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Abstract

A previously undescribed triterpenoid saponin, 3-*O*-[α-L-rhamnopyranosyl-(1→2)-{β-D-glucopyranosyl-(1→6)-}β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-sophoradiol (**1**), in addition to twenty-nine known constituents (**2-30**) were isolated from the aerial parts of *Genista numidica* Spach. Structures elucidation was performed by comprehensive 1D- and 2D-NMR analyses and HRESIMS. The extracts, fractions and isolated compounds were evaluated for their antibacterial, antioxidant and tyrosinase inhibitory activities. The experimental findings indicated that genistin (**16**), isosalipurpol (**27**), and koaburaside (**29**) have moderate to low antibacterial activity against *E. faecalis*, *S. aureus*, *S. epidermidis* and *P. aeruginosa* bacteria with MICs ranging from 31.2 to 125 µg/mL. Compounds **19** and **27** exhibited a good antiradical activity potential (IC$_{50}$11.8 and 11.1 µg/mL, respectively). Only compounds **23**, **27** and **28** exhibited low inhibitory effect against mushroom tyrosinase (IC$_{50}$ from 90.2 to 225.6 µg/mL).

KEYWORDS: *Genista numidica*; Fabaceae; Triterpenoid saponins; Flavonoids; Antibacterial activity; Antioxidant activity; Antityrosinase activity.

1. Introduction

Genista is an important genus of the Fabaceae (Leguminosae) family with approximately 100 species distributed mainly in the Mediterranean region and in Western Asia (Noccioli et al., 2011). This genus is represented in Algeria by 23 species, eleven of which being endemic to Algeria (Quezel and Santa, 1963). Many *Genista* species show interesting biological properties such as hypoglycemic (Rauter et al., 2009), antiinflammatory, antiulcer (Rainova et al., 1988), and cytotoxic activity against different human cancer cell lines (Bontempo et al., 2013; Rigano et al., 2009). All phytochemical analysis on *Genista* have revealed the presence of flavonoids, isoflavones and alkaloids (Serrilli et al., 2010; Kacem et al., 2014). The methanolic extracts of the flowers and the leaves of *Genista numidica* Spach. showed the presence of flavonoid and these extracts exhibited DPPH radical scavenging activity (Ati et al., 2017). The chemical composition and the antimicrobial activity of the essential oil of aerial parts of *G. numidica* showed that the main constituents of the essential oils are rich in fatty acid. The oils showed no significant antibacterial activities against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Lograda et al., 2009). The aim of the present study focuses on the isolation and structure elucidation of chemical constituents from *G. numidica* aerial parts and evaluation of their antibacterial, antioxidant, and tyrosinase inhibitory activities.

2. Results and discussion

The 80% MeOH extract of dried aerial parts of *G. numidica* was fractionated and purified by a combination of chromatographic methods to

