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ORIGINAL ARTICLE

Bioactivities of extracts from *Chamaecyparis obtusa* branch heartwood

Takuya Morikawa · Tatsuya Ashitani · Nobuhiro Sekine · Norihisa Kusumoto · Koetsu Takahashi

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Abstract In order to find new utilization method of woody wastes, we examined the bioactivities of extracts from branch heartwood of *Chamaecyparis obtusa* (hinoki) and compared to extracts from trunk heartwood. The bioactivities examined were antifungal activities against four fungi (*Trametes versicolor*, *Fomitopsis palustris*, *Trichoderma virens*, *Rhizopus oryzae*), and bioassay with brine shrimp (*Artemia salina*) which shows any allelopathic activities not measurable with fungi. Antifungal activities were observed in the hexane and ethyl acetate extracts of branch and trunk heartwood. The hexane and ethyl acetate extracts of branch and hexane extract of trunk showed strong lethality against brine shrimp. The yields of the active extracts of branch were much more than that of trunk. The identified compounds in the active extracts of branch were germacra-1-(10),5-dien-4β-ol, *t*-cadinol, *t*-muurolol, hinokiresinol, and hinokinin. Hinokiresinol and *t*-muurolol showed strong antifungal activities. Hinokiresinol showed bioactivities against *T. virens*, *R. oryzae* and brine shrimp. Germacra-1-(10),5-dien-4β-ol was lethal to brine shrimp. Germacra-1-(10),5-dien-4β-ol and hinokiresinol were minor components in trunk heartwood, but major components in branch. These qualitative and quantitative results suggest that the branch heartwood could be a valuable chemical resource because it contains large amounts of antifungal and allelopathic compounds.

Keywords *Chamaecyparis obtusa* · Branch heartwood · Antifungal activity · Brine shrimp · Hinokiresinol

T. Morikawa · T. Ashitani · K. Takahashi
The United Graduate School of Agricultural Science, Iwate University, Morioka 020-8550, Japan

T. Morikawa (✉) · T. Ashitani · N. Sekine · N. Kusumoto · K. Takahashi
Faculty of Agriculture, Yamagata University, 1-23, Wakaba-machi, Tsuruoka, Yamagata 997-8555, Japan
e-mail: morikawa@tds1.tr.yamagata-u.ac.jp

N. Sekine · N. Kusumoto
Institute of Wood Technology, Akita Prefectural University, Noshiro 016-0876, Japan

Introduction

Chamaecyparis obtusa Endl. (hinoki) is an important Japanese conifer tree, as is *Cryptomeria japonica*. Hinoki heartwood is highly resistant to termites [1] and wood decay fungi [2–4], and its bioactivity is originated from various extractive components, such as mono- and sesquiterpenoids. The distribution of such components is not homogeneous in the heartwood [5].

The color of the heartwood of hinoki is an important factor in its commercial value. In general, the trunk heartwood is pale red, while the branch heartwood is a more vivid shade of red [6]. The pale red color of the trunk heartwood is due to certain contents and composition of extractable components [7]. Thus, it is likely that the extractable components differ between the trunk and the branch heartwood, since they are different colors. To date, however, there have been no reports on the bioactivity of branch heartwood extracts, although there are many reports on the durability of hinoki as related to extractable compounds in the trunk. At present, branches of hinoki are regarded as waste material. It would be useful, therefore, to develop a method to use this material. In this study, we determined the bioactivities of extracts from branch heartwood of hinoki, and compared them with the bioactivities of extracts from trunk heartwood. Three major wood decay fungi, *Trametes versicolor* (white-rot),

Fomitopusis palustris (brown-rot), and *Trichoderma virens* (soft-rot) were applied to the antifungal tests. Similarly, a typical zygomycete fungus of *Rhizopus oryzae* was applied to investigate antifungal activity as non-wood decay fungi. In addition, the brine shrimp (*Artemia salina*) was known as good test animals for monitoring allelopathic compounds [8–11]. We, therefore, also performed the bioassay test for screening of allelopathic activities against harmful organism other than fungi.

