Transformation of Mangiferin to Norathyriol by Human Fecal Matrix in Anaerobic Conditions: Comprehensive NMR of the Xanthone Metabolites, Antioxidant Capacity, and Comparative Cytotoxicity Against Cancer Cell Lines

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Abstract
Several natural drugs (termed prodrugs) when administered orally undergo transformation by intestinal bacteria, producing metabolites, which may be more active than the parent compound. Mangiferin (I) is reported to have very low bioavailability in the upper gastrointestinal tract and reaches the large intestine, where it may be metabolized by the indigenous bacteria. Therefore, the aim of this study was to conduct pilot anaerobic fermentation studies with fecal inocula from human volunteers (n = 3) to identify possible metabolic end products of mangiferin by the gastrointestinal metabolome. The major metabolite identified was deglycosylated mangiferin, namely, norathyriol (II) with an increase in homomangiferin (III) which was a minor contaminant of I. Mangiferin metabolites were identified and quantitated in the fermentation broths by high performance liquid chromatography (HPLC)–diode array detection–electrospray ionization-mass spectrometry, and structures confirmed unequivocally by nuclear magnetic resonance, after purification by semipreparative HPLC. Cell culture assays with 2 human cancer cell lines Caco-2 (colon cancer) and A240286S (non-small lung adenocarcinoma) showed that while the substrate mangiferin (I) and homomangiferin (III), a minor metabolite, are non-cytotoxic (half-maximal inhibitory concentration [IC₅₀] ≥ 100 µM), the major metabolite norathyriol (II) is cytotoxic against Caco-2 cells (IC₅₀ = 51.0 µM), whereas it is cytostatic against A240286S cells with a similar IC₅₀ (51.1 µM).

Keywords
mangiferin, metabolism, fecal bacteria, norathyriol, HPLC-ESI-MS, NMR

Received: December 2nd, 2019; Accepted: January 15th, 2020.

Many natural products are available worldwide as potential cancer chemoprotective agents and are present in vegetables, fruits, plant extracts, and herbs. The xanthone glycoside mangiferin (1,3,6,7-tetrahydroxanthone-C2-beta-D-glucoside) has already been reported to have strong antioxidant, antiviral, antitumor as well as other activities.¹⁻¹⁰ As for many other glycosides, mangiferin may also exert its activity as a prodrug, following metabolism at a specific site in the body into several other metabolites, which may have stronger biologic activities than the parent compound itself.

Between 5% and 7% of drugs approved worldwide are reported to be or are classified as prodrugs.¹¹ Several glycosides of natural products are prodrugs and an example are the senosides, which have been used for many years as laxatives.
Sennosides taken orally are hydrolyzed by the colonic microflora to liberate the active rhein anthrones. The intestinal microflora produces a variety of glycosidases, and therefore, a number of synthetic glycosides have been examined as potential colon-specific delivery systems, in particular corticoids. A similar strategy was applied to the colon-specific delivery of corticosteroids used to treat inflammatory bowel disease. Davis reported that all drugs which are absorbed into the bloodstream are subjected to hepatic metabolism. Most administered drugs are rapidly and completely absorbed in the upper gastrointestinal tract and therefore do not come into contact with the bacterial flora of the large bowel. However, Biopharma is providing an increasing number of new drug candidates that have low solubility, low permeability, or both. Drugs that display these properties enter the large bowel and are subjected to metabolism by the indigenous bacterial flora therein and the metabolites may be absorbed by the colonic mucosa.

Several researchers have reported that the bioavailability of orally administered mangiferin is very low, and in rats, it has been shown to be as low as 1.2%, but there is no data on blood levels of its major metabolite norathyriol in humans. Pure norathyriol is extremely expensive, and therefore, we sought a local source for the production of sufficient material to assess the chemopreventive capacity of this compound against human cancer cell lines. Human intestinal bacteria are reported to deglycosylate C-glucosidic derivatives such as anthrones, flavones, and xanthones. Therefore, in this study, we screened a very small number of fecal samples from 3 male volunteers (Table 1) to establish a source of biologic material, whereby norathyriol could be produced in high yield from mangiferin by fermentation in anaerobic conditions.

Fecal inocula of V1 consistently degraded mangiferin (I) to norathyriol (II) with the production of minor amounts of homomangiferin (III) after 120 hours of incubation.

