Antioxidant activity-guided isolation of constituents from *Euphorbia gaditana* Coss. and their antioxidant and tyrosinase inhibitory activities

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Graphical Abstract

Euphorbia gaditana Coss.
(Euphorbiaceae)

31 compounds

2 previously
unreported

Tyrosinase activity

Compound 1: 89.78 ± 0.93 $\mu\text{g/mL}$

Compound 2: 52.39 ± 0.69 $\mu\text{g/mL}$

Antioxidant activity:

DPPH, CUPRAC for all
isolated compounds (1-31)

Highlights

- A phytochemical study of *Euphorbia gaditana* was carried out.
- Two previous unreported compounds type acetophenone and α -pyrone, along with 29 known compounds were identified.
- An antioxidant activity-guided isolation was performed using DPPH and CUPRAC assays.
- All extracts and isolated compounds were evaluated for their antioxidant activity.
- The tyrosinase inhibitory activity was estimated for the two previous unreported molecules.

**Antioxidant activity-guided isolation of constituents from *Euphorbia gadihana*
Coss. and their antioxidant and tyrosinase inhibitory activities**

Mohamed Ibrahim Badaoui^a, Abdulmagid Alabdul Magid^b, Laurence Voutquenne-
Nazabadioko^b, Mohammed Benkhaled^a, Dominique Harakat^b, Anthony Robert^b
and Hamada Haba^{a,*}

*^aLaboratoire de Chimie et Chimie de l'Environnement (LCCE), Département de Chimie, Faculté
des Sciences de la Matière, Université de Batna-1 05000, Algeria*

^bUniversité de Reims Champagne Ardenne, CNRS, ICMR UMR 7312, 51097 Reims, France

* Corresponding author: Hamada Haba Tel: +213 33 31 90 15; Fax: +213 33 31 90 15; E-mail
address: haba.hamada@yahoo.fr; hamada.haba@univ-batna.dz

ABSTRACT

Two previously unreported compounds, 4-*O*-(2''-*O*-galloyl- β -D-glucopyranosyl)-3,5-dihydroxyacetophenone (**1**) and 5-isopropyl-2-oxo-3,6-dihydropyran-4-carboxylic acid (**2**), along with twenty-nine known compounds (**3-31**) were isolated from the aerial parts of *Euphorbia gaditana* Coss. Their structures were elucidated based on extensive spectroscopic analysis 1D and 2D-NMR, mass spectrometry HR-ESI-MS, optical rotation $[\alpha]_D$, acid hydrolysis and the comparison of NMR data with those described in literature. The antioxidant activity-guided study was conducted using DPPH and CUPRAC methods started from the extracts to bioactive isolated molecules. Most of the isolates (**1-31**) showed a good to excellent antioxidant activity compared to the standards BHT and ascorbic acid. Furthermore, **1** and **2** exhibited moderate tyrosinase inhibitory activity (IC_{50} 89.78 ± 0.93 and 52.39 ± 0.69 $\mu\text{g/mL}$, respectively) compared to the standard kojic acid (IC_{50} 25.23 ± 0.78 $\mu\text{g/mL}$).

Keywords: Euphorbiaceae; *Euphorbia gaditana* Coss.; 4-*O*-(2''-*O*-galloyl- β -D-glucopyranosyl)-3,5-dihydroxyacetophenone; 5-isopropyl-2-oxo-3,6-dihydropyran-4-carboxylic acid; antioxidant activity; tyrosinase inhibitory activity

1. Introduction

The largest *Euphorbia* genus of the Euphorbiaceae family comprises about 2000 plants distributed worldwide. The species of this genus, from annuals to trees, are characterized by their latex (Shi et al., 2008). In the past years, a considerable attention has been paid to *Euphorbia* species due to their use in folk medicines to cure skin diseases, gonorrhea, migraines, intestinal parasites and as wart cures, and for their diverse pharmacological effects of their secondary

metabolites, including antiproliferative, antimicrobial, antioxidant, antiviral and anti-inflammatory activities (Benabdelaziz et al., 2018; Mouffouk et al., 2019; Özbilgin and Citoğlu, 2012; Shi et al., 2008; Singla and Pathak, 1990). This large range of pharmacological properties is due to a huge diversity of their chemical constituents such as diterpenes, triterpenes, sesquiterpenes, coumarins, phenols and flavonoids (Aichour et al., 2014; Benmerache et al., 2017; Haba et al., 2009; Haba et al., 2013; Özbilgin and Citoğlu, 2012; Liu et al., 2019).

Euphorbia gaditana Coss. is an annual plant grown in northern Algeria, Tunisia and southern Spain. Previously, the petroleum ether extract of *E. gaditana* has been studied and allowed the identification of two new diterpenes (Flores-Giubi et al., 2017). However, the present investigation is mainly focused on the EtOAc and *n*-BuOH extracts of this plant to discover other secondary metabolites particularly flavonoids and polyphenolic compounds following antioxidant activity-guided isolation.

