JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Study on Human Intestinal Bacterium *Blautia* sp. AUH-JLD56 for the Conversion of Arctigenin to (−)-3′-Desmethylarctigenin

Ming-Yue Liu,[†] Meng Li,[†] Xiu-Ling Wang,* Peng Liu, Qing-Hong Hao, and Xiu-Mei Yu

College of Life Sciences, Agricultural University of Hebei, No. 289, Lingyusi Street, Baoding 071001, Hebei Province, China

ABSTRACT: *Arctium lappa* L. (*A. lappa*) is a popularly used vegetable as well as herbal medicine. Human intestinal microflora was reported to convert arctiin, the lignan compound with highest content in the dried fruits of *Arctium lappa*, to a series of metabolites. However, the specific bacterium responsible for the formation of 3′-desmethylarctigenin (3′-DMAG), the most predominant metabolite of arctiin by rat or human intestinal microflora, has not been isolated yet. In the present study, we isolated one single bacterium, which we named *Blautia* sp. AUH-JLD56, capable of solely biotransforming arctiin or arctigenin to (−)-3′-DMAG. The structure of the metabolite 3′-DMAG was elucidated by electrospray ionization mass spectrometry (ESI-MS) and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The biotransforming kinetics and maximum biotransforming capacity of strain AUH-JLD56 was investigated. In addition, the metabolite 3′-DMAG showed significantly higher 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity than that of the substrate arctigenin at the concentrations tested.

KEYWORDS: *arctigenin, 3′-desmethylarctigenin (3′-DMAG), bacterium isolation, microbial biotransformation, DPPH radical-scavenging activity*

INTRODUCTION

Arctium lappa L. (*A. lappa*) is a plant popularly used as a vegetable (root and leaf) as well as a herbal medicine (seed and root). The constituents of the root of *A. lappa* were reported to have hepatoprotective, anti-inflammatory, and free radical-scavenging activities.[1] Arctiin, which is the most abundant lignan in the seeds of *A. lappa*,[2] has been reported to have much wider bioactivities, including antiausteric,[3] antiallergic,[4] anti-inflammatory,[5] antiasthmatic,[6] and antidiabetic effects,[7] chemopreventive effect against cancer,[8] and ameliorative effect to attenuate the deterioration of renal function,[9] etc. Arctigenin, an aglycone of arctiin, was reported to be a lead structure for the inhibition of human immunodeficiency virus.[10] Since almost all herbal medicines and vegetables are administered orally, the intestinal microflora plays an essential role in arctiin metabolism. Studies have shown that arctiin is able to be metabolized to a series of metabolites, such as arctigenin, 3′-desmethylarctigenin (3′-DMAG), dihydroxyenterolactone, enterolactone, and so on by both rat[11,12] and human intestinal bacteria.[13] In 2007, Professor Hattori and his research team isolated the first human intestinal bacterium *Eubaterium* sp. ARC-2 (EF413640) capable of transforming arctigenin. Bacterium strain ARC-2 was reported to be able to convert the metabolite arctigenin to seven different metabolites, including three monodesmethylarctigenins, three didesmethylarctigenins, and dihydroxyenterolactone.[14] In the same year, Professor Hattori and his team reported another human intestinal bacterium *Ruminococcus* sp. END-1 (EF451052) capable of oxidizing enterodiol to enterolactone.[15] Their subsequent work demonstrated that strain END-1 could convert arctiin to (−)-dihydroxyenterolactone, indicating strain END-1 had not only oxidation activity but also demethylation of a plant lignan arctiin.[16]

According to Nose et al.,[11] when the substrate arctiin was incubated with rat intestinal microflora, the maximum amount of the corresponding aglycone arctigenin was obtained after 1 h of incubation. Then, the major metabolite AM2 (i.e., 3′-DMAG) increased gradually. Their experiments suggested that 3′-DMAG was the most predominant metabolite by incubation of arctiin with rat intestinal microflora. Similar to those reported by Nose et al., Professor Hattori and his team[13] also observed that incubation of arctiin with rat intestinal bacterial mixture led to metabolites 2 (i.e., arctigenin) and metabolite 3 (i.e., 3′-DMAG). From the recent studies in our laboratory, we found that the most predominant metabolite was also 3′-DMAG by incubation of arctiin with human intestinal microflora. Therefore, we suggest that the metabolite 3′-DMAG after *A. lappa* consumption may be beneficially associated with human health. Study of the bioactivity of the main metabolite 3′-DMAG seems important.

Although human bacterium ARC-2 was reported to be capable of converting arctiin to 3′-DMAG, while strain ARC-2 also can convert arctiin to the other six metabolites mentioned previously. Among all of the seven metabolites produced by incubation of arctiin with strain ARC-2, four metabolites, i.e., metabolite 2, metabolite 3, metabolite 5, and metabolite 6 (i.e., 3′-DMAG), were just in small fractions (see ref 14, Figure 2), which may reflect the impossibility of synthesizing 3′-DMAG by incubation of the substrate arctiin with bacterium strain ARC-2. To date, no particular bacteria responsible for the formation of 3′-DMAG from arctiin or arctigenin have been isolated and identified.

