BIOAVAILABILITY AND METABOLISM OF ROSEMARY INFUSION POLYPHENOLS USING CACO-2 AND HEPG2 CELL MODEL SYSTEMS

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ABSTRACT

BACKGROUND: *Rosmarinus officinalis* is an aromatic plant used in folk medicine due to the therapeutic properties associated to its phenolic composition, rich in rosmarinic acid (RA) and caffeic acid (CA). To better understand the bioactivity of these compounds, their absorption and metabolism were assessed in human Caco-2 and HepG2 cells, as small intestine and liver models, respectively, using RA and CA standards, as well as a rosemary infusion and ferulic acid (FA).

RESULTS: Test compounds were partially up-taken and metabolized by Caco-2 and HepG2 cells, although higher metabolization rate was observed after hepatic incubation than intestinal. CA was the compound best absorbed followed by RA and FA, showing metabolites percentages of 30.4%, 11.8% and 4.4% in Caco-2 and 34.3%, 10.3% and 3.2% in HepG2 cells, respectively. RA in the rosemary infusion showed improved bioavailability compared to pure RA. Methyl derivatives were the main metabolites detected for CA and RA after intestinal and hepatic metabolism, followed by methyl-glucuronidates and glucuronidates. RA was also minimally hydrolyzed into CA while FA only was glucuronidated. Rosemary polyphenols followed the same biotransformation pathways as the standards. In addition, phase II derivatives of luteolin were observed.

CONCLUSION: Rosemary polyphenols are partially metabolized in both intestine and liver.

KEY WORDS: Rosemary polyphenols; rosmarinic acid; caffeic acid; ferulic acid; Caco-2 & HepG2 cells; absorption and metabolism

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L) is a widespread aromatic plant commonly consumed as a tea in traditional cuisine and in folk medicine to treat various illnesses due to its therapeutic properties. Rosemary is used as medicinal herb against many illnesses, especially headaches, respiratory diseases and several neuropsychiatric disorders [1-4] . These medicinal benefits are usually achieved through fresh or dried rosemary tea consumption.

Phytochemical characterization in rosemary extracts has shown that the main bioactive compounds in rosemary are polyphenols. Extracted by supercritical fluid, microwave or infusion, the main phenolic compounds present in rosemary extracts are hesperidin, homoplantaginin, luteolin-glucuronide, rosmarinic acid, rosmarinic acid-3-O-glucoside, luteolin 3-*O*-(O-acetyl)-β-D-glucuronide, cirsimaritin, and rosmanol [5-7]. A recent study characterized forty-nine compounds in rosemary tea belonging to six families, namely flavonoids, phenolic acids, phenolic terpenes, jasmonate, phenolic glycosides, and lignans, being rosmarinic acid the main compound in addition to caffeic acid and luteolin derivatives, among others [5]. This composition contrasts with that reported after the extraction of rosemary phenols with ethanol or methanol solvents, having been described flavonoids, hydroxycinnamic acids, and tannins, as well as carnosic acid and carnosol as the main compounds [8-10].

It is well known that *in vivo* biological activity of polyphenols depends on the absorption and metabolic fate of the compounds. Few bioavailability studies have been developed using rosemary phenols, the majority having been focused on the absorption, distribution and elimination of carnosic acid [11-13] . It is noteworthy that the study by Romo Vaquero et al., (2013) focused on the bioavailability and metabolism of diterpenes from ethanolic rosemary extract [9]. However, to the best of the author's knowledge, there are no reports on the bioavailability and metabolism of *Rosmarinus officinalis* tea polyphenols.

Therefore, the aim of this work was to examine the bioavailability and metabolism of polyphenols in a rosemary infusion as well as its main hydroxycinnamic acids rosmarinic acid (RA) and caffeic acid (CA) using a human intestinal Caco-2 cells and hepatic HepG2 cells, as *in vitro* models of small intestine and liver, respectively. The metabolite ferulic acid (FA) was also studied to evaluate the structure-metabolism relationship, which in turn would contribute to a better understanding of the mechanisms of action of *Rosmarinus officinalis* tea.

EXPERIMENTAL

Chemicals

Acetonitrile, formic acid, sodium chloride, disodium hydrogen phosphate anhydrous, potassium dihydrogen phosphate and ascorbic acid were acquired from Panreac (Barcelona, Spain). Phenolic compounds (caffeic, ferulic and rosmarinic acids along with luteolin), antibiotics (gentamicin, penicillin, and streptomycin) and dimethyl sulfoxide were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain). DMEM F-12 medium, trypsin and fetal bovine serum (FBS) were from Biowhitaker (Innogenetics, Madrid, Spain). All reagents were of analytical or chromatographic grade.

Preparation of *Rosmarinus officinalis* infusion and quantification of its phenolic composition

Rosmarinus officinalis infusion was prepared as described elsewhere[5]. Briefly, 50 g of rosemary (leaves and branches) were placed into 100 mL of boiled water. The leaves and branches steeped for 15 min were then filtered and made to 100 mL. The infusion was kept at -20°C until chromatographic analysis. For HPLC analysis, rosemary infusion was filtered (0.45 μm) prior to injection into the HPLC system. The phenolic content of rosemary infusion was determined by HPLC-DAD as previously described[5], using an Agilent 1200 liquid chromatographic system equipped with a thermostatic autosampler, quaternary pump and diode-array detector (DAD). The column used for chromatographic separation was a

Superspher 100 RP18 column (250 mm x 4.6 mm, 4 µm i.d., Agilent Technologies) preceded by an ODS RP18 guard column in a thermostatic oven at 30 °C. 20 µL of filtered rosemary infusion was injected and separated by using a mobile phase consisting of water (solvent A) and acetonitrile (solvent B), both containing 1% formic acid (all HLPC quality), at a flow rate of 1 mL/min. The solvent gradient changed from 10% to 20% solvent B over 20 min, 20%-25% solvent B over 10 min, 25%-35% solvent B over 10 min, isocratically for 10 min, returning to the initial conditions over 10 min and maintaining isocratically over 5 min. Chromatograms were recorded at 324 nm according to the maxima spectra of phenolic compounds. Calibration curves of pure standards of [rosmarinic acid, caffeic acid and luteolin] were used for quantification of these compounds as well as other compounds with similar structures. Thus, rosmarinic acid calibration curve was used for quantification of rosmarinic acid and homoplantaginin, whereas luteolin calibration curve was used to quantify luteolin, luteolin-3-*O*-glucuronide and luteolin-3'-*O*-(*O*-acetyl)-β-D-glucuronide.

Cell cultures and treatments

The human colon adenocarcinoma cell line Caco-2 was obtained from Scientific Instrument Center at University of Granada (Spain) while the human hepatoma cell line HepG2 cells was kindly provided by Dr. Paloma Martín-Sanz (Instituto de Bioquímica, CSIC, Madrid, Spain). Both cellular lines were seeded at the density of 5-6 x 10^6 cells/plate in 100 mm plates and maintained in DMEM F-12 medium supplemented with 50 mg/L of each gentamicin, penicillin and streptomycin, in addition to 2.5% and 10% Fetal Bovine Serum (FBS) to keep HepG2 and Caco-2 cells, respectively. The cells grew up in a humidified incubator containing 5% CO_2 and 95% air at 37°C. The culture medium was changed every second day, and the cells were usually split 1:6 when they reached confluence.

