Diameter of zones of inhibition (mm) | |
|---|---|---|
| Bacterial pathogen | Taxodone | [b]SM |
| *Listeria monocytogenes* ATCC 19166 | 14.2 ± 0.5a | 14.0 ± 0.7b |
| *Salmonella typhimurium* KCTC 2515 | 10.2 ± 0.7c | 13.0 ± 0.2c |
| *Salmonella enteritidis* KCTC 2021 | 9.8 ± 0.4d | 14.0 ± 0.6b |
| *Escherichia coli* ATCC 8739 | 10.3 ± 0.2c | 24.0 ± 0.7a |
| *Escherichia coli* O157:H7 ATCC 43888 | 9.4 ± 0.3c | 15.0 ± 0.9b |
| *Staphylococcus aureus* ATCC 6538 | 12.2 ± 0.5b | 14.0 ± 0.6b |
| *Staphylococcus aureus* KCTC 1916 | 12.6 ± 0.7b | 14.0 ± 0.9b |
| *Enterobacter aerogenes* KCTC 2190 | 10.2 ± 0.3c | 13.0 ± 0.2c |

[a] Diameter of inhibition zones of taxodone (tested volume 50 $\mu$g/disc); [b] Streptomycin (10 $\mu$g/disc).

Values followed by the different letters (a, b, c and d) are significantly different ($P$ >0.05).

**Table 2.** Determination of MIC and MBC of taxodone against foodborne pathogens isolated from *M. glyptostroboides*

| | | Taxodone | |
|---|---|---|---|
| Bacterial pathogen | Gram- | MIC[a] | MBC[b] |
| *Listeria monocytogenes* ATCC 19166 | + | 250 | 250 |
| *Salmonella typhimurium* KCTC 2515 | – | 500 | 500 |
| *Salmonella enteritidis* KCTC 2021 | – | 1000 | <2000 |
| *Escherichia coli* ATCC 8739 | – | 500 | 500 |
| *Escherichia coli* O157:H7 ATCC 43888 | – | 1000 | <2000 |
| *Staphylococcus aureus* ATCC 6538 | + | 500 | 500 |
| *Staphylococcus aureus* KCTC 1916 | + | 250 | 250 |
| *Enterobacter aerogenes* KCTC 2190 | – | 500 | 500 |

[a]Minimum inhibitory concentration (values in $\mu$g/ml).
[b]Minimum bactericidal concentration (values in $\mu$g/ml).

