Discovery of β-Glucosidase Inhibitors from a Chemically Engineered Extract Prepared through Ethanolysis

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Abstract: A series of vegetal extracts have been chemically altered by ethanolysis. The effect of the reaction on the inhibition of the enzyme β-glucosidase properties of the mixtures was studied using thin layer chromatography (TLC) with biodetection. Glucosidase inhibitory activity guided fractionation of one of the produced chemically engineered extracts led to the isolation of apigenin and ethyl *p*-cumarate. Both compounds were generated during the chemical modification step.

Keywords: Chemically engineered extracts, Ethanolysis, TLC biodetection, β-glucosidase inhibition.

INTRODUCTION

The chemical transformation of the components of crude natural extracts represents a clear way of altering the properties of those natural mixtures. Sometimes unintended chemical alteration occurs during extraction or purification. In solution, natural products may react with the extraction solvent, or be activated by the solvent to react with other components present in the solution leading to the formation of derivatives or to the breakdown of some natural molecules into various components [1].

In other cases chemical alteration is deliberate as in the case of chemically engineered extracts [2]. This approach focuses on the chemical transformation of a significant proportion of the molecules present in a natural extract without previous knowledge of the exact composition of the starting material. Since the preparation of chemically engineered extracts involves the transformation of a complex mixture of natural products (natural extract) into another mixture of natural and semisynthetic molecules, it is important to use methods that allow the clear comparison of the biomolecular properties of both mixtures. In this context the use of analytical thin layer chromatography (TLC) biodetection, based on fungal growth [3] or enzyme activity [4-8], facilitates the visualization of the bioactivity profiles of the starting mixture and the derived chemically engineered extract. The combination of chromatography and biochemical staining represents a helpful tool to spot the bioactive members within the complex mixtures and to guide the isolation of the bioactive compounds generated during the chemical transformation.

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To date chemical transformation of natural extracts with hydrazine [9], bromine [10] or arylsulfonyl chlorides [11, 12] has led to the isolation of semisynthetic molecules with antifungal activity and enzyme inhibitors. Concerning the use of solvents as reagents (solvolysis), a few reports describe the positive effect of acid hydrolysis on the antioxidant [13, 14] or antifungal [15] properties of different plant extracts. However, there are no examples of the use of alcoholysis to alter the biomolecular properties of extracts.

Clifford and co-authors suggested methanolysis as a fast derivatization method of phenylpropanoids from natural extracts [16]: the treatment of methanolic mixtures with tetramethylammonium hydroxide and the consequent formation of the corresponding methyl esters allowed identification of the type of phenylpropanoid present.

Furthermore, since ethanol is present in chloroform as stabilizer [17] ethyl esters of fatty acids were isolated, together with several ethanolysis products during the chloroform extraction of maple syrup [18].

Here we report the preparation of chemically engineered extracts by ethanolysis. The effect of the reaction on β-glucosidase inhibitory properties of the mixtures was studied using TLC with biodetection.

Glycosidases are responsible for the hydrolysis of poly and oligosaccharides into monomers or the cleavage of bonds between sugars and noncarbohydrate aglycone. They are involved in several important biological processes, such as digestion, biosynthesis of glycoproteins, and the lysosomal catabolism of glycoconjugates. Glycosidase inhibitors have been subject of extensive interest [19] because of their potential as drugs for the treatment of diabetes [20], cancer [21], viral infection [22, 23], and hereditary lysosomal storage diseases [24]. Several glycosidase inhibitors have been

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discovered from natural sources showing the potential of natural products as a source of such compounds [21, 25, 26].

β-Glucosidase activity guided fractionation of one of the produced chemically engineered extracts led to the isolation of apigenin and ethyl *p*-cumarate. Both compounds were generated during the chemical modification step.

MATERIALS AND METHODS

Chemical reagents were purchased from commercial sources and were used without further purification unless noted otherwise. Solvents were analytical grade or were purified prior to use by fractional distillation.