The purpose of our study is to isolate a specific single bacterium capable of metabolizing arctiin or arctigenin to 3′-DMAG in an efficient way. In the present study, we isolated

Received: September 5, 2013
Revised: November 16, 2013
Accepted: November 17, 2013
Published: November 18, 2013

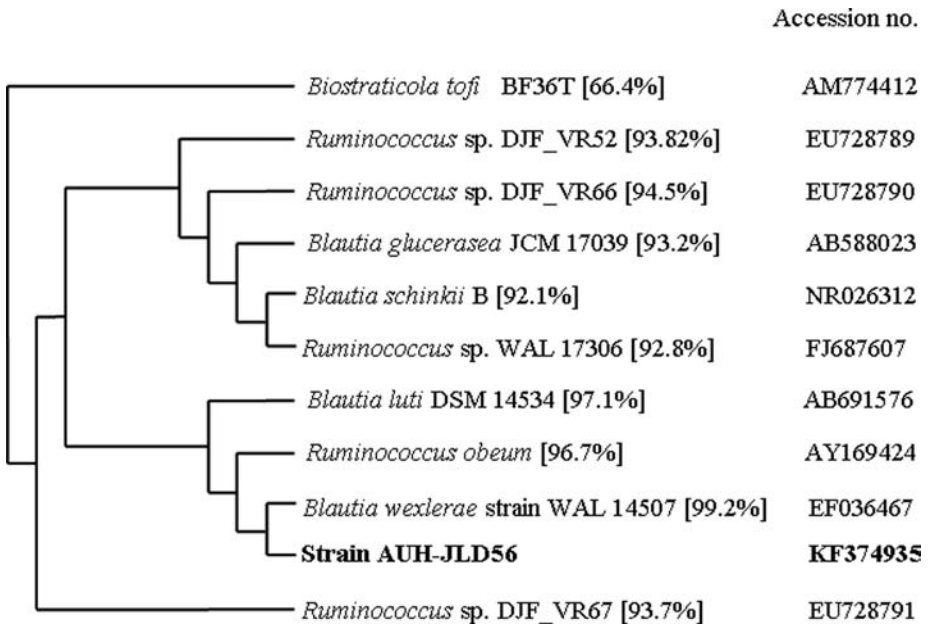

Figure 1. Dendrogram showing the phylogenetic affiliation of the newly isolated human intestinal bacterium strain AUH-JLD56.

one human intestinal bacterium, which we named AUH-JLD56, capable of converting arctiin or arctigenin to 3′-DMAG solely and efficiently. The biotransforming properties of strain AUH-JLD56 and the free radical-scavenging activity of arctigenin and the metabolite 3′-DMAG were studied as well.

MATERIALS AND METHODS

Chemicals. The standard arctigenin was purchased from Senbeijia Biological Company (Nanjing, China). The authentic enterolactone was purchased from Sigma-Aldrich (Shanghai, China).The authentic racemic equol was from LC Laboratories. DPPH was purchased from Sigma-Aldrich. Acetonitrile, methanol, and acetic acid were of high-performance liquid chromatography (HPLC) grade. All other reagents were of analytical grade. Brain heart infusion (BHI) powder was from Difco Co.

Preparation of Arctigenin. The seeds of *Arctium lappa* L. (*A. lappa*) were purchased from Huaxing Pharmaceutical Store (Baoding, China). The substrate arctigenin used in this study was prepared by acid hydrolysis of arctiin extracted from the seeds of *A. lappa* by 95% methanol. The air-dried seeds of *A. lappa* (10 g) were crushed in a pulverizer and transferred to a 250 mL flask followed by the addition of 80 mL of 95% methanol. After being extracted by ultrasonication for 15 min at room temperature, the extract was filtered and evaporated to dryness followed by hydrolyzation in 20 mL of hydrochloric acid (1 mol/L) in a water bath at 80 °C for 2 h. The hydrolyzate was cooled down, and the reactant was neutralized with 1 mol/L NaOH and extracted with 100% ethyl acetate (1:1). After the ethyl acetate was evaporated to dryness, the dried hydrolyzate (i.e., the crude arctigenin) was ready to be used as the substrate. The compound arctigenin was identified by comparing both the UV spectrum and the retention time in HPLC profiles with that of the authentic arctigenin. We purified the hydrolyzate by using a Waters HPLC instrument equipped with a photodiode array detector and a Kromasil C_{18} reversed phase column (8 mm × 250 mm). The dried hydrolyzate was redissolved in 100% methanol and filtered by an organic membrane before being injected into an HPLC instrument. The eluting solution consisted of 10% acetonitrile in 0.1% acetic acid (A) and 90% acetonitrile in 0.1% acetic acid (B). Elution was carried out as follows: A/B at 60:40 (v/v); the flow rate was 2 mL/min. We collected the peak corresponding to the hydrolyzate for a further detection of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity.

Bacterial Isolation and Identification. Fresh feces of a healthy woman was collected in 5 mL of BHI liquid medium, which was covered with 2 mL of sterilized mineral oil. Bacterium isolation and identification methods were as we described previously.[17] The 16S rRNA gene sequence has been submitted to NCBI GenBank with the accession number KF374935. The isolated bacterium, which we named strain AUH-JLD56 was cultured in BHI liquid medium in an anaerobic chamber (Concept 400, Ruskinn, UK) containing 5% CO_2, 10% H_2, and 85% N_2, at 37 °C.

