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Caffeic Acid Derivatives in Artichoke Extract are Metabolised to Phenolic Acids *in vivo*

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The purpose of this study was to investigate the absorption and metabolism of hydroxycinnamates from artichoke extract by determining the urinary excretion of the conjugates. Ten healthy, non smoking volunteers (5 female, 5 male) were given three capsules containing artichoke extract every 4 h (0, 4, 8 h) following two days of a low-polyphenol diet. One capsule contained 320 mg of artichoke extract equivalent to 34.3 ± 0.6 mg/g hydroxycinnamates (caffeic acid derivatives) and 5.6 ± 0.1 mg/g flavonoids. Polyphenols and phenolic acids present in the artichoke extract were not detected in the urine either as conjugates or aglycones. However, ferulic, isoferulic, dihydroferulic and vanillic acid were identified as major metabolites after β-glucuronidase treatment of urine. The amount excreted as well as the ratio to that of creatinine, a biomarker for the general excretion rate, increased significantly on the study day compared to the pre-supplementation day. Thus, the caffeic acid esters found in the artichoke extract capsule are absorbed, metabolised and excreted as methylated phenolic acids such as ferulic, isoferulic, dihydroferulic and vanillic acid.

Keywords: Artichoke, phenolic acids, absorption, metabolism, methylation, ferulic acid

INTRODUCTION

Artichoke (*Cynara scolymus* L.) extract is rich in phenolic acids and flavonoids.[1] Artichoke has been used as a remedy since the 4th century BC. The *in vitro* effects of artichoke extract are well described and are mostly accounted for by its phenolic constituents. Hepatoprotective and antioxidative activities in cultured rat hepatocytes exposed to several agents have been described.[2,3] Artichoke extract also showed antioxidative activity in *in vitro* test systems using human blood cells[4] and low-density lipoprotein.[5] Artichoke extract also inhibited cholesterol biosynthesis in cultured rat hepatocytes[6] and showed choleretic and cholesterol lowering properties in rats.[7] As active compounds for the described effects, luteolin-7-glucoside and particularly its aglycones, luteolin, are identified[6] but the antioxidative and

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hepatoprotective activities are mainly accounted for the caffeic acid derivatives, such as chlorogenic acid and 1,3-dicaffeoyl quinic acid.[3] Recent human and animal studies using artichoke leaf extract begin to show possible physiological health benefits *in vivo*. In randomised placebo-controlled double-blind human trial an increase of choleresis and bile excretion were found after administration of artichoke extract.[8] In a six week multicenter post-marketing surveillance (PMS) study,[9] which was performed in 553 dyseptic patients, artichoke extract showed strong carminative, antiemetic and spasmolytic effects. The results of this study were confirmed by a six month study with a similar study design.[10]

The purpose of this study was to determine the bioavailability and metabolism of phenolic compounds from artichoke extract by investigating the urinary excretion of absorbed compounds.

MATERIAL AND METHODS

Materials

Ferulic acid, isoferulic acid, chlorogenic acid, luteolin-7-glucoside, luteolin-4-glucoside, luteolin, phloretin (HPLC standards) were obtained from Extrasynthése, Genay, France. Methanol, acetonitrile, acetic acid, acetone were obtained by Rathburn Chemicals Ltd, Walkerburn, Scotland. Hydrochloric acid and trichloracetic acid were from BDH Laboratory Supplies, Poole, England. Vanillic acid (purity > 99%), Thymol, L-ascorbic acid, simulated gastric fluid (without pepsin), and β-glucuronidase (EC 3.2.1.31) Type L-II from limpets was purchased from Sigma Chemicals Co., Steinheim, Germany. 3-(4-Hydroxy-3-methoxy phenyl)-propionic acid (dihydroferulic acid) (purity 98%) was obtained from Lancaster, UK. Creatinine reagent was obtained from Audit Diagnostics, UK. The artichoke extract capsules (Hepar SL® forte) used in the study were provided by Sertuerner Arzneimittel GmbH, Germany.

