

## Pharmacokinetic of <sup>3</sup>H-deacetylasperulosidic acid in mice

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### ABSTRACT

**Background:** An investigation was conducted to determine the fate of the iridoid derivative deacetylasperulosidic acid (DAA) after oral application to mice.

**Methods:** DAA was extracted from *Morinda citrifolia* leaf and purified by preparative HPLC. The identity was verified by MS and NMR spectroscopy. A sample of DAA was radioactively labelled with tritium and applied to mice by gavage. The pharmacokinetic of the radioactivity was investigated in blood, organs, urine and feces. Metabolites were isolated in blood and urine by HPLC and identified by LC-MS. In vitro incubation of DAA with mouse duodenum and liver homogenate and human fecal bacteria was performed and possible metabolites were separated by HPLC.

**Results:** DAA was rapidly absorbed and excreted mainly via the kidneys with a half-life of 30 minutes. Radioactivity was present in all organs with highest concentrations in kidney and liver. Almost 100% of the radioactivity isolated from urine and organs could be identified as unchanged DAA. Additionally, no metabolism could be observed after in vitro incubation of DAA with mouse small intestine or liver homogenate. However, a total breakdown of the molecule was observed after incubation of DAA with human intestinal bacteria.

**Conclusion:** The absorption and excretion of glycosides such as DAA in mammals without hydrolysis is a potential defense mechanism of animals against the toxicity of these compounds.

**Keywords:** Deacetylasperulosidic acid, DAA, tritium label, pharmacokinetic, iridoid, metabolism

## BACKGROUND

Iridoids are a chemical group of secondary plant metabolites with hundreds of different derivatives, which occur in many species of angiosperms, particularly within the superorder of Sympetalae. Most iridoids share a cyclopentanoid monoterpene aglycone attached to a sugar moiety [1]. Some plants use iridoids as precursors for the synthesis of alkaloids [2], but also as a defense against predators [3]. The toxicity of the iridoids depends on its hydrolysis by glycosidases present in the GI-tract or other tissues of animals. The enzymes can also occur in the plant, mostly in the leaves, where they are stored in vacuoles apart from the iridoids. Upon the chewing of the plants by predators, the glycosidases come into contact with the iridoids and aglycones are formed. The lactone ring of iridoid aglycones can open to form highly reactive dialdehydes, which are able to form adducts with proteins and other functional macromolecules. This can cause toxic effects to the intestinal wall and inner organs of the plant predators. It also results in denaturation of plant proteins, with subsequent abolishment of their nutritional value [4], which causes the animals to avoid such plants for food purposes. The reactivity of iridoid aglycones depends on its chemical structure. Free carboxyl groups in the C6-position of the six-membered lactone ring favor the ring opening reaction and enhance the reactivity of the aglycone. For example, genipin, the aglycone of geniposid with a methyl ester group at C6 is a relatively stable molecule, whereas the aglycone of the free acid, geniposidic acid, is much less stable [5].

## INTRODUCTION

Very little information is available regarding the metabolism and pharmacokinetics of iridoids in mammals. Su et al [6] studied the pharmacokinetic of aucubin via different routes of application to rats. The authors concluded that the bioavailability of the compound from the GI-tract was limited after oral application, which might be partly due to the hydrolysis in the acidic environment of the stomach and poor absorption because of the hydrophilicity of aucubin. In a study using radioactive aucubin, it was demonstrated that radioactivity—most likely the aglycone—bound covalently to serum albumin, resulting in a constant blood level over a long time period [7]. The unspecific reaction of the highly reactive aucubin aglycone with proteins is unavoidably associated with toxicity, which is in accordance with the fact that aucubin containing plants, such as *Aucuba japonica*, are toxic [8]. The pharmacokinetics of a geniposide containing decoction and the aglycone genipin was studied in rats [9]. Genipin was rapidly converted to its sulfate conjugate after oral and parenteral administration, which was detected in the serum. No geniposide was observed in the serum after oral administration of a decoction of *Gardenia jasmoides*. In contrast to this observation, the presence of geniposide in rat serum was observed after oral application of a *Gardenia jasminoides* extract by Qu et al. [10].

A variety of iridoid containing plants is devoid of toxicity and used as herbal remedies or food plants [11]. Among these plants is *Morinda citrifolia* L. (Noni), a tropical plant belonging to the family of Rubiaceae, which has been used by native Polynesian people since their early time of migrations about 3000 years ago [12, 13]. Noni fruits and leaves contain up to 16 different iridoids [14, 15] of which more than 90% belong to two compounds only (asperulosidic acid (AA) and desoxyasperulosidic acid (DAA)). A placebo controlled double blind clinical trial with human volunteers did not show any adverse effects after consumption

of 750 ml of a noni fruit juice (Tahitian Noni™ juice) per day over a period of 28 days [14]. This amount was equivalent to the daily ingestion of approximately 700 mg DAA.

Iridoids are thought to be the active principles of many medicinal plants with adaptogenic properties, leading to immunomodulation, inhibition of inflammatory diseases and increase of physical endurance [16]; however, their mechanism of action is still unclear. It is also unclear whether cleavage of the glycosidic bond is an essential prerequisite of their biological activity and how the hydrolysis of the glycosides by glycosidases present in the intestinal mucosa or gut bacteria, which leads to reactive iridoid aglycones capable of causing tissue damage, is avoided.

In order to better understand the lack of toxicity of iridoids, in particular DAA, we investigated the pharmacokinetics and metabolism of radioactively labeled DAA in mice.

## MATERIALS AND METHODS

### Chemicals

1 mg of authentic DAA was kindly provided by Jarakae Jensen, Morinda Inc., Provo, Utah, USA. This amount was used for the characterization of RT-values in HPLC, and UV-, MS and NMR-spectra. Methanol (MeOH), ethanol (EtOH), HPLC grade water, and trifluoroacetic acid (TFA) for spectroscopy were obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile (MeCN), was purchased from J.T. Baker (Netherlands). 4-Nitrophenyl- $\beta$ -D-glucopyranoside (PNG) and all other chemicals were purchased from Sigma-Aldrich.