We report in this paper chemical constitution of EtOAc and *n*-BuOH extracts, obtained from the 70% aqueous-ethanol extract of *E. gaditana* aerial parts, consisting of two previously unreported compounds, an acetophenone glycoside (**1**) and an α -pyrone derivative (**2**), along with the previously described eighteen flavonoids (**3-20**) and eleven phenolic compounds (**21-31**). In addition, the antioxidant capacity of the extracts of different solvents (PE, CHCl₃, EtOAc and *n*-BuOH), fractions and isolated compounds was evaluated using DPPH and CUPRAC methods. Furthermore, the tyrosinase inhibitory activity of all the extracts and **1** and **2** was investigated.

2. Results and discussion

2.1. Phenolic and flavonoid contents

The aerial parts of *E. gaditana* were macerated in 70% aqueous-ethanol and the obtained extract was fractionated by liquid-liquid partition, with petroleum ether, chloroform, ethyl acetate, and *n*-butanol. The obtained extracts i.e. PE, CHCl₃, EtOAc and *n*-BuOH were investigated for their total phenolic and flavonoid contents (TPC, TFC). The results of TPC are expressed by gallic acid equivalent (GAE) as (mg GAE/g Extract), and those of TFC are given by quercetin equivalent (QE) as (mg QE/g Extract) (Table 1). Indeed, the most rich extract in flavonoids was the EtOAc extract (91.31 ± 1.35 mg QE/g), followed by the *n*-BuOH extract (37.16 ± 2.40 mg QE/g), and then CHCl₃ and PE extracts (11.70 ± 1.51 and 1.17 ± 0.25 mg QE/g, respectively). In addition, the determination of total phenolic contents indicated that the EtOAc extract has the highest content of phenolic compounds (189.87 ± 1.32 mg GAE/g) even more than the *n*-BuOH extract (109.26 ± 1.43 mg GAE/g). However, the CHCl₃ and PE extracts yielded low phenolic contents (29.39 ± 1.98 and 6.32 ± 1.43 mg GAE/g, respectively) (Table 1).

Insert table 1 here

2.2. Chemical constituents

The EtOAc and *n*-BuOH extracts were chosen for further purification based on their total phenolic contents, antioxidant activity, TLC and HPLC profiles. The purification of EtOAc and *n*-BuOH extracts using different chromatographic methods (VLC, CC, TLC, MPLC, and HPLC) afforded thirty-one compounds. All the structures were elucidated based on 1D, 2D-NMR, (-) and (+)-HR-ESI-MS techniques and the measurement of optical rotation. Besides, the NMR data of previously described compounds (**3-29**) were compared with those reported in the literature.

The previously unreported compounds were elucidated as 4-*O*-(2''-*O*-galloyl- β -D-glucopyranosyl)-3,5-dihydroxyacetophenone (**1**) and 5-isopropyl-2-oxo-3,6-dihydropyran-4-carboxylic acid (**2**) (Fig. 1), whereas the known compounds have been identified as (-) catechin

(3) (Son et al., 1989), quercetin (4) (Chang et al., 2009), myricetin (5) (Sakushima et al., 1983), 3-*O*- β -D-glucopyranosyl quercetin (6) (Chang et al., 2009), 3-*O*- α -L-arabinopyranosyl kaempferol (7) (Cui et al., 2018), 3-*O*- α -L-arabinopyranosyl quercetin (8), 3-*O*- α -L-arabinopyranosyl myricetin (9) (El-Toumy et al., 2010), 3-*O*- α -L-rhamnopyranosyl quercetin (10) (Chang et al., 2009), 3-*O*- α -L-rhamnopyranosyl myricetin (11) (El-Toumy et al., 2010), 3-*O*- β -D-glucuronopyranosyl kaempferol (12) (Satake et al., 2007), 3-*O*-(6''-*O*-methyl- β -D-glucuronopyranosyl) kaempferol (13) (Zaghloul, 1993), 3-*O*- β -D-glucuronopyranosyl quercetin (14) (Satake et al., 2007), 3-*O*- β -D-glucuronopyranosyl myricetin (15) (Hilbert et al., 2014), 3-*O*-(6''-*O*-methyl- β -D-glucuronopyranosyl) myricetin (16) (Gomaa-Darwish et al., 2016), 3-*O*-(2''-*O*-galloyl- α -L-rhamnopyranosyl) quercetin (17) (Isobe et al., 1981), 3-*O*-(3''-*O*-galloyl- α -L-rhamnopyranosyl) quercetin (18) (Lin et al., 2002), 3-*O*-(2''-*O*-galloyl- α -L-rhamnopyranosyl) myricetin (19) (Lee et al., 2017), 3-*O*-(3''-*O*-galloyl- α -L-rhamnopyranosyl) myricetin (20) (sun et al., 1991), trilobatin (21) (Dugé de Bernonville et al., 2009), citrusin E (22) (Matsubara et al., 1991), (*Z*)-*p*-coumaroyl-1-*O*- β -D-glucopyranoside (23) (Fons et al., 1998), 3,5-dihydroxyacetophenone-4-*O*- β -D-glucopyranoside (24) (Cho et al., 2013), 4-*O*-caffeoylquinic acid methyl ester (25) (Zhu et al., 2005), 4-*O*-feruloylquinic acid methyl ester (26) (Li et al., 1998), chlorogenic acid (27) (Satake et al., 2007), 5-*O*-feruloylquinic acid methyl ester (28) (Menozzi Smarrito et al., 2008), 3,4,5-trihydroxyacetophenone (29) (Li and Seeram, 2011), ethyl gallate (30) (Chen et al., 2018), and (*Z*)-*p*-coumaric acid (31) (Swisłocka et al., 2012) (Fig. S1).