HPLC Analysis

HPLC was undertaken using a Waters system consisting of Controller 600, Autosampler 717plus, Photodiode array detector 996, on-line degasser. Samples were analysed on a Novo-Pak C18 column, 4.6 × 250 mm, with 4 µm particle size and a guard column of the same material 4.6 × 15 mm. Mobile phase A consisted of methanol/water/5N HCl (10/89.9/0.1 v/v/v) and mobile phase B of acetonitrile/water/5N HCl (50/49.9/0.1 v/v/v). The gradient started 0 to 5 min 90% A and 10% B, 5 to 50 min linear gradient 100% B, 50 to 55 min 100% B, from 55.1 min 90% A and 10% B. Run time was 60 min followed by a 10 min delay prior to the next injection. Injection volume of the urine samples was 50 µL and of the standard solution and the artichoke extract, 10 µL.

Sample Preparation

The artichoke extract was weighed (± 0.01 mg) in a 50 mL volumetric flask, 10 mL of water was added and the suspension was sonicated for 15 min (Camlab Transsonic T460, 35 kHz). After addition of 30 mL methanol sonication was continued for further 30 min. The suspension was made up to 50 mL with water at 20 °C and an aliquot of the suspension was filtered (0.45 µm pore size) and analysed by HPLC. Identification was undertaken according to retention time, UV/visible spectra and spiking with commercially available relevant standards (chlorogenic acid, luteolin-7-glucoside). Most caffeic acid derivatives were tentatively assigned due to reports in the literature.[11] Caffeic acid derivatives in the extract were quantified relative to chlorogenic acid and the glycosides of luteolin, including luteolin-7-glucoside, relative to luteolin-7-glucoside.

For the human studies, urine samples were acidified to pH 4 with acetic acid and treated with β-glucuronidase (approx. 1 mg/ml) for 1 h at 37 °C. Samples were filtered and phloretin

was used as internal standard (10 μg/ml). After hydrolysis with β-glucuronidase, components were identified according to retention time, UV/visible spectra and spiking with commercially available relevant standards (ferulic acid, isoferulic acid, dihydroferulic acid, vanillic acid). Standard curves were obtained from the authentic standard compounds. Wavelengths used for quantification were: ferulic (RT: 31.5 min) and isoferulic acid (RT: 33.5 min) and artichoke hydroxycinnamates (324 nm), dihydroferulic acid (280 nm; RT: 28.4 min), vanillic acid (295 nm; RT: 21.8 min), luteolin-7-glucoside (RT: 32.1 min) and luteolin derivative (350 nm). Coefficient of variance was < 2%.

Blood samples, containing acid-citric-dextrose as anticoagulant, were centrifuged for 30 min at 3000 rpm (10 °C). Prior to separation of the isolated plasma, L-ascorbic acid (~0.5 mg/ml) was added as an antioxidant. After acidification with acetic acid, β-glucuronidase treatment was undertaken at pH 4 for 1 h. For deproteinization and extraction of the plasma, trichloracetic acid in methanol (10%) and acetone were added. After centrifugation (30 min, 3500 rpm, 5 °C) the supernatant was collected and freeze-dried. The remaining residue after freeze-drying was dissolved in methanol/water (20/80 v/v) and analyzed by HPLC.

Creatinine Measurement

Urinary creatinine levels were measured with a Hitachi 911E Random Access Automatic Clinical Chemistry Analyser by monitoring the formation rate of a coloured complex formed by creatinine and picrate in alkaline solution.

Gastric Juice Treatment

A similar amount of the artichoke extract powder (100 mg) was added to a similar volume (50 mL) of simulated gastric juice (see Materials section) or of water and incubated at 37 °C for 2 h. After cooling, the filtered solutions were analysed by HPLC.

