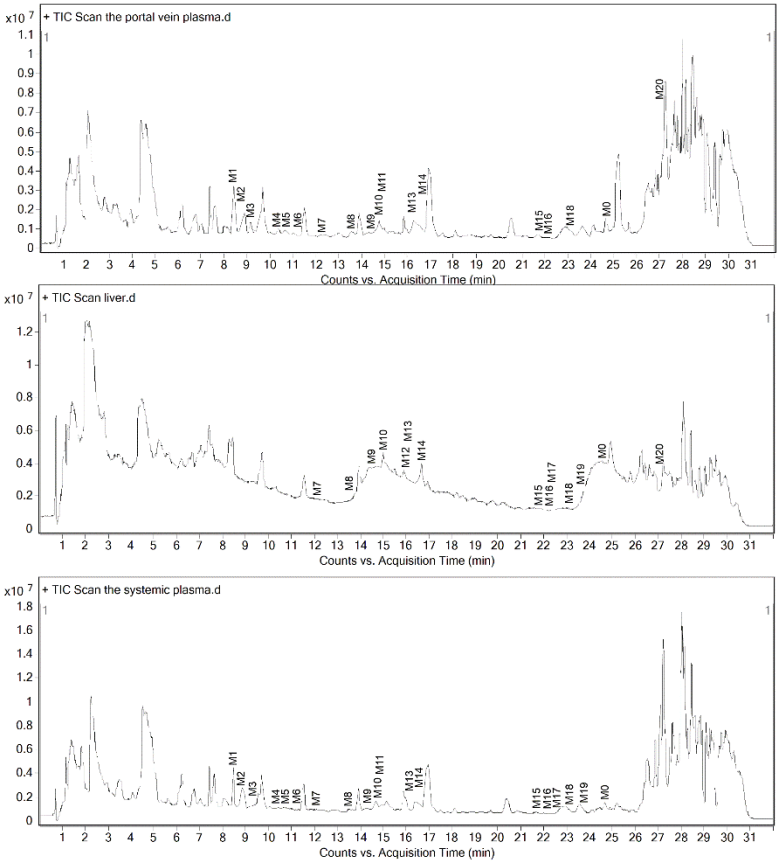

Figure S1. Total ion chromatograms of portal vein plasma (a), the liver (b), and systemic plasma (c) of rats after the oral administration of calycosin.

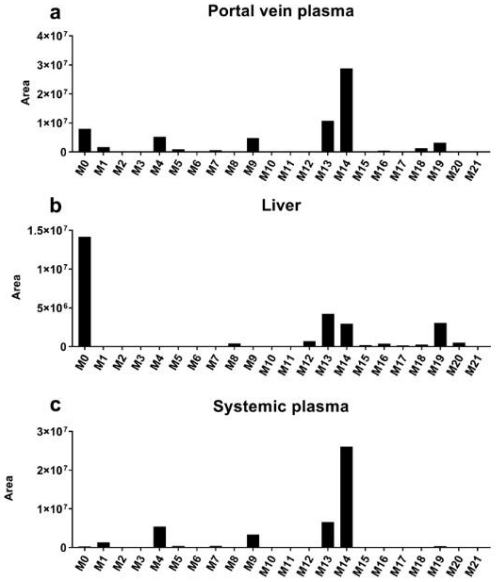

Figure S2. The peak areas of calycosin and its metabolites in portal vein plasma (a), the liver (b) and systemic plasma (c) after the oral administration of 76.4 mg/kg calycosin as detected by HPLC-Q-TOF.

