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Yli-Öyrä, J, Juvonen, R, Lehtonen, M, Herrala, M, Finel, M, Räisänen, R & Rysä, J 2024, 'Anthraquinone biocolourant dermocybin is metabolized whereas dermorubin is not in in vitro liver fractions and recombinant metabolic enzymes', Basic & Clinical Pharmacology & Toxicology, vol. 134, no. 6, pp. 846-857. https://doi.org/10.1111/bcpt.14013

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Received: 25 October 2023 | Revised: 29 February 2024 | Accepted: 8 April 2024

DOI: 10.1111/bcpt.14013

ORIGINAL ARTICLE

BCPT Basic & Clinical Pharmacology & Toxicology

Anthraquinone biocolourant dermocybin is metabolized whereas dermorubin is not in in vitro liver fractions and recombinant metabolic enzymes

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Funding information
Research Council of Finland, Grant/Award Number: 346744; Strategic Research Council at Research Council of Finland, Grant/Award Numbers: 327178, 327194, 352460, 352466

Abstract

Fungal anthraquinones dermocybin and dermorubin are attractive alternatives for synthetic dyes but their metabolism is largely unknown. We conducted a qualitative in vitro study to identify their metabolism using human liver microsomes and cytosol, as well as recombinant human cytochrome P450 (CYP), UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) enzymes. Additionally, liver microsomal and cytosolic fractions from rat, mouse and pig were used. Following incubations of the biocolourants with the enzymes in the presence of nicotinamide adenine dinucleotide phosphate, UDP-glucuronic acid, 3′-phosphoadenosine-5′-phosphosulfate (PAPS) or S-adenosyl methionine (SAM) to enable CYP oxidation, glucuronidation, sulfonation or methylation, we observed several oxidation and conjugation metabolites for dermocybin but none for dermorubin. Human CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A7 catalysed dermocybin oxidation. The formation of dermocybin glucuronides was catalysed by human UGT1A1, 1A3, 1A7, 1A8, 1A9, 1A10 and 2B15. Human SULT1B1, 1C2 and 2A1 sulfonated dermocybin. Dermocybin oxidation was faster than conjugation in human liver microsomes. Species differences were seen in dermocybin glucuronidation between human, rat, mouse and pig. In conclusion, many CYP and conjugation enzymes metabolized dermocybin, whereas dermorubin was not metabolized in human liver fractions in vitro. The results indicate that dermocybin would be metabolized in humans in vivo.

KEYWORDS
anthraquinone, biotransformation, *Cortinarius sanguineus*, fungi, LC-MS

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1 | INTRODUCTION

Fungal anthraquinones are promising bio-based alternatives for synthetic dyes that can cause a variety of toxic effects both to humans and to the environment.[1,2] Biocolourants attract industrial interest due to their potential as textile dyes and components in biodegradable packaging.[3] The fruiting bodies of bloodred webcap (*Cortinarius sanguineus*) have been shown to contain at least 15 anthraquinone dyes, comprising approximately 6% of its dry weight.[4] The most commonly present are emodin, dermocybin and dermorubin (Figure 1) in respective dry-weight proportions of 63%, 31% and 4%. Recently, it has been shown that they work well in waterless CO_2 dyeing of polyester[5] and in the dyeing of cellulosic fibres.[6]

In textiles and food packaging, the dyes could get in touch with skin and gastrointestinal tract, and, therefore, studying their innate toxicity and skin sensitizing properties is warranted. According to our previous studies, the toxicity of dermocybin and dermorubin in vitro is low,[5] and they do not possess skin sensitizing or mutagenic properties, unlike emodin, which is known to be mutagenic after metabolic activation.[7] Glucuronidation was determined its main metabolic pathway in an in vivo study conducted with rats and UDP-glucuronosyltransferase (UGT) 1A9 as the main metabolizing enzyme.[8] Additionally, UGT2B subfamily was able to glucuronidate its 3-OH group (Figure 1). In an in vitro study conducted with human and rat liver and intestinal microsomes, three emodin glucuronides were formed, out of which emodin-3-O-β-D-glucuronide comprised more than 98%.[9] The metabolism of other anthraquinones of *C. sanguineus* are unknown. Given that emodin becomes mutagenic after metabolic activation, information of other anthraquinones should also be obtained to assess their overall toxicity.

Dermorubin and dermocybin are attractive sustainable alternatives for toxic synthetic dyes, due to their low toxicity in in vitro studies.[5] When assessing the safety of dyes or other chemicals, data on their kinetics and especially metabolism are essential, since it is known that metabolism can alter the overall toxicity of chemical substances, including biocolourants. In this study, we set out to identify in vitro metabolites of the previously unstudied *C. sanguineus* dyes dermocybin and dermorubin in liver microsomal and cytosolic fractions, as well as human recombinant cytochrome P450 (CYP), UGT, sulfotransferase (SULT) and catechol-O-methyl transferase (COMT) enzymes. Additionally, we studied possible inter-species differences in the human, rat, mouse and pig liver microsomes and cytosol.

2 | MATERIAL AND METHODS

2.1 | Dyes

Dermorubin (98.08%) and dermocybin (99.74%) were extracted from the *C. sanguineus* fungal bodies. The anthraquinones were separated using multiple liquid–liquid partition as described before[10,11] and dissolved into sterile dimethyl sulfoxide (DMSO) in 1 mM concentration. The purity and chemical structures of the separated compounds were confirmed by high-performance liquid chromatography coupled with diode-array detection and electrospray ionization tandem mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.[5]

2.2 | Human and animal cytosolic and microsomal fractions

Human cytosolic and microsomal fractions from a mixed-gender pool of 50 donors were purchased from Xenotech (Kansas City, KS, USA). Cytosolic fraction contains the enzymes for reduction, methylation and sulfonation. Microsomes contain enzymes that catalyse glucuronidation, oxidation and reduction.

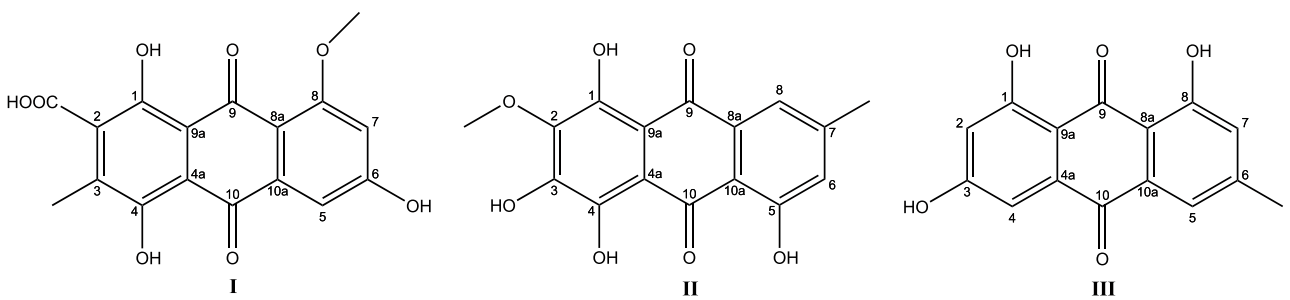


FIGURE 1 The chemical structures of (**I**) dermorubin, (**II**) dermocybin and (**III**) emodin present in *Cortinarius sanguineus*. Free aromatic carbons, as well as methyl and methoxy groups, are possible targets for cytochrome P450 (CYP) reactions, whereas hydroxyl groups are typical targets of conjugation.

