DOI:10.1021/jf102689k

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Phytochemicals from *Acacia confusa* Heartwood Extracts Reduce Serum Uric Acid Levels in Oxonate-Induced Mice: Their Potential Use as Xanthine Oxidase Inhibitors

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In this study, the antihyperuricemic effect of *Acacia confusa* heartwood extracts and their phytochemicals on potassium oxonate (PO)-induced acute hyperuricemia was investigated for the first time. All treatments at the same dosage (100 mmol/kg) were administered to the abdominal cavity of PO-induced hyperuricemic mice, and serum uric acid level was measured at 3 h after administration. In experimental mice, serum uric acid level was significantly suppressed by the administration of *A. confusa* heartwood extracts and their major phytochemicals, (−)-2,3-*cis*-3,4-*cis*-3,3′,4,4′,7,8-hexahydroxyflavan, (−)-2,3-*cis*-3,4-*cis*-4′-methoxy-3,3′,4,7,8-pentahydroxyflavan, melanoxetin, transilitin, and okanin, relative to the PO group. The direct inhibitory effect of these five compounds on xanthine oxidase (XOD) activity was examined using isothermal titration calorimetry (ITC). Among them, melanoxetin showed a more remarkable inhibitory effect on XOD activity than allopurinol, a clinical drug used for XOD inhibitor. To further understand the stereochemistry between XOD and melanoxetin (or allopurinol), structure-based molecular modeling was performed. Melanoxetin undergoes extended interactions in the hydrophobic region via the 3′,4′-dihydroxyphenyl moiety, thus accounting for its higher binding affinity to XOD than allopurinol. These results indicate that *A. confusa* heartwood extracts and their major phytochemicals exhibit strong XOD inhibitory effects, which reduce serum uric acid levels while inhibiting uric acid generation in purine metabolism.

KEYWORDS: *Acacia confusa*; xanthine oxidase; hyperuricemia; melanoxetin; isothermal titration calorimetry; molecular docking

INTRODUCTION

Excess uric acid (hyperuricemia) in the body has been associated with gout, chronic kidney disease, and metabolic syndrome (*1*−*3*). The optimal treatment of hyperuricemia-induced clinical disorders requires long-standing reduction in serum uric acid. The urate-lowering drugs used to treat chronic gout are uricostatic agents, which are xanthine oxidase inhibitors, and uricolytic agents. Medications for urate-lowering therapy are still needed for the management of hyperuricemia (*4*).

In recent years, there has been increasing interest in the search for more effective or novel bioactive compounds for antihyperuricemic agents from a wide variety of traditional herbal plants (*5*−*7*). Xanthine oxidase is an important enzyme that catalyzes the oxidation of hypoxanthine to xanthine and uric acid from xanthine in the purine metabolism, and approximately half of the antihyperuricemic agents are xanthine oxidase inhibitors. Therefore, xanthine oxidase is a good molecular target for research and development of new therapeutic agents against hyperuricemic syndromes. Recently, there have been more and more studies on the enzyme-catalyzed reaction using isothermal titration calorimetry (ITC) (*8*−*11*). Like all chemical reactions, these reactions occur with some heat release or absorption in direct proportion to the rate, making calorimetric techniques very suitable for them (*12*, *13*). In this study, the xanthine−xanthine oxidase reaction was investigated for the first time using ITC.

The genus *Acacia*, an evergreen tree, comprises more than 1300 species and occurs in almost all habitat types (*14*). Many *Acacia* species have been reported to have important uses in traditional medicine; chemical constituents found in the various species have different medicinal actions (*15*−*23*). *Acacia confusa* Merr. (Leguminosae), a native species in Taiwan, is widely distributed on the hills and lowlands of Taiwan and has been traditionally used as a medicine (*20*). In our previous studies, it was found that ethanolic extracts of the heartwood contain large amounts of flavonoids and show excellent antioxidant and anti-inflammatory activities (*20*, *21*). Flavonoids are naturally occurring plant compounds that have been demonstrated to possess xanthine

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 Published on Web 08/31/2010 © 2010 American Chemical Society

oxidase inhibitory property (*24*). The consumption of flavonoids has also been reported to be associated with the protective effects of certain diets and herbs against hyperuricemia and gout (*25*, *26*). Therefore, ethanolic extracts of *A. confusa* heartwood may be good candidates for further development as clinically used anti-hyperuricemic agents. To the best of our knowledge there is only one report on the hypouricemic effect of ethanolic extracts from *A. confusa* heartwood. It was proven that the heartwood extract of *A. confusa* and its constituents exhibited excellent XOD-inhibitory performance using in vitro assay (*27*). However, at this juncture we have no definite information that the ethanolic extract from *A. confusa* heartwood and its constituents possess hyperuricemic activity in vivo. Therefore, in this study we investigated the hypouricemic effect of ethanolic extracts and their major phytochemicals from *A. confusa* heartwood in mice for the first time.