1 Reductive metabolites of calycosin (M0): M7, M11, and M16

The protonated molecule at *m/z* 287.0905 ($C_{16}H_{15}O_5^+$, -3.13 ppm) given by M16 at 22.19 min was 2 Da higher than that of calycosin. Previous studies revealed that isoflavanones, the structurally saturated derivatives in the C2–C3 bond of isoflavones, possess fragmented pathways that differ from isoflavones because of their less stable C-ring. The base peaks of isoflavanones typically corresponded to $[M+H-B_{ring}]^+$, and the RDA reaction of isoflavanones was prone to occur in the C2–C3 and C3–C4 bonds. For M16, the fragment ion at *m/z* 163.0386 ($C_9H_7O_3^+$) was generated by the elimination of the B-ring as the base peak, and the fragment ion at *m/z* 137.0589 ($C_8H_9O_2^+$) was produced via the RDA reaction through the breakup of C2–C3 and C3–C4 bonds. Therefore, it was reasonably deduced that M16 was 7,3'-dihydroxy-4'-methoxyisoflavanone.

M11 exhibited $[M+H]^+$ at *m/z* 463.1224 ($C_{22}H_{23}O_{11}^+$, -2.38 ppm) at 14.67 min. After eliminating a $C_6H_8O_6$ (176 Da) unit from the precursor ion, the fragment ion at *m/z* 287.0914 ($C_{16}H_{15}O_5^+$) was presented as the base peak. The fragment ion at *m/z* 287.0914 ($C_{16}H_{15}O_5^+$) underwent two classical fragmentations of isoflavanones, thereby yielding the product ions at *m/z* 163.0386 ($C_9H_7O_3^+$) and 137.0588 ($C_8H_9O_2^+$), which corresponded to those of M16. Therefore, M11 was tentatively identified as the glucuronidated metabolite of M16 and named 2,3-dihydrocalycosin-glucuronide.

M7, which has a formula with one less CH_2 unit than M11, elicited the protonated molecule at *m/z* 449.1076 ($C_{21}H_{21}O_{11}^+$, −0.45 ppm) at 12.04 min. Similar to that of M11, the fragment ion at *m/z* 273.0752 ($C_{15}H_{13}O_5^+$) appeared as the base peak through

eliminating a $C_6H_8O_6$ (176 Da) group from the precursor ion. Subsequently, the fragment ion at *m/z* 163.0388 ($C_9H_7O_3^+$) was derived from the cleavage of the B-ring, and the fragment ion at *m/z* 123.0448 ($C_7H_7O_2^+$) was 14 Da (CH_2) less than that of M11 at m/z 137.0589 ($C_8H_9O_2^+$) through RDA fragmentation. Thus, M7 was tentatively identified as the C4′ demethylated product of M11 and named 7,3',4'-trihydroxyisoflavanone-glucuronide.

2 The phase II metabolites of calycosin (M0): M1, M4, M5, M8, M13, M14, M17, M18, and M20

The precursor ion of M13 at *m/z* 365.0323 ($C_{16}H_{13}O_8S^+$, −0.82 ppm) was observed at 16.14 min. After a SO_3 (80 Da) unit was neutrally eliminated from the precursor ion, the fragment ion at *m/z* 285.0755 ($C_{16}H_{13}O_5^+$) was detected as the base peak. Moreover, the product ions at *m/z* 270.0518 ($C_{15}H_{10}O_5^+$), 253.0488 ($C_{15}H_9O_4^+$), 225.0542 ($C_{14}H_9O_3^+$), and 137.0228 ($C_7H_5O_3^+$) were consistent with those of calycosin. Considering that the C3′-OH of calycosin is more easily sulfated than C7-OH, we tentatively determined M13 as calycosin-3′ -sulfate.

M4 displayed a quasi-molecular ion at *m/z* 444.9891 ($C_{16}H_{13}O_{11}S_2^+$; −0.67 ppm) at 10.28 min. The precursor ion of M4 underwent a continuous loss of SO_3 (80 Da) units, thereby generating the fragment ions at *m/z* 365.0322 ($C_{16}H_{12}O_8S^+$) and 285.0756 ($C_{16}H_{13}O_5^+$). The product ions at *m/z* 270.0524 ($C_{15}H_{10}O_5^+$), 253.0499 ($C_{15}H_9O_4^+$), and 137.0233 ($C_7H_5O_3^+$) were the same as those of calycosin. Thus, M4 was tentatively identified as calycosin-7,3′ -di-sulfate.