2.3. Structure elucidation of **1** and **2**

Compound **1** was isolated as a brown amorphous powder. The (+)-HR-ESI-MS showed a pseudomolecular ion peak at m/z 505.0953 [$M+Na$]⁺ (calcd C₂₁H₂₂O₁₃Na, 505.0958), which

corresponded to the molecular formula $C_{21}H_{22}O_{13}$ requiring 11 degrees of unsaturation. The UV spectrum of **1** exhibited maxima absorption bands at 220 and 278 nm revealing the presence of a phenolic derivative (Jancovicova et al., 2007). The 1H -NMR spectrum (Table 2) displayed signals for a methyl group at δ_H 2.60 (3H, s), and four aromatic protons belonging to two symmetrical 1,3,4,5-tetrasubstituted benzene rings at δ_H 5.98 (2H, s, H-2/H-6) and 7.07 (2H, s, H-2''/H-6''), respectively. A signal of an anomeric proton at δ_H 5.21 (1H, d, J = 8.1 Hz), with a signal at δ_H 5.12 (1H, dd, J = 9.5, 8.1 Hz) and other protons in the range (3.5–4.0 ppm) coupled with the correlations observed in the 1H - 1H -COSY spectrum (Figs. S6 and S7) and the large coupling constants between H-1'/H-2', H-2'/H-3', H-3'/H-4' and H-4'/H-5' indicated the presence of β -glucopyranosyl moiety. These structural features suggested **1** to be an acetophenone glucoside (Cho et al., 2013; Huang et al., 2017). The HSQC correlations allowed evidently the assignment of the protons at δ_H 2.60, 3.53, 3.74, 3.77, 3.96, 5.12, 5.98 and 7.07 attached to the carbons at δ_C 33.0 (C-8), 71.6 (C-4'), 78.5 (C-5'), 76.1 (C-3'), 62.3 (C-6'), 74.9 (C-2'), 99.7 (C-1'), 96.3 (C-2 and C-6) and 110.3 (C-2'' and C-6'') respectively. In addition to these carbons, the ^{13}C -NMR spectrum (Table 2) exhibited two signals at δ_C 107.3 (C-1) and 121.3 (C-1'') ascribable to non-protonated aromatic carbons, and four signals of oxygenated aromatic carbons at δ_C 140.0 (C-4''), 146.5 (C-3'', C-5''), 164.9 (C-4), and 165.5 (C-3, C-5). The non-protonated sp^2 carbon signal resonating at 167.6 ppm was attributed to a conjugated carbonyl group (C-7''), whereas another carbonyl signal at δ_C 205.3 (C-7) suggested the presence of a ketone functionality. The HMBC spectrum showed a correlation from H-1' to C-4 suggesting that the β -glucopyranosyl moiety is linked to C-4 via an oxygen bridge. Moreover, H-2' and H-2''/H-6'' also displayed HMBC correlations to C-7'', indicating that C-2' of the β -glucopyranosyl moiety is linked to C-7'' of the galloyl moiety through an ester linkage. Furthermore, the HMBC spectrum presented

cross-peaks from H-2/H-6 and H₃-8 to C-7 supporting the presence of a function ketone on C-7 (Fig. S12). The NOESY spectrum displayed correlation from H-2/H-6 to H₃-8, thus confirming the structure of **1**. Based on the above evidence and comparison of the NMR data of **1** with those of the previously isolated analogue (Huang et al., 2017), **1** was elucidated as 4-*O*-(2''-*O*-galloyl- β -D-glucopyranosyl)-3,5-dihydroxyacetophenone, and was named gadiacetophenone.

Insert fig. 1 here

Compound **2** was isolated as white crystal. The (+)-HR-ESI-MS spectrum displayed a pseudomolecular ion peak at m/z 207.0628 $[M+Na]^+$ (calcd C₉H₁₂O₄Na, 207.0633) corresponding to the molecular formula C₉H₁₂O₄, indicating that the molecule possessed four degrees of unsaturation. The IR spectrum indicated absorption bands at 3451 cm⁻¹ (OH), 1752cm⁻¹ (pyrone carbonyl), 1690 cm⁻¹ (carbonyl acid), and 1645 cm⁻¹ (double bond). The ¹H-NMR spectrum (Table 2) showed signals at δ_H 1.09 (6H, d, J = 7.0 Hz, CH₃-8, CH₃-9), 3.33 (2H, t, J = 1.8 Hz, H-3), 4.95 (2H, t, J = 1.8 Hz, H-6) and 3.87 (1H, m, H-7). The ¹H-¹H-COSY spectrum revealed correlations from the doublet at δ_H 1.09 to H-7 suggesting the presence of an isopropyl group. The ¹³C-NMR spectrum (Table 2) exhibited signals corresponding to nine carbon atoms including five sp³ at δ_C 20.1 (C-8, C-9), 29.8 (C-7), 32.8 (C-3) and 68.2 (C-6), two non-protonated sp² at δ_C 121.0 (C-4) and 152.8 (C-5) and two carbonyls at δ_C 168.1 (C-10) and 173.3 (C-2). In addition, the HSQC spectrum allowed the assignment of the methyl protons at δ_H 1.09 to C-8 and C-9, the methylene protons at δ_H 3.33 to the carbon at 32.8 (C-3), the methine proton at δ_H 3.87 to the carbon at δ_C 29.8 (C-7) and the oxymethylene protons at δ_H 4.95 to the carbon at 68.2 (C-6). The HMBC spectrum also showed correlations from H₃-8 and H₃-9 to C-7 and C-5, H-7 to C-8, C-9, C-6, C-4 and C-5, H₂-3 to C-4, C-5, C-10 and C-2, and H₂-6 to C-4, C-5 and C-2 (Figs. S22 and S23). These spectroscopic features suggested that **2** is a derivative of α -

