Cytotoxicity of *Coprinopsis atramentaria* extract, organic acids and their synthesized methylated and glucuronate derivatives

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Abstract

Coprinopsis atramentaria is a wild edible mushroom whose methanolic extract revealed a marked antioxidant activity; *p*-hydroxybenzoic (HA), *p*-coumaric (CoA) and cinnamic (CA) acids were identified in the extract. In the present work, it was evaluated the cytotoxicity of *C. atramentaria* extract, previously identified organic acids and their synthesized derivatives (methylated compounds and protected glucuronides). Among all the tested cell lines (MCF-7- breast adenocarcinoma, NCI-H460- non-small cell lung carcinoma, HCT15- colon carcinoma, HeLa- cervical carcinoma and HepG2- hepatocellular carcinoma), the extract presented good activity for the first three cell lines mentioned; in most of the cases methylated and glucuronide derivatives showed a higher activity than the corresponding parental compounds. The substitution of the carboxylic group (in parental organic acids) for an ester (in methylated derivatives) increased the cytotoxicity for tumor cell lines. Acetylated glucuronide derivatives showed higher cytotoxicity when compared with the corresponding parental acids.

1. Introduction

Mushrooms consumption is increasing because of their unique and subtle flavor (Sadler, 2003), nutritional properties (Kalac, 2009) and richness on bioactive compounds. The scientific community, searching for new therapeutic alternatives, studied many different species and has found variable therapeutic activities such as anti-inflammatory and antibiotic effects (Lindquist, Niedermeyer, & Jülich, 2005; Poucheret, Fons, & Rapior, 2006; Alves et al., 2012; Heleno et al., 2013b). Moreover, mushrooms are a source of compounds with antitumor properties, including low-molecular-weight (e.g. quinones, sesquiterpenes, steroids) and high-molecular-weight (e.g. homo and heteropolysaccharides, glycoproteins) compounds (Ferreira, Vaz, Vasconcelos, & Martins, 2010). Our attention is being focused on the capacity of organic acids (including phenolic acids) from mushrooms to inhibit the growth of different tumor cell lines.

Coprinopsis atramentaria (Bull.: Fr.) Redhead, Vilgalys & Moncalvo, commonly known as the common ink cap or inky cap, is a widespread wild edible mushroom found in Europe and North America. Previously known as *Coprinus atramentarius*, it is the second best known ink cap and previous member of the genus *Coprinus* after *Coprinus comatus*. Clumps of mushrooms arise after rain from spring to autumn, commonly in urban and disturbed habitats such as vacant lots and lawns, as well as grassy areas. This mushroom, previously characterized by us for its nutritional composition showed a notable antioxidant activity; phenolic acids were identified in its extract (Heleno, Barros, Queiroz, Santos-Buelga, & Ferreira, 2012) and have been reported as common compounds in mushrooms (Vaz et al., 2011).

Dietary phenolic compounds, including phenolic acids, are widely considered to contribute to health benefits in humans (Ho, Lee, & Huang, 1992). Nevertheless, there

are very few studies dealing with cytotoxicity of phenolic acids and related compounds. Cinnamic and protocatechuic acids revealed cytotoxicity for a lung cancer cell line, and were implied in the capacity of *Clitocybe alexandri* to induce cell cycle arrest and apoptosis in the same cell line (Vaz, Almeida, Ferreira, Martins, & Vasconcelos, 2012). Cinnamic acid was also pointed out as possessing antitumor activity against human malignant tumors, including melanoma, glioblastoma, adenocarcinoma of the prostate and lung (Liu, Hudgins, Shack, Yin, & Samid, 1995) and colon adenocarcinoma (Ekmekcioglu et al., 1998). Drupanin and baccharin, two cinnamic acid derivatives, were reported as inhibitors of the growth of HeLa-60 cell lines by inducing apoptosis (Akao et al., 2003).

However, little is known about phenolic acids bioactive forms *in vivo* and the mechanisms by which they may contribute toward disease prevention. In fact, many studies have ignored the question of their metabolism (Scalbert & Williamson, 2000; Rechner et al., 2002; Manach, Williamson, Morand, Scalbert, & Remesy, 2005; Nardini et al., 2009). After absorption from the gastrointestinal tract, these molecules circulate in human plasma as conjugated forms namely glucuronide and methylated derivatives (Rechner et al., 2002; Piazzon et al., 2012).