M5 and M14 shared the same formula of $C_{22}H_{20}O_{11}$ with the precursor ions at *m/z*

461.1077 (−0.22 ppm) and 461.1069 (−1.95 ppm) at 10.77 and 16.76 min, respectively. After losing a $C_6H_8O_6$ unit (176 Da), the fragment ions at *m/z* 285.0752/285.0753 ($C_{16}H_{13}O_5^+$) were observed in the MS/MS spectra of M5 and M14. Their following fragment ions at *m/z* 270.0516/270.0515 ($C_{15}H_{10}O_5^+$), 253.0491/253.0489 ($C_{15}H_9O_4^+$), and 137.0228/137.0226 ($C_7H_5O_3^+$) were consistent with those of calycosin. Considering that the polarity of calycosin- 3′ -glucuronide is poorer than that of calycosin-7-glucuronide, we respectively identified M5 and M14 as calycosin-7-glucuronide and calycosin-3′ -glucuronide.

M1 and M8 showed comparable protonated molecule at *m/z* 541.0643 and 541.0644 at 8.55 and 13.79 min, respectively, and their formulas contained one more $C_6H_8O_6$ (176 Da) and SO_3 (80 Da) moieties than that of calycosin. After the $C_6H_8O_6$ and SO_3 groups were removed consecutively, the fragment ions at *m/z* 365.0324 ($C_{16}H_{13}O_8S^+$) and 285.0752/ 285.0756 ($C_{16}H_{13}O_5^+$) were observed. Thus, M1 and M8 were regarded as glucuronidated and sulfated calycosin. ClogP values are utilized to differentiate the polarity of the compounds and estimate the retention time of the isomers in a reversed phase column. Using ChemBioDraw Ultra14.0 (PerkinElmer Inc., MA, USA) for calculation, we obtained the ClogP values of −1.96 and −1.61 for calycosin-7-sulfate-3′-glucuronide and calycosin-7-glucuronide-3′-sulfate, respectively. M1 and M8 were tentatively identified as calycosin-7-sulfate-3′-glucuronide and calycosin-7-glucuronide-3′-sulfate, based on their retention times at 8.55 and 13.79 min, respectively.

The quasi-molecular ion of M20 at *m/z* 299.0914 (0.00 ppm) showed the elemental

composition of $C_{17}H_{14}O_5$, which contained one more CH_2 unit than calycosin. The retention time of M20 at 27.20 min was slightly longer than that of calycosin (M0) at 24.93 min. After a C_2H_4 (28 Da) unit was eliminated, the fragment ion at *m/z* 271.0604 ($C_{15}H_{11}O_5^+$) was generated as the base peak, indicating that two methoxyl groups were vicinal to each other. The product ions at *m/z* 253.0488 ($C_{15}H_9O_4^+$) and 137.0236 ($C_7H_5O_3^+$) were consistent with those of calycosin. Thus, M20 was partially identified as calycosin-3′ -methyl ether.

The quasi-molecular ions of M17 at *m/z* 379.0481 ($C_{17}H_{15}O_8S^+$, −0.26 ppm) and M18 at *m/z* 475.1230 ($C_{23}H_{23}O_{11}^+$, -1.05 ppm) were detected at 22.59 and 23.08 min, respectively. After SO_3 (80 Da) and $C_6H_8O_6$ (176 Da) units were eliminated, the fragment ions at *m/z* 299.0907/299.0914 ($C_{17}H_{15}O_5^+$) were identical to the molecular ion of M20. Other ions at *m/z* 271.0606/271.0592 ($C_{15}H_{11}O_5^+$), 253.0495/253.0488 ($C_{15}H_9O_4^+$), and 225.0553/225.0548 ($C_{14}H_9O_3^+$) were also overlapped with those of M20. Thus, M17 and M18 were tentatively regarded as calycosin-7-sulfate-3′-methyl ether and calycosin-7-glucuronide-3′-methyl ether, respectively.

