

Microbial Metabolism Part 9.¹⁾ Structure and Antioxidant Significance of the Metabolites of 5,7-Dihydroxyflavone (Chrysin), and 5- and 6-Hydroxyflavones

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5,7-Dihydroxyflavone (chrysin) (**1**) when fermented with fungal cultures, *Aspergillus alliaceous* (ATCC 10060), *Beauveria bassiana* (ATCC 13144) and *Absidia glauco* (ATCC 22752) gave mainly 4'-hydroxychrysin (**4**), chrysin 7-*O*- β -D-4-*O*-methylglucopyranoside (**5**) and chrysin 7-sulfate (**6**), respectively. *Mucore ramannianus* (ATCC 9628), however, transformed chrysin into six metabolites: 4'-hydroxy-3'-methoxychrysin (chrysoeriol) (**7**), 4'-hydroxychrysin (apigenin) (**4**), 3',4'-dihydroxychrysin (luteolin) (**8**), 3'-methoxychrysin 4'-*O*- α -D-6-deoxyallopypyranoside (**9**), chrysin 4'-*O*- α -D-6-deoxyallopypyranoside (**10**), and luteolin 3'-sulfate (**11**). Cultures of *A. alliaceous* (ATCC 10060) and *B. bassiana* (ATCC 13144) metabolized 5-hydroxyflavone (**2**) into 5,4'-dihydroxyflavone (**12**) and 4'-hydroxyflavone 5-*O*- β -D-4-*O*-methylglucopyranoside (**13**), respectively. 6-Hydroxyflavone (**3**) was transformed into 6-hydroxyflavanone (**14**), flavone 3-*O*- β -D-4-*O*-methylglucopyranoside (**15**) and ($\pm$)-flavanone 6-*O*- β -D-4-*O*-methylglucopyranoside (**16**) by cultures of *Beauveria bassiana* (ATCC 13144). The structures of the metabolic products were elucidated by means of spectroscopic data. The significance of the metabolites as antioxidants in relation to their structure is briefly discussed.

Key words flavonoid; microbial metabolism; *Beauveria bassiana*; *Aspergillus alliaceous*; *Absidia glauco*; *Mucore ramannianus*

Flavonoids are an important class of polyphenolic natural products that are biosynthesized from phenylalanine.²⁾ They are widely distributed in the plant kingdom but confined mainly to higher plants. Mosses and liverworts are some of the lower plant groups that contain flavonoids.³⁾ Rare detections of these compounds have been made in fungi.⁴⁾ Animals are not able to biosynthesize flavonoids.⁵⁾ Besides imparting vivid colors to various parts of plants, flavonoids play many roles in the development and growth of plants.⁶⁾ Several biological activities including anticarcinogenic,^{7–9)} anti-inflammatory^{7,10,11)} and antioxidant^{12,13)} have been attributed to this class of compounds. Flavonoids are widely consumed in the form of herbal preparations or dietary supplements by humans. Although most of the flavonoids are considered to be safe, their efficacy is yet to be determined by means of clinical trials.¹⁴⁾ Moreover, potential toxicities of flavonoids as well as their drug interactions have not been well studied.¹⁴⁾ With the publicity given to the beneficial effects, the consumption of dietary supplements containing flavonoids has increased significantly. It has been pointed out that the excess use of these compounds could have drastic effects, as high concentrations of flavonoids may act as mutagens, pro-oxidants and inhibitors of hormone metabolizing enzymes.¹⁵⁾

Microorganisms may be utilized as models of drug metabolism to predict the fate of xenobiotics in mammalian systems.^{16,17)} Since this method often gives sufficient quantities of metabolites, complete chemical structure and pharmacological activities could be determined. Also, they can be used as analytical standards to detect and characterize particular flavonoid metabolites which are present in very small quantities in biological fluids.¹⁷⁾ In our ongoing research on microbial transformation of flavonoids, 5,7-dihydroxyflavone (chrysin), and 5- and 6-hydroxyflavones were screened using 40 different microorganisms. Of the organ-

isms which effected transformation, scale up studies was carried out with selected cultures to isolate the maximum number of metabolites in reasonable yields. Structure elucidation of the metabolites of the three flavonoids, **1**–**3** and their possible impact on the antioxidant activity in relation to structure are discussed.

