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ORIGINAL ARTICLE

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Antioxidant compounds from *Eucalyptus grandis* biomass by subcritical liquid water extraction

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Abstract The effectiveness of subcritical liquid water processing for extraction of antioxidants from the leaves of *Eucalyptus grandis* biomass was evaluated by determining the capability of the processed samples to scavenge peroxynitrite radicals in vitro, as compared with the extracts obtained by conventional extraction. Pyrogallol (**1**), 5-hydroxymethyl-2-furaldehyde (**2**), and 4,4,6,6-tetramethyl-3,5-dioxo-cyclohex-1-enecarboxylic acid (**3**) were identified as the major products obtained from the subcritical liquid water extracts. Among these, pyrogallol (**1**) exhibited stronger antioxidant activity than butylated hydroxytoluene, which was used as a standard for the antioxidant activity measurement.

Key words Subcritical liquid water · *Eucalyptus grandis* · Antioxidant activity · Peroxynitrite

Introduction

The genus *Eucalyptus* (Myrtaceae), which is native to Australia, is now grown in many parts of the world and is known for its rich source of bioactive compounds.[1] For many years, the bark and leaves of selected species have been used to treat colds, influenza, toothache, snakebite, fever, diarrhea, and many other complaints.[1,2] Although the genus is mainly grown for the production of essential oil, paper, and charcoal, it also produces a number of flavonoids, tannins, and triterpenes.[3] In the past, research on eucalyptus was mainly focused on tannins and triterpenes. However, eucalyptus is also a source for several unique secondary metabolites, which show a variety of biological activities, such as those of antioxidants, antibacterials, HIV inhibitors, attachment inhibitors, and others.[4–8] Previous studies in our laboratory and by others have indicated that the leaves of the species *Eucalyptus grandis* contain large amounts of secondary metabolites.[9,10] Among these, the phenol compound classes known as phloroglucinols and G-inhibitors have been found to show significant antioxidant activities.[11,12] In production of essential oil, paper, and charcoal, however, the leaves are usually discarded. In this research, we concentrated our efforts on effective utilization and extraction of *E. grandis* leaves.

The term "subcritical liquid water" refers to water at temperatures between its boiling temperature (100°C) and its critical temperature (374°C) and at a pressure high enough to maintain it in the liquid state. Under such conditions, the dielectric constant of water is low, which means that its polarity is also low.[13] As a result, the solubility of organic compounds in subcritical liquid water is higher than in normal water.

Research on subcritical liquid water processing (SLWP) with other plant species such as noni (*Morinda citrifolia*), oregano (*Origanum vulgare* L.), and rosemary (*Rosmarinus officinalis*) has shown that this technique can be used extensively to obtain extracts with significant antioxidant activity.[14–16] Furthermore, this process is more environmentally friendly, quicker, and less expensive than various other solvent-extraction techniques. It presents virtually no disposal costs and can be considered a clean alternative to conventional organic solvents. However, to our knowledge there has been no report of application of SLWP to *Eucalyptus grandis* leaves for extraction of antioxidants. The goal of the present investigation was to extract phenol and G-inhibitor antioxidants using SLWP and to study the differences in antioxidant activities between normal and SLWP-extracted samples of *E. grandis*. We also investigated the use of SLWP for effective utilization of *E. grandis* leaf biomass.

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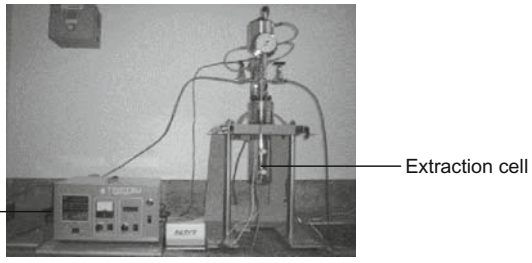


Fig. 1. Instrument used for subcritical liquid water processing (SLWP)

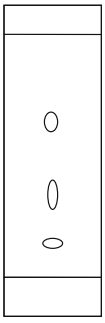


Fig. 2. Thin-layer chromatography of 160°C SLWP sample. Compound **1**, Rf = 0.66 (*top*); compound **2**, Rf = 0.52 (*middle*); and compound **3**, Rf = 0.26 (*bottom*). Silica gel $60F_{254}$, eluted with ethyl acetate, visualized under ultraviolet light (254 nm) and after spraying with vanillin sulfate

Materials and methods

Collection of *Eucalyptus grandis* leaves

Leaves of *Eucalyptus grandis* were collected from the specimen sample deposited at the Shizuoka University agricultural farm. The leaves were collected during January 2006, and were washed and preserved at –20°C until use.