pyrone (Onocha et al., 1995) with a carboxylic acid functionality. Due to the previous HMBC correlations from H₃-8 and H₃-9 to C-5 and H-7 to C-4, and NOESY effects observed between H₃-8/H₃-9 and H₂-6, the isopropyl group and the carboxylic acid functionality are linked to ethylenic carbons C-5 and C-4, respectively (Figs. S20 and S23). All the above data were consistent with the structure of **2** which was characterized as 5-isopropyl-2-oxo-3,6-dihydropyran-4-carboxylic acid and named gadipyrene.

Several plants revealed the presence of α -pyrone derivatives with different skeletons such as monocyclic α -pyrone (e.g., djalonenol, boronolide, deacetylboronolide) (Onocha et al., 1995; Davies-Coleman et al., 1987), monobenzo- α -pyrone (e.g., aesculetin, fraxin) (Liu et al., 2005), dibenzo- α -pyrone (e.g., autumnariol, sarolactone, 3-epialtenuene) (Mao et al., 2014) or a moiety of a complex structure (e.g., cryptorigidifoliol F, cryptorigidifoliol I) (Liu et al., 2015). Compound **2** is the first example of α -pyrone possessing a double bond between C-4 and C-5, which might be biosynthesized via the pathways involved in the biosynthesis of α -pyrone such as the β -oxidation of linoleic acid and the condensation of malonyl-CoA derivatives in polyketide synthase (Schäberle 2016). Hence, gadipyrene (**2**) could be of importance for the elucidation of biogenesis of α -pyrone.

Insert table 2 here

2.3.1. Identification of D-glucose

The acid hydrolysis of **1** was carried to establish the configuration of the monosaccharide residue. 10 mg of **1** was refluxed with 5 mL of 2N HCl for 4h, and then neutralized with 0.5M KOH. After extraction with CH₂Cl₂ (3 $\times$ 5 mL), the aqueous solution was evaporated to get D-

glucose (1.6 mg, $[\alpha]^{20}_D +25.8^\circ$ (c 0.11, H₂O) (Budavari et al., 1989), which was confirmed by TLC comparison with an authentic sample.

2.4. Biological studies

The phytochemical investigation of *E. gaditana* afforded numerous secondary metabolites especially flavonoids and phenolics from the AcOEt and *n*-BuOH extracts, which is compatible with the TFC and TPC measurements. It's well known that natural polyphenol compounds furnished a good antioxidant activity (Badaoui et al., 2019; Mouffouk et al., 2019; Rice-Evans et al., 1996). For this reason, and in order to isolate bioactive compounds, an antioxidant activity-guided isolation has been conducted. The antioxidant capacity of all the extracts has been evaluated using DPPH and CUPRAC methods. The EtOAc and *n*-BuOH extracts have been selected for a phytochemical and biological study, due to their richness in polyphenolic compounds and according to their antioxidant capacity (DPPH IC₅₀ 8.28 ± 0.88 and 11.23 ± 0.66 µg/mL; CUPRAC A_{0.5} 6.44 ± 1.09 and 15.94 ± 1.07 µg/mL, respectively). Furthermore, each extract was fractionated, and the major fractions obtained were tested using DPPH and CUPRAC assays. Fractions Ac1, Ac2, Ac3, Bu1, Bu2 and Bu3 have been chosen for chromatographic purifications based on their IC₅₀ and A_{0.5} values (Table 3).

Insert table 3 here

The selected fractions were subjected to a phytochemical investigation and 31 compounds were isolated and tested for their antioxidant capacity by DPPH and CUPRAC methods. Compound **1** exhibited an excellent antioxidant activity (DPPH IC₅₀ 3.25 ± 0.09 µg/mL, CUPRAC A_{0.5} 7.09 ± 0.1 µg/mL) compared to the standards BHT and ascorbic acid (DPPH IC₅₀ 12.99 ± 0.41 and 13.94 ± 2.81 µg/mL; CUPRAC A_{0.5} 8.97 ± 3.94 and 52.59 ± 1.98 µg/mL,

respectively). Compound **2** was not active at the tested concentration (200 µg/mL). In addition, the majority of the known compounds (**3-31**) indicated good to excellent antioxidant activity (Table 3).