Results and Discussion

Forty fungal strains were used in the initial screening of the flavonoids **1**–**3**. The two-stage standard procedure was used in all the screening experiments.¹⁸⁾ Selection of fungal cultures for scale up studies was made in such a manner as to obtain all the transformed products with maximum yield. Chrysin (**1**) was converted to eight derivatives whilst 5-hydroxyflavone (**2**) and 6-hydroxyflavone (**3**) were transformed into two and three metabolites respectively (Table 1).

Metabolites, **4**, **6**–**8**, **11**, **12**, **14** and **15** being known compounds were identified by comparison with published data as, 4'-hydroxychrysin (apigenin),^{19,20)} chrysin 7-sulfate,²¹⁾ 4'-hydroxy-3'-methoxychrysin (chrysoeriol),^{19,20)} 3',4'-dihydroxychrysin (luteolin),^{19,20)} luteolin 3'-sulfate, 5,4'-dihydroxyflavone,^{19,20)} ($\pm$)-6-hydroxyflavanone,²²⁾ and flavone 3-*O*- β -D-4-*O*-methylglucopyranoside.²³⁾ respectively. ($\pm$)-6-hydroxyflavanone (**14**) indicated a non-stereoselective process of hydroxylation of the olefinic bond in the starting material (**3**). However, due to the non availability of spectral information, the structure of compound **11** was elucidated by a detailed study of its high resolution spectroscopic data.

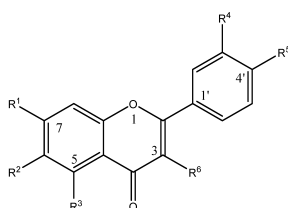
Metabolite **5** (221.0 mg, 44.2% yield), a white solid, was shown to have a molecular formula C₂₂H₂₂O₉ (HR-ESI-MS). IR absorption bands at 3408, 2925, 1620 and 1665 cm⁻¹ suggested the presence of –OH, –CH, –C=C and –C=O functional groups, respectively. The chemical shifts and the *J* values for aromatic protons observed in the ¹H-NMR spec-

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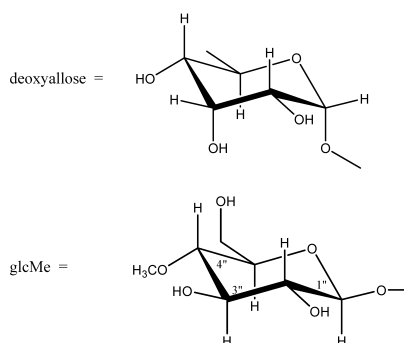
Table 1. Microbial Transformation of Chrysin and 5- and 6-Hydroxyflavones

Substrate	Microorganism	Metabolite	Yield (%)	Antioxidant activity IC ₅₀ (μM)
Chrysin (1)	<i>Aspergillus alliaceous</i> (ATCC 10060)	4'-Hydroxychrysin (apigenin) (4)	4.1	N/A
	<i>Beauveria bassiana</i> (ATCC 13144)	Chrysin 7- <i>O</i> -β-D-4- <i>O</i> -methylglucopyranoside (5)	44.2	N/A
	<i>Absidia glauco</i> (ATCC 22752)	Chrysin 7-sulfate (6)	5.74	N/A
	<i>Mucore ramannianus</i> (ATCC 9628)	4'-Hydroxy-3'-methoxychrysin (chrysoeriol) (7),	1.4	N/A
		4'-Hydroxychrysin (apigenin) (4),	3.0	N/A
		3',4'-Dihydroxychrysin (luteolin) (8),	10.2	11.0
		3'-Methoxychrysin 4'- <i>O</i> -α-D-6-deoxyallopypyranoside (9),	3.2	N/A
		Chrysin 4'- <i>O</i> -α-D-6-deoxyallopypyranoside (10),	18.7	N/A
		Luteolin 3'-sulfate (11)	10.2	N/A
		5,4'-Dihydroxyflavone (12)	0.46	N/A
5-Hydroxyflavone (2)	<i>Aspergillus alliaceous</i> (ATCC 10060)	4'-Hydroxyflavone 5- <i>O</i> -β-D-4- <i>O</i> -methylglucopyranoside (13)	98	N/A
6-Hydroxyflavone (3)	<i>Beauveria bassiana</i> (ATCC 13144)	(±)-6-Hydroxyflavanone (14),	8.3	N/A
		Flavone 3- <i>O</i> -β-D-4- <i>O</i> -methylglucopyranoside (15),	1.6	N/A
		(±)-Flavanone 6- <i>O</i> -β-D-4- <i>O</i> -methylglucopyranoside (16)	0.8	N/A

N/A: No noticeable activity.



