Potential of sugi wood diterpenes as an Alzheimer's disease preventive and therapeutic drug by the β-amyloid toxicity reduction effect

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Abstract

Alzheimer's disease is the most common form of dementia, and neurological disorder from beta amyloid (Aβ) peptide is regarded as a cause of Alzheimer's disease. In this study, the authors evaluate Aβ toxicity reduction effects of major diterpenes isolated from sugi wood by bioassay using *Caenorhabditis elegans* and examined its relationship with the antioxidative activity. Among the six kinds of sugi wood diterpenes: ferruginol, 6,7-dehydroferruginol, sandaracopi-marinal, sandaracopimarinol, abietadiene, abietatriene and phyllocladenes, prominent Aβ toxicity reduction effects were found in ferruginol and 6,7-dehydroferruginol, and their activity was almost equivalent to that of the four kinds of anti-Alzheimer active agents that have been reported so far: ginkgolide A, morin, rosmarinic acid and carnosic acid. Antioxidative activity similar to other anti-Alzheimer active agents was found in ferruginol and 6,7-dehydroferruginol in a 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test, which suggested that antioxidative activity is concerned with expression of the Aβ toxicity reduction effect of sugi wood diterpenes.

Keywords: *Cryptomeria japonica*, Terpenoids, Alzheimer's disease

Introduction

The aging rate in Japan reached 28.1% in 2019 (the rate of population of 65 years or older among the entire population) and a super-aging society that is unprecedented anywhere around the globe is coming [1]. Dementia is one of the most serious social problems accompanied with aging, and the number of patients in 2025 is estimated to be over 7 million. Among them, the number of Alzheimer's disease patients is estimated to be 4,660,000, and it is predicted that the majority of future dementia patients will suffer from Alzheimer's disease [2]. Beta amyloid (Aβ) is said to be the etiology of Alzheimer's disease, which is supported by many evidences (amyloid hypothesis), but the pathology and mechanism of Alzheimer's disease remain unclear [3].

Aβ is a peptide that is constantly biosynthesized as a metabolite, and several types that have 36–43 amino acid residues are known. Highly agglutinative Aβ42 is particularly produced excessively in an Alzheimer's disease patient's brain, and aggregates called senile plaque are formed in the brain of a patient with serious symptoms [4]. Based on the above-mentioned findings, treatment strategies that targeted Aβ42 and its aggregates have been focused. Aβ42 polymerizes with radical formation reaction by oxidation of tyrosine, which is a constituent

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Yamaji *et al. J Wood Sci*   (2020) 66:76

Page 2 of 6

amino acid, as a starting point, and expresses neuronal cell toxicity during the process in which it aggregates and deposits [5]. Furthermore, it has been reported that the reactive oxygen species concentration in tissues rises by polymerization of Aβ and that increased oxidation stress caused by polymerized Aβ is deeply involved with the expression of neurotoxicity [6].

The authors isolated and identified several kinds of diterpene from volatile compounds discharged from the sugi wood-drying process and reported that some of these have prominent antioxidative effects [7]. In this study, the authors evaluated the Aβ toxicity reduction effect of major diterpenes isolated from sugi wood by bioassay using *Caenorhabditis elegans* and moreover examined their relationship with the antioxidative activity.

Materials and methods
Samples and reagents
Sugi wood (*Cryptomeria japonica*) diterpene (Fig. 1): ferruginol (I), 6,7-dehydroferruginol (II), sandaracopimarinal (III), sandaracopimarinol (IV), abietadiene (V), abietatriene (VI) and phyllocladenes (VII-i and -ii; mixture of phyllocladene and isophyllocladene) were isolated

and identified according to a previous report [7]. The chemical structure and purity of each compound were confirmed by Gas chromatography (GC) and ^{1}H/^{13}C Nuclear magnetic resonance (NMR). The isolated diterpenoids are more than 95% pure. Ginkgolide A was purchased from Funakoshi Co.; rosmarinic acid was purchased from SIGMA-ALDRICH; carnosic acid and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were purchased from Tokyo Chemical Industry Co., Ltd. and morin and 2,6-Di-*tert*-butyl-4-methylphenol (BHT) were purchased from Fujifilm Wako Pure Chemical Corporation. These purchased compounds and reagents are also more than 95% pure.

