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Antiplatelet Activity of *Thujopsis dolabrata* var. *hondai*-Derived Component Against Platelet Aggregation

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Abstract The steam distillate obtained from *Thujopsis dolabrata* var. *hondai* sawdust was fractionated by centrifugal thin-film evaporation, and the fractions were then investigated for antiplatelet activity using washed rabbit platelets. The biologically active constituent of *T. dolabrata* var. *hondai* sawdust was isolated by silica gel column and HPLC chromatographies and characterized as carvacrol by various spectral analyses. Carvacrol inhibited platelet aggregation induced by collagen, arachidonic acid, and platelet activating factor with IC_{50} values of 12.6, 2.5, and 385.3 μM, respectively. However, carvacrol had no effect on thrombin, calcium ionophore A23187, or phorbol 12-myristate 13-acetate induced platelet aggregation. Carvacrol was a much more potent inhibitor, as antiplatelet agents, compared with aspirin. These results suggest that carvacrol isolated from *T. dolabrata* var. *hondai* sawdust may be useful as a lead compound for inhibiting arachidonic acid-induced platelet aggregation.

Key words: *Thujopsis dolabrata* var. *hondai*, carvacrol, platelet aggregations, antiplatelet agents

Platelet aggregation is a complex phenomenon that probably involves several intracellular biochemical pathways. Platelets activated by a number of physiological agonists, such as collagen, arachidonic acid (AA), thrombin, or platelet activating factor (PAF), undergo a complex cascade of events that result in shape change, secretion, formation of AA metabolites, and aggregation [17]. Since platelets readily aggregate in response to a variety of endogenous substances and secrete various substances that cause further aggregation, they can initiate thrombus formation and precipitate thromboembolism, leading to ischemic diseases. In addition, the interactions between platelets and blood vessel walls are important in the development of thrombosis and cardiovascular diseases [5, 8, 15]. When blood vessels are damaged, platelet aggregation occurs rapidly to form hemostatic plugs or arterial thrombi at the sites of vessel injury or in regions where blood flow is disturbed. These thrombi are the source of thromboembolic complications of atherosclerosis, heart attacks, stroke, and peripheral vascular disease [13]. Therefore, the inhibition of platelet function represents a promising approach for the prevention of thrombosis.

Plant extracts may be an alternative to currently used medicinal sources, because they constitute a rich source of bioactive chemicals [9, 18, 20]. Since many of them are largely free from adverse effects and have excellent pharmacological actions, they could lead to the development of new classes of possibly safer and antiplatelet agents [10, 15, 22]. Additionally, some flavonoids and polyphenols are found to have effective inhibitory activities of platelet aggregation induced by collagen [12, 22]. Therefore, much effort has been focused on the plants for potentially useful products as commercial antiplatelet agents or lead compounds. However, relatively little work has been done on the inhibitory activities of platelet aggregation by *Thujopsis dolabrata* sawdust despite its excellent antibacterial [10], hypocholesterolemic [6], insecticidal [2], and rodent-repellent effects [1]. Active compound isolated from *T. dolabrata* sawdust may be a good source as a lead compound for antiplatelet agent.

Chemicals

The sawdust of *Thujopsis dolabrata* var. *hondai* was kindly provided by Prof. Sang-Hyun Lee at the Institute of Agricultural Science & Technology, College of Agriculture, Chonbuk National University, Chonju, Korea and subjected

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to steam distillation. Collagen, AA, and thrombin were obtained from Chrono-Log Co. (Havertown, PA, U.S.A.). Aspirin, PAF, calcium ionophore A23187, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Other chemicals were of analytical grade.