DAA was isolated from *Morinda citrifolia* leaf, harvested on the island of Fatu Hiva, French Polynesia, according to a method of Lavarone et al. [17]. Briefly, 100g of dry pulverized plant material was extracted with a 25% sodium chloride solution in water by stirring at room temperature for 48 hours. After filtration of the eluate, the extract was applied to a column (50 x 5cm) filled with activated charcoal to absorb organic contents of the extract, including iridoids. The column was washed with distilled water, until no further NaCl could be detected in the eluate by precipitation with AgNO<sub>3</sub>. The column was then eluted with 500 ml each of a sequence of aqueous ethanol solutions (10, 30, 50 and 80%). The eluates were collected separately and the presence of DAA was verified by HPLC analysis. Nearly the entire amount of DAA was present in the 80% ethanol fraction, which was evaporated to dryness. The residue was dissolved in 3 ml distilled water and DAA was isolated after purification by preparative HPLC. The whole procedure was repeated several times until about 1 g of DAA was obtained. The identity of the isolated DAA was confirmed by MS and NMR data.

Tritium labeled DAA (5 mCi) was obtained from Hartmann Analytics (Braunschweig, Germany). Labelling was performed by treatment of 10mg DAA with tritium gas. Loosely bound tritium was removed by the provider. Final purification of the labelled DAA was performed by us using preparative HPLC. The radioactive DAA was adjusted with non-radioactive DAA to a specific activity of 100  $\mu$ Ci/mg ( $9.6 \times 10^6$  Bq/mmol).

## METHODS

### Equipment

Radioactive samples were diluted with a scintillation cocktail (Aquasafe 300+, Zinsser Analytic, Frankfurt, Germany) and counted in DPM mode in a Liquid Scintillation Counter (Beckmann, Fullerton, CA) equipped with a Ba-133 internal standard source.

Preparative fractionation was carried out on a Merck Hitachi LaChrom HPLC system (Merck, Germany) equipped with a Model L 7150 Preparation Pump, Model 7200 Autosampler, Model L 7400 UV Detector, Model D 7000 Interface and a fraction collector Foxy Jr. (Teledyne Isco, Inc., USA). Separation of DAA was performed on a 250 x 4.6 mm Nucleosil 100RP-18 column (Macherey-Nagel, Düren, Germany) with a gradient of acetonitrile/water containing 0.1% TFA (0-5 min, 100% H<sub>2</sub>O; 5-30 min, 79% H<sub>2</sub>O; 30-37 min, 100% MeCN). The flow rate was 9 ml/min and the injection volume was 300 µl.

Analytical separations were performed on a Thermo Finnigan Surveyor HPLC system (Thermo Finnigan Analytische Systeme GmbH, Germany) equipped with an auto sampler, PDA detector and LC pump with an integrated degasser, including a Nucleosil-100 RP-18 10 µm HPLC column (250mm x 4.6 mm) (Macherey-Nagel, Düren, Germany). A flow rate of 0.8 ml/min and an injection volume of 20 µl were used for the separation which was performed by a gradient of MeCN-H<sub>2</sub>O containing 0.1% TFA (0-5 min, 100% H<sub>2</sub>O; 5-40 min, 70% H<sub>2</sub>O; 40-45 min, 5% H<sub>2</sub>O; 45-50 min, 100% MeCN; 50-55 min, 100% MeCN). Eluates of radioactive samples were collected in fractions of 15 – 60 sec directly in 20 ml plastic scintillation vials (Zinsser, Analytics, Frankfurt, Germany), mixed with 10 ml scintillation cocktail and counted.

LC-MS analysis was performed on a Varian 1200L Triple Quadrupole MS system equipped with two Varian ProStar model 210 HPLC pumps. Samples of 10 µl were directly injected into the system (70 % MeCN, 30 % H<sub>2</sub>O containing 0.1% formic acid, 0.2 ml/min). For the positive electrospray ionization mode (ESI+), the needle and shield voltages were set at 4500 and 600 V, respectively. Scanning was performed in one second intervals through 50 to 800m/z. The detector voltage was set to 950 V.

NMR measurements were carried out with a Bruker WM 400 (<sup>1</sup>H, 400 MHz; <sup>13</sup>C, 100,6 MHz) or a Bruker WM 500 (<sup>1</sup>H, 500MHz; <sup>13</sup>C, 125,8 MHz) instrument. The samples were diluted in DMSO-d<sub>6</sub>.

## ANIMAL EXPERIMENTS

Female C57Bl/6 mice were purchased from Charles River, Sulzfeld, Germany. The animals were housed in the animal facility of the University Clinic, Hamburg in groups of five in macrolon cages at 22 °C and a light/dark cycle of 12 h with unlimited access to food and water. All animal experiments were approved by the veterinary department of the University Medical Clinic of Hamburg, Germany and authorized by the state department of veterinary affairs, Hansestadt Hamburg, Germany.

For pharmacokinetic studies, mice with an average weight of 20g received 0.2 ml of <sup>3</sup>H-DAA in water (1.5x10<sup>6</sup> DPM, 0.5 mg DAA) by oral gavage. Animals scheduled for collection times of 15-240 minutes were kept in glass beakers covered with aluminum foil. The bottom contained a round piece of filter paper for absorption of urine. These animals had no access to food or water during the sampling time. Mice scheduled for the 24 h time point were kept in a special glass device, which allowed the separation of urine and feces. These animals had free access to water but no access to feed. After the sampling times (15, 30, 60, 90, 120, 240 minutes and 24 hours), mice were killed by carbon dioxide, the abdomen and chest were opened and a blood sample (0.2-0.5 ml) was taken by heart puncture with a syringe. Liver, kidneys, spleen, stomach, intestine, bladder and brain were removed. The bladder was opened with a scissor and washed several times with small volumes of saline.

The fresh weight of all organs was measured before the tissues were solubilized in 5 ml of 10N HNO<sub>3</sub> (63%) at 50°C. Clear solutions were obtained within 2-5 hours and neutralized by addition of 5 ml of 10N KOH (56%). The solutions were adjusted with water to 20ml. An aliquot of 1 ml was added to 10 ml of scintillation cocktail and the radioactivity was counted in the liquid scintillation counter.