The experiments were carried out in 60 mm plates after seeding at the density of 2.0-2.5 x 10^6 cells/plate, approximately. When Caco-2 and HepG2 cells reached 80% confluence, they

were changed to FBS-free medium the day previous to the assay to avoid potential interference of the serum. To evaluate the bioavailability and metabolism of caffeic acid (CA), ferulic acid (FA), rosmarinic acid (RA) standards and polyphenols in the rosemary infusion in both Caco-2 and HepG2 cells, 100 μM of each standard or the phenolic content of the infusion were incubated for 2 or 23 h in serum-free culture medium. Control plates, with cells in culture medium without polyphenols, were also maintained to observe potential changes in the culture medium due to normal cell metabolism. Pure RA, CA and FA standards were dissolved in 1% DMSO in deionized water and diluted with FBS-free culture medium to prepare the test solutions (100 μM of each standard and 0.1% DMSO in the cell culture medium). Likewise, rosemary infusion was subsequently diluted in 1% DMSO in deionized water and then in FBS-free culture medium to prepare test solution (100 μM of total phenolic compounds of the infusion and 0.1% DMSO in the cell culture medium). Additionally, all treatments and controls contained 500 μM of ascorbic acid to protect test compounds from oxidation. After the incubation times finished, media and cells were processed independently. Medium was collected and stored at -20°C until analysis. Cells were washed twice with 0.01 M phosphate-buffered saline solution (PBS; pH 7.4) and harvested by scraping. Plates corresponding to a particular condition were combined in an eppendorf vial. After centrifugation at 1250 rpm for 5 min at 4 °C, supernatants were removed and cell pellets resuspended in 200 μL of PBS. Cells were sonicated for 10 min at room temperature and centrifuged at 7000 rpm for 10 min at 4 °C. Supernatants were kept frozen at −20 °C. All metabolic experiments were repeated three times. Media and intracellular content were separately subjected to HPLC-DAD and LC-MS analyses.

Analysis of metabolism experiments in Caco-2 and HepG2 cells

HPLC Procedure

All extracellular culture samples and cell lysates from experiments carried out with Caco-2 and HepG2 cells were analyzed by HPLC using an Agilent 1200 Liquid Chromatographic System (Agilent Technologies) equipped with diode array UV-Vis detector and a thermostatic autosampler (4°C). Agilent ChemStation software system controlled the equipment and carried out data processing. Elution of samples was performed at a flow rate of 1.0 mL/min using as mobile phase deionized water (solvent A) and acetonitrile (solvent B), both containing 1% formic acid. 20 μL of sample were analyzed in reversed mode with a Kinetex 100 RP18 column (250 x 4.6 mm i.d., 5 μm particle size, Phenomenex) with holder AJO-9000 and pre-column AJO5768C18 maintaining the oven temperature at 30°C. The solvent gradient changed from 10% to 20% solvent B over 20 min, 20% to 25% B over 10 min, 25% to 35% solvent B over 10 min, then maintained isocratically for 10 min, and returning to the initial conditions over 10 min (10% B) and followed by 5 min of maintenance. Chromatograms were acquired at 324 nm. Phenolic compounds were quantified using standard calibration curves. Standards were prepared in serum-free culture media in a range of concentrations from 1.0 μM to 100 μM obtaining a linear response for all standard curves, as checked by linear regression analysis with R^2 values greater than 0.999 (n=3). RA, CA, FA and luteolin were the standards used to quantify the metabolites formed in extracellular medium samples. Percentage of standards recovery added to the culture medium (varying from 90.5 to 100%), limits of detection (ranging from 0.02 to 0.04 μmol/L), limits of quantification (0.056-0.111μmol/L), and precision of the assay (as the coefficient of variation, ranging from 0.1 to 0.23 %) were considered acceptable and allowed quantification of phenolic compounds and their metabolites (quantified as equivalents of the respective parent molecules). Due to the lack of standards for some phase II metabolites, they were tentatively quantified by using calibration curves corresponding to their phenolic precursors.

Metabolites identification by HPLC-ESI-QToF analysis

Analysis were performed on an Agilent 1200 series LC system coupled to an Agilent G6530A Accurate-Mass Quadrupole Time-of-Flight (Q-ToF) with ESI-Jet Stream Technology (Agilent Technologies). Chromatographic conditions (eluents, column, flow rate, gradient, etc.) were the same as described above. The QToF acquisition conditions were as follows: drying gas flow (nitrogen, purity > 99.9%) and temperature were 10 L/min and 350°C, respectively; sheath gas flow and temperature were 11 L/min and 350°C, respectively; nebulizer pressure was 45 psi; cap voltage was 3500 V; nozzle voltage was 500 V. Mass range selected was from 100 up to 970 m/z in negative mode and fragmentor voltage of 100 V. Data were processed by using Mass Hunter Workstation Software.

Statistical analysis

Statistical analysis of data was performed using the software SPSS 24.0 (SPSS Inc., IL, USA). Homogeneity of variances was checked by the Levene test and comparisons were carried out using one-way ANOVA. The level of significance was $p<0.05$. Results are expressed as mean ± standard deviation (SD).

RESULTS

Characterization and quantification of phenolic content of rosemary infusion

In accordance with Achour et al., (2017)[5], the main phenolic constituent of the rosemary infusion was rosmarinic acid (RA) which accounted for 40% of the total phenolic content in the infusion, however lower contents of luteolin-3-*O*-glucuronide (up to 24 %), luteolin-3'-*O*-(O-acetyl)-β-D-glucuronide (up to 15%), caffeic acid (CA) (up to 10%) and homoplantaginin (6%) were observed. Luteolin was the minor component in rosemary extract (about 5% of the phenolic rosemary infusion).

Evaluation of bioavailability and metabolism of the rosemary infusion and its main hydroxycinnamic acids by Caco2 and HepG2 cells

Figures 1 and 2 show the chromatographic profile of culture media after incubation of Caco-2 and HepG2 cells, respectively, with 100 µM RA (Figures 1A, 1B, 2A and 2B), 100 µM CA (Figures 1C, 1D, 2C and 2D), 100 µM ferulic acid (FA) (Figures 1E, 1F, 2E and 2F) or rosemary infusion (Figures 1G, 1H, 2G and 2H) containing about 100 µM of the phenolic compounds.

Metabolite Identification

HPLC analysis of cell culture media collected after incubation of RA, CA, FA as well as polyphenols in the rosemary infusion in Caco-2 and HepG2 cells showed, in both cell models, a number of news peaks with absorbance at 324 nm that were not present in the original culture medium, nor in the control plates without polyphenols (data not shown). The appearance of these new peaks was concomitant with a decrease in the contents of their respective parent compounds (Figures 1 and 2), suggesting that the additional peaks were phenolic metabolites.

Subsequently, samples were analyzed by high-resolution mass spectrometry using an ESI-QToF detector in the negative ion mode and selected ion monitoring (SIM) and afterward subjected to LC-MS^2 measurements by collision-induced dissociation mass spectrometry to complete the characterization of the metabolites. UV-spectra evaluation further supported polyphenol metabolite characterization. Table 1 shows the retention time (RT), UV absorption maximum from DAD, quasimolecular ion $[M-H]^-$ after negative ionization, MS/MS fragment ions and tentative identification of the metabolites. Identified chromatographic peaks generated after the metabolism of CA, FA, RA standards and polyphenols in the rosemary infusion were labeled as M1, M2, and so on according to their elution order.

Most constituents displayed similar spectral behavior with maximum absorption peak at 314-330 nm and a shoulder at 294-300 nm, being characterized as hydroxycinnamic acid

derivatives. In addition, rosemary infusion contained a minor amount of luteolin derivatives with two maximum absorption peaks at 248-266 and 338-350 nm.