As far as we know, nothing has been reported on cytotoxicity of *C. atramentaria* extract and glucuronide or methylated derivatives of phenolic acids. Detailed knowledge concerning the conjugative and metabolic events following the ingestion of these organic acids is crucial to the understanding of their bioactivity effect, in order to clarify the role of the metabolites in the pharmacology of the parental substance. Therefore, in the present work, it was evaluated the cytotoxicity of *C. atramentaria* extract, organic acids (*p*-hydroxybenzoic, *p*-coumaric and cinnamic acids) and their synthesized derivatives: methylated derivatives and protected glucuronides.

2. Materials and methods

2.1. Wild mushroom and preparation of the extract

Samples of *Coprinopsis atramentaria* (Bull.: Fr.) Redhead, Vilgalys & Moncalvo were collected in Bragança (Northeast Portugal). After taxonomic identification of the sporocarps (Oria de Rueda, 2007), specimens were deposited at the herbarium of School of Agriculture of Polytechnic Institute of Bragança. The samples were lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), reduced to a fine powder (20 mesh) and kept at room temperature inside a desiccator until further analysis (within a week).

The powder (~10 g) was extracted with methanol (250 ml) at -20 °C for 6 h. The extract was sonicated for 15 min, centrifuged at 4000g for 10 min and filtered through Whatman No.4 paper. The residue was then re-extracted with three additional 150 ml portions of methanol. The combined extracts were evaporated (rotary evaporator Büchi R-210; Flawil, Switzerland) at 40 °C to dryness.

2.2. Organic acids

p-Hydroxybenzoic acid (HA), *p*-coumaric acid (CoA) and cinnamic acid (CA) were identified in *C. atramentaria* extract (Heleno et al., 2012). For cytotoxicity assays, these compounds were purchased from Sigma (St. Louis, MO, USA; CAS numbers 99-96-7, 501-98-4 and 140-10-3, respectively).

2.3. Acetylated glucuronide derivatives (protected forms of p-*coumaric,* p-*hydroxybenzoic and cinnamic acid glucuronides)*

2.3.1. Synthesis of 2,3,4-tri-O-acetyl-1-p-coumaroyl-D-glucuronic acid methyl ester (CoAGP).

p-Coumaric acid (0.100 g, 0.609 mM), acetobromo-α-D-glucuronic acid methyl ester (0.362 g, 0.914 mM) and potassium carbonate (0.084 g, 0.609 mM) were dissolved in 10 ml of DMSO under argon and the mixture was stirred for 24h. The reaction mixture was diluted with 50 ml of ethyl acetate and then washed with water (7×10 ml). The organic layer was dried over $MgSO_4$ and the solvent was evaporated. The product obtained was purified by a column chromatography using silica gel 60A (60-200 micron) and a mixture of ethyl acetate/petroleum ether (55/45, v/v) as eluent. The product was then crystallized with acetone and was isolated as a white solid (0.095 g, 33%). M.p. = 201-202ºC ^{1}H NMR (300 MHz, $CDCl_3$): δ= 2.03 (s, 3H), 2.06 (s, 3H), 2.07 (s, 3H), 3.75 (s, 3H), 4.25 (d, *J*= 9.3 Hz, 1H), 5.27 (ap t, *J*= 9.3 and 8.1 Hz, 1H), 5.28 (t, *J*= 9.3 Hz, 1H), 5.38 (t, *J*= 9.3 Hz, 1H), 5.90 (d, *J*= 8.1 Hz, 1H), 6.14 (d, *J*= 16 Hz, 1H),6.84 (d, *J*= 8.7 Hz, 2H), 7.38 (d, *J*= 8.7 Hz, 2H), 7.61 (d, *J*= 16 Hz, 1H) [*]. ^{13}C NMR (75.4 MHz, $CDCl_3$): 20.48 (CH_3), 20.56 (CH_3), 20.58 (CH_3), 53.15 (OCH_3), 69.08 (CH), 70.13 (CH), 71.81 (CH), 72.84 (CH), 91.35 (CH), 112.85 (CH), 115.97 (2×CH), 126.33 (C), 130.47 (2xCH), 147.39 (CH), 158.69 (C), 164.91 (C=O), 167.26 (C=O), 169.45 (C=O), 169.55 (C=O), 169.96 (C=O). HRMS (ESI) calcd. for $C_{22}H_{24}NaO_{12}$ (M^++Na) 503.1160, found 503.1164.

