Microbial glycosylation of antitubercular agent chlorflavonin

Jie Ren, Jixun Zhan*

Department of Biological Engineering, Utah State University, 4105 Old Main Hill, Logan, UT 84322-4105, USA

Correspondence: jixun.zhan@usu.edu

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ABSTRACT

Flavonoids have shown health-benefiting properties such as antioxidative and anti-inflammatory activities and are commonly used as nutraceuticals and pharmaceuticals. Although flavonoids are predominantly identified from plants, several filamentous fungal species have also been reported to produce bioactive flavonoids, such as chlorflavonin from *Aspergillus candidus*, a novel halogenated flavonoid with potent antifungal and antitubercular (anti-TB) activities. Unfortunately, the low water-solubility of this molecule may hinder its bioavailability. Glycosylation is an effective method to enhance the polarity of natural products and alter their physicochemical properties. This work focuses on the development of novel water-soluble chlorflavonin derivatives to combat the threat of drug-resistant tuberculosis. In this study, we first increased the production titer of chlorflavonin in *A. candidus* NRRL 5214 by optimizing the fermentation and purification processes. Next, chlorflavonin-5-*O*-*β*-D-glucuronopyranoside (**1**) and chlorflavonin-7-*O*-4"-*O*-methyl-*β*-D-glucopyranoside (**2**) were produced from chlorflavonin using *Streptomyces chromofuscus* ATCC 49982 and *Beauveria bassiana* ATCC 7159, respectively. Compared to chlorflavonin (4.38 ± 0.54 mg/L in water), the water solubility of the two new glycosides was determined to be 117.86 ± 4.81 mg/L (**1**) and 124.34 ± 9.13 mg/L (**2**), respectively. This study provides a promising method to create water-soluble glycosides of chlorflavonin for the development of novel anti-TB drugs.

Key words: Flavonoids; Chlorflavonin; Antifungal; Antitubercular; Glycosylation

Flavonoids represent an important class of plant secondary metabolites with a variety of biological functions in the developmental processes of hosts (1), including protection of plants against UV radiation and phytopathogens, auxin transport, signaling, and coloration (2-4). Flavonoids also possess antioxidant, antiviral, antimicrobial, anticancer, anti-proliferative, anti-diabetic, anti-inflammatory and hepatoprotective properties (5, 6). Therefore, flavonoids are important agents to prevent and treat various diseases such as cancers, oxidant stress, obesity, diabetes, pathogenic bacteria, hypertension, hyperlipidemia, inflammations, cardiovascular diseases, neurological disorders and osteoporosis (7, 8).

Flavonoids are predominantly identified from plants in stems, fruits, seeds, and other organs, and more than 9,000 structural identities have been characterized to date (9). However, several filamentous fungal species were also reported to produce uncommon flavonoids, such as chlorflavonin (3′-chloro-2′,5-dihydroxy-3,7,8-trimethoxyflavone) from *Aspergillus candidus* and *Acanthostigmella* sp. (10, 11), aspergivones A (5-*O*-methyl-chlorflavonin) and aspergivones B (5-*O*-methyl-dechlorochlorflavonin) from *A. candidus* (8), 5′-hydroxychlorflavonin from *Aspergillus* sp. AF119 (12), as well as dechlorochlorflavonin and a bromine-containing analog CJ-19,784 (3′-bromine-2′,5-dihydroxy-3,7,8-trimethoxyflavone) from *Acanthostigmella* sp. (11). Among them, chlorflavonin has attracted many researchers' attention due to its antifungal and antibacterial activities.

The World Health Organization reported that resistance of bacteria to antibiotics is a major global health challenge and it is important to explore new antibacterial agents

(13). Chlorflavonin is a novel halogenated flavonoid with stronger antifungal activity against *Candida albicans* and *Aspergillus fumigatus* than the well-known antifungal agent amphotericin B (11). Meanwhile, chlorflavonin exhibited significant *in vitro* inhibitory activity against *Mycobacterium tuberculosis* with an MIC_{90} of 1.56 μM, but without cytotoxicity to human cell lines MRC-5 and THP-1 up to 100 μM. More interestingly, chlorflavonin showed synergistic effects with the first-line antibiotics isoniazid and delamanid on intracellular activity against infected human macrophages. Therefore, the development of novel chlorflavonin derivatives is promising to combat the threat of drug-resistant tuberculosis (TB), which is considered to be the main approach to end the global TB pandemic (14).

Creating structural analogs of bioactive products is critical for the development of new drugs. For example, a recent study showed that the new chemically synthesized chlorflavonin analog, namely bromflavonin, exhibited improved anti-TB activity with an MIC_{90} of 0.78 μM (15). On the other hand, chlorflavonin, as well as many natural flavonoids, have low water-solubility, which hinders their bioavailability and health benefits (16, 17). Glycosylation is an effective method to enhance their polarity and then alter physicochemical properties and find their new application in human diseases (18, 19). In this work, we demonstrate that microbial strains can be used as an effective tool to attach various sugar moieties to chlorflavonin and generate more water-soluble glycosides.

MATERIALS AND METHODS

General experimental procedures Agilent 1200 HPLC instrument with an Agilent Eclipse Plus-C_{18} column (5 μm, 250 mm × 4.6 mm) was used to analyze and purify the products. The samples were eluted with methanol-water (65:35 to 95:5, v/v, over 15 min, containing 0.1% formic acid) at a flow rate of 1 mL/min. Low-resolution ESI-MS spectra were obtained on an Agilent 6130 single quadrupole LC-MS to confirm the molecular weights of chlorflavonin and its glycosides. Purified compounds were dissolved in deuterated dimethyl sulfoxide or methanol (DMSO-d_6 or CD_3OD) to collect the NMR spectra on a Bruker Avance III HD Ascend-500 NMR instrument (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR) located in the Department of Chemistry and Biochemistry, Utah State University. The chemical shift (δ) values are given in parts per million (ppm). The coupling constants (J values) are reported in hertz (Hz). Compounds were purified through chromatography using normal phase silica gel (40-60 μm, VWR® Agela Technologies, USA), Sephadex™ LH-20 (Cytiva, USA), and HPLC with the gradient elution of methanol-water (20:80 to 95:5, v/v, over 30 min). All solvents were purchased from Fisher Scientific. Milli-Q water was used throughout this study.