Study Design

Ethical permission was obtained from the Guy's Research Ethics Committee [99/06/17]. Ten non-smoking healthy volunteers, 5 female, 5 male, were recruited, mean age of 29.2 years (23–43), mean body mass index (BMI) of 23.8 kg/m^2 (18–31). One volunteer was excluded during the study due to confounding of criteria. Exclusion criteria were smoking, pregnancy, being outside of the age range (20–45), blood donating or blood receiving less than 3 month prior to the study, simultaneous participation in another drug trial, history of drug abuse or alcoholism, consumption of more than 21 (male)/14 (female) units of alcohol a week, prescription drugs within the past 4 weeks, anyone who on screening has a clinically relevant history of disorders including chronic diseases influencing absorption, metabolism or excretion (e.g. hepatitis, Crohn's disease, chronic diarrhoea, celiac disease), drug hypersensitivity, intolerance or hypersensitivity against artichoke, infections or inflammatory processes or acute gastrointestinal symptoms. The permitted foods for the low-hydroxycinnamate diet were potato, sweet corn, mushroom, carrot, banana, any kind of meat or fish, tea, water and milk, pasta, cheese, non-flavoured milk products, butter and margarine. Tea consumption was allowed because it contains mainly flavanols and their polymers and gallic acid derivatives but minimal hydroxycinnamates.[11] For the first two days of the study, the volunteers consumed a low-polyphenol diet and their 24 h urine was collected each day. After administration of three artichoke extract capsules (Hepar SL® forte) with water in the morning, collection of the individual urine samples commenced. Later a further three capsules were administered at 4 h and 8 h. Collection of the individual urine samples and the adherence to the low-hydroxycinnamate diet were continued until 24 h after the first capsule administration.

Thymol was used for preservation of the urine samples (~1 g/L), which were then stored at

−70 °C. Blood samples were taken intravenously prior to the first administration of the artichoke capsules (control) and 1 h after the second administration.

RESULTS

Prior to supplementation the artichoke capsules were analysed for their phenolic constituents by HPLC with photodiode array detection. A typical chromatogram of the extracted artichoke powder is shown in Figure 1. The main phenolic compounds detected, were the hydroxycinnamate chlorogenic acid and the flavonoid luteolin-7-glucoside. In addition, detection of several caffeoyl quinic acid derivatives was suggested from their relative elution times and their spectral characteristics, as reported in the literature. These are not commercially available and so tentative assignments of identity are 4-caffeoyl quinic acid, 3,5-dicaffeoyl quinic acid, 1,3-dicaffeoyl quinic acid and 3-caffeoyl quinic acid (Figure 1). Each was quantified relative to chlorogenic acid. A second luteolin-related derivative was also identified and quantified relative to luteolin-7-glucoside. The total amount of caffeoyl quinic acid derivatives calculated with reference to chlorogenic acid was 34.4 ± 0.6 mg/g artichoke extract, of which 11.8 ± 0.1 mg/g was chlorogenic acid, and the total amount of luteolin glycosides relative to luteolin-7-glucoside 5.6 ± 0.1 mg/g, of which 4.4 ± 0.1 mg/g was luteolin-7-glucoside ($n = 2$). Within nine capsules ingested, a total amount of 123.9 mg total caffeoyl quinic acid derivatives (as chlorogenic

