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ORIGINAL ARTICLE

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Inhibition activity of essential oils obtained from Japanese trees against *Skeletonema costatum*

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Abstract The growth inhibition activities of essential oils obtained from *Cryptomeria japonica*, *Chamaecyparis obtusa*, and *Pinus thunbergii* were examined against the bacillariophyceae *Skeletonema costatum*, also known as red tide plankton. The essential oils were extracted from the heartwood, leaves, and bark of these typical indigenous Japanese conifers. The essential oils from *C. japonica* bark and *P. thunbergii* heartwood possessed strong growth inhibition activity. The chemical compositions of these essential oils were analyzed by gas chromatography/flame ionization detection (GC-FID) and gas chromatography/mass spectrometry (GC-MS). α-Terpineol and longifolene were the main components of the essential oil from *P. thunbergii* heartwood. The *C. japonica* bark essential oil was mainly composed of α-terpineol, δ-cadinene, isophyllocladene, and ferruginol. Ferruginol and longifolene showed more potent growth inhibition against *S. costatum* than hinokitiol (β-thujaplicine), which is known to be a strong antifungal compound among wood components. Ferruginol and longifolene were important factors for the growth inhibition activity of the essential oils from *C. japonica* bark and *P. thunbergii* heartwood, respectively. These results suggest the possibility of using *C. japonica* bark and *P. thunbergii* heartwood for the control of red tide plankton.

Key words Essential oil · Inhibition activity · Red tide plankton · *Skeletonema costatum* · Woody waste

Introduction

Red tide is a serious environmental problem in the world's oceans and is caused by the explosive growth of plankton. *Skeletonema costatum*, *Heterosigma akashiwo*, and *Chattonella antiqua* are the major species causing red tide, and they are known as red tide plankton (hereafter, red tide). *S. costatum* is known to be a major species of red tide in eutrophic regions and is the cause of huge economic losses in aquaculture.[1,2] Physical, chemical, and biological methods have been examined for the control of red tide; among them, direct red tide collection, UV radiation,[3] the addition of chemical reagents and clay,[4,5] and algicidal viruses[6] and *Ruditapes philippinarum* have been used to eliminate red tide.[7] It has been reported that sesquiterpenes and fatty acids that are produced by other red tide plankton species, brown alga, or both inhibited the growth of red tide plankton.[8–10] In addition, there have been several reports on the inhibition activities of plant components against freshwater algal growth. For example, juglone, catechin, and some phenolic compounds had inhibition activities against the growth of *Microcystis aeruginosa*.[11–13] It has been reported that algae are a key life form in freshwater, and the ability of some natural products to inhibit microalgae may be of ecotoxicological significance.[14] Similarly, the control of marine microalgae using natural products is environmentally essential.

The essential oils obtained from woody plants are recognized as safe natural inhibitors with various applications because they have various bioactivities, e.g., antimicrobial, antifungal, insecticidal, and insect-repelling activities, among others.[15–17] The essential oils obtained from the wood, leaves, and bark of *Cryptomeria japonica* have inhibitory effects against bacteria, fungi, and mites.[18–22] Yoshitome et al.[23] have reported that components of *C. japonica* and *Chamaecyparis obtusa* bark suppress the growth of plants such as *Brassica rapa* var. *peruviridis*. However, there are no reports concerning inhibitory effects of essential oils against red tide plankton.

The aim of this report was to investigate the activity of essential oils from *C. japonica*, *Ch. obtusa*, and *Pinus thunbergii* against the red tide plankton *S. costatum* and to develop a new chemical utilization method of woody materials. In addition, the chemical compositions of the essential oils were analyzed by gas chromatography/mass spectrometry (GC-MS) and each active component was also examined.

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Materials and methods

Plant materials

Cryptomeria japonica and *Chamaecyparis obtusa* were obtained from the Yamagata Field Science Center (Faculty of Agriculture, Yamagata University, Japan), and *Pinus thunbergii* was obtained from Yamagata Prefecture, Japan. The heartwood of each tree specimen was ground using a Willey mill. Leaves were cut into pieces of about 1–2 cm, and bark was cut into pieces of about 1 cm^2.