Caenorhabditis elegans and management
C.elegans CL4176 strains were provided by Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN) (Fig. 2). Aβ synthetic gene with the temperature dependency is incorporated in CL4176 strain, toxic Aβ1-42 is expressed in a muscle tissue-specific manner by raising breeding temperature from 16 to 25 °C and it becomes the paralysis [8]. The *C.elegans* were bred and preserved on a Nematode Growth Medium (NGM) storage plate at 16 °C with *Escherichia coli* OP50
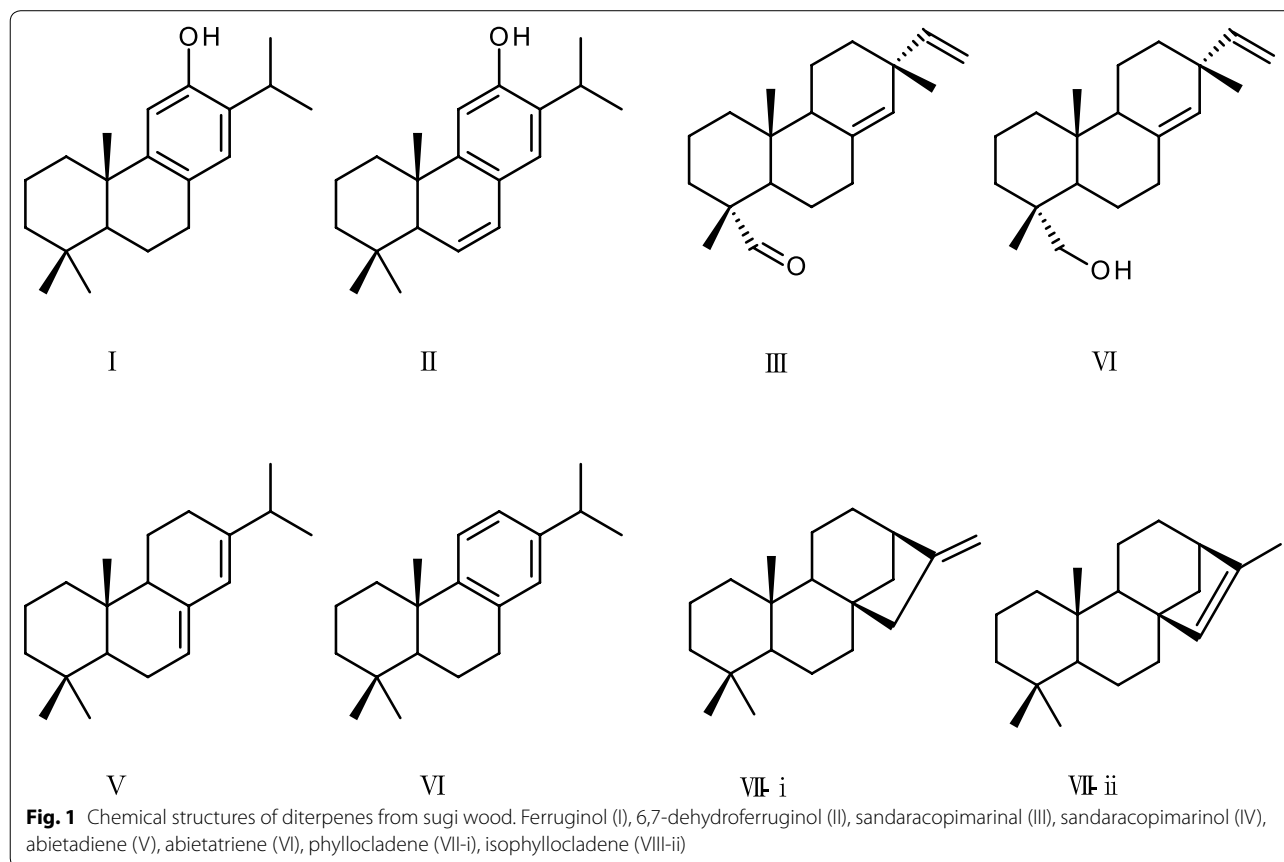