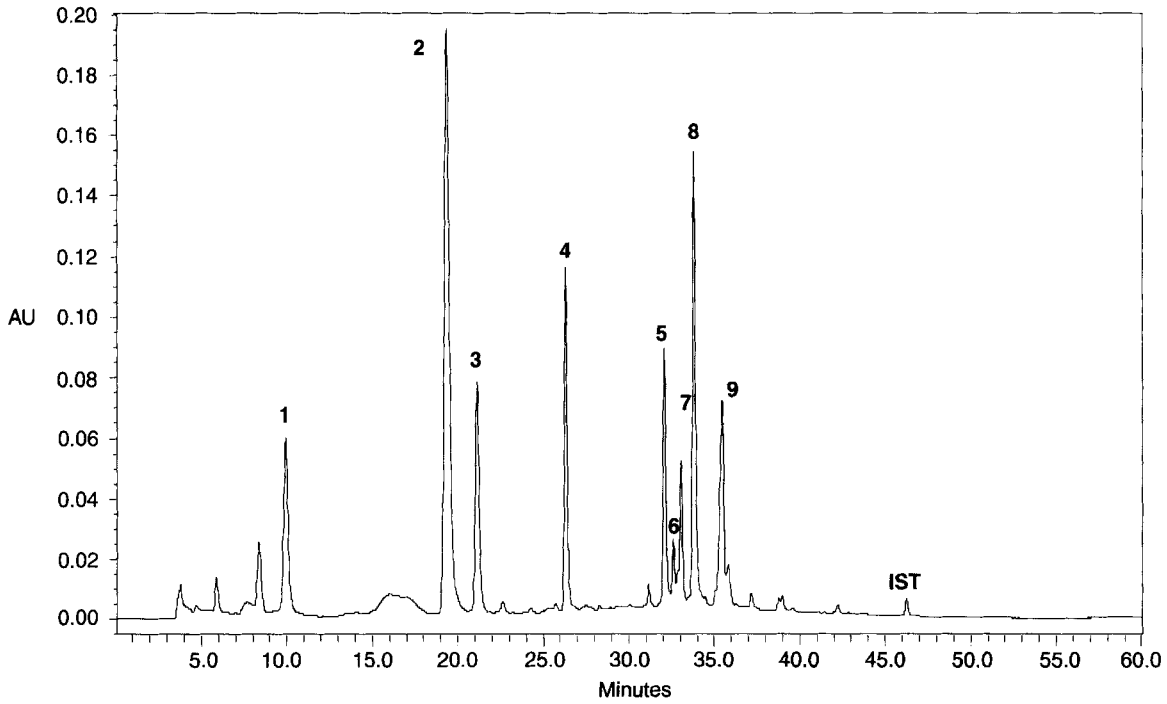

FIGURE 1 HPLC analysis with photodiode array detection of the phenolic composition of artichoke extract. A 70% methanolic extract was subjected to HPLC with photodiode array detection at 330 nm as described in Material and Methods. The observed peaks correspond to 3-caffeoyl quinic acid (1), chlorogenic acid (2), 4-caffeoyl quinic acid (3), 1,3-dicaffeoyl quinic acid (4), luteolin-7-glucoside (5), luteolin glycoside (6), caffeoyl quinic acid derivative 1 (7), 3,5-dicaffeoyl quinic acid (8), caffeoyl quinic acid derivative 2 (9). Phloretin (IST) was added as internal standard.

acid) and 20.1 mg of total luteolin glycosides (as luteolin-7-glucoside) were administered.

Prior to monitoring urinary elimination of the ingested capsules, the potential effects of the acidic environment of the gastric lumen on the stability of the phenolics were examined *in vitro*. The artichoke extract was treated with simulated gastric juice for 2 h and subjected to HPLC analysis. No changes in the phenolic profile were observed.

The pharmacokinetics of urinary excretion of phenolic conjugates were monitored after ingestion of the artichoke extract capsules. The identification of polyphenols and metabolites was undertaken before and after β-glucuronidase treatment. No caffeoyl quinic acid derivatives and luteolin or its conjugates were detected. However, ferulic and isoferulic acid as O-methylated products of caffeic acid were found in higher amounts in the urine post-supplementation after β-glucuronidase treatment, as well as to a lesser extent vanillic acid (Figure 2). In addition, a further metabolite was detected post-supplementation and was identified as dihydroferulic acid (Figure 2).