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Animal cytosolic and microsomal fractions from mice, rats and pigs were prepared previously in-house as described in Juvonen et al[12] and Lang and Nebert,[13] respectively. The pig liver samples were from 8-month-old female pigs and rodent liver samples were from DBA/2N/Kuo mice and Wistar rats,[12] all from the colony of National Laboratory Animal Centre from Kuopio University.

2.3 | Human recombinant CYP, SULT and UGT enzymes

Human recombinant CYP enzymes 1A1, 1A2, 1B1, 2A6, 2A13, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A7 were from BD Biosciences Discovery Labware (Woburn, MA, USA). Individual SULT enzymes 1A1, 1A2, 1A3, 1B1, 1C2, 1C4, 1E1 and 2A1 were from CYPEX (Dundee, Scotland, UK). UGT 2B10 and 2B15 (marked as 2B10C and 2B15C) were purchased from Corning Life Sciences (Corning, New York, USA). Other UGT enzymes, 1A1, 1A3, 1A6–10, 2A1–3, 2B4, 2B7, 2B10, 2B15 and 2B17, were in-house recombinant products.[12,14,15]

2.4 | Incubation with microsomes or cytosol

The tests were conducted using 10 μM dermocybin or dermorubin, an intermediate concentration that allows the detection of metabolites but does not saturate the reactions. In the test tubes, a 100 μl solution containing 10 μM dermocybin or dermorubin, 100 mM K-phosphate buffer pH 7.4, 5 mM $MgCl_2$ (Thermo Fisher Scientific, Waltham, MA, USA) and 40 μg human microsomal protein (oxidation, reduction, glucuronidation) or 20 μg human cytosol (sulfonation, methylation) was mixed. The reactions were started by adding the final 10 μl of enzyme-specific cofactor nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system for CYP (Roche Diagnostics, Mannheim, Germany), 5 mM UDP-glucuronic acid (UDPGA) for UGT, 100 μM 3′-phosphoadenosine-5′-phosphosulfate (PAPS) for SULT or 1 mM S-adenosylmethionine (SAM) for COMT (UDPGA and SAM from Sigma-Aldrich, Saint Louis, MO, USA). Two blanks were prepared without enzymes or cofactor. The reaction tubes were incubated for 1 h at 37°C, reactions were stopped by adding 300 μl acetonitrile (HPLC-grade, Merck, Darmstadt, Germany), and the samples were centrifuged for 5 min at 10 000 *g*. Supernatant containing the remaining dyes and their metabolites was transferred to a clean tube and stored at −80°C.

The oxidation and conjugation reactions of different species were studied using liver microsomal or cytosolic protein fractions of pooled human, rat, mouse or a pig liver sample. Ten to forty micrograms of microsomal or cytosolic protein from each species was added. Otherwise, the incubation conditions were the same as described above.

2.5 | Incubations with human recombinant enzymes

Oxidation reactions were studied in a total volume of 100 μl: 100 mM K-phosphate buffer pH 7.4, 10 μM dermocybin, 20 nM recombinant CYP1A1, 1A2, 1B1, 2A6, 2A13, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 or 3A7 enzyme and 10% NADPH regenerating system. Glucuronidation reaction mixture contained 100 mM K-phosphate buffer pH 7.4, 5 mM $MgCl_2$, 10 μM dermocybin, 20–50 μg recombinant UGT1A1, 1A3, 1A6–10, 2A1–3, 2B4, 2B7, 2B10C, 2B10, 2B15C, 2B15 or 2B17 enzyme, 5 mM $MgCl_2$, and 500 μM UDPGA. Sulfonation reactions were studied by incubating 100 mM K-phosphate buffer pH 7.4, 5 mM $MgCl_2$, 10 μM dermocybin, 0.2 mg/ml SULT1A1, 1A1*2, 1A2, 1A3, 1B1, 1C2, 1C4, 1E1 or 2A1 enzymes and 10 μM PAPS. Two blanks were used in each assay and missing either enzymes or cofactor. The tubes were incubated for 1 h at 37°C, the reaction was stopped with 300 μl acetonitrile, and the samples were prepared for LC-MS analysis as described above.

2.6 | Mass spectrometry analysis

Two high-resolution mass spectrometry (HRMS) equipment were used for the analysis employing an untargeted metabolite profiling method developed earlier.[16,17] For human microsomal and cytosolic samples that were used for metabolite screening, a liquid chromatography connected to a heated electrospray ionization source and a quadrupole time-of-flight mass spectrometry (LC-qTOF-MS) (1290 LC and 6540 qTOF, Agilent Technologies, Santa Clara, CA, USA) was used to acquire the data using both ionization polarities, that is, positive and negative ionization (ESI+ and ESI−). For recombinant metabolic enzymes and samples from different species, an ultra-high-performance liquid chromatography (UHPLC, Vanquich Flex UHPLC system Thermo Fisher Scientific, Waltham, MA, USA) coupled to HRMS (Q Exactive Classic, Thermo Scientific) was utilized.

The supernatant solution (2 μl) was injected on a reversed-phase column (Zorbax Eclipse XDB-C18,

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2.1 × 100 mm, 1.8 μm; Agilent Technologies). For LC-qTOF-MS, column temperature was 50°C, and the trey was kept at 4°C, and for UHPLC-HRMS, the column temperature was 40°C, and sample tray 10°C. In both instruments, the mobile phase flow rate was 400 μl/min and consisted of water (eluent A) and methanol (eluent B), both containing 0.1% (v/v) of formic acid, delivered with the following gradient conditions: 0–10 min: 2 → 100% B, 10–14.5 min: 100% B, 14.5–14.51 min: 100 → 2% B; 14.51–16.5 min: 2% B.