MATERIALS AND METHODS

Chemicals. Allopurinol, potassium oxonate, xanthine, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO). The other chemicals and solvents used in this experiment were of HPLC grade.

Plant Materials. The heartwood of *A. confusa* was sampled from the experimental forest of National Taiwan University in Nan-Tou County. The species was identified by Sheng-You Lu of the Taiwan Forestry Research Institute, and a voucher specimen (AC006) was deposited at the School of Forestry and Resource Conservation, National Taiwan University. The materials were air-dried at ambient temperature (25 °C) and stored in a refrigerator at 4 °C prior to treatments.

Extraction, Isolation, and Quantification. Heartwoods were cut into small pieces and soaked in ethanol at ambient temperature for 7 days. The extracts were decanted and filtered through Whatman no. 2 filter paper, and the filtrates were concentrated in a rotary evaporator and then lyophilized. The phytochemicals from crude extracts were separated and purified by semipreparative HPLC on a model PU-980 pump (Jasco, Japan) equipped with an MD-910 photodiode array detector (Jasco) and a 250 mm × 10.0 mm i.d., 5 μm, Luna RP-18 column (Phenomenex, Torrance, CA). The mobile phase was solvent A, 100% MeOH, and solvent B, ultrapure water. Elution conditions were 0−20 min of 15−30% A to B (linear gradient) and 20−50 min of 30−50% A to B (linear gradient) at a flow rate of 4 mL/min. ESI-MS data were collected by a Finnigan MAT-95S mass spectrometer, and NMR spectra were recorded by a Bruker Avance 500 MHz FT-NMR spectrometer. The structures of compounds **1**−**5** were identified by ESI-MS and NMR.

The phytochemicals were quantified by analytical HPLC on a model PU-980 pump with a 250 mm × 4.6 mm i.d., 5 μm, Luna RP-18 column. The mobile phase was solvent A, 100% MeOH, and solvent B, ultrapure water. Elution conditions were 0−25 min of 10−35% A to B (linear gradient), 25−55 min of 35−50% A to B (linear gradient), and 55−60 min of 50−100% A to B (linear gradient) at a flow rate of 1.0 mL/min using a detector, an MD-910 photodiode array at 254 nm wavelength. For the preparation of the calibration curve, standard stock solutions of compounds were prepared in methanol, filtered through Millipore filters (0.45 μm), and appropriately diluted to obtain the desired concentrations in the quantification range. The calibration graphs were plotted after linear regression of the peak areas versus concentrations.

Animals. Male ICR mice with a body weight of about 30 g (6 weeks old) were purchased from the Laboratory Animal Center of the Medical College of National Taiwan University (Taipei, Taiwan). Mice were given a standard laboratory diet and distilled water ad libitum and kept on a 12 h light/dark cycle at 22 ± 2 °C. This study was conducted according to institutional guidelines and approved by the Institutional Animal Care and Utilization Committee of Taipei Medical University, Taiwan.

Potassium Oxonate (PO)-Induced Hyperuricemia in Mice. For the hyperuricemia study, PO, the uricase inhibitor, was employed to induce acute hyperuricemia according to the method of Chien et al. (*28*) with slight modifications. Fifty-four mice were randomly assigned to nine groups for treatment (n = 6 per group): (1) vehicle group; (2) PO group; (3) PO + AP group; (4) PO + CE group; (5) PO + ACH1 group; (6) PO + ACH2 group; (7) PO + MXT group; (8) PO + TST group; (9) PO + OK group. Briefly, mice were injected intraperitoneally (ip) with PBS containing PO (250 mg/kg) 1 h before the testing samples were administered to increase the serum uric acid level. For comparative study, the same dosages at 100 mmol/kg of allopurinol (AP, 13.6 mg/kg), crude extract (CE, 30.6 mg/kg), (−)-2,3-*cis*-3,4-*cis*-3,3′,4,4′,7,8-hexahydroxyflavan (ACH1, 30.6 mg/kg), (−)-2,3-*cis*-3,4-*cis*-4′-methoxy-3,3′,4,7,8-pentahydroxyflavan (ACH2, 32.0 mg/kg), melanoxetin (MXT, 30.2 mg/kg), transilitin (TST, 31.6 mg/kg), and okanin (OK, 28.8 mg/kg) dissolved in DMSO were delivered ip for 1 h post PO administration.