[*] The proton of the OH group was not detected in the proton NMR spectrum.

2,3,4-tri-*O*-acetyl-1-*p*-hydroxybenzoyl-D-glucuronic acid methyl ester (*HAGP*) and 2,3,4-tri-*O*-acetyl-1-cinnamoyl-D-glucuronic acid methyl ester (*CAGP*) were obtained according to the procedure described by Heleno et al. (2013a).

2.4. Methylated derivatives of p-coumaric, p-hydroxybenzoic and cinnamic acids

2.4.1. Synthesis of methyl 3-(4-hydroxyphenyl)acrylate (CoAM1), methyl 4-hydroxybenzoate (HAM1) and methyl 3-phenylacrylate (CAM)

p-Coumaric (CoA), *p*-hydroxybenzoic (HA) and cinnamic (CA) acids (0.100 g) were stirred at room temperature with 7.5 ml of methanol, containing 0.2 ml of concentrated H_2SO_4 for 5 days (Reis et al., 2010). The solvent was then partially evaporated under reduced pressure, and the solution was neutralized with aqueous 1 M Na_2CO_3. The mixture was extracted with diethyl ether (3×15 ml). The organic phases were combined, washed with water (3×10 ml), dried over $MgSO_4$, and the solvent was evaporated.

The products: CoAM1, HAM1 and CAM were isolated as yellow solids (0.080 g, 74%; 0.075 g, 68%; 0.083 g, 75%, respectively). CoAM1 M.p.= 120-122ºC ^{1}H NMR (400 MHz, DMSO-d_6): δ= 3.68 (s, 3H), 6.38 (d, *J*= 16 Hz, 1H), 6.78 (d, *J*= 8.8 Hz, 2H), 7.54 (d, *J*= 8.8 Hz, 2H), 7.55 (d, *J*= 16Hz, 1H), 9.99 (OH). ^{13}C NMR (75.4 MHz, DMSO-d_6): 51.19 (OCH_3), 113.89 (CH), 115.73 (2xCH), 125.04 (C), 130.28 (2xCH), 144.72 (CH), 159.82 (C), 167.02 (C=O). HRMS (EI-TOF) calcd. for $C_{10}H_{10}O_3$ (M^+) 178.0630, found 178.0630.

HAM1 and CAM are known and commercially available reagents (Sigma, St. Louis, MO, USA; CAS numbers 99-76-3 and 103-26-4, respectively). Mp are in agreement with the ones reported by Sigma Aldrich HAM1= 128-130ºC, CAM= 44-46ºC.

2.4.2. Synthesis of methyl 3-(4-methoxyphenyl)acrylate (*CoAM2*) *and methyl p-anisate* (*HAM2*)

p-Coumaric and *p*-hydroxybenzoic acids (0.300 g), dimethyl sulphate (1 equiv.) and potassium carbonate (2 equivs) were stirred with acetone (50 ml) at 45-50ºC for 24 h (Deng et al., 2006). The solvent was evaporated. The residue was suspended in an ammonia solution (3.6% w/v; 7 ml) and extracted with ethyl acetate (3×3 ml). The

combined ethyl acetate extracts were dried over $MgSO_4$, and the solvent was evaporated. The products CoAM2 and HAM2 were isolated as white solids, (0.213g, 61%; 0.224g, 62%). *CoAM2* M.p. = 87-89ºC ^{1}H NMR (300 MHz, $CDCl_3$): δ= 3.80 (s, 3H), 3.85 (s, 3H), 6.32 (d, *J*= 15.9 Hz, 1H), 6.91 (d, *J*= 8.7 Hz, 2H), 7.48 (d, *J*= 9.0 Hz, 2H), 7.66 (d, *J*= 15.9 Hz, 1H). ^{13}C NMR (75.4 MHz, CDCl3): 51.57 (OCH_3), 55.36 (OCH_3), 114.30 (2xCH), 115.24 (CH), 127.10 (C), 129.71 (2xCH), 144.52 (CH), 161.37 (C), 167.77 (C=O). HRMS (EI-TOF) calcd. for $C_{11}H_{12}O_3$ (M^+) 192.0786, found 192.0790.

HAM2 is a known and commercially available reagent (Sigma, St. Louis, MO, USA; CAS number 121-98-2). Mp are in agreement with the one reported by Sigma Aldrich HAM2= 52-54ºC.