Production and Identification of the Metabolite of Arctigenin by Strain AUH-JLD56. The metabolite of arctigenin by strain AUH-JLD56 was determined by both the UV spectrum and the retention time in HPLC profiles. The procedures were as follows: 100 μL of the culture broth was extracted with 1 mL of ethyl acetate and evaporated to dryness using a DK-VC010 Concentrator (Daiki, Seoul, South Korea). The residue was redissolved in 100 μL of 100% of methanol, and 10 μL of the solution was used for HPLC detection. The samples were analyzed on an HPLC instrument. The eluting solution consisted of 10% acetonitrile in 0.1% acetic acid (A) and 90% acetonitrile in 0.1% acetic acid (B). Elution was carried out as follows: A/B at 60:40 (v/v). The flow rate was 1 mL/min. For the preparation of the metabolite of arctigenin in large quantities, 10% of preprepared one-day-old bacterial seed was inoculated in a 250 mL flask containing 100 mL of BHI liquid medium, followed by the addition of the stock solution of crude arctigenin (200 mM in 100% methanol) at a final concentration of 0.8 mmol/L. The bacterial culture was then continuously incubated in a biochemical incubator at 37 °C for 2 days. The culture broth was extracted with an equal volume of ethyl acetate, followed by evaporation to dryness with a rotary vacuum evaporator (Yarong Co. China). The dried metabolite was redissolved in 100% of methanol and purified on a preparative HPLC equipped with a Kromasil C_{18} reversed phase column (8 mm × 250 mm). The flow rate was 2 mL/min. The metabolite of arctigenin by strain AUH-JLD56 was determined by using a UV spectrum, electrospray ionization mass spectrometry (ESI-MS), and ^1H and ^{13}C nuclear magnetic resonance spectroscopy (NMR). The ESI-MS was obtained on a Bruker Daltonic Apex-Ultra mass spectrometer (Massachusetts, USA). The ^1H and ^{13}C NMR spectra in $CDCl_3$ were obtained from a 400 MHz on a Bruker AVANCE NMR spectrometer (Karlsruhe, Germany). The optical rotation was detected on A21171-Autopol III Rudolph Research Analytical Automatic Polarimeter (New Jersey, USA).

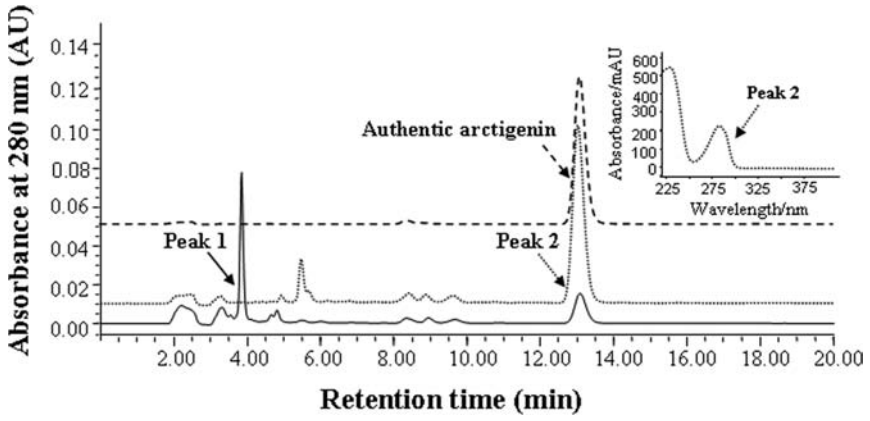

Figure 2. HPLC elution profiles of the crude extract of *Arctium lappa* (solid line) and the hydrolyzate of the crude extract of *Arctium lappa* (dotted line). The insert shows the UV spectrum of the hydrolyzate. AU, absorbance units.

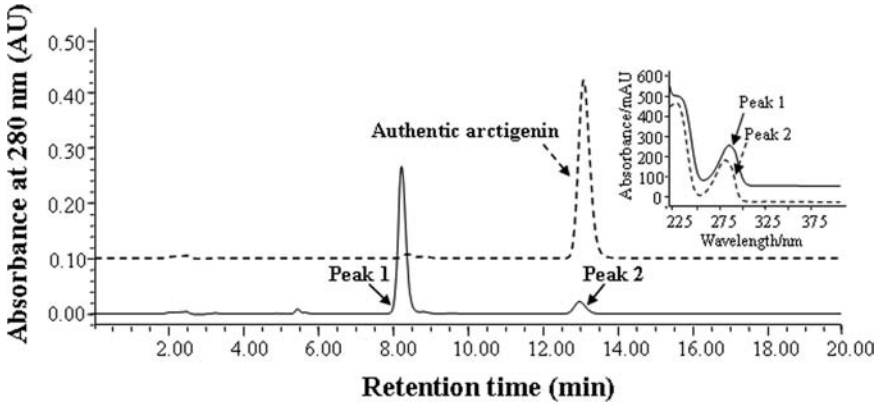

Figure 3. HPLC elution profiles of arctigenin after 2 days of incubation with strain AUH-JLD56 (solid line). The inserts show UV spectra of arctigenin (dashed line) and the metabolite of arctigenin (solid line). AU, absorbance units.