The number and a structural variation of the isolated flavonoids lead us to study the relationship structure-activity. Quercetin (**4**) gave a higher antioxidant activity than myricetin (**5**); so, we conclude that the third hydroxyl group in B ring of the flavonol decreases the antioxidant capacity (Fig. S1). Moreover, the type of sugar on C-3 of flavonols also affects the antioxidant activity. Comparing the effect of sugars on C-3 on the antioxidant activity of **6, 8, 10** and **14**, it was found that the antioxidant activity decreases from arabinose to glucose, from glucose to rhamnose and from rhamnose to glucuronic acid. This effect is corroborated by the antioxidant capacity observed for **9, 11** and **15**. Comparison of **12** with **13**, and **15** with **16** revealed that glucuronic acid displays a higher activity than its methyl ester. Furthermore, the comparison of **17** and **18** with **10**, and of **19** and **20** with **11**, indicated that the addition of the galloyl group to rhamnose of the 3-*O*-rhamnosyl flavonoids increases their antioxidant activity, especially when the galloyl group is linked to C-3 instead of C-2 of rhamnose (Table 3).

The tyrosinase activity (Table 3) was also evaluated for the extracts, major fractions as well as for **1** and **2**. Indeed, **1** and **2** displayed moderate tyrosinase inhibitory activity with IC₅₀ values of 89.78 ± 0.93 and 52.39 ± 0.69 µg/mL, respectively, when compared to kojic acid (IC₅₀ = 25.23 ± 0.78 µg/mL).

3. Conclusion

In summary, two previously undescribed compounds named gadiacetophenone (**1**) and gadipyronone (**2**), along with twenty-nine previously reported compounds (**3-31**), comprising

seventeen flavonoids and eleven phenolic compounds, were isolated and identified from the aerial parts of *Euphorbia gaditana* Coss. Among the known constituents, thirteen compounds (**9**, **15-22**, **24-26** and **28**) possessing several skeletons like flavonol, acetophenone glycoside, hydroxycinnamic acid glycoside and chlorogenic acid, are found for the first time in *Euphorbia* species. The antioxidant activity, evaluated by DPPH and CUPRAC methods for extracts, fractions and all the isolated compounds showed in general good to excellent antioxidant activity. Furthermore, the two undescribed compounds **1** and **2** showed a moderate inhibition of the tyrosinase enzyme comparing to the standard kojic acid.

4. Experimental

4.1. General experimental procedures

UV spectra were measured on a Shimadzu UV/Vis U-2450 spectrophotometer. IR spectra were obtained using a Nicolet Impact 410 FTIR spectrometer. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. 1D and 2D-NMR spectra were recorded in CH₃OH-*d*₄ on Bruker Avance DRX III 500 instrument using standard Bruker microprograms (Karlsruhe, Germany). (-) and (+)-HR-ESI-MS were obtained from Micromass Q-TOF micro-instrument (Manchester, UK). Flash chromatography was performed on a Grace Reveleris system equipped with dual UV and ELSD detection using Grace cartridges (Silica gel or RP-C₁₈), and the monitored wavelengths were at 205 and 254 nm. The medium-pressure liquid chromatography (MPLC) was employed using a Buchi pump system AP250/500 (Buchi, France), with a RP-C₁₈ silica gel MERCK column (15 × 230 and 26 × 460 mm). Preparative HPLC (PLC 2050 Gilson) was equipped with a RP-C₁₈ column (Phenomenex 250 -15 mm, Luna 5 m, Interchim, France), it was used as a semi-preparative HPLC with a binary gradient eluent (H₂O pH 2.4 with TFA;

CH₃CN) and a flow rate of 5 mL/min; the chromatogram was monitored at 205, 254, 300, and 360 nm. Semi-preparative HPLC was performed on an apparatus equipped with an ASI-100 Dionex autosampler, an Ultimate 3000 pump ThermoFisher Scientific, a diode array detector UVD 340S and a Chromeleon software (Dionex, ThermoFisher Scientific, France). RP-C₁₈ column (Phenomenex 250 × 4.6 mm, Luna 5 μm, Interchim, France) was used for a semi-preparative HPLC with a binary gradient eluent (H₂O pH 2.4 with TFA; CH₃CN) and a flow rate of 5 mL/min; the chromatogram was monitored at 205, 254, 300, and 360 nm. An HPLC analytic Ultimate 3000 ThermoFisher Scientific equipped with LPG 3400SD pump, WPS3000SL injector, UV-DAD-3000 detector (ThermoFisher Scientific, France); and an Interchim Uptisphere strategy column C-18-HQ 250 × 4.6 mm (Interchim, France) was used. Analytical TLC was performed using silica gel plates (Merck Kieselgel 60 F254) and RP-C₁₈ (Kieselgel 60 F254S) plates and visualized at 254 and 366 nm and by spraying the dried plates with 50% of H₂SO₄, followed by heating.

The bioactivity assays were carried out at the Center of biotechnology Research (Algeria) on a 96-well microplate reader, Perkin Elmer Multimode Plate Reader EnSpire (Perkin Elmer, France). Folin-Ciocalteu reagent (FCR), 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), ascorbic acid, ammonium acetate were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). Copper (II) chloride, neocuproine, L-DOPA and tyrosinase enzyme were purchased from Biochem Chemopharma (Biochem Chemopharma, France). All other chemicals and solvents were of analytical grade.