Materials and methods

Plant materials

Hinoki trees were harvested from the Yamagata Field Science Center (Faculty of Agriculture, Yamagata University, Japan) located in Tsuruoka City in western Yamagata Prefecture in October 2007. Trunk log (ca. 28 cm i.d. × 35 cm) and branches (ca. 5 cm i.d. × 20 cm) were collected.

Extraction and isolation

The branch and trunk heartwood were separated from the logs, and crushed using a Wiley mill. The heartwood samples were extracted at ambient temperature for 7 days by successive extraction with hexane, ethyl acetate, and methanol. Each solvent was removed by evaporation to yield each extract. The hexane extract of branch heart wood was applied to silica gel (60 N, spherical 63–210 μm, neutral; Kanto Chemical Co., Inc., Japan) column chromatography and hexane eluted fraction was collected as namely H-1. Further, the column was eluted with a gradation of hexane/ethyl acetate (100/1 to ethyl acetate only) to isolate germacra-1-(10),5-dien-4β-ol, *t*-cadinol, *t*-muurolol, and hinokinin. The ethyl acetate extract was also fractionated by silica gel 60 N column chromatography with hexane and acetone to isolate hinokiresinol and hinokinin.

The above compounds, except for germacra-1-(10), 5-dien-4β-ol, were identified by comparison to gas–liquid chromatographic (GC) mass spectroscopy (MS) data of standard compounds stocked in our laboratory. Germacra-1-(10),5-dien-4β-ol was identified by comparison of its nuclear magnetic resonance (NMR) spectra data with published data [12]. NMR was measured with a JEOL JNM-EX400 (^{1}H 400 MHz/^{13}C 100 MHz) spectrometer (JEOL Ltd., Japan).

Germacra-1-(10),5-dien-4β-ol

^{1}H-NMR δ: 4.95 (bs, J = 11.3 Hz), 2.51 (dddd, J = 14.7, 11.9, 11.9, 3.5 Hz), 5.25 (d, J = 15.6 Hz), 5.17 (dd, J = 1.5, 9.2 Hz), 0.79 (d, J = 6.8 Hz), 0.83 (d, J = 6.7 Hz), 1.54 (s), 1.19 (s). ^{13}C-NMR δ: 128.8, 23.7, 41.3, 73.1, 140.1, 125.7, 52.8, 39.6, 25.9, 132.6, 33.0, 20.6, 18.9, 16.7, 30.7.

GC analysis

GC-FID analysis was performed with a HITACHI G-3000 gas chromatograph (HITACHI Ltd., Japan) under the following conditions: DB-1 capillary column (30 m × 0.32 mm i.d.; 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA); a column temperature was started at 100 °C (1 min) and increased with a gradient of 4 °C/min up to 280 °C (15 min); injection temperature of 250 °C and detection temperature of 250 °C. Helium was used as the carrier gas with a column head pressure of 50 kPa. GC–MS data were measured with a Shimadzu QP-5000 GC–MS (SHIMADZU Corp., Japan): DB-1 capillary column (30 m × 0.32 mm i.d.; 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA); a column temperature was started at 100 °C (1 min) and increased with a gradient of 4 °C/min up to 280 °C (15 min); injection temperature of 250 °C; and interface temperature of 250 °C. The acquisition mass range was 50–450 amu. Helium was used as the carrier gas with a column head pressure of 50 kPa. The identification of peaks, except the isolated compounds, on the chromatogram was based on comparison of mass spectra with those in the NIST 62 Mass spectral library.

