Human gut microbiota metabolism of dietary sesquiterpene lactones: Untargeted metabolomics study of lactucopicrin and lactucin conversion *in vitro* and *in vivo*.

Carlos J. García[a]*; David Beltrán*[a] *and Francisco A. Tomás-Barberán*[ab]*

[a]Research group of Quality, Safety, and Bioactivity of Plant Foods. CEBAS-CSIC, P.O. Box 164, Murcia 30100, Spain. [b] Foods for Health Chair, UCAM Catholic University Murcia, Spain.

*Contact. E-mail: fatomas@cebas.csic.es

Abbreviations: **ABB**, anaerobe basal broth; **EIC**, extracted ion chromatogram; **ESI**, electrospray ionization; **FbI**, find by ion; **MFE**, molecular feature extraction; **MPP**, mass profiler professional; **NB**, nutrient broth; **PLS-DA**, partial least square discriminant analysis; **PVDF**, polyvinylidene fluoride; **QTOF**, quadrupole time of flight; **RFE**, recursive feature extraction; **Rt**, retention time; **UPLC**, ultra-performance liquid chromatography; **VIP**, variable importance in projection.

Keywords. Escarole / Gut microbiota metabolism / Lettuce (*Lactuca sativa*) / Sesquiterpene lactones / Untargeted metabolomics

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Scope: Gut microbiota converts dietary phytochemicals into metabolites and modulates their health effects. The microbial metabolism of dietary terpenoids, as the sesquiterpene lactones of leafy vegetables, is unknown.

Methods & Results: *In vitro* fermentation of lactucopicrin, lactucin, and romaine lettuce with gut microbiota from independent donors, showed their extensive metabolism through untargeted metabolomics of the fecal incubations. Dehydroxylations and double bond hydrogenations were the main catabolic reactions. Isomers of dihydrolactucopicrin, tetrahydrolactucopicrin, and deoxylactucin, were observed after lactucopicrin metabolism. Tetrahydrolactucin and hexahydrolactucin were also found after lactucin metabolism. Lettuce fermentation showed similar metabolic conversions. Phase II conjugates of most of these metabolites were detected in the urine of healthy volunteers after escarole salad intake. Glucuronides, and sulfates, of dihydrolactucopicrin, tetrahydrolactucopicrin, dihydrolactucin, and deoxylactucin, were detected in the urine although with large inter-subject variability.

Conclusion: This is the first report on the gut microbiota metabolism of sesquiterpene lactones in humans, and one of the first reports to describe that dietary terpenoids of widely consumed leafy vegetables are extensively catabolized by human gut microbiota. A large inter-subject variation in the metabolism of sesquiterpene lactones also reflects differences in gut microbiota composition. It suggests that inter-individual differences in their health effects should be expected.

1 Introduction

Gut microbiota is known to metabolize dietary phytochemicals leading to metabolites that are better absorbed than the parent compounds, persist in the body for long times, and show relevant bioactivity.[1] This has been extensively demonstrated for (poly)phenols. Active microbial metabolites have been identified after the metabolism of soy isoflavones,[2] flaxseed lignans,[3] hops and beer flavanones,[4,5] tea, cocoa, and wine proanthocyanidins,[6] and pomegranate, nuts, and berries ellagitannins and ellagic acid.[7] The metabolism of other phytochemicals by gut microbiota has been less studied so far. Thus, the metabolism of glucosinolates by gut microbiota has just been reported.[8] However, the human gut microbial metabolism of terpenoid phytochemicals in general, and sesquiterpene lactones, in particular, has not been studied so far to the best of our knowledge. Other terpenoid molecules, as bile acids, are known to be metabolized by the gut microbes through dehydroxylation leading to secondary bile acids,[9] and saponin glycosides are hydrolyzed by gut microbiota leading to the corresponding partially deglycosylated derivatives and finally to aglycones.[10]

Lettuce, endive, and escarole are rich in sesquiterpene lactones that are primarily located in the latex and are responsible for their bitterness and relevant biological effects.[11-13]

In the present study, we aimed to evaluate the gut microbiota metabolism of lettuce and escarole terpenoids, and particularly sesquiterpene lactones, using an *in vitro* fermentation model and then assess the metabolites produced in humans by the untargeted metabolomics analysis of their occurrence in urine after the intake of curly escarole.

2 Experimental Section

2.1 Chemicals

Authentic standards of lactucin, 11,13-dihydro-lactucin, lactucopicrin, and 11,13-dihydro-lactucopicrin were from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, and 0.1% (v/v) formic acid in water (J.T.Baker, Deventer, Netherlands), and formic acid (Panreac, Barcelona, Spain). Nutrient Broth (NB) was from Oxoid (Basingstoke, Hampshire, UK). L-Cysteine hydrochloride (Panreac Química, Barcelona, Spain).

2.2 Plant material

i) Romaine lettuce (*Lactuca sativa*) cultivar was selected from a previous study of enzymatic browning susceptibility. [14] Lettuce heads were processed as described previously with minor modifications.[14] Four lettuce heads were used as independent replicates. A total of 10 leaves per head from the middle part of the head were processed, and midribs were carefully excised (1.5 cm). Samples of 30 g of midribs were directly frozen in liquid nitrogen and stored at −80 °C. Midribs were then freeze-dried and milled into powder using a blender (Oster Professional BPST02-B) before being added to the fermentation medium.

ii) Curly escarole (*Cichorium endibia* var *crispa*) was purchased from a supermarket in Murcia on the same day of intake.

2.3 Collection of human fecal samples

A total of 3 healthy donors of stool samples, two males (age 30 both) and a female (age 28), were recruited at the Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC, Murcia, Spain) and gave written informed consent. These volunteers were classified by their gut microbiota metabolic

ability in one of the three urolithin metabotypes previously described.[15] They indicate that the volunteers have different microbial gut ecologies, although the production of urolithin metabolites from ellagic acid used for the stratification has no relevance for lettuce or escarole metabolism.[16] Nevertheless, we wanted to show if the differences observed in the gut microbiota of the different urolithin metabotypes affected sesquiterpene lactone metabolism. Each volunteer provided a fresh stool sample early in the morning to do the fermentation experiments. Samples were stored at 4 °C and further processed within one hour of donation.