Identification of rosmarinic acid (RA) conjugates after incubation with Caco-2 and HepG2 cells

After incubation of RA with Caco-2 cells, eight new peaks were detected at RT of 9.5, 24.7, 32.2, 33.3, 38.6, 39.2, 39.4 and 40.1 min (labeled CA, M12, M18, M19, M22, M23, M24 and M25, respectively) after 2 and 23 h incubation (Figure 1A and 1B). The peak with RT at 9.5 min was CA, which was confirmed by comparison with the corresponding commercial standard. The chromatographic peak obtained at 24.7 min (M12) showed the same UV and MS spectrum as the RA standard and thus was assigned as a RA isomer. M18 and M19 exhibited a similar UV spectrum as RA (maximum at 330 nm and shoulder at 294 nm) and their LC-MS analysis ($[M-H]^-$ at *m/z* 373.0929 and fragment ion at *m/z* 359 corresponding to RA) allowed their identification as methyl derivatives of RA. To end, M22, M23, M24 and M25 showed a UV spectrum that hardly changed compared to RA (maximum at 328 nm and shoulder at 294 nm) while LC-MS analysis showed a quasimolecular ion at *m/z* 387.1085 plus fragment ion at m/z 359 corresponding to the loss of two methyl moieties. Attending to these results, M22, M23, M24 and M25 could be identified as dimethyl conjugates of RA.

Likewise, incubation of RA with Hep2 cells after 2 and 23 h generated 10 and 16 new peaks, respectively (Figure 2A and 2B). All metabolites formed after short incubation were also found after 23 h and additionally, 6 new metabolites were observed after the longer incubation period. All chromatographic peaks showed UV spectra compatible with hydroxycinnamic acids (λ_{max} at 320-330 nm and shoulder at 294-298 nm). By order of elution, apart from caffeic acid (CA), RA isomer (M12), two isomers of methyl-RA (M18 and M19) and two isomers of dimethyl-RA (M24 and M25) were tentatively identified as metabolites with chromatographic peaks eluting at 16.4, 20.9, 21.1, 23.1, 26.0, 26.5, 28.6, 29.9, 35.7 and 37.1

min (labelled FA, M9, M10, M11, M13, M14, M15, M16, M20 and M21). The peak observed at 9.6 min corresponded to FA, which was confirmed thanks to the corresponding commercial standard and UV and MS spectra. Identification of M9, M11 and M13 at 20.9, 23.1 and 26.0 min, respectively, showed a shift of the UV absorption maximum to shorter wavelengths (hypsochromic effect) (Table 1). Attending to their quasimolecular parent ion at m/z 549.1250 and fragment ion at m/z 373 and 359, these metabolites were identified as methyl-RA glucuronides with the loss of glucuronide and methylglucuronide moieties, respectively. Likewise, the chromatographic peak eluting at 21.1 min (labeled as M10) showed similar UV spectrum than the former glucuronide derivatives identified (M9, M11 and M13) suggesting to contain a glucuronide moiety. LC-MS analysis confirmed this after showing a quasimolecular ion at *m/z* 535.1093 and fragment ion at m/z 359, corresponding to RA after losing the glucuronide moiety. M10 was unequivocally identified as RA glucuronide. Finally, chromatographic peaks labeled as M14, M15, M16, M20 and M21, which were observed at 26.5, 28.6, 29.9, 35.7 and 37.1 min, respectively, with a maximum at 320-328 nm and a shoulder at 292-296 nm showed a MS spectrum ($[M-H]^-$ at *m/z* 387.1085 and fragment ion at *m/z* 359) which allowed their unambiguous identification as dimethyl-RA glucuronides.

Identification of caffeic acid (CA) conjugates after incubation with Caco-2 and HepG2 cells

After incubation of CA with Caco-2 cells, seven peaks were detected at RT of 5.5, 6.7, 9.7, 10.1, 11.4, 16.4 and 18.2 min (labeled M1, M2, M4, M5, M6, FA and M7 respectively) (Figure 1C and 1D). The chromatographic peak observed at 16.4 min corresponded to FA as aforementioned, whereas the chromatographic peak eluting at 18.2 min (labelled as M7) was assigned to *iso*ferulic acid according to the commercial standard. Peaks M1, M2, M4, M5 and M6 exhibited a shift of the UV absorption maximum to shorter wavelengths (hypsochromic effect), compatible with a chemical structure containing glucuronide moiety. LC-MS analysis allowed identifying M1 and M5 ($[M-H]^-$ at *m/z* 355.0671 and fragment ion at *m/z* 179) as CA

glucuronides, while M2, M4 and M6 ($[M-H]^-$ at *m/z* 369.0827 and fragment ion at *m/z* 193) were described as FA glucuronides.

Incubation of CA with HepG2 cells provided, in addition to the seven metabolites described above (M1, M2, M4, M5, M6, FA and M7), a new chromatographic peak labeled as M3, which appeared at 7.4 nm. This peak showed a UV spectrum (λ_{max} at 314 nm and shoulder at 296 nm) and MS spectrum ($[M-H]^-$ at *m/z* 355.0671 and fragment ion at *m/z* 179) which allowed its identification as CA glucuronide (Figure 2C and 2D).

Identification of ferulic acid (FA) conjugates after incubation with Caco-2 and HepG2 cells

Three peaks (M2, M4 and M6) were formed after incubation of FA with Caco-2 and HepG2 cells (Figures 1E, 1F, 2E and 2F) which were FA glucuronides, as aforementioned.

Identification of *Rosmarinus officinalis* infusion metabolites after incubation with Caco-2 and HepG2 cells

After incubation of rosemary tea with Caco-2 cells, 18 new peaks were detected. Most have already been described above: M1 and M3 as CA glucuronides, M2, M4 and M6 as FA glucuronides, ferulic acid labeled as FA, M10 as RA glucuronide, M12 as isomer of RA, M13 as methyl-RA glucuronide, M14, M15, M20 and M21 as dimethyl-RA glucuronide, M18 as methyl-RA and M23, M24 and M25 as dimethyl-RA. Apart from these metabolites, a new chromatographic peak at 30.2 min labeled as M17, which showed UV spectrum with λ_{max} at 266 and 340 nm compatible with the chemical structure of luteolin, was detected. LC-MS analysis showed a quasimolecular ion $[M-H]^-$ at *m/z* 475.0882 and fragment ions at *m/z* 299 and 285 corresponding to methyl-luteolin and luteolin, respectively (Table 1). Both UV and MS spectra allowed identifying M17 as methyl-luteolin glucuronide.

To end, incubation of rosemary infusion with HepG2 cells, showed 19 metabolites. Apart from M1, M2, M3, M4, M6, FA, M10, M11, M12, M13, M14, M15, M16, M17, M18, M21 and

M23 (see Table 1), two new peaks appeared at RT (min) 18.9 and 41.8 labeled as M8, and M26. Both new peaks, showed a UV spectrum with λ_{max} at 248-266 nm and 340-348 nm characteristic of luteolin derivatives. Chromatographic peak M8 provided an $[M-H]^-$ ion at *m/z* 637.1046 plus fragment ions at *m/z* 461 and 285 corresponding to the loss of glucuronide and diglucuronide moieties, respectively, which allowed identifying M8 as luteolin diglucuronide. However, M26 provided a $[M-H]^-$ ion at *m/z* 299.0561 plus fragment ion at *m/z* 285 corresponding to luteolin, which allowed confirming its identification as the methyl conjugate of luteolin.

Quantification of rosemary infusion and hydroxycinnamic acids metabolites in Caco-2 cells

The amount of unmetabolized phenols and the respective metabolites in the extracellular culture medium was quantified after 2 and 23 h incubation with Caco-2 cells. Differences were observed due to the incubation time as well as the chemical structure of the phenolic compounds. Results are summarized in Table 2.