Fig. 1 Chemical structures of diterpenes from sugi wood. Ferruginol (I), 6,7-dehydroferruginol (II), sandaracopimarinal (III), sandaracopimarinol (IV), abietadiene (V), abietatriene (VI), phyllocladene (VII-i), isophyllocladene (VIII-ii)

Yamaji *et al. J Wood Sci*   (2020) 66:76

Page 3 of 6

Fig. 2 *Caenorhabditis elegans* CL4176

as feed, and moved to new storage plates once in 4 days. *E. coli* OP50 that was inoculated into 8 mL of Luria–Bertani (LB) media and cultured at 25 °C overnight was used as bacterial cell suspension according to the usual manner. For the storage plate for *C. elegans*, 10 μL of *E. coli* OP50 suspension was dropped onto an NGM plate, spread with a spreader and cultured at 25 °C for one night before use.

Aβ toxicity reduction test (*C. elegans* paralysis test)
Preparation of a test plate, 10 μL of *E. coli* OP50 suspension and 10 μL of sample solution (0.5 mg/mL dimethyl sulfoxide solution, as molarity of I:1.7 μM, II:1.8 μM, III:1.8 μM, IV:1.7 μM, V:1.8 μM, VI:1.8 μM, VII:1.8 μM, ginkgolide A:1.2 μM, rosmarinic acid:1.4 μM, carnosic acid:1.5 μM, morin:1.7 μM) were dropped onto an NGM plate, and spread with a spreader. Only DMSO was used for a control plate instead of the sample solution. Twenty five L3 larvae of synchronized *C. elegans* CL4176 that was grown at 16 °C were taken to each of two plates and moved to an incubator of 25 °C to have Aβ genes expressed. Observation was started 20 h after raising temperature to 25 °C and the number of paralyzed individuals was counted every two hours. Aβ toxicity reduction effects of each sample were assessed by statistically analyzing data obtained from the test plate and control plate using the Kaplan–Meier method (log-rank test).

Radical scavenging activity test
DPPH was dissolved in ethanol at the concentration of 0.2 mM. Each diterpenoid sample was dissolved in ethanol at a concentration of 10 mM. BHT was used as a positive control. First, 0.2 mL of sample solution, 1.0 mL of DPPH solution, 0.2 mL of 0.1 M Tris–HCL (pH 7.4) buffer, and 0.6 mL of ethanol were mixed in a screw tube. Then, the screw tubes were shaken, and allowed to react at room temperature under dark conditions. After 30 min, the absorbance was measured at 517 nm by UV mini 1240 (SHIMADZU, Japan). Based on the data obtained by each concentration, concentration for 50% inhibition concentration (IC50) was calculated.

GC
GC was performed on a 7890B GC System (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness,