	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	C-2,3 dihydro
1	OH	H	OH	H	H	H	-
2	H	H	OH	H	H	H	-
3	H	OH	H	H	H	H	-
4	OH	H	OH	H	OH	H	-
5	glcMe	H	OH	H	H	H	-
6	OSO ₃ H	H	OH	H	H	H	-
7	OH	H	OH	OMe	OH	H	-
8	OH	H	OH	OH	OH	H	-
9	OH	H	OH	OMe	deoxyallose	H	-
10	OH	H	OH	H	deoxyallose	H	-
11	OH	H	OH	OSO ₃ H	OH	H	-
12	H	H	OH	H	OH	H	-
13	H	H	glcMe	H	OH	H	-
14	H	OH	H	H	H	H	C-2,3 dihydro
15	H	H	H	H	H	glcMe	-
16	H	glcMe	H	H	H	H	C-2,3 dihydro



trum, as well as the ¹³C-NMR data were similar to those of chrysin. These indicated that the chrysin architecture remained largely unchanged during transformation. The ¹³C-NMR spectrum additionally showed five carbon resonances (δ 60–80) in the aliphatic region indicative of the presence of a glycosyl moiety. The anomeric proton at δ 5.11 showed a one bond correlation (HMQC) with the methine carbon at δ 99.5 and three bond correlations (HMBC) with the quaternary carbon at δ 163.1 (C-7), which in turn exhibited two-

bond correlations to H-6 (6.47) and H-8 (6.87) suggesting *O*-glucosylation at C-7. A *trans*-diaxial relationship was inferred by the large coupling constant (8.4 Hz) between the anomeric proton and H-2'' establishing a β-glycosidic linkage. *trans*-Diaxial relationships were suggested between H-2''/3'', H-3''/4'', H-4''/5'' protons, as large coupling constants, (*J*_{2''/3''}, *J*_{3''/4''}, *J*_{4''/5''}) were observed between the respective pairs of protons. The downfield shift of C-4'' (δ 78.8) in the ¹³C-NMR spectrum together with HMBC correlations showed the presence of an *O*-methyl group at C-4''. Compound **5** was thus identified as chrysin 7-*O*-β-D-4-*O*-methylglucopyranoside.

Compound **10** (93.6 mg, 18.7% yield), was assigned the molecular formula, C₂₂H₂₂O₉, (HR-ESI-MS). The ¹H-NMR data of **10** were similar to those of **1**, except for the B-ring protons which showed *para*-substitution [δ 8.00 (2H, d, *J*=8.0 Hz), 7.18 (2H, d, *J*=8.0 Hz)]. The additional five carbon resonances (δ 60–80) observed in the aliphatic region of the ¹³C-NMR spectrum indicated the presences of a glycosyl moiety. Correlations observed in the HMBC spectrum permitted the assignment of the glycosyl group at C-4''. Identification of the sugar moiety was by the analysis of ¹H-, ¹³C-NMR and their correlation spectra. The anomeric proton at δ 5.55 was coupled (HMQC) to the methine carbon at δ 98.4 and to the proton at δ 3.78 (H-2'') with a small coupling constant (*J*<1 Hz). This suggested that the anomeric proton was equatorially disposed. H-2'' was coupled to H-3'' with a small coupling constant which in turn was similarly coupled to H-4''. Therefore, H-2'' and H-3'' were required to be equatorial. The large coupling constant (7.2 Hz) between H-4'' and H-5'' suggested that they were *trans*-diaxial oriented. H-5'' was coupled to the methyl doublet implying that the sugar was a 6-deoxy sugar. On acid hydrolysis, **10** afforded deoxyallose whose structure was supported by the ¹H-NMR coupling pattern and optical rotation^{24,25} and hence, the sugar was identified as D-6-deoxyallose. The chemical shift of the anomeric carbon (δ 98.5) and the coupling constant of the anomeric proton suggested the presence of an α-*O*-glycosidic bond. The spectroscopic data of the aglycon formed were similar in all respects to those of 4'-hydroxychrysin (**4**). The structure of compound **10** was determined as chrysin 4'-*O*-α-D-6-deoxyallopypyranoside.