^{1}H NMR (nuclear magnetic resonance) spectra were recorded on a Bruker avance II at 300 MHz in $CDCl_3$, in the presence of TMS (tetrametylsilane) (0.00 ppm) as the internal standard. ^{13}C NMR spectra were recorded on the same apparatus at 75 MHz with $CDCl_3$ as solvent and reference (76.9 ppm); ^{13}C NMR assignments were made on the basis of chemical shifts and proton multiplicities on COSY ^{1}H-^{1}H (correlation spectroscopy), HSQC (heteronuclear single quantum coherence), and HMBC (heteronuclear multiple bonding correlation).

HRMS (high resolution mass spectrometry) analysis was performed using a Bruker micrOTOF-Q II spectrometer. MS and MS-MS Parameters: Source type: ESI (Electrospray ionization), Ion polarity: Negative and positive, Set nebulizer: 0.4 Bar, Set dry heater: 180 °C, Set dry gas: 4.0 L/min, Set capillary: 4500 V, Set end plate offset: -500 V, Set collision Cell RF: 150.0 Vpp. ISC collision energy for MS-MS experiments: 30 eV.

TLC was carried out with silica gel 60 F_{254} pre-coated aluminum sheets (Merck, Darmstadt, Germany). Depending on their solubility, samples were spotted as solutions in DCM (dichloromethane) or DCM:MeOH mixtures on 0.5 cm bands with a 0.5 cm separation between bands. Column chromatography was performed using Analtech silica gel 150 Å pore, 35-75μm particles. Molecular exclusion column chromatography was performed on Sephadex® LH-20. Reversed-phase TLC was carried out on commercial aluminum plates RP-18 Merck F_{254}. TLC images were captured with a Chromadoc-IT equipment fit up with visible, UV 254 nm and 365 nm lamps and Olympus 5.1 megapixel digital camera.

β-Glucosidase (β-Glc) from almonds (number product 49290), conduritol β-epoxide, and α-cyclodextrin were purchased from Sigma (St. Louis, MO, USA). Agar was purchased from Britania (Buenos Aires, Argentina). Sodium acetate, sodium phosphate, ferric chloride hexahydrate and glacial acetic acid were purchased from Cicarelli (San Lorenzo, Argentina). Esculin hydrate was purchased from Aldrich (Milwaukee, WI, USA). *p*-nitrophenol-β-*O*-D-glucopyranoside was purchased from Fluka (Buchs, Switzerland).

Plant material. Plants were collected in Pergamino, Buenos Aires province, Argentina, during November 2005. Voucher specimens were deposited at the Universidad Nacional de Rosario Herbarium. Collected plants were: *Urtica urens* L. Urticaceae (UU), ID MO010; *Lamium amplexicaule* L. Laminaceae (LA), ID MO001; *Solanum diflorum Vell.* Solanaceae (SD), ID MO005; *Sonchus oleraceus* L. Asteraceae (SO), ID MO013; *Brassica rapa* L. Brasicaceae (BR) ID MO003; and *Oenothera affinis* Cambess. Onagraceae (OA), ID OM012.

Crude Extracts. Plant material was dried at 30°C for several days and then milled with a laboratory grinder. A sample (200 g) of dried and powdered material was refluxed with 3 × 4 L of methanol for 45 min. The extracts were combined after filtration, and the solvent was evaporated under reduced pressure by rotary evaporation.

Natural Hexane, Dichloromethane and Butanol Subextracts

Typical procedure: 30 g of crude extract was dissolved in 1 L of MeOH: H_2O (9:1) and extracted with hexane (2 x 500 mL). The MeOH was evaporated from the aqueous phase under reduced pressure and H_2O was added to a final volume of 200 mL. The resulting solution was extracted with DCM (3 x 200 mL) and then *n*-butanol (4 x 200 mL). The organic phases (hexane, dichloromethane and butanol) were dried with anhydrous Na_2SO_4, the solvents were evaporated at reduced pressure to give the corresponding natural hexane, dichloromethane and butanol subextracts.