For qTOF-MS, electrospray ionization source was operated using the following conditions: drying gas (nitrogen) temperature 325°C and flow 10/min, sheath gas temperature 350°C and flow 11 L/min, nebulizer pressure 45 psi, capillary voltage 3500 V, nozzle voltage 1000 V, and fragmentor voltage 100 V. Data acquisition was performed using 2 GHz extended dynamic range mode across a mass range of *m/z* 50–1600. Scan rate was 2.5 Hz. Data acquisition was in centroid mode with an abundance threshold of 150 counts.

In UHPLC-MS, the following ionization source settings were utilized: spray voltage (3500 V), sheath gas (40), auxiliary gas (10), and sweep gas (2) (flow rates as arbitrary units for ion source). The capillary temperature was set to 300°C and the probe heater temperature to 425°C. The S-lens RF level was set to 50 V. A full scan range from 120 to 1200 (*m/z*) was used with the resolution of 70 000 (m/Δm, full width at half maximum at 200 u). Automatic injection time was used, and automated gain control (AGC) was targeted at 1 000 000 ions. The detector was calibrated before the sample sequence and subsequently operated at high mass accuracy (<2 ppm).

2.7 | Data handling and statistical analysis

The metabolites were identified based on their retention time (RT), accurate mass and isotopic pattern. The quantification of dermocybin and dermorubin was based on external standard method. The peak area difference between blank and reaction samples indicated the consumption that was calculated by comparing the dye quantities in the full reaction and blank reaction using the equation: consumption = 100% − (quantity in reaction/quantity in blank) * 100%. GraphPad Prism version 9.4.1 (GraphPad Prism Inc., San Diego, CA, USA) was used for graph creation. Duplicate samples were used in all experiments in order to identify the types of metabolites derived from dermocybin or dermorubin. Results are expressed as mean of the duplicate samples. The study was conducted in accordance with the *Basic & Clinical Pharmacology & Toxicology* policy for experimental and clinical studies.[18]

3 | RESULTS

Dermorubin was not consumed during the microsomal and cytosolic incubations, and therefore, no metabolites could be detected in LC-HRMS method. On the other hand, several metabolites were detected for dermocybin, and they are presented in Table 1. Negative mode ESI produced a higher ionization efficiency than positive mode, and therefore, it was used for detection and their quantitative analysis.

TABLE 1 Detected metabolites of dermocybin in the negative ionization mode.

Product	Structure	RT (min)	Calculated *m/z*	Detected *m/z* (min–max)
D	$C_{16}H_{12}O_7$	9.47	315.0510	315.0511–315.0516
$DdCH_3$	$C_{15}H_{10}O_7$	7.33	301.0354	301.0355–301.036
DO1	$C_{16}H_{12}O_8$	5.54	331.0459	331.0460–331.0468
DO2	$C_{16}H_{12}O_8$	7.37	331.0459	331.0461–331.047
DO3	$C_{16}H_{12}O_8$	8.64	331.0459	331.0461–331.0468
DS1	$C_{16}H_{12}O_{10}S$	7.13	395.0078	395.0078–395.0079
DS2	$C_{16}H_{12}O_{10}S$	8.40	395.0078	395.0081–395.0111
DG1	$C_{22}H_{20}O_{13}$	7.09	491.0831	491.0825–491.0846
DG2	$C_{22}H_{20}O_{13}$	7.84	491.0831	491.0826–491.0841
DG3	$C_{22}H_{20}O_{13}$	8.15	491.0831	491.0829–491.0844

Abbreviations: D, Dermocybin; $DdCH_3$, demethylated dermocybin; DO1–3, hydroxylated dermocybin; DS1–2, sulfonated dermocybin; DG1–3, glucuronidated dermocybin; RT, retention time.

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3.1 | Human liver microsomal and cytosolic incubations of dermocybin and dermorubin

Dermocybin and dermorubin were incubated in oxidation, glucuronidation, sulfonation or methylation conditions with human liver samples for 1 h at 37°C to quantify the dye consumption and to identify the formed metabolites. Authentic dermocybin and dermorubin standards were used for quantitation, and the formed metabolites identified using their accurate mass, isotopic pattern and retention time in LC-MS analysis.

These characteristics were utilized when dermorubin (*m/z* 343.0459) and its metabolites were identified from the LC-HRMS data. No formation of its demethylation (*m/z* 329.0303), hydroxylation (*m/z* 359.0409), sulfonation (*m/z* 423.0028) or glucuronidation (*m/z* 519.0780) metabolites was detected, and, therefore, dermorubin was not studied further.

Similar analysis was conducted for dermocybin (*m/z* 315.0510) and its demethylation (*m/z* 301.0354), hydroxylation (*m/z* 331.0459), reduction (*m/z* 317.0812), glucuronidation (*m/z* 491.0831), sulfonation (*m/z* 395.0078) and methylation (*m/z* 329.0667) metabolites. Dermocybin consumption in CYP oxidation reactions involving hydroxylation and demethylation was 25%, whereas only 7%–18.3% was consumed in the conjugation reactions. No methylation or reduction products were discovered. Based on the structure of dermocybin, it was hypothesized that hydroxylation could occur in C6 and C8 and in the methyl group of C7 (Figure 2A). Demethylation could take place in methoxy group of C2. Conjugation could occur in the hydroxy groups of dermocybin: C1, C3, C4 and C5 (Figure 2B).