Table 1. Brief Profiles of the Human Volunteers (N = 3).

| Parameters          | Volunteer 1 | Volunteer 2 | Volunteer 3 |
|---------------------|-------------|-------------|-------------|
| Age                 | 32          | 62          | 59          |
| Gender              | Male        | Male        | Male        |
| Body mass index     | 22.8        | 24          | 21          |
| Diet                | Low-fat     | Normal      | Normal      |
| Smoker              | No          | No          | Yes         |
| Alcohol consumption | No          | Moderate    | Average     |
| Sports              | Moderate    | Moderate    | Moderate    |
| Medication          | None        | Beta blocker| None        |

BMI, Body mass index.

Following isolation and purification of II and III, cell culture assays with 2 human cancer cell lines, Caco-2 (colon cancer) and A240286S (lung adenocarcinoma), showed that while mangiferin (I) and its methyl derivative homomangiferin (III) were non-cytotoxic (half-maximal inhibitory concentration [IC_{50}] > 100 µM) against both cell lines, the major metabolite norathyriol (II) was cytotoxic against Caco-2 cells (IC_{50} = 51.0 µM); whereas, it was only cytostatic against A240286S cells with a similar IC_{50} (51.1 µM).

The data shows that the transformation of mangiferin to bacterial metabolites was extremely variable even in this very small pilot study using a fecal matrix donated by 3 male volunteers. V1 metabolized mangiferin (Figure 1) extremely rapidly with over 80% conversion to metabolites in 12 hours and was virtually complete at 120 hours. With V2, metabolism (Figure 2) was incomplete at 120 hours and reached a maximum of 32% at 48 hours with no increase thereafter. On the other hand, the metabolism of mangiferin was not detected with fecal inocula from V3.

![Figure 1](image_url). Transformation at 120 hours of mangiferin (I) to norathyriol (II) and homomangiferin (III) by volunteer 1.
and mangiferin remained unchanged after 120 hours. The data for the time-course experiments are shown in Figure 2.

These results were extremely reproducible when conducted in triplicate and on 3 different occasions. Therefore V1, V2, and V3 can be considered as ultrarapid, intermediate, and non-metabolizers of mangiferin, respectively.20 The major metabolite in the fermentation broths produced by V1 and V2 was initially tentatively identified as norathyriol (II) and the minor metabolite as homomangiferin (III) by high performance liquid chromatography (HPLC)–diode array detection–electrospray ionization (ESI)-mass spectrometry (MS) (Table 2).

The ultraviolet spectra of mangiferin (I) (258, 318, and 366 nm), norathyriol (II) (254, 326, and 364 nm), and homomangiferin (III) (258, 318, and 364 nm) were very similar.

Fermentation of mangiferin with the ultrarapid metabolizer V1 was repeated 10 times, and following fractionation of the fermentation broths by SPE on C18 columns, fractions enriched in the metabolites were subjected to semipreparative HPLC. The structures of mangiferin and its bacterial metabolites are depicted in Figure 9. The fermentation data for V1 is very similar to the in vitro data of Huang et al23 who reported that after 12 hours of incubation with human intestinal bacteria, extensive conversion of mangiferin to norathyriol occurred. The bacterial species of the intestinal microflora responsible for deglycosylation of mangiferin has been identified as Bacteroides sp. MANG,18,19 but studies on human populations, in terms of the incidence and abundance of this species, have not been conducted.

There is only 1 report of a pharmacokinetic study on mangiferin in human plasma. A detailed study was conducted by Hou et al15 in which human volunteers were administered a single dose of mangiferin (0.1, 0.3, and 0.9 g, respectively, to 3 groups of 7 volunteers) with 200 mL of water. Analyses of plasma samples by HPLC-ESI-MS revealed maximal concentrations of mangiferin at 1 hour of 19.94 ± 3.47, 34.70 ± 6.83, and 38.64 ± 6.75 ng/mL with increasing dose, which is equivalent to 47.25, 82.23, and 91.56 nM. At this time point, these values represent 0.10%, 0.06%, and 0.02% of the administered doses of mangiferin. They concluded that the pharmacokinetics of mangiferin in plasma were not linear. However, no attempt was made to evaluate the presence of possible glucuronide or sulfate derivatives in plasma, feces, or urine.