Biotransformation Kinetics of Arctigenin by AUH-JLD56. In order to study the biotransforming kinetics of strain AUH-JLD56, we inoculated 10% of the preprepared culture of strain AUH-JLD56 to BHI liquid medium containing 0.4 mmol/L of arctigenin in the anaerobic chamber and sampled after every 4 h of incubation time. The bacterial culture of strain AUH-JLD56 containing arctigenin was sampled for 24 h. All experiments were performed in triplicate.

Antioxidant Activity: DPPH Assay. The in vitro antioxidant test was performed as we described previously[18] with a modification in reaction temperature. The compounds that we detected include the substrate arctigenin, the metabolite 3′-DMAG, authentic enterolactone, and equol. The purity of the authentic enterolactone was 98% and that of the authentic equol was 99%. The purity of the synthesized arctigenin by acid hydrolysis of arctiin extracted from the seeds of *A. lappa* and that of 3′-DMAG was 99%. The concentrations of each compound were 0.025 mmol/L, 0.050 mmol/L, 0.100 mmol/L, and 0.500 mmol/L, respectively. Here, we used ethanol as the solvent. The reaction temperature was 20 °C, and the reaction time was 48 h. All experiments were performed in triplicate. Statistical analyses were carried out by one-way ANOVA of variance using SPSS, version 13 for Windows.

RESULTS

Identification of Human Intestinal Bacterium Strain AUH-JLD56. A newly isolated bacterium, which we named strain AUH-JLD56, from human feces was determined to be an elliptical shaped, gram-positive, anaerobic bacterium. The 16S rRNA gene sequence (1,409 bp) of strain AUH-JLD56 had 99.2% similarity to that of *Blautia wexlerae* strain WAL 14507 (accession number EF036467). The phytogenetic affiliation of strain AUH-JLD56 is shown in Figure 1. The genus *Blautia* is a newly described genus. Most of the species in the genus of *Blautia* were from the genus *Ruminococcus* due to the obvious differences existing in biochemical characteristics. The 16S rRNA gene sequence had 99.2% similarity to that of *Blautia wexlerae* strain WAL 14507; however, obvious differences were detected in most of the biochemical traits. For example, strain AUH-JLD56 is urease negative, while that of *Blautia wexlerae* strain WAL 14507 is positive;[19] *Blautia wexlerae* strain WAL 14507 is able to use most of the carbohydrates in the API kit, while strain AUH-JLD56 is not able to use most of the carbohydrates. Therefore, we refer to strain AUH-JLD56 as *Blautia* sp. strain AUH-JLD56.

Preparation of Arctigenin. The HPLC elution profiles detected a major component eluting at 3.6 min (peak 1 in Figure 2) from the extract of the seeds of *Arctium lappa* L. (*A. lappa*), a result we inferred to be arctiin, the glycoside of arctigenin. After being hydrolyzed by hydrochloric acid, another major component eluting at 13.1 min (peak 2 in Figure 2) was detected from the hydrolyzate of the crude extract of the seed of *A. lappa*. Peak 2 in Figure 2 gave absorbance maxima at 228 and 279 nm, which were identical to that of the authentic arctigenin eluting at 13.2 min. Therefore, based on the HPLC retention time and UV spectrum, we inferred peak 2, the hydrolyzate of the crude extract of the seeds of *A. lappa* was arctigenin. After the eluting solution was evaporated to dryness, we got the weight (268 mg) of the peak corresponding to the hydrolyzate. The final yield for the preparation of actigenin

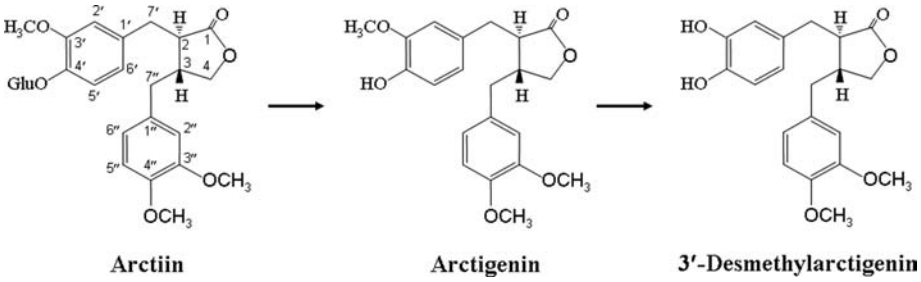

Figure 4. Proposed metabolic pathway of arctiin by strain AUH-JLD56 under anaerobic conditions.

from *A. lappa* was 2.68%. The purity of the obtained actigenin was more than 99%.

Identification of the Metabolite of Acrtigenin Produced by Strain AUH-JLD56. Human intestinal bacterium strain AUH-JLD56 is the first isolated bacterium which is capable of solely converting arctigenin to 3′-DMAG. In fact, when we initially isolated bacterium strain AUH-JLD56, we noticed that it was able to convert arctiin to 3′-DMAG, indicating that strain AUH-JLD56 showed not only demethylation but also deglucosylation activity. However, the maximal concentration of the substrate arctiin that strain AUH-JLD56 could transform was lower than 1.0 mmol/L. Therefore, we prepared arctigenin from arctiin by acid hydrolysis and used arctigenin as the substrate for strain AUH-JLD56. Surprisingly, we found that the capability to produce 3′-DMAG from arctigenin was significantly higher than that from arctiin.