Yamaji *et al. J Wood Sci*   (2020) 66:76

Page 4 of 6

Agilent Technologies). The oven temperature started at 60 °C and increased at 3.0 °C/min to at 240 °C. The injector and the detector temperatures were both 250 °C.
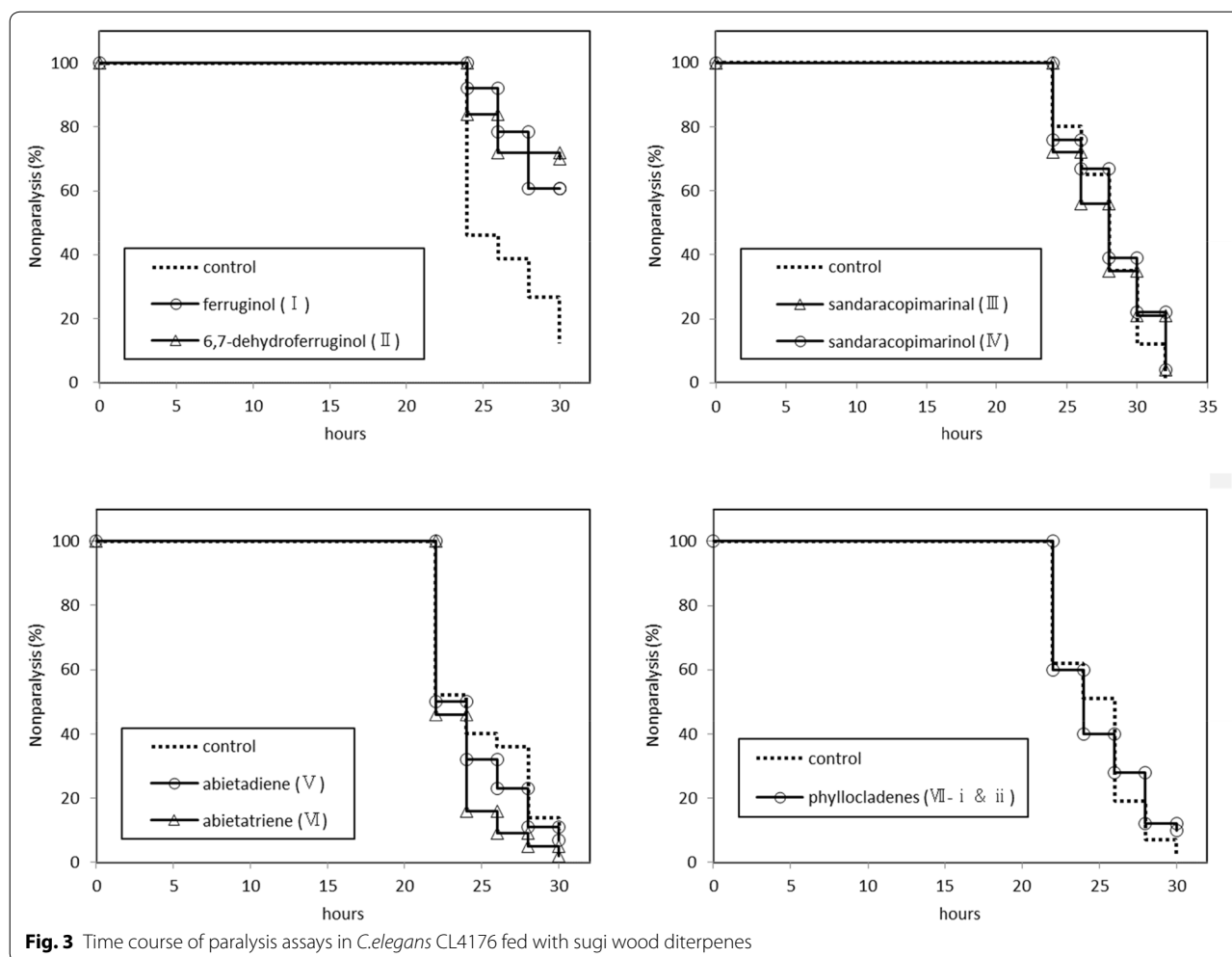
NMR

NMR spectra (^{1}H: 400 MHz, ^{13}C: 100 MHz) were recorded on a JEOL AL400 FT-NMR spectrometer. Each purified diterpenoid (50 mg) was dissolved in 0.7 mL chloroform-*d*. The experimentally measured spectra were compared with data from the previous report [7].

Results and discussion

To examine the Aβ toxicity reduction effect of sugi wood diterpenes, each diterpenoid at the final concentration of 0.5 mg/mL was given to *C. elegans* and paralysis rates were observed (Fig. 3). As a result of examining the difference in non-paralysis rates, significantly positive effects were found in the experimental plot of ferruginol and 6,7-dehydroferruginol ($p < 0.05$). These results indicated that expression of paralysis from Aβ toxicity in *C.elegans*

CL4176 was suppressed by administering ferruginol and 6,7-dehydroferruginol. On the other hand, significant differences were not found in experimental plots of other sugi wood diterpenes, and it has been revealed that the Aβ toxicity reduction effect is a property peculiar to ferruginol and related compounds. This study reports the anti-Alzheimer's disease effects from extracts of sugi wood for the first time.