As yet, there are no reports on the plasma concentrations of the major metabolite of mangiferin, namely, norathyriol in human plasma. However, an intervention study was conducted by Bock et al,24 with mature female pigs administered 74 mg/kg of mangiferin/day in the form of a Cyclopia genistoides (bush) extract for 11 days. Mean plasma concentrations of norathyriol ranged from 7.8 to 11.8 µM equivalent to 2.03 and 3.07 µg/mL, respectively, on days 9 and 11.

The data reported by these authors show that approximately 80% of the administered dose of mangiferin was unaccounted for, based on the ratio of intervention and biospecimen levels. In a further study, aimed at the identification of further bacterial metabolites of norathyriol, Bock et al25 detected 4 phenolic acid metabolites ostensibly derived from mangiferin in pig feces, but they were below the limits of quantitation. The fate of the remainder is as yet unknown. Fermentation studies with human bacterial microflora of norathyriol are underway in our laboratories to establish whether or not complete metabolism through to CO₂ is evident.

### Table 2. High Performance Liquid Chromatography–Electrospray Ionization-Mass Spectrum Data in Negative Ion Mode of Mangiferin (I) and Its Bacterial Metabolites, Norathyriol (II) and Homomangiferin (III)

| No | Molecular formula | Retention time (minutes) | [M − H]⁻ | [2M − H]⁻ |
|----|-------------------|--------------------------|----------|----------|
| I  | C₁₉H₁₉O₁₁         | 20.04                    | 421.1    | 843.1    |
| II | C₁₃H₇O₆           | 36.01                    | 259.1    | 519.1    |
| III| C₂₀H₂₀O₁₁         | 22.20                    | 435.1    | 871.2    |

The ²H and ¹³C NMR data for mangiferin (I) was almost identical to that previously described by us.² The ¹H NMR data for norathyriol (II) is very consistent with that of Qin et al²¹ taking into account solvent effects. Unfortunately, Qin et al²¹ did not report ¹⁳C data for norathyriol. Our NMR data on homomangiferin (III) indicated the presence of 2 rotamers. This is due to hindered rotation caused by the methoxy group located at C3. Our data for III is very consistent, again taking into consideration solvent effects, with that reported by Wei et al²² following organic synthesis, who also detected 2 distinct rotamers of homomangiferin. Our NMR data on I–III presented here represents the most uniform, comprehensive, precise, and unambiguous assignments for these xanthones.
In conclusion, mangiferin can be fermented in vitro to a major (>95%) metabolite namely norathyriol by the fecal matrix. Norathyriol is less effective as an antioxidant compared with the substrate mangiferin (Table 3) but has a far greater (49%; 13.0 µg/mL) anticarcinogenic capacity, as tested against colon and lung cancer cell lines. Screening of the general human population, with regard to the efficacy of the fecal metabolome to produce norathyriol from mangiferin, in tandem with its bioavailability, is therefore warranted.
Reagents and Materials

Mangiferin was purified and characterized as described by Barreto et al.\(^2\) and an authentic standard was also obtained from Extrasynthese (Lyon Nord, Genay, France) for comparison. Acetic acid, acetonitrile, brain heart infusion broth (BHI), K\(_2\)HPO\(_4\), KH\(_2\)PO\(_4\) were obtained from Merck (Darmstadt, Germany); methanol from Carl Roth (Karlsruhe, Germany); Duran bottles, L-cysteine–hydrochloric acid (HCl), and sodium formaldehyde sulphoxylate from Sigma-Aldrich (Steinheim, Germany); sachets of ascorbic acid, AnaeroGen (Nr. 68061), and indicators of anaerobiosis (Nr. BR0055B) from Oxoid Ltd (Basingstoke, UK); anaerobic churn (Big Model Typ DIAB

Figure 5. \(^1\)H nuclear magnetic resonance spectrum at 600 MHz of norathyriol (II).

Figure 6. \(^13\)C nuclear magnetic resonance spectrum at 150 MHz of norathyriol (II).
10003) from Dinkelberg Analytics (Gablingen, Germany), and Sep-Pak C18 cartridges for solid-phase extraction (Supelclean LC-18 SPE in 6 mL tubes, 500 and 5000 mg; Supelco, Bellefonte, PA, USA). All other chemicals used were of analytical grade. Cell culture experiments were performed with human non-small lung carcinoma epithelial tumor (A240286S) and epithelial colon cancer (Caco-2) cell lines.