The HPLC elution profile detected one single metabolite eluting at 8.3 min (peak 1 in Figure 3), which was produced from arctigenin by bacterium strain AUH-JLD56 (Figure 3). The metabolite gave absorbance maxima at 225 and 280 nm. The ESI-MS spectrum showed a $[M + H]^+$ ion at m/z 359, 14 mass units ($-CH_3$) less than that of the substrate arctigenin, suggesting that the metabolite was a demethylated product. For further structural identification, the metabolite was purified and subjected to ^1H and ^{13}C NMR analyses. Both the ^1H and ^{13}C NMR data were identical to those for 3′-desmethylarctigenin reported previously.[13] Therefore, the metabolite of arctigenin by strain AUH-JLD56 was accurately identified as 3′-DMAG. The proposed metabolic pathway of the substrate arctiin by strain AUH-JLD56 was summarized in Figure 4.

^1H NMR ($CDCl_3$, 400 MHz,): δ 2.46−2.64 (4H, m, H-2, 3, 7″), 2.85 (2H, d, J = 5.5 Hz, H-7′), 3.81−3.84 (6H, s, -OCH_3*2), 3.88 (1H, dd, J = 8.54, 7.6 Hz, H-4), 4.13 (1H, dd, J = 8.54, 7.8 Hz, H-4), 6.48 (1H, d, J = 1.94 Hz, H-2″), 6.51 (1H, dd, J = 8.19, 1.94 Hz, H-6′), 6.57 (1H, dd, J = 8.19, 1.94 Hz, H-6″), 6.66 (1H, d, J = 1.94 Hz, H-2′), 6.75 (1H, d, J = 8.19 Hz, H-5′), 6.77 (1H, d, J = 8.19 Hz, H-5″).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 179.9 (C-1), 149.0 (C-3″), 147.8 (C-4″), 144.0(C-3′), 142.9 (C-4′), 130.0 (C-1″), 129.9 (C-1′), 121.7 (C-6′), 120.7 (C-6″), 116.1 (C-2′), 115.2 (C-5′), 111.8 (C-2″), 111.4 (C-5″), 71.8(C-4), -Me 55.9, -Me 55.8, 46.6(C-2), 40.9 (C-3), 38.2 (C-7″), 34.0(C-7′).

Chirality Study of Biosynthesized 3′-DMAG. The metabolite 3′-DMAG obtained by incubation of the substrate arctigenin with human intestinal bacterium strain AUH-JLD56 made the plane-polarized light rotated, indicating that the biosynthesized 3′-DMAG is an optically active substance. The rotating direction of the metabolite 3′-DMAG was determined to be left due to the negative value observed from the analyzer. The specific rotation ($[\alpha]^{26}_D$) of 3′-DMAG was −65.61° (c = 1.9 × 10^{-3}, MeOH), which was significantly higher than the previously reported $[\alpha]^{25}_D$ (−42.8°; c = 0.13, MeOH) of biosynthesized 3′-DMAG by human intestinal microflora[13] or $[\alpha]^{23}_D$ (−40.3°; c = 0.197, MeOH) of biosynthesized 3′-DMAG by human intestinal bacterium strain ARC-2.[14]

Biotransformation Kinetics of Arctigenin by Strain AUH-JLD56. The biotransformation kinetics of arctigenin by strain AUH-JLD56 showed that arctigenin was converted to 3′-DMAG drastically within the first 12 h of incubation in an anaerobic chamber. The biotransformation process reached the highest amount after about 16 h of incubation, and the average biotransformation rate of the substrate arctigenin by strain AUH-JLD56 was 96.3% (Figure 5).

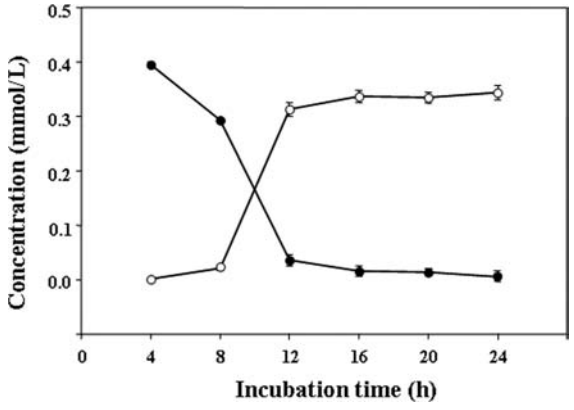

Figure 5. Biotransformation kinetics of arctigenin (−●−) by strain AUH-JLD56 in BHI liquid medium. Initial concentration of arctigenin was 0.4 mmol/L; the metabolite 3′-desmethylarctigenin is also shown (−○−).

Biotransformation Capacity of Arctigenin by Strain AUH-JLD56. Bacterium strain AUH-JLD56 was tested in its capacity to convert different amounts of the substrate arctigenin added in BHI liquid medium. The results showed that the average bioconversion rate of arctigenin was 95.6% when the concentration of the substrate arctigenin was or was lower than 2.4 mmol/L; when the concentration of arctigenin was more than 2.4 mmol/L but lower than 3.6 mmol/L, the average bioconversion rate of arctigenin was 90.5%. However, when the concentration of arctigenin was over 3.6 mmol/L, the biotransforming capacity of arctigenin by strain AUH-JLD56 was sharply dropped to 54.4% (Figure 6).