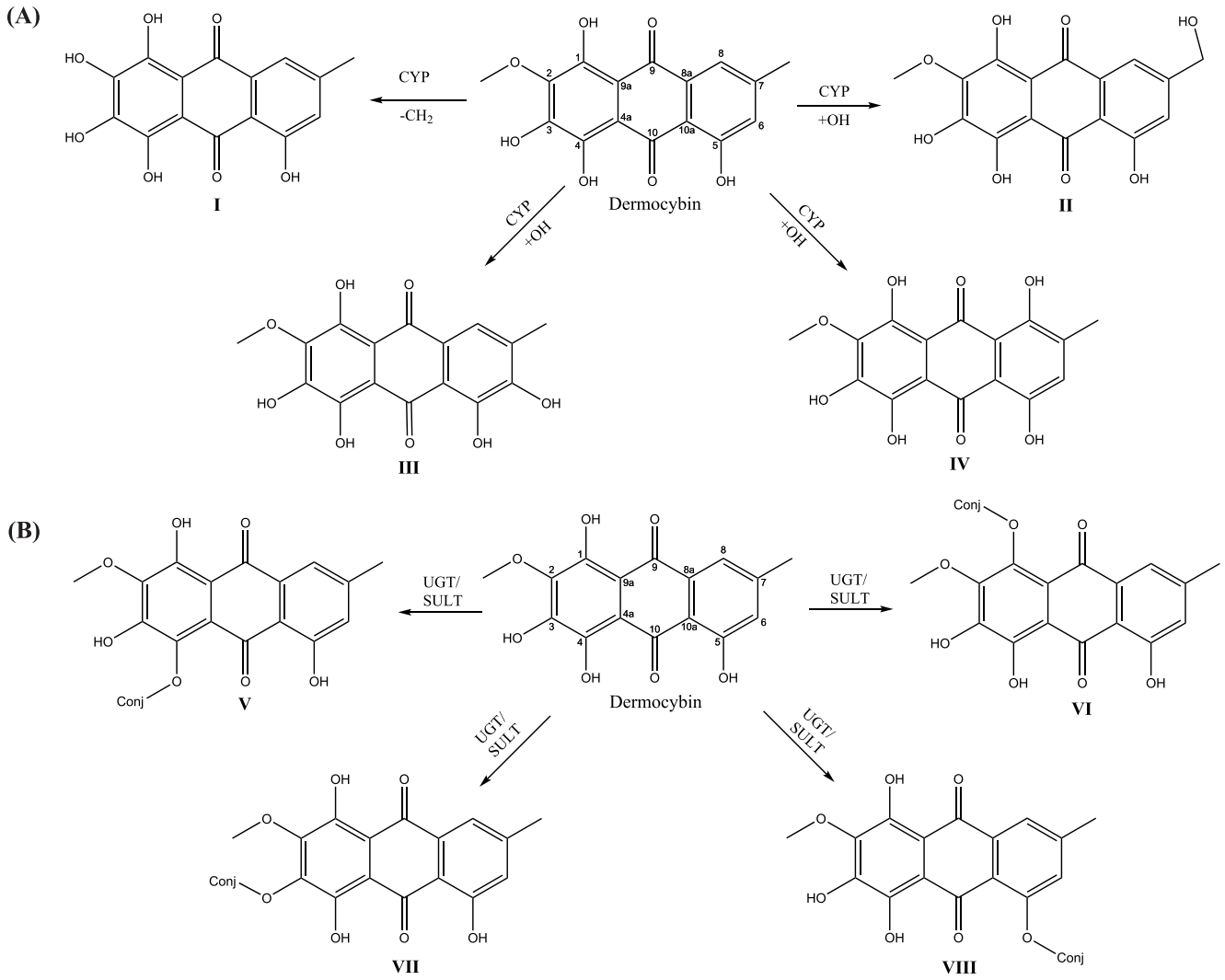


FIGURE 2 Predicted metabolites of dermocybin in liver incubations. Panel (A) shows prediction of oxidation reactions for **I** demethylation or **II–IV** hydroxylation. Panel (B) shows a prediction of conjugation reactions **V–VIII** mediated by SULT or UGT enzymes. Conj, glucuronide or sulfate conjugate; CYP, cytochrome P450; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase.

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Indeed, three hydroxylated dermocybin metabolites with retention times 5.55, 7.37 and 8.64 min and one demethylation metabolite with retention time 7.33 min were detected. Correspondingly, in conjugation reactions, two sulfonation (RTs 7.13 and 8.4) and three glucuronidation metabolites (RT 7.06, 7.8 and 8.15 min) were detected.

3.2 | Oxidation metabolites of dermocybin by human CYPs

Dermocybin oxidation was studied with recombinant human CYPs to reveal enzymes catalysing the reaction. We found that several CYP enzymes catalysed the oxidation of dermocybin. 20 nM CYP1A2, 2B6, 2C9, 2D6, 2E1, 3A4 and 3A7 consumed 50%–60% of 10 μM dermocybin in 1 h incubation, whereas 2A13 did not transform it at all. A more detailed description of the CYP oxidation is shown in Figure 3.

The same oxidation metabolites that were identified following microsomal incubation were also discovered in the incubations with recombinant CYPs. The retention times of the three hydroxylated metabolites were 5.55, 7.37 and 8.64 min (Figures 4A–C and 5), whereas the metabolite at RT 7.3 min was an O-demethylation metabolite (1,2,3,4,5-pentahydroxy-7-methylanthracene-9,10-dione; Figure 4D).

According to peak areas, the abundance of metabolites, from the most to least abundant, were hydroxyl metabolite (RT 8.64), hydroxyl metabolite (RT 7.37), demethylation metabolite RT 7.3 and a hydroxyl metabolite (RT 5.55). The formation of metabolites RT 5.55 and 7.3 was catalysed by several CYPs, whereas metabolite RT 8.64 was produced mainly by CYP1B1 and to a small extent by CYP1A1. The formation of metabolite RT 7.37 min was catalysed particularly by CYP1B1 and CYP2C9. CYP1B1 was the most capable human CYP, producing three dermocybin oxidation metabolites (RT 5.55, 7.37 and 8.64; Figure 5). A further example of the chromatogram of the metabolites formed in the reactions catalysed by CYP1A1 is available in Figure S3.

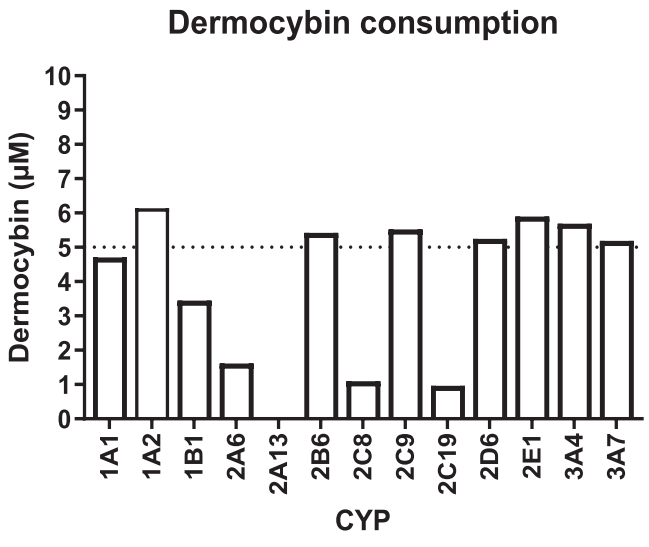


FIGURE 3 Consumption of 10 μM dermocybin in 1 h incubation with 20 nM human recombinant cytochrome P450 (CYP) enzymes. The dashed line depicts 50% consumption. Data are shown as mean, $n = 2$.

3.3 | Conjugation of dermocybin by human UGTs and SULTs

UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) enzymes conjugate hydroxyl-containing organic compounds with glucuronide and sulfate groups, respectively. Dermocybin hydroxyls of carbons C1 and C3–C5 could be conjugated as presented in Figure 2.