Strains, media, and culture conditions *Aspergillus candidus* NRRL 5214, *Streptomyces coeruleorubidus* NRRL B-2569, and *Streptomyces* sp. NRRL S-1521 were kindly provided by the United States Department of Agriculture ARS Collection (NRRL). *Streptomyces chromofuscus* ATCC 49982, *Actinomadura hibisca* P157-2 (ATCC 53557), and *Beauveria bassiana* ATCC 7159 were purchased from the

American Type Culture Collection (ATCC). Actinomycete strains were routinely grown in Yeast-Malt (YM) medium consisting of yeast extract (4 g/L), malt extract (10 g/L), and glucose (4 g/L) at 28 ℃. *A. candidus* NRRL 5214 and *B. bassiana* ATCC 7159 were grown in Potato Dextrose Broth (PDB) at 28 ℃.

Quantitative analysis and optimization of chlorflavonin production Standard curves of chlorflavonin and dechlorochlorflavonin were used to calculate their production titers in fermentation broth (FB), cell extract supernatant (CS), and cell extract precipitate (CP) fractions at 350 nm in the *A. candidus* NRRL 5214 culture. Quantitative analysis experiments were conducted in 250-mL Erlenmeyer flasks, containing 50 mL of PDB with or without the supplementation of NaCl. The experimental processes for quantitative analysis were described in the flow chart (Fig. 1). Briefly, *A. candidus* was cultivated in PDB medium at 28 ℃ using a rotary shaker operating at 250 rpm. Following cultivation, the culture was filtered through cotton to yield two fractions, namely, the filtrate (fermentation broth fraction of FB fraction) and the mycelial solids. The filtrate was dried *in vacuo* at 39 ℃ and the residue was dissolved in methanol and dimethyl sulfoxide (1:1 v/v) for HPLC quantitative analysis. The mycelial solids were soaked in methanol and sonicated for 1 h. After centrifugation at 4,000 ×g for 10 min, the cell debris was discarded, and the cell extract was dried *in vacuo* on a rotavapor at 39 ℃. The residue was recovered in methanol at room temperature, which was then centrifuged at 13,000 ×g for 10 min. The supernatant (cell extract supernatant fraction or CS fraction) and precipitate (cell extract precipitate fraction or CP fraction) were subjected to HPLC quantitative analysis. Specifically, the

supernatant was directly used for HPLC, and the precipitate was dissolved in a mixture of methanol and dimethyl sulfoxide (1:1, v/v) for analysis.

To optimize the production titer of chlorflavonin in CP fraction, cultures with different NaCl concentrations (0.5%, 1%, 1.5%, 2%, 3%, 4.5%, and 6%) were tested after 10 days of fermentation. Furthermore, the effect of cultivation time (6, 8, 10, 12, 14, 16, 18, and 20 days) was also investigated, by calculating production titer of chlorflavonin for each 3 days after 6 days of fermentation. HPLC analysis with a gradient mobile phase of methanol-water from 65% to 95% over 15 min was used for quantitative analysis. All crude extracts after drying *in vacuo* were redissolved in a DMSO-methanol mixture (20:80, v/v) and directly measured by using standard curves to calculate the titers. All samples were performed in three replicates.

Screening of different microorganisms for the glycosylation of chlorflavonin To test the ability of microorganisms to glycosylate chlorflavonin, we screened five strains, namely, *S. coeruleorubidus* NRRL B-2569, *Streptomyces* sp. NRRL S-1521, A. *hibisca* P157-2, *S. chromofuscus* ATCC 49982, and *B. bassiana* ATCC 7159. These microorganisms were grown in 50 mL of YM or PDB medium in a rotary shaker at 250 rpm and 28 ℃ for 3 days. In total 4 mg of chlorflavonin dissolved in 50 μL of DMSO was added into each 50 mL of culture. The cultures were incubated under the same conditions for an additional 3 days. Then the fermentation broth (1 mL) was sampled and centrifuged at 15,000 ×g for 10 min. The supernatant was analyzed by HPLC at 350 nm.

Isolation of chlorflavonin and its glycosylated products To isolate sufficient chlorflavonin for biotransformation, *A. candidus* NRRL 5214 was cultivated in six 2-L Erlenmeyer flasks, each containing 500 mL of PDB + 2.5% NaCl medium. After 14 days of fermentation, cultures were treated by following the procedures described in the flow chart (Fig. 1). All CP fractions were combined and dried for normal phase silica gel column chromatography, eluted with chloroform-methanol (10:1, v/v). Chlorflavonin-containing fractions were combined and subjected to reverse phase HPLC with gradient elution of methanol-water (65-95%, v/v, 0-15 min) for purified chlorflavonin. To isolate the biotransformation products of chlorflavonin for structure elucidation, *S. chromofuscus* ATCC 49982 and *B. bassiana* ATCC 7159 were cultivated in 1-L Erlenmeyer flasks, containing 250 mL of YM or PDB medium. In total, 20 mg of chlorflavonin was added for each biotransformation. After 3 days, both cultures were centrifuged at 4,000 ×g for 10 min to collect the supernatant. The collected fermentation broths were dried under reduced pressure at 39 ℃, and the residue was dissolved in 15 mL of 50% methanol-water (v/v). The samples were subjected to fractionation using a silica gel column, eluted with a gradient of chloroform-methanol (1:0 to 0:1, v/v). The product-containing fractions were filtered and subjected to fractionation using a Sephadex LH-20 column, eluted with methanol-water (1:1, v/v). The target fractions were combined and further separated by reverse phase HPLC, and eluted with methanol-water (65-95%, 0-15 min) containing 0.1% formic acid (v/v) to yield product **1** (3.0 mg). For isolating compound **2**, procedures were similar except isocratic elution methanol-water (53%, 0-15 min) was used to yield

product **2** (3.5 mg). Product **1** was dissolved in methanol-d_4 and product **2** was dissolved in DMSO-d_6, and then subjected to NMR analysis. Their chemical structures were characterized by NMR spectra and arranged in Table 1.

Determination of the water-solubility of dechlorochlorflavonin, chlorflavonin and the glycosides The purified dechlorochlorflavonin, chlorflavonin and produced glycosides were tested for their water solubility as previously described (18). Standard curves were established by using purified dechlorochlorflavonin, chlorflavonin, chlorflavonin-5-*O*-*β*-D-glucuronopyranoside (**1**), and chlorflavonin-7-*O*-4"-*O*-methyl-*β*-D-glucopyranoside (**2**) to quantify the water solubility of each compound. Briefly, the purified dechlorochlorflavonin, chlorflavonin and glycosides were each mixed with 300 μL of distilled water in an Eppendorf tube at 25 ℃. An ultrasonic cleaner was used to facilitate the dissolution. After 30 min of sonication, the samples were centrifuged at 13,000 × g for 10 min to separate the solution from the undissolved compounds. The supernatant of each sample was analyzed by HPLC to determine the compound concentrations in the solution. All tests were performed in triplicate and water solubility of each sample are expressed as the mean ± standard deviation (SD).