2.4 Collection of human urine samples

A total of 5 healthy volunteers, three males (age 28, 32, and 43) and two females (age 28 and 32), were recruited as donors of urine samples at CEBAS-CSIC, Murcia, Spain. Volunteers ingested 250 g of fresh escarole as part of a regular salad and collected their urine for 24 h after the intake. Institutional ethical approval was not necessary as experiments were carried out with freely available foodstuff, and only urine samples were collected, as advised by the Catholic University of Murcia Ethical Committee. The volunteers gave written informed consent. Urine samples (24 h) were collected and stored at 4 °C until delivered at CEBAS-CSIC Institute. Samples were vortexed and then centrifuged at 18,000 g for 10 min (1-16K Microcentrifuge, Sigma Laborzentrifugen GmbH, Osterode am Hanz, Germany), followed by 1-2 dilution with UPLC grade water. Aliquots of 2 mL of the homogenized urine samples were vortexed for 2 min and filtered by a 0.22 µm PVDF filter (Merck Millipore, Cork, Ireland) before injection in UPLC-ESI-QTOF-MS.

2.5 Conversion experiments by human fecal cultures

Preparation of fecal suspensions and subsequent fermentation experiments were performed under anoxic conditions in an anaerobic chamber (Concept 400, Baker Ruskinn Technologies, Ltd, Bridgend, South Wales, United Kingdom) with an atmosphere consisting of $N_2/H_2/CO_2$ (85:5:10) at 37 °C. Aliquots of stool samples (10 g) were diluted 1/10 (w/v) in Nutrients Broth supplemented with 0.05%

L-cysteine hydrochloride and homogenized by a stomacher in filter bags. Aliquots of fecal suspensions (50 µL) were inoculated into 5 mL of fermentation medium anaerobe basal broth (ABB, Oxoid, Basingstoke, Hampshire, UK) containing either 15 µM lactucopicrin (0.25% DMSO in the final culture medium) or 15 µM lactucin (1% MeOH in the final culture medium). Freeze-dried romaine lettuce powder (0.2 g) was also fermented independently. Three replicate cultures were prepared in parallel from each fecal suspension. Two different controls were used; one without fecal suspension and another without the substrates, either lactucopicrin or lactucin. Samples (5 mL) were collected after five days of incubation at 37 °C and extracted with 5 mL of ethyl acetate LC-MS (Scharlau, Barcelona, Spain) using a refrigerated thermoblock shaker (VWR lnternational, LLC, Radnor, PA, USA) at 20 °C for 10 min at 1500 rpm. Samples were centrifuged at 3500g for 10 min at 4 °C. The organic phase was evaporated under reduced pressure in a speed vacuum concentrator (Savant SPD121P, Thermo Scientific, Waltham, MA, USA), re-dissolved in 500 µL of methanol, and filtered through a 0.22 µm PVDF filter (Merck Millipore, Cork, Ireland). Samples were diluted 1:2 in methanol before the UPLC-ESI-QTOF-MS analysis.

2.6 UPLC-ESI-QTOF-MS analysis

A UPLC system (Agilent 1290 Infinity, Agilent Technologies, Waldbronn, Germany) coupled with a Mass QTOF detector (6550 Accurate, Agilent), electrospray ionization via Jet Stream Technology and a C18 column (Poroshell 120, 3 x 100 mm, 2.7 µm pore size) was used. Chromatographic and mass spectrometry conditions were the same as those described previously.[17] Briefly, 3 µL of the samples dissolved in methanol were injected and separated on the column with a mobile phase composed of water + 0.1% formic acid (A) and ACN+0.1% formic acid (B) at a flow rate of 0.4 mL min^{-1}. Nitrogen was used as a nebulizer (35 psi, 9 L min^{-1}) and drying gas (280 °C, 9 L min^{-1}, sheath gas temperature 400 °C, and sheath gas flow 12 L min^{-1}). Spectra were acquired in the range *m/z* 100–1100 in the negative mode, with a fragmentor voltage (energy) of 100 V and an acquisition rate of 1.5 spectra s^{-1}.

2.7 Untargeted metabolomics data treatment

Raw data were acquired in profile mode from 6550 iFunnel Q-TOF LC/MS Agilent Technologies. Files .d acquired were pre-processed by the Agilent metabolomics software platform using Profinder version B.10.0. A specific methodology was set to be as representative as possible of the total number of ions in samples. The parameters for pre-processing were established based on the batch recursive feature extraction (RFE) for small molecules option. RFE includes a molecular feature extraction (MFE) and then uses mass and retention time from the results to perform a targeted feature extraction referred to as Find by Ion (FbI). The RFE was programmed based on i) Extraction parameters for in input data: Rt (1.000-25.000 minutes), restrict *m/z* (100.0000-1100.0000), peak filter with height >=1000, ion species (negative ions –H and +HCOO); ii) Alignment parameters: Rt tolerance (0.30 minutes); Mass tolerance (± 5 ppm + 2.00 mDa); iii) MFE filters: Absolute height (>=10000 counts), Score (>=80.00 %); iv) FbI: Extraction Ion Chromatogram (EIC) tolerance (symmetric ppm ± 10 and ± 1.5 minutes), EIC peak integration and filtering (Agilent integrate algorithm); vi) FbI filters (Absolute height (>=10000 counts) , Score FbI (>=80.00 %). After pre-processing data, file .profinder was exported to Mass profiler professional (MPP) for processing data and data analysis. The data processing step included and Pareto scaling and a log transformation before data analysis.