4.2. Plant material

The plant *Euphorbia gaditana* Coss. was collected in May 2015 in Seriana from semi-arid region of Batna (North eastern of Algeria). It was identified by Prof. Bachir Oudjehih from the Agronomic Institute of the University of Batna-1 (Algeria). A voucher specimen number (908/LCCE) was deposited at the Faculty of Sciences of Matter, University of Batna-1, Algeria.

4.3 Extraction and isolation

The aerial parts of *E. gaditana* (1 kg of dried powder) were macerated in 70% aqueous-ethanol (2×12 L, 48h) at room temperature, and then filtered by filter paper and the aqueous-ethanol solution was concentrated under reduced pressure to remove EtOH. The aqueous solution was successively extracted with PE (3×300 mL), CHCl_3 (3×300 mL), EtOAc (3×300 mL) and *n*-BuOH (3×300 mL). The obtained organic phases were evaporated to dryness under reduced pressure at 45 °C to provide 1.3 g of PE, 30 g of CHCl_3 , 7.1 g of EtOAc and 23.1 g of *n*-BuOH extracts. All the extracts and fractions were stored in darkness at room temperature before fractionation and purification. The EtOAc and *n*-BuOH extracts were chosen for further purification based on their antioxidant activity, TLC and HPLC profiles.

The EtOAc extract (7.1 g) was subjected to vacuum liquid chromatography (VLC) over RP- C_{18} and eluted successively with (20, 40, 60, 80, and 100% MeOH in H_2O , 1 L each) to provide five major fractions (Ac1-Ac5). Ac1 (2.2 g) was applied to a flash chromatography of silica gel and eluted with mixtures of CH_2Cl_2 -MeOH- H_2O (10:0:0-7:3:0-70:30:5) to obtain fraction Ac1-E (195 mg) which was further purified by flash chromatography over RP- C_{18} and eluted with MeOH- H_2O (5-20% MeOH) to yield **3** (19.4 mg). Fraction Ac2 (2 g) was fractionated by preparative HPLC over RP- C_{18} . The elution was carried out by CH_3CN - H_2O (20-30% CH_3CN , in 45 min) to obtain fractions Ac2-C (144 mg), Ac2-D (192), Ac2-E (356 mg), Ac2-F (268 mg),

Ac2-G (157 mg) and Ac2-H (100 mg); then, fractions (Ac2-C, Ac2-D, Ac2-E, Ac2-G and Ac2-H) were purified by flash chromatography of a silica gel and eluted with CH₂Cl₂-MeOH-H₂O (100:0:0-70:30:0-70:30:5). Indeed, fraction Ac2-C provided compounds **9** (10 mg) and **24** (7 mg), fraction Ac2-D gave compound **5** (5.8 mg), fraction Ac2-E afforded compound **30** (43.5 mg) and sub-fraction Ac2-E-g (34 mg) which was further purified using preparative HPLC to yield **6** (2.5 mg), and fraction Ac2-G provided compounds **2** (5.8 mg) and **31** (7 mg), whereas, fraction Ac2-H furnished **29** (2.5 mg). Fraction Ac2-F was purified by flash chromatography of silica gel and eluted with CH₂Cl₂-MeOH (0-35% MeOH, 30 min) to afford sub-fraction Ac2-F-k (141 mg), which was purified by preparative HPLC using CH₃CN-H₂O as eluent (20-35% CH₃CN, 30 min) to furnish **1** (5.8 mg). Fraction Ac3 was subjected to preparative HPLC over RP-C₁₈ and eluted with CH₃CN-H₂O (20-35% CH₃CN, 30 min) to obtain **4** (7 mg), **8** (19.6 mg), **10** (24 mg), **11** (6.7 mg), **17** (17.5 mg), in addition to sub-fractions Ac3-24 (27 mg), Ac3-26 (18 mg) and Ac3-31 (18 mg). These sub-fractions were further purified with semi-preparative HPLC using CH₃CN-H₂O as eluent (20-35% CH₃CN) to yield **19** (3.8 mg, *t_R* 3.05 min) and **20** (2.6 mg, *t_R* 11.52 min) from sub-fraction Ac3-24; **21** (3 mg, *t_R* 26.55 min) from sub-fraction Ac3-26 and **18** (2.5 mg, *t_R* 22.10 min) from sub-fraction Ac3-31.

The *n*-BuOH extract (22 g) was applied to VLC over RP-C₁₈. The elution was performed with MeOH-H₂O (0:100-100:0) to afford six fractions (Bu0-Bu5). Fraction Bu1 (3.4 g) was fractionated by flash chromatography over RP-C₁₈ and eluted with MeOH-H₂O (5-40% MeOH, 30 min) to obtain **27** (17 mg) and several sub-fractions. Sub-fractions Bu1-C and Bu1-D were combined (64 mg) and purified by semi-preparative HPLC using CH₃CN-H₂O as eluent (5-25% MeCN, 30 min) to yield **23** (1.5 mg, *t_R* 25.03 min). Sub-fraction Bu1-Q (316 mg) was purified by flash chromatography over silica gel and eluted with CH₂Cl₂-MeOH-H₂O (100:0:0-70:30:0-