Effect on DPPH Radical-Scavenging Activity. We detected the in vitro antioxidant activity by using the stable DPPH radical. The result showed that the DPPH radical-scavenging activity of the metabolite 3′-DMAG was significantly stronger ($p < 0.01$) than that of the substrate when the concentrations ranged from 0.025 mmol/L to 0.100 mmol/L.

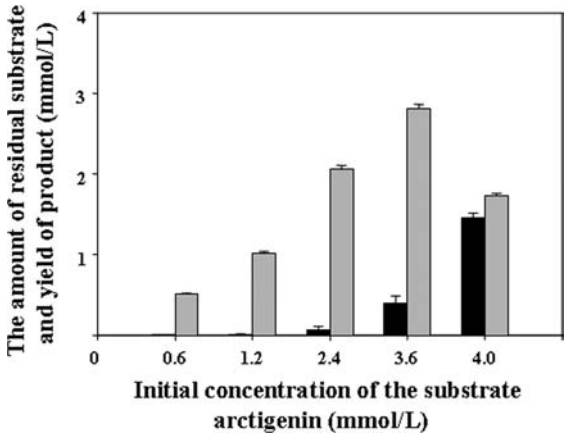

Figure 6. Bioconversion capacity of strain AUH-JLD56 after 3 days of incubation with different concentrations of the substrate arctigenin. Arctigenin (black bars); 3′-desmethylarctigenin (gray bars).

No obvious difference was detected when the concentration was 0.500 mmol/L, indicating the concentration of the compound may be in an oversaturated situation compared with the concentration of DPPH in the same reaction system (Figure 7). Equol, one microbial metabolite by intestinal

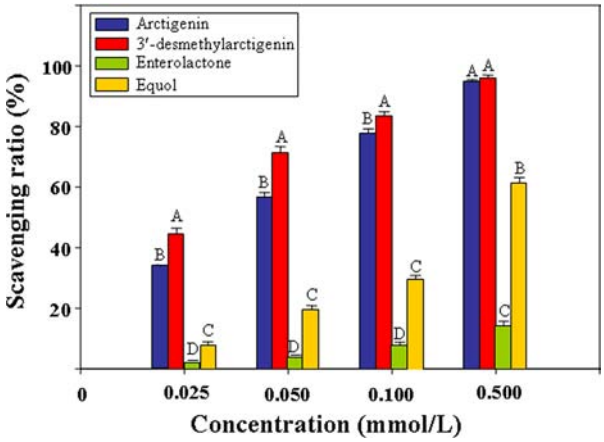

Figure 7. DPPH radical-scavenging capacity of arctigenin (dark blue bars), 3′-desmethylarctigenin (red bars), enterolactone (green bars), and equol (yellow bars) at different concentrations.

microflora or by some specific intestinal bacteria, was demonstrated to have the strongest free radical-scavenging activity.[18] Enterolactone, one metabolite of arctigenin by human or rat intestinal microflora,[11−13] was reported to have estrogenic, antiestrogenic,[20−22] and antioxidative activity.[23] Therefore, in order to have an accurate comparison, the substrate arctigenin, the metabolite 3′-DMAG, enterolactone, and equol were investigated for their DPPH radical-scavenging activity. The results showed that DPPH radical-scavenging activity of the metabolite 3′-DMAG was significantly stronger than that of the other three tested compounds, including arctigenin, enterolactone, and equol. On the contrary, the authentic enterolactone showed extremely low DPPH radical-scavenging activity at the concentrations tested (Figure 7). The average IC_{50} values of 3′-DMAG, arctigenin, and equol were 0.027 mmol/L, 0.048 mmol/L, and 0.304 mmol/L, respectively. Since the DPPH radical-scavenging activity was extremely low at the tested concentrations, the IC_{50} of enterolactone was not given.

DISCUSSION

Recently, Professor Hattori and his research team investigated the binding affinity of arctigenin and its seven metabolites, which were produced by incubation of arctiin with human intestinal flora.[13] Their results showed that didesmethylarctigenins had stronger estrogen receptor alpha (ER_α) binding affinity than most of the monodesmethylarctigenins. However, compound 6 (i.e., 3′-DMAG), which is a monodesmethylarctigenin, showed a similar ER_α binding affinity compared to that of didesmethylarctigenins when the concentration of the compound was over 5×10^{-4} mol/L. In addition, the IC_{50} of compound 6 was the same as that of compound 4, a didesmethylarctigenin.[24] It is worthy to noticing here that the metabolite 3′-DMAG obviously prolonged the lifespan of *C. elegans* (Zhang, C. H., et al., unpublished observation).

In order to know whether the metabolite 3′-DMAG has any cytotoxicity, we tested the antimicrobial activity of the substrate arctigenin, the metabolite 3′-DMAG, enterolactone, and equol (data not shown). However, none of them showed antimicrobial activity at the concentration of 3.0 mmol/L. When the concentration of the tested compounds was increased to 5.0 mmol/L, both enterolactone and equol showed a weak antimicrobial activity to *Staphylococcus aureus* (ATCC27217) and *Salmonella paratyphi* (CMCC50001) but not to *Escherichia coli* (CICC10372). However, neither the substrate arctigenin nor the metabolite 3′-DMAG was shown any antimicrobial activity.