2.8 Multivariate data analysis

Partial least square discriminant analysis (PLS-DA) was used as the classification method for modeling the discrimination between the fecal fermentation samples of both sesquiterpenes lactones collected from the three different donors.

Multivariate analysis was performed using Mass Professional Profinder 15.0 (Agilent Technologies, Waldbronn, Germany). The validation type used was N-Fold. The classes in the input data were divided into three random equal parts, and the process was repeated ten times, with a different part being used for testing in every iteration.

3 Results and Discussion

3.1 Untargeted metabolomics discovery of metabolites

After data processing of the total sample dataset, a total of latent 13218 variables were detected for the lactucopicrin conversion study and 11829 for that of lactucin. The calculated PLS-DA model based on the 12 model samples gave: i) Lactucopicrin: two significant components, according to cross-validation, describing 61.2% of the variation in X (R2X 0.612), 99.9% of the variation in the response Y (class) (R2Y 0.999), and predicting 92.5% of the variation in the response Y(class), according to cross-validation (Q2Y 0.925); ii) Lactucin: two significant components, according to cross-validation, describing 62.1% of the variation in X (R2X 0.621), 99.5% of the variation in the response Y (class) (R2Y 0.995), and predicting 72.2% of the variation in the response Y(class), according to cross-validation (Q2Y 0.722).

VIP score criteria were used to select the most discriminant variables. The VIP scores obtained were: i) Lactucopicrin PLS-DA model: 1.4 for tetrahydrolactucopicrin (the higher value than contributed to the model 1.8); ii) Lactucin PLS-DA model: 2.8 and 0.75 for tetrahydro-lactucin and hexahydro-lactucin respectively (the higher values than contributed to the model 2.9).

3.2 *In vitro* fermentation of lactucopicrin, lactucin, and lettuce sesquiterpene lactones

Lactucopicrin and lactucin were submitted to fermentation with fecal microbiota from three healthy subjects independently. A sample of freeze-dried romaine lettuce containing both sesquiterpene lactones was also fermented, and UPLC-QTOF MS analyzed the metabolites produced and compared with the control. The results of the analyses are summarized in **Table 1**. Lactucopicrin was reduced (**R**) by the gut microbiota of the three volunteers to produce 11β,13-dihydrolactucopicrin, identified by comparison with an authentic standard (**Figure 1**). Four other isomers with the same formula and MS-MS fragments were detected, indicating that different isomers could be associated with the hydrogenation of one of the three double bonds, or the two C=O groups available in the molecule (**Figure 2**). Untargeted metabolomics analysis was also able to identify three new isomers of tetrahydrolactucopicrin not previously described and produced during the *in vitro* fermentation (**Figure 2**). Gut microbiota also released the 4-hydroxyphenyl acetic ester in lactucopicrin by an esterase activity (**E)**, and a fragment corresponding with the hydroxyphenyl acetic acid residue ($C_8H_8O_3$) was observed in all cases as well as the dehydroxylated phenyl-acetaldehyde (C_8H_8O) (**Figure 1, Table 1**). However, the hydroxyphenyl acetic metabolites produced during the *in vitro* fermentation of lactucopicrin are not characteristic metabolites of the degradation of this sesquiterpene lactone since hydroxy-phenyl acetic acids are also produced by the gut microbiota metabolism of phenolic compounds and aromatic amino acids.[18, 19]

Lactucin was reduced by the gut microbiota to produce tetrahydro-lactucin and hexahydro-lactucin. These two metabolites were also identified for the first time by untargeted metabolomics. The study highlights that untargeted metabolomics can be used in studies of metabolites conversion by gut microbiota, in samples in which the matrix is very complex with a very high number of mass signals, to discover new metabolites produced by the gut microbiota metabolism.

However, the lactucin released from lactucopicrin was not observed in the fermentation medium, suggesting that it is readily converted by bacterial dehydroxylases (**DH**) into three different isomers of deoxy-lactucin, deoxy-dihydrolactucin, and deoxy-tetrahydro-lactucin (**Figure 1, Table 1**).

In the *in vitro* fermentation of lactucin, no other conversion metabolites were observed apart from those produced by hydrogenation (reduction) of the double bond.

Deoxylactucin was the most common metabolite ($C_{15}H_{16}O_4$), although dehydrolactucin (lactucin – H_2O) ($C_{15}H_{14}O_4$), deoxy-dihydrolactucin ($C_{15}H_{18}O_4$), and its decarboxylated derivative ($C_{14}H_{18}O_2$) were also observed (**Figure 1**).

Both dehydroxylation (**DH**), to substitute a hydroxyl by hydrogen in the sesquiterpene molecule, and double bond reduction (**R**) (dihydro-derivatives) are characteristic metabolic reactions of strict anaerobe bacteria that colonize the colon.[20] Although the gut microbiota metabolism of dietary terpenoids remains almost unknown, it is well established that bile acids are converted by gut microbiota 7-α-dehydroxylase activity into secondary bile acids such as deoxycholic and lithocholic acids. Strains of the Ruminococcaceae and the Lachnospiraceae have been shown to contain the 7-α-dehydroxylase genes.[21] The reduction of double bonds can be achieved by strains of the Eggerthelaceae, as it happens in the reduction of digoxin by some *Eggerthella lenta* strains, leading to an inactive metabolite.[20,22]

3.2 Determination of sesquiterpene lactone gut microbiota metabolites in humans

The metabolism of sesquiterpene lactones in humans was studied by analyzing the urinary metabolites excreted after the intake of a regular food with a significant content of these phytochemicals. Thus, curly escarole was selected as one of the highest dietary sources of sesquiterpene lactones (containing: Lactucopicrin and its 15-oxalate, 11,13-dihydrolactucopicrin, lactucin, lactucin sulfate, dihydrolactucin, dihydrolactucin sulfate, 8-deoxylactucin, and 8-deoxylactucin sulfate) (**Table 2**), in agreement with previous reports.[11] Five healthy volunteers ingested fresh escarole (250 g) in a salad as part of their usual diet. The 24 h urine was collected

after the intake, and the sesquiterpene lactone metabolites analyzed by UPLC-QTOF-MS. In the urine of all the volunteers, two isomers of dihydrolactucin, their sulfates, and glucuronides were detected. However, lactucin sulfate, a component of escarole, was not detected in any of them. Two isomers of deoxylactucin, one glucuronide conjugate, and tetrahydro-lactucin sulfate were also detected (**Table 2**). Derivatives of lactucopicrin were detected in all the volunteers as well, including 11β,13-dihydrolactucopicrin glucuronide, two isomers of tetrahydrolactucopicrin glucuronide, and lactucopicrin 15-oxalate glucuronide.