Measurement of Serum Uric Acid Level. The blood samples were collected by retro-orbital bleeding at 3 h after PO administration. Blood samples were centrifuged at 1400*g* at 4 °C for 15 min, and the uric acid level in serum supernatants was determined using a commercial kit from Randox Laboratories (U.K.).

Determination of Xanthine Oxidase-Inhibitory Activity Using Isothermal Titration Calorimetry. Enzyme catalytic rates are determined by measuring the change in thermal power after titration of the substrate through a stirred injection syringe. Calorimetric assays were done on an iTC200 (MicroCal Inc.) equilibrated with a sample cell and a reference cell maintained at 37 °C through the continuous addition of thermal power. Sample and reference cells (200 μL) were filled with 0.01 μM xanthine oxidase in PBS buffer containing 20 μM inhibitors and water alone, respectively. After a 120 s wait, 10 successive injections of 0.2−2 μL 600 μM xanthine in PBS buffer were made every 120 s. Stirring speed was 1000 rpm, and thermal power was recorded every 2 s. Total molar enthalpy, $\Delta Happ$, for the complete hydrolysis of xanthine was −176.20 kcal/mol determined in a separate ITC experiment (**Figure 1a**).

Computational Docking Studies. The X-ray crystal structure of bovine xanthine oxidase (PDB code 3B9J) was retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb) (*29*), and its C chain was used for docking studies of melanoxetin and allopurinol. The substrate 2-hydroxy-6-methylpurine and all water molecules except two conserved water molecules in the active site that are close to Arg880 were removed from the C chain. Protonation of the protein was performed at physiological pH using SYBYL software, and the resulting structure was directly used in the docking experiments. The three-dimensional structures of ligand molecules were built and optimized by energy minimization using the Tripos force field in the software package SYBYL 6.5 (Tripos, Inc., St. Louis, MO). Docking experiments were performed using the GOLD 3.1 program (*30*) on a Silicon Graphics Octane workstation with dual 270 MHz MIPS R12000 processors. The GOLD program utilizes a genetic algorithm (GA) to perform flexible ligand docking. In the present study, for each of the 50 independent GA runs, a maximum number of 100,000 GA operations were performed on a single population of 100 individuals. Operator weights for crossover, mutation, and migration were set to be 95, 95, and 10, respectively. The GoldScore fitness function was applied for scoring the docking poses of compounds. The annealing parameters for hydrogen bonding and van der Waals were set to be 4.0 and 2.5 Å, respectively. The active site of xanthine oxidase for docking studies was defined with a sphere of 12 Å radius from the methyl carbon atom of the substrate 2-hydroxy-6-methylpurine. The best docking solution (with the highest GOLD fitness score) for a compound was chosen to represent the predicted binding mode to the enzyme.

Statistical Analysis. All data are expressed as mean ± SEM (n = 6). The significance of difference was calculated by using the Scheffe test, and results with $P < 0.0001$ were considered to be statistically significant.

RESULTS

Extraction Yield and Quantification of Major Phytochemicals in Heartwood Extracts of *A. confusa*. The yield of heartwood extracts was 5.1%. The major constituents of ethanolic extracts from *A. confusa* heartwood were quantified by HPLC (**Figure 2**). The five major constituents (**Figure 3**) in ethanolic heartwood extracts were found to be (−)-2,3-*cis*-3,4-*cis*-3,3′,4,4′,7,8-hexahydroxyflavan (**1**), (−)-2,3-*cis*-3,4-*cis*-4′-methoxy-3,3′,4,7,8-pentahydroxyflavan (**2**), melanoxetin (**3**), transilitin (**4**), and okanin (**5**). The contents of (−)-2,3-*cis*-3,4-*cis*-3,3′,4,4′,7,8-hexahydroxyflavan (**1**), (−)-2,3-*cis*-3,4-*cis*-4′-methoxy-3,3′,4,7,8-pentahydroxyflavan (**2**), melanoxetin (**3**), transilitin (**4**), and okanin (**5**) are 18.8, 5.7, 61.1, 40.8, and