In the present study, the results clearly showed that at the concentrations tested (from 0.010 to 0.100 mmol/L), the DPPH radical-scavenging activity of the metabolite 3′-DMAG was significantly higher than that of all the other three tested compounds mentioned previously. However, enterolactone, which is a final metabolite of arctiin by human intestinal microflora with stronger ER_α binding affinity, almost showed no DPPH radical-scavenging activity at the concentrations tested (Figure 7). Our study provided the first evidence that the metabolite 3′-DMAG had a significantly higher DPPH radical-scavenging activity than that of arctigenin. Phytoestrogenic equol, a metabolite of isoflavone daidzein by microflora or by isolated specific bacteria, was demonstrated to be of the strongest superoxide anion and DPPH radical-scavenging activity among soy isoflavones and all of the other isoflavone metabolites.[18] In humans, oxidative stress is an important factor in aging-associated diseases such as cancer, diabetes, Parkinson's disease, etc. The strong DPPH radical-scavenging activity detected in the present study (much stronger than that of enterolactone) provided a rationale for the development of 3′-DMAG as an effective, protective, or curative agent against the damage exerted by oxidative stress in the future.

DPPH, which is a well-known stable free radical, is composed of different crystalline forms.[25] In the present study, we discovered that the stability of DPPH in ethanol was influenced by the variety and the quantity of each crystalline form. Since the ratio of each crystalline form of DPPH may differ from different production batches, the stability of DPPH from different production batches may also be different. In order to solve this problem, we detected both the stability and the DPPH radical-scavenging activity at different temperatures (20 and 37 °C). We found that DPPH in ethanol was much more stable at lower temperatures. Furthermore, the DPPH radical-

scavenging activity of equol detected at 20 °C was just a little lower than that of equol detected at 37 °C.[18] No significant difference was found between the DPPH radical-scavenging activities of equol detected at different temperatures. Therefore, we suggested that it should be more accurate to detect the DPPH radical-scavenging activity at 20 °C with a relatively longer reaction time.

AUTHOR INFORMATION

Corresponding Author

*Phone: 86-312-7528257. Fax: 86-312-7528265. E-mail: wxling2000@hebau.edu.cn.

Author Contributions

†M.-Y.L. and M.L. contributed equally to this work.

Funding

This work was supported by an NSFC grant from the National Natural Science Foundation of China (no. 31170058) and a grant from the Service Center for Experts and Scholars of Hebei Province (CPRC027).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

3′-DMAG, 3′-desmethylarctigenin; ESI-MS, electrospray ionization mass spectrometry; DPPH, 1,1-diphenyl-2-picrylhydrazyl; IC_{50}, inhibitor concentration

REFERENCES

(1) Predes, F. S.; Ruiz, A. L.; Carvalho, J. E.; Foglio, M. A.; Dolder, H. Antioxidative and *in vitro* antiproliferative activity of *Arctium lappa* root extracts. *BMC Complement Altern. Med.* **2011**, *11*, 25.

(2) Sun, W. J.; Sha, Z. F.; Gao, H. Determination of arctiin and arctigenin in Fructus Arctii by reverse-phase HPLC. *Yao Xue Xue Bao* **1992**, *27*, 549−551.

(3) Tezuka, Y.; Yamamoto, K.; Awale, S.; Lia, F.; Yomoda, S.; Kadota, S. Anti-austeric activity of phenolic constituents of seeds of *Arctium lappa*. *Nat. Prod. Commun.* **2013**, *8*, 463−466.

(4) Knipping, K.; van Esch, E. C.; Wijering, S. C.; van der Heide, S.; Dubois, A. E.; Garssen, J. *In vitro* and *in vivo* anti-allergic effects of *Arctium lappa* L. *Exp. Biol. Med. (Maywood, NJ, U. S.)* **2008**, *233*, 1469−1477.

(5) Lee, S.; Shin, S.; Kim, H.; Han, S.; Kim, K.; Kwon, J.; Kwak, J. H.; Lee, C. K.; Ha, N. J.; Yim, D.; Kim, K. Anti-inflammatory function of arctiin by inhibiting COX-2 expression via NF-κB pathways. *J. Inflammation (London, U. K.)* **2011**, *7*, 8−16.

(6) Lee, J. H.; Lee, J. Y.; Kim, T. D.; Kim, C. J. Antiasthmatic action of dibenzylbutyrolactone lignans from fruits of Forsythia viridissima on asthmatic responses to ovalbumin challenge in conscious guinea-pigs. *Phytother Res.* **2011**, *25*, 387−395.

(7) Lu, L. C.; Zhou, W.; Li, Z. W.; Li, Z. H.; Yu, C. P.; Li, C. W.; Luo, M. H.; Xie, H. Effects of arctiin on stereptpzotoxin-induced diabetic retinopathy in Sprague-Dawley rats. *Planta Med.* **2012**, *78*, 1317−1323.

(8) Kang, K.; Lee, H. J.; Kim, C. Y.; Lee, S. B.; Tunsag, J.; Batsuren, D.; Nho, C. W. The chemopreventive effects of Saussurea salicifolia through induction of apoptosis and phase II detoxification enzyme. *Biol. Pharm. Bull.* **2007**, *30*, 2352−2359.