Preparation of Chemically Engineered Subextracts

Typical reaction procedure: 200 mg of plant extract and 370.6 mg of $NH_2OH.HCl$ were dissolved in 10 mL of EtOH. The resulting solution was stirred under reflux for 4 hours. The EtOH was removed under reduced pressure and the obtained residue was suspended in DCM (100 mL) and washed with water (3 x 100 mL). The 300 ml of aqueous solution obtained were extracted with 100 mL of DCM. The remaining aqueous fraction was alkalinized to pH = 12 with NaOH and extracted with DCM (3 x 100 mL). Then, the aqueous fraction was acidified to pH = 1 with HCl (36 %w/w) and extracted again with DCM (3 x 100 mL). All organic fractions were combined and dried with anhydrous Na_2SO_4. The resulting solution was concentrated by rotary evaporation to obtain the modified subextracts.

Preparation of Control Extract

273 mg of butanol subextract of *Lamium amplexicaule* L. were dissolved in 137 mL of DCM and washed with water (3 x 137 mL). The 411 mL of aqueous solution was extracted with 137 mL of DCM. The remaining aqueous fraction was alkalinized to pH 12 with NaOH and extracted with DCM (3 x 137 mL). Finally, the aqueous fraction was acidified to pH 1 with HCl and extracted with DCM (3 x 137 mL). All dichloromethane solutions were combined, dried with anhydrous Na_2SO_4, filtered and concentrated under reduced pressure.

Scaling-up of the reaction: 249 g of crude extract, were used to prepare the hexane, dichloromethane and butanol natural subextracts following the typical procedure applied in the first fractionation. Obtained yields: 14.2%, 6.0% and 20.7% for hexane, dichloromethane and butanol natural subextracts respectively. 51.5 g of butanol subextract and 95.3 g of $NH_2OH.HCl$ were dissolved in 500 mL of EtOH. The resulting solution was stirred at reflux for 29 h. After

this time, the solvent was removed under reduced pressure and the crude reaction mixture was dissolved in 500 mL of DCM and washed with water (3 x 500 mL). The 1.5 L of aqueous solution was extracted with 500 mL of DCM. The remaining aqueous fraction was alkalinized to pH = 12 with NaOH and extracted with DCM (3 x 500 mL). Finally, the aqueous fraction was acidified to pH = 1 with HCl 36% w/w and extracted with DCM (3 x 500 mL). All dichloromethane solutions were combined, dried with anhydrous Na_2SO_4, filtered, and concentrated by rotary evaporation to obtain 4.45 g of modified butanol subextract (8.6% yield).

β-glucosidase TLC Assay

The amount of extract loaded on the TLC plate was 250 μg of extract/band. After elution, the solvents were removed under air current and the plate was subjected to autographic analysis. The methodology developed by our group activity was used [5]. Conduritol β-epoxide was used as positive control.

Staining solution for β-glucosidase. Agar (52.5 mg) was dissolved at 80 °C in sodium acetate buffer (0.1 M, pH 5, 7.5 mL). The solution was allowed to cool to 60 °C, ferric chloride solution (0.5% w/v in 0.1 M acetate buffer, 0.9 mL) was added. At 40 °C, β-glucosidase solution in 0.1 M acetate buffer pH = 5 (2.5 U/mL, 25 μL) was added and the mixture was carefully homogeneized by shaking.

Typical procedure for the detection of β–glucosidase activity. An aliquot of approximately 6.5 mL of β-glucosidase staining solution was distributed over the TLC layer (10 × 5 cm). After the staining solution had solidified, the TLC plate was incubated at 37 °C for 120 min and immersed in 0.2% w/v solution of esculin in 0.1 M acetate buffer at 37 °C for 120 min. Clear bands (representing areas exhibiting inhibition of β-glucosidase activity) were observed against a dark brown background.