After collection of feces, the filter papers were removed from the glass beakers and eluted with methanol until the eluate was free of radioactivity. The glass beakers were rinsed with methanol which was combined with the filter eluate. The total eluates were evaporated to a small volume, which was combined with the eluate of the bladder and adjusted with water to 10 ml. An aliquot of 1 ml was mixed with 10 ml of scintillation cocktail and the radioactivity was measured. The collected feces boli were solubilized with HNO<sub>3</sub> as outlined for the organ samples.

## IN VITRO EXPERIMENTS

### Stability of DAA at different pH-values:

1mg/ml DAA was treated with 0.1 N HCl (pH = 1) or 0.1 N NaOH (pH = 13) at 37°C. Aliquots of 100 µl were removed after 0 min, 30 min, 60 min, 120 min, 4 h, 6 h, 12h and 24 h and subjected to HPLC for analysis. In another experiment 1mg of DAA was heated in 8% H<sub>2</sub>SO<sub>4</sub> at 95°C for 15 minutes and 20 µl were analyzed by HPLC.

**Incubation of DAA with mouse duodenum:** A piece of mouse duodenum (ca. 15 cm) was excised directly after the animal was killed with CO<sub>2</sub>. The luminal and serosal side were rinsed twice with ice cold Tyrode's solution and the intestine was tied at one end. The test compound (1mg DAA containing 1µCi of <sup>3</sup>H or 1mg PNG) dissolved in 1 ml Tyrode's solution was filled into the lumen with an Eppendorf pipette and the open end of the intestine was tied. The serosal side was rinsed twice with Tyrode's solution and the intestine was placed in a 50 ml plastic cup, containing 10 ml of Tyrode's solution. The cup was filled with oxygen and placed in a shaking incubator at 37°C. Samples of 100 µl were removed after one hour from the internal and external fluid and subjected to HPLC analysis. After the end of the incubation time the intestine was rinsed with Tyrode's solution and homogenized with an ultraturax. After centrifugation the precipitate was washed several times with saline and finally solubilized in concentrated HNO<sub>3</sub>. The clear solution obtained was neutralized with KOH and the radioactivity was counted as outlined before.

**Incubation of DAA with mouse liver homogenate:** 10 ml of ice cold Hank's buffer containing 10 mM dithiothreitol was added to 1 g of freshly excised mouse liver and the tissue was homogenized with an Ultraturax. The homogenate was filled into 1 ml Eppendorf cups and centrifuged at 4°C and 13,000 g for 30 minutes. The supernatant was removed and subjected to Eppendorf cups. The liver homogenate samples were either directly used or stored at -80°C. Incubation was performed by mixing 50 µl of a solution containing either 50 µg DAA (1µCi) or 50 µg PNG with 10µl of liver homogenate. The reaction mixtures were incubated 90 minutes at 37°C. Thereafter the samples were cooled on ice and 50 µl of ice cold methanol was added. After 5 minutes on ice, the samples were centrifuged at 4°C and 13,000 g for 10 minutes. 20 µl of the supernatants were removed and subjected to HPLC

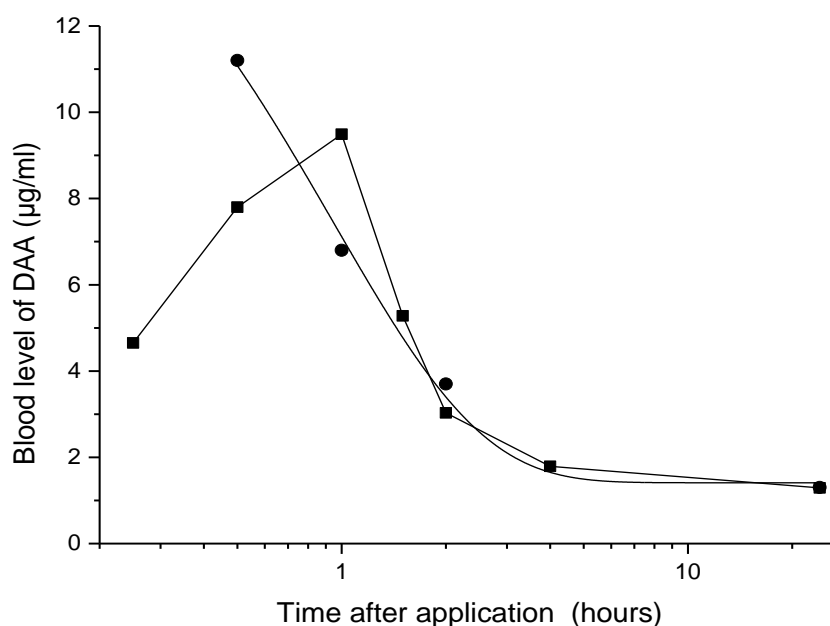
analysis. The precipitates were washed with ice cold methanol several times and solubilized in HNO<sub>3</sub> in the process described above.

**Incubation of DAA with human intestinal bacteria:** A fresh sample of human feces (1g) from a healthy volunteer was inoculated in 100 ml of Nutrient broth (Sigma, Aldrich) and incubated in a rotating incubator at 37°C for 24 hours. 100 µl of the bacterial suspension was transferred to an Eppendorf cup and 1 µg of DAA (1 µCi) in 10 µl of water was added. The mixture was incubated in an Eppendorf Thermomixer® at 37°C. Samples of 20 µl were removed at 2 and 24 hours and mixed with 20 µl of ice cold methanol. After 5 minutes on ice, the samples were centrifuged at 4°C and 13,000 g for 10 minutes. 20 µl of the supernatants were removed and subjected to HPLC analysis.