Metabolite **9** (16.0 mg, 3.2% yield) was obtained as a white solid with a molecular formula, $C_{22}H_{22}O_{10}$ as determined by HR-ESI-MS. Comparison of this compound with metabolite **10** revealed significant similarity except for the presence of an *O*-methyl group, the position of which was determined as C-3' by correlation NMR spectra. Compound **9** was therefore identified as 3'-methoxychrysin 4'-*O*- α -D-6-deoxyallopypyranoside.

Compound **13** (490 mg, 98% yield) the second metabolic product of **2** had a molecular formula, $C_{22}H_{22}O_9$ (HR-ESI-MS data). Very similar chemical shifts and *J*-values of aromatic protons together with similar ^{13}C -NMR data, suggested that this compound and metabolite **12** had the same flavonoid framework. However, the presence of resonances between δ 3.05 and 5.05 in the 1H -NMR and five carbon resonances between δ 100.2 and 60.9 in the ^{13}C -NMR spectra revealed the presence of a glycosyl moiety. The coupling constant of the anomeric proton at δ 5.05 (1H, d, $J=7.8$ Hz, H-1'') was consistent with a β -configuration of the glycosyl unit. The three bond correlation of the anomeric proton with the quaternary carbon at δ 160.5 (C-5) together with its three-bond correlation to H-7 (δ 7.64) suggested *O*-glucosylation at C-5. The large coupling constants observed between H-2''/3'', H-3''/4'', H-4''/5'' protons showed *trans*-diaxial relationships. HMBC correlations and the downfield shift of C-4'' (δ 79.6) in the ^{13}C -NMR spectrum revealed the presence of an *O*-methyl group at C-4''. Metabolite **13** was thus, characterized as 4'-hydroxy-5-*O*- β -D-4-*O*-methylglucopyranoside.

Metabolite **16** (4 mg, 0.8% yield) of **3** with a molecular mass of 416 was assigned a molecular formula $C_{22}H_{24}O_8$ (HR-ESI-MS data). Its spectroscopic data were in close agreement with those of metabolite **14** with the exception of additional NMR resonances due to an *O*-methylated glucopyranoside unit which was indicated by the excess of 176 mass units. 1H -NMR coupling constants together with NMR correlation data confirmed the structure of **16** as ($\pm$)-flavanone 6-*O*- β -D-4-*O*-methylglucopyranoside, again indicating a non-stereoselective hydrogenation process.

The DPPH assay conducted on the metabolites showed that only 3',4'-dihydroxychrysin (luteolin) (**8**) had significant antioxidant activity as compared to the literature value.²⁶⁾ All the other metabolites showed no noticeable activity.

Conclusion

In this study, *Beauveria bassiana* (ATCC 13144) transformed 6-hydroxyflavone (**3**) to 6-hydroxyflavanone (**14**), flavone 3-*O*- β -D-4-*O*-methylglucopyranoside (**15**), and ($\pm$)-flavanone 6-*O*- β -D-4-*O*-methylglucopyranoside (**16**). The starting compound (**3**) was reduced during functionalization (Phase I) to yield compound **14** followed by conjugation in Phase II conversion to yield metabolite **16**. It was also susceptible to several conversion steps, namely, deoxygenation, oxygenation and conjugation to form metabolite **15**. In contrast, 5-hydroxyflavanone (**2**) and chrysin (**3**) did not undergo reduction but yielded the oxygenated products during the functionalization phase. Similar results were obtained with our previous microbial conversion of the flavonoids 3-hydroxyflavone and 7-hydroxyflavone.²³⁾ However, conjugation seems to be the preferred reaction in most of these conversions. Oxygenation and conjugation products obtained with compounds, **1**–**3** together with many other similar microbial

metabolites of flavonoids reported, are paralleled in mammals^{27–29)} indicating the use of microbes to mimic mammalian metabolism. Conjugation is the most common final step in mammalian metabolism of intact flavonoids.³⁰⁾