β-glucosidase Quantitative Assays

Microplate assay. The method reported by Arnaldos *et al.* [27] based in the continuous measurement of the hydrolized *p*-nitrophenol-β-*O*-D-glucopyranoside in presence of α-ciclodextrin was used. Wells were filled with β-glucosidase solution in 0.1 M acetate buffer pH 6.8 (0.2 U/mL, 20 μL), α-cyclodextrin solution in 0.1 M acetate buffer pH 6.8 (3 mM, 130 μL) and 3 μL of test compound solutions in DMSO (dimethylsulfoxide) (or 3 μL of DMSO in control wells). The final volume (240 μL) was completed with 0.1 M phosphate buffer pH 6.8. The microplate was incubated at 37°C for 30 min. After that incubation time, the enzymatic reaction was initiated by addition of *p*-nitrophenol-β-*O*-D-glucopyranoside (19.5 mM, 20 μL in 0.1 M phosphate buffer pH 6.8). The plate was shaken for 1 s and the increase in absorbance at 405 nm was monitored at 37 °C for 10 min.

Aliquots of ½ seriated dilutions (3 μL) starting in 100 mM for compounds **1** and **2**, and in 50 mM for conduritol β-epoxide were added on each well. The three most concentrated solutions of compound **2** were not taken into account due to the precipitation of it on the wells. Wells containing the corresponding volume of DMSO without inhibitor were used as reference of maximum enzymatic rates. IC_{50} were calculated using Prism V5.01 (GraphPad Software Inc.).

Bioautographic Assay. Aliquots of 1/10 seriated dilutions (10 μL) starting in 1mM for compounds 1 and 2 and conduritol β-epoxide were spotted over a TLC plate. After the solvent was removed under air current, the plate was subjected to the β-glucosidase authography assay reported by Salazar *et al.* [5].

Bioguided Fractionation of Modified Butanolic Subextract of *Lamium amplexicaule* L. (*Laminaceae*)

The 4.45 g of modified butanol subextract of *L. amplexicaule* were chromatographed on Silica gel (hexane:ethyl acetate gradient, 98:2 to 90:10). The active fractions (3 g, 67.4%) were chromatographed on Sephadex® (Hexane: $CHCl_3$: MeOH, 1:1:2). Six fractions were obtained (M_1-M_6), M_2 (1.14 g) was enriched in the compound **1** and M_4 (94 mg) was enriched in the compound **2**.

Isolation of Compound 1. Fraction M_2 was separated on Sephadex® (Hex: $CHCl_3$: MeOH (1:1:2). The active fraction (372 mg) was separated by successive preparative chromatography (DCM: MeOH, 9:1 and 98:2) to obtain compound **1** (11.8 mg, 0.27%).

^{1}H RMN (300 MHz, $CDCl_3$) δ=7.63ppm (1H, *d*, *J*=15.9 Hz, =C**H**-), 7.42 (2H, *d*, *J*=8.7 Hz, Ar-**H**), 6.84 (2H, *d*, *J*=8.7 Hz, Ar-**H**), 6.30 (1H, *d*, *J*=15.9Hz, =C**H**-), 4.26 (2H, *q*, *J*=7.1Hz, -C$\mathbf{H_2}$CH$_3$), 1,33 (3H, *t, J*$_1$=*J*$_2$=7.1 Hz, -CH$_2$C$\mathbf{H_3}$).

^{13}C RMN (75 MHz, $CDCl_3$) δ =167.91 (C, **C**=O), 158.16 (C, **Ar**), 127.52 (C, **Ar**); 144.74 (**C**H, vinyl), 115.95 (**C**H, vinyl), 130.28 (2 CH, **Ar**), 116.22 (2 CH, **Ar**); 60.79 (CH_2, ethyl), 14.34 (CH_3, ethyl). HRMS: found m/z = 191.07186, calculated m/z for $C_{11}H_{11}O_3$ (M-1) 191.07137 (-2.6 ppm error).