**Cell Cultures**

The A240286S (non-small lung cancer adenoma) and Caco-2 (colon cancer) cell lines utilized in this study were obtained from the in-house collection of the German Cancer Research Center (Heidelberg, Germany).

![Figure 7](image1.png)

**Figure 7.** $^1$H nuclear magnetic resonance spectrum at 600 MHz of homomangiferin (III).

![Figure 8](image2.png)

**Figure 8.** $^{13}$C nuclear magnetic resonance spectrum at 150 MHz of homomangiferin (III).
Stool Sampling

Stool samples from each volunteer were collected on the same day. Aliquots (approximately 500 mg) prepared in an atmosphere of nitrogen were stored at –80°C for use in the in vitro experiments.

Anaerobic Fermentation of Mangiferin

The BHI broth was prepared by dissolving the powdered material in double distilled water at a concentration of 37 g/L fortified with the reducing agents sodium formaldehyde sulfoxylate (0.3 g/L) and L-cysteine–HCl (0.5 g/L). The BHI broths (100 mL in 100 mL Duran bottles) were sterilized at 120°C for 20 minutes. After adding 0.5 mg/mL of mangiferin (purified from Mango bark as described by Barreto et al) in ethanol (5.0 mL) to the BHI broths, the medium was steamed for 30 minutes to remove residual oxygen and allowed to cool before inoculation with freshly voided fecal matrix (100 mg) from each human volunteer. The bottles, with open caps, were placed in an anaerobic churn (neolAB, Heidelberg, Germany) containing an indicator of anaerobiosis (Anaerobic indicator, BR0055B), which was generated by 2 freshly opened sachets of ascorbic acid (AnaeroGen, Nr. 68061). The churn was sealed and fermentation was commenced at 37°C for 120 hours in triplicate. Samples of the fermentation broths (2.0 mL) at various time points were withdrawn from the Duran bottles inside the anaerobic churn, using a syringe connected by a tubular system, coupled to the anaerobic container, and centrifuged in Eppendorf tubes at 13 000 rpm for 5 minutes. Supernatants were aspirated and aliquots (10 µL) were analyzed by HPLC–ESI-MS for the presence of mangiferin and its bacterial metabolites.

HPLC–ESI-MS

HPLC–ESI-MS was conducted on an Agilent 1100 HPLC, coupled to an Agilent single quadrupole mass-selective detector (HP 1101; Agilent Technologies, Waldbronn, Germany). Chromatographic separation of methanol extracts was conducted using a C18, Gemini reverse-phase (5 µm), column (250 × 4.6 mm I.D.) Phenomenex (Aschaffenburg, Germany). The mobile phase consisted of 2% acetic acid in doubly distilled water (solvent A) and acetonitrile (solvent B) with the following gradient: initially 95% A for 10 minutes; to 90% A in 1 minutes; to 80% A in 9 minutes; to 60% A in 10 minutes; to 40% A in 10 minutes; to 0% A in 5 minutes; and continuing at 0% A until completion of the run. Detection of phenolic compounds was by means of UV absorbance (A) at 257, 278, 320, and 340 nm at 30°C. Mass spectra in negative ion mode were generated under the following conditions: fragmentor voltage, 100 V; capillary voltage, 2500 V; nebulizer pressure, 30 psi;

Table 3. Antioxidant Capacites of Mangiferin (I) and Norathyriol (II) as Determined by the DPPH, FRAP, and ORAC In Vitro Assays.

| Compound        | DPPH assay IC50 (µM) | FRAP assay EC50 (µM) | ORAC assay (ORAC unit) |
|-----------------|----------------------|----------------------|------------------------|
| Mangiferin (I)  | 41.4                 | 281                  | 3.36                   |
| Norathyriol (II)| 114.0                | 7154                 | 1.38                   |

DPPH, 2,2’-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical absorbance capacity; IC50, half-maximal inhibitory concentration; EC50, half-maximal effective concentration.