Other chemicals

For antioxidant activity analysis, peroxynitrite was prepared according to a previously described method.[17] All other chemicals used were purchased from Wako (Osaka, Japan).

SLWP instrument setup and extraction method

The extraction system was equipped with a temperature controller unit from Taiatsu Techno (Tokyo, Japan) (Fig. 1). Extractions were carried out using distilled water. Pressures were measured at 160° to 300°C in a 300-ml capacity cell that contained 200 ml of water under a nitrogen atmosphere. Before the extraction, the *E. grandis* leaves were blended into an appropriate particle size range (250–500 μm) using a mixer. The cell was equipped with a stirrer to provide uniform processing of the sample. The stirrer speed was maintained at 300 rpm in each extraction.

Randomly selected extraction temperatures of 300°, 200°, and 160°C were used. It is known that pressure does not significantly affect extraction efficiency.[18] An initial pressure of 3.0 MPa was used in each extraction, and this was later increased gradually as the temperature was raised in order to maintain the water in the liquid state. The maximum pressures observed at the above temperature conditions were 7.20, 4.91, and 4.35 MPa, respectively.

Leaves of *E. grandis* that had been preserved at –20°C (60.00 g) were processed with 200 ml of water. The extraction procedure was as follows:

1. The sample was loaded into the cell,
2. The cell was filled with 200 ml of water and sealed,
3. The initial pressure (3.0 MPa) was applied with nitrogen gas,
4. The temperature was gradually raised up to the set value (with a temperature controller unit) from the initial room temperature. The holding time was set to 3 min. The typical heating period required to raise the temperature of the reactor from 25° to 200°C was about 30 min,
5. After extraction, the cell was cooled to room temperature and depressurized.

The cell was rinsed between extractions to prevent contamination between extractions. The collected samples were protected from light and preserved at 4°C.

After the extraction, the aqueous layer was filtered and the filtrate was further treated with ethyl acetate (300 ml × 3) to separate the extraction components into organic and aqueous phases. The organic layer was then dehydrated over anhydrous Na_2SO_4 and concentrated under vacuum in readiness for separation of component compounds. The yields of concentrated samples were: 290 mg (160°C), 231 mg (200°C), and 15.5 mg (300°C).

The SLWP sample extracted at 160°C was selected for further investigation because of its comparatively high yield and the clear and concentrated spots observed in thin-layer chromatography (TLC) analysis (Fig. 2). For measurement of antioxidant activity, the aqueous layer obtained from 60.0 g of *E. grandis* leaves by SLWP (at 160°C) was freeze-dried (Eyela FDU-2000, Tokyo Rikakikai, Japan) and redissolved in 200 ml of methanol to a final concentration of 1 mg/ml.

Isolation and determination of compounds

TLC analysis was performed on silica gel glass plates ($60F_{254}$, Merk) using ethyl acetate as eluent. The spots were detected by spraying with vanillin sulfate and then examining under ultraviolet (UV) light (254 nm). A hexane/ethyl acetate solvent system (7:3; 6:4; 5:5; 4:6; 3:7, and 0:10) was used for the separation of components by column chromatography. The structures of the isolated compounds were elucidated by using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) and by comparison of spectroscopic data with literature values. NMR spectra were recorded on a Jeol JNM-EX 270 spectrometer (^{1}H 270 MHz; ^{13}C 67.8 MHz) and on Jeol JNM-LA 500 spectrometer (^{1}H 500 MHz; ^{13}C 125.65 MHz). Mass spectrometry was carried out in the electrospray ionization (EI) positive-ion mode with a Jeol JMS-DX 303HF mass

spectrometer. CD_3OD was used as solvent for the spectral analysis.