As for the anti-Alzheimer's disease activity of extractives from plants, the effects of natural compounds such as ginkgolide A [9], morin [10], rosmarinic acid [11] and carnosic acid [12] have been reported. In this study, to examine how the Aβ toxicity reduction effect of ferruginol is effective in comparison with the above anti-Alzheimer active compounds, a simultaneous comparison in the *C. elegans* CL4176 paralysis test was performed with commercial reagents of each active compound as a positive control. As a result of examining the difference in non-paralysis rates, significant differences were not found between the positive control-added plate

Fig. 3 Time course of paralysis assays in *C.elegans* CL4176 fed with sugi wood diterpenes

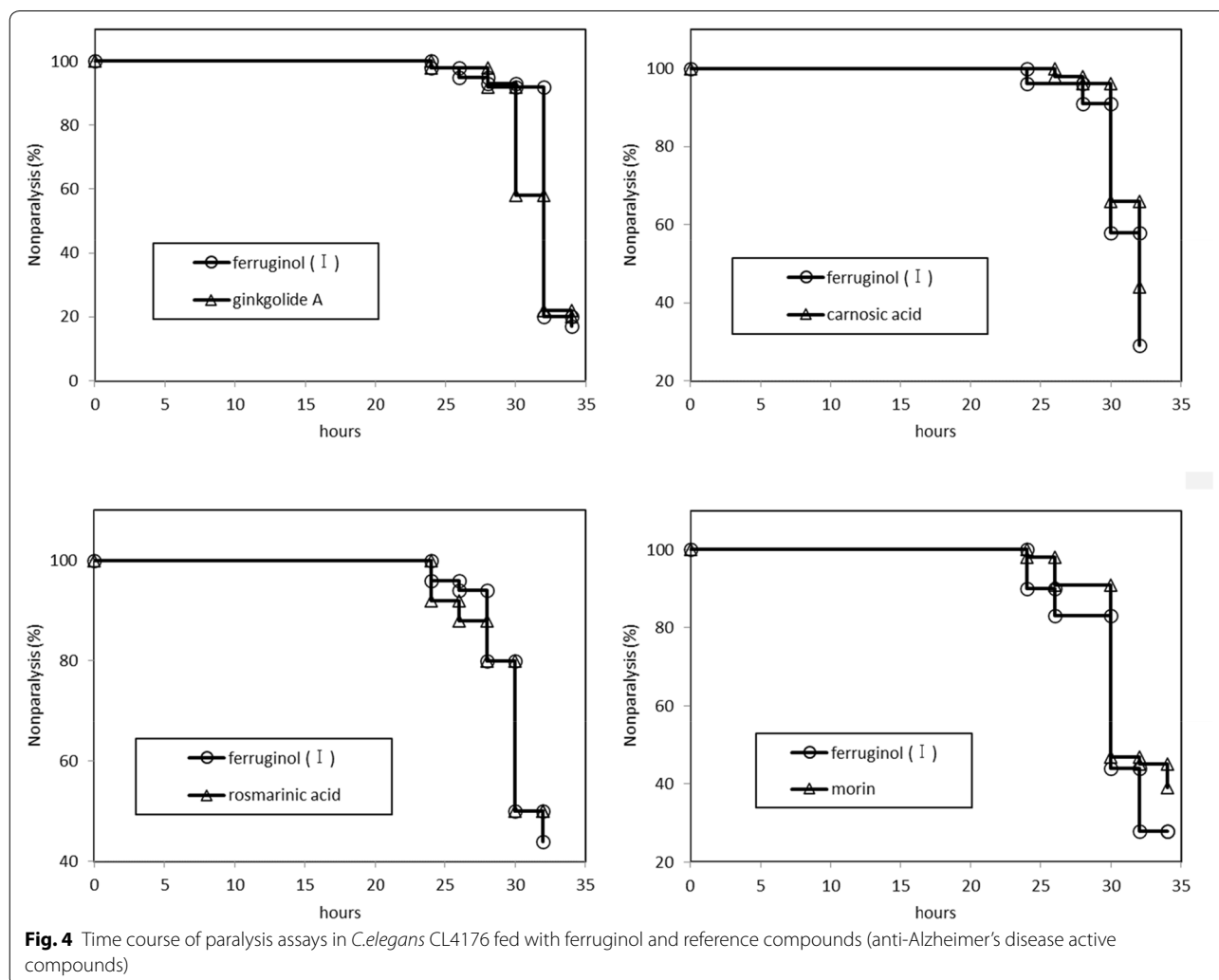Yamaji *et al. J Wood Sci*   (2020) 66:76

Page 5 of 6

and ferruginol-added plate in either experiment system (Fig. 4). These results suggest that ferruginol has anti-Alzheimer's disease effects that are equal to other natural active agents reported in the previous studies.

