Mutagenic, Antimutagenic and Antioxidant Activities of a New Class of β-Glucoside Hydroxyhydroquinone from *Anagallis monelli* Growing in Tunisia

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A new skeleton of an O-heteroside natural substance named zinolol, the first representative of a new class of aminated hydroxyhydroquinone, has been isolated from the whole plant *Anagallis monelli*. Its structure has been established by one and two dimensional NMR spectroscopic procedures. Antioxidant, mutagenic, antimutagenic activities were realised and positive results were recorded.

Key words   *Anagallis monelli*; new O-heteroside; mutagenic; antimutagenic; antioxidant

As a part of our investigation into some medicinal plants growing in Tunisia[1—4] and in order to continue our search on potential biological natural compounds, we report below the isolation, the structure elucidation and biological activities of a new O-heteroside zinolol, identified in the methanolic extract of the whole *Anagallis monelli* ssp. *linifolia* (L.) (Primulaceae). The Tunisian flora counts six species of *anagallis*, the most preponderant one is *arvensis*.[5] According to our bibliographic studies and contrary to the *arvensis* species, no much is known on the chemistry of *Anagallis monelli*. We note that in folk medicine the decoction of the genre *Anagallis* has fungistatic proprieties.[6] Occurrence of saponins in *arvensis* species[7,8] explains its traditional uses as antifungic. Saponins from *Anagallis arvensis* were also mentioned as antiviral agent against herpes simplex virus and poliovirus.[9] With an aim to focus in the biological interest of the species *monelli*, mutagenic and antimutagenic activities were realised based on the SOS Chromotest and antioxidant activities were realised based on the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) tests. The structure of zinolol was established by the spectroscopic means (1 and 2D NMR experiments). The presence of *N*-methylaminomethylene group on the aromatic ring of the 2-(*O*-β-D-glucosyl)hydroxyhydroquinone enables us to confirm the originality of this natural substance, named systematically, 2-(*O*-β-D-glucosyl)-6-(methylaminomethyl)hydroxyhydroquinone. The mutagenic potential of zinolol in the SOS chromotest shows significant activities; in the same way antioxidant capacity of this substance was noteworthy.

Results and Discussion

Structure Elucidation   Zinolol was isolated as amorphous solid. Negative ion electrospray ionization mass spectrum of this substance shows the pseudomolecular pick [M−H]⁻ at *m/z* 330 and confirms the molecular formula $C_{14}H_{21}NO_8$. The ^{13}C-NMR spectrum exhibits the presence of 14 carbon atoms whose multiplicity assignments were made by DEPT 135 experiment. Observation of five peaks at 62.5, 71.2, 75.7, 78.3 and 78.7 ppm relative respectively to carbons $C_{6'}$, $C_{4'}$, $C_{2'}$, $C_{3'}$ and $C_{5'}$ prove the presence of a glucosyl ring. $H_{6'}$–$C_{6'}$; $H_{4'}$–$C_{4'}$; $H_{2'}$–$C_{2'}$; $H_{3'}$–$C_{3'}$ and $H_{5'}$–$C_{5'}$ correla-

tions observed on the CHCorr spectrum confirm also the presence of the five glucosyl protons $H_{6'}$, $H_{4'}$, $H_{2'}$, $H_{3'}$ and $H_{5'}$. the anomeric proton $H_{1'}$ was also observed at 4.49 ppm. The anomeric coupling constant value of 7.2 Hz confirms the β configuration of the glucosyl moiety.[10]

The H–H COSY correlations $H_{1'}$–$H_{2'}$; $H_{2'}$–$H_{3'}$; $H_{4'}$–$H_{5'}$ and $H_{5'}$–$H_{6'}$ and those of the HMBC spectrum represented in Table 1 permit us to establish the total skeleton of the *O*-β-D-glucosyl moiety. Based on the HMBC spectrum the aromatic aglycone structure was established. Apparition of the methylene group ($H_{1''a}$ and $H_{1''b}$ δ respectively 3.87 and 4.02 ppm) as doublet (d) for each proton at the position 1″ was probably due to the hydrogen bond. Position of the methyl-
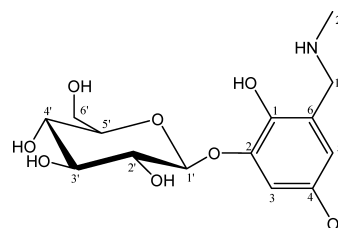

Fig. 1.   Structure of Zinolol

Table 1. ^1H- and ^{13}C-NMR Chemical Shifts of Zinolol in CD₃OD and ^{13}C, ^1H Correlations (HMBC)