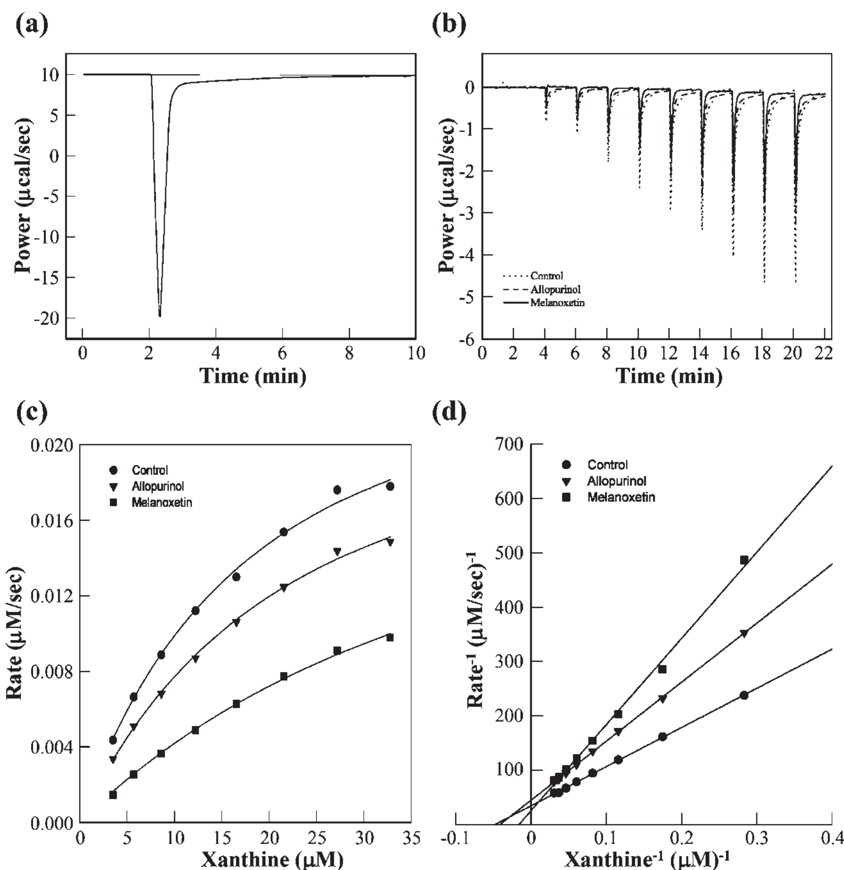

Figure 1. Characterization of melanoxetin or allopurinol interaction with xanthine oxidase by ITC. (**a**) Calorimetric thermogram for the xanthine oxidase reaction. 7.5 μL of 600 μM xanthine in PBS buffer was injected into the 200 μL reaction cell containing 0.01 μM xanthine oxidase at 37 °C (arrow). The ΔH_{app} can be determined by allowing the reaction to proceed to completion and then integrating the signal to obtain the total molar enthalpy. (**b**) Calorimetric thermogram for the reaction of xanthine oxidase on xanthine with or without inhibitors: dotted line, control; dashed line, containing 20 μM allopurinol; solid line, containing 20 μM melanoxetin. (**c**) Michaelis–Menten plot of xanthine oxidase activity with xanthine as substrate plus inhibitors or not. (**d**) Lineweaver–Burk plot of xanthine oxidase activity with xanthine as substrate plus inhibitors or water alone.

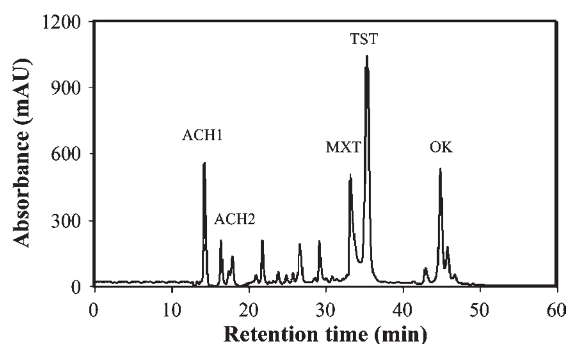

Figure 2. HPLC chromatogram of crude extracts of *A. confusa* heartwood. The phytochemicals from crude extracts were separated by HPLC on a 250 mm × 4.6 mm i.d., 5 μm, Luna RP-18 column (Phenomenex, Torrance, CA) with a MeOH/H_2O solvent system. Elution conditions were 0–25 min of 10–35% A to B (linear gradient), 25–55 min of 35–50% A to B (linear gradient), and 55–60 min of 50–100% A to B (linear gradient) at a flow rate of 1 mL/min.