70:30:5) to give two sub-fractions Bu1-Q-c and Bu1-Q-d. Bu1-Q-c (27 mg) was further purified by semi-preparative HPLC eluting with MeCN-H₂O (17-21% CH₃CN, 25 min) to afford **25** (5.5 mg, *t_R* 8.25 min) and **22** (3 mg, *t_R* 11.54 min). Sub-fraction Bu1-Q-d (10.5 mg) gave, after precipitation in MeOH, **28** (7.7 mg). Fraction Bu2 (5.3 g) was purified by flash chromatography of a silica gel and eluted with CH₂Cl₂-MeOH-H₂O (100:0:0-70:30:0-70:30:5) to provide several sub-fractions. Sub-fraction Bu2-C (11.3 mg) was purified by preparative HPLC (15-35% CH₃CN, 30 min) to give **26** (2.6 mg). Sub-fraction Bu2-E (600 mg) was applied to flash chromatography of RP-C₁₈ and eluted with MeOH-H₂O (15-25% MeOH, 30 min) to afford sub-fraction Bu2-E-h (68 mg) which was purified by semi-preparative HPLC over RP-C₁₈ using CH₃CN-H₂O as eluent (15-25%) to furnish **16** (44 mg, *t_R* 12.13 min). Sub-fraction Bu2-J (80 mg) was purified by preparative HPLC and eluted with CH₃CN-H₂O (15-35 CH₃CN, 30 min) to give **15** (6 mg). Fraction Bu3 (2.6 g) was chromatographed over flash chromatography of silica gel. The elution was carried out with CH₂Cl₂-MeOH-H₂O (100:0:0-70:30:0-70:30:5) to provide two sub-fractions Bu3-I (20 mg) and Bu3-R (84 mg) which were purified by preparative HPLC using CH₃CN-H₂O as eluent (25-45% CH₃CN) and (15-25% CH₃CN), respectively, to yield **7** (1.3 mg) and **13** (1mg) from sub fraction Bu3-I, and **12** (5.4 mg) and **14** (7.3 mg) from sub-fraction Bu3-R.

4.3.1. Spectral data

Gadiacetophenone (4-O-(2"-O-galloyl-β-d-glucopyranosyl)-3,5-dihydroxyacetophenone) (1): Brown amorphous powder; $[\alpha]_D^{20}$ -13.1° (C 0.25, MeOH); UV $\lambda_{\max}$ (MeOH): 220, 278 nm; ¹H NMR (500 MHz, CH₃OH-*d*₄) and ¹³C NMR (125 MHz, CH₃OH-*d*₄), see [Table 2](#); (+)-HR-ESI-MS *m/z* 505.0953 [M+Na]⁺ (calcd C₂₁H₂₂O₁₃Na, 505.0958).

Gadipyrone (5-isopropyl-2-oxo-3,6-dihydropyran-4-carboxylic acid) (2): White crystal; UV λ_{max} (MeOH): 220 nm; IR ν : 3451, 1752, 1690 and 1645 cm^{-1} ; ^1H NMR (500 MHz, $\text{CH}_3\text{OH}-d_4$) and ^{13}C NMR (125 MHz, $\text{CH}_3\text{OH}-d_4$), see [Table 2](#); (+)-HR-ESI-MS m/z 207.0628 $[\text{M}+\text{Na}]^+$ (calcd $\text{C}_9\text{H}_{12}\text{O}_4\text{Na}$, 207.0633).

4.4. Chemical screening of plant extracts

The total phenolic and flavonoid contents of PE, CHCl_3 , EtOAc and *n*-BuOH extracts were established using Folin-Ciocalteu and trichloroaluminum methods (See supplementary material).

4.5. Antioxidant activity

The antioxidant activity of the extracts, fractions and isolated compounds was evaluated by DPPH free radical scavenging and Cupric reducing antioxidant capacity (CUPRAC) assays (See supplementary material).

4.6. Tyrosinase inhibitory activity

The extracts, fractions and **1** and **2** were tested for their tyrosinase inhibitory activity (See supplementary material).

Statistical analysis

All data on bioassays were the average of triplicate analyses. The data were recorded as mean values $\pm$ standard deviation. The IC_{50} and $\text{A}_{0.5}$ values were calculated by linear regression analysis.

Supplementary material

General methods, UV, IR, HR-ESI-MS, ^1H and ^{13}C NMR, COSY, HSQC, HMBC and NOESY spectra of the previously unreported compounds **1** and **2** are available online.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Fig. 1. Chemical structures of the two previously unreported compounds **1** and **2** isolated from *E. gaditana*.

Table 1. Phenolic and flavonoid contents in *E. gaditana* extracts

Extracts	TPC (mg GAE ^x /g)	TFC (mg QE ^y /g)
PE	6.32 ± 1.43	1.17 ± 0.25
CHCl₃	29.39 ± 1.98	11.70 ± 1.51
EtOAc	189.87 ± 1.32	91.31 ± 1.35
<i>n</i>-BuOH	109.26 ± 1.43	37.16 ± 2.40

^xGallic acid equivalent, ^yquercetin equivalent

Table 2. ^1H and ^{13}C NMR data of compounds **1** and **2** (500 and 125 MHz, $\text{CH}_3\text{OH}-d_4$)