The analysis showed that *in vivo* gut microbiota mainly reduced one or two double bonds of the naturally occurring sesquiterpene lactones. Secondarily, they also catalyzed the dehydroxylation of the molecules, that were later conjugated by phase II metabolism to yield sulfate and glucuronide conjugates (**Figure 1**). The results observed *in vivo* were generally consistent with those found during the *in vitro* fermentation. Interestingly, some escarole phytochemicals were absorbed without gut microbial metabolism, as they were probably absorbed in the upper part of the gastrointestinal tract, conjugated by phase II metabolism, and then excreted in the urine, as is the case of lactucopicrin 15-oxalate-glucuronide. However, it was only observed in one volunteer.

The number of isomers detected for some metabolites (**Table 2**) exceeds the number of expected metabolites when considering the phytochemicals occurring in lettuce and escarole. For instance, the number of dihydrolactucin glucuronides and sulfates expected should be two (the 8-glucuronide and the 15-glucuronide), as only two hydroxyls susceptible to conjugation were present, and the gut microbiota dehydroxylation will even potentially reduce this number. The number of isomers in the urine samples, however, was four for dihydrolactucin sulfate and six for dihydrolactucin glucuronide (**Table 2** and Supplementary **Table 1**). This shows that besides of the dehydroxylation produced by the gut microbiota, that leads to several deoxylactucin derivatives (8-deoxylactucin, 15-deoxylactucin), and the reduction of the double bonds leading to dihydro derivatives (**Figure 1**) (dihydro-lactucin and dihydro-lactucopicrin), they can also suffer the

hydroxylation of double bonds by human Phase I metabolism (CYP oxidation), as has already been reported for terpenes such as limonene.[24] In the case of R-limonene, the exocyclic (preferentially) and endo-cyclic double bonds were hydroxylated. As lettuce and escarole sesquiterpene lactones have several double bonds, they can also lead to different hydroxylated isomers (**Figure 3**). Thus, deoxy-derivatives such as deoxylactucin can lead to several CYP metabolites with a molecular formula identical to that of dihydro-lactucin, and several isomers are feasible that would explain the large number some phase II metabolites observed in the urine analyses (**Table 2** and Supplementary **Table 1**). The same discussion would also be valid for the isomers of tetrahydro-lactucin and hexahydro-lactucin that despite showing just one isomer in the *in vitro* fecal incubation, a large number of phase II isomers was observed in the urine samples (**Table 2** and suppl. **Table 1**).

When looking at the metabolites produced and excreted by each of the five healthy volunteers, a large inter-individual variability was observed (**Suppl. Table 1**). These results, although exciting and pointing out to further research on the topic, have to be considered preliminary due to the small number of volunteers and the lack of metabolite quantification. The inter-individual variability of polyphenol metabolism due to differences in gut microbiota composition, phase I and phase II metabolism, and transport (genetic polymorphisms) has recently been highlighted.[25-26] These studies suggested that this variability should be considered in the clinical trials with dietary phytochemicals, and the present study also shows that inter-individual variability should be considered in clinical trials with terpenoids as well. This study shows that in the case of sesquiterpene lactone metabolism, both gut microbiota, and human Phase I and II metabolism have a relevant role in the inter-individual variability observed (**Suppl. Table 1**). The relevance of gut microbiota and human metabolism in the inter-individual variability observed in the bioavailability of phytochemicals has already been reported for gut microbiota metabolites of ellagic acid (Phase II metabolism of urolithins)[27] and proanthocyanidins (Phase II metabolites of valerolactones).[28]

The present results suggest that gut microbiota can also metabolize other relevant sesquiterpene lactones from herbal medicinal products to reduce double bonds and remove hydroxyls, therefore modulating their efficacy.[12,13] Isoalantolactone was metabolized in rats through oxidation, hydration, demethylation, hydrogenation, and SH_2 addition, as well as different conjugations.[23] Thus, the present study suggests that differences in gut microbiota, and phase I and phase II metabolism need to be considered when looking at the effectiveness of foods, drugs, and nutraceuticals containing sesquiterpene lactones in humans.

4. Concluding remarks

The conversion of phytochemicals by the human gut microbiota still hides many unknown metabolites, which undoubtedly could have an essential role in health. Their metabolic modification and bioactivity are still unknown. This study reveals that sesquiterpene lactones, a large family of phytochemicals widely distributed in the diet of the majority of the population, are extensively metabolized by the human gut microbiota, producing bioavailable metabolites which biological activity is unidentified. Further studies of the biological effects of these novel metabolites should be completed to understand the impact of sesquiterpene lactones from dietary leafy greens in human health.

According to reports from FAO, lettuce and escarole are two of the most produced crops in the world, with a total production above 28 million tons, involving a significant consumption.[29] Therefore, it is crucial to know how the human gut microbiota transforms lettuce sesquiterpenes and how this affects their bioavailability and their health effects.

In summary, this study shows that the dehydroxylation and the double bond reduction are the most common conversions of lettuce sesquiterpene lactones *in vitro. In vivo,* human gut microbiota mainly reduced one or two double bonds of the sesquiterpene lactones, and the

metabolites were then conjugated by phase II metabolism to produce sulfate and glucuronide derivatives. Dihydro, tetrahydro, and hexahydro derivatives were the primary conversions found both *in vitro* and *in vivo*, suggesting that the reduction of terpenes by gut microbiota was the main catabolic activity.