Figure 9. Structures of the substrate mangiferin (I) and its bacterial metabolites, norathyriol (II) and homomangiferin (III).
drying gas temperature, 350°C, and mass range, 100-1500 D. Instrument control and data handling were by means of an HP Chemstation operating in the Microsoft Windows software environment.

**Chromatography on C18 SPE Columns**

Fermentation of mangiferin with the ultrarapid metabolizer V1 was repeated 10 times and completed fermentation broths were placed in 50 mL Falcon tubes and spun at 5000 rpm for 15 minutes. The supernatants were decanted and applied to C18 columns for subfractionation. The columns were preconditioned with methanol (50 mL) and double distilled water (50 mL) and were not allowed to dry. Elution was performed with solvent mixtures containing increasing concentrations of methanol (0%, 5%, 25%, 50%, and 100%) in 2% acetic acid, resulting in 5 fractions. The solvent was removed on a freeze drier (Christ, Gefriertrocknungsanlagen, Osterode, Germany). Individual phenolic compounds in relevant fractions were purified by semipreparative HPLC for structure elucidation by spectroscopic analyses.

**Semipreparative HPLC**

Semipreparative HPLC was conducted on an HP 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) fitted with an Agilent C18 reverse-phase Zorbax Eclipse Plus Phenyl Hexyl column (250 × 10 mm I.D.) under the conditions described by Owen et al.\textsuperscript{26} but at 30°C.

**Nano-ESI-MS**

Mangiferin and the bacterial metabolites were analyzed as described by Owen et al.\textsuperscript{27} after purification by semipreparative HPLC.

**NMR Spectroscopy**

NMR spectra for all compounds were recorded on a Bruker Avance II(III) spectrometer equipped with a 5 mm inverse(normal)-configuration probe with triple(z)-axis gradient capability at a field strength of 14.1(9.4) T operating at 600–1(400.1) and 150.9(100.6) MHz for \textsuperscript{1}H and \textsuperscript{13}C nuclei, respectively, at 303 K. The xanthones were poorly soluble in CD\textsubscript{3}OD (99.8% D), typically 3–10 mg in 4.0 mL, so that a 3:1 mixture of CD\textsubscript{3}OD/dimethyl sulfoxide-$d_6$ was required. Conventional 1-dimensional (1D) and 2-dimensional (2D) Fourier transform techniques were employed as necessary to achieve unequivocal signal assignments and structure proof for all compounds independently. In addition to 2D shift-correlation experiments (\textsuperscript{1}H-\textsuperscript{1}H correlation spectroscopy with long-range connectivities; C-H correlation via \textsuperscript{1}H-\textsuperscript{13}C), extensive use was made of \textsuperscript{1}H-coupled \textsuperscript{13}C spectra and selective \textsuperscript{1}H-decoupling to determine long-range \textsuperscript{13}C coupling constants and to assign all quaternary carbons unambiguously. Where necessary, stereochemical assignments were made with the aid of 1D nuclear Overhauser difference spectra or 2D rotating frame Overhauser effect spectroscopy experiments. Detailed analysis of resolution-enhance spectra was performed using Bruker’s WIN-NMR (peak picking, integration, multiplet analysis) and WIN-DAISY (spin system simulation and iteration) software for PCs. \textsuperscript{1}H and \textsuperscript{13}C chemical shifts (\(\delta\)) are reported in ppm relative to CH\textsubscript{3}OD (\(\delta = 3.30\) ppm for \textsuperscript{1}H) or CD\textsubscript{3}OD (49.0 for \textsuperscript{13}C) or internal standard Me\textsubscript{4}Si (TMS, \(\delta = 0.0\)) in mixed solvents.

**MTT Assay**

The cytotoxicity of the xanthones (I–III) was determined by the MTT assay as described by Stibarova et al.\textsuperscript{28}

**Antioxidant Assays**

The antioxidant assays were conducted exactly as described by Maia et al.\textsuperscript{29}

**Statistics**

The concentration of xanthones producing 50% cytotoxicity (IC\textsubscript{50}) against the cancer cell lines was determined using the Table Curve program (Jandel Scientific, Chicago, IL, USA).

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The authors should like to express their thanks for financial support from the CNPq, CAPES/DAAD Sandwich Doctorate program (Brazil).

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**Supplemental Material**

Supplemental material for this article is available online.

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