obtain a new triterpenoid saponin (**1**) (Figure 1) and twenty-nine known compounds (**2-30**). The known compounds were elucidated as kaikasaponin III (**2**) (Kinjo et al., 1991), soyasaponin I (**3**) (Tantry et Khan, 2013), sophoraflavoside II (**4**) (Mohamed et al., 1995), kudzusaponin B_1 (**5**) (Arao et al., 1996), phaseoside IV (**6**) (Lu et al., 2013), dehydrosoyasaponin I (**7**) (Mohamed et al., 1995), baptisiasaponin I(**8**) (Udayama et al., 1998), kakkasaponin III (**9**) (Lu et al., 2013), 3,7-bis-α-L-rhamnopyranosyl-aromadendrin (**10**) (Venditti et al., 2016), neoliquiritin (**11**) (Li et al., 1992), 7-O-β-D-glucopyranosyl-4′dihydroxyflavone (**12**) (Nascimento and de Oliveira, 2004), cynaroside (**13**) (Furkan et al., 2012), genistein (**14**) and daidzin (**15**) (Shimoda et al., 2006), genistin (**16**) (Yuldashev et al., 1990), sissotrin (**17**) (Fu et al., 2012), 8-C-β-D-glucopyranosyl-genistein (**18**) and 8-C-β-D-glucopyranosyl-orobol (**19**) (Sato et al., 2006), 8-C-β-D-glucopyranosyl-genistein-4′-O-β-D-glucopyranoside (**20**) (Watanabe et al., 1993), 4′,7-di-O-β-D-glucopyranosyl-genistein (**21**) (Watanabe et al., 1993), 4′,7-di-O-β-D-glucopyranosyl-5-O-methylgenistein (**22**) (Watanabe et al., 1993), isoliquiritigenin (**23**) (Ma et al., 2005), neoisoliquiritigenin (**24**) (Veitch et al., 2003), isoliquiritin (**25**) (Wang et al., 2008), 4,4′-di-O-β-D-glucopyranosyl-isoliquiritigenin (**26**) (Ji et al., 2016), isosalipurpol (**27**) (Zapesochnaya et al., 2003), licoagroside B (**28**) (Li et al., 2000), koaburaside (**29**) (Burns et al., 2007) and tyramine (**30**) (Forino et al., 2016) (Figure S1).Their spectroscopic data were in perfect agreement with those reported in the literature.
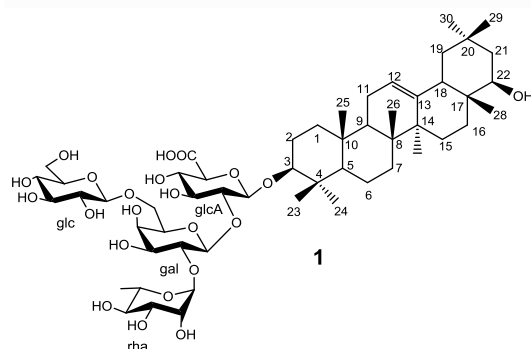

Fig. 1.
Structure of compound **1** isolated from *Genista numidica*.

Compound **1** exhibited in the HRESIMS spectrum (positive-ion mode) a pseudo-molecular ion peak at m/z1111.5658 $[M+Na]^+$ (calcd for 1111.5665, $C_{54}H_{88}O_{22}Na$). The ^{1}H and ^{13}C NMR spectra of the aglycone part exhibited eight methyl groups at δ_H 1.13 (s, H-23), 0.90 (s, H-24), 1.00 (s, H-25), 1.02 (s, H-26), 1.15 (s, H-27), 0.86 (s, H-28), 0.94 (s, H-29) and 1.03 (s, H-30), one olefinic proton signal at δ_H 5.27 (1H, J = 3.5 Hz, H-12) and two olefinic carbon signals at δ_C 123.8 (C-12) and 145.2 (C-13), characteristic of an oleanene-type skeleton (Boutaghane et al., 2013). Futhermore, other characteristic signals were observed such as two oxygen bearing methine proton signals at δ_H 3.13 (dd, J=11.8, 4.4 Hz) and δ_H 3.42 (t, J=10.2 Hz, H-22). The HMBC correlation between the two methyl groups H-23 and H-24 and C-3 (δ_C 92.2) confirmed the location of the first secondary alcoholic function at C-3. The second secondary alcoholic function at C-22 (δ_C 76.0) was deduced by the COSY correlations observed between H-22 and H-21(δ_H 1.34, 1.46) and the HMBC cross-peaks between H-28 and C-22. In the ROESY spectrum, correlations observed between H-3/H-5 (δ_H 0.78) and H-5/H-9 (δ_H 1.58) indicated their α-orientation and thus the β-orientation of the oxygen at C-3. In the same manner, the ROESY correlations between CH_3-25/CH_3-24, CH_3-25/CH_3-26, H-26/H-1, and H-18 (δ_H 2.07, dd, J=13.2, 3.5 Hz)/CH_3-30 indicated their β-axial orientation. The ROESY experiment revealed also several correlations between CH_3-23/H-5, CH_3-23/H-9, H-9/CH_3-27 and H-22/CH_3-29 indicating the α-orientation of CH_3-23, CH_3-27, CH_3-29 and H-22 and thus the β-orientation of the oxygen at C-22. Taken together, these data were indicative of a typical sophoradiol (Manabu et al., 1998). This assumption was confirmed by analysis of the COSY, ROESY, HSQC and HMBC spectra which allowed the full assignment of the proton and carbon resonances of the aglycone (Table S1). The ^{1}H NMR spectrum of **1** displayed in the sugar region signals for four anomeric protons at δ_H 5.20 (d, J=1.5 Hz), 4.84 (d, J=7.7 Hz), 4.51 (d, J=7.9 Hz) and 4.46 (d, J=7.7 Hz), which gave correlations, in the HSQC spectrum, with four anomeric carbon signals at δ_C 102.1, 102.7, 104.5 and 105.9, respectively. Complete assignments of the resonances of each sugar were achieved by extensive 2D-NMR analyses