## RESULTS

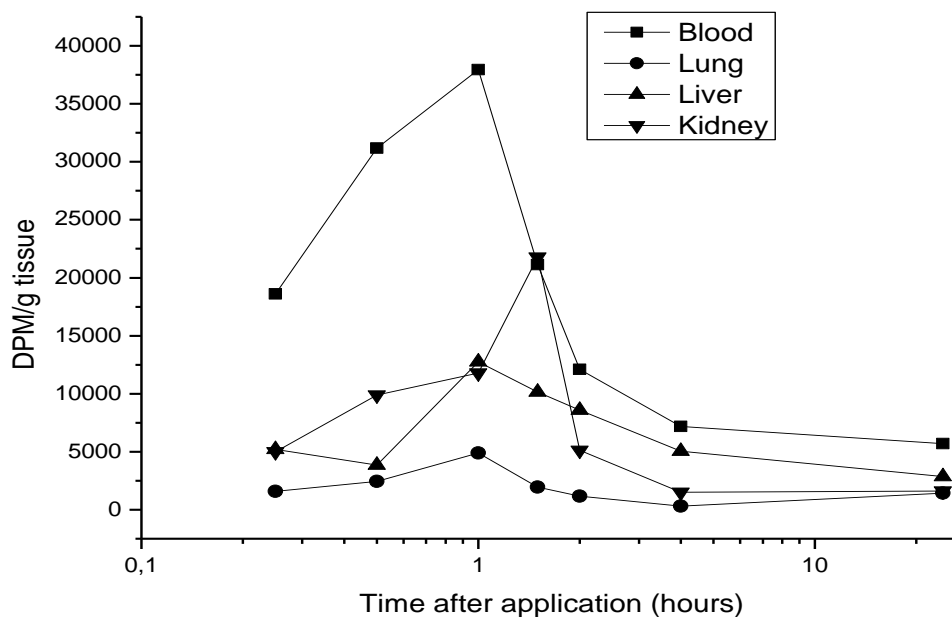
The stability of DAA was tested in aqueous solution at various pH-values. At 37°C the compound was stable in the range between pH 1-13 over a time period of 24 hours. After heating of DAA with 8% sulfuric acid for 15 minutes at 95°C, the glycoside was destroyed; however, no aglycone could be isolated.

The blood concentration of tritium after oral gavage of  $1.5 \times 10^6$  DPM <sup>3</sup>H -DAA (equivalent to 25 mg/kg bw) expressed in µg/ml for two independent experiments is shown in Fig 1. Similar results were obtained for the two independent experiments, although only one animal per time point was used (due to a direction of our institute) to keep the number of animals for experiments as low as possible. Maximum serum concentrations (equivalent to 10 µg DAA/ml) were reached one hour after application in the second experiment, whereas in the first experiment the highest radioactivity was already reached after 30 minutes, which was the first sampling time. A half live of  $\approx 0.5$  hours was calculated from these plots using a first order logarithmical curve fitting.

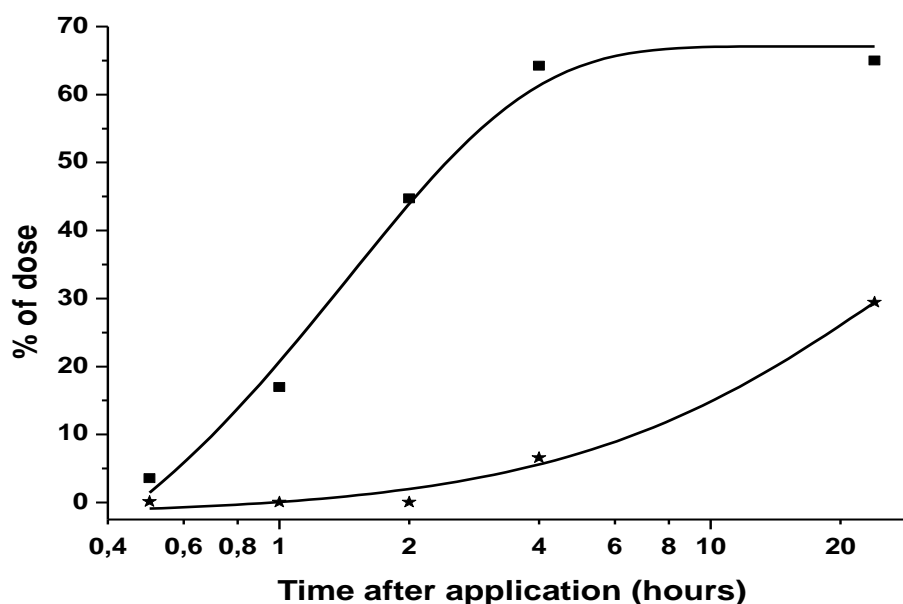


**Fig.1** Blood level of <sup>3</sup>H-DAA in mice after application of 25 mg ( $1.5 \times 10^6$  DPM) / kg bw ( -●- first experiment; -■- second experiment)

Fig. 2 shows the  $^3\text{H}$ -concentration of lung, kidney, liver and blood from the experiment starting with a sampling time of 15 minutes. Highest organ concentrations were observed in kidney and liver followed by lung. The data for heart, brain and muscle were also determined but are not shown in this figure. Their values were below that of the lungs and appear to resemble the blood content of these organs. The time course of the  $^3\text{H}$ -concentrations in the organs followed that of the blood, with the exception of kidneys, which showed a delay in the maximum concentration. The quantitative excretion of  $^3\text{H}$  in urine and feces is shown in Fig.3. More than 60% of the radioactivity was excreted in urine within the first four hours, whereas no radioactivity was found in feces before this time. The total excretion of  $^3\text{H}$  in urine and feces after 24 hours was above 90%.