Isolation and Identification

The sawdust of *T. dolabrata* var. *hondai* (30 kg) was extracted by steam distillation. The steam distillate (300 g) was fractionated by centrifugal thin-film evaporation, and then the fractions I (white color, 19.5 g), II (blue color, 98 g), III (red color, 85.2 g), and IV (black color, 97 g) were tested for their antiplatelet activity. Fraction I was isolated by silica gel column chromatography, Develosil 60-5 (Nomura Chemical, Japan), and Cosmosil $_5C_{18}$ columns (Nacalai Tesque, Japan) as previously described by Ahn *et al.* [1]. Structural determination of the active isolate was made by spectroscopic analyses. ^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker AVNCE 200 spectrometer (TMS as an internal standard), and GC mass spectra on a Shimadzu GC/MS-QP5050 spectrometer.

Platelet Aggregation

Platelets were obtained as platelet-rich plasma (PRP) from fresh rabbit blood according to the washing procedures described previously [12]. Platelet aggregation was measured using an aggregometer (470-vs, Chrono-log Co. PA, U.S.A.) as previously described [14]. Each inhibition rate was obtained from the maximal aggregation induced by respective agonists. The extents of inhibition of platelet aggregation are expressed as % inhibition (**X**), using the following equation: **X**=[(A-B)/A]×100; A: maximal aggregation of control, B: maximal aggregation of sample treated washed platelets.

Four fractions (I-IV) obtained from the steam distillate of *T. dolabrata* sawdust were tested for the inhibitory effect on platelet aggregation, using washed rabbit platelets (Table 1). Of the fractions, fraction I showed strong inhibitory effect (88%) on collagen-induced platelet aggregation, but the other fractions (II-IV) exhibited no or weak inhibitory effects. Due to the strong activity of fraction I, purification of the biologically active component was carried out by silica gel column and HPLC chromatographies. Five isolates (A-E), described in Materials and Methods, exhibited inhibitory activity against collagen. Compound D strongly inhibited platelet aggregation induced by collagen. These results indicated that the compound D was the most effective antiplatelet compound. Various spectroscopic analyses characterized the structure of the compound D as carvacrol. The ^{13}C- and ^{1}H-NMR spectra of authentic carvacrol were found to be the same as those for the carvacrol isolated from *T. dolabrata* var. *hondai* sawdust [1, 2].

Table 1. Effects of various fractions obtained from the steam distillate of *Thujopsis dolabrata* sawdust on platelet aggregation induced by collagen.

Sample[a]	Inhibition (%)
Steam distillate	65±3.2
Fraction I	88±5.1
II	19±3.2
III	10±0.9
IV	34±2.4

[a]Injected sample concentration: 300 μg/ml. Values are presented as means± S.D.

Table 2. IC_{50} (μM) of carvacrol on platelet aggregation induced by various agonists in washed rabbit platelets.

Agonists	Carvacrol	Aspirin
Collagen	12.6±1.3	>200
AA	2.5±0.7	35.6±2.7
Thrombin	>200	>200
PAF	>200	>200
A23187	>200	>200
PMA	>200	>200

Washed rabbit platelets were preincubated with carvacrol, DMSO (0.5% control), or aspirin (50 μM) at 37°C for 3 min in the presence of 1 mM $CaCl_2$, and platelet aggregation was then induced by the addition of collagen (1 μg/ml), AA (100 μM), thrombin (0.1 unit/ml), PAF (10 nM), A23187 (2 μM), or PMA (20 μM). The 50% inhibitory concentration (IC_{50}) values were calculated from at least three separate experiments. Values are presented as means±S.D.

The inhibitory activities of carvacrol against platelet aggregation induced by collagen (1 μg/ml), AA (100 μM), PAF (10 nM), thrombin (0.1 unit/ml), calcium ionophore A23187 (2 μM), and PMA (20 μM) were compared with those of aspirin as antiplatelet agents (Table 2). Carvacrol inhibited platelet aggregation induced by collagen, AA, and PAF with IC_{50} values of 12.6, 2.5, and 385.3 μM, respectively. However, carvacrol had no or weak inhibitory effect on thrombin, calcium ionophore A23187, and PMA-induced platelet aggregations. Carvacrol was most effective in inhibiting arachidonic acid-induced platelet aggregation, followed by collagen or PAF: The commonly used aspirin served as a standard of comparison for the antiplatelet activity. Aspirin inhibited platelet aggregation induced by AA with IC_{50} value of 35.6 μM (Table 2). However, aspirin had no or weak inhibitory effect on collagen, thrombin, PAF, calcium ionophore A23187, and PMA. Therefore, carvacrol in the AA-induced platelet aggregation was about 14 times stronger than aspirin.