Skeletonema costatum (NIES-16) was obtained from the National Institute for Environmental Studies (NIES), Japan, and was maintained under 2000 lux (12:12-h light/dark cycle) at 20°C in a NKsystem BIOTRON (Nippon Medical & Chemical Instruments). The cells were grown in f/2 medium (1 l seawater, 75 mg $NaNO_3$, 6 mg $NaH_2PO_4{\cdot}2H_2O$, 10 mg $Na_2SiO_3{\cdot}9H_2O$, 440 µl $Na_2EDTA{\cdot}2H_2O$, 316 µl $FeCl_3{\cdot}6H_2O$, 1.2 µl $CoSO_4{\cdot}7H_2O$, 2.1 µl $ZnSO_4{\cdot}7H_2O$, 18 µl $MnCl_2{\cdot}4H_2O$, 0.7 µl $CuSO_4{\cdot}5H_2O$, and 0.7 µl $Na_2MoO_4{\cdot}2H_2O$).

Preparation of essential oils

The essential oils of *C. japonica*, *Ch. obtusa*, and *P. thunbergii* were obtained from the heartwood, leaves, and bark of each tree species. The samples (raw weight: 0.2–0.25 kg) were immersed in 2 l of distilled water and submitted to hot water distillation for 8 h at 100°C according to the procedure reported by Yatagai and Takahashi.[24] The collected essential oils were separated using ethyl acetate in a separating funnel. The yields of heartwood, leaf, and bark essential oils from *C. japonica*, *Ch. obtusa*, and *P. thunbergii* were 2.8, 6.5, and 1.5 g/kg; 6.4, 11.5, and 1.6 g/kg; and 0.56, 4.0, and 0.1 g/kg, respectively.

Analysis of essential oils and compounds

Gas chromatography/flame ionization detection (GC-FID) analysis was performed with a Hitachi G-3500 gas chromatograph 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 from 50° to 300°C (10 min) at 5°C/min; an injection temperature of 250°C; and a detection temperature of 250°C. GC/MS data were collected with a Shimadzu QP-5000 GC-MS under the following conditions: DB-1 capillary column (0.32 mm i.d. ×30 m; 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA); column temperature from 40° (2 min) to 150°C (0 min) at 5°C/min and from 150° (0 min) to 320°C (2 min) at 15°C/min; an injection temperature of 230°C; a detection temperature of 250°C; and an acquisition mass range of 450–50 amu using helium as the carrier gas (3.6 ml/min). Most components were identified by comparison of the experimental GC-MS data with authentic compounds. Nuclear magnetic resonance (NMR) spectra were measured with a JEOL JNM-EX400 (^{1}H 400 MHz/^{13}C 100 MHz) spectrometer.

Authentic compounds

α-Terpineol and β-caryophyllene were obtained from Kanto Chemical, Japan. Hinokitiol (β-thujaplicine) was obtained from Osaka Organic Chemical Industry, Japan. Phyllocladene and ferruginol were isolated from *C. japonica* in previous studies.[25,26] 6,7-Dehydroferruginol was synthesized from sugiol by a chemical conversion.[27] δ-Cadinene was isolated from essential oil of *C. japonica* bark by column chromatography. Longifolene was isolated from an *n*-hexane extract of *P. thunbergii* heartwood by column chromatography. δ-Cadinene and longifolene were identified by comparison of the MS and the NMR data with previous reports.[28–30] Isophyllocladene was synthesized from phyllocladene by a chemical conversion according to a previous report.[31] Phyllocladene was isolated from *C. japonica* heartwood. Phyllocladene (10 mg) was dissolved in benzene in a round-bottom flask, and *p*-toluenesulfonic acid was added to the solution. The mixture was heated under reflux for 1 h. The cooled mixture was washed with 1% NaOH (aq.) and water and was evaporated to give the crude product (8.9 mg). The crude product was recrystallized in methanol. Isophyllocladene was identified by comparison of the NMR and MS data with previous reports.[32,33]