(COSY, ROESY, HSQC and HMBC) (Table S1). One α-L-rhamnopyranosyl (rha) was easily identified by equatorial anomeric proton (δ_H 5.20, d, J = 1.5 Hz), a methyl doublets (δ_H 1.28, d, J=6.2 Hz), a small coupling constant between H-2 and H-3 ($J_{H2eq,H3ax}$=3.3 Hz) and a large coupling constants between H-3 and H-4 ($J_{H3ax,H4ax} \geq 9.5$ Hz). Starting from the anomeric proton signals at δ_H 4.51, the NMR signals belonging to the same system were assigned to a β-D-glucopyranosyl (glc),H-2, H-3 and H-4 exhibit large vicinal couplings ($\geq$ 7.9 Hz) indicating that all are axials. A β-D-galactopyranose unit (gal) was identified starting from the anomeric proton at δ_H 4.84, d, J=7.7 Hz) characterized by the large coupling constants $J_{H1,H2}$ and $J_{H2,H3}$ ($\geq$ 7.7 Hz) and the small coupling constant between H-3 and H-4 ($J_{H3,H4}$=3.5 Hz). The considerably downfield shift of gal-C-2 and gal-C-6 (δ_C 77.3 and δ_C 69.4, respectively) suggested that gal was 2,6-disubstituted. The sugar unit with anomeric proton at δ_H4.46 (d, J=7.7 Hz) corresponded to a β-D-glucuronic acid unit (glcA), characterized by a five spin system possessing large coupling constants ($J_{H1,H2}$, $J_{H2,H3}$, $J_{H3,H4}$, and $J_{H4,H5} \geq$ 7.7 Hz), a doublet corresponding to proton glcA-H-5 ($J_{H4,H5}$=9.6 Hz) and a carbonyl glc-A-C-6 at δ_C 178.6 coupled with glcA-H-5 of the same sugar in the HMBC spectrum. The NMR data showed lowfield position of glcA-C-2 (δ_C 78.5) indicating it to be substituted at this position. The HMBC correlation between rha-H-1/gal-C-2, glc-H-1/gal-C-6, gal-H-1/glcA-C-2 and glcA-H-1/aglycone-C-3 indicated that the tetra-saccharide (rha-(1→2)-{glc-(1→6)-}gal-(1→2)-glcA) was linked to the C-3 of the aglycone. In addition, ROESY correlations confirming the interglycosidic linkage and the point of attachment of the tetra-saccharide at the H-3 of the aglycone were observed between glc-H-1/gal-H$_2$-6, rha-H-1/gal-H-2, gal-H-1/glcA-H-2, and glcA-H-1/aglycone-H-3. Consequently, the structure of **1** was concluded to be 3-O-[α-L-rhamnopyranosyl-(1→2)-{β-D-glucopyranosyl-(1→6)-}β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-sophoradiol (Figure 1).