RESULTS

Enhanced production of chlorflavonin in *A. candidus* NRRL 5214 *A. candidus* NRRL 5214 produces both chlorflavonin and dechlorochlorflavonin in the PDB medium (Fig. S1); however, researchers are more interested in chlorflavonin due to its significant anti-TB activities (14, 15). Aside from the limited supply and high price of commercially available chlorflavonin, its low production yield makes it difficult to

obtain sufficient amounts for structural modification. Our previous studies suggest that exogenous chloride may facilitate the production of chlorflavonin (20, 21). To test this assumption, we supplemented 1% NaCl in PDB during fermentation. We separated the cultures into three fractions, including fermentation broth (FB), cell extract supernatant (CS), and cell extract precipitate (CP), for quantitative analysis of dechlorochlorflavonin and chlorflavonin (Fig. 1).

In the PDB medium, the production titer of dechlorochlorflavonin (1.59 ± 0.2 mg/L) is around eight times higher than chlorflavonin (0.18 ± 0.05 mg/L) in FB fraction, probably due to the higher water solubility of dechlorochlorflavonin (Fig. 2A). Furthermore, the overall production titer of dechlorochlorflavonin (4.42 ± 0.4 mg/L) is nearly twice that of chlorflavonin (2.24 ± 0.28 mg/L) in PDB medium (Fig. 2B). By adding 1% NaCl into the PDB medium, the overall production titer of chlorflavonin doubled to 4.94 ± 0.26 mg/L, which is much higher than that of dechlorochlorflavonin (0.32 ± 0.05 mg/L) (Fig. 2C). These results showed that the production titer of chlorflavonin can be increased by supplementing 1% NaCl in PDB. Moreover, around 75% chlorflavonin production was in the CP fraction of PDB + 1% NaCl medium, which was used as a basis of comparison for optimizing NaCl concentration and fermentation time.

Then, the effect of different NaCl concentrations on chlorflavonin production was investigated. As shown in Fig. 2D, the chlorflavonin production titer in the CP fraction exhibited the highest value of 4.65 ± 0.05 mg/L at 2.5% NaCl. However, further increase of NaCl resulted in a dramatic decrease in chlorflavonin production. This is

probably because high NaCl concentration has an inhibitory effect on the growth of *A. candidus* NRRL 5214. Furthermore, the influence of fermentation time was also investigated at the concentration of 2.5% NaCl. The titer of chlorflavonin in the CP fraction reached 5.71 ± 0.06 mg/L after 14 days, representing a 4-fold increase compared to 1.29 ± 0.05 mg/L at 6 days (Fig. 2E). However, further fermentation led to a decrease on the production titer, probably due to degradation of chlorflavonin. Thus, we isolated chlorflavonin from the CP fraction in PDB medium after 14 days of fermentation supplemented with 2.5% NaCl for the following glycosylation work.

Screening different microorganisms for glycosylating chlorflavonin

Microorganisms, especially actinomycetes, are well-known for producing structurally diverse bioactive natural products and contain various biosynthetic enzymes. We speculated that some of these strains may have versatile glycosyltransferases (GTs) that can introduce the sugar moiety to chlorflavonin. To this end, chlorflavonin was incubated with five different strains, including four actinomycetes (*S. coeruleorubidus* NRRL B-2569, *Streptomyces* sp. NRRL S-1521, *A. hibisca* P157-2, *S. chromofuscus* ATCC 49982) and a fungal strain (*B. bassiana* ATCC 7159). HPLC analysis revealed that two more polar metabolites, at 8.2 min for product **1** and 7.8 min for product **2**, were biosynthesized from chlorflavonin by *S. chromofuscus* ATCC 49982 and *B. bassiana* ATCC 7159, respectively (Fig. 3A). However, no products were detected from the other three strains. The UV absorption patterns of the products were both similar to that of chlorflavonin, suggesting that these two polar products are derivatives of the substrate (Figs. 3B and 3C).

Structural characterization of biotransformed products of chlorflavonin by *S. chromofuscus* ATCC 49982 and *B. bassiana* ATCC 7159 Compound **1** was isolated as a light yellow, amorphous powder. To elucidate the chemical structure, **1** was analyzed by NMR (Figs. S2-S6). The ^{13}C NMR analysis (Fig. S3) presented 24 peaks in the spectrum. In addition to the 18 signals belonging to the substrate, 6 additional carbon signals at δ_C 170.0, 105.3, 76.9, 76.7, 74.6, and 72.8 were found in the spectra, together with the additional proton signals at δ_H 3.57-5.00 in the ^1H NMR spectrum (Fig. S2), suggesting that a sugar moiety had been added to chlorflavonin. Unlike the common sugar glucose, this sugar moiety has a quaternary carbon signal at δ_C 170.0, indicating the presence of a carboxyl group in the sugar moiety. Both the ^1H and ^{13}C signals of this sugar moiety are consistent with a glucuronic acid moiety. Moreover, the ^1H NMR spectrum showed a doublet at δ_H 5.00, indicative of an anomeric proton with a coupling constant of 7.7 Hz, and the chemical shift along with the *J*-coupling value were consistent with that of *β*-D-glucuronic acid (17, 18). The correlation of the anomeric H-1" signal at δ_H 5.00 to C-5 signal at δ_C 154.6 in the HMBC spectrum (Fig. S6) revealed that the glucuronic acid moiety was located at C-5. The above data together with the detailed analysis of its 2D NMR spectra confirmed its structure as chlorflavonin-5-*O*-*β*-D-glucuronopyranoside (Fig. 4) and all signals were assigned accordingly (Table 1).