A direct relationship between metabolization rate and incubation time was observed for RA, CA and FA in the rosemary infusion and the standards. The percentage of unmetabolized compounds detected in the culture medium after 2 h incubation represented 97.9, 96.7 and 99.7 % for RA, CA and FA, respectively, which decreased to 88.2, 69.6 and 95.6 % for RA, CA, and FA, respectively, after 23 h incubation. Similar behavior was observed with the rosemary infusion, showing 86.6 and 80.6% of unmetabolized compounds after short and long term incubation, respectively.

Regarding the nature of the metabolites formed, CA was mainly methylated to form FA and its isomer *iso*FA (M7) (1.75% and 26.10% after 2 and 23 h of incubation, respectively), which were subsequently glucuronidated into M2, M4 and M6 (FA glucuronides), amounting to 1.62 and 2.03% of the total metabolites after 2 and 23 h incubation, respectively. Glucuronidated conjugates of CA (M1 and M5) were also detected after 23 h incubation,

accounting for 2.31% of the total metabolites. FA was minimally transformed into its glucuronidated conjugates, forming M2, M4 and M6 (FA glucuronides), amounting to 0.33 and 4.38% after 2 and 23 h of incubation, respectively. RA was minimally hydrolyzed to form free CA (0.08 and 0.08% after 2 and 23 h of incubation, respectively), but the main RA pathway was methylation to form monomethyl (M18 and M19) and dimethyl derivatives of RA (M22, M23, M24 and M25), which reached percentages of 1.03 and 10.66% after 2 and 23 h of incubation, respectively. Minimal amount of one isomer of RA (M12) was detected, accounting up to 1.0% of the total metabolites quantified. To conclude, phenols in rosemary tea showed a metabolic behavior similar to that described for the corresponding standards. Thus, phase II derivatives of CA (4.13 and 4.75% after 2 and 23 h of incubation, respectively) were counted after the incubation of rosemary infusion, which could derive from CA already present in the rosemary infusion (up to 10% of the total phenolic content of the infusion), or RA hydrolysis to form free CA. Apart from the slight isomerization of RA (3.24 and 4.90% after 2 h and 23 h of incubation, respectively), RA was partially transformed into its phase II derivatives: methyl conjugates (M18, M23, M24 and M25, up to 3.70%), glucuronidated conjugates (M10, up to 1.77%) and methyl-glucuronidated conjugates (M13, M14, M15 and M21, up to 2.67%) after the longer incubation. Separately, as aforementioned, rosemary tea contains a minor amount of luteolin and luteolin derivatives, which represented the 44% of the total amount of rosemary infusion polyphenols. Consequently, methyl conjugates (up to 0.31%) and methyl-glucuronidated of luteolin (up to 1.44%) were detected after 23 h incubation.

Quantification of rosemary infusion and hydroxycinnamic acids metabolites in HepG2 cells

Free and conjugated metabolites were quantified in the extracellular culture medium after 2 and 23 h of incubation with HepG2 cells (Table 2). As shown in Table 3, a direct relationship between metabolization rate and incubation time was observed, although the phenol metabolization degree was different between pure RA, CA and FA and the acids in the

rosemary infusion. After the short incubation, pure standards showed comparable amounts of unmetabolized compounds (97.34%, 96.14% and 99.55% corresponding to RA, CA and FA, respectively), however, after the long incubation time, higher differences were observed, amounting to 89.67%, 65.73% and 96.80% corresponding to RA, CA and FA, respectively. However, in the rosemary infusion, the unmetabolized phenolic fraction added up to 86.45% and 76.54% after short and long incubation time, respectively.

Regarding biotransformation pathways followed by the tested compounds and to shorten only the percentages of the metabolites formed after long incubation time are here presented, whereas data of the short incubation times are listed in Table 3. Methyl conjugates (FA and M7, 24.71% of the total metabolites) were the main metabolites obtained from CA, followed by glucuronidated conjugates (M1, M3 and M5) and methyl-glucuronidated conjugates (M2, M4 and M6) (8.44% and 1.12%, respectively). Glucuronidated conjugates (M2, M4 and M6) were the only metabolites identified (3.20% of the total metabolites) when FA was assayed. RA was partially transformed into its isomer (M12) (2.41% of the total metabolites) and phase II derivatives; monomethyl (M18 and M19) and dimethyl derivatives (M24 and M25) amounted to 4.20%, glucuronidated derivatives (M9, M11, M13, M14, M15, M16, M20 and M21) added up to 3.35% and the glucuronidated conjugate (M10) amounted to 0.07% of the total metabolites. RA was also slightly hydrolyzed to form free CA (0.19% of the total metabolites), which was subsequently methylated to FA (0.11% of the total metabolites). As a final point, when rosemary infusion was incubated with HepG2 cells, a higher percentage of phase II derivatives of CA was observed compared to pure RA standard (5.30 and 0.11%, respectively), due to the amount of CA contained in rosemary infusion (up to 10% of the total phenolic content in the infusion). Although RA and its isomer M12 (4.79% of the total metabolites) were partially hydrolyzed to contribute to the formation of phase II derivatives of CA, the main pathway followed by RA and its isomer (M12) was the transformation into phase II derivatives already described when RA was incubated as pure standard. Thus, methyl-glucuronidated

(M10, M14, M15, M16 and M21, 5.42% of the total metabolites), followed by glucuronidated (M10, 3.32% of the total metabolites) and methyl conjugates (M18 and M23, 2.71% of the total metabolites) were the metabolites registered after rosemary infusion incubation with HepG2 cells. Additionally, luteolin derivatives present in the rosemary tea provided a minor amount of metabolites, such as methyl-luetolin glucuronide (M17, 1.27%) and methyl-luteolin (M26, 0.65%).

DISCUSSION

Considering that the biological activity of polyphenols partially depends on the intestinal and hepatic uptake and metabolism, the absorption of rosemary polyphenols and the extent to which they are conjugated and metabolized across the small intestine and liver were investigated using human Caco-2 and HepG2 cells, as intestinal and hepatic *in vitro* models, respectively. To our knowledge, there is no bioavailability study on phenols in rosemary infusion, such as the beverage is usually consumed. There are few studies using enriched rosemary polyphenol extracts obtained by supercritical fluid extraction [14], which composition differs from that of the present rosemary infusion. In addition, to establish the effects of the plant matrix on the *in vitro* absorption and metabolism of RA and CA, the uptake of the pure compounds compared to the same compounds in the rosemary infusion was studied using the aforementioned human cell models. In addition, FA as a metabolite derived of both RA and CA was also tested to evaluate the influence of the different chemical structure on the bioavailability and metabolism of hydroxycinnamic acids. After the ingestion of μM to mM levels of polyphenol aglycones and glycosides, which are the usual forms present in plant foods, concentrations in the low μM to nM range are found in the circulatory system, while most of the consumed polyphenols are not absorbed in the small intestine, passing into the colon, where they are degraded by the action of the local microbiota[15, 16]. Bearing this in mind, 100 μM of each pure standard and rosemary infusion was used to carry out the bioavailability

experiments, which is a physiological concentration likely to be present in the intestinal lumen. This concentration, however, is likely higher than what could be expected in the blood after the intake of rosemary tea, but we used the same concentration in the incubation with HepG2 cells to allow for comparison in these *in vitro* assays. All test samples (standards and rosemary infusion) were incubated in both cell lines (Caco-2 and HepG2 cells) for 2 and 23 h to mimic the physiological time of permanence in the gastrointestinal tract and the liver and to force the formation of metabolites, respectively.