Isolation of compound 2. Fraction M_4 (94mg) was separated by size exclusion chromatography with Sephadex® (Hex: $CHCl_3$:MeOH, 1:1:2) and analyzed by TLC autography. The biologically active fraction (41mg) was purified on preparative chromatography (DCM:MeOH, 9:1). The active fraction (30mg) was separated by reversed-phase TLC (MeOH:H_2O, 7:3) to obtain apigenin (**2**) (4 mg, 0.09%).

^{1}H RMN (300 MHz, $CDCl_3$) δ= 7.82p (2H, *d*, *J*=8.9Hz, Ar-**H** ring B), 6.94 (2H, *d*, *J*=8.9Hz, Ar-H ring B), 6.56 (1H, *s*, H$_\alpha$ C=O), δ 6.46 (1H, *d*, *J*=2.1Hz, Ar-H ring A), δ 6.25 (1H, *d*, *J*=2.1Hz, Ar-H ring A). ^{13}C RMN (75 MHz, $CDCl_3$:CD_3OD (1:1) δ =182.34 (C, C=O), 164.66 (C, Ar A ring), 157.78 (C, Ar A ring), 103.95 (C, Ar A ring), 160.91 (C, Ar A ring), 164.25 (C, Ar C ring), 161.41 (C, Ar B ring), 121.74 (C, Ar B ring), 127.90, (CH, Ar B ring), 115.56 (CH, Ar B ring), 102.48 (CH, C$_\alpha$ C=O C ring), 98.83, (CH, Ar A ring), 93.78 (CH, Ar A ring). HRMS: found m/z = 271.06051, calculated m/z for $C_{15}H_{11}O_5$ (M+1) 271.06010 (-1.5 ppm error).

RESULTS

Natural and Modified Subextracts

Aiming at the chemical modification of the components of natural extracts we used as starting material a set of 18 subextracts from six species of weeds (*Solanum diflorum*

Vell., *Sonchus oleraceus* L., *Brassica rapa* L., *Urtica urens* L., *Lamium amplexicaule* L., and *Oenothera affinis* Cambess.). The subextracts were obtained by partition of the six crude extracts with solvents of different polarity such us hexane (natural hexane subextract), dichloromethane (natural dichloromethane subextract) and butanol (natural butanol subextract).

Each 'natural subextract' was stirred in refluxing ethanol in the presence of hydroxylamine hydrochloride in refluxing ethanol. The crude mixture obtained after evaporation was dissolved in DCM and washed with water to produce the 'modified extracts' (hexane, dichloromethane and modified butanol subextracts).

Biodetection

Differences between the biological properties of the modified and natural subextracts were studied by thin layer chromatography with biodetection, a technique particularly suited for the analysis of mixtures. This methodology allows the evaluation of inhibitory properties of a sample spotted onto a TLC plate covered with a gel that contains enzyme, substrate, and a revealing reagent for the product.

The bioactivity profile of the modified mixtures was clearly different from the profile of the natural subextracts. Over a total of 18 bioactivity measurements for the natural extracts, 12 positive events were observed (66%). All of them resulted from the β-glucosidase inhibition produced by the natural, hexane and dichloromethane, subextracts. Quite differently, a significant part of the active modified mixtures (43%) were butanol subextracts prepared from *Sonchus oleraceus*, *Lamium amplexicaule* and *Oenothera affinis*. All these inhibition bands, detected only in the modified extracts, could be caused by molecules produced by transformation of less active molecules during the reaction.

The inhibition of the enzyme β-glucosidase by the butanol modified subextract of the weed *Lamium amplexicaule* was particularly interesting since it showed three clear inhibition bands that were absent in the starting natural subextract, suggesting the generation of at least three different active compounds (Fig. **1**). The band with higher Rf value (0.49, DCM:MeOH, 97:3) was the most defined and the band with lower Rf value (0.10, DCM:MeOH, 97:3) presented a dark center (Fig. **1**). A third and more diffuse band, was observed at an intermediate Rf value (0.21, DCM:MeOH, 97:3). In order to find out whether the active compounds were reaction products or they had actually been generated during the reaction work up, the unmodified natural butanol subextract of *L. amplexicaule* was subjected to the work-up procedure previously applied to the reaction crude. None of the three bands were seen on the bioauthography of this control extract indicating that they were generated during the reaction with ethanol/NH_2OH.