This study also showed that untargeted metabolomics techniques could be applied to complex sample matrices, such as fecal fermentation cultures and urine samples, for metabolite profiling. Finally, this study concluded that multivariate models for data analysis could be used to discover new metabolites as tetrahydrolactucopicrin, tetrahydrolactucin, and hexahydrolactucin were tentatively identified.

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Figure captions

Figure 1. *In vitro* gut microbiota metabolism of lactucopicrin, lactucin, and lettuce. (**E**) Esterase activity; (**R**) Reduction (dihydrogenation); (**DH**) dehydroxylation. **Green color**, food phytochemicals added to the fermentation medium. **Black color**, metabolites produced. **Red color**, feasible metabolites not detected.

Figure 2. Possible isomers of dihydrolactucopicrin and tetrahydrolactucopicrin.

Figure 3. Phase I metabolism of 8-deoxy-lactucin. Potential metabolites obtained by double bond hydroxylation by CYP.

Compound	Formula	[M-H] observed	error	V1	V2	V3	Rt (min)
Deoxylactucin	$C_{15}H_{16}O_4$	259.0996	1.01	x	x	x	12.95
Deoxylactucin [b]	$C_{15}H_{16}O_4$	259.0975	0.39	x	x	x	16.9
Deoxylactucin	$C_{15}H_{16}O_4$	259.0976	-0.08	x	x	x	16.92
Tetrahydrolactucin [c]	$C_{15}H_{20}O_5$	279.1237	1.79	x	x	x	9.80
Hexahydrolactucin [c]	$C_{15}H_{22}O_5$	281.1403	4,97	x	x	x	8.27
Dehydro-deoxylactucin	$C_{15}H_{14}O_4$	257.0818	-0.41	x	-	-	16.81
Dehydro-deoxylactucin	$C_{15}H_{14}O_4$	257.0822	1	x	x	x	18.23
Dihydrolactucopicrin	$C_{23}H_{24}O_7$	411.1448	-0.79	x	x	-	16.13

Dihydrolactucopicrin	$C_{23}H_{24}O_7$	411.1458	1.99	x	x	x	16.28
Dihydrolactucopicrin	$C_{23}H_{24}O_7$	411.1445	-1.35	x	x	-	16.32
Dihydrolactucopicrin	$C_{23}H_{24}O_7$	411.1449	-0.29	x	x	-	16.43
11β,13-Dihydrolactucopicrin [a]	$C_{23}H_{24}O_7$	411.1452	0.55	x	x	x	16.87
Tetrahydrolactucopicrin[d]	$C_{23}H_{26}O_7$	413.1603	0.95	x	x	x	15.30
Tetrahydrolactucopicrin[d]	$C_{23}H_{26}O_7$	413.1609	1.58	x	x	x	16.13
Tetrahydrolactucopicrin	$C_{23}H_{26}O_7$	413.16	-0.22	x	x	x	16.71
Hydroxyphenyl acetic acid	$C_8H_8O_3$	151.0398	-1.56	x	x	x	8.36
Hydroxyphenyl acetic acid	$C_8H_8O_3$	151.0399	-0.9	x	x	x	10.19
Hydroxyphenyl acetic acid	$C_8H_8O_3$	151.0397	-2.81	x	-	-	16.13
Phenylacetaldehyde	C_8H_8O	119.0494	0.94	x	x	x	8.79
Phenylacetaldehyde	C_8H_8O	119.0503	0.21	x	x	x	11.25

Table 1. Metabolites identified in *in vitro* fermentation samples after incubations with Romaine lettuce and with commercial standards of lactucopicrin and lactucin.

According to Sumner et al. 2007 the identification level was 2. [30]
V1=Metabotype A; V2=Metabotype B; V3=Metabotype 0. [7]
[a] According to Sumner et al. 2007 the identification level was 1 for this metabolite (identified by comparison with authentic standards). [30]
[b] Detected in fecal fermentation samples of lettuce, lactucopicrin and lactucin and in the urine samples (**Table 2**).
[c] Metabolite of gut microbiota conversion of lactucin; [d]Metabolite of gut microbiota conversion of lactucopicrin
Table 2. Metabolites identified in escarole and in the urine samples collected for 24h after the intake of 250 g fresh escarole by five healthy volunteers.

Compound	Formula	$[M-H]^-$ observed	error	Detected in escarole	The ratio of occurrence in volunteers	Rt (min)	Number of isomers