Atom	δ ^{13}C (ppm)	δ ^1H (ppm); *J* (Hz)	HMBC
1	139.1	—	$H_{1''a}$–C_1, $H_{1''b}$–C_1
2	152.0	—	H_3–C_2
3	104.1	6.12 (1H, d); J_{3-5}=3.00	H_5–C_3
4	156.3	—	H_3–C_4
5	109.8	6.32 (1H, d); J_{5-3}=3.00	H_3–C_5, $H_{1''a}$–C_5, $H_{1''b}$–C_5
6	131.3	—	$H_{1''a}$–C_6, $H_{1''b}$–C_6
1′	108.1	4.49 (1H, d); $J_{1'-2'}$=7.2	$H_{2'}$–$C_{1'}$
2′	75.7	3.48 (1H, m)	$H_{1'}$–$C_{2'}$, $H_{3'}$–$C_{2'}$
3′	78.3	3.46 (1H, m)	$H_{2'}$–$C_{3'}$, $H_{4'}$–$C_{1'}$
4′	71.2	3.49 (1H, m)	$H_{5'}$–$C_{4'}$, $H_{3'}$–$C_{4'}$
5′	78.7	3.30 (1H, m)	$H_{4'}$–$C_{5'}$, $H_{3'}$–$C_{5'}$
6′	62.5	3.84 (2H, m)	$H_{5'}$–$C_{6'}$
1″a	46.7	4.04 (1H, d); $J_{1a''-1b''}$=16.2	H_5–$C_{1''}$
1″b	46.7	4.02 (1H, d); $J_{1b''-1a''}$=16.2	—
2″	30.1	2.23 (3H, s)	$H_{1''a}$–$C_{2''}$, $H_{1''b}$–$C_{2''}$

Table 2. Mutagenicity and Antimutagenicity Results of Zinolol to *Escherichia coli* PQ37 against the Mutagen Nifuroxazide

	Concentration (mg/ml)	U_β	U_P	R	IF	Results
Nifuroxazide	0	2.5	7.86	0.31	—	—
	+	34.75	5.05	6.88	22.19	—
Mutagenic activity	50	1.81	4.9	0.36	1.16	Non mutagen
	10	2.19	5.27	0.41	1.32	Non mutagen
	2	1.61	4.4	0.36	1.18	Non mutagen
						Genotoxcicity inhibition (%)
Antimutagenic activity	50	13.17	4.15	3.17	10.23	56.44
	10	10.76	3.9	2.75	8.89	62.76
	2	6.24	3.65	1.70	5.48	78.85

Table 3. $ABTS^{+\cdot}$ and $DPPH^\cdot$ Inhibition Percentage in the Presence of Different Concentrations of Zinolol

	ABTS % inhibition				DPPH
Concentration (mg/ml)	Time (min)				
	5	10	15	20	30
1	97.0±0.1	97.0±0.3	97.1±0.3	97.8±0.3	97.8±0.1
0.5	97.7±0.7	97.7±0.6	97.8±0.6	97.7±0.7	86.1±1.6
0.25	82.5±4.7	89.1±3.1	94.6±2.3	97.6±1.6	76.8±1.1
0.125	69.8±7.8	79.7±6.8	87.1±5.7	91.8±4.6	71.4±0.8
0.06	78.2±9.5	84.3±9.1	88.8±8.3	89.1±4.4	69.3±1.6
0.03	70.1±5.4	79.8±7.4	83.2±6.5	87.2±6.2	25.8±0.5

aminomethyl fragment attached to the carbon C_6 was determined *via* the HMBC correlations $H_{1''a}$–C_6; $H_{1''b}$–C_6; $H_{1''a}$–C_1; $H_{1''b}$–C_1; $H_{1''a}$–C_5 and $H_{1''b}$–C_5. From the HMBC spectrum the long-range heteronuclear correlations $H_{2''}$–$C_{1''}$ and $H_{1''a,b}$–$C_{2''}$ were observed. The NH nitrogen group position was also confirmed based on the COSY 2D relay experiment. On the ^1H spectrum the methyl group appears as a singulet this multiplicity was explained by the exchange of the amine proton. The COSY relay experiment shows methyl group correlations $H_{1''a}$–$H_{2''}$; $H_{1''b}$–$H_{2''}$, that allows us to identify the full coupled spin network $\{-CH_{2''}-NH-CH_{1''}\}$.