Incubation of the tested compounds and rosemary infusion in human Caco-2 cells showed that RA, CA and FA were partially metabolized mainly by the action of phase II enzymes, showing an incubation-time dependent relationship. Structural differences among the rosemary polyphenols brought about variations in the type of metabolites formed in Caco-2 cells. CA metabolites were mainly methyl derivatives followed by methylglucuronide and glucuronides in similar extension, whereas FA was conjugated into glucuronide derivatives and RA into methyl derivatives by phase II enzymes. Therefore, RA underwent hydrolysis to yield free CA by cellular carboxylesterase. It is noteworthy that RA was partially transformed into an isomer of RA. The same polyphenols in the rosemary infusion followed similar biotransformation pathways as the pure standards, although with slight modifications. RA in rosemary infusion was mainly methylated although methyl-glucuronidated and glucuronidated conjugates of RA were detected after short and long incubation times. Likewise, RA was slightly hydrolyzed into free CA, which was subsequently conjugated by phase II enzymes forming FA. Additionally, due to the content of luteolin derivatives in the rosemary infusion, methylated and methyl-glucuronidated derivatives of this flavonoid were also detected.

No sulfated conjugates were identified after the incubation of any of the standards either the rosemary infusion in Caco-2 cells, contrary to that reported by Kern et al. (2003) using ferulic, sinapic and *p*-coumaric acid [17]. We cannot discard that low levels of sulfated metabolites might have been formed, yet not detected under the present experimental

conditions. In agreement with our results, no sulfation of CA was detected in Caco-2 cells by Kern et al[17]. Methylation was the preferential pathway for intestinal metabolism of pure RA and CA, but not for FA due to the absence of catechol moiety that disables the recognition by the catechol-*O*-methyl transferase (COMT). Similarly, RA was predominantly methylated when incubated as rosemary tea, although also glucuronidated, pointing out the plant matrix effect on RA metabolization pathways.

All compounds detected in the cell lysates were below the limit of quantification, indicating that no intracellular accumulation in colonic and hepatic cells of RA, CA and FA, as pure standards and in a rosemary tea, occurred.

ROSEMARY INFUSION POLYPHENOLS

In the present study only metabolization rates were determined, not uptake, since Caco-2 cells were not differentiated. The proportion of unconjugated compounds was high after their incubation with Caco-2 cells (97.9%, 96.6%, 99.7% and 86.6% after 2 h and 88.2%, 69.5%, 95.6% and 80.6% after 23 h incubation for RA, CA, FA and rosemary infusion, respectively). Previous studies have reported low absorption rates for RA [18], CA [19] and FA [20] in differentiated Caco-2 cells and extensive metabolization of the absorbed compounds. Baba et al (2004) also confirmed low RA bioavailability in rats, with an urinary recovery up to 5.5% of the ingested RA mainly in the form of free hydroxycinnamic acids (CA, FA and *m*-coumaric acid to a lower extent) and their phase II derivatives, followed by phase II RA derivatives and free RA [21]. Moreover, approximately 83% of the total metabolites were excreted between 8 to 18 h after RA intake, compatible with colonic absorption [21]. The same research group also evaluated the bioavailability of RA after the intake of *Perilla frutescens* extract in humans [22]. Results in humans showed great similarity to those obtained in rats, since the total urinary excretion of phase II derivatives of RA, CA and FA and free standards resulted in a recovery of 6.3% of the

phenols ingested from *Perilla frutescens* extract. Considering the low bioavailability observed for RA in studies carried out in rats [21] and in humans [22], and for RA, CA and FA in *ex vivo* studies carried out in differentiated Caco-2 cells [18, 19, 23], together with the high metabolization rate described for absorbed hydroxycinnamic acids, suggest that the metabolite percentages observed in the present study could correspond with cellular uptake of these hydroxycinnamic acids, which was poor after 2 h of incubation.

Comparing the metabolization rate of the pure standards after 23 h of incubation with Caco-2 cells, CA was the most bioavailable compound (30.44% of the total metabolites), followed by RA (11.75%) and FA (4.38%). Previous studies with nitrocatechols [24], alkyl gallates [25] and hydroxytyrosol derivatives [26, 27] have described a direct relationship between the lipophilic nature of the compound and its uptake and biotransformation. Attending to these studies, the absorption of RA and FA should have been higher than that of CA; however, the tendency here observed was contrary, suggesting that the chemical structure of the compound strongly determines its bioavailability and the mechanisms ruling its intestinal absorption. In this sense, it is important to note that Intestinal absorption mechanisms of CA, FA and RA have been previously reported using Caco-2 cells [19, 20, 23, 28]. Pure RA and CA may be absorbed by paracellular diffusion in Caco-2 cells although CA showed low affinity for monocarboxylic acid transporter (MCT) [19, 28] whereas FA was absorbed by the colonic epithelium following a combination of mechanisms such as passive transcellular diffusion and MCT1 and sodium-dependent MCT facilitated transport [20, 23].

It is well known that any phenolic compound that enters the bloodstream reaches the liver where it is subsequently metabolized. Therefore, hepatic uptake and metabolism of RA, CA and FA as standards as well as in rosemary infusion was evaluated in HepG2 cells, also enabling to understand the phenol biotransformation. The present results revealed a time-dependent uptake and metabolism of the standards in HepG2, which was low after 2 h incubation (2.66%, 3.86% and 0.45% for RA, CA and FA, respectively) and moderate after 23 h

incubation (10.33%, 34.27% and 3.2% for RA, CA and FA, respectively). In general, these values were slightly higher than those described after incubation with Caco-2 cells, although still modest. Therefore, it may be concluded that hydroxycinnamic acids in rosemary show limited uptake in HepG2 cells.

Biotransformation pathways were in agreement with those previously described in Caco-2 cells, although with certain differences. Thus, methylation was the preferential pathway followed by CA and RA, although glucuronidation rates were higher than in Caco-2 cells. FA, lacking the catechol moiety, was only glucuronidated. In addition, RA was slightly transformed into its isomer and hydrolyzed to form CA, which was subsequently methylated to yield FA. Hydrolysis and isomerization reactions of RA showed higher yield after incubation with HepG2 than with Caco-2 cells. However, RA in the rosemary infusion incubated with HepG2 cells was preferably glucuronidated, although methyl-glucuronides and methyl conjugates were also detected to a lower extent. Contrarily, RA incubated as pure compound or rosemary infusion with Caco-2 cells, yielded methyl conjugates as the most abundant metabolites. Isomerization and hydrolysis of RA in rosemary infusion occurred in a higher rate than in a pure solution. Finally, luteolin derivatives of rosemary infusion provided phase II derivatives in similar extension than after its incubation with Caco-2 cells. The present results were in agreement with Mateos et al. (2006) that described limited uptake and metabolism for CA and FA in HepG2 cells [29].

The outcome that intestinal and hepatic uptake and metabolism of RA was higher when the compound formed part of the rosemary infusion compared to the pure standard points out the effects of the plant matrix on the RA metabolization pathways. This different bioavailability suggests that the bioactivity of dietary phenolic compounds might be different than that of the pure compound, which might be of relevance when considering the intake of pure phenolic compounds as supplements versus the consumption of a food or beverage with a complex phenolic composition. It is well known that plant matrices may influence

phytochemical metabolism altering transporters of the compounds [30] or due to the interaction with other components of the plant matrix [18].

It is also well known that polyphenols that reach the liver can be secreted *via* the biliary pathway into the duodenum and be partially re-absorbed [31]. In addition, polyphenols not absorbed in the small intestine pass into the large intestine, where they are metabolized by the colonic microbiota and partly absorbed. Both pathways enhance the permanence of polyphenols in the body as well as their recovery and, therefore, favor the phenol bioactivity. Taking into account the aforementioned bioavailability studies with RA in rats [21] and humans [22] and the reported delayed urinary excretion, it is likely that RA is mainly absorbed by the colon, in agreement with the limited bioavailability here observed in human enterocyte cells *in vitro*.