Compound Identification

In order to identify the molecules responsible for the observed inhibition, a new collection of vegetal material was necessary. Extraction and solvents partition were scaled-up: starting from 249 g of crude extract of newly collected vegetal material; 51.5 g of natural butanol subextract were obtained (20.7%). This butanol subextract was then refluxed in ethanol/hydroxylamine hydrochloride under the same reaction conditions described before. TLC analysis with biodetection at 4 h did not show any inhibition band, consequently $NH_2OH.HCl$ was added and the reaction was continued for other 29 h. Despite the extended reaction time, only the inhibition bands with highest and lowest Rf values were detected. This could be due to differences in the composition of the newly collected plan material [28].

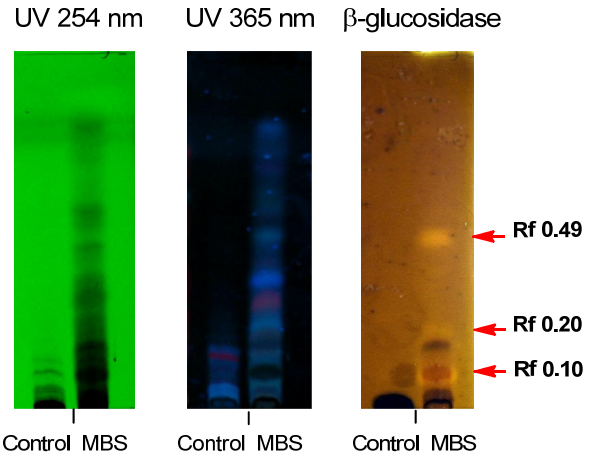

Fig. (1). TLC chromatogram of *L. amplexicaule* modified butanol subextract observed under different wavelengths and stained with the β-glucosidase autographic assay. Bands with β-glucosidase activity are indicated with the correspondent Rf. Solvent DCM:MeOH (97:3).

Bioactivity guided fractionation of the new modified extract through silica gel chromatography followed by gel filtration on Sephadex® and successive reverse phase chromatography led to the isolation of two compounds that concentrated the inhibitory properties of bands with Rf = 0.49 and Rf = 0.10 respectively. The spectroscopic ^1H NMR information for the less polar compound showed two correlated doublets, one located at δ 7.42 ppm (J = 8.7 Hz, 2H) and the other at δ 6.84ppm (J = 8.7 Hz, 2H) suggesting the presence of a *para* substituted aromatic ring. It was also observed by another pair of correlated doublets located at δ = 7.63 ppm and 6.30 ppm that corresponded to two vinyl protons. Their coupling constant (J = 15.9 Hz) agree with the presence of a *trans* substituted double bond. Finally, the presence of one quartet for 2H at δ = 4.26 ppm and one triplet for 3H at δ = 4.26 ppm, suggest the presence of an ethyl ester moiety in the structure. One signal at δ = 167.91 ppm in the ^{13}C spectrum confirms the presence of a quaternary carbon signal typical of an ester carbonyl carbon. The correlations observed in the HMBC between the ^{13}C signal at δ = 167.91 ppm and the ^1H signals at δ = 7.63ppm and 6.30 ppm indicated the presence of an α-β unsaturated carbonyl group. High resolution mass spectrometry (HRMS) analysis showed the presence of a signal with an m/z = 191.07186 that could correspond to the $[M - H]^-$ ion for the molecular formula $C_{11}H_{11}O_3$. All this information suggested that the compound responsible for the inhibitory band with Rf = 0.49 was the ethyl ester of the 4-hydroxycinnamic acid (Fig. **2**).