Solvent extraction of *Eucalyptus grandis* leaves

Leaves of *E. grandis* (60 g) were extracted with 200 ml of methanol in a 300-ml flask for 7 days with constant stirring. The extract was then filtered (Whatman no. 1) and the filtrate was concentrated under vacuum using a rotary evaporator. The resulting concentrate (312 mg) was used for measurement of antioxidant activity after suitable dilution (1 mg/ml).

Antioxidant activity test

The antioxidant activity of each sample was evaluated by measuring the ability of the samples to scavenge the free radicals of peroxynitrite in vitro. The tests were carried out by a method described in a previous report.[17]

Results and discussion

Comparison of SLWP and conventional extraction

The performance of SLWP was compared with conventional extraction in terms of extraction yield and by measuring their peroxynitrite antioxidant activities. The extraction yields were observed to be 290 mg for SLWP and 312 mg for solvent extraction.

However, SLWP extracts exhibited 1.4 times higher antioxidant activity than those of the conventional extract. The antioxidant activities of the SLWP extract and the conventional solvent extract were 56.72 ± 0.94 (%) and 40.23 ± 1.20 (%), respectively (n = 3).

Isolation and determination of compounds present in SLWP samples

To investigate the reason for the difference between the antioxidant activities of the extracts, the 160°C SLWP sample was further analyzed by TLC and the components were separated by column chromatography. On TLC analysis, three major spots were detected and are referred to as compounds **1**, **2**, and **3** (Rf values: 0.66, 0.52, and 0.26, respectively) (Fig. 2).

Structure elucidation of 1, 2, and 3 by spectral analysis

Compounds **1** (Rf = 0.66) and **2** (Rf = 0.52) were assigned as pyrogallol (105 mg) and 5-hydroxymethyl-2-furaldehyde (132 mg), respectively, by comparison of ^{1}H and ^{13}C NMR spectra with those of authentic commercially available samples. Compound **3** (Rf = 0.26) was characterized as 4,4,6,6-tetramethyl-3,5-dioxo-cyclohex-1-enecarboxylic acid (15 mg) from ^{1}H and ^{13}C NMR spectra and EI mass spectra and by comparison with the literature data (Fig. 3).[19]

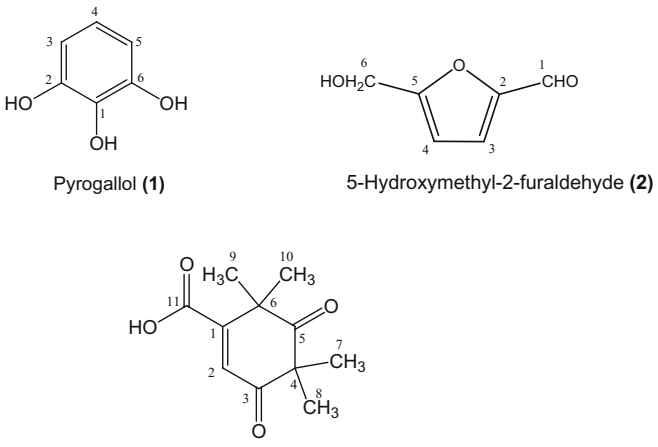


Fig. 3. Isolated compounds from 160°C SLWP sample

*Pyrogallol (**1**).* ^{1}H NMR δ (CD_3OD): 6.31 (2H, d, 3 and 5-CH), 6.49 (1H, t, 4-CH), 5.14 (3H, broad peak, 1, 2, and 6-OH). ^{13}C NMR δ (CD_3OD): 109.1 (3 and 5-C), 120.9 (4-C), 134.9 (1-C), 147.7 (2 and 6-C).

*5-Hydroxymethyl-2-furaldehyde (**2**).* ^{1}H NMR δ (CD_3OD): 9.53 (1H, s, 1-CHO), 7.37 (1H, d, 3-CH), 6.57 (1H, d, 4-CH), 4.83(2H, s, 6-CH_2). ^{13}C NMR δ (CD_3OD): 57.6 (6-C), 110.8 (4-C), 124.7 (3-C), 153.9 (2-C), 163.2 (5-C), 179.4 (1-C).