Compound **2** was obtained as a yellow, amorphous powder. The NMR analysis (Figs. S7- S11) was performed to further elucidate the chemical structure of **2**. Among the 24 carbon signals in the ^{13}C NMR spectrum (Fig. S8), 17 signals belonging to the skeleton of chlorflavonin were determined to be similar to those present in **1**. The seven

263 remaining carbon signals at δ_C 100.0, 73.3, 76.2, 78.9, 75.7, 60.1, and 59.6 in the ^{13}C
264 NMR spectrum, together with the proton signals in the range of δ_H 3.05-5.10 in the ^{1}H
265 NMR spectrum (Fig. S7), further suggested that a typical methyl glucose was added to
266 chlorflavonin. The methoxy carbon signal was observed at δ_C 59.6, and the HMBC
267 spectrum of **2** revealed the correlation of methoxy group at δ_H 3.46 to C-4" at δ_C 78.9
268 (Fig. S11). Therefore, both the ^{1}H and ^{13}C signals of this sugar moiety supported the
269 presence of a 4"-*O*-methyl-glucose moiety. Moreover, the ^{1}H NMR spectrum showed
270 a doublet at δ_H 5.10, corresponding to the anomeric proton of this sugar moiety with a
271 coupling constant of 7.8 Hz, which is consistent with that of 4"-*O*-*β*-D-methyl-glucose
272 (18). Compared to the skeleton of chlorflavonin, one less methoxy group was observed
273 in product **2** (Fig. S8). Moreover, the HMBC spectrum of **2** revealed the correlations of
274 5-OH at δ_H 12.3 to C-5, C-6, C-10 at δ_C 155.9, 98.5, and 106.3, respectively (Fig. S11),
275 as well as the correlations of 2'-OH at δ_H 10.09 to C-1', C-2', C-3' at δ_C 119.7, 150.9,
276 and 121.6, respectively, which further confirmed that 4"-*O*-*β*-D-methyl-glucose was not
277 attached to the 5-OH and 2'-OH positions. The HMBC spectrum of **2** revealed the
278 correlation of H-1" at δ_H 5.10 to C-7 at δ_C 156.0 (Fig. S11), which confirmed that **2** has
279 a 4"-*O*-*β*-D-methyl-glucose moiety at C-7. Based on the above spectral evidence, the
280 compound was identified to be a new compound, namely chlorflavonin-7-*O*-4"-*O*-
281 methyl-*β*-D-glucopyranoside with all assigned signals in Table 1. Moreover, the
282 proposed biosynthetic pathway of **2** from chlorflavonin is shown in Fig. 4.

283 **Water solubility test of dechlorochlorflavonin, chlorflavonin and its glycosides**

284 Water solubility may affect the bioavailability of bioactive natural products in the

285 human body (22). Thus, the water solubility of dechlorochlorflavonin, chlorflavonin,
286 as well as chlorflavonin glycosides **1** and **2** were determined by HPLC. The water
287 solubility of each compound was measured in triplicate (n = 3) and computed to be
288 53.61 ± 1.39 mg/L, 4.38 ± 0.54 mg/L, 117.86 ± 4.81 mg/L, and 124.34 ± 9.13 mg/L,
289 respectively. The water solubility of chlorflavonin glycosides **1** and **2** were both around
290 27 times higher than the substrate chlorflavonin (Fig. 5), indicating that microbes can
291 convert chlorflavonin into more water-soluble glycosides. The two microorganisms
292 identified in this study could be used to glycosylate other bioactive natural products to
293 create new analogs with enhanced water solubility.

294 **DISCUSSION**

295 In 2020, around 1.5 million people died from the bacterial pathogen *M.*
296 *tuberculosis* which causes tuberculosis (TB) (23). However, the normal first-line
297 (isoniazid and rifampicin) and second-line (capreomycin, streptomycin, and
298 cycloserine) therapies cannot tackle the emerging challenge of multi-drug resistance
299 (MDR) of *M. tuberculosis* (15). Although new anti-TB drugs, including bedaquiline,
300 delamanid, and pretomanid, have been clinically approved (24), their applications are
301 still limited due to potential side effects and long treatment periods (14, 25-27). Thus,
302 discovering new drugs for combating against drug-resistant TB is urgent to alleviate
303 the transition of *M. tuberculosis* strains from MDR into extensively drug resistant
304 (XDR), which further hinders the World Health Organization's goal to end the global
305 TB pandemic by the year 2035 (15, 28).

306 Chlorflavonin is a natural product that exhibits significant *in vitro* inhibitory
307 activity against *M. tuberculosis*. Furthermore, chlorflavonin showed synergistic effects
308 with other antibiotics (such as isoniazid and delamanid) against infected human
309 macrophages, with better intracellular activity than streptomycin. Therefore,
310 chlorflavonin could be a promising chemotherapy for TB patients with highly specific
311 and selective antitubercular activity (14). In this study, we optimized the production of
312 chlorflavonin from *A. candidus* NRRL 5214 by supplementing 2.5% NaCl into the PDB
313 medium after 14 days of fermentation. The results showed that the production titer of
314 unwanted dechlorochlorflavonin was significantly decreased, leading to the
315 development of an easier downstream process for obtaining chlorflavonin for future
316 studies. More importantly, through microbial glycosylation, we generated two more
317 water-soluble glycosides of chlorflavonin using a bacterial strain and a fungal strain,
318 respectively. These two glycosides contain different sugar moieties, one having a
319 glucuronic acid moiety attached to the 5-OH group and the other with a 4"-*O*-methyl-
320 *β*-D-glucose moiety linked to the 7-OH position, further demonstrating that
321 microorganisms are a useful resource of biocatalytic tools for natural product
322 derivatization. The significantly increased water solubility of the two glycosides
323 indicates that glycosylation is an effective tool to improve the water solubility of
324 bioactive molecules.

325 Unlike product **1**, the sugar moiety of product **2** is not attached onto one of the
326 original two hydroxyl groups of chlorflavonin, but at the 7-OH group, which was
327 originally occupied by a methyl group in the substrate. *B. bassiana* ATCC 7159 has

328 been reported to catalyze a variety of reactions, including *O*-demethylation (29). We
329 have recently also characterized a versatile *O*-glycosyltransferase from this strain,
330 which can transfer a *β*-D-glucose or 4"-*O*-methyl-*β*-D-glucose moiety to various
331 substrates (16). Therefore, we propose that chlorflavonin was first demethylated at the
332 7-OCH_3 group to give a free hydroxyl group, to which the 4"-*O*-methyl-*β*-D-glucose
333 moiety was attached by the dedicated glycosyltransferase BbGT in *B. bassiana* ATCC
334 7159.