Antifungal test

We obtained the wood decay fungi, *Trametes versicolor* (white-rot) (NBRC:30340), *Fomitopusis palustris* (brown-rot) (NBRC:30339), and zygomycete fungi, *Rhizopus oryzae* (NBRC:31005A) from the Natural Institute of Technology and Evaluation Biological Resource Center (NBRC), Tokyo, Japan. The wood decay fungi, *Trichoderma virens* (soft-rot) (MAFF:645007) was provided by the National Institute of Agrobiological Sciences (Tokyo, Japan). These fungi are routinely chosen for antifungal tests according to Japan Industrial Standard (JIS) K1517 and Z2911. Antifungal tests were carried out as described in previous reports [13, 14]. Test samples were dissolved in acetone and applied at a concentration of 5.0 μg/cm^2 to the surface of potato dextrose agar (PDA) medium in Petri dishes (90 mm diameter) and then air-dried. A pre-cultured fungus colony was cut with a cork-borer (5 mm diameter) and the trimmed colony section was placed in the center of the medium. The fungi were cultured in the dark in an incubator at 25.5 °C. For the control, pure acetone (300 μl) was similarly applied to the PDA medium. This was carried out in parallel with the test sample. Each experiment was repeated three times. When mycelia reached the edge of the

Petri dish in one of the controls, the diameter of the mycelial growth in the treatment Petri dish was measured. The antifungal activity was evaluated as a percentage of relative growth rates, calculated as follows:

$$\text{Antifungal activity } (\%) = 100 \times (1 - D_a/D_b)$$

where D_a is the total average of the mycelia diameter of each sample, and D_b is the average diameter of the mycelia in the control.

Brine shrimp test

The lethal activities of the samples against brine shrimp were determined as described in a previous report [8, 9]. Commercially available eggs of brine shrimp were hatched in artificial seawater for 24 h at 26 °C. Then, 3–4 ml seawater was added to 10-ml vials, and 10 brine shrimp larvae were added to each vial. The samples were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml. Aliquots of each sample solution (50, 25, or 5 μl) were added to the vials, corresponding to 100, 50 and 10 μg/ml, respectively. The volume was adjusted with seawater to 5 ml/vial. For blank tests, DMSO was added to each vial. Each experiment was repeated three times, and the control was carried out in parallel with the sample treatments. After 24 h at 26 °C in dark conditions, the number of dead larvae was counted. LC50 values were calculated from the number of dead larvae.

Results and discussion

Bioactivity of successive extracts

The extract yields are shown in Table 1. Quantity of hexane extractive components in branch heartwood (yield 15.3 %) was about 6 times more than that of trunk heartwood (2.6 %). Ethyl acetate extract was also contained more in branch (10.9 %) as 12 times quantities of trunk (0.9 %).

Antifungal activities of extracts of hinoki heartwood are shown in Table 2. The hexane and ethyl acetate extracts of branch and trunk heartwood showed antifungal activities. The antifungal activities were stronger against *F. palustris* than against the other fungi. The hexane extracts of branch heartwood showed stronger activities against *T. virens* as antifungal activities 42.0 ± 4.2 % and *R. oryzae*, 31.6 ± 4.1 %, than the activities with *T. virens*, 24.8 ± 4.1 %, *R. oryzae*, 22.0 ± 4.8 % at trunk heartwood extractives. The ethyl acetate extracts of branch heartwood showed slightly stronger activities against four fungi than that of trunk heartwood. The hexane extracts of branch should be under the investigation because of its high yield and activities as in the next section.

Table 1 Yields of the successive extracts from hinoki heartwood

Solvent	Yield (%)
Branch heartwood extract	
Hexane	15.3
Ethyl acetate	10.9
Methanol	1.9
Trunk heartwood extract	
Hexane	2.6
Ethyl acetate	0.9
Methanol	1.2

Yields were calculated based on dry-weight of the heartwood

Brine shrimp test is a good bioassay method for monitoring bioactive compounds against animals [8–11]. We, therefore, performed brine shrimp test in order to screening of allelopathic activities against harmful organism other than fungi. The activities of the extracts against brine shrimp larvae are shown in Table 2. The hexane extracts of branch and trunk heartwood showed strong lethal activities to brine shrimp larvae. Methanol extracts from both branch and trunk heartwood had no bioactivity. The ethyl acetate extract from branch heartwood showed certain activity against brine shrimp larvae, but that from trunk heartwood did not. These results suggest that the characteristic allelopathic compounds were present in branch heartwood at the fractions of the ethyl acetate extract.