Platelets play an important role in the hemostatic process, and their aggregation can cause arterial thrombosis [17]. Accordingly, bioactive chemicals with antiplatelet activity can be useful therapeutic agents. The bioactive chemicals derived from plants display numerous biological activities [3, 11, 19], including antiplatelet activity [4, 12, 21, 22]. Many *Thujopsis* species are a rich source of terpenoids [7].

It has previously been reported that *Thujopsis* species have antimicrobial [10], hypocholesterolemic [6], and rodent-repellent effects [1] in toxicological and pharmacological investigations. Furthermore, carvacrol and β-thujaplicine isolated from *T. dolabrata* sawdust had broad insecticidal and acaricidal activities against agricultural, stored-product, and medical arthropod pests (*Reticulitermes speratus*, *Lasioderma serricorne*, *Callosobruchus chinensis*, *Sitophilus oryzae*, *Plutella xylostella*, *Myzus persicae*, *Blatella germanica*, and *Tetranychus urticae*) [2]. The insecticidal activity of carvacrol is attributable to fumigant action. As a naturally occurring insecticide, carvacrol could be a new useful preventive agent against damage caused by these arthropod pests. In this study, *T. dolabrata* sawdust-derived carvacrol has been suggested to be an indication of at least one of the pharmacological actions for inhibiting AA-induced platelet aggregation.

Arachidonic acid is a membrane-derived fatty acid that is metabolized by cyclooxygenase to prostaglandin (PG) endoperoxide intermediates such as prostaglandin (PG) H_2 [17]. In platelets, endoperoxides are further metabolized to thromboxane A_2 (TXA_2) and PGs [16]. In this study, carvacrol showed potent inhibitory effect, especially on AA-induced platelet aggregation. TXA_2, which strongly induces platelet aggregation [22], is synthesized from arachidonic acid *via* the COX pathway, suggesting that carvacrol may at least have an effect on the COX pathway in platelets. Based on our limited data together with some earlier findings, the inhibitory action of *T. dolabrata* sawdust-derived material showed superiority and usefulness as antiplatelet agents, although the *in vivo* efficacy and their clinical usefulness remain to be evaluated.