For eight out of nine volunteers a significant increase of ferulic acid excretion (as glucuronide) and for all nine volunteers a significant increase in isoferulic, dihydroferulic and vanillic acid excretion was found after administration of nine artichoke extract capsules on the study day (Table I). Taking the mean of the molar increase of ferulic, isoferulic, dihydroferulic and vanillic acid excretion on the study (18.80 μmol) and an estimated molar intake of caffeic acid from the artichoke extract capsules (337 μmol) the estimated absorption rate for caffeic acid would be approximately 5.6%, based on urinary excretion up to 24 h. A ratio was calculated between the determined molar amounts of the metabolites and the determined molar amounts of creatinine in the individual elimination samples, to study a standardised increase of ferulic, isoferulic, dihydroferulic and vanillic acid excretion. The maximum of the molar metabolites/creatinine ratios appeared between 4 and 20 h after the first administration of the artichoke extract capsules; thus in some volunteers the metabolism is much faster than in others. An increase in the molar metabolites/creatinine ratios was observed 1–3 h after the first administration of the artichoke capsules. Three volunteers showed a clear correlation of the molar ratios of the metabolites and the creatinine excreted and the time points of the artichoke capsule administration (Figure 3).

After β-glucuronidase treatment of the plasma, a small amount of ferulic acid ranging from 12–43 nM was detected and identified in the post-supplementation sample according to retention time, spectroscopic matching and spiking.

TABLE I Increase of 24 h urinary ferulic, isoferulic, dihydroferulic and vanillic acid excretion on the study day relative to the pre-study day

Volunteer	Ferulic acid increase	Isoferulic acid increase	Dihydroferulic acid increase	Vanillic acid increase
V1	+1.07 mg	+0.29 mg	+1.19 mg	+2.00 mg
V2	+1.19 mg	+0.19 mg	+0.70 mg	+2.51 mg
V3	no change	+0.63 mg	+1.43 mg	+0.78 mg
V4	+0.48 mg	+0.29 mg	+1.10 mg	+0.15 mg
V5	+0.72 mg	+0.17 mg	+1.31 mg	+0.25 mg
V7	+3.11 mg	+0.40 mg	+1.91 mg	+0.32 mg
V8	+0.60 mg	+0.24 mg	+1.39 mg	+1.17 mg
V9	+1.26 mg	+0.60 mg	+1.56 mg	+0.67 mg
V10	+0.87 mg	+0.29 mg	+1.03 mg	+0.40 mg