*4,4,6,6-Tetramethyl-3,5-dioxo-cyclohex-1-enecarboxylic acid (**3**).* EI MS *m/z*: 210. ^{1}H NMR δ (CD_3OD): 1.28 (6H, s, 9 and 10-CH_3), 1.50 (6H, s, 7 and 8-CH_3), 6.33 (1H, s, 2-CH). ^{13}C NMR δ (CD_3OD): 24.2 (7 and 8-C), 26.6 (9 and 10-C), 47.5 (6-C), 57.8 (4-C), 124.5 (2-C), 162.1 (1-C), 173.3 (11-C), 203.4 (3-C), 215.0 (5-C).

Antioxidant activity of individual compounds

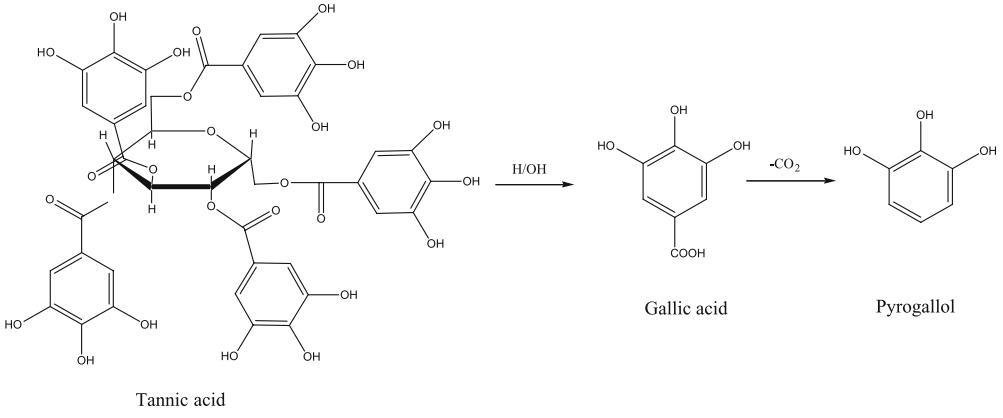
Antioxidant activities of all isolated compounds were measured to determine their individual influence on total antioxidant activity of the SLWP sample (Table 1). Butylated hydroxytoluene (BHT) was used as a standard for the antioxidant activity measurement. Pyrogallol exhibited the strongest antioxidant activity. At 100 μM concentration, the activity of pyrogallol was almost four times higher than that of BHT. At 200 μM and 400 μM, the activity of pyrogallol was around double that of BHT. 5-Hydroxymethyl-2-furaldehyde and compound **3** did not exhibit much activity and had lower activities than BHT. These results revealed that the antioxidant activity of the 160°C SLWP sample was largely attributed to pyrogallol, followed by 5-hydroxymethyl-2-furaldehyde and compound **3**. Formation of these compounds could be a reason for the increase in antioxidant activity of the SLWP sample of *Eucalyptus grandis*.

Table 1. Antioxidant activities of isolated individual compounds and butylated hydroxytoluene (BHT) against peroxynitrite free radicals

Compound concentration	Activity (%)			
	1	2	3	BHT
100 μM	46.68 ± 0.24	2.87 ± 0.56	2.15 ± 0.76	11.83 ± 0.75
200 μM	66.19 ± 0.76	8.36 ± 0.83	8.01 ± 0.91	34.65 ± 0.66
400 μM	82.59 ± 0.04	17.55 ± 0.26	13.89 ± 0.57	41.08 ± 0.24

Data given as mean ± standard deviation; $n = 3$
1, Pyrogallol; **2**, 5-hydroxymethyl-2-furaldehyde; **3**, 4,4,6,6-tetramethyl-3,5-dioxo-cyclohex-1-enecarboxylic acid

Fig. 4. Formation mechanism for pyrogallol (**1**)



Mechanism for the formation of each compound in SLWP

The type of compounds isolated by SLWP suggests that they were not originally present in the untreated plant, but seemed to be the degradation products formed due to the high reactivity of subcritical liquid water. In order to confirm this assumption, we compared solvent-extracted *E. grandis* sample with that of SLWP sample by TLC. The solvent-extracted sample showed no spots corresponding to compounds **1**, **2**, and **3**, indicating these compounds were transformed products. Therefore, we hypothesized the following possible pathways for the formation of the three compounds.