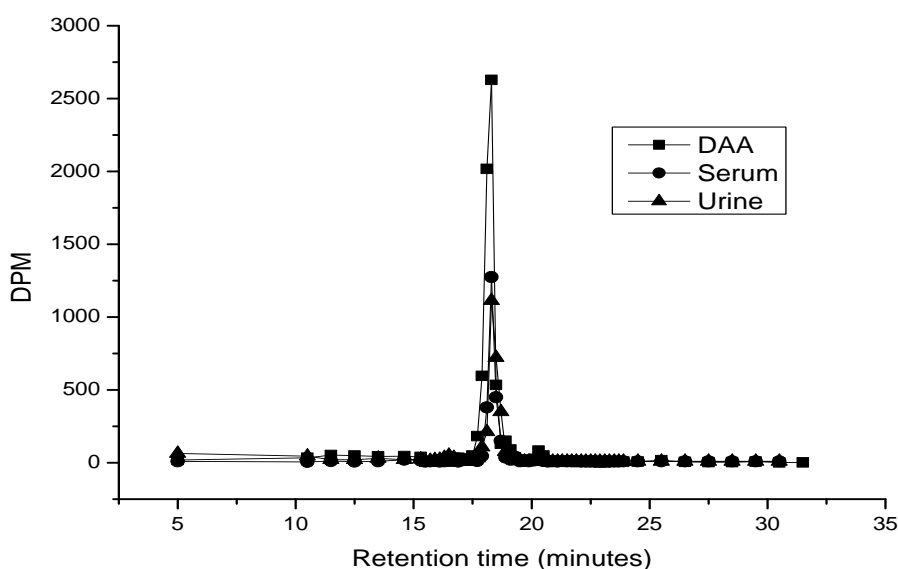


**Fig.2** Organ distribution of  $^3\text{H}$ -DAA in mice after application of 25 mg ( $1.5 \times 10^6$  DPM) / kg bw

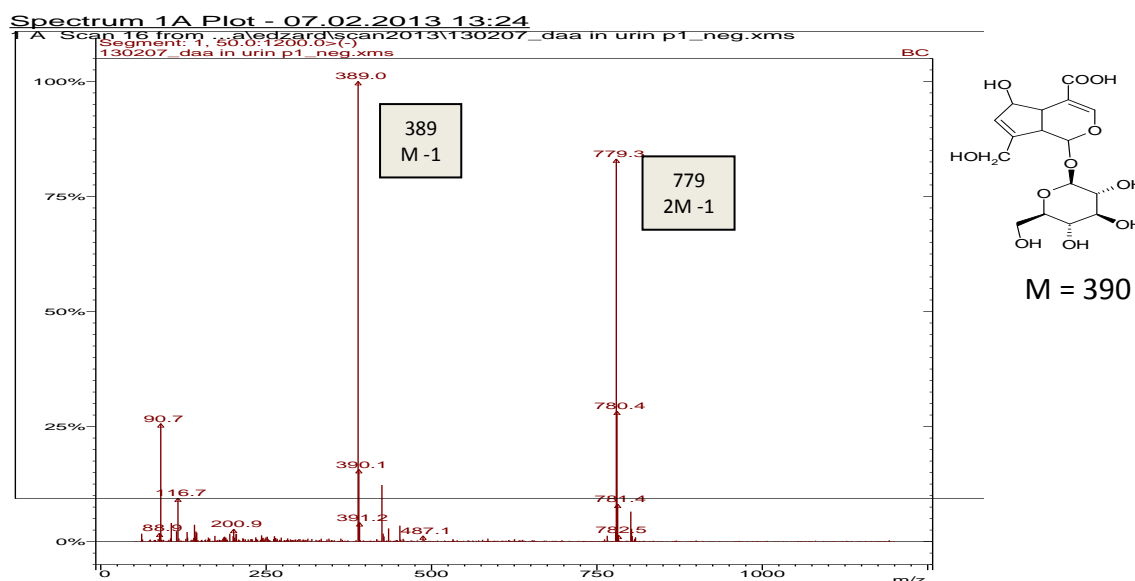


**Fig.3** Excretion of  $^3\text{H}$ -DAA in urine (-■-) and feces (-★-) of mice after application of 25 mg ( $1.5 \times 10^6$  DPM) / kg

HPLC-radiochromatograms performed with samples of serum and urine of mice after oral application of  $^3\text{H}$ -DAA (25mg/kg bw) are shown in Fig.4. Both chromatograms contained only one peak with a retention time of 18.3 minutes, which is identical with that of DAA. In order to identify the compound related to the peak, the fraction was collected and applied to HPLC-MS in the positive and negative mode. The resulting mass spectra are shown in Fig. 5 and confirm that the peak consisted indeed of DAA. Peaks with a molecular mass of 389 Da (M-1) and 779 Da (2M-1) and 413 (M+23) and 803 (2M+23) were observed in the negative and positive MS modes, respectively. Moreover, the fragmentation patterns of the urine metabolite of DAA were identical with that of a DAA standard (data not shown). The conclusion from this experiment is that after oral application of DAA to mice, the compound is absorbed and excreted in the urine without metabolism.



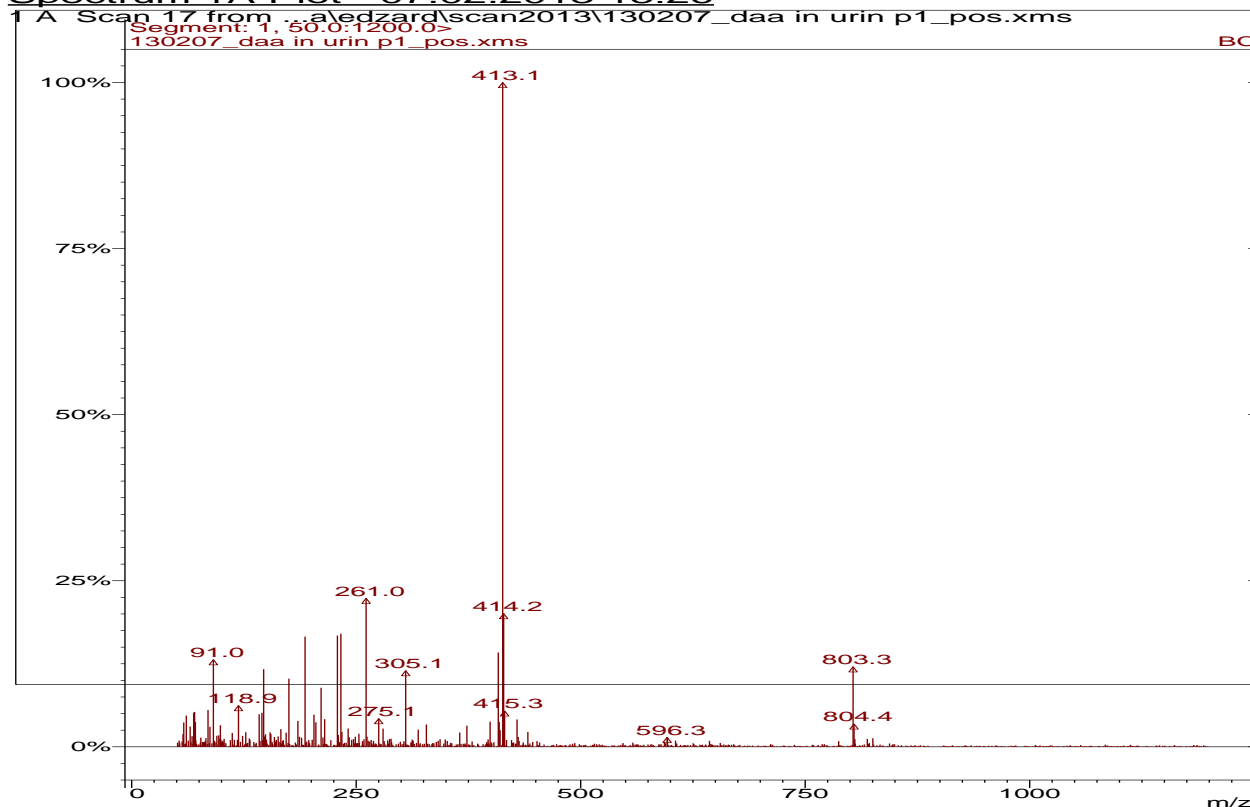
**Fig.4** Superimposition of HPLC-chromatograms of authentic DAA and serum and urine after oral application of  $^3\text{H}$ -DAA to mice. Details are described under materials and methods.