13.8 mg/g of crude extract, respectively. The results obtained herein are similar to those obtained by Wu et al. (*20*), who found that the main constituents of EtOAc fraction from crude extract of *A. confusa* heartwood were melanoxetin (53.7 mg/g of crude extract), transilitin (40.8 mg/g of crude extract), and okanin (9.3 mg/g of crude extract).

Antihyperuricemic Effect in Hyperuricemic Mice. The antihyperuricemic effect of *A. confusa* heartwood extracts and their phytochemicals on PO-induced hyperuricemia in mice is shown in **Figure 4**. In vehicle control mice, serum uric acid level was 1.44 ± 0.43 mg/dL. In hyperuricemic animals, serum uric acid level was elevated to 4.46 ± 0.52 mg/dL 3 h after ip administration of PO. Therefore, at 3 h after PO injection, the serum uric acid level showed a > 3-fold increase as compared with the vehicle control ($P < 0.0001$). Administration of allopurinol (100 mmol/kg) reduced significantly the serum uric acid level by 79% relative to the PO group. At an equimolar dose (100 mmol/kg), animals treated with (−)-2,3-*cis*-3,4-*cis*-3,3′,4,4′,7,8-hexahydroxyflavan, (−)-2,3-*cis*-3,4-*cis*-4′-methoxy-3,3′,4,7,8-pentahydroxyflavan, melanoxetin, transilitin, and okanin showed significant reductions in uric acid by 66, 72, 75, 65, and 69%, respectively, relative to the PO group ($P < 0.0001$).

Calorimetric Thermogram for Reaction of Xanthine Oxidase on Xanthine with or without Inhibitors. We used ITC to further examine the direct inhibitory effect of these five compounds on xanthine oxidase activity. As seen in **Figure 1b**, a sample cell containing 0.01 μM xanthine oxidase with 20 μM inhibitors or water alone was allowed to reach thermal equilibrium (0–120 s), and 600 μM xanthine was injected every 120 s. The negative deflection indicates that this reaction is exothermic. Increasing the amount of xanthine injected to the sample cell increases the thermal power generated by the enzyme. In the presence of allopurinol, a well-known competitive xanthine oxidase inhibitor, the

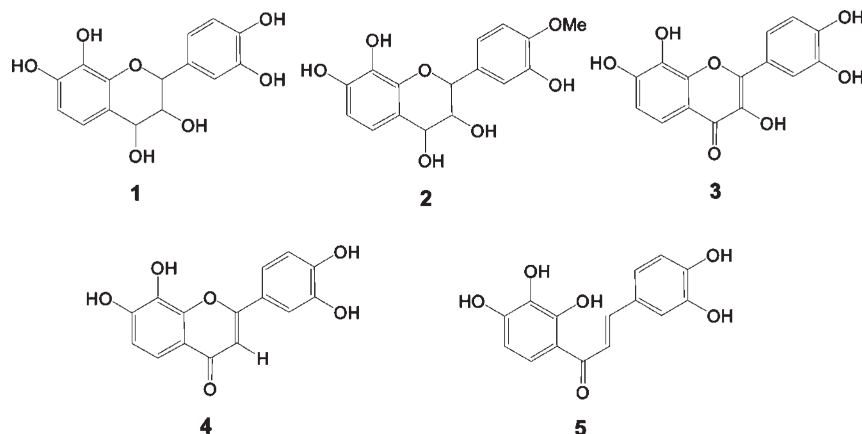

Figure 3. Flavonoids isolated from crude extracts of *A. confusa* heartwood: **1**, (−)-2,3-*cis*-3,4-*cis*-3,3′,4,4′,7,8-hexahydroxyflavan (ACH1); **2**, (−)-2,3-*cis*-3,4-*cis*-4′-methoxy-3,3′,4,7,8-pentahydroxyflavan (ACH2); **3**, melanoxetin (MXT); **4**, transilitin (TST); **5**, okanin (OK).