Flavonoids are associated with many biological properties, among which the antioxidant activity is well covered. Most of the results have been based on *in vitro* experiments. The antioxidant nature of these compounds has been defined by the ability to donate electrons to free radicals, due to the presence of a catechol group in the B-ring.³¹⁾ This feature is considered to be highly important in addition to the presence of a double bond between C-2 and C-3 in conjugation with the 4-oxo-group, and the ability to chelate ions like copper and iron.³¹⁾ The flavonoid, quercetin meets all the criteria to be a good antioxidant *in vitro*. It is reflected in experiments such as the DPPH assay which indicated a 2.2 μM IC₅₀ value.³²⁾ Examination of the structures of the metabolites of compounds **1**–**3**, which have been *O*-methylated, *O*-glucosylated or sulfated, showed that their effectiveness as antioxidants, compared to the starting compounds (**1**–**3**), should decrease due to their reduced ability to donate electrons. Although the metabolite 3',4'-dihydroxychrysin (luteolin) (**8**) has the necessary structural features to show the ability to donate electrons,³²⁾ the substantial formation of conjugated products implied its rapid inactivation. This was supported by the DPPH assay conducted on the metabolites which showed significant antioxidant activity only by 3',4'-dihydroxychrysin (luteolin) (**8**).²⁶⁾ These results tend to support the view that although flavonoids exhibit powerful antioxidant properties *in vitro*, such an activity has yet to be clarified *in vivo*.³³⁾ Thus, as has been pointed out,¹¹⁾ it is essential to establish how flavonoids are absorbed in the intestine, their bioavailability, and the properties of the circulating metabolites to consider them as effective antioxidants. Further, the formation of conjugates facilitates rapid elimination of flavonoids, resulting in the observed lower concentrations of the parent compounds and their metabolites in the plasma and in organs as compared to antioxidants like ascorbic acid and α -tocopherol which are present in high concentrations. Hence, in competition, the former is expected to play an insignificant role as far as antioxidant activity is concerned.²⁹⁾ However, in the light of new data it has been pointed out that even though flavonoids and their *in vivo* metabolites may not act as antioxidants, they are present in sufficient amounts *in vivo* to have pharmacological activity at receptors, transcription factors and enzymes.²⁹⁾ The formation of conjugated products may be viewed as a safety mechanism which prevents the accumulation of flavonoids to levels harmful to the body¹⁵⁾ by aiding in rapid elimination and retaining the amounts necessary to have beneficial effects.

Experimental

General Experimental Procedures IR spectra were measured in $CHCl_3$ on an ATI Mattson Genesis series FT-IR spectrophotometer. UV spectra were run on a Hewlett Packard 8452A diode array spectrometer. Optical rotations were measured with a Jasco DIP-370 digital polarimeter. 1H - and ^{13}C -NMR were obtained in $CDCl_3$ and $DMSO-d_6$ on a Varian Unity Inova 600 spectrometer unless otherwise stated. HR-ESI-MS data were acquired using a Bruker GioApex 3.0.

Substrates Chrysin (**1**), 5-hydroxyflavone (**2**) and 6-hydroxyflavone (**3**) were purchased from Aldrich Co. (Milwaukee, Wisconsin) and their authenticity was confirmed by NMR data.

Organisms and Metabolism Forty culture samples from the microbial

collection of The National Center for Natural Products Research of The University of Mississippi were used in the initial screening stage of the experiment to identify organisms capable of converting the flavonoids, 1—3 to their respective metabolites in good yield. Initial screening was carried out in 125 ml Erlenmeyer flasks containing 25 ml medium α .¹⁸ A two-stage procedure was used in all experiments.¹⁸ Each compound was administered separately in dimethylformamide (0.5 mg/ml) solution to 24 h old stage II cultures and incubated for 14 d on a rotary shaker (New Brunswick Model G10-21) at 100 rpm. The progress of each reaction was monitored at 7-d intervals using precoated Si gel 60 F₂₅₄ TLC plates (E. Merck) with *p*-anisaldehyde as the spray reagent. Preparative scale fermentations were carried out in five 21 flasks, each containing 100 mg of substrate in 500 ml medium α . EtOAc or EtOAc/(Me)₂CHOH–EtOAc (2 : 9) was used to extract the combined culture filtrates. The residues obtained by the evaporation of solvents were column chromatographed (Silica gel 60 F₂₅₄) to isolate the metabolites. Purification of the compounds was by repeated column and preparative thin layer (Silica gel 60 F₂₅₄) chromatography, where appropriate. Culture and substrate controls were run along with the above experiments.¹⁸