As mentioned above, branch heartwood contained greater quantities of active extracts, hexane and ethyl acetate extracts, than trunk heartwood. Therefore, the branch heartwood of hinoki is likely more resistant to wood decaying fungi and other attacking organisms to hinoki than its trunk heartwood. Moreover, it would be also considered that these effects are protecting hinoki branch against wide spectrum of harmful organism in the living hinoki tree from the viewpoint of chemical ecology.

Active components in extracts from branch heartwood

Figures 1 and 2 show total ion chromatograms of the hexane and ethyl acetate extracts from branch and trunk heartwood. The retention time and content of each compound are shown in Table 3. Sesquiterpenoids were present in both extracts, and the composition of the extracts was similar, except for germacra-1-(10),5-dien-4β-ol. Germacra-1-(10),5-dien-4β-ol was present at high levels in the branch extract. A lignan, hinokinin, was present in both branch and trunk extracts. A norlignan, hinokiresinol, was a characteristic compound on the chromatogram of the branch heartwood extract.

The fractions and compounds separated from the active extracts were used in bioactivity tests against fungi and

Table 2 Antifungal activities (%) and result of brine shrimp test (LC_{50} μg/ml) of the successive extracts from hinoki heartwood

Solvent	Antifungal activities (%)				LC_{50} μg/ml
	White-rot fungi *Trametes versicolor*	Brown-rot fungi *Fomitopsis palustris*	Soft-rot fungi *Trichoderma virens*	Other fungi *Rhizopus oryzae*	Brine shrimp *Artemia salina*
Branch heartwood extract					
Hexane	24.3 ± 9.5	50 ± 1.8	42.0 ± 4.2	31.6 ± 4.1	61.0
Ethyl acetate	26.8 ± 5.1	49.0 ± 3.8	42.0 ± 3.7	26.7 ± 4.7	92.0
Methanol	10.3 ± 3.2	23.4 ± 8.5	17.9 ± 14.0	13.1 ± 12.2	ND
Trunk heartwood extract					
Hexane	26.2 ± 9.8	45.2 ± 6.4	24.8 ± 4.1	22.0 ± 4.8	72.0
Ethyl acetate	20.6 ± 8.3	44.6 ± 4.8	36.9 ± 4.9	25.3 ± 9.7	ND
Methanol	14.0 ± 12.6	32.4 ± 4.0	27.0 ± 8.9	16.8 ± 9.6	ND