335 In summary, this work demonstrates an approach for improved production of
336 chlorflavonin in *A. candidus* NRRL 5214. Direct supply of exogenous sodium chloride
337 at the concentration of 2.5% in the fermentation broth leads to the production of
338 chlorflavonin at 5.71 ± 0.06 mg/L under optimized conditions. Glycosylation is a useful
339 tool for structural modification of natural products for bioactive agents. These new
340 molecules provide promising candidates for *in vivo* testing of the bioavailability and
341 anti-TB activity in future studies. Additional chlorflavonin glycosides can be prepared
342 by other glycosylating strains. Therefore, this work provides effective biocatalytic tools
343 to facilitate the creation of new chlorflavonin analogs to combat the drug resistant *M.*
344 *tuberculosis* strains.

345 Supplementary data to this article can be found online at https://doi.org/

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453 **Table 1.** ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data for compounds **1** and **2.**

Position	**1** (CD_3OD)		**2** (DMSO-d_6)	
	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
2	156.1, C		155.7, C	
3	142.8, CH		139.6, CH	
4	176.3, C		178.6, C	
5	154.6, CH		155.9, CH	
6	102.4, CH	7.12 (1H, s)	98.5, CH	6.70 (1H, s)
7	158.1, CH		156.0, CH	
8	134.4, CH		129.1, CH	
9	152.2, C		149.0, C	
10	111.7, C		106.3, C	
1'	121.3, C		119.7, C	
2'	152.6, C		150.9, C	
3'	123.2, CH		121.6, CH	
4'	133.3, CH	7.52 (1H, dd, *J*=8.0, 1.5 Hz)	132.2, CH	7.60 (1H, dd, *J*=8.0, 1.1 Hz)
5'	121.4, CH	7.00 (1H, t, *J*=7.0 Hz)	120.2, CH	7.01 (1H, t, *J*=7.9 Hz)
6'	130.5, CH	7.41 (1H, dd, *J*=7.7, 1.5 Hz)	129.5, CH	7.42 (1H, dd, *J*=7.6, 1.1 Hz)
1"	105.3, CH	5.00 (1H, d, *J*=7.7 Hz)	100.0, CH	5.10 (1H, d, *J*=7.8 Hz)
2"	74.6, CH	3.68 (2H, m, overlapped)	73.3, CH	3.29 (1H, m)
3"	76.9, CH	3.57 (1H, t, *J*=9.0 Hz)	76.2, CH	3.43 (1H, m)
4"	72.8, CH	3.68 (2H, m, overlapped)	78.9, CH	3.05 (1H, t, *J*=9.2 Hz)
5"	76.7, CH	4.04 (1H, d, *J*=9.7 Hz)	75.7, CH	3.50 (2H, m, overlapped)
6"	170.0, C		60.1, CH_2	3.64 (1H, m), 3.50 (2H, m, overlapped)
3-OCH_3	61.1, CH_3	3.73 (3H, s)	60.1, CH_3	3.72 (3H, s)
7-OCH_3	57.0, CH_3	3.97 (3H, s)		
8-OCH_3	62.0, CH_3	3.84 (3H, s)	61.2, CH_3	3.74 (3H, s)
4"-OCH_3			59.6, CH_3	3.46 (3H, s)

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459 **Figure legends**

460 **FIG. 1.** Flow chart for obtaining dechlorochlorflavonin (dcfv) and chlorflavonin (cfv)
461 and glycosides preparation of cfv. Three fractions are fermentation broth (FB), cell
462 extract supernatant (CS), and cell extract precipitate (CP), which are outlined in the
463 green boxes. Two main fractions for the isolation of dcfv and/or cfv are outlined in the
464 dashed line boxes.

465 **FIG. 2.** Production of dechlorochlorflavonin (dcfv) and chlorflavonin (cfv) in *A.*
466 *candidus* NRRL 5214. Cultures were treated for product analysis after 10 days of
467 growth. Data are presented as the mean ± SD from three independent experiments. (A)
468 Production titer of dechlorochlorflavonin and chlorflavonin in fermentation broth (FB),
469 cell extract supernatant (CS), and cell extract precipitate (CP) in PDB or PDB + 1%
470 NaCl. Statistical analysis was performed by using two-tailed *t* test, where ** indicates
471 p-value < 0.01, * indicates p-value < 0.05, n.s. indicates no significant difference ($p >$
472 0.05). (B) Overall production titer of dechlorochlorflavonin and chlorflavonin in PDB,
473 and their distribution in three fractions. (C) Overall production titer of
474 dechlorochlorflavonin and chlorflavonin in PDB + 1% NaCl, and their distribution in
475 the three fractions. (D) The effects of different NaCl concentrations on the titer of
476 chlorflavonin in the CP fraction. (E) The effects of fermentation time on the titer of
477 chlorflavonin in the CP fraction from PDB medium with 2.5% NaCl.

478 **FIG. 3.** Screening of microbes for the ability to biotransform chlorflavonin. (A) HPLC
479 analysis (350 nm) of biotransformation of chlorflavonin by five microorganisms. (i)
480 chlorflavonin + YM medium; (ii) chlorflavonin + *S. coeruleorubidus* NRRL B-2569;