2.4.3. Synthesis of 3-(4-methoxyphenyl)acrylic acid (CoAM3) and 4-methoxybenzoic acid (HAM3)

Methyl 3-(4-methoxyphenyl)acrylate (CoAM2) and methyl *p*-anisate (HAM2) (0.100 g), NaOH (3 equivs) were stirred in ethanol at 65ºC for 3h. The solvent was evaporated. The residue was dissolved in water and adjusted to pH=3 with a solution of HCl 37%. The residues was then filtered and the products CoAM3 and HAM3 were isolated as white solids (0.050 g, 54% and 0.074 g, 80%, respectively).

CoAM3 and HAM3 are known and commercially available reagents (Sigma, St. Louis, MO, USA; CAS numbers 830-09-1 and 100-09-4, respectively). Mps are in agreement with the ones reported by sigma Aldrich. CoAM3= 175-177ºC, HAM3= 185-187ºC.

2.5. Cytotoxicity assays

Five human tumor cell lines were used: MCF-7 (breast adenocarcinoma) from DSMZ (Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen

GmbH), NCI-H460 (non-small cell lung carcinoma), HCT15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma) from ECACC (European Collection of Cell Cultures). Cells were routinely maintained as adherent cell cultures at 37 °C, in a humidified air incubator containing 5% CO_2. Cells were cultured in: i) RPMI-1640 medium supplemented with10% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine, for the MCF-7, NCI-H460 and HCT15 cell lines and ii) DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin, for HeLa and HepG2 cells. To assess the cytotoxicity of the extract/compounds, the Sulforhodamine B assay was performed according to a procedure previously described by the authors (Abreu et al., 2011). For this, each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT15 cells or 1.0×10^4 cells/well for HeLa and HepG2 cells) in 96-well plates.

For the evaluation of toxicity towards liver cells, a primary cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, according to a procedure previously established by some of us (Abreu et al., 2011), these cells were designated as PLP2 cells. Cell culture was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were sub-cultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultured in DMEM medium with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.

Cells were treated for 48h with the different extract/compound solutions (the extract was dissolved in water at 8mg/ml; methylated and glucuronide compounds were dissolved in DMSO at 100 mM and 40 mM, respectively) and the procedure for SRB assay was followed, as described above. The results were expressed in GI_{50} values

(concentrations that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was performed followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$, coupled with Welch's statistic. This treatment was carried out using SPSS v. 18.0 program.

3. Results and Discussion

3.1. Synthesis of acetylated glucuronide and methylated derivatives

p-Coumaric acid (CoA) was reacted with acetobromo-α-D-glucuronic acid methyl ester (1.5 equiv.) according to the procedure described by our group (Heleno et al., 2013a), affording the corresponding 2,3,4-tri-*O*-acetyl-1-*p*-coumaroyl-D-glucuronic acid methyl ester (*CoAGP*, **Scheme 1**), with 33% yield after purification, which is in agreement with the yields previously reported for *HAGP* and *CAGP* (38% and 32%, respectively; Heleno et al., 2013a). As far as we know, this is the first report on the synthesis of protected glucuronide derivatives of CoA. Nevertheless, there are studies describing the synthesis of protected glucuronide derivatives of *p*-hydroxybenzoic (HA) and cinnamic (CA) acids (*HAGP* and *CAGP*; Heleno et al., 2013a), ferulic acid (25% yield; Piazzon et al., 2012) and of ethyl-4-hydroxybenzoate (Zhang et al., 2012). The yields obtained in the chemical synthesis of these compounds are considered good, since the *in vivo* glucuronidation that occur in animal metabolism involve specific enzymes.

CoA, HA and CA were reacted with methanol and sulphuric acid according to the procedure described by Reis et al. (2010), to afford the corresponding *CoAM1*, *HAM1* and *CAM* in good yields (74%, 68% and 75%, respectively). An esterification reaction

was carried out in order to prepare the carboxylic ester derivatives, the *para*-hydroxyl groups were unreacted under these conditions for *HAM1* and *CoAM1*. These methylated compounds are common metabolites obtained *in vivo* from organic acids. *HAM1* and *CAM* are commercially available compounds, but our group synthesized them to perform the biological assays. Otherwise, as far as we know, *CoAM1* is a new compound, being fully characterized herein.