These reactions were studied using 16 recombinant human UGT and nine human SULT enzymes. When 10 μM dermocybin was incubated for 1 h in the presence of UGT or SULT and their corresponding cofactor, three glucuronides (RT 7.09, 7.85 and 8.15 min) and two sulfates were detected (RT 7.13 and 8.4 min).

Multiple UGT enzymes catalysed the glucuronidation (Figure S1). The most abundant metabolite was RT 8.15 min, catalysed by five UGTs: 1A1, 1A3, 1A7, 1A9 and 1A10. Formation of RT 7.85 was catalysed by UGTs 1A7, 1A9 and 1A10. Glucuronidation of RT 7.09 was catalysed by seven UGT enzymes, even though its peak areas were the smallest. A chromatogram of the metabolites formed in the reaction catalysed by UGT1A10 is available in Figure S4.

Two sulfonation metabolites were detected with retention times 7.13 and 8.4 min (Figure S2). SULT1B1 catalysed the formation of metabolite RT 7.13, which was also more abundant than RT 8.4, whose formation was catalysed by SULTs 1C2 and 2A1.

3.4 | Metabolism of dermocybin in different species

Liver microsomes and cytosol of rat, mouse and pig were used to compare oxidation, glucuronidation and sulfonation of dermocybin in different species. An individual and a pooled human microsome sample were also present in the comparisons. In oxidation reactions, less than twofold differences in dermocybin consumption were observed between the species (Figure 6).

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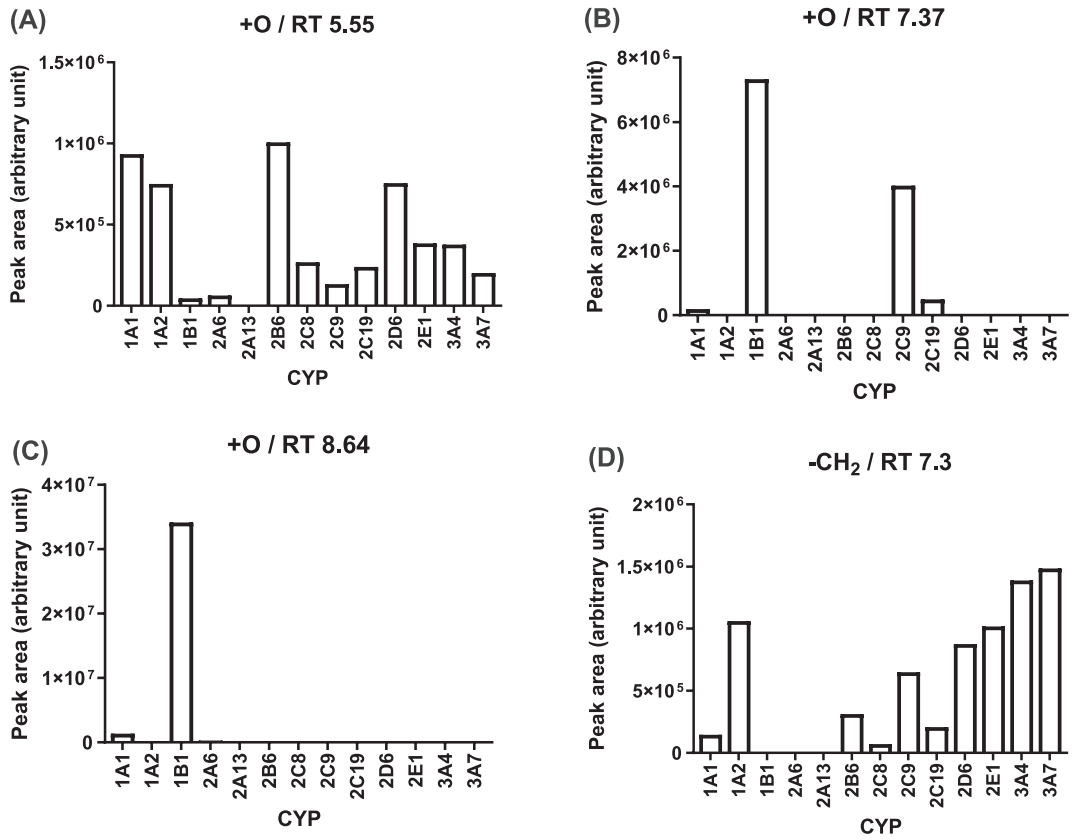


FIGURE 4 Oxidation metabolites of dermocybin by cytochrome P450 (CYP) enzymes. 10 μM dermocybin was incubated for 1 h at 37°C with 20 nM human CYPs in the presence of NADPH. Panels A–D show oxidation metabolites with indicated retention times (RT, min). The scale of y-axis varies between the panels. +O, hydroxylated metabolite; $-CH_2$, demethylation metabolite; RT, retention time. Data are shown as mean, $n = 2$.

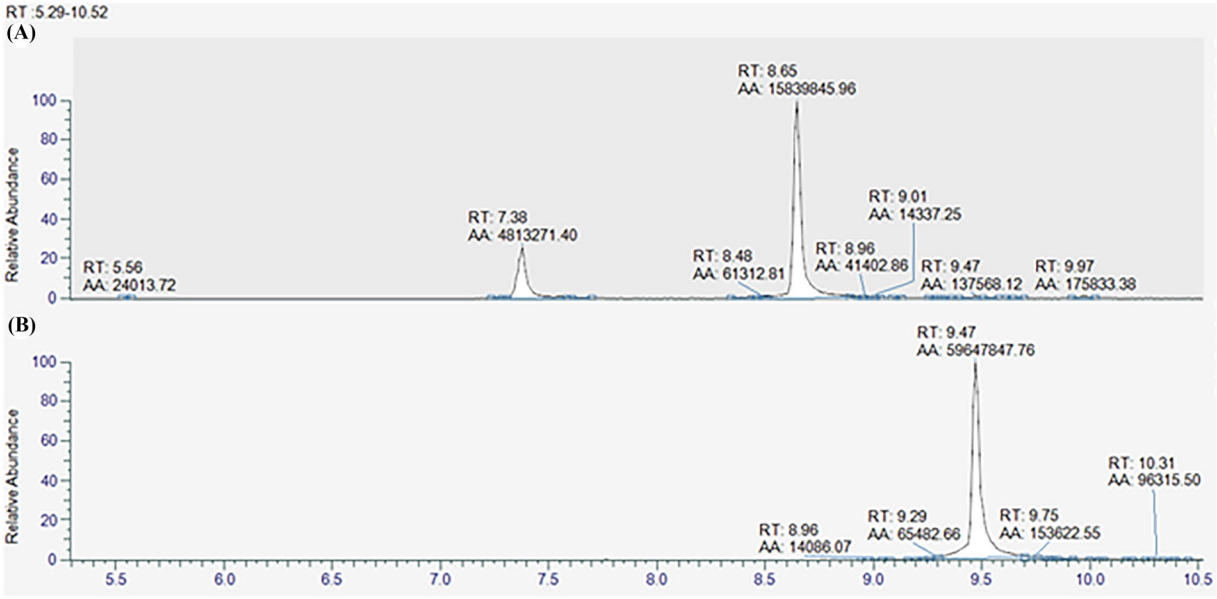


FIGURE 5 The extracted ion chromatograms of (A) dermocybin oxidation metabolites (m/z 331.0459) in a reaction catalysed by cytochrome P450 (CYP) 1B1 and (B) parent compound dermocybin (m/z 315.0510), expressed as relative abundance. Blanks only contained the parent compound. AA, peak area; RT, retention time.