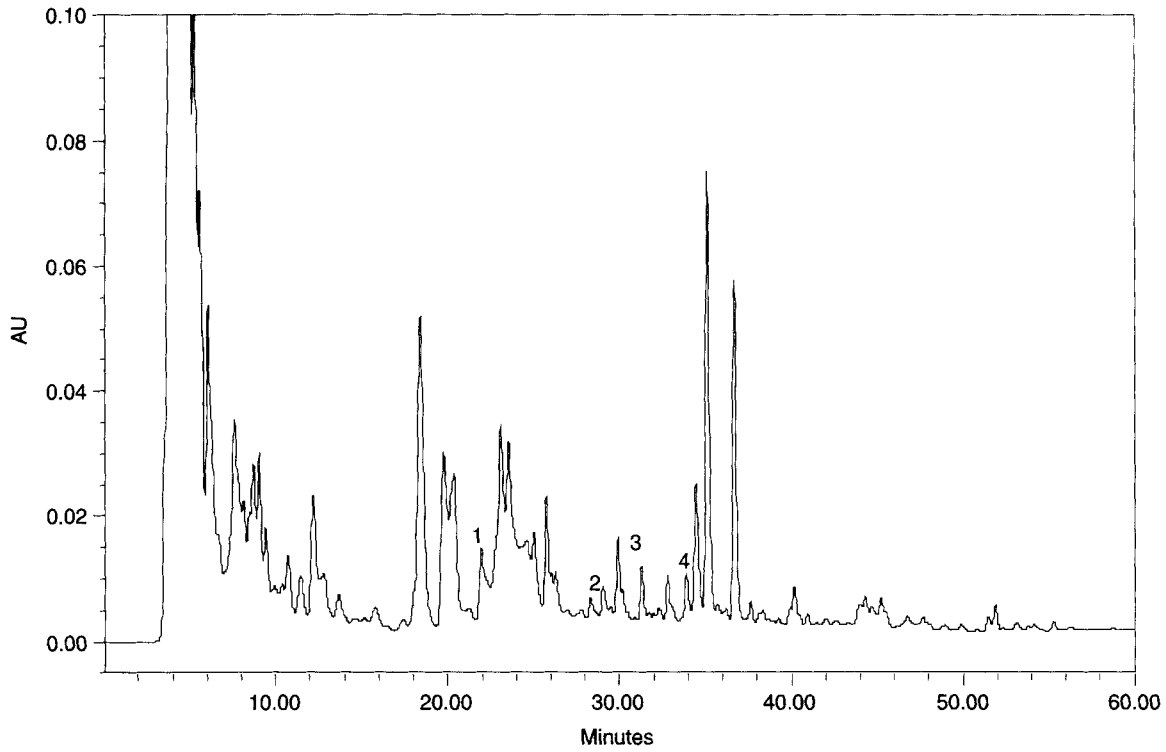

FIGURE 2 HPLC analysis with photodiode array detection of the urinary excretion of phenolics after ingestion of artichoke extract. Details in Materials and Methods. Chromatogram is shown at 280 nm after β-glucuronidase treatment of the urine (volunteer V3). The observed peaks correspond to vanillic acid (1), dihydroferulic acid (2), ferulic acid (3), isoferulic acid (4).

DISCUSSION

The appearance of ferulic, isoferulic, dihydroferulic and vanillic acid and its conjugates in the urine after the administration of caffeic acid-rich artichoke extract capsules is evidence for the absorption and metabolism of the caffeoyl quinic acid constituents. To derive ferulic and isoferulic acid from the caffeoyl quinic acid derivatives the ester linkage is cleaved and the 3-hydroxy-group or 4-hydroxygroup of the caffeic acid is O-methylated (Figure 4). Reduction of ferulic acid results in dihydroferulic acid, from which vanillic acid could be formed after β-oxidation. The very low concentrations of ferulic detected in post-supplementation plasma after β-glucuronidase treatment are interpreted as a consequence of the relatively low dose of hydroxycinnamates administered (only approx. 20 mg of caffeic acid per administration).

Acidic gastric juice treatment did not cleave the ester linkage of the caffeoyl quinic acids nor the glycoside linkage of the luteolin glycosides found in the artichoke extract. This implies that the most likely site for cleavage of the caffeoyl quinic acid derivatives is the large intestine. Other researchers investigating extracts of other human body fluids such as small intestine epithelium, liver, plasma showed no esterase activity on chlorogenic acid (5-caffeoyl quinic acid) while an extract of the colonic microflora provided an esterase activity.[12] In addition, in an *in vitro* model using the small intestine from rats to study absorption and metabolism only minimal absorption of chlorogenic acid (0.1%) was found,[13] which could be taken as another pointer towards the

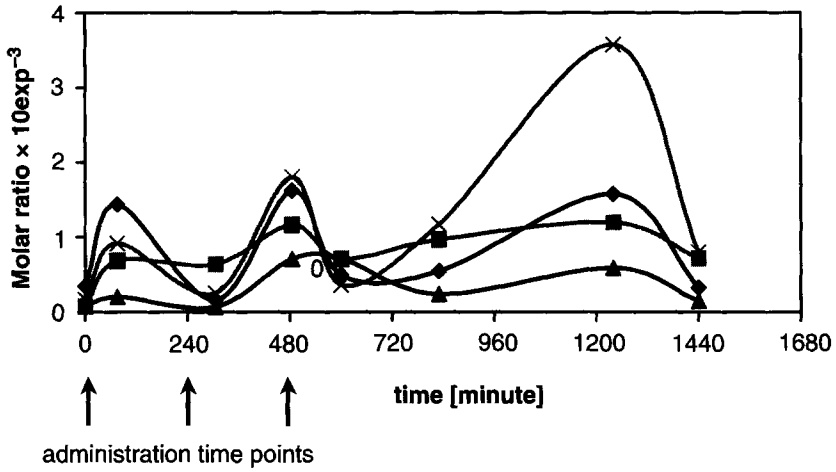

FIGURE 3 Molar ratios of ferulic acid/creatinine (-◆-), isoferulic acid/creatinine (-■-), dihydroferulic acid/creatinine (-▲-) and vanillic acid/creatinine (-✳-) monitored 24 h post-administration of artichoke extract (volunteer V2).