The study of the Noe effects in zinolol permit us to confirm the β configuration of the glucosyl moiety *via* the correlations $H_{1'}$–$H_{5'}$ and $H_{1'}$–$H_{3'}$. Our deep bibliographic study on hydroquinones shows the skeleton originality of the present natural substance, and confirms that it is the first representative of a new class of hydroxyhydroquinone. The water solubility of zinolol encouraged us to focus on its biological activity and especially on its prevention of mutagenicity and radical toxicity. Indeed hydroxyhydroquinone derivatives were endowed by many activities; they show inhibition of HIV-1 proteinase[11] and activities of hydratase[12] and amine oxidase.[13]

E. coli **Mutagenicity Assay** The genotoxicity of zinolol was studied using the SOS chromotest (Table 2). At different concentrations and even with high concentration (50 mg/ml) our substance seems cause no mutagenic effects. Compared with the positive control (IF=22.19 at a concentration of 1 mg/ml) zinolol induction factor was constantly feeble (IF≈1). According to Mesch–Sundermann, a compound with an IF lower then 2.00 was classified as non genotoxic.[14] Therefore we can prove that zinolol is a non genotoxic com-

pound.

Antimutagenic Activity This test was based on the inhibition of the SOS inducing factor (IF), after co-incubation of the positive mutagenic compound nifuroxazide and the tested substance zinolol. Results were expressed as percentage of genotoxicity inhibition relative to positive nifuroxazide (Table 2). Application of zinolol with exponential value concentrations shows genotoxicity inhibition rising from 56.44 to 78.85%. As shown in Table 2 unexpected results were recorded, zinolol was more active with feeble concentrations it shows maximum genotoxicity inhibition of 78.85% at a concentration of 2 mg/ml. Complementary experiments are necessary to explain the concentration related inhibitory effects of zinolol, perhaps in the presence of another positive chemical mutagen.

Radical Cation $ABTS^{+\cdot}$ Scavenging Activity Results of our investigation on $ABTS^{+\cdot}$ cation radicals scavenging activity, represented by Table 3, show maximal inhibition percentage of 97.8% at a zinolol concentration of 1 mg/ml. At feeble concentration (0.03 mg/ml) and immediately after 5 min of incubation zinolol displays a value of 70% of $ABTS^{+\cdot}$ inhibition. Compared to Trolox (97.9% inhibition at 1 mg/ml and after 5 min of incubation), zinolol, is gifted of a very powerful antioxidant activity against $ABTS^{+\cdot}$ cation radicals.

$DPPH^\cdot$ Scavenging Capacity The result of antioxidant activity of zinolol, against free radical $DPPH^\cdot$ and after 30 min of incubation, was represented by Table 3. Compared to Trolox as a standard reference product (99.3% inhibition at 1 mg/ml and after 30 min of incubation), we note that our natural substance shows also powerful inhibition activity. The increase of the inhibition percentage from 25.8 to

97.1%, shows the dose-dependence of the zinolol antioxidant effect.

The *in vitro* test of zinolol against toxic radical entities ABTS$^{+\cdot}$ and DPPH$^{\cdot}$ proves the efficiency of this substance as an antioxidant agent. Indeed the remarkable activity of zinolol is awaited. Based on the structure–activity relationship of polyphenols compounds,[15] oxidation of hydroquinone skeleton results with the formation of the quinone stable form.

Along this study we are focusing on the interest of a new natural substance, isolated from the plant *Anagallis monelli*, as a strong agent against the genotoxicity and the oxidative effects caused by free radicals. The safety of zinolol and the genotoxicity–anticancer activity relationship[16] were two reasonable reasons to continue our research and to focus on the anticancer effects of our natural compounds.

Experimental

General Experimental Procedures ^{1}H-, ^{13}C-NMR and two-dimensional NMR spectra were obtained with Bruker WP 300 spectrometer at 300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR. Measurements were made in CD$_3$OD at 27 °C, and the resonance of residual solvent were used as internal references. Hxcoqf and inv4lplrndqf heteronucleair pulse programs were used respectively for CHCorr and HMBC experiments; 2D homonuclear correlation pulse programs, Cosyqf45 and Cosyqfrl were used for COSY experiments. NOESY experiment was performed based on the noesyph pulse program. ES-MS spectrum was recorded in negative switching mode, using a HP 1100 MSD apparatus. FT-IR spectrum was measured on a Perkin-Elmer 157G infrared spectro-photometer. Polax-2L instrument was used to determine the optical rotation. Optical density values were mesured on Hitachi Fluorescence (model 4010) and Shimadzu UV-2101 PC Spectrophotometers respectively for genotoxicity and antioxidant activities.

Plant Material Aerial parts (leaves, stems and fruit) of the plant *Anagallis monelli* (1200 g) were collected at the beginning of May 2002 in Sousse (centre of Tunisia). A voucher specimen was deposited at the herbarium in the Faculty of Sciences, University of Monastir, Tunisia.