Although *in vitro* cell culture models are of great value to study the uptake and metabolism of different phenolic compounds, allowing for comparison of the metabolic pathways underwent by structurally related compounds and the potential influence of the food matrix as seen in the present experiments, these results should be confirmed in human studies, needed to evaluate the bioavailability of rosemary phenols and the influence of the colonic microbiota in their metabolism.

In summary, RA, CA and FA, as pure standards and in a rosemary tea, were partially absorbed and extensively metabolized in both Caco-2 and HepG2 cells, with higher metabolization rate after hepatic incubation than with intestinal Caco-2 cells. Structural differences among the rosemary polyphenols brought about variations in the type of metabolites formed in Caco-2 and HepG2 cell models. The main metabolites detected after intestinal and hepatic metabolism of CA and RA were methyl derivatives followed by methyl-glucuronides and glucuronides to a lower extent, but not sulfated derivatives, whereas FA yielded only glucuronidated metabolites. In addition, RA was partially hydrolyzed into CA, which was subsequently conjugated by phase II enzymes. The rosemary infusion matrix enhanced the bioavailability of RA compared to that of pure compound, which could turn into

a greater bioactivity. In conclusion, rosemary polyphenols are partially absorbed and metabolized at intestinal and hepatic level.

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TABLES

Table1. Chromatographic and spectroscopic characteristics of rosemary infusion polyphenols and ferulic acids as well as the metabolites formed after incubation with Caco-2 and HepG2 cells.

Number Peak	RT (min)	λ max	Molecular Formula	$[M-H]^-$ (m/z)	Fragment ions	Identified Compound
CA	9.5	324, 298sh	$C_9H_8O_4$	179.0350	135	Caffeic acid
FA	16.4	324, 296sh	$C_{10}H_{10}O_4$	193.0506	179; 149	Ferulic acid
LGlcA	20.2	255, 346	$C_{21}H_{18}O_{12}$	461.0725	285	Luteolin glucuronide (Isomer 1)
RA	25.6	328, 294sh	$C_{18}H_{16}O_8$	359.0772	135; 161; 179	Rosmarinic acid
Hp	25.8	272, 332	$C_{22}H_{22}O_{11}$	461.1089	161; 283; 297	Homoplantaginin
LGlcA	27.5	267-350	$C_{21}H_{18}O_{12}$	461.0725	285	Luteolin glucuronide (Isomer 2)
LAGlcA	32.2	266, 338	$C_{23}H_{20}O_{13}$	503.0831	285; 399	Luteolin-3'-*O*-(*O*-acetyl)-β-d-glucuronide (Isomer 1)
LAGlcA	32.9	264, 332	$C_{23}H_{20}O_{13}$	503.0831	285; 399	Luteolin-3'- *O* -(*O*-acetyl)-β-d-glucuronide (Isomer 2)
L	34.6	250, 350	$C_{15}H_{10}O_6$	285.0405	146; 191; 263	Luteolin
M1	5.5	320	$C_{15}H_{16}O_{10}$	355.0671	179	Caffeic acid glucuronide (Isomer 1)
M2	6.7	328, 300sh	$C_{16}H_{18}O_{10}$	369.0827	193	Ferulic acid glucuronide (Isomer 1)
M3	7.4	314, 296sh	$C_{15}H_{16}O_{10}$	355.0671	179	Caffeic acid glucuronide (Isomer 2)
M4	9.7	308	$C_{16}H_{18}O_{10}$	369.0827	193	Ferulic acid glucuronide (Isomer 2)
M5	10.1	314, 294sh	$C_{15}H_{16}O_{10}$	355.0671	179	Caffeic acid glucuronide (Isomer 3)
M6	11.4	328, 300sh	$C_{16}H_{18}O_{18}$	369.0827	193	Ferulic acid glucuronide (Isomer 3)
M7	18.2	324, 296sh	$C_{10}H_{10}O_4$	193.0506	149	*iso*Ferulic acid
M8	18.9	266, 340	$C_{27}H_{26}O_{18}$	637.1046	461; 285	Luteolin diglucuronide
M9	20.9	322, 296sh	$C_{25}H_{26}O_{14}$	549.1250	373, 359	Methyl rosmarinic acid glucuronide
M10	21.1	320, 294sh	$C_{24}H_{24}O_{14}$	535.1093	359	Rosmarinic acid glucuronide
M11	23.1	320, 294sh	$C_{25}H_{26}O_{14}$	549.1250	373, 359	Methyl rosmarinic acid glucuronide
M12	24.7	328, 294sh	$C_{18}H_{16}O_8$	359.0772	135; 161; 179	Rosmarinic acid (isomer)

M13	26.0	326, 294sh	$C_{25}H_{26}O_{14}$	549.1250	373; 359	Methyl rosmarinic acid glucuronide
M14	26.5	328, 296sh	$C_{26}H_{28}O_{14}$	563.1406	359	Dimethyl rosmarinic acid glucuronide
M15	28.6	320, 294sh	$C_{26}H_{28}O_{14}$	563.1406	359	Dimethyl rosmarinic acid glucuronide
M16	29.9	320, 294sh	$C_{26}H_{28}O_{14}$	563.1406	359	Dimethyl rosmarinic acid glucuronide
M17	30.2	266, 340	$C_{22}H_{20}O_{12}$	475.0882	299; 285	Methyl luteolin glucuronide
M18	32.2	330, 294sh	$C_{19}H_{18}O_8$	373.0929	359	Methyl rosmarinic acid
M19	33.3	330, 294sh	$C_{19}H_{18}O_8$	373.0929	359	Methyl rosmarinic acid
M20	35.7	324, 292sh	$C_{26}H_{28}O_{14}$	563.1406	359	Dimethyl rosmarinic acid glucuronide
M21	37.1	324, 292sh	$C_{26}H_{28}O_{14}$	563.1406	359	Dimethyl rosmarinic acid glucuronide
M22	38.6	328, 294sh	$C_{20}H_{20}O_8$	387.1085	359	Dimethyl rosmarinic acid
M23	39.2	328, 294sh	$C_{20}H_{20}O_8$	387.1085	359	Dimethyl rosmarinic acid
M24	39.4	328, 294sh	$C_{20}H_{20}O_8$	387.1085	359	Dimethyl rosmarinic acid
M25	40.1	328, 294sh	$C_{20}H_{20}O_8$	387.1085	359	Dimethyl rosmarinic acid
M26	41.8	248, 348	$C_{16}H_{12}O_6$	299.0561	285	Methyl luteolin

Abbreviations: RT = Retention Time; RA = Rosmarinic Acid; CA: Caffeic acid; FA: Ferulic acid; Glc: Glucoside; GlcA: Glucuronide; Hp: Homoplantaginin; LGlcA: Luteolin glucuronide; LAGlcA: Luteolin-3'-*O*-(*O*-acetyl)-β-d-glucuronide; L: Luteolin

Table 2. Concentrations of free molecules and their metabolites in the extracellular culture media after 2 h and 23 h of incubation of Caco-2 cells in the presence of 100 μM of each hydroxycinnamic acid rosmarinic acid (RA), caffeic acid (CA) and ferulic Acid (FA) and rosemary infusion containing 100µM of phenolic compounds