*Formation of pyrogallol (**1**)*

Eucalyptus is a rich source of tannins.[3] It is known from past research that under high temperature and pressure in SLWP, compounds tend to undergo hydrolysis.[20,21] We expect that the tannins present in *E. grandis* undergo hydrolysis to form gallic acid, which later undergoes decarboxylation to form pyrogallol (Fig. 4).

*Formation of 5-hydroxymethyl-2-furaldehyde (**2**)*

We confirmed that during SLWP, cellulose and polysaccharides present in eucalyptus undergo thermal degradation reactions and dissociate to form 5-hydroxymethyl-2-furaldehyde.[22]

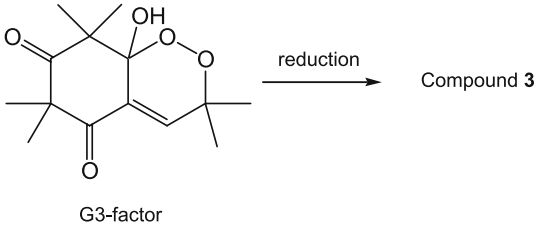


Fig. 5. Formation mechanism for compound **3**

*Formation of 4,4,6,6-tetramethyl-3,5-dioxo-cyclohex-1-enecarboxylic acid (**3**)*

Eucalyptus is also a rich source of secondary metabolites such as the G inhibitor class of compounds,[23] including one known as G3 factor. Synthetic studies by Andre-Barres et al.[19] revealed that G3 factor undergoes reduction reaction to form **3**. Based on this observation, we speculated that the reduction reaction would occur in SLWP to form **3** from G3 factor of *E. grandis* (Fig. 5). In order to confirm this assumption, we synthetically prepared G3 factor as described before[24] and subjected the purified G3 factor to SLWP under the same conditions used for SLWP at 160°C. We obtained a major fraction, which on TLC analysis gave the same Rf value as **3**. Furthermore, isolation and spectral analysis of the fraction revealed similar spectral patterns to confirm the formation of **3**. For a description of the detailed reduction reaction pathway, refer to Andre-Barres et al.[19]

*4,4,6,6-Tetramethyl-3,5-dioxo-cyclohex-1-enecarboxylic acid (synthetically confirmed sample) (**3**).* ^{1}H NMR δ (CD_3OD): 1.31 (6H, s, 9 and 10-CH_3), 1.52 (6H, s, 7 and 8-CH_3), 6.50 (1H, s, 2-CH). ^{13}C NMR δ (CD_3OD): 24.0 (7 and 8-C), 26.2 (9 and 10-C), 47.5 (6-C), 58.5 (4-C), 129.2 (2-C), 155.2 (1-C), 169.1 (11-C), 202.7 (3-C), 214.2 (5-C).

Conclusions

We expect that the results of our study will be helpful to understanding different reaction modes of SLWP and this technique will be utilized in future not only for extraction but also as a tool to perform hydrolysis, degradation, oxidation, and reduction reactions.

The results in this study suggested that organic solvent-free SLWP gives comparable amounts and quality of extracts, as determined from the antioxidant activity, when compared with conventional solvent extraction. Further isolation and analysis of SLWP samples gave three compounds of which pyrogallol showed the strongest antioxidant activity. The three compounds separated were not originally present in the leaves of *E. grandis*; rather, they were formed by hydrolysis, thermal decomposition, and reduction reactions, respectively.

Based on the observed results, we believe that this technique can be utilized as a fast, inexpensive, and environment-friendly tool for processing biomass materials and for performing different types of reactions. Furthermore, because decomposition of long-chain and heavy compounds takes place in SLWP, plant materials like wood, which are difficult to degrade otherwise, would be degraded easily. In addition, many important and novel intermediates as well as final products can be isolated by the use of this method. This research highlights SLWP as a very promising technique for analysis, degradation, and synthesis purposes. Further research on application of SLWP for wood biomass processing and respective analysis conditions is presently underway.

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