Microbial Transformation of Chrysin (1) by *A. alliaceous* (ATCC 10060) The combined (Me)₂CHOH–EtOAc (2 : 9) extract of the culture filtrates when flash chromatographed with CHCl₃ enriched with MeOH, afforded metabolite 4. The structure was elucidated by spectroscopic data.

4'-Hydroxychrysin (4) was obtained as a pale yellow solid (20.6 mg, 4.1% yield). *R*_f 0.38 [MeOH–CHCl₃ (1 : 9)]. It was identified by comparison with literature data.^{19,20}

Microbial Transformation of Chrysin (1) by *B. bassiana* (ATCC 13144) The methanol soluble fraction of the combined (Me)₂CHOH–EtOAc (2 : 9) extract of the culture filtrates gave compound 5 on recrystallization. The structure was determined by spectroscopic data.

5-Hydroxyflavone 7-*O*- β -D-4-*O*-methylglucopyranoside (5) was a pale yellow solid (221.0 mg, 44.2% yield) with a *R*_f 0.34 [MeOH–CH₂Cl₂ (3 : 1)]; [α]_D²³ –13.9° (*c*=0.11, MeOH).

Microbial Transformation of Chrysin (1) by *A. glauco* (ATCC 22750) Flash chromatographic separation of the combined (Me)₂CHOH–EtOAc (2 : 9) extract of the culture media resulted in the isolation of the metabolite 6. CHCl₃ enriched with MeOH was used as the eluent.

Chrysin 7-sulphate (6) was isolated as a pale yellow solid (28.7 mg, 5.74% yield). *R*_f 0.24 [MeOH–CHCl₃ (3 : 17)]. The compound was identified by comparison with published data.²¹

Microbial Transformation of Chrysin (1) by *M. ramannianus* (ATCC 9628) Compounds 4 (14.8 mg, 3.0% yield) and 7—9 were isolated by Flash Chromatography of the combined (Me)₂CHOH–EtOAc (2 : 9) extracts of the culture media using CHCl₃ enriched with MeOH, followed by repeated column chromatography. The structures were elucidated by spectroscopic data.

4'-Hydroxy-3'-methoxychrysin (7) (6.9 mg, 1.4% yield), a white solid with a *R*_f 0.53 [MeOH–CHCl₃ (1 : 9)]. The metabolite was identified by comparison with published data.^{19,20}

4'-Hydroxychrysin (4), isolated as a pale yellow solid (14.8 mg, 3.0% yield); *R*_f 0.38 [MeOH–CHCl₃ (1 : 9)] was identified with the aid of literature data.^{19,20}

3',4'-Dihydroxychrysin (8) (51.0 mg, 10.2% yield), a white solid with an *R*_f 0.24 [MeOH–CHCl₃ (1 : 9)] was identified by comparison with literature data.^{19,20}

3'-Methoxychrysin 4'-*O*- α -D-6-deoxyallopypyranoside (9) (16.0 mg, 3.2% yield), was obtained as a white solid. *R*_f 0.14 [MeOH–CHCl₃ (1 : 9)]; [α]_D²³ –45.5° (*c*=0.08, MeOH). Spectroscopic analysis confirmed the structure of 9.

Chrysin 4'-*O*- α -D-6-deoxyallopypyranoside (10) (93.6 mg, 18.7% yield), with a *R*_f 0.12 [MeOH–CHCl₃ (1 : 9)] was obtained as a white solid. [α]_D²³ –76.3° (*c*=0.12, MeOH). Identification was by means of spectroscopic data.