Extraction and Isolation Dried and finely powdered *Anagallis monelli* aerial part (1200 g) was immersed in MeOH (4×2 l) at room temperature for a week. Methanolic extract was filtered then evaporated to dryness. The MeOH extract (360 g) was extracted with AcOEt (1 l). Removal of the solvent from the AcOEt solution gave a methanolic residue symbolized E1 (147 g) and an oily substance (212 g). Extraction of the oily substance with petroleum ether yield an ethyl acetate residue symbolized E2 (127 g) and a petroleum ether extract (86 g). The ethyl acetate residue E2 was fractioned by silica gel column chromatography (CC) eluted with a step-gradient of AcOEt in petroleum ether. Fifteen fractions (F1 to F15) were collected. Repeated CC separations of the twelfth fraction (2764 mg) afforded in pure form the natural compound zinolol (126 mg).

Chemicals The mutagen Nifuroxazide, the *o*-nitrophenyl-β-D-galactopyranoside and *n*-nitrophenyl-phosphate, for colorimetric evaluation of β-galactosidase and alkaline phosphatase, were purchased from Sigma. (ABTS), (DPPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were also obtained from Sigma. All other chemicals were of analytical grade purity.

***E. coli* Mutagenicity Assay** Genotoxicity assay of zinolol was carried out using SOS chromotest. This test is based on using the genetically modified *Escherichia coli* PQ37 strain, in which the *lacZ* is under the control of the *sfiA* geneb.[17] In short, genotoxicity in samples was detected measuring the activation of SOS response of tester organism by evaluating β-galactosidase induction and alkaline phosphatase expression. The β-galactosidase and alkaline phosphatase activities were calculated according to Quillardet and Hofnung.[18] Units$=$U$_{(\beta,p)}=$A$_{420}\times10^3/$t (A$_{420}=$optical density at 420 nm for the tested molecule at a determined concentration; t: substrate conversion time in min). The induction factor (IF) was calculated as the ratio R/R$_o$ with R$=$U$_\beta$/U$_P$ (U$_\beta=$β-galactosidase activity and U$_P=$alkaline phosphatase activity at zinolol determined concentration; R$_o=$β-galactosidase activity/alkaline phosphatase activity at test compound concentration zero). A positive control, Nifuroxazide was used as reference genotoxic compound.

Antimutagenic Activity The antimutagenic activity was carried out with the same method as that described for the study of the mutagenic activity, except the incubation of the mutagen agent at the same time as the tested substance.[19] The same concentrations of molecules used for the study of the mutagenic activity, are used for the study of the antimutagenic activity. All experiments were performed in triplicate.

Radical Cation ABTS$^{+\cdot}$ Scavenging Activity The standard method described by Miller *et al.*[20] has been adopted with minor modifications. This assay assesses the total radical scavenging capacity based on the ability of a compound to scavenge the stable ABTS radical (ABTS$^{+\cdot}$). The blue green ABTS radical form was produced through the reaction between ABTS and potassium persulfate in water. A concentrated ABTS$^{+\cdot}$ stock solution was diluted with phosphate buffered saline (PBS), pH 7.4 to a final absorbance of 0.70±0.02 with a wavelength of 734 nm and at a temperature of 37 °C. Solutions with different diluted concentrations of zinolol, were prepared in ethanol. Ten microliters of an antioxidant containing studied solution were added to 990 ml ABTS$^{+\cdot}$ solution and the absorbance at 734 nm was measured. Sample absorbance was compared to a blank where 10 μl of the solvent were added to 990 ml of the ABTS$^{+\cdot}$ solution. Absorbance was measured at 5, 10, 15 and 20 min after addition of the antioxidant. Results were expressed in inhibition percentage *versus* samples concentrations (mmol) at different times.

DPPH$^{\cdot}$ Scavenging Capacity The DPPH radical scavenging capacity of zinolol was measured from the bleaching of purple colored ethanol solution of DPPH. Method described by Hatano *et al.*[21] has been used. 0.5 ml of each sample concentration was mixed with a same volume of DPPH ethanolic solution. After incubation of 30 min in darkness at room temperature, absorbance was read at 520 nm wavelength. A mixture of 0.5 ml of DPPH solution and 0.5 ml of ethanol was taken as a blank. Decrease in absorption induced by the tested compounds was compared to that of the positive control Trolox. IC$_{50}$ values calculated denote the concentration (microgram of natural substance per milliliter of ethanol) required to scavenge 50% of DPPH radicals. All measurements were performed in triplicate. Results were expressed in inhibition percentage *versus* samples concentrations (mg/ml) at 30 min.

Zinolol: Amorphous solid, mp 164—166 °C. $[\alpha]_D^{25}$ −375° (*c*=0.04, methanol). IR (KBr) γ_{max}: 3348, 2923, 1699, 1603, 1471 cm^{-1}. UV λ_{max} (MeOH) nm (ε): 244 (10450). ^{1}H- and ^{13}C-NMR data, see Table 1. ES-MS [M−H]$^-$ at *m/z* 330 (C$_{14}$H$_{21}$NO$_8$, 331).

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