				[c]			
11β,13-Dihydrolactucin [a]	$C_{15}H_{18}O_5$	277.1077	-1.85	x	5/5	9.4	1
Dihydrolactucin isomers	$C_{15}H_{18}O_5$	277.1077	-1.85	-	4/5	12.6/16.8	2
Dihydrolactucin-8-sulfate	$C_{15}H_{18}O_8S$	357.0647	-1.08	x	4/5	8.2	1
Dihydrolactucin-sulfate isomers	$C_{15}H_{18}O_8S$	357.0647	-1.08	-	5/5	8.3/10.1/18.0	3
Dihydrolactucin-glucuronide isomers	$C_{21}H_{26}O_{11}$	453.1411	2.41	-	5/5	7.7/8.3/10.3/11.5 13.5/18.2	6
Lactucin [a]	$C_{15}H_{16}O_5$	275.0921	-1.7	x	-	10.2	1
Lactucin-8-sulfate	$C_{15}H_{16}O_8S$	355.094	-0.96	x	-	8.4	1
Lactucin sulfate isomers	$C_{15}H_{16}O_8S$	355.094	-0.96	-	5/5	2.9/4.4/10.2/13.8 16.2/17.4	6
8-Deoxylactucin [b]	$C_{15}H_{16}O_4$	259.0975	0.39	x	4/5	16.8	1
Deoxylactucin isomers	$C_{15}H_{16}O_4$	259.0975	0.39	-	3/5	14.8/17.7	2
8-Deoxylactucin sulfate	$C_{15}H_{16}O_7S$	339.0541	-0.61	x	3/5	13.3	1
Deoxylactucin glucuronide	$C_{22}H_{24}O_{10}$	447.1291	-1.63	-	2/5	13.7/14.1	2
Tetrahydrolactucin-sulfate	$C_{15}H_{20}O_8S$	359.0593	-1.67	-	5/5	8.4/9.2/13.7/17.7	4
Tetrahydrolactucin-glucuronide	$C_{21}H_{28}O_{11}$	455.1562	0.72	-	5/5	6.5/8.0/11.1/15.7/17.2	5
Hexahydrolactucin sulfate	$C_{15}H_{22}O_8S$	361.0972	2.57	-	4/5	6.6/13.9/16.3	3
Hexahydrolactucin glucuronide	$C_{21}H_{30}O_{11}$	457.1715	1.52	-	5/5	6.8/7.7/10.1/13.2 15.4/16.8/18.2	7
8-Desacetylmatricarin-sulfate	$C_{15}H_{18}O_7S$	341.0693	-2	x	-	13.3	1
Lactucopicrin [a]	$C_{23}H_{22}O_7$	409.1285	-1.57	x	-	16.6	1
Lactucopicrin isomers	$C_{23}H_{22}O_7$	409.1283	-1.57		4/5	5.6/10.0	2
11β,13-Dihydrolactucopicrin [a]	$C_{23}H_{24}O_7$	411.144	-2.3	x	-	16.8	1
Dihydrolactucopicrin-sulfate	$C_{23}H_{24}O_{10}S$	491.1025	-1.4	-	2/5	15.4	1
Dihydrolactucopicrin-glucuronide	$C_{29}H_{32}O_{13}$	587.1786	2.94	-	4/5	14.1	1
Tetrahydrolactucopicrin-glucuronide isomers	$C_{29}H_{34}O_{13}$	589.1928	0.56	-	5/5	6.3/11.3/14.1/14.3	4
Lactucopicrin-15-oxalate	$C_{25}H_{22}O_{10}$	481.1127	-2.03	x	-	18.2	1
Lactucopicrin-15-oxalate-glucuronide	$C_{31}H_{30}O_{16}$	657.145	-0.92	-	1/5	11.1	1

According to Sumner et al. 2007 the identification level was 2. [30]

[a] According to Sumner et al. 2007 the identification was level 1 (identified by comparison with authentic standards). [30]

[b] Detected in fecal and urine samples

[c] Escarole extract sesequiterpene lactones were used as surrogate standards. [31]

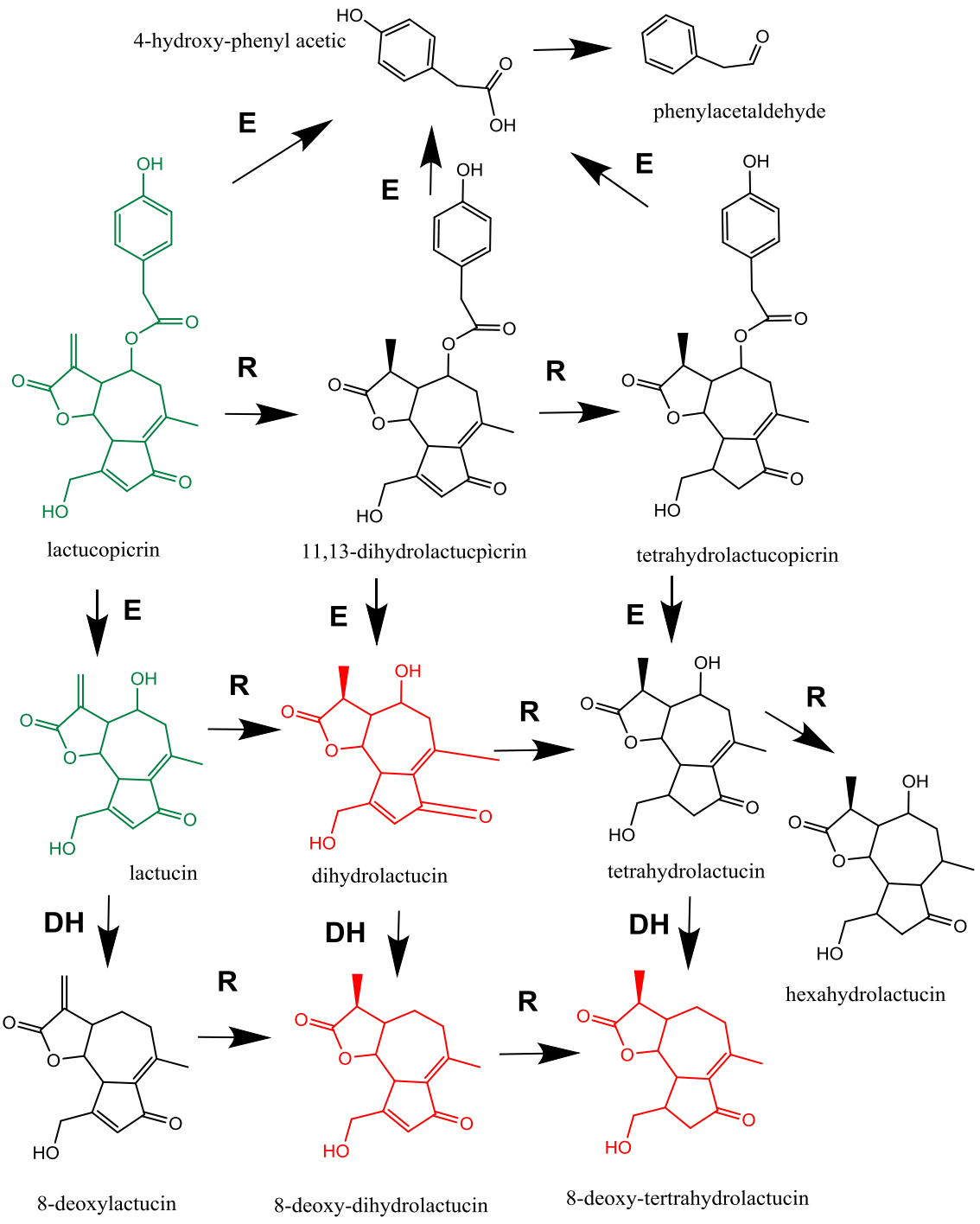
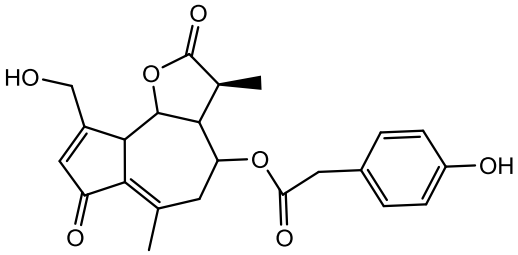
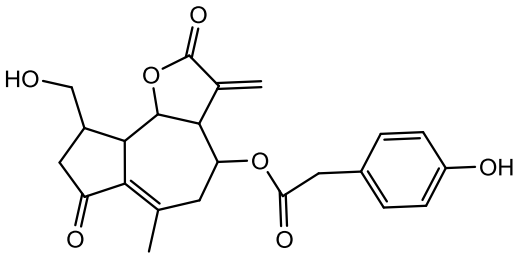
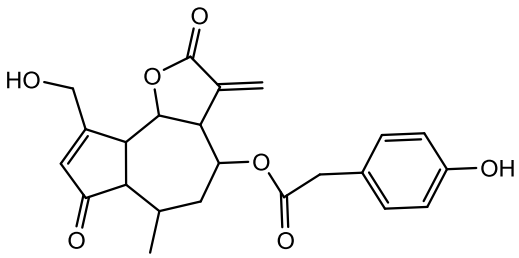