CoA and HA were reacted with dimethyl sulphate (1 equiv.) and potassium carbonate (2 equiv.) (Deng et al., 2006) to give *CoAM2* and *HAM2* in good yields (61% and 62% respectively), affording in both cases dimethylated derivatives. These compounds are possible methylated metabolites also circulating in the organism; *HAM2* is a commercially available reagent, while *CoAM2* is a new compound fully characterized herein.

CoAM2 and *HAM2* were reacted with NaOH (3 equiv.) in ethanol to perform a regioselective hydrolysis of the ester group affording *CoAM3* and *HAM3* in moderate to good yields (54% and 80%, respectively), with a free carboxylic group. Despite *CoAM3* and *HAM3* are commercially available compounds, they were synthesized in order to complete the series of methylated metabolites and compare the bioactivity of the different possible methylations of each parental organic acid.

3.2. Cytotoxicity of C. atramentaria extract

C. atramentaria extract showed high cytotoxicity for MCF-7, NCI-H460 and HCT15 cell lines (**Table 1**). Furthermore, it did not show any activity against liver primary cells (GI_{50} > 400 µg/ml). The obtained values are relevant in comparison with other reports on cytotoxicity of mushroom extracts for tumor cell lines. Vaz et al. (2010) reported the following GI_{50} values of *Clitocibe alexandri* ethanolic extract: 24.8±2.3 µg/ml (NCI-

H460), 17.9±1.3 µg/ml (MCF7), 21.7±2.3 µg/ml (HCT-15) and 26.0±1.3 µg/ml (AGS, gastric cancer). The same authors described GI_{50} values of *Suillus collinitus* methanolic extract of 62.5±6.3 µg/ml, 25.2±0.16 µg/ml, 103.2±9.9 µg/ml and 79.2±15.5 µg/ml, respectively for each of the previous mentioned cell lines (Vaz et al., 2012b).

It should be highlighted that the *C. atramentaria* methanolic extract showed higher activity than individual compounds. This can be due to additive and synergistic effects of compounds such as *p*-hydroxybenzoic (4.71 mg/100 g dry weight), *p*-coumaric (0.82 mg/100 g) and cinnamic (1.70 mg/100 g) acids previously identified in the extract (Heleno et al., 2012). Besides the organic acids previously mentioned, *C. atramentarius* is well known to produce coprine, a cyclopropyl amino acid that generates a metabolite, which inhibits alcohol dehydrogenase and, therefore, it cannot be simultaneously consumed with alcohol. All these compounds could be responsible for the cytotoxicity showed by the extract. Regarding the other cell lines, HeLa and HepG2, the extract did not show activity at the highest tested concentration (400 µg/ml), which indicates that the compounds present in the extract are not active for these cell lines, or that antagonist effects could occur.

3.3. Cytotoxicity of p-coumaric acid and derivatives

p-Coumaric acid (CoA) revealed very weak cytotoxicity for three cell lines, and no activity for HeLa and HePG2 cells. Nevertheless, the activity of its glucuronide derivative (CoAGP) was moderate for all the tumor cell lines, being more active against HePG2 cells (**Table 1**). The methylated derivatives CoAM1 and CoAM2 also presented moderate activity against all the tested tumor cell lines. This could be attributed to the presence of ester groups; in both compounds the carboxylic group of the parental molecule was replaced by an ester; CoAM1 has a hydroxyl group in the *para* position,

while CoAM2 presents a methoxyl group in the same position. However, CoAM3 that remains the carboxylic group of CoA but with a methoxyl group in the *para* position showed a very weak activity.

3.4. Cytotoxicity of p-hydroxybenzoic acid and derivatives

p-Hydroxybenzoic acid (HA) did not show activity against none of the tumor cell lines (**Table 1**). This in in agreement with Vaz et al. (2012a) that also reported the lack of activity of HA against NCI-H460 cells at the highest tested concentration (3000 μM). Nonetheless, its glucuronide derivative, HAGP, gave a moderate activity against the tested cell lines, with the exception of HePG2 cells in which this compound revealed a good activity. The methylated derivatives, HAM1 and HAM2 showed very weak activity, with the exception of HAM1 for HeLa cells that presented a moderate activity; HAM3 had no activity. Once more, the presence of an ester group increased the growth inhibitory activity of the compounds; HAM1 has the carboxylic group replaced by an ester group and a hydroxyl group in the *para* position, while HAM2 has also a carboxylic group replaced by an ester group and a methoxy group in the *para* position. HAM3 that remains with the carboxylic group, revealed no activity.