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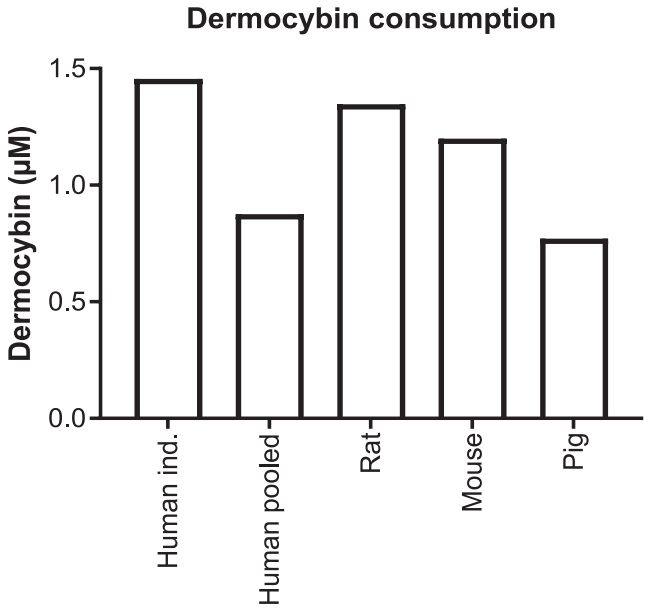


FIGURE 6 Dermocybin consumption in 1-h oxidation incubations in liver microsomes of indicated species. Ind., individual. Data are shown as mean, $n = 2$.

In the species incubations, the same metabolites were formed as in the human enzyme assay (Figure 7). Three distinct hydroxylation and one demethylation metabolite were detected in human, rat and mouse liver incubations. The demethylation metabolite was not formed in pig liver. The most abundant oxidation metabolites were RT 7.37 and 8.64 min in all species.

Dermocybin glucuronidation was studied in human, rat, mouse and pig microsomes, whereas sulfonation was studied in the cytosolic samples from human, rat, mouse and pig. Three glucuronides with retention times of 7.06, 7.85 and 8.15 min were detected. All of them were formed only in the mouse liver incubation (Figure 8). Glucuronide metabolite RT 8.15 was formed in all species. As a summary, marked species differences can be observed in dermocybin glucuronidation.

No sulfonation products were observed in human samples (Figure 9), but two metabolites with retention times 7.13 and 8.4 min were formed in rat liver, whereas mouse and pig incubations produced one sulfonation metabolite RT 8.4 min.

4 | DISCUSSION

Natural anthraquinones obtained from *C. sanguineus* such as dermocybin and dermorubin could potentially be produced following the principles of circular economy. Previously, we have studied that they can be utilized in supercritical CO_2 dyeing that does not involve the use of fresh water and that they exhibit low toxicity.[5] In the present work, we studied the previously unknown metabolic properties of dermocybin and dermorubin. We found that dermorubin was not metabolized in in vitro incubation system by human liver microsomal or cytosolic fractions. Dermocybin, on the other hand, was metabolized via oxidation and conjugation reactions, forming three different hydroxylation and one demethylation metabolites in CYP enzyme reactions and three glucuronide and two sulfate conjugates by UGT and SULT enzymes, respectively. The same metabolites were also formed when dermocybin was incubated with rat, mouse or pig liver fractions. However, especially in conjugation reactions, we observed marked interspecies differences and, interestingly, also between an individual and a pooled human sample of 50 donors. It is likely that the pooled human sample is a better indicator of the average metabolizing capacity.

According to the molecular structures of dermocybin and dermorubin, both CYP oxidation and different types of conjugation reactions are possible. CYP reactions could take place in the methoxy and methyl groups or in the free aromatic carbons both in dermocybin and dermorubin.[19] Both molecules also contain several hydroxyl groups that are typical targets for glucuronidation or sulfonation. The carboxylic acid group of dermorubin could also be glucuronidated to an acyl conjugate. In addition, dermocybin contains a catechol structure that is known to be methylated by COMT.

We observed CYP oxidation, glucuronidation and sulfonation with dermocybin but not with dermorubin, and, according to dermocybin consumption, oxidation reactions were faster than conjugation in the liver fractions. Typically, it is considered that UGT and SULT conjugations are faster than CYP oxidation, as adding a glucuronide or sulfone structure to a hydroxyl group is faster than carbon atom oxidation in CYP reactions. Further, Liu et al reported in their study that the glucuronidation rate of emodin, a structurally similar anthraquinone, was five times higher than its metabolism via phase I oxidation reactions.[20] Therefore, our observation of the CYP reactions being faster than conjugation in in vitro liver incubations was unexpected. However, when reactions are studied in in vitro versus in vivo situation, the rates of oxidation and conjugation reactions may differ. Secondly, secondary metabolites such as conjugates can be formed from the oxidation metabolites. This can be studied by enabling the occurrence of several reactions in the same incubation tube and identifying the formed metabolites in future.

The main structural difference between dermocybin and dermorubin is the carboxylic acid substituent present in dermorubin at position C2. It is negatively charged