The antibacterial potential of the 80% MeOH extract and fractions VLC-I to VLC-VI obtained from the aerial parts of *G. numidica* was evaluated by a bioautography assay against *Staphylococcus aureus* CIP 53.154. Only fraction VLC-III exhibited antimicrobial activity. This fraction was then tested against 22 micro-organisms by the minimum inhibitory concentration (MIC) determination method in solid media. Considering criteria for endpoint determination and evaluation of antimicrobial activity proposed by Cos et al. (2006) and Rios and Recio (2005), a good antimicrobial activity was observed against 3 bacteria (MICs $\leq$ 100 µg/mL) (Table S2). Fraction VLC-III showed a high antibacterial activity against *Shigella sonnei* (MIC 31.2 µg/mL), a good antibacterial activity against *Staphylococcus epidermidis* and *Micrococcus luteus* (MICs 62.5 µg/mL) and low *Bacillus subtilis* and *Enterococcus faecalis* (MICs 125 µg/mL) (Table S2). Fraction VLC-III showed low antifungal activity against *Candida tropicalis*, *Candida kefyr* and *Cryptococcus neoformans* (MICs 125 µg/mL) (Table S2). Subsequently, fraction VLC-III was purified by combination of chromatographic methods and a second bioautography assay was applied against *S. aureus* with isolated compounds. Compounds **16**, **27** and **29** showed a good antibacterial activity against *S. aureus*. Thus, the MICs of compounds **16, 27** and **29** against *E. faecalis*, *S. aureus*, *S. epidermidis, Escherichia coli*, and *Pseudomonas aeruginosa* were determined (Table S3). Compound **16** was the most active against *S. epidermidis* (MIC of 31.2 µg/mL) and *S. aureus* (MIC 62.5 µg/mL) and *P. aeruginosa* (MIC 125 µg/mL). Compound **29** exhibited also low antibacterial activity against *E. faecalis* and *S. epidermidis* (MICs 125 µg/mL). Compound **27** showed low antibacterial activity against *E. faecalis* (MIC 125 µg/mL). However, Rios and Recio (2005) proposed that only concentrations below 10 µg/mL in the case of isolated compounds could be considered as very interesting.

The antioxidant properties of the 80% MeOH and CHCl$_3$ extracts and fractions VLC-I to VLC-VI were investigated by lipid peroxidation inhibition activity, DPPH radical scavenging activity, ABTS cation radical scavenging activity and cupric reducing antioxidant capacity (CUPRAC) assays. The 80% MeOH extract, fraction VLC-II and VLC-III showed moderate antioxidant properties in the four tests with IC$_{50}$ ranging from 14.57 to 45.32 µg/mL (Table S4). Consequently, compounds **10-30** obtained from these fractions VLC-II and VLC-III were evaluated for their DPPH radical

scavenging activity (Table S4). Compounds **19** and **27** exhibited good activity (IC_{50} 11.8 and 11.10 µg/mL, respectively). The other compounds showed low or no antiradical activity (IC_{50} not achieved at the concentration of 200 µg/mL).

The 80% MeOH extract and fractions VLC-II and VLC-III showed low antityrosinase activity (Table S4). Among the compounds isolated from fractions VLC-II and VLC-III (**10**-**30**), only **15**, **16**, **23**, **27** and **28** exhibited low to moderate antityrosinase effect (IC_{50} 230.0, 197.0, 90.2, 110.3 and 225.6 µg/mL, respectively) (Table S4).

3. Conclusion

A new triterpenoid saponin (**1**) and twenty-nine known compounds (**2**-**30**) were isolated from the aerial parts of *Genista numidica* Spach. Genistin (**16**), isosalipurpol (**27**), and koaburaside (**29**) have moderate to low antibacterial activity against *E. faecalis*, *S. aureus*, *S. epidermidis* and *P. aeruginosa* bacteria with MICs ranging from 31.2 to 125 µg/mL. Compounds **19** and **27** exhibited good DPPH radical scavenging activity (IC_{50} 11.8 and 11.1 µg/mL, respectively). Compounds **23**, **27** and **28** exhibited low effect against mushroom tyrosinase (IC_{50} from 90.2 to 225.6 µg/mL).