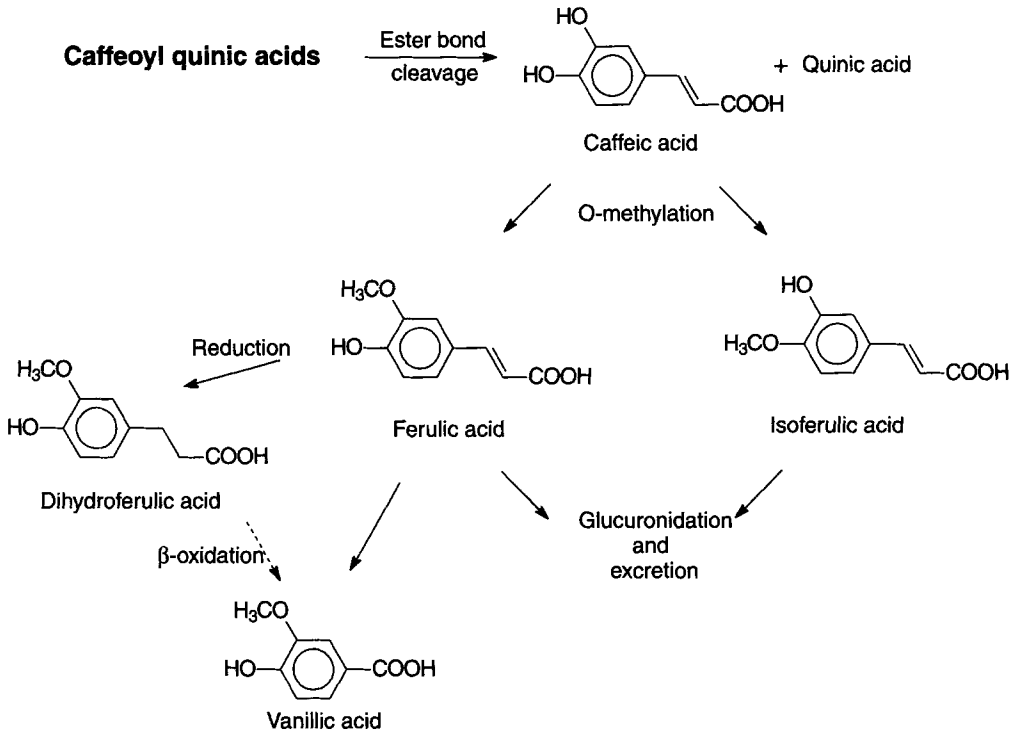

FIGURE 4 Derivation of ferulic, isoferulic, dihydroferulic and vanillic acid from metabolism of caffeoyl quinic acids.[12,16]

large intestine as the most likely place for absorption and metabolism of the caffeoyl quinic acid derivatives. The gut microflora might also be responsible for the O-methylation of caffeic acid to yield ferulic and isoferulic acid[14] as well as the liver. The findings are consistent with early studies on absorption and metabolism of caffeic acid forming ferulic and isoferulic acid after

3-O-methylation or 4-O-methylation.[15] These authors also detected vanillic acid as a metabolite of caffeic acid while the appearance of dihydroferulic acid is not mentioned.

The identified metabolites of polyphenols after artichoke extract ingestion should be considered for further investigation for possible biological actions in humans rather than the crude extract, whose polyphenols seem not to be absorbed unmetabolized.

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