Isophyllocladene

Colorless needles. mp: 107.5°–108.4°C; EI-MS: m/z 272 (M^+, $C_{20}H_{32}$, 24%), 257 (5), 161 (15), 134 (18), 133 (28), 121 (25), 120 (100), 119 (37), 107 (45), 106 (62), 105 (42), 95 (21), 94 (29), 93 (33), 91 (53), 81 (24), 79 (27), 77 (25), 69 (21), 67 (23), 55 (38), 53 (15); ^{1}H-NMR ($CDCl_3$): δ 0.71 (s, 3 H, CH_3), 0.79 (s, 3 H, CH_3), 0.83 (s, 3 H, CH_3), 1.68 (d, 3 H, J = 1.45 Hz), 5.33 (s, H); ^{13}C-NMR ($CDCl_3$): δ 15.01 (CH_3), 15.5 (CH_3), 18.6 (CH_2), 19.2 (CH_2), 20.3 (CH_2), 21.9 (CH_3), 24.6 (CH_2), 33.2 (C), 33.7 (CH_3), 37.3 (C), 37.8 (CH_2), 39.1 (CH_2), 42.1 (CH_2), 42.3 (CH), 47.9 (C), 53.3 (CH), 55.0 (CH_2), 56.0 (CH), 129.3 (CH), 140.6 (C).

Longifolene

Colorless oil. EI-MS: m/z 204 (M^+, $C_{15}H_{24}$, 21%), 189 (29), 161 (74), 147 (25), 135 (39), 133 (46), 119 (51), 109 (43), 107 (69), 103 (76), 95 (62), 94 (86), 93 (78), 92 (29), 91 (100), 81 (36), 79 (79), 77 (51), 67 (48), 55 (73); ^{1}H-NMR ($CDCl_3$): δ 0.88 (s, 3 H, CH_3), 0.93 (s, 3 H, CH_3), 0.97 (s, 3 H, CH_3), 1.14 (m, 1 H), 1.53 (m, 10 H), 2.06 (d, 1 H, J = 3.9 Hz), 2.60 (d, 1 H, J = 4.6 Hz), 4.48 (s, 1 H), 4.73 (s, 1 H); ^{13}C-NMR ($CDCl_3$): δ 21.1 (CH_2), 25.5 (CH_2), 29.8 (CH_2), 30.1 (CH_3), 30.5 (CH_3), 30.6 (CH_3), 33.6 (C), 36.4 (CH_2), 43.4 (CH_2), 44.02 (C), 45.1 (CH), 47.9 (CH), 62.1 (CH), 98.9 (CH_2), 168.1 (C).

δ-Cadinene

Colorless oil. EI-MS: m/z 204 (M^+, $C_{15}H_{24}$, 36%), 189 (12), 161 (100), 134 (67), 133 (20), 119 (81), 105 (77), 93 (23), 91 (52), 81 (47), 77 (24), 55 (31), 53 (17); ^{1}H-NMR ($CDCl_3$): δ

0.77 (d, 3 H, J = 6.8 Hz), 0.94 (d, 3 H, J = 7.1 Hz), 1.04 (td, 1 H, J = 10.5, 2.4), 1.15 (m, 1 H), 1.59 (m, 1 H), 1.63 (s, 3 H, CH_3), 1.65 (s, 3 H, CH_3), 1.89 (m, 1 H), 1.93 (s, br, 1 H), 1.97 (s, br, 2 H, 2.51), 2.03 (m, 1 H), 2.50 (d, 1 H, J = 7.8 Hz), 2.69 (ddd, 1 H, J = 12.8, 4.4, 2.6), 5.45 (s, 1 H)

Growth inhibition activity

The 5-ml vials for testing were prepared for each sample, and then three vials were prepared at each concentration (100, 10, and 1 mg/l) for a total of nine vials. One milligram of each sample was weighed out, and 1 ml of acetone (1 mg/ml) was added. Different amounts of this solution (500, 50, and 5 µl) were added to 5-ml volumes of f/2 medium to obtain solutions with final concentrations of 100, 10, and 1 mg/l. After 2 h of volatilization of the acetone, *S. costatum* was added to each vial (starting cell density: 6.5×10^4–15.0×10^4 cell/ml). The volume was adjusted with culture medium to 5 ml/vial. The cell count of each vial was measured daily for 6 days. Cells were counted microscopically using counting chambers (Hirschmann Laborgerate, Eberstadt, Germany). The number of cells in 576 masses in the counting chambers was determined (N), and the counted result was converted to cell density per milliliter (the counted area of the counting chambers was 0.0025 $mm^2 \times 0.10$ mm). The cell density (D) per milliliter was defined as:

$$D = (N/576) \times 1000/(0.0025 \times 0.10) \quad (1)$$

where N is the number of cells in 576 masses

Inhibition activities (IA) were calculated using Eq. 2. A higher IA value means stronger growth inhibition:

$$\mathrm{IA} = 100 \times (1 - D_s/D_c) \quad (2)$$

where D_s is the cell density of each sample vial on the final day and D_c is the cell density of the control (untreated) vial on the final day.