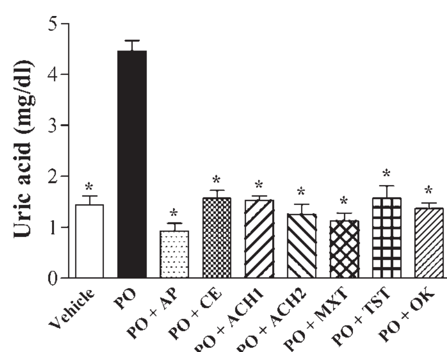

Figure 4. Uric acid lowering effect by the *A. confusa* extracts and their phytochemicals in PO-induced hyperuricemic mice. Results are mean ± SEM of six rats. ∗, $P < 0.0001$, compared with the PO group.

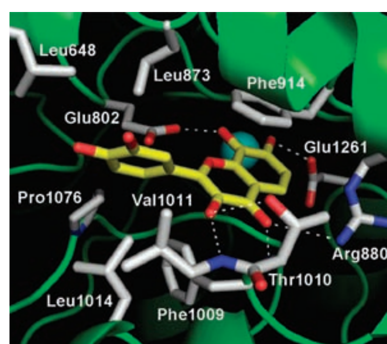

Figure 5. Predicted binding mode of melanoxetin docked into the active site of xanthine oxidase using the program GOLD 3.1. Melanoxetin (yellow) and the amino acid residues (gray) interacting with melanoxetin are shown as stick structures. Hydrogen atoms are omitted for clarity. The dashed lines indicate hydrogen-bonding interactions.

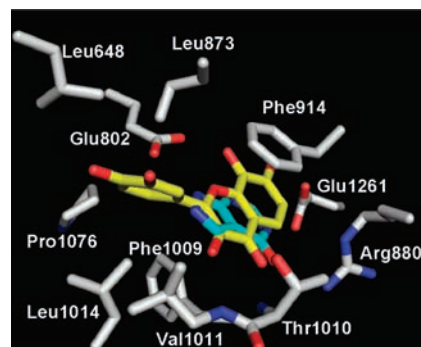

Figure 6. Superposition of the docked structures of melanoxetin (yellow carbons) and allopurinol (cyan carbons) in the active site of xanthine oxidase.

thermal power generated by each injection was obviously decreased. Among the five isolated compounds, melanoxetin showed a remarkable inhibitory effect on xanthine oxidase activity relative to allopurinol. This indicated that the inhibitory effect of melanoxetin was stronger than that of allopurinol.

Data shown in **Figure 1b** were further converted into the enzyme reaction rate according to the equations mentioned by Todd and Gomez (*13*). After fitting to the Michaelis−Menten plot (**Figure 1c**) and Lineweaver−Burk plot (**Figure 1d**), kinetic constants for xanthine oxidase reacted on xanthine with or without inhibitors could be determined. The Michaelis constant (K_m) of xanthine oxidase reacting on xanthine at 37 °C was 21.0 μM. After the addition of 20 μM allopurinol or melanoxetin to the enzyme-catalyzed reaction, K_m values were changed to 24.5 and 34.6 μM, respectively, indicating that melanoxetin possesses an inhibitory effect superior to that of allopurinol by decreasing the affinity of xanthine to xanthine oxidase as well as the catalytic rate.

Molecular Docking Studies of Melanoxetin and Allopurinol. In addition to possessing uric acid-lowering activity in vivo, melanoxetin also inhibits xanthine oxidase activity in vitro. We were interested in visualizing the effects of melanoxetin on xanthine oxidase to gain insights into the observed activities and elucidate the possible different responses from allopurinol; a computational docking study using the GOLD 3.1 program was performed to predict the binding mode of melanoxetin in the active site of xanthine oxidase. The docking model, as depicted in **Figure 5**, indicates that the bicyclic benzopyranone ring of melanoxetin is sandwiched between Phe914 and Phe1009 and makes aromatic interactions ($\pi-\pi$ effects) with the two phenylalanines. As revealed by the reported crystal structures of xanthine oxidase bound with aromatic substrates or inhibitors, such sandwiching aromatic interactions ($\pi-\pi$ effects) might be important for ligand recognition by xanthine oxidase. The carbonyl and three hydroxyl groups on the benzopyranone ring can form hydrogen bonds with several active-site residues including Arg880, Thr1010, Val1011, Glu802, and Glu1261. In this regard, decreasing the number of hydroxyl groups on the benzopyranone ring would be expected to result in a reduction in inhibitory activity against xanthine oxidase. This hypothesis, in fact, has been supported by our previous study on the flavone