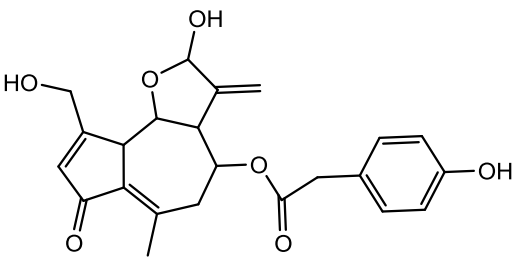
Figure 1

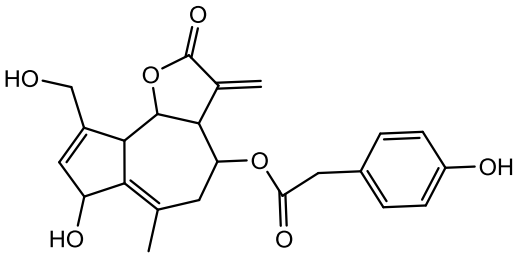
11,13-dihydrolactucpicrin



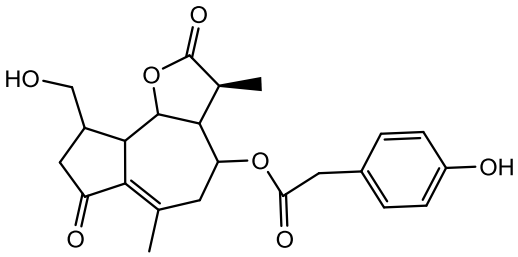


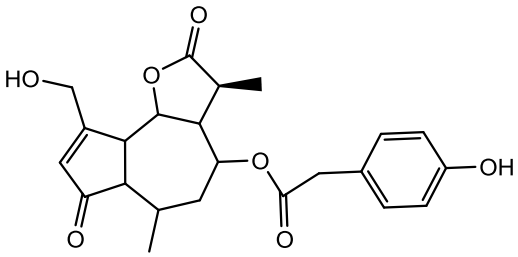


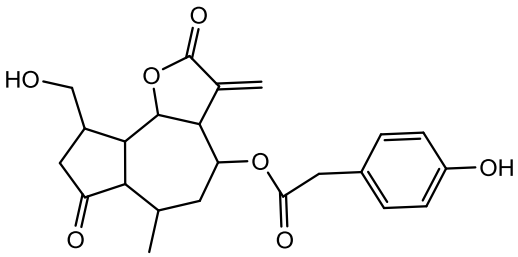




dihydrolactucopicrin isomers



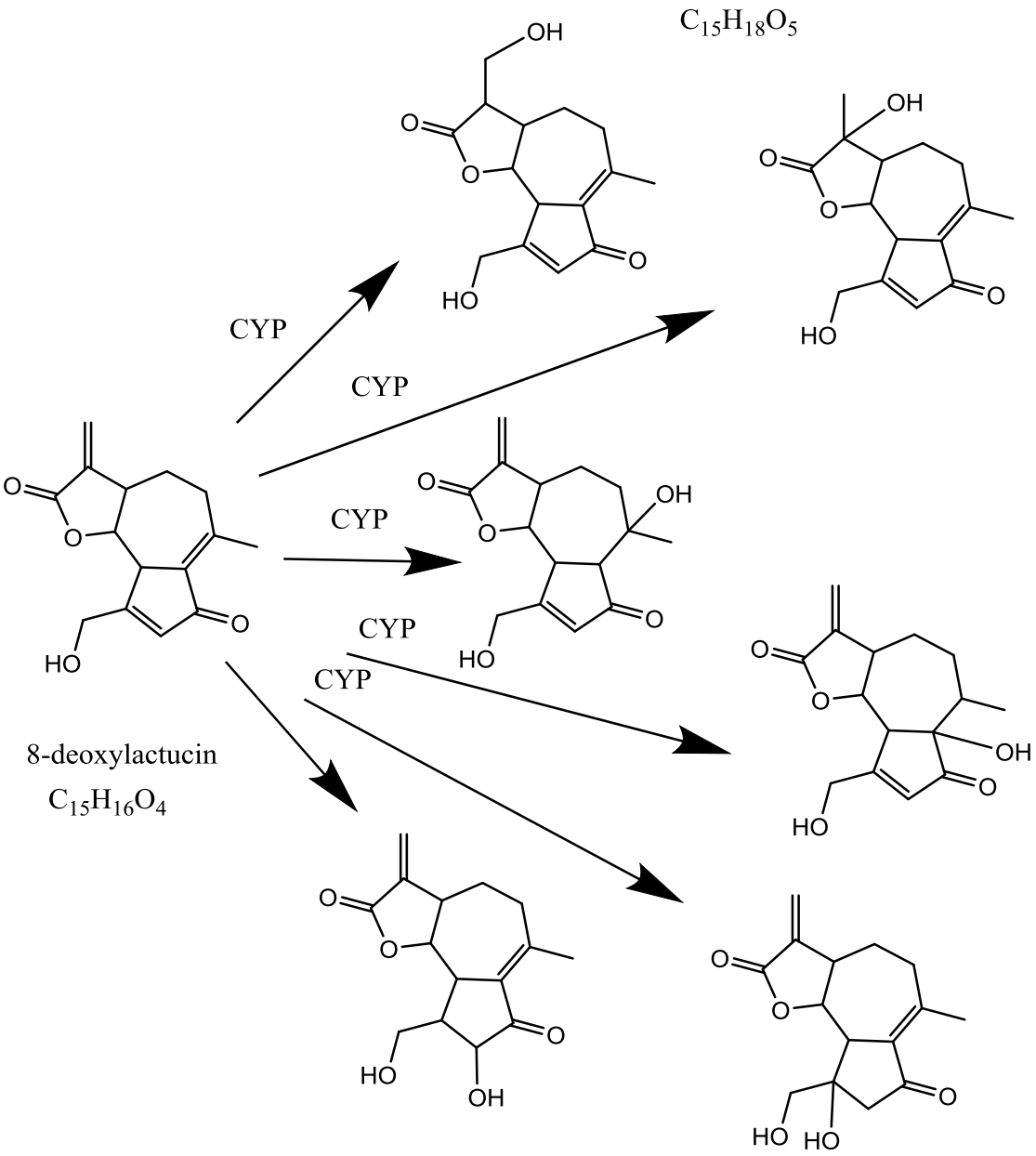




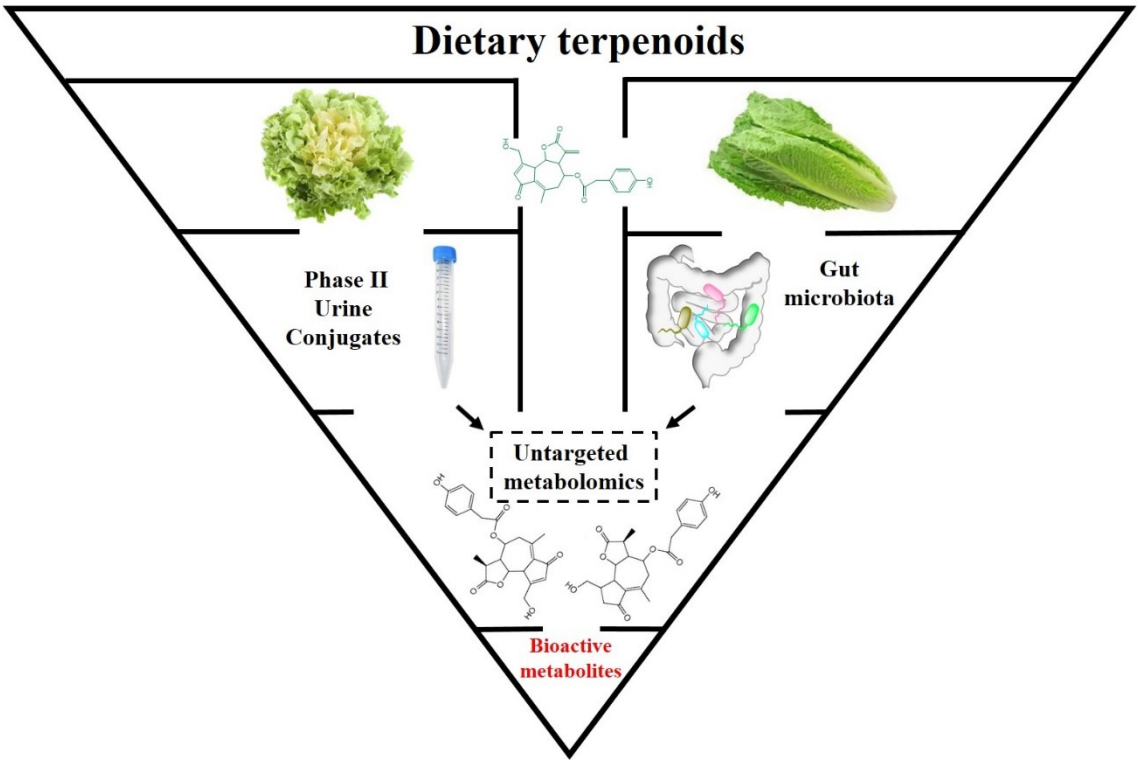
tetrahydrolactucopicrin isomers

Figure 2

Figure 3



Graphical abstract



TEXT: Although the transformation of dietary phenolic compounds by human gut microbiota has been studied, the metabolism of food terpenoids remains unknown. The present study has shown that lettuce and escarole terpenoids are transformed by gut microbiota into a new family of derivatives with potential biological effects that should be studied in further work