Statistical analysis

Test samples were compared using analysis of variance (ANOVA). Significant differences ($P < 0.01$) between the blank (untreated) and treated cultures were analyzed by the Tukey-Kramer method.

Results and discussion

Effects of each essential oil

S. costatum cells were completely inhibited at concentrations from 10 to 100 mg/l of all essential oils examined in this study (data not shown). The inhibition activities of acetone as solvent were almost the same as the blank test (no solvent). Thus, we used acetone as a solvent in this study. At a concentration 1 mg/l, the essential oils were dissolved uniformly in medium because no emulsifications in the medium were observed. The results of the tests at a concentration of 1 mg/l are shown in Fig. 1. In the tests of essential oil from *C. japonica*, strong inhibition activity was observed for the bark essential oil (cells were destroyed) ($P < 0.01$), with lower inhibition activities observed for the heartwood (28.0%) and leaf essential oils (14.1%). In the case of *Ch. obtusa*, essential oils from each part had only weak inhibition activities (heartwood: 34.5%, bark: 23.1%, and leaves: 18.7%). Regarding the *P. thunbergii* essential oils, the heartwood essential oil exhibited high inhibition activity (85.1%, $P < 0.01$), followed by leaves (49.1%) and bark (17.9%). Thus, we found that the *C. japonica* bark and *P. thunbergii* heartwood essential oils had strong inhibition activities. Therefore, the components of these two essential oils were analyzed.

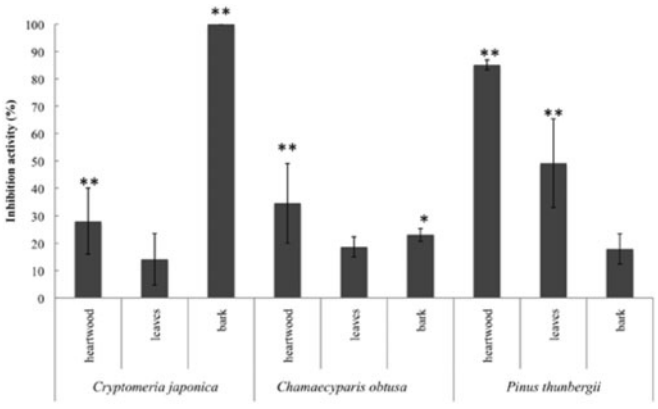

Fig. 1. Inhibition activities of essential oils against *Skeletonema costatum* at day 6. Means ± SE of three replicate experiments are given. Sample concentration was 1 mg/l. *$P < 0.05$; **$P < 0.01$, compared with control

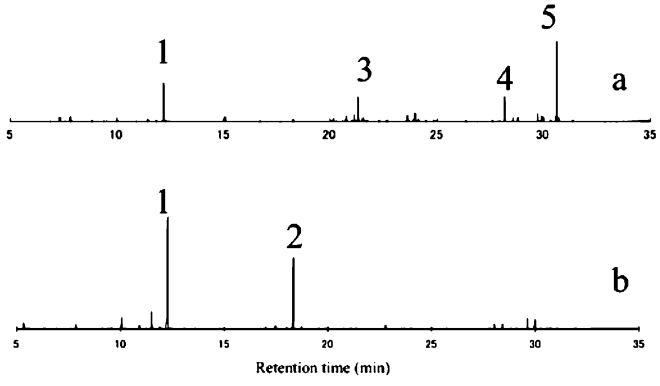

Fig. 2. Gas chromatograms of essential oils obtained from *Cryptomeria japonica* bark (**a**) and *Pinus thunbergii* heartwood (**b**). *1*, α-terpineol; *2*, longifolene; *3*, δ-cadinene; *4*, isophyllocladene; *5*, ferruginol