**Fig.5A** Mass spectrum (negative mode) of radioactive metabolite isolated from urine of mice after application of 25 mg ( $1.5 \times 10^6$  DPM) / kg bw



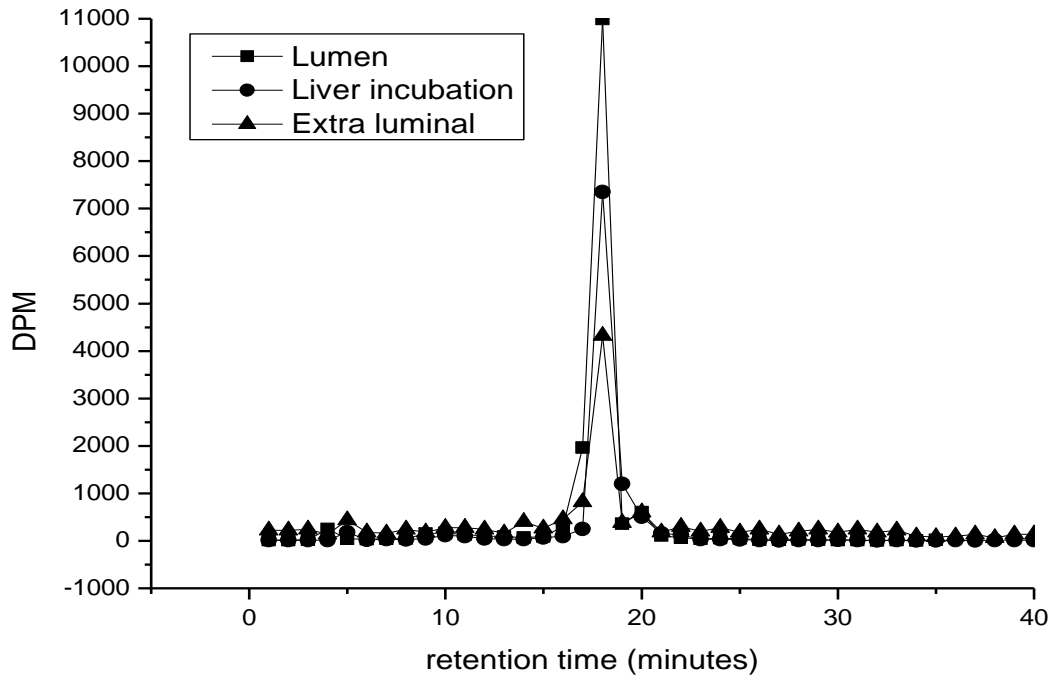
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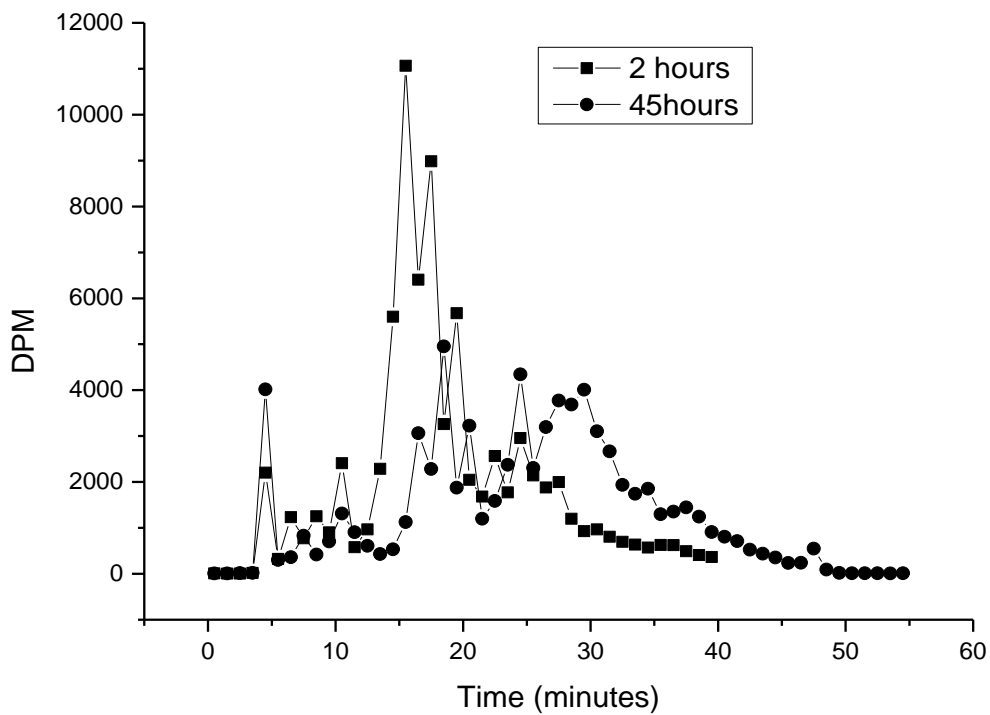
**Fig.5B** Mass spectrum (positive mode) of radioactive metabolite isolated from urine of mice after application of 25 mg ( $1.5 \times 10^6$  DPM) / kg bw