	Incubation Time	2 h		23 h		P
Acid	Type	µM	%	µM	%	
RA	Free	92.62 ± 0.35	97.87	80.67 ± 3.05	88.25	0.003
	Caffeic Acid (Hydrolysis)	0.08 ± 0.02	0.08	0.07 ± 0.01	0.08	N.S.
	Ferulic Acid	-		-		-
	RA isomer	0.96 ± 0.02	1.02	0.92 ± 0.08	1.01	N.S.
	Glucuronidated	-		-		-
	Methylated	0.97 ± 0.04	1.03	9.75 ± 0.25	10.66	<0.001
	Methyl-glucuronidated	-		-		-
	TOTAL	94.64 ± 0.42	100	91.40 ± 3.40	100	N.S.
CA	Free	92.32 ± 0.37	96.63	62.30 ± 1.32	69.56	<0.001
	Glucuronidated	-	-	2.07 ± 0.29	2.31	<0.001
	Methylated	1.67 ± 0.04	1.75	23.38 ± 1.30	26.10	<0.001
	Methyl-glucuronidated	1.55 ± 0.23	1.62	1.82 ± 0.18	2.03	N.S.
	Total	95.54 ± 0.63	100	89.57 ± 3.08	100	0.020
FA	Free	95.44 ± 1.38	99.67	88.61 ± 6.32	95.62	N.S.
	Glucuronidated	0.31 ± 0.02	0.33	4.06 ± 0.34	4.38	<0.001
	TOTAL	95.75 ± 1.40	100	92.66 ± 6.66	100	N.S.
Rosemary Infusion	Free	80.95 ± 2.40	86.58	74.71 ± 6.31	80.65	N.S.
	RA isomer	3.02 ± 0.38	3.24	4.54 ± 0.23	4.90	0.004
	Phase II derivatives of CA	3.86 ± 0.63	4.13	4.40 ± 1.34	4.75	N.S.
	RA glucuronides	1.65 ± 0.72	1.77	1.52 ± 0.22	1.64	N.S.
	RA methylated	0.54 ± 0.07	0.58	3.43 ± 0.54	3.70	<0.001
	RA methyl-glucuronid	2.49 ± 0.52	2.67	2.42 ± 0.61	2.61	N.S.
	Luteolin methylated	-		0.28 ± 0.07	0.31	0.002
	Luteolin methyl-glucuronid	0.96 ± 0.03	1.03	1.33 ± 0.12	1.44	0.008
	TOTAL	93.49 ± 4.76	100	92.63 ± 9.46	100	N.S.

Values are the mean ± SEM, n=3. P values correspond to one-way ANOVA.

Table 3. Concentrations of free molecules and their metabolites in the extracellular culture media after 2 h and 23 h of incubation of HepG2 cells in the presence of 100 μM of each hydroxycinnamic acid rosmarinic acid (RA), caffeic acid (CA) and ferulic Acid (FA) and rosemary infusion containing 10µM of phenolic compounds

Incubation Time		2 h		23 h		P
Acid	**Type**	**µM**	**%**	**µM**	**%**	
RA	**Free**	88.04 ± 0.14	97.34	82.58 ± 1.39	89.67	0.010
	Caffeic Acid (Hydrolysis)	0.14 ± 0.01	0.15	0.17 ± 0.08	0.19	N.S.
	Ferulic Acid	0.05 ± 0.01	0.06	0.10 ± 0.02	0.11	0.015
	RA isomer	1.55 ± 0.15	1.72	2.22 ± 0.57	2.41	N.S.
	Glucuronidated	-	-	0.07 ± 0.01	0.07	-
	Methylated	0.20 ± 0.03	0.22	3.86 ± 0.06	4.20	<0.001
	Methyl glucuronidated	0.46 ± 0.05	0.51	3.08 ± 0.10	3.35	<0.001
	TOTAL	90.44 ± 0.38	100	92.09 ± 2.23	100	0.041
CA	**Free**	93.87 ± 1.89	96.14	58.18 ± 0.77	65.73	<0.001
	Glucuronidated	0.07 ± 0.02	0.07	7.47 ± 1.58	8.44	0.001
	Methylated	0.95 ± 0.05	0.97	21.87 ± 2.62	24.71	<0.001
	Methyl-Glucuronidated	2.74 ± 0.48	2.81	0.99 ± 0.35	1.12	0.004
	TOTAL	97.64 ± 2.44	100	88.52 ± 5.32	100	0.026
FA	**Free**	91.37 ± 1.36	99.55	87.75 ± 0.13	96.80	0.010
	Glucuronidated	0.41 ± 0.13	0.45	2.90 ± 0.47	3.20	<0.001
	TOTAL	91.78 ± 1.49	100	90.66 ± 0.60	100	N.S.
Rosemary Infusion	**Free**	79.62 ± 1.96	86.45	65.08 ± 3.83	76.54	<0.001
	RA isomer	3.17 ± 0.37	3.44	4.07 ± 0.94	4.79	N.S.
	Phase II derivatives of CA	3.88 ± 0.39	4.21	4.51 ± 0.33	5.30	0.023
	RA glucuronides	1.74 ± 0.02	1.90	2.82 ± 0.20	3.32	0.001
	RA methylated	0.64 ± 0.33	0.70	2.30 ± 0.13	2.71	0.001
	RA methyl-glucuronid	2.21 ± 0.26	2.40	4.61 ± 0.98	5.42	0.006
	Luteolin methylated	-	-	0.55 ± 0.07	0.65	-
	Luteolin methyl-glucuronid	0.82 ± 0.10	0.89	1.08 ± 0.06	1.27	0.017
	TOTAL	92.10 ± 3.43	100	85.02 ± 6.55	100	0.008

Values are the mean ± SEM, n=3. P values correspond to one-way ANOVA.

Figure legends

Figure 1: HPLC chromatograms of extracellular medium in contact with Caco-2 cells obtained after 2 and 23 h (left and right pictures, respectively) incubation with 100µM of the following compounds (A,B): Rosmarinic Acid, (C,D): Caffeic Acid, (E,F): Ferulic Acid, (G,H): Rosmarinus officinalis tea containing 100µM of total phenols. RA: rosmarinic acid; CA: caffeic acid; FA: ferulic acid; Hp: homoplantaginin; LGlcA: luteolin glucuronide; LAGlcA: luteolin-3'-o-(2''-o-acetyl)-β-d-glucuronide; L: luteolin.

Figure 2: HPLC chromatograms of extracellular medium in contact with HepG2 cells obtained after 2 and 23 h (left and right pictures, respectively) incubation with 100µM of the following compounds (A,B): Rosmarinic Acid, (C,D): Caffeic Acid, (E,F): Ferulic Acid, (G,H): Rosmarinus officinalis tea containing 100µM of the total phenols. RA: rosmarinic acid; CA: caffeic acid; FA: ferulic acid; Hp: homoplantaginin; LGlcA: luteolin glucuronide; LAGlcA: luteolin-3'-o-(2''-o-acetyl)-β-d-glucuronide; L: luteolin.

Figure 1.