3 Phase II metabolites of 7,3′,4′-trihydroxylisoflavone (M12): M2, M6, M9, M10, and M19

M10 was detected at 14.63 min with a precursor ion at *m/z* 351.0161 ($C_{15}H_{11}O_8S^+$, −2.28 ppm). When a SO_3 unit (80 Da) was eliminated, the fragment ion at *m/z* 271.0590 ($C_{15}H_{11}O_5^+$), identical to the protonated molecule ion of M12, was detected as the base peak. The product ions at *m/z* 225.0542 ($C_{14}H_9O_3^+$) and 137.0237 ($C_7H_5O_3^+$) were also consistent with those of M12. Thus, M10 was tentatively identified as 7,3′,4′-

trihydroxyisoflavone-sulfate.

M2 exhibited a protonated molecule at *m/z* 430.9738 ($C_{15}H_{11}O_{11}S_2^+$, 0.23 ppm) at 8.83 min. When two SO_3 (80 Da) moieties were lost, the product ions at *m/z* 351.0175 ($C_{15}H_{11}O_8S^+$) and 271.0601 ($C_{15}H_{11}O_5^+$) were observed in the MS/MS spectra, where the fragment ion at *m/z* 271.0601 ($C_{15}H_{11}O_5^+$) was detected as the base peak, and a typical RDA fragment ion at *m/z* 137.0238 ($C_7H_5O_3^+$) was found. Therefore, M2 was tentatively identified as 7,3′,4′-trihydroxyisoflavone-di-sulfate.

M6 and M9, a pair of isomers, showed protonated molecule at *m/z* 447.0920 ($C_{21}H_{19}O_{11}^+$, -0.45 ppm) and 447.0921 ($C_{21}H_{19}O_{11}^+$, -0.22 ppm) with similar fragmented patterns at 11.31 and 14.38 min, respectively. After eliminating a $C_6H_8O_6$ (176 Da) moiety, the fragment ions at *m/z* 271.0594/271.0593 ($C_{15}H_{11}O_5^+$) were identical to the precursor ion of M12 and detected as the base peak. The product ions at *m/z* 253.0492/253.0494 ($C_{15}H_9O_4^+$), 225.0538/225.0540 ($C_{14}H_9O_3^+$), and 137.0236/137.0223 ($C_7H_5O_3^+$) were also overlapped with those of M12. Therefore, M6 and M9 were tentatively determined as 7,3′,4′-trihydroxyisoflavone-glucuronide.

M19, which contained one more CH_2 (14 Da) unit than M12, displayed a $[M+H]^+$ at *m/z* 285.0755 ($C_{16}H_{13}O_5^+$, -0.70 ppm) at 23.71 min. The precursor ion of M19 yielded a series of product ions at *m/z* 253.0487 ($C_{15}H_9O_4^+$), 225.0539 ($C_{14}H_9O_3^+$), and 137.0229 ($C_7H_5O_3^+$), consistent with those of M12. The presence of the product ion at *m/z* 137.0229 ($C_7H_5O_3^+$) hindered the methylation from occurring at the C7 position of M12. If a CH_2 unit was added to the C4′ position of M12, the chemical structure of M19 would become the same as that of calycosin. Thus, M19 was tentatively determined as

the 7,3′,4′ -trihydroxyisoflavone-3′ -methyl ether.

4 Phase II metabolite of daidzein (M15): M3

M3 displayed a quasi-molecular ion at *m/z* 431.0972 ($C_{21}H_{19}O_{10}^{+}$, −0.23 ppm) at 9.30 min. The characteristic fragment ion at *m/z* 255.0650 ($C_{15}H_{11}O_{4}^{+}$) was derived from the precursor ion through the elimination of a $C_6H_8O_6$ moiety (176 Da). The fragment ions at *m/z* 199.0745 ($C_{13}H_{11}O_{2}^{+}$) and 137.0226 ($C_7H_5O_3^{+}$) agreed with those of M15. A previous study revealed that the glucuronide group is preferably conjugated at the C7 position of M15. Thus, M3 was tentatively identified as daidzein-7-glucuronide.