In order to further confirm this finding, radioactive DAA was subjected to the luminal site of a freshly excised piece of mouse small intestine, which was placed in a bath of Tyrode's solution at 37°C (for details see materials and methods). After one hour of incubation, samples were taken from the intra and extra luminal site and subjected to HPLC.  $^3\text{H}$ -DAA was also incubated with a homogenate prepared from mouse liver. After one hour of incubation protein was precipitated by the addition of ice cold 10% perchloric acid and the supernatant was subjected to HPLC. A superimposition of the three radio-chromatograms is shown in Fig. 6. The retention times of all three peaks were identical with that of DAA, which shows that neither enzymes of the intestinal wall nor the liver are able to metabolize DAA. 4-Nitrophenyl- $\beta$ -D-glucopyranoside was run in parallel as a positive control. The compound was hydrolyzed to p-nitrophenol and glucose by both, mouse intestine and liver homogenate (data not shown), demonstrating the activity of glucosidases in the preparations. We also analyzed the protein precipitates of the intestine and liver for possible attachment of radioactivity; however, we could not observe any of it.

Next, we investigated the possibility of human intestinal bacteria to metabolize DAA. For this purpose, a suspension of bacteria from human feces was inoculated in nutrient broth with  $^3\text{H}$ -DAA ( $2.2 \times 10^6$  DPM). After 2 and 24 hours, samples were taken and subjected to HPLC (for details see materials and methods). The radiochromatograms are shown in Fig. 7. A considerable breakdown of DAA already occurs after only two hours and is nearly complete after 24 hours. Several radioactive peaks could be detected in the chromatograms, which demonstrate the capability of human bacteria to metabolize DAA.



**Fig. 6** Superimposition of HPLC chromatograms of radioactivity after in vitro incubation of mouse intestine and liver with <sup>3</sup>H-DAA (50µg, 1µCi). Details are described under “Materials and Methods”



**Fig. 7** HPLC chromatograms of <sup>3</sup>H-DAA (1 µCi) after incubation with human intestinal bacteria. Details are described under” Materials and Methods”.

## DISCUSSION

Iridoids are terpenoids synthesized mainly by plants, but some also occur in animals (Dobler et al. 2011). In plants, iridoids mainly occur as glycosides located in the leaves. However, some plants also store iridoids in fruits. Among these are noni (*Morinda citrifolia*) [15], blueberries (*Vaccinium myrtillus*), cranberries (*Vaccinium macrocarpon*) [18] and Cornelian cherries (*Cornus mas*) [19]. However, iridoids are relatively stable molecules; for example, in the presence of glycosidases, iridoid aglycones can be formed, which in some cases are highly reactive and able to inactivate functional macromolecules. Plants use these reactions for defense purposes against predators [4]. The intestinal mucosae of herbivorous animals contain glycosidases, which are able to hydrolyze glycosides occurring in plants. This reaction makes the sugar moieties available for digestion and energy production. The released aglycones are mostly lipophilic molecules which enter the circulatory system of the animals, where they are further metabolized or excreted after conjugation with glucuronic acid, sulfate or other phase II metabolites.

Aglycones of iridoids containing a six-membered lactone ring can undergo a ring opening reaction leading to a dialdehyde, which reacts with free amino groups to form Schiff's bases, followed by a sequence of secondary reactions. A well-known example is the formation of blue colored adducts of genipin with amino acids [20]. The aglycone of geniposidic acid was also used as a crosslinking agent for proteins. Because free amino groups are essential for functional macromolecules, such reactions may lead to an inactivation of biologically active molecules [21].

The widespread distribution of iridoids in the plant kingdom made it necessary for herbivorous animals to develop strategies to overcome the toxicity of such compounds. Indeed, many iridoid containing plants are of low toxicity. Such plants are often used as food or feed or for medicinal purposes [11, 16].

The noni plant (*Morinda citrifolia* L.), which grows in most tropical areas worldwide, contains over 16 different iridoids, of which deacetylasperulosidic acid and asperulosidic acid make up over 90% of the total iridoid content [15]. The iridoids in noni do not only occur in the leaves but also in the fruits, which may contain up to 1% of DAA in dry mass (own unpublished results). Noni fruit juice was traditionally used by ancient Polynesian tribes as food, herbal medicine and tonic [12, 13] and is very popular today as a wellness drink worldwide. There is no literature data available about the stability of the DAA aglycone.

We did not observe hydrolysis of DAA by treatment under acidic or alkaline conditions in the range of pH 1-13 at 37°C. It can therefore be concluded that no hydrolysis of DAA occurs in the acidic environment of the stomach. In contrast, aucubin, an iridoid with a similar structure like DAA, but lacking the carboxy-group, was demonstrated to get hydrolyzed under stomach conditions [7]. The relative stability of DAA confirms our observation that the DAA concentration in noni juice (pH 3-4) remains constant even after storage over several years at room temperature. After heating of DAA with 8% sulfuric acid at 95°C for 15 minutes, the molecule disappeared. However, we were not able to isolate an aglycone, which suggests, that the DAA aglycone is very unstable.

Because of its high reactivity, the DAA aglycone is expected to immediately damage the intestinal wall after hydrolysis by intestinal glucosidases. However, this contradicts with the fact that the consumption of 750 ml Noni fruit juice per day over a period of four weeks was well tolerated in a study with human volunteers [14] and the experience of millions of people

worldwide, many of whom drink noni juice regularly up to amounts of several hundred ml per day. Experiments with rats demonstrated that quantities of even 80 ml per kg body weight did not cause any toxic effects [22]. The lack of toxicity suggests that after oral ingestion, either no aglycone of DAA is formed or it is immediately deactivated.