Analysis of essential oils

TIC gas chromatograms of the *C. japonica* bark and *P. thunbergii* heartwood essential oils by GC-MS analysis are shown in Fig. 2. A quantitative analysis of each essential oil by GC-FID is shown in Table 1. The *C. japonica* bark essential oil contained α-terpineol (28.7%), δ-cadinene (19.8%),

isophyllocladene (7.7%), and ferruginol (15.7%), as shown in Fig. 2a and Table 1. Isophyllocladene was not found in the *C. japonica* bark extract by the solvent-extraction method.[26] We assumed that isophyllocladene was formed by isomerization of phyllocladene or dehydration of phyllocladenol during hot-water distillation. Sesquiterpenes were identified as small peaks. Therefore, the main components of the *C. japonica* bark essential oil used in this study were α-terpineol, δ-cadinene, isophyllocladene, and ferruginol. The *P. thunbergii* heartwood essential oil was composed mainly of the monoterpene α-terpineol and the sesquiterpene longifolene, as shown in Fig. 2b. The amount of α-terpineol and longifolene was remarkably high in *P. thunbergii* heartwood essential oil, comprising 49.4% and 30.5%, respectively. Longifolene is known to be a characteristic component of *P. thunbergii* heartwood.[34,35] Diterpenes were not observed as main peaks on the chromatogram obtained from the *P. thunbergii* heartwood essential oil.

Table 1. Constituents of components in essential oils of *Pinus thunbergii* heartwood and *Cryptomeria japonica* bark

	Compound	Retention time[a] (min)	Content[b] (%)
C. japonica bark	α-Terpineol	11.1	28.7
	δ-Cadinene	20.1	19.8
	Isophyllocladene	30	7.7
	Ferruginol	36.2	15.7
Total			71.9
P. Thunbergii heartwood	α-Terpineol	11.1	49.4
	Longifolene	17.2	30.5
Total			79.9

[a] Conditions of gas chromatography/flame ionization detection (GC-FID) analysis are described in the Materials and methods
[b] Contents were calculated by the calibration curve method with GC-FID analysis

Effects of the essential oil components against S. costatum

The results of the growth inhibition assays for the five components contained in *C. japonica* bark and *P. thunbergii* heartwood essential oils and, in addition, caryophyllene; 6,7-dehydroferruginol; and hinokitiol, are shown in Fig. 3. The additional three compounds were used to characterize the active components in the essential oil. Isophyllocladene and δ-cadinene showed no inhibition activity. α-Terpineol and 6,7-dehydroferruginol (α-terpineol: 10.4%, 6,7-dehydroferruginol: 20.5%) had weak inhibition activities. Caryophyllene and hinokitiol showed mild activity (29.8% and 29.3% $P < 0.01$). Longifolene and ferruginol possessed strong inhibition activities (longifolene: all cells were destroyed, ferruginol: 87.5% $P < 0.01$). Hinokitiol is known to be a strong bioactive compound against fungi, bacteria,[36] and plants.[37] However, the inhibition activity of hinokitiol was not strong. In this study, longifolene and ferruginol had stronger activities than those of hinokitiol.

From the above results, the activities of the *C. japonica* bark and *P. thunbergii* heartwood essential oils were consid-