ND not determined

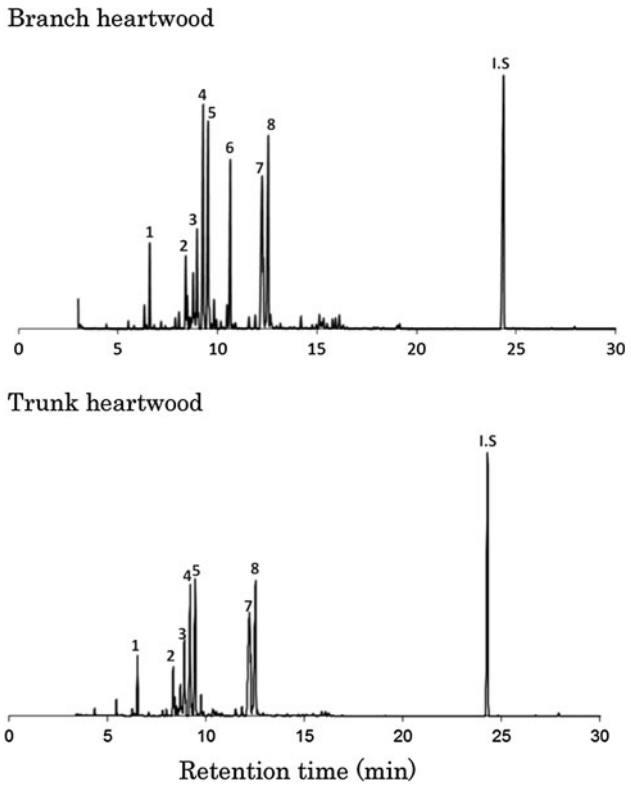

Fig. 1 Total ion chromatograms of hexane extract of branch heartwood (*upper panel*) and trunk heartwood (*lower panel*). *Numbers 1–8* refer to compounds shown in Table 3. *I.S* internal standard (heneicosan)

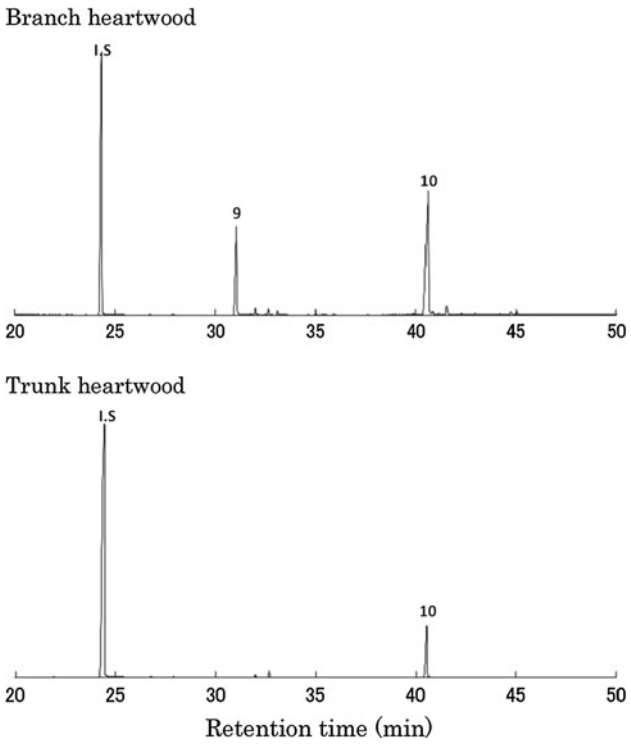

Fig. 2 Total ion chromatograms of ethyl acetate extract of branch heartwood (*upper panel*) and trunk heartwood (*lower panel*). *Numbers 9–10* refer to compounds shown in Table 3. *I.S* internal standard (heneicosan)

brine shrimps. Hinokitiol (*β*-thujaplicin) which is well known as strong antifungal compound was used as the positive control [15, 16]. As shown in Table 4, the H-1 had no, or very little, antifungal activity against all fungi. H-1 was hexane eluted fraction by silica gel column chromatography. As determined by GC–MS analysis, H-1 was mixture of hydrocarbon compounds, which consisted of *β*-elemene, copaene, α-muurolene, *γ*-cadinene, and *δ*-cadinene. *t*-Muurolol showed strong antifungal activities against *T. versicolor* (antifungal activities 52.5 ± 16.2 %) and *T. virens* (60.1 ± 6.2 %). However, *t*-cadinol, a stereoisomer of the *t*-muurolol, showed little antifungal activity. Germacra-1-(10),5-dien-4*β*-ol was a characteristic compound of the branch heartwood, and it showed weak antifungal activity against all fungi tested. The norlignan, hinokiresinol, showed strong activity against *T. virens*