481 (iii) chlorflavonin + *Streptomyces* sp. NRRL S-1521; (iv) chlorflavonin + *A. hibisca*
482 P157-2; (v) chlorflavonin + *S. chromofuscus* ATCC 49982; (vi) chlorflavonin + *B.*
483 *bassiana* ATCC 7159. (B) UV spectra comparison of chlorflavonin and product **1**. (C)
484 UV spectra comparison of chlorflavonin and product **2**.
485 **FIG. 4.** Chemical structures (with HMBC correlations) of the produced chlorflavonin
486 glycosides **1** and **2** and proposed biosynthetic pathway of compound **2**.
487 **FIG. 5.** Water solubility of dechlorochlorflavonin (dcfv), chlorflavonin (cfv),
488 chlorflavonin-5-*O*-*β*-D-glucuronopyranoside (**1**), and chlorflavonin-7-*O*-4"-*O*-methyl-
489 *β*-D-glucopyranoside (**2**). Data are presented as the mean ± SD from three independent
490 experiments.
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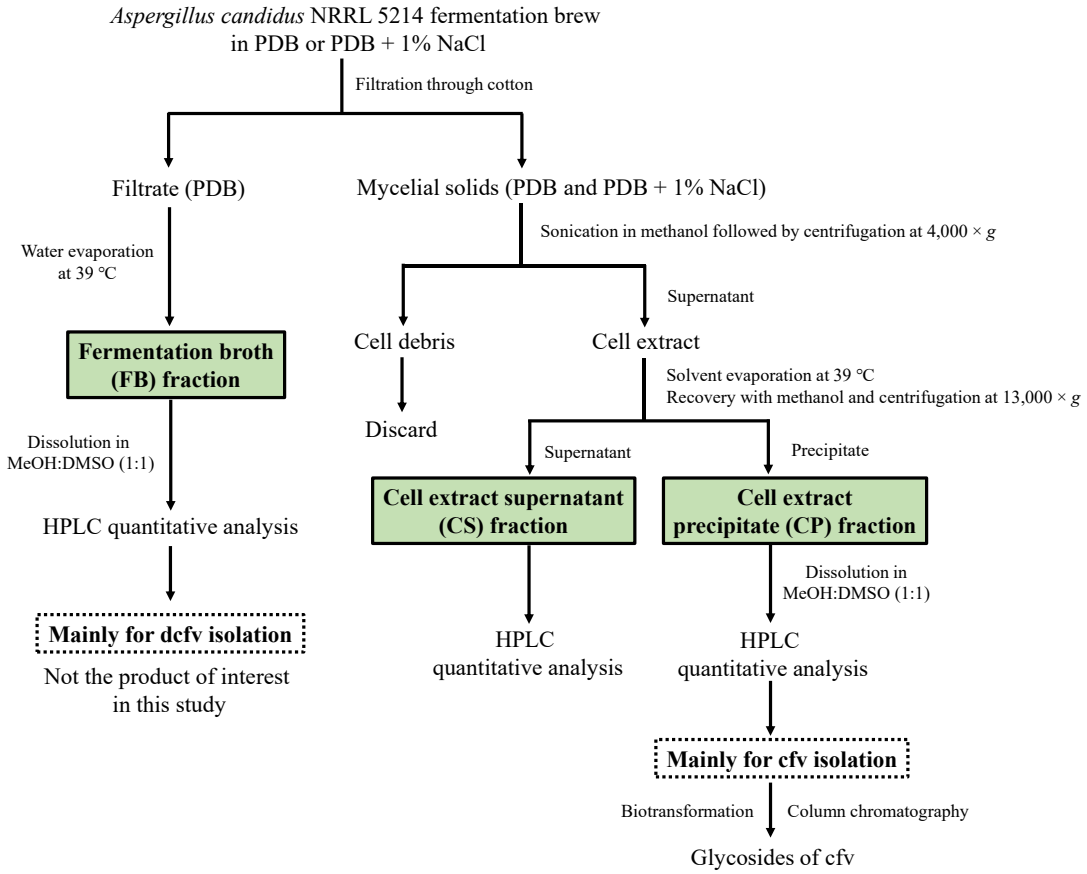

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505 **FIG. 2.**

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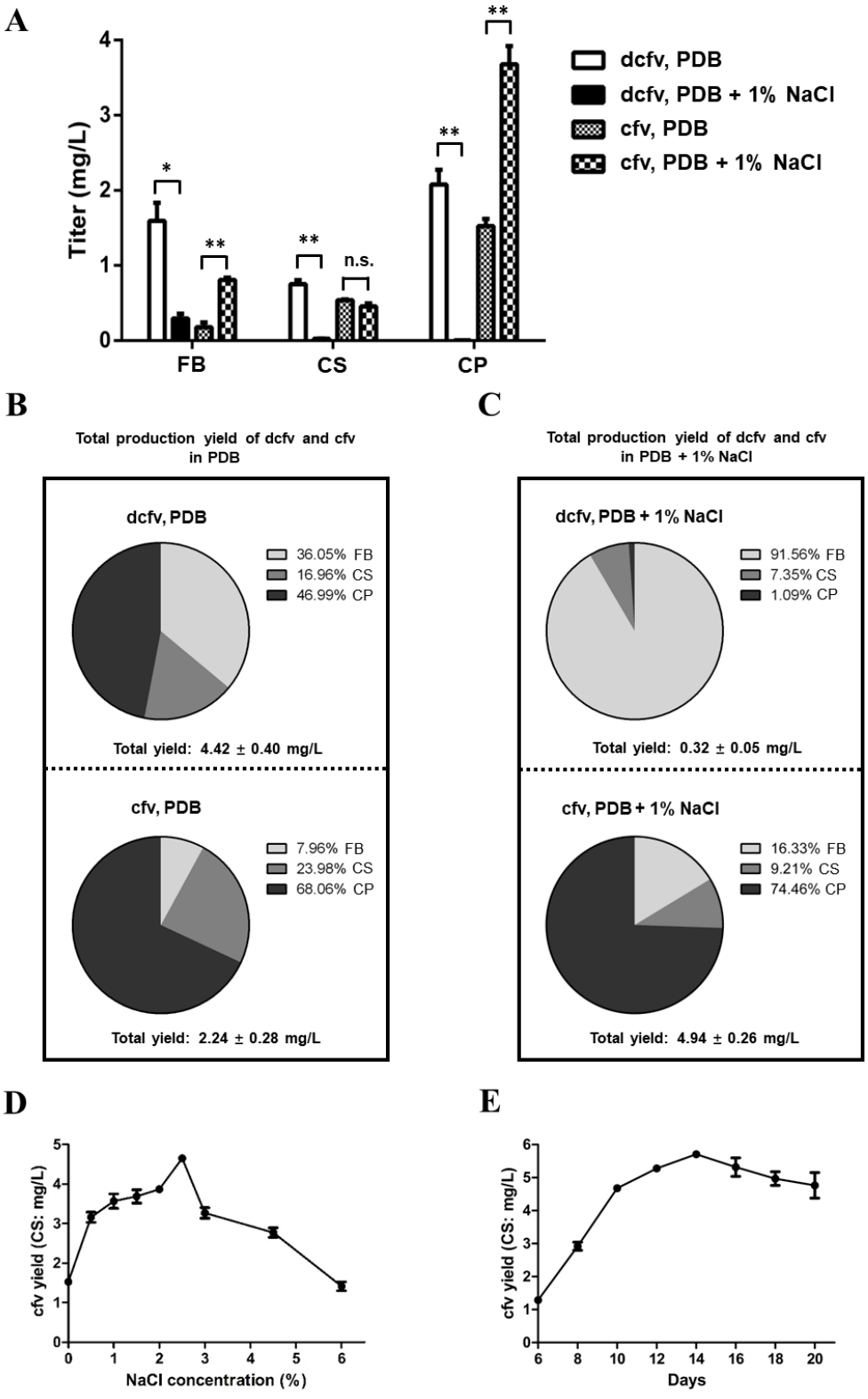
A
Titer (mg/L)
FB
CS
CP
dcfv, PDB
dcfv, PDB + 1% NaCl
cfv, PDB
cfv, PDB + 1% NaCl
n.s.
B
Total production yield of dcfv and cfv in PDB
dcfv, PDB
36.05% FB
16.96% CS
46.99% CP
Total yield: 4.42 ± 0.40 mg/L
cfv, PDB
7.96% FB
23.98% CS
68.06% CP
Total yield: 2.24 ± 0.28 mg/L
C
Total production yield of dcfv and cfv in PDB + 1% NaCl
dcfv, PDB + 1% NaCl
91.56% FB
7.35% CS
1.09% CP
Total yield: 0.32 ± 0.05 mg/L
cfv, PDB + 1% NaCl
16.33% FB
9.21% CS
74.46% CP
Total yield: 4.94 ± 0.26 mg/L
D
cfv yield (CS: mg/L)
NaCl concentration (%)
E
cfv yield (CS: mg/L)
Days

FIG. 2.