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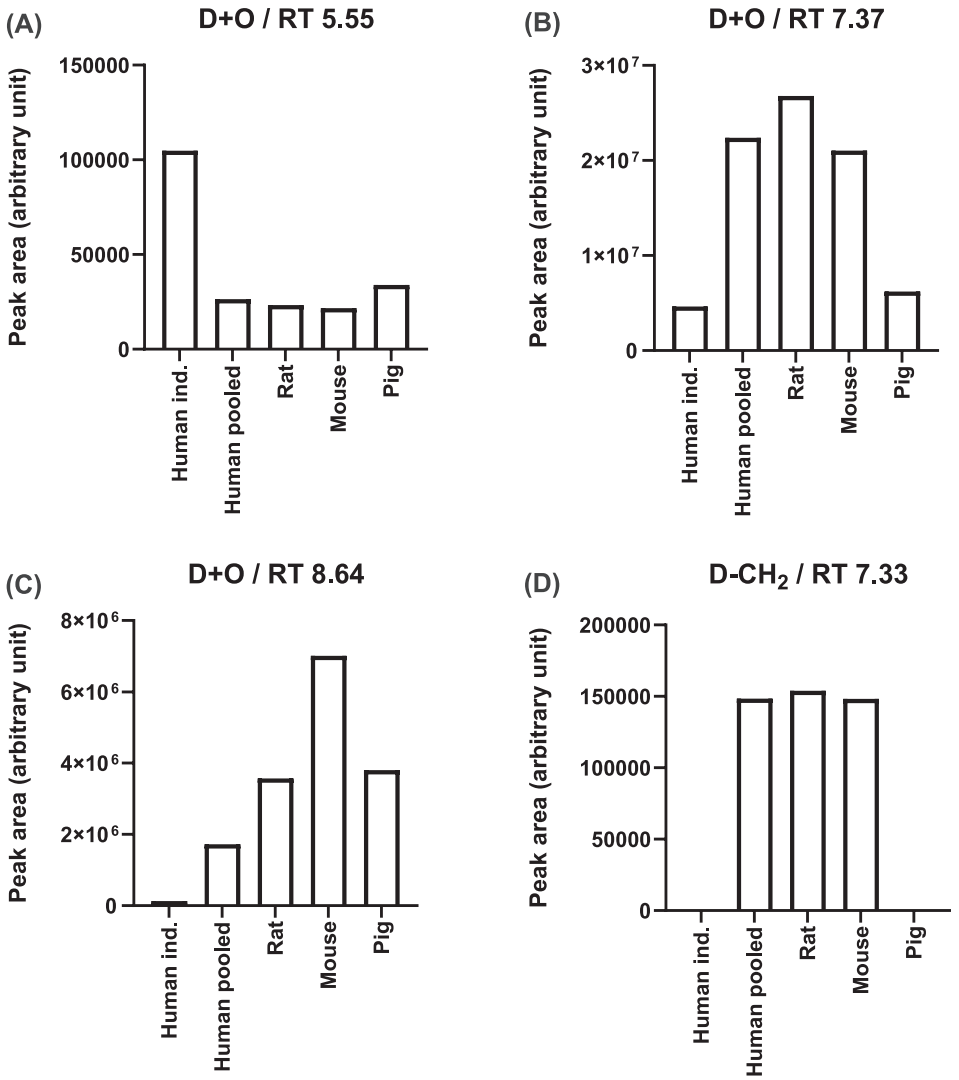


FIGURE 7 Dermocybin oxidation metabolites detected in LC-HRMS in CYP reaction conditions of individual human, pooled human, rat, mouse and pig liver microsomes. The same metabolites were seen also in positive ionization mode. The scale of y-axis varies between the panels. +O, oxidation; $-CH_2$, demethylation; D, dermocybin; Ind., individual; RT, retention time. Data are shown as mean, $n = 2$.

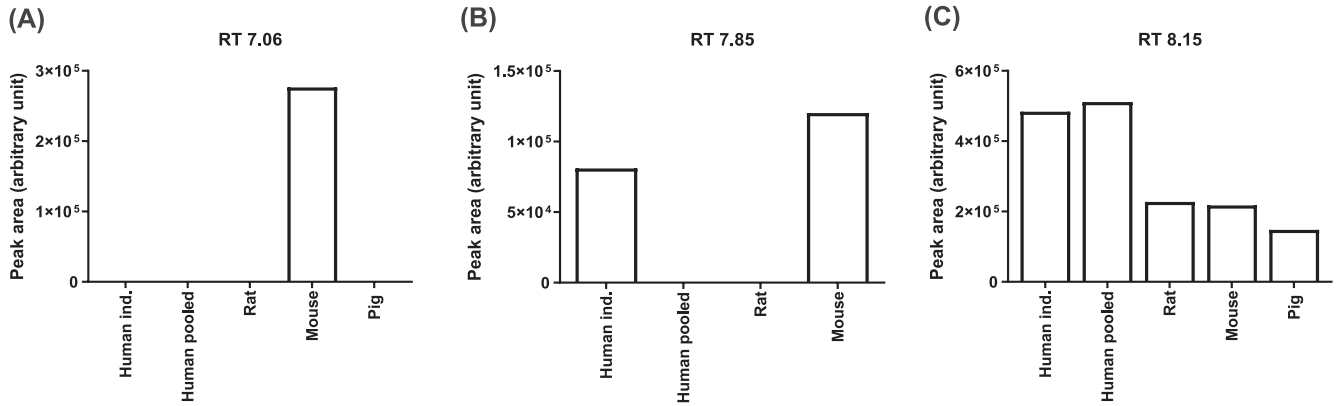


FIGURE 8 Glucuronidation metabolites of dermocybin formed in incubation with liver microsomes of indicated species. The scale of y-axis varies between the panels. Ind., individual; RT, retention time. Data are shown as mean, $n = 2$.

at pH 7.4, whereas dermocybin does not contain a carboxylic acid and is neutral. It is possible that the negatively charged substitution causes the difference in the ability of dermorubin and dermocybin to enter the active sites of CYP, UGT and SULT enzymes. Similarly, rhein, a natural anthraquinone that contains a carboxylic acid group, is not glucuronidated by UGT enzymes[9] contrary to, for example, emodin that does not contain an acid

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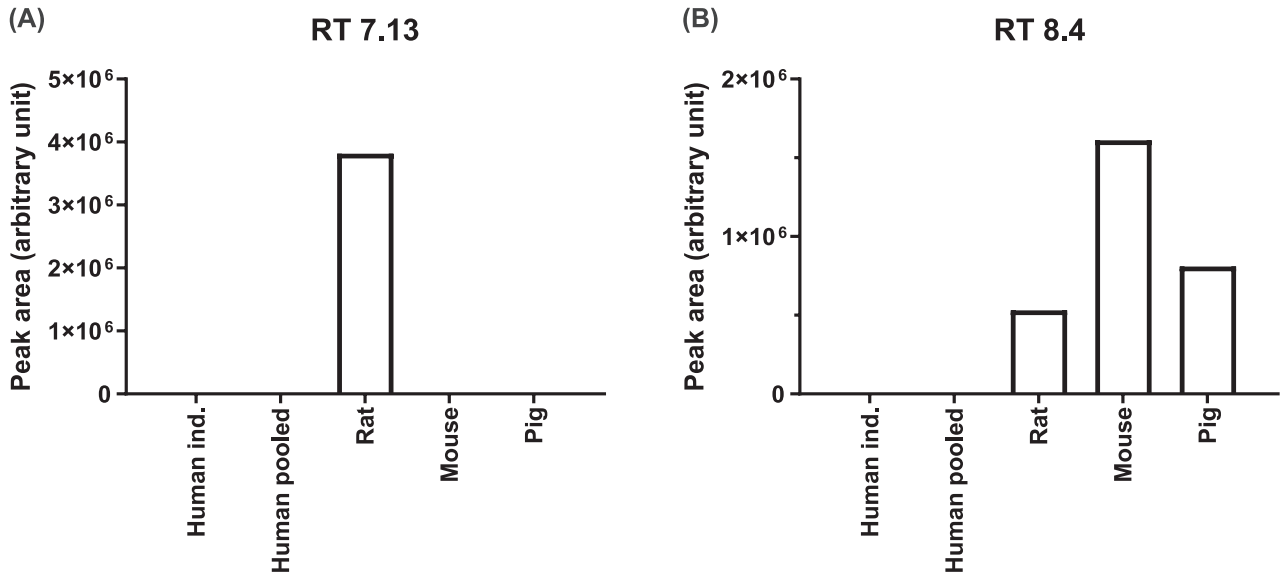