(9) Wu, J. G.; Wu, J. Z.; Sun, L. N.; Han, T.; Du, J.; Ye, Q.; Zhang, H.; Zhang, Y. G. Ameliorative effects of arctiin from *Arctium lappa* on experimental glomerulonephritis in rats. *Phytomedicine* **2009**, *16*, 1033−1041.

(10) Eich, E.; Pertz, H.; Kaloga, M.; Schulz, J.; Fesen, M. R.; Mazumder, A.; Pommier, Y. (−)-Arctigenin as a lead structure for inhibitors of human immunodeficiency virus type-1 integrase. *J. Med. Chem.* **1996**, *39*, 86−95.

(11) Nose, M.; Fujimoto, T.; Takeda, T.; Nishibe, S.; Ogihara, Y. Structural transformation of lignan compounds in rat gastrointestinal tract. *Planta Med.* **1992**, *58*, 520−523.

(12) Wang, W.; Pan, Q.; Han, X. Y.; Wang, J.; Tan, R. Q.; He, F.; Dou, D. Q.; Kang, T. G. Simultaneous determination of arctiin and its metabolites in rat urine and feces by HPLC. *Fitoterapia* **2013**, *86*, 6−12.

(13) Xie, L. H.; Ahn, E. M.; Hattori, M. Transformation of arctiin to estrogenic and antiestrogenic substances by human intestinal bacteria. *Chem. Pharm. Bull.* **2003**, *51*, 378−384.

(14) Jin, J. S.; Zhao, Y. F.; Norio, N.; Teruaki, A.; Nobuko, K.; Masao, H. Isolation and characterization of a human intestinal bacterium, *Eubacterium* sp. ARC-2, capable of demethylating arctigenin, in the essential metabolic process to enterolactone. *Biol. Pharm. Bull.* **2007**, *30*, 904−911.

(15) Jin, J. S.; Kakiuchi, N.; Hattori, M. Enantioselective oxidation of enterodiol to enterolactone by human intestinal bacteria. *Biol. Pharm. Bull.* **2007**, *30*, 2204−2206.

(16) Jin, J. S.; Hattori, M. Further studies on a human intestinal bacterium *Ruminococcus* sp. END-1 for transformation of plant lignans to mammalian lignans. *J. Agric. Food Chem.* **2009**, *57*, 7537−7542.

(17) Wang, X. L.; Hur, H. G.; Lee, J. H.; Kim, K. T.; Kim, S. I. Enantioselective synthesis of *S*-equol from dihydrodaidzein by a newly isolated anaerobic human intestinal bacterium. *Appl. Environ. Microbiol.* **2005**, *71*, 214−219.

(18) Liang, X. L.; Wang, X. L.; Li, Z.; Hao, Q. H.; Wang, S. Y. Improved *in vitro* assays of superoxide anion and 1,1-diphenyl- 2-picrylhydrazyl (DPPH) radical-scavenging activity of isoflavones and isoflavone metabolites. *J. Agric. Food Chem.* **2010**, *58*, 11548−11552.

(19) Liu, C. X.; Finegold, S. M.; Song, Y. L.; Lawson, P. A. Reclassification of *Clostridium coccoides*, *Ruminococcus hansenii*, *Ruminococcus hydrogenotrophicus*, *Ruminococcus luti*, *Ruminococcus productus* and *Ruminococcus schinkii* as *Blautia coccoides* gen. nov., comb. nov., *Blautia hansenii* comb. nov., *Blautia hydrogenotrophica* comb. nov., *Blautia luti* comb. nov., *Blautia producta* comb. nov., *Blautia schinkii* comb. nov. and description of *Blautia wexlerae* sp. nov., isolated from human faeces. *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 1896−1902.

(20) Adlercreutz, H.; Mousavi, Y.; Clark, J.; Höckerstedt, K.; Hämäläinen, E.; Wähälä, K.; Mäkelä, T.; Hase, T. Dietary phytoestrogens and cancer: *in vitro* and *in vivo* studies. *J. Steroid Biochem. Mol. Biol.* **1992**, *41*, 331−337.

(21) Mueller, S. O.; Simon, S.; Chae, K.; Metzler, M.; Korach, K. S. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ERalpha) and ERbeta in human cells. *Toxicol. Sci.* **2004**, *80*, 14−25.

(22) Schöttner, M.; Gansser, D.; Spiteller, G. Lignans from the roots of *Urtica dioica* and their metabolites bind to human sex hormone binding globulin (SHBG). *Planta Med.* **1997**, *63*, 529−532.

(23) Prasad, K. Antioxidant activity of secoisolariciresinol diglucoside-derived metabolites, secoisolariciresinol, enterodiol, and enterolactone. *Int. J. Angiol.* **2000**, *9*, 220−225.

(24) Jin, J. S.; Lee, J. H.; Hattori, M. Ligand binding affinities of arctigenin and its demethylated metabolites to estrogen receptor alpha. *Molecules* **2013**, *18*, 1122−1127.

(25) Kiers, C. T.; De Boer, J. L.; Olthof, R.; Spek, A. L. The crystal structure of a 2,2-diphenyl-1-picrylhydrazyl (DPPH) modification. *Acta Crystallogr., Sect. B* **1976**, *32*, 2297−2305.