The onset of Alzheimer's disease is presumably associated with enhancement of oxidation stress in a brain [13]. In this study, to examine the relationship between Aβ toxicity reduction effect and the antioxidative activity of sugi wood terpenoid, the DPPH radical scavenging activity of major sugi wood diterpenes was measured. Six kinds of purified diterpenes, four kinds of anti-Alzheimer's disease active compounds and BHT (positive control), which is a commercial anti-oxidant, were used for samples and 50% inhibition concentration (IC50) was calculated (Table 1). Among the sugi wood diterpenes used for the experiment, the only two compounds that presented prominent activity were ferruginol and 6,7-dehydroferruginol (IC50 = 7.2 mM and 0.9 mM, respectively), and antioxidative activity was not found in

Table 1 IC50 of sugi wood diterpenes and reference compounds in DPPH radical scavenging activity

Sugi wood diterpenes	IC50 (mM)	Reference compounds	IC50 (mM)
BHT (positive control)	1.47	Ginkgolide A	n. d
Ferruginol (I)	7.24	Carnosic acid	0.50
6,7-Dehydroferruginol (II)	0.90	Rosmarinic acid	0.03
Other diterpenes (III, IV, V, VI, VII-i and -ii)	n. d	Morin	0.45

high concentration of 10 mM for other diterpenes. Aβ toxicity reduction effect and antioxidative activity were found in ferruginol and 6,7-dehydroferruginol, while no activities were found in the other sugi wood diterpenes, and antioxidative activity was found in the three kinds of positive controls. These findings suggest the relationships

Fig. 4 Time course of paralysis assays in *C.elegans* CL4176 fed with ferruginol and reference compounds (anti-Alzheimer's disease active compounds)

Yamaji *et al. J Wood Sci*   (2020) 66:76

Page 6 of 6

between Aβ toxicity reduction effect of ferruginol/6,7-dehydroferruginol and antioxidative activity.

As the mechanism of antioxidative reaction by ferruginol, the process that the radical of the object substance is removed by ferruginol being oxidized to sugiol through quinone methide intermediates has been proposed [14]. The results obtained in this study suggest that ferruginol suppresses Aβ polymerization and oxidation stress by scavenging Aβ radical based on a similar mechanism. It is necessary to clarify the reaction mechanism of Aβ toxicity reduction effect by ferruginol in detail.

Conclusions
The two kinds of diterpenes, ferruginol and 6,7-dehydroferruginol, which were isolated from volatile compounds discharged from sugi wood-drying process improved disorder of motor function (paralysis) of *C.elegans* CL4176 caused by the Aβ toxicity. Comparison of Aβ toxicity reduction effect by ferruginol and the known anti-Alzheimer's disease active compounds revealed no significant difference and it was regarded that their activities were approximately same level. Furthermore, the antioxidative activity in the DPPH radical scavenging test was found only in ferruginol and 6,7-dehydroferruginol that presented Aβ toxicity reduction effect, suggesting that the antioxidative activity of these compounds is the important factor to relieve the toxicity of Aβ. The active diterpenes revealed in this study are skin permeable and volatile, so they can be applied transdermally or nasally. In the future, it is expected to be used as a therapeutic or preventive drug for Alzheimer's disease due to the Aβ toxicity reduction effect.

Abbreviations
Aβ: Beta amyloid; GC: Gas chromatography; NMR: Nuclear magnetic resonance; DPPH: 1,1-Diphenyl-2-picrylhydrazyl; BHT: 2,6-Di-tert-butyl-4-methylphenol; NGM: Nematode growth medium; LB medium: Luria–Bertani medium; IC50: 50% Inhibition concentration.

Authors' contributions
YY performed isolation of diterpenoids and biological assay. TW managed *C.elegans* and gave advice for handling and evaluating biological assay. HK designed this study and prepared the manuscript. All authors read and approved the final manuscript.

Availability of data and materials
The datasets during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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