Experimental Section

3.1. General experimental procedures

The optical rotations were recorded on a Perkin-Elmer 341 Polarimeter. The UV spectra were obtained in MeOH on a Shimadzu UV-2450 spectrophotometer. IR spectra were obtained using a Shimadzu IR-470 spectrometer. 1D and 2D NMR spectra were recorded in CD_3OD on a Bruker Avance DRX III 500 spectrometer (^{1}H at 500 MHz and ^{13}C at 125 MHz). ESI-MS experiments were performed using a Micromass Q-TOF instrument. Silica gel 60 F_{254} pre-coated aluminium plates (0.2 mm, Merck) were used for TLC analysis. The TLC spots were visualized under UV light (254 and 366 nm) followed by spraying with 50% H_2SO_4 and heating. Column chromatography was carried out on Kieselgel 60 (63-200 mesh) Merck. An Armen instrument equipped with an AP 250/500 pump, ACC 250/500 sampler, and a Merck UV-detector K-2501 was used for preparative HPLC. A Lichrospher RP18 prepacked column (Merck 250x50mm, 12µm) was used with binary gradient eluent (H_2O and CH_3CN) and a flow rate of 40 mL/min; the chromatogram was monitored at λ 250 nm. Semi-preparative HPLC were performed on a Dionex apparatus equipped with an ASI-100 autosampler, an Ultimate 3000 pump, a diode array detector UVD 340S and Chromeleon software. RP18 column (Phenomenex 250 x 15 mm, Luna 5µ) was used for semi-prep HPLC with binary gradient eluent (H_2O (pH 2.4 with TFA); CH_3CN) and a flow rate of 5 mL/min; the chromatogram was monitored at 205, 210, 250, and 360 nm. High Performance Flash chromatography was performed on a Grace Reveleris system equipped with dual UV and ELSD detection using Grace® cartridges (Silica gel or RP-C_{18}) and a flow rate of 30 mL/min. The chromatograms were monitored at 205, 225, 250, and 360 nm. Biological assays were read on a Fluostar Omega microplate reader (BMG Labtech).

3.2. Chemicals and drugs

Butylatedhydroxylanisole (BHA), butylatedhydroxyltoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH$^•$), 2,2′-azinobis(3-ethyl-benzothiazoline-6-suphonic acid) diammonium salt (ABTS$^{•+}$), *β*-carotene-linoleic acid and kojic acid were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Steinheim, Germany). All other chemicals and solvents used were of analytical grade.

3.3. Plant material

The aerial parts of *Genista numidica* Spach were collected in the flowering season at Taza national park- Jijel (North Eastern Algerian) in May 2012. The plant material was authenticated by Pr. Gerard De Bélair (University of Annaba, Algeria). A voucher specimen (ZKGn05/12) was deposited in the Herbarium of Biology Department, (Mentouri University, Constantine).

3.4. Extraction and isolation

The powdered dried aerial parts (500 g) of *G. numidica* were extracted by $CHCl_3$ (10L). The solvent was evaporated and the powder dried, then macerated in MeOH 80% (3×5 L, 24h). After removal of the solvent, the residue (25 g) was subjected to *vacuum* liquid chromatography (VLC) over RP-18 eluted successively with