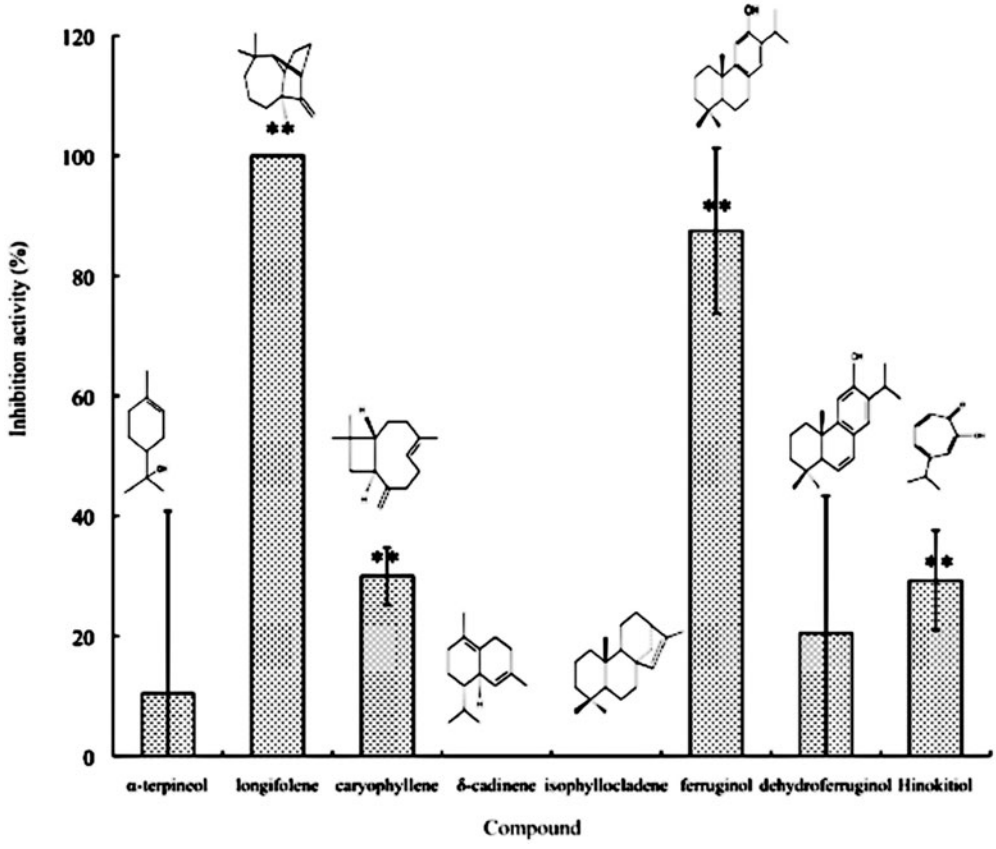

Fig. 3. Inhibition activities of essential oil components against *S. costatum* at day 6. Means ± SE of three replicate experiments are given. Sample concentration was 1 mg/l. $^{**}P < 0.01$ compared with control

ered to be mainly caused by ferruginol and longifolene, respectively. Ferruginol also has antitermitic and antifungal activities.[38–41] This study provides new knowledge about the inhibition of red tide plankton growth by ferruginol. There have been no reports about any notable bioactivity of longifolene. This is the first report of notable inhibition of red tide plankton by longifolene. Ferruginol and longifolene were important factors for the growth inhibition activity of the essential oils from *C. japonica* bark and *P. thunbergii* heartwood, respectively.

Ferruginol and 6,7-dehydroferruginol are abietane-type compounds.

Different antitermite and antifungal activities between ferruginol and 6,7-dehydroferruginol have been found, and ferruginol had much higher activities than 6,7-dehydroferruginol in both cases.[41,42] In this study also, the presence of the 6,7-double bond in the ferruginol structure strongly influenced the inhibition activities against *S. costatum*. Sesquiterpene hydrocarbons longifolene, δ-cadinene, and caryophyllene have the same molecular formula; however the activity of the compounds was not same. Longifolene is a tricyclic compound, whereas δ-cadinene and caryophyllene are bicyclic compounds. In addition longifolene and caryophyllene have an exo-double bond, but δ-cadinene has end-double bonds only. These differences in the respective structures might affect the inhibition activities of the sesquiterpenes. From Fig. 3, it is suggested that the strong activities of ferruginol and longifolene against *S. costatum* are caused by their respective structures. Thus, it was shown that the inhibition activities of ferruginol and longifolene are special and are sensitive to structural changes.

Conclusions

Essential oils from *C. japonica* bark and *P. thunbergii* heartwood showed strong growth inhibition against *S. costatum*. The *C. japonica* bark essential oil contained mainly α-terpineol, δ-cadinene, isophyllocladene, and ferruginol. The *P. thunbergii* heartwood essential oil contained mainly α-terpineol and longifolene. It was revealed that ferruginol and longifolene were the essential oil components exhibiting strong activity against *S. costatum*. These results indicate the possibility of a new use for components of woody plants to control red tide plankton growth. Forest ecology might have an effect on marine ecology through the chemical components of trees.

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