The ^1H NMR spectrum of the more polar compound (Rf = 0.10) showed two doublets, one at 7.82 ppm (J = 8.9 Hz, 2H) and another at 6.94 ppm (J=8.9 Hz, 2H) corresponding

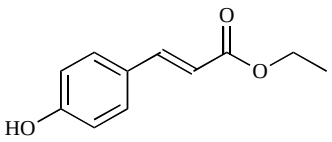

Fig. (2). Structure of compound **1** isolated from a modified by ethanolysis butanol subextract of *Lamium amplexicaule* L.

to a *para* substituted aromatic ring. In addition one singlet was observed at δ 6.56 ppm (1H) corresponding to an α,β unsaturated vinyl proton. Finally, two doublets at δ = 6.46 ppm (J = 2.1 Hz) and at δ = 6.25 ppm (J = 2.1 Hz) corresponding to a second aromatic ring with *meta* substitution were present. ^{13}C NMR spectra, HMQC and HMBC experiments indicated the presence of 8 quaternary carbon signals, accompanied by other seven ^{13}C signals. One signal at δ = 182.34 ppm, corresponded to an α,β unsaturated ketone carbonyl carbon. The HRMS of this compound showed an [M + H] with m/z = 271.06051 coincident with a molecular formula $C_{15}H_{11}O_5$. These data indicate that this compound is the 5,7-dihydroxy-2-(*p*-hydroxyphenyl)-4-cromenona, known as apigenin (Fig. **3**).

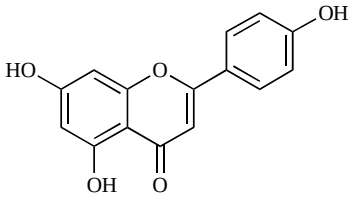

Fig. (3). Structure of compound **2** isolated from the modified butanol subextract of *Lamium amplexicaule* L.

It is worth mentioning that HRMS analysis of the natural butanol subextract of *L. amplexicaule* did not show the presence of compounds **1** and **2**, suggesting that both molecules were generated during the chemical modification step with ethanol/hydroxylamine hydrochloride.

DISCUSSION

Although free apigenin has been reported in numerous plant species [29,30], this flavone is usually forming part of glycosides [31]. Most of them are 7-O-glucosides, 7-O-cumaril-glucosides and 7-O-ferulil-glucosides [30-33] such as those found in the family *Lamiaceae* [34]. Presumably, some of those compounds could have been the starting material for the generation of the isolated bioactive molecules during the ethanolysis of the natural butanol subextract.

There are no reports about inhibition of β-glucosidase by ethyl coumarate or apigenin. However, inhibition of α-glucosidase by different esters of cinnamic acid derivatives was reported by Adisakwattana *et al.*, who observed that the 4-methoxy-trans-ethyl cinnamate was one of the best competitive inhibitors of the studied series [35]. Tandera *et al.* reported that some flavonoids, including apigenin inhibit α-glucosidases [36].

The inhibitory potency of both compounds was initially compared to that of the known inhibitor conduritol β-epoxide [37] using β-glucosidase TLC autography. Ethyl coumarate produced inhibition down to 0.01 μmol/band. It is ten times less active than conduritol β-epoxide and apigenin that showed inhibition at 0.001 μmol/band. The inhibitory properties of **1**, **2**, and conduritol β-epoxide were also compared using a microplate assay based on the quantification of hydrolysis of *p*-nitrophenyl glucoside [27]. The observed IC_{50} values were 2.079 mM for ethyl *p*-coumarate, 0.245mM for apigenin and 0.398mM for conduritol-β-epoxid**e**.

CONCLUSION

In summary, the results demonstrate that biomolecular properties of natural extracts can be altered by reaction with ethanol/hydroxylamine hydrochloride. The ethanolysis reaction increased the inhibition properties of several natural subextracts against the enzyme β-glucosidase.

Bioactivity guided fractionation of a modified butanol subextract of *Lamium amplexicaule* led to the identification of two active compounds: ethyl *p*-coumarate and apigenin. None of these compounds were detected in the inactive starting natural extract, suggesting that they were produced by ethanolysis of one or more natural components.

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