1			2		
Position	δ_{H} , m (<i>J</i> in Hz)	δ_{C} , type	Position	δ_{H} , m (<i>J</i> in Hz)	δ_{C} , type
1	-	107.3 (C)	2	-	173.3 (CO)
2	5.98, s	96.3 (CH)	3	3.33, t (1.8)	32.8 (CH ₂)
3	-	165.5 (C)	4	-	121.0 (C)
4	-	164.9 (C)	5	-	152.8 (C)
5	-	165.5 (C)	6	4.95, t (1.8)	68.2 (CH ₂)
6	5.98, s	96.3 (CH)	7	3.87, m	29.8 (CH)
7	-	205.3 (CO)	8	1.09, d (7.0)	20.1 (CH ₃)
8	2.60, s	33.0 (CH ₃)	9	1.09, d (7.0)	20.1 (CH ₃)
1'	5.21, d (8.1)	99.7 (CH)	10	-	168.1 (CO)
2'	5.12, dd (9.5, 8.1)	74.9 (CH)			
3'	3.74, t (9.1)	76.1 (CH)			
4'	3.53, t (9.0)	71.6 (CH)			
5'	3.57, m	78.5 (CH)			
6'a	3.77, dd (12.1, 5.1)	62.3 (CH ₂)			
6'b	3.96, dd (12.1, 1.9)				
1''	-	121.3 (C)			
2''	7.07, s	110.3 (CH)			
3''	-	146.5 (C)			
4''	-	140.0 (C)			
5''	-	146.5 (C)			
6''	7.07, s	110.3 (CH)			
7''	-	167.6 (CO)			

Table 3. Antioxidant (DPPH and CUPRAC assays) and tyrosinase inhibitory activity of *E. gaditana* extracts, fractions, and compounds (**1-31**)

Extracts/ Fractions/ Compounds	Antioxidant activities		Tyrosinase activity
	DPPH assay IC ₅₀ (μg/mL)	CUPRAC assay A _{0.5} (μg/mL)	IC ₅₀ (μg/mL)
PE extract	> 200	> 200	> 200
CHCl₃ extract	> 200	61.2 ± 0.35	> 200
EtOAc extract	8.28 ± 0.88	6.44 ± 1.09	> 200
<i>n</i>-BuOH extract	11.23 ± 0.66	15.94 ± 1.07	> 200
Ac1	3.98 ± 0.43	13.38 ± 0.78	> 200
Ac2	2.86 ± 0.29	8.72 ± 0.26	35.39 ± 2.92
Ac3	4.78 ± 0.44	11.68 ± 0.09	> 200
Ac4	30.82 ± 2.57	59.41 ± 1.37	> 200
Bu0	56.27 ± 1.13	107.89 ± 1.45	> 200
Bu1	5.27 ± 0.73	12.68 ± 0.44	> 200
Bu2	3.59 ± 0.13	10.85 ± 0.29	32.16 ± 2.18
Bu3	6.15 ± 0.1	22.11 ± 0.24	15.23 ± 0.87
Bu4	4.09 ± 0.2	21.40 ± 0.12	88.25 ± 1.16
1	3.25 ± 0.09	7.09 ± 0.1	89.78 ± 0.93
2	NA	NA	52.39 ± 0.69
3	3.40 ± 0.06	4.20 ± 0.42	-
4	0.89 ± 0.13	2.20 ± 0.26	-
5	3.0 ± 0.14	6.32 ± 0.07	-
6	3.32 ± 0.34	9.79 ± 1.15	-
7	31.34 ± 1.11	72.98 ± 1.29	-
8	1.12 ± 0.19	8.13 ± 0.34	-
9	2.61 ± 0.11	10.37 ± 0.09	-
10	3.52 ± 1.20	11.59 ± 0.45	-
11	2.87 ± 0.21	11.01 ± 0.11	-
12	9.94 ± 0.86	18.02 ± 0.71	-
13	18.36 ± 0.76	21.38 ± 0.72	-
14	5.59 ± 0.39	11.19 ± 0.24	-
15	8.12 ± 0.12	11.88 ± 0.39	-
16	8.6 ± 0.38	12.77 ± 0.35	-
17	3.34 ± 0.27	10.52 ± 0.75	-
18	3.18 ± 0.33	10.13 ± 0.89	-
19	2.8 ± 0.58	10.64 ± 0.36	-
20	2.51 ± 0.23	10.30 ± 0.78	-
21	19.49 ± 0.77	19.44 ± 1.19	-
22	> 200	83.84 ± 1.54	-

23	153.7 ± 0.8	75.43 ± 0.38	-
24	98.7 ± 2.6	11.64 ± 0.55	-
25	5.78 ± 0.09	8.10 ± 0.67	-
26	30.84 ± 1.23	40.32 ± 1.33	-
27	8.23 ± 0.2	10.43 ± 0.68	-
28	17.99 ± 1.11	13.04 ± 0.28	-
29	39.3 ± 1.94	5.83 ± 0.5	-
30	1.01 ± 0.07	2.25 ± 0.15	-
31	> 200	16.0 ± 0.84	-
BHT	12.99 ± 0.41	8.97 ± 3.94	-
Ascorbic acid	13.94 ± 2.81	52.59 ± 1.98	-
Kojic acid	-	-	25.23 ± 0.78

IC₅₀ values are defined as the concentration of 50% inhibition percentages, A_{0.5} values are defined as the concentration at absorbance A=0.5 and calculated by linear regression analysis and expressed as Mean ± SD (n=3). NA: not absorbed.