Table 3 Constituents isolated compounds from branch of hinoki

No.	Compound	Retention time (min)	Relative rate (GC%)[a]	
			Branch	Trunk
1	β-Elemene	9.3	1.9	3.1
2	Copaene	11.4	2.4	3.6
3	α-Muurolene	12.0	3.4	5.3
4	γ-Cadinene	12.4	12.1	11.3
5	δ-Cadinene	12.7	11.6	13.5
6	Germacra-1-(10),5-dien-4β-ol	13.8	6.7	T
7	*t*-Cadinol	15.7	14.6	19.9
8	*t*-Muurolol	16.9	13.0	17.7
9	Hinokiresinol	34.8	1.6	T
10	Hinokinin	44.7	7.2	1.4

T trace

[a] Relative rate (%) = 100 × each component peak area/total diterpenoids peak area (GC-FID)

Table 4 Antifungal activities (%) of isolated compounds from branch of hinoki

	White-rot fungi *Trametes versicolor*	Brown-rot fungi *Fomitopsis palustris*	Soft-rot fungi *Trichoderma virens*	Other fungi *Rhizopus oryzae*
H-1	15 ± 4.9	16.1 ± 4.7	11.2 ± 2.7	23.6 ± 4.5
Germacra-1-(10),5-dien-4β-ol	18.2 ± 3.0	21.3 ± 3.1	13.6 ± 4.9	15.5 ± 8.1
t-Cadinol	20.4 ± 2.8	20.5 ± 5.2	29.6 ± 3.3	19.5 ± 2.9
t-Muurolol	52.5 ± 16.2	31.9 ± 4.1	60.1 ± 6.2	24.8 ± 5.3
Hinokiresinol	18.4 ± 6.4	36.5 ± 3.2	74.2 ± 4.9	45.2 ± 11.1
Hinokinin	37.7 ± 2.6	32.4 ± 32.6	50.4 ± 10.9	29.8 ± 4.6
Hinokitiol	93.4 ± 11.7	92.7 ± 13.1	50.1 ± 4.7	57.1 ± 3.6

(74.2 ± 4.9 %), and its activity was stronger than that of the positive control, hinokitiol. The lignan, hinokinin, which was present in both branch and heartwood extracts, also showed certain activity against *T. virens* (50.4 ± 10.9 %).

The activities of the fractions and compounds against brine shrimp larvae are shown in Table 5. Hinokinin did not show strong activity, but sesquiterpenes did. In particular, germacra-1-(10),5-dien-4β-ol showed a strong lethal effect of the LC_{50}, 4 μg/ml. Norlignan hinokiresinol had also strong activity (LC_{50} 6 μg/ml). These LC_{50} were similar values as LC_{50} 5 μg/ml of gallic acid known as strong allelopathic compound, which was reported by Sheikh et al. [10]. Hinokiresinol and germacra-1-(10),5-dien-4β-ol were characteristic compounds in branch heartwood. Germacra-1-(10),5-dien-4β-ol had weak activities against fungi, while it showed a strong lethal effect at brine shrimp larvae. Thus, it was suggested that germacra-1-(10),5-dien-4β-ol possibly has bioactivity against other organism than fungi. From the above results, the bioactivities of branch heartwood to protect them are attributable to sesquiterpenes and hinokiresinol.

Table 5 Results of brine shrimp test of isolated compounds from branch of hinoki

	LC_{50} (μg/ml)
H-1	15.0
Germacra-1-(10),5-dien-4β-ol	4.0
t-Cadinol	7.0
t-Muurolol	6.0
Hinokiresinol	6.0
Hinokinin	ND
Hinokitiol	ND

ND not determined

Conclusion

The sesquiterpene compound *t*-muurolol showed strong growth inhibiting activity against wood-rotting fungi. This compound was commonly found in both branch and trunk extracts. The norlignan hinokiresinol showed the activity against sac fungi. In addition, sesquiterpenes and hinokiresinol had lethal activity against brine shrimp larvae showing potential defensing effect to wide spectrum of organisms. These active sesquiterpenes are more abundant in branch heartwood than in trunk heartwood. The active compounds hinokiresinol and germacra-1-(10),5-dien-4β-ol were characteristic components of branch heartwood. At present, the branch wood of hinoki is wasted because it is considered to be a valueless part. However, the results in this paper indicated that the branch heartwood could be a valuable chemical resource, because it contains large amounts of antifungal and allelopathic compounds.