3.5. Cytotoxicity of cinnamic acid and derivatives

Cinnamic acid (CA) has also a weak activity against NCI-H460, HCT15 and HeLa cell lines, and no activity for the other cell lines (**Table 1**). Vaz et al. (2012a) reported a GI_{50} value of cinnamic acid for NCI-H460 cells (845.9±97.5 μM) similar to the one described herein. Otherwise, Ekmekcioglu et al. (1998) reported that CA has antiproliferative effects against colon cancer cells with a maximal effect at 8.0 mM with GI_{50} values between 4.0-5.0 mM. The glucuronide derivative (CAGP) revealed a

moderate activity against all the cell lines. The methylated derivative presented low activity for HCT15 cell line and no activity for the other tested cell lines.

CAM has also the carboxylic group replaced by an ester and has no groups in *para* position. In this case the presence of the ester group did not increased the activity.

It should be highlighted that despite the high GI_{50} values obtained for the individual compounds (much higher than the values for ellipticine), they did not present any toxicity for non-tumor cells PLP2; meaning that we could increase the therapeutic doses without toxicity effects. This is very important regarding the high toxicity revealed by the positive control/antitumor compound (in which the range between therapeutic and toxic doses is very short).

Overall, the extract showed higher activity than individual compounds, maybe due to synergistic effects among the present molecules. This study allowed a comparison between parental molecules and derived metabolites; it is important to understand the behavior of organic acids (including phenolic acids) regarding their bioactivity after metabolism, and this study, gives clues on this matter. Organic acids are extensively metabolized in humans, being methylation and glucuronation two of the conjugated forms occurring in the organism and giving methylated and glucuronide metabolites, respectively. In this work, it was proved that the substitution of the carboxylic group (in parental organic acids) for an ester (in methylated derivatives) increased the cytotoxicity of the parental compounds. The acetylated glucuronide derivatives had considerably increase the cytotoxicity when compared with the respectively parental compounds.

Although future studies should be carried out in order to clarify specific mechanistic pathways, the present study contributes to compare the bioactivity of organic acids and derivative compounds, including possible human metabolites.

Acknowledgements

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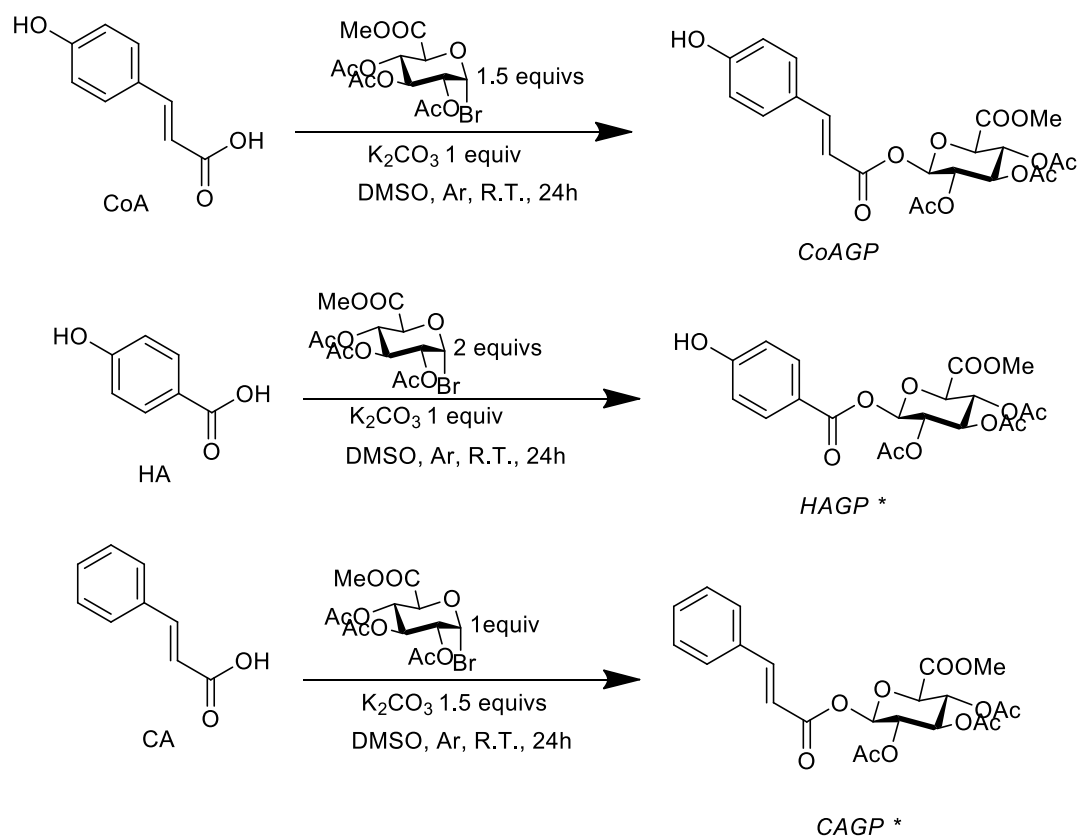
Scheme 1. Glucuronidation of *p*-coumaric, *p*-hydroxybenzoic and cinnamic acids. CoA- *p*-coumaric acid; CoAGP- *p*-coumaric acid glucuronide protected form; HA- *p*-hydroxybenzoic acid; HAGP- *p*-hydroxybenzoic acid glucuronide protected form; CA-cinnamic acid; CAGP- cinnamic acid glucuronide protected form.