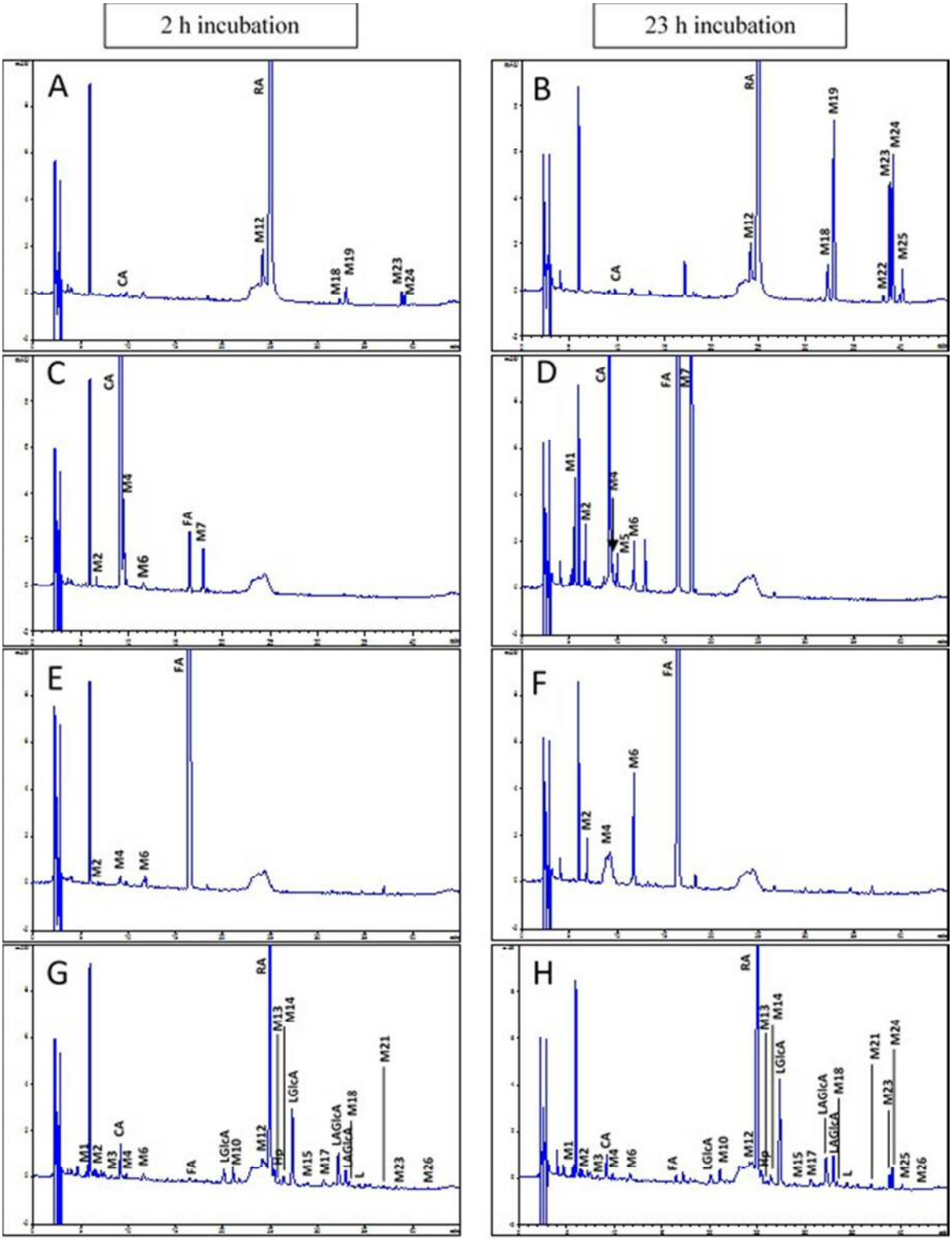
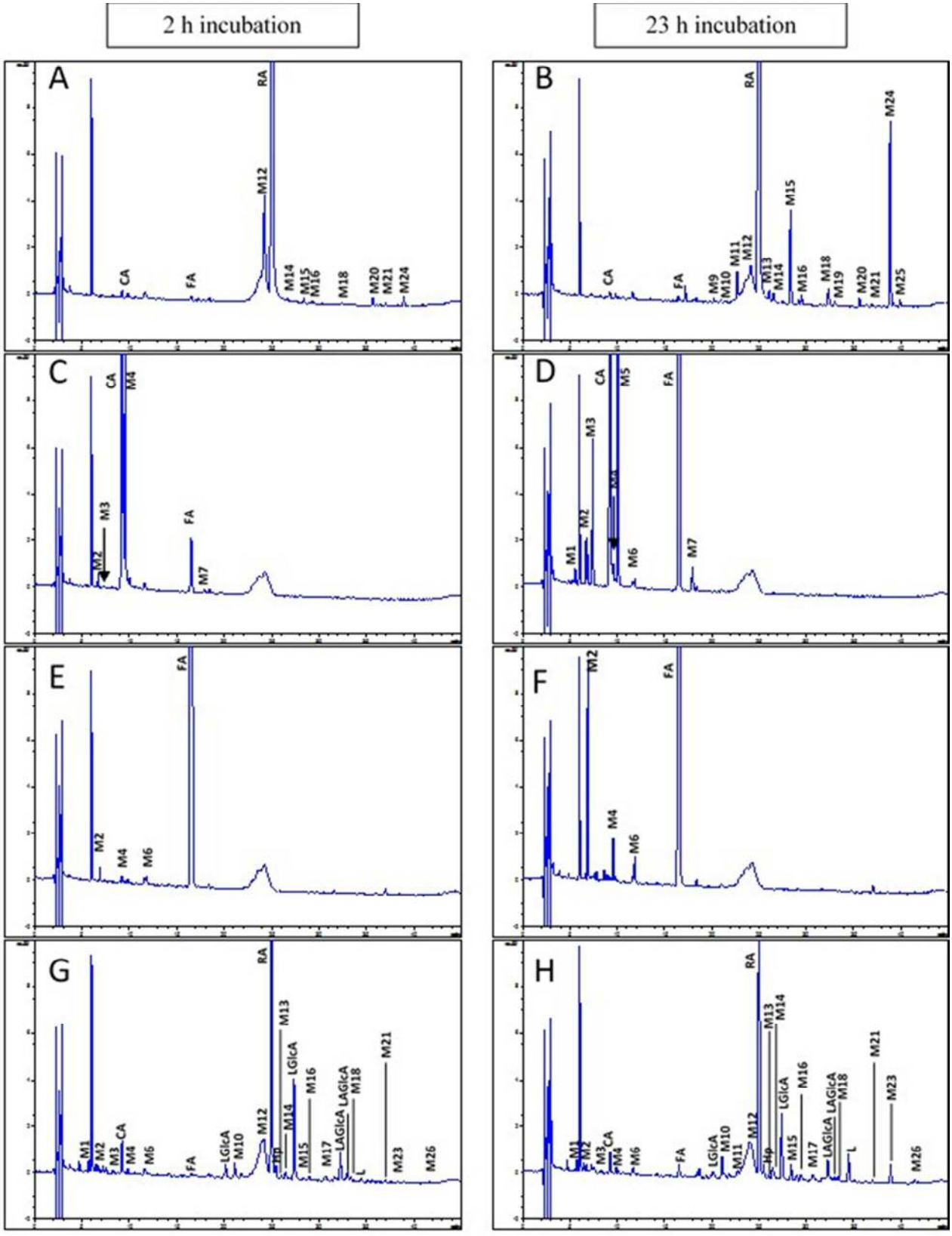

2 h incubation
23 h incubation
A
RA
M12
CA
M18
M19
M23
M24
B
RA
M19
M12
M23
M24
CA
M18
M22
M25
C
CA
M4
M2
M6
FA
M7
D
CA
FA
M7
M1
M4
M2
M6
M5
E
FA
M2
M4
M6
F
FA
M6
M2
M4
G
RA
M13
M14
LGlcA
M21
M1
M2
M3
CA
M4
M6
FA
LGlcA
M10
M12
Hp
M15
M17
LAGlcA
LAGlcA
M18
L
M23
M26
H
RA
M13
M14
LGlcA
M21
M24
LAGlcA
M18
M23
M1
M2
M3
CA
M4
M6
FA
LGlcA
M10
M12
Hp
M15
M17
LAGlcA
L
M25
M26

Figure 2.


2 h incubation
23 h incubation
A
B
C
D
E
F
G
H
RA
CA
FA
M1
M2
M3
M4
M5
M6
M7
M9
M10
M11
M12
M13
M14
M15
M16
M17
M18
M19
M20
M21
M23
M24
M25
M26
LGlcA
LAGlcA
Hp
L