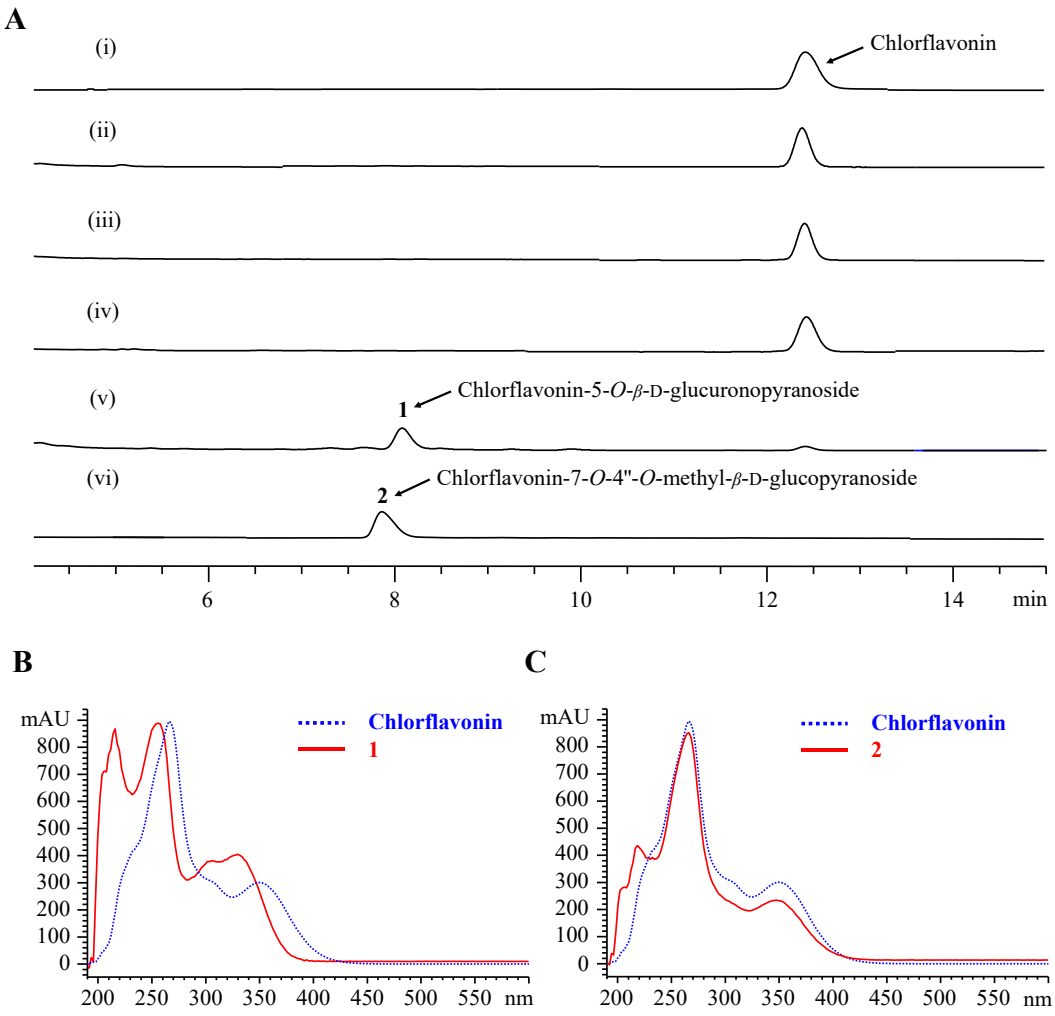
A
(i)
Chlorflavonin
(ii)
(iii)
(iv)
(v)
1
Chlorflavonin-5-O-β-D-glucuronopyranoside
(vi)
2
Chlorflavonin-7-O-4''-O-methyl-β-D-glucopyranoside
6
8
10
12
14
min
B
mAU
800
700
600
500
400
300
200
100
0
Chlorflavonin
1
200
250
300
350
400
450
500
550
nm
C
mAU
800
700
600
500
400
300
200
100
0
Chlorflavonin
2
200
250
300
350
400
450
500
550
nm

FIG. 3.