* Synthesis and structural characterization previously reported by the authors (Heleno et al., 2013).
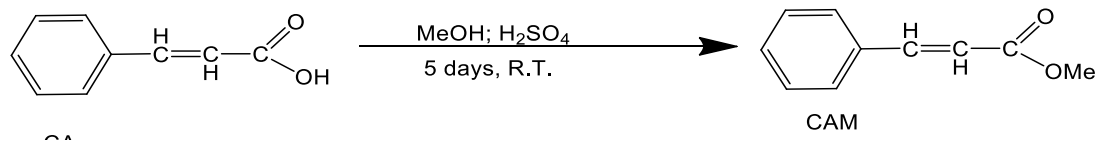
CoA → (MeOH; H₂SO₄, 5 days R.T.) → CoAM1

CoA → (MeO₂SO₂, 1 equiv, K₂CO₃, 2 equivs, acetone, 50ºC, 24h) → CoAM2 → (NaOH, 3 equivs, EtOH, 65ºC, 3h) → CoAM3

HA → (MeOH; H₂SO₄, 5 days R.T.) → HAM1

HA → (MeO₂SO₂, 1 equiv, K₂CO₃, 2 equivs, acetone, 50ºC, 24h) → HAM2 → (NaOH, 3 equivs, EtOH, 65ºC, 3h) → HAM3

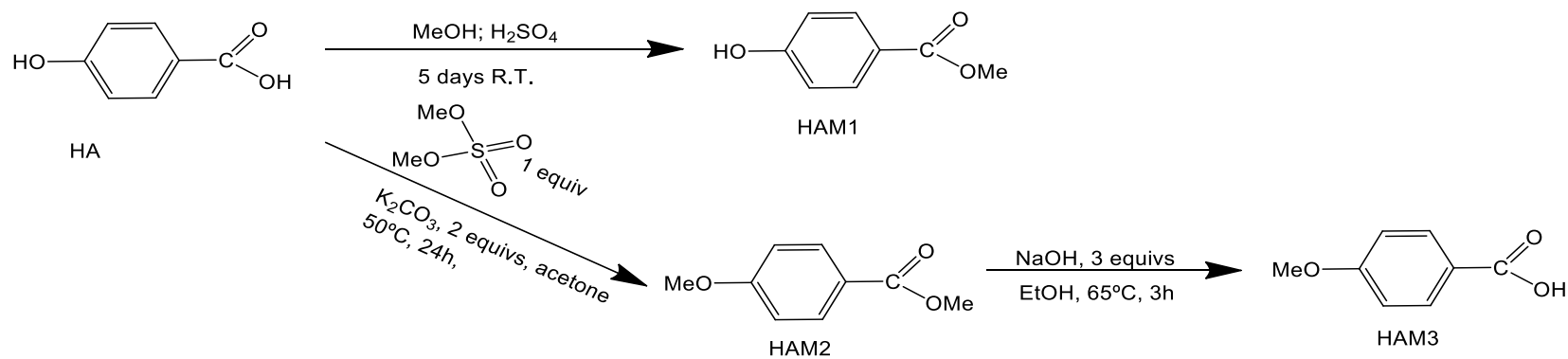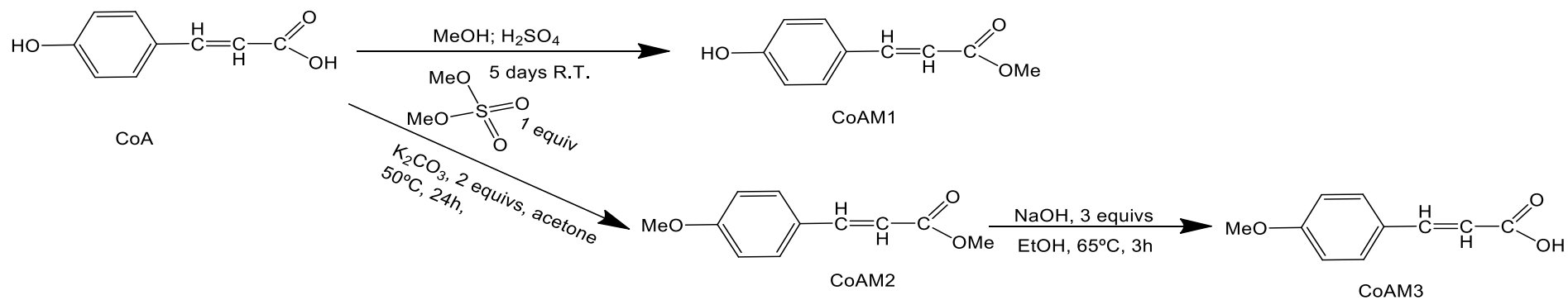CA → (MeOH; H₂SO₄, 5 days, R.T.) → CAM