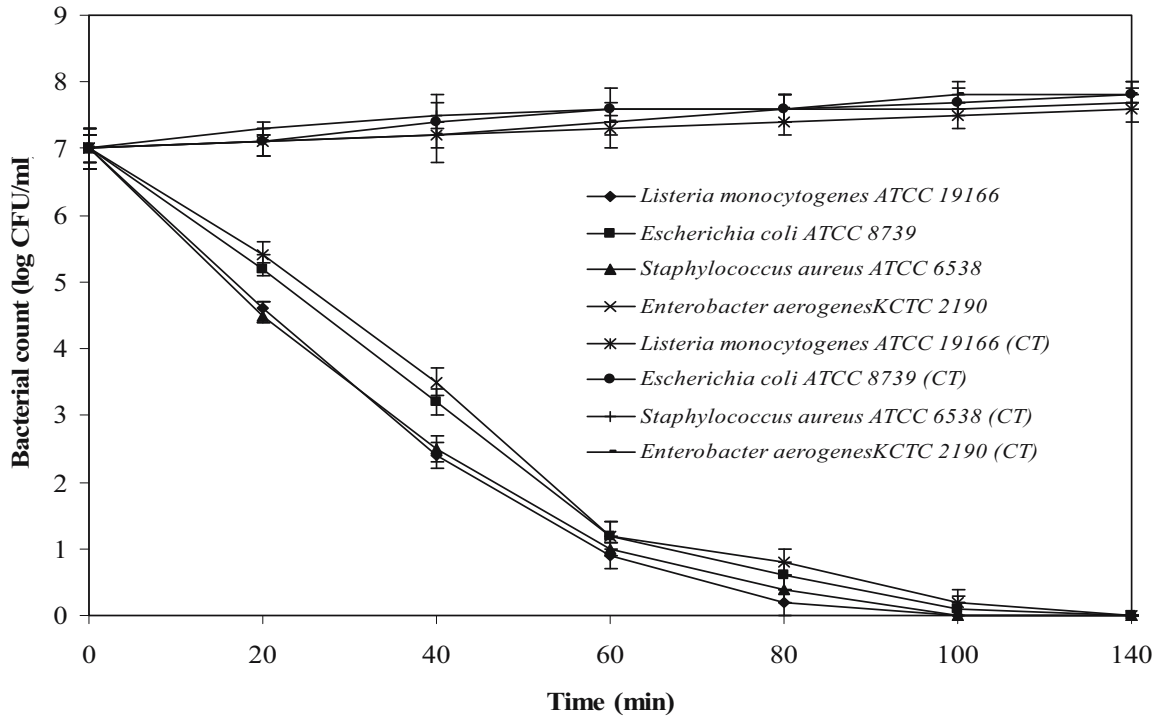


**Figure 2.** Effect of taxodone (250 $\mu$g/ml) on viability of the tested bacterial pathogens isolated from *M. glyptostroboides* (CT: control without treatment).

*S. aureus* ATCC 6538 and *E. aerogenes* KCTC 2190. The effect of taxodone on the growth of tested bacteria demonstrated reduced viability at the used concentration (figure 2). At 80 min exposure, nearly 80–90% inhibition of all the tested strains was observed. Exposure of 100 min of the compound revealed complete inhibition of CFU numbers against both gram-positive bacteria such as *L. monocytogenes* ATCC 19166 and *S. aureus* ATCC 6538. However, *E. coli* ATCC 8739 and *E. aerogenes* KCTC 2190 showed less susceptibility as compared with other bacterial pathogens and complete inhibition of both the strains was observed at 140 min exposure time (figure 2). Similar to our findings, some of the terpenoid compounds (geranylgeraniol, teprenone and phytol) also exerted an inhibitory effect against the foodborne pathogenic bacterium of *Staphylococcus aureus* (Inoueb *et al*. 2005).

Food safety is a fundamental concern of both consumers and the industry of food, especially as the number of reported cases of food-associated infections continues to increase and is rapidly changing (Alzoreky and Nakahara 2003). Recurring outbreaks of foodborne illness caused by foodborne pathogenic bacteria have sustained the demand for preservation systems that limit the proliferation of foodborne pathogens in refrigerated, minimally processed and ready-to-eat foods. In this regard, plant-based antimicrobials could be potential alternatives in the food industry to control foodborne pathogens.

Utilization of antibacterial activity of bioactive secondary metabolites as natural antibacterial agents may offer many new applications for food industry. Natural food preservatives targeted at food and food products that are easily contaminated by bacteria are highly desired. The availability of natural compounds developed by exploring novel chemistry, activation of plants' natural resistance mechanisms and natural products will contribute to sustainable food and food products. The results described in this study clearly indicate that taxodone, an abietane-type diterpenoid, isolated form *M. glyptostroboides*, possessed the potential to control foodborne pathogens, and these findings are in strong agreement with previous reports (Singh and Singh 2003; Murthy *et al*. 2005). Thus, recent findings reinforce the suggestions that *M. glyptostroboides*–derived antibacterials might act on a broad spectrum of bacteria and could be included as an effective addition to traditional antibacterial agents and treatments for their possible applications in the food industry because low mammalian toxicity and environmental impact as well as low residues in food are very important features of plant-based natural compounds.. However, studies concerning safety, toxicology, combined use with traditional medicines and legislation are still needed.

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