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REFERENCES

1. Ahn, Y. J., S. B. Lee, T. Okubo, and M. Kim. 1995. Antignawing factor of crude oil derived from *Thujopsis dolabrata* S. et. Z. var. *hondai* sawdust against mice. *J. Chemical Ecology* **21:** 263-271.
2. Ahn, Y. J., S. B. Lee, H. S. Lee, and G. H. Kim. 1998. Insecticidal and acaricidal activity of carvacrol and β-thujaplicine derived from *Thujopsis dolabrata* var. *hondai* sawdust. *J. Chemical Ecology* **24:** 81-90.
3. Balandrin, M., J. Klocke, E. S. Wurtele, and W. H. Bollinger. 1985. Natural plant chemicals: Sources of industrial and medicinal materials. *Science* **228:** 1154-1160.
4. Chang, F. R., J. L. Wei, C. M. Teng, and Y. C. Wu. 1998. Antiplatelet aggregation constituents from *Annona purpurea*. *J. Nat. Prod.* **61:** 1457-1461.
5. Dinerman, J. L. and J. L. Mehta. 1990. Endothelial, platelet and leukocyte interactions in ischemic heart disease: Insights into potential mechanisms and their clinical relevance. *J. Am. Cardiol.* **16:** 207-222.
6. Enomoto, H., Y. Yoshikuni, Y. Yasutomi, K. Ohata, K. Sempuku, K. Kitaguchi, Y. Fujita, and T. Mori. 1997. Hypocholesterolemic action of tricyclic diterpenoids in rats. *Chem. Pharm. Bull.* **25:** 507-510.
7. Hasegawa, S. and Y. Hirose. 1982. Terpenoids from the seed of *Thujopsis dolabrata* var. *dolabrata*. *Phytochemistry* **21:** 643-646.
8. Hirsh, J. 1987. Hyperactive platelets and complications of coronary artery disease. *N. Engl. J. Med.* **316:** 1543-1544.
9. Hwang, Y. H. and H. S. Lee. 2002. Antibacterial activity of *Pinus densiflora* leaf-derived components toward human intestinal bacteria. *J. Microbiol. Biotechnol.* **12:** 610-616.
10. Ito, M., M. Hamada, M. Arakawa, and F. Abe. 1980. Antimicrobial activities of Hiba oil, Thujopsene and its various derivatives. *Bokin Bobai* **8:** 3-6.
11. Kim, M. K. and H. S. Lee. 2002. Growth responses of various flower extracts toward human intestinal microflora. *Food Sci. Biotechnol.* **11:** 444-447.
12. Lin, C. N. and C. M. Lu. 1996. Novel antiplatelet constituents from Formosan *Moraceous plants*. *J. Nat. Prod.* **59:** 834-838.
13. Packham, M. A. 1994. Role of platelets in thrombosis and hemostasis. *Can. J. Physiol. Pharm.* **72:** 278-284.
14. Rho, M. C., Y. H. Park, S. Sasaki, M. Ishibashi, K. Kondo, J. Kobayashi, and Y. Ohizumi. 1996. The mode of rabbit platelet shape change and aggregation induced by theonezolide-A, a novel polyketide macrolide, isolated from the Okinawan marine sponge *Theonella* sp. *Can. J. Physiol. Pharm.* **74:** 193-199.
15. Ross, R. 1986. The pathogenesis of atherosclerosis: An update. *N. Engl. J. Med.* **314:** 488-500.
16. Samuelsson, B., M. Goldyne, E. Granstrom, M. Hamberg, S. Hammarstrom, and C. Malmsten. 1978. Prostaglandins and thromboxanes. *Annu. Rev. Biochem.* **47:** 997-1029.
17. Siess, W. 1989. Molecular mechanisms of platelet activation. *Physiol. Rev.* **69:** 58-178.
18. Singh, D. P., A. Kumar, and M. B. Tyagi. 2003. Biotoxic cyanobacterial metabolites exhibiting pesticidal and mosquito larvicidal activities. *J. Microbiol. Biotechnol.* **13:** 50-56.
19. Son, D. J., S. E. Lee, and B. S. Park. 2003. Inhibitory effects of naturally occurring flavonoids on human intestinal bacterium, *Clostridium perfringens*. *Food Sci. Biotechnol.* **12:** 180-182.
20. Song, M. H., K. Selvam, H. Y. Jeong, and K. S. Chae. 2003. Inhibition of asexual sporulation and growth of *Aspergillus niger* and *Aspergillus oryzae* by propylamine. *J. Microbiol. Biotechnol.* **13:** 146-148.
21. Tsai, W. J., W. C. Hsin, and C. C. Chen. 1996. Antiplatelet flavonoids from seeds of *Psoralea corylifolia*. *J. Nat. Prod.* **59:** 671-672.
22. Tsai, I. L., W. Y. Lin, C. M. Teng, T. Ishikawa, S. L. Doong, M.W. Huang, Y. C. Chen, and I. S. Chen. 2000. Coumarins and antiplatelet constituents from the root bark of *Zanthoxylum schinifolium*. *Planta Medica* **66:** 618-723.