compound 7,3′,4′-trihydroxyflavone. The 3′,4′-dihydroxyphenyl moiety at C-2 of melanoxetin is inserted into the hydrophobic region to interact with residues Leu648, Leu873, Val1011, Leu1014, and Pro1076. It was believed that these hydrophobic interactions might significantly contribute to the observed greater inhibitory activity of melanoxetin against xanthine oxidase compared with allopurinol. According to the docking studies, melanoxetin has a higher GOLD fitness score (68.20) to xanthine oxidase than allopurinol (49.13). As shown in **Figure 6**, the allopurinol molecule occupies the same binding site as the benzopyranone ring of melanoxetin. In contrast, melanoxetin makes extended interactions in the hydrophobic region via the 3′,4′-dihydroxyphenyl moiety, thus accounting for its higher binding affinity to xanthine oxidase than allopurinol.

DISCUSSION

Earlier studies have shown that flavonoids, active constituents of *A. confusa* heartwood, possess potent antioxidant and anti-inflammatory properties (*20*, *21*). Antioxidant, anti-inflammatory, and xanthine oxidase inhibitory activities of flavonoids make the medicinal plants ideal candidates for a triad of ailments including hyperuricemia, ROS, and gouty arthritis (*31*).

We have previously shown that *A. confusa* heartwood extract decreased significantly the urate level in hyperuricemic mice at a dosage of 100 mmol/kg. At the same dosage (100 mmol/kg), animals treated with (−)-2,3-*cis*-3,4-*cis*-3,3′,4,4′,7,8-hexahydroxyflavan, (−)-2,3-*cis*-3,4-*cis*-4′-methoxy-3,3′,4,7,8-pentahydroxyflavan, melanoxetin, transilitin, and okanin all showed significant reductions of uric acid by 66, 72, 75, 65, and 69%, respectively. Wang et al. (*32*) reported that the major compound of *Cinnamomum osmophloeum* leaves, cinnamaldehyde, showed good inhibitory activity for reducing xanthine oxidase activity. Therefore, administration of cinnamaldehyde reduced significantly the serum uric acid level by 60% compared with the PO group at a dosage of 150 mg/kg. Zhu et al. (*33*) also found that administration of quercetin and rutin reduced significantly the serum uric acid level by 36 and 32%, respectively, at a dosage of 150 mg/kg. Comparisons of the aforesaid results indicated that *A. confusa* heartwood extracts and their major flavonoid constituents have an excellent effect in reducing the urate levels. Among the flavonoid constituents examined, melanoxetin was comparable with allopurinol, showing excellent effect in reducing urate levels. It was concluded from the present study that melanoxetin showed a potent inhibitory effect on xanthine oxidase in a competitive mode, and its inhibitory activity could be stronger than that of allopurinol in in vitro assay. On the basis of their antihyperuricemic effects, the structure−activity relationships of flavonoids (compounds **1**−**5**) were also illustrated in this study. Surprisingly, with the same number of hydroxyl groups in the A- and B-rings, melanoxetin had a greater antihyperuricemic effect than transilitin, revealing that the hydroxyl group at the C3 position enhanced the antihyperuricemic effect. Moreover, with the same number of hydroxyl groups in the A- and C-rings, 3′-OCH_3 might have better antihyperuricemic effect than 3′-OH. It is of great interest that the antihyperuricemic effect of melanoxetin could be equal to that of a clinically used drug. In this sense, the dietary use of *A. confusa* heartwood extracts and their constituents may provide some choices for prevention and/or treatment of hyperuricemia.

In conclusion, this study demonstrated that *A. confusa* heartwood extracts and their major constituents possessed in vivo potent hypouricemic effects in hyperuricemic mice pretreated with oxonate. These hypouricemic effects are partly due to inhibition of xanthine dehydrogenase/xanthine oxidase activities. It is therefore suggested that *A. confusa* heartwood extracts and their flavonoid compounds may represent a new type of hypouricemic agents and may have a potent hypouricemic effect in clinical use.

ACKNOWLEDGMENT

We thank Shou-Ling Huang (Department of Chemistry, National Taiwan University) for NMR spectral analyses. We also thank Si-Lin Yang, Dr. Sen-Sung Cheng, and Min-Jay Chung of the Experimental Forest of National Taiwan University for providing specimens.

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Received for review July 11, 2010. Revised manuscript received August 21, 2010. Accepted August 22, 2010. We thank the Forestry Bureau of the Council of Agriculture for financial support.