References

1. Matsushima N, Kang HY, Sameshima K, Takamura N (1990) The complexity of termicidal activity in hinoki (*Chamaecyparis obtusa* Endl) wood. Mokuzai Gakkaishi 36(7):559–564
2. Kondo R, Imamura H (1986) Antifungal compounds in heartwood extractives of hinoki (*Chamaecyparis obtusa* Endl). Mokuzai Gakkaishi 32(3):213–217
3. Kinjo K, Doufuku Y, Yaga S (1988) Termicidal substances from the wood of *Chamaecyparis obtusa* Endl. Mokuzai Gakkaishi 34(5):451–455
4. Kinjo K, Yaga S (1986) Study on the cultivation culture media of bacidiomycetes IV. Antifungal activity of hinoki. Mokuzai Gakkaishi 32(8):632–634

5. Takaku N, Choi DH, Mikame K, Okunishi T, Suzuki S, Ohashi H, Umezawa T, Shimada M (2001) Lignans of *Chamaecyparis obtusa*. J Wood Sci 47(6):476–482
6. Masataka A, Yasuhiro M, Fumihiko M (2005) Development of new cultivars from Keichiku Hinoki. Annual report of Fukuoka Forest Research and Extension Center, pp 12–13
7. Hideo O, Hideyuki H, Mitsuko Y, Moritani Y (1987) Phenolic heartwood constituents and heartwood color of Japanese Cypress. Research bulletin of the Faculty College of Agriculture Gifu University 52:131–139
8. Ohira T, Yatagai M (1994) Allelopathic compounds produced by forest plants II. The relationships between the inhibition effects on plant growth and killing activities of brine shrimp on phenolic compounds. Mokuzai Gakkaishi 40(5):541–548
9. Tanaka K, Manabe M, Matsuura S (1979) Biological test using brine shrimp (part2). Rept Natl Food Res Inst 34:84–88
10. Sheikh C, Hossain MS, Easmin MS, Islam MS, Rashid M (2004) Evaluation of in vitro antimicrobial and in vivo cytotoxic properties of some novel titanium-based coordination complexes. Biol Pharm Bull 27(5):710–713
11. Ali N, Ahmed G, Shah SWA, Ismail Shah, Ghias M, Imran Khan (2011) Acute toxicity, brine shrimp cytotoxicity and relaxant activity of fruits of *Callistemon citrinus* curtis. BMC Compl Altern Med 11:99
12. Medarde M, Gordaliza M, Lucas MJ (1995) Structure elucidation of germacrane alcohols from *Juniperus communis* subsp. Hemisphaerica. J Nat Prod 58(7):1059–1064
13. Sekine N, Ashitani T, Murayama T, Shibutani S, Hattori S, Takahashi K (2009) Bioactivity of latifolin and its derivatives against termites and fungi. J Agric Food Chem 57(13):5707–5712
14. Kusumoto N, Ashitani T, Murayama T, Ogiyama K, Takahashi K (2010) Antifungal abietane-type diterpenes from the cones of *Taxodium distichum* Rich. J Chem Ecol 36(12):1381–1386
15. Yamaguchi T, Fujita K, Sakai K (1999) Biological activity of extracts from *Cupressus lusitanica* cell culture. J Wood Sci 45(2):170–173
16. Saito K, Okabe T, Fukui T, Inamori Y (1997) Antibacterial activity of hinokitiol against methicillin-resistant *Staphylococcus aureus* (MRSA) II. Applications to the disinfections of hospital environments. Mokuzai Gakkaishi 43(10):882–891