Table 1. Cytotoxicity of *Coprinopsis atramentaria* extract*,* organic acids and their synthesized methylated and glucuronide derivatives on various human tumor cell lines and in liver primary cells.

	MCF7 (breast carcinoma)	NCI-H460 (non-small lung carcinoma)	HCT15 (colon carcinoma)	HeLa (cervical carcinoma)	HepG2 (hepatocellular carcinoma)	PLP2 (liver cells)
Extract	53.10±4.72	15.13±1.35	36.44±3.30	>400	>400	>400
CoA	1152.84±58.65^a	1082.50±81.32^a	1177.46±32.54^a	>1250^a	>1250^a	>1250
CoAGP	334.62±8.20^c	384.21±16.85^c	284.99±2.39^c	205.56±6.93^c	134.35±14.68^d	>500*
CoAM1	295.42±6.41^c	282.75±8.44^c	274.94±12.72^c	199.09±13.44^c	680.46±49.02^b	>1250
CoAM2	333.35±10.87^c	344.91±26.36^c	315.56±15.85^c	247.98±2.16^b	632.90±6.68^c	>1250
CoAM3	1013.97±36.21^b	915.79±81.70^b	995.72±23.16^b	>1250^a	>1250^a	>1250
HA	>1250^a	>1250^a	>1250^a	>1250^a	>1250^a	>1250
HAGP	257.66±18.12^c	310.92±1.80^c	195.49±0.75^d	192.39±7.72^d	57.78±5.65^c	>500*
HAM1	1140.70±4.51^b	1060.69±70.13^b	942.55±2.33^c	507.38±31.06^c	1169.42±64.37^b	>1250
HAM2	>1250^a	>1250^a	1149.49±5.69^b	850.73±14.75^b	>1250^a	>1250
HAM3	>1250a	>1250^a	>1250^a	>1250^a	>1250^a	>1250
CA	>1250^a	887.24±21.76^b	847.44±7.57^b	1073.01±54.84^b	>1250^a	>1250
CAGP	306.40±16.69^b	311.12±12.34^c	174.65±7.89^c	121.42±2.79^c	289.92±11.25^b	>500*
CAM	>1250^a	>1250^a	1195.99±3.87^a	>1250^a	>1250^a	>1250
Ellipticine	0.91±0.04	1.42±0.00	1.91±0.06	1.14±0.21	3.22±0.67	2.06±0.03

The results are expressed as GI$_{50}$ values (μg/ml for extract and μM for compounds) corresponding to the extract/compound concentration, which inhibited 50% of cell growth. Results are from three independent experiments (performed in triplicate), and are expressed as mean ± standard deviation (SD). Different letters in each column, and for each compound series, mean significant differences between GI$_{50}$ values (p<0.05).*Due de low solubility of the compound, the maximum concentration tested was 500 μM.