FIGURE 9 Sulfonation metabolites of dermocybin formed in the liver cytosolic samples of indicated species. The scale of y-axis varies between the panels. Ind., individual; RT, retention time. Data are shown as mean, $n = 2$.

group and is mainly metabolized by glucuronidation.[8] Considering our observation that oxidation was the major reaction in these in vitro incubations of dermocybin, and dermorubin was neither oxidized nor conjugated, it is evident that substituents can have a major effect on the metabolism. However, in CYP reactions, new hydroxyl groups are formed in the structure of dermocybin, and it is possible that UGT enzymes would be able to conjugate those in an in vivo situation. Further studies are needed to examine this possibility, as well as the role of intestinal fractions in the metabolism of dermocybin and dermorubin.

In this study, metabolic activation of dermocybin or dermorubin was not assessed. It is known, however, that emodin is mutagenic in Ames test in the presence of S9, meaning that it is bioactivated.[21] Some anthraquinones can be bioactivated per se via several mechanisms such as excessive reactive oxygen species (ROS) production via redox cycling, epoxidation and catechol formation.[22] CYP and reduction enzymes play an important role in these reactions. Dermorubin oxidation did not take place, and, therefore, it is most probably not bioactivated. Additionally, due to its carboxylic acid group, it is likely water soluble enough to be excreted to urine from the body as such, similarly to rhein.[9]

Two-electron reduction metabolites were not observed in neither dermorubin nor dermocybin incubations. ROS formation might take place either as one-electron reduction, which produces a reactive intermediate, or in two-electron pathways that lead to anthraquinone reduction.[23] Dermocybin could be inherently reactive as it contains a catechol structure that is known to be metabolized into reactive semiquinones in one-electron reduction.[24] We did not observe any such metabolites in LC–MS analysis. Nevertheless, dermocybin could be bioactivated to semiquinones in the same way as, for example, tolcapone, another catechol.[25] A risk-alleviating factor is that we did observe oxidation, glucuronidation and sulfonation of dermocybin mediated by several CYP, UGT and SULT enzymes, which in most cases minimizes its reduction to reactive semiquinones and reduces the risk of toxicity.

In our study, oxidation of dermocybin occurred more than glucuronidation or sulfonation in human, rat, mouse and pig. No notable species differences were observed in its oxidative metabolism. The glucuronidation rate of dermocybin was slower than oxidation in all the tested species, and they differed somewhat among themselves in the glucuronidation position. Mouse liver microsomes glucuronidated dermocybin into three, mouse and human to two and other species to one glucuronide. In vitro glucuronidation of emodin is at least five times faster than its oxidation in rat liver microsomes, as measured by Liu et al.[26] The group showed that glucuronidation is somewhat higher in rat, mouse and guinea pig than in human liver microsomes.

Altogether this data contributes the previous in vitro safety data on dermorubin and dermocybin.[5] European chemical legislation requires in vivo toxicity testing for chemical substances such as biocolourants when entering the market and their production exceeds certain limit. Consequently, it is important to know the metabolism of xenobiotics in different species to increase the reliability of the toxicity assessment. The present study is a

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qualitative discovery-state study rather than a quantitative measurement of the metabolism of the studied dyes. In the future, bioactivation of dermocybin could be studied, though it did not present mutagenicity in the Ames test even when S9 was present.[5] In addition, recombinant CYP, SULT and UGT enzymes were the most common genetic forms of the enzymes, and the effects due to genetic variation cannot be ruled out. Other questions to be answered are (1) what the exact molecular chemical structures of the dermocybin metabolites are, (2) do secondary metabolites form in vivo from these compounds and (3) how dermocybin oxidation in liver microsomes is reduced by selective CYP inhibitors.

5 | CONCLUSIONS

We have conducted a qualitative study on the in vitro metabolism of two anthraquinone dyes obtained from *C. sanguineus* fungus, dermocybin and dermorubin, using liver microsomal and cytosolic fractions of human, rat, mouse and pig, as well as human recombinant CYP, UGT and SULT enzymes. Dermorubin was not metabolized in human liver microsomes, whereas dermocybin was oxidized and glucuronidated by liver microsomes of human, rat, mouse and pig and sulfonated by liver cytosol fractions of human, rat, mouse and pig. Many hepatic and extrahepatic CYP and conjugation enzymes took part in dermocybin metabolism. Oxidation of dermocybin was catalysed by human CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A7, whereas its conjugation was catalysed via glucuronidation by UGT1A1, 1A3, 1A7, 1A8, 1A9, 1A10 and 2B15 and sulfonation by SULT1B1, 1C2 and 2A1. Altogether, it can be concluded that the metabolic rate and oxidative metabolites of dermocybin are similar in in vitro liver microsomes between human, rat, mouse and pig, but their glucuronidation pattern differs. According to these results, it is likely that dermocybin would be metabolized in human in vivo.

AUTHOR CONTRIBUTIONS

Johanna Yli-Öyrä, Jaana Rysä, Mikko Herrala, Risto O. Juvonen and Riikka Räisänen designed the research. Johanna Yli-Öyrä, Marko Lehtonen and Risto O. Juvonen performed the research and analysed the data. Moshe Finel and Riikka Räisänen contributed to analytical methods. Johanna Yli-Öyrä wrote the first draft of the manuscript. Jaana Rysä, Mikko Herrala, Marko Lehtonen and Risto O. Juvonen have done major contribution on writing the manuscript. All authors have read and approved the manuscript.

ACKNOWLEDGEMENTS

We thank Mrs Miia Reponen for expert technical assistance.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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