Acid Hydrolysis of Chrysin 4'-*O*- α -D-6-Deoxyallopypyranoside (10): A solution of 10 (8 mg) in 1.2 ml 2 N HCl–MeOH was refluxed for 4 h. On cooling an off-white precipitate was formed which was identified as 4'-hydroxychrysin (4) by means of spectroscopic data. The supernatant was purified by preparative layer chromatography (CH₂Cl₂–MeOH, 9 : 1) to give a white compound which was identified by spectroscopic data as D-6-deoxyallose sugar. *R*_f 0.37 [MeOH–CHCl₃ (1 : 9)]; [α]_D²⁷ –3.9° (*c*=0.25, MeOH).^{24,25}

Luteolin 3'-sulfate (11) (51.0 mg, 10.2% yield), a white solid with an *R*_f 0.26 [MeOH–CHCl₃ (1 : 3)] was identified by a detailed study of its spectroscopic data.^{19,20}

Microbial Transformation of 5-Hydroxyflavone (2) by *A. alliaceous*

(ATCC 10060) The combined (Me)₂CHOH–EtOAc (2 : 9) extracts of the culture filtrates were flash chromatographed with hexane enriched with EtOAc, to give the metabolite 12.

5,4'-Dihydroxyflavone (12) was isolated as a light yellow solid (2.3 mg, 0.46% yield). *R*_f 0.28 [EtOAc–hexane (3 : 7)]. It was identified by comparison with published data.^{19,20}

Microbial Transformation of 5-Hydroxyflavone (2) by *B. bassiana* (ATCC 13144) Recrystallization of the MeOH soluble portion of the combined (Me)₂CHOH–EtOAc (2 : 9) extracts yielded 13.

4'-Hydroxyflavone 5-*O*- β -D-4-*O*-methylglucopyranoside (13) was isolated as a white solid (490 mg, 98% yield). *R*_f 0.34 [MeOH–CHCl₃ (3 : 17)]; [α]_D²³ –49.4° (*c*=0.08, MeOH). Spectroscopic analysis confirmed the structure of 11.

Microbial Transformation of 6-Hydroxyflavone (3) by *B. bassiana* (ATCC 13144) The brown solid obtained from the EtOAc extract of the combined culture filtrates was subjected to silica gel column chromatography (Si gel 230–400 mesh: E. Merck, 30 g, column diameter: 20 mm) with CH₂Cl₂ and increasing amounts of MeOH. The fractions obtained were purified by repeated column and preparative layer chromatography (CH₂Cl₂–MeOH, 3 : 2) to obtain three compounds, 14—16 which were characterized by spectroscopic data.

6-Hydroxyflavanone (14) was a white solid (41.4 mg, 8.3% yield). *R*_f 0.52 [MeOH–CH₂Cl₂ (1 : 19)]; [α]_D²⁷ 0° (*c*=0.2, MeOH). Identification of the metabolite was by comparison with published data.²²

Flavone 3-*O*- β -D-4-*O*-methylglucopyranoside (15) was separated as a white solid (8 mg, 1.6% yield). *R*_f 0.18 [MeOH–CHCl₃ (1 : 19)]. It was identified by comparing the spectral data with of the literature values.²³

($\pm$)-Flavanone 6-*O*- β -D-4-*O*-methylglucopyranoside (16) was purified as a white solid (4 mg, 0.8% yield). *R*_f 0.18 [MeOH–CH₂Cl₂ (1 : 19)]; [α]_D²⁷ 0° (*c*=0.2, MeOH). Identification was by detailed study of spectroscopic data.

The free radical scavenging activity of the metabolites was analyzed by the DPPH assay as described by S. Burda *et al.*³⁴) 1 ml of 10^{–4} M solution in methanol of each metabolite was added to 2 ml of DPPH solution (10 mg/l in MeOH), vortexed and kept for 10 min in the dark at room temperature. Absorbance of each solution was measured at 517 nm and the scavenging percentage of activity was calculated. Quercetin was used as a positive control.²⁶) Only 3',4'-dihydroxychrysin (luteolin) (8) showed significant antioxidant activity as compared to the literature value (IC₅₀ (μ M) 11.0).²⁶) All other metabolites showed no noticeable activity.

Supplementary Material Spectroscopic data of metabolites 4—16 are available as supplementary material.

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