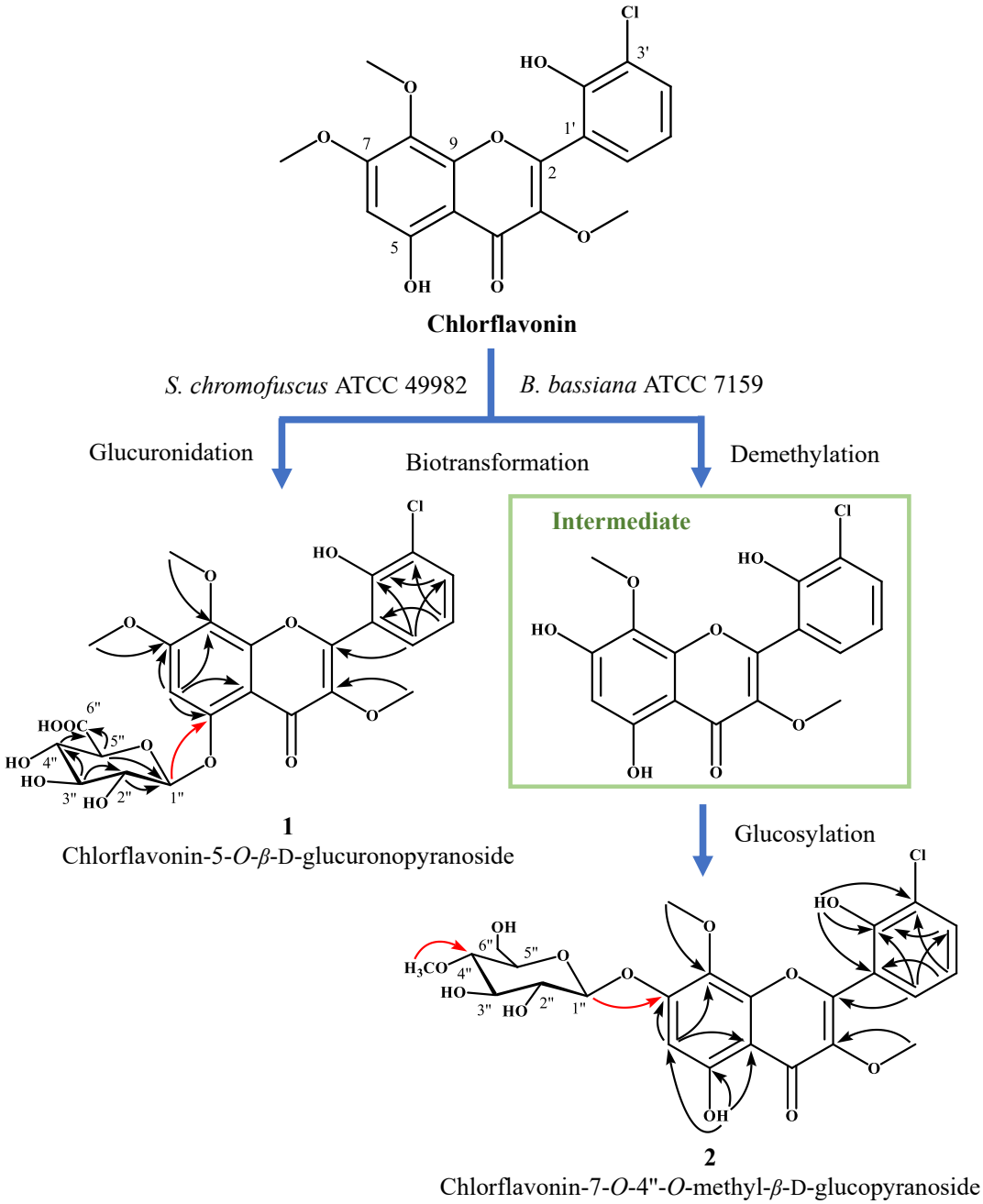
Chlorflavonin
S. chromofuscus ATCC 49982
B. bassiana ATCC 7159
Glucuronidation
Biotransformation
Demethylation
Intermediate
1
Chlorflavonin-5-O-β-D-glucuronopyranoside
Glucosylation
2
Chlorflavonin-7-O-4"-O-methyl-β-D-glucopyranoside

FIG. 4.

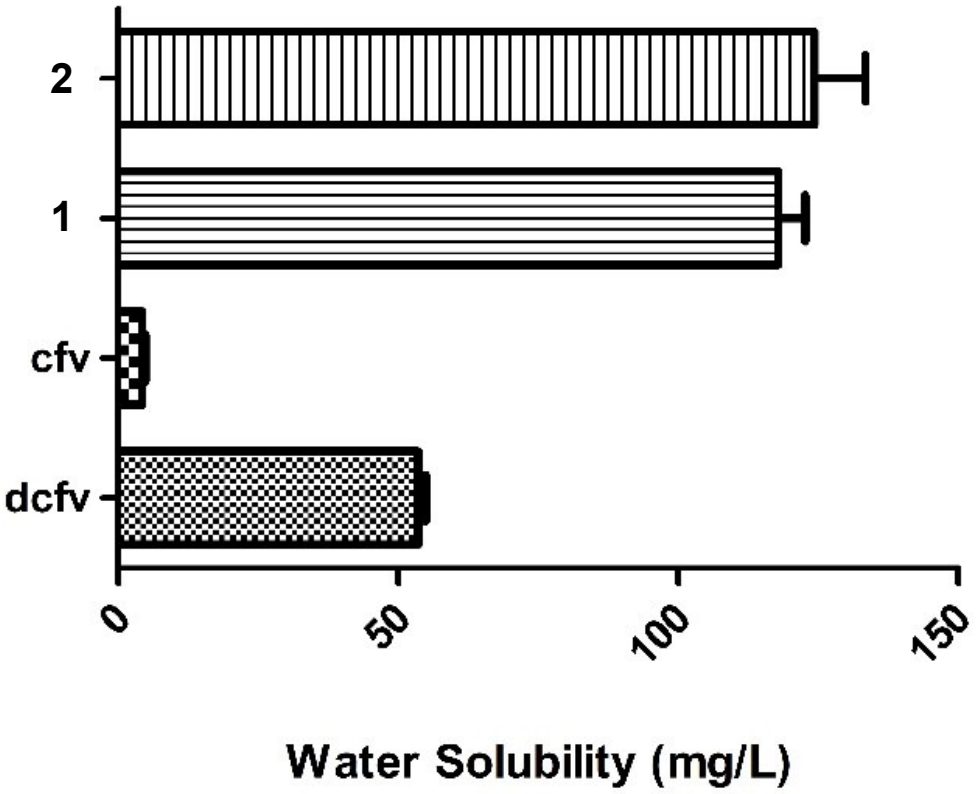

FIG. 5.

542 **Supplementary figure legends**

543 **Figure S1.** Flavonoids produced from *Aspergillus candidus* NRRL 5214.

544 **Figure S2.** ^{1}H NMR spectrum of product **1** (Methanol-d_4, 500 MHz).

545 **Figure S3.** ^{13}C NMR spectrum of product **1** (Methanol-d_4, 125 MHz).

546 **Figure S4.** ^{1}H-^{1}H COSY spectrum of product **1** in Methanol-d_4.

547 **Figure S5.** HSQC spectrum of product **1** in Methanol-d_4.

548 **Figure S6.** HMBC spectrum of product **1** in Methanol-d_4.

549 **Figure S7.** ^{1}H NMR spectrum of product **2** (DMSO-d_6, 500 MHz).

550 **Figure S8.** ^{13}C NMR spectrum of product **2** (DMSO-d_6, 125 MHz).

551 **Figure S9.** ^{1}H-^{1}H COSY spectrum of product **2** in DMSO-d_6.

552 **Figure S10.** HSQC spectrum of product **2** in DMSO-d_6.

553 **Figure S11.** HMBC spectrum of product **2** in DMSO-d_6.

554