After incubation of  $^3\text{H}$ -DAA with mouse small intestine *in vitro*, no cleavage of the glucoside could be observed and the native compound was detected in the extra luminal fluid, demonstrating a possible absorption of the iridoid (Fig.6), which would make the liver the next target for the compound. We therefore investigated, whether glucosidases of the liver are able to cleave the glucoside by incubation of mouse liver homogenate with  $^3\text{H}$ -DAA. Again, no cleavage occurred (Fig 6). In both cases, 4-nitro- $\beta$ -glucopyranoside was hydrolyzed, demonstrating the activity of glucosidases in the intestine and the liver preparation. We also could not detect any radioactive material attached to the proteinaceous matter of the intestinal wall or liver tissue, which might have been formed by reaction of the hypothetical DAA aglycone with amino acids such as lysine. This latter finding also confirms the absence of metabolism of DAA by murine enzymes. In contrast, after treatment of  $^3\text{H}$ -DAA with human intestinal bacteria *in vitro*, a rapid breakdown of the molecule occurred (Fig.7).

The *in vitro* experiments were confirmed *in vivo* by oral application of  $^3\text{H}$ -DAA to mice. The radioactivity was rapidly absorbed and excreted with a half-life of approx. 30 minutes via the kidneys (Fig.1). Highest concentrations of  $^3\text{H}$  were observed in blood at all time points and the kinetic in organs followed that of the blood. Among different organs, highest concentrations of tritium were found in kidney and liver (Fig.2) which is in accordance with the high blood flow through these organs. An HPLC analysis of serum and urine showed only one peak with a retention time identical with that of DAA (Fig.4). An HPLC-MS analysis of the urine metabolite confirmed that the peak was indeed DAA. From these experiments, it can be concluded that the glucoside DAA passes the stomach without degradation and is absorbed without cleavage and excreted unchanged in the urine. The short half-life of the excretion suggests that the absorption takes place in the upper parts of the intestine, which avoids the contact of the compound with intestinal bacteria. Nevertheless, more than 20% of the radioactivity was excreted via feces after 24 hours. Because we did not analyze this radioactivity, we cannot decide whether it is DAA or metabolites thereof. However, the breakdown of DAA by human intestinal bacteria suggests that feces may contain metabolites of DAA. Because we did not detect any metabolites in the serum or urine of the animals, these metabolites are either not absorbed or tightly bound to the fecal compartment.

Glycosides are hydrophilic molecules and therefore expected to be poorly or not absorbed as such. It is well known that many glycosides of secondary plant metabolites are hydrolyzed by intestinal glycosidases [23]. The free aglycones are mostly lipophilic compounds and much better absorbed compared to the parent glycosides. After conjugation with glucuronic acid or sulfate they are excreted via the kidneys or bile. In the case of iridoids with highly reactive aglycones, hydrolysis of the glycosides would lead to toxic compounds, able to damage the intestinal wall or—after absorption—the inner organs of herbivorous animals. A way out for the animals could be to develop glycosidases, with reduced or lacking affinity to such glycosides. According to our findings, this seems to be case for DAA. However, the compounds would then reach the deeper parts of the intestine, where they come into contact with bacteria, able to hydrolyze it, which could finally nevertheless result in toxic effects to the animals. Therefore, the ideal solution for the animal is to absorb the iridoid

glycosides in the small intestine, possibly by an active transport mechanism, and excrete it unchanged. This is what we observed in our experiments with DAA. We also investigated the pharmacokinetics of a variety of other iridoids (geniposide, geniposidic acid, loganin and loganic acid) and found that they are also excreted, at least partly, unchanged via urine (these results will be published in a separate publication). Absorption of iridoid glucosides including DAA have also been reported by other authors [24, 25]. Evidence for a possible cleavage of geniposide has been published by Hou et al [9]. The authors reported about the presence of a sulfate conjugate of genipine in the plasma of rats after oral application of a decoction of the fruits of *Gardenia Jasminoides*, containing geniposide. The hypothesis was based on the fact that pure glucuronidase from bovine liver did not hydrolyze the metabolite observed in the plasma of rats, whereas a mixture of glucuronidase and sulfatase from *Helix pomatia* was effective. However, it is known that this enzyme preparation also contains  $\beta$ -glucosidase activity [26]. Thus, the experiment does not exclude the presence of geniposide in the rat plasma.

The biological properties of iridoids are the result of its contact with systemic targets, such as enzymes and cellular receptors. Anti-inflammatory activities are typical for many iridoids [11]. Studies performed in vitro [27, 28] showed that the inhibition of lipoxygenases and cyclooxygenases by iridoids required the presence of  $\beta$ -glucosidase. However, the glucosidases used by these authors were from almonds and it is unclear whether the investigated iridoids would have been hydrolyzed by mammalian glucosidases. Our investigation clearly shows that at least DAA is stable in the presence of intestinal and liver glucosidases from mice. It is therefore likely that DAA exerts its biological properties via a direct interaction with functional macromolecules.

The absorption of intact iridoid glycosides could be an adaptive process of animals to avoid the toxicity of potential food plants. Further investigations are needed to evaluate the mode of action of biologically active iridoids. Because of the multitude of chemical structures among plant derived iridoids and their different chemical reactivities, it is likely and expected that there is more than just one mode of action.

## CONCLUSION

After oral application of the iridoid glucoside deacetylasperulosidic acid (DAA), the compound was absorbed without cleavage and the intact glucoside was excreted via the kidneys. It was demonstrated that neither intestinal nor liver glucosidases of mice were able to hydrolyze DAA—however, the molecule was broken down by intestinal bacteria. The rapid absorption and excretion of the unchanged glucoside of DAA is possibly an evolutionary adaptive process to avoid the toxic effects of the DAA aglycone formed after hydrolysis of the glycosidic bond.

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**Authors' Contributions:** All authors contributed to this study.

**Abbreviations:** AA, asperulosidic acid; DAA, deacetylasperulosidic acid; DPM, decays per minute; EtOH, ethanol; HPLC, high performance liquid chromatography; MeOH, methanol,

MeCN, acetonitrile; NMR, nuclear magnetic resonance; MS, mass spectroscopy; PNG, 4-Nitrophenyl- $\beta$ -D-glucopyranoside, TFA, trifluoroacetic acid;

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