MeOH-H$_2$O (0:10, 3:7, 4:6, 6:4, 8:2 and 10:0), to give six fractions (VLC-I to VLC-VI, respectively). Fraction VLC-II (3.25 g) was purified by preparative HPLC (5-20% CH$_3$CN in 60 min) and fraction per min was collected (frs II-1 to II-60) to give compounds **21** (Rt 8 min, 40 mg), **22** (Rt 15 min, 65 mg), **19** (Rt 20 min, 6 mg), **17** (Rt 27 min, 22 mg), **18** (Rt 38 min, 11 mg) and **30** (Rt 10.2 min, 5 mg). Fractions II-26 to II-31 were combined and purified by semi-prep HPLC (10% CH$_3$CN) to afford **28** (Rt 3.9 min, 5 mg) and **20** (Rt 8.8 min, 15 mg). Fraction VLC-III (1.23 g) was purified by preparative HPLC (15% CH$_3$CN in 90 min) and fraction per min was collected (frs III-1 to III-90) to give compounds **29** (Rt 8.5 min, 7 mg), **15** (Rt 10 min, 7 mg), **10** (Rt 15 min, 10 mg), **16** (Rt 33 min, 14 mg), **13** (Rt 21 min, 12 mg), **12** (Rt 26 min, 18 mg), **14** (Rt 36 min, 5 mg), **27** (Rt 58 min, 72 mg) and **11** (Rt 74 min, 13 mg). Fractions III-72 to III-78 were pooled and purified by semi-prep HPLC (15-50% CH$_3$CN, in 20 min) to afford **26** (Rt 14.5 min, 5 mg), **25** (Rt 15.2 min, 15 mg), **24** (Rt 15.5 min, 15 mg) and **23** (Rt 17.3 min, 5 mg). Fraction VLC-IV (1.3 g) was subjected to High Performance Flash Chromatography (HPFC), over RP-C$_{18}$ (80 g cartridge), using a binary gradient of CH$_3$CN–H$_2$O (10-80% CH$_3$CN in 30 min) to yield 120 fractions (fr per 15 seconds; IV-1 to IV-120). Fractions IV-64 to IV-120 were combined (796 mg) and purified by semi-preparative HPLC (25-45% CH$_3$CN, in 20 min) to furnish compounds **5** (Rt 12.6 min, 10 mg), **4** (Rt 13.3 min, 15 mg). Fraction VLC-V (900 mg) was fractionated by HPFC on silica gel (12 g cartridge) using a binary gradient of CHCl$_3$–MeOH (0–35% MeOH), in 36 min to provide 140 fractions (fraction per 15 seconds, V-1 to V-140) and fractions V-108 to V-118 were pooled (125 mg) and purified by semi-preparative HPLC (40-60% CH$_3$CN in 25 min) to adduce the compounds **2** (Rt 12.5 min, 13 mg), **7** (Rt 14.9 min, 2 mg), **8** (Rt 15.5 min, 5 mg), **6** (Rt 16.9 min, 3.4 mg) and **9** (Rt 17.4 min, 2 mg). Fraction VLC-VI (780 mg) was fractionated by HPFC on silica gel (4 g cartridge) using a binary gradient of CHCl$_3$-MeOH (0-5% for 10 min and 5-35% MeOH for 20 min) to give 120 fractions (VI-1 to VI-120; fraction per 15 seconds). Fractions VI-60 to VI-71 were combined (33 mg) and purified by semi prep HPLC (40-60% CH$_3$CN, in 20 min to give

1 (Rt 16.2 min, 5 mg) and **3** (Rt 14.5 min, 4 mg).

3.4.1. 3-O-[α-L-rhamnopyranosyl-(1→2)-{β-D-glucopyranosyl-(1→6)-}β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-sophoradiol (1)

Amorphous powder; $[\alpha]_D^{20}$ -9 (*c* 0.25 MeOH); ^{1}H-NMR (500 MHz, CD$_3$OD) and ^{13}C-NMR (125 MHz, CD$_3$OD), see table S1; HRESIMS: *m/z* 1111.5658 [M+Na]$^+$(calcd for 1111.5665, C$_{54}$H$_{88}$O$_{22}$Na).

3.4.2. Acid Hydrolysis.

A part of the saponin mixture (Fraction VLC-VI) (100 mg) was refluxed with 10 mL of 2 N HCl for 4 h. After extraction with EtOAc (3×7 mL), the aqueous layer was neutralized to pH 6 with 50 mM KOH and freeze-dried to provide the monosaccharide residue. Four sugars were identified and compared with authentic samples by TLC using MeCOEt-iso-PrOH-Me$_2$CO-H$_2$O (20:10:7:6) as glucuronic acid, glucose, galactose and rhamnose. The sugar mixture (50 mg) was dissolved in pyridine (1 mL) and subjected to a preparative TLC using the same solvent. The optical rotation of each purified sugar was measured to reveal D-glucose, D-galactose, D-glucuronic acid and L-rhamnose.

3.5. Antibacterial assay

3.5.1. Evaluation of the antibacterial activity of the isolated compounds against *Staphylococcus aureus* by bioautography

To identify the most promising compounds in terms of antimicrobial activity, an immersion bioautography method was adopted (Abedini et al., 2016; Benmerache et al.,2017). Compounds **1-30** were solubilized in methanol to obtain a solution of 2 mg/mL and 25 µL of each solution was spotted onto Merck 60 F$_{254}$ pre-coated silica gel plates (10 × 10 cm). Methanol and Gentamicin (50 µg) were also spotted on the plates as negative and positive control, respectively. The TLC plates were directly dried without migration and sterilized. The plates were then covered by Mueller-Hinton (MH) agar medium containing a *Staphylococcus aureus* 53.154 suspension (10^5 bacteria/mL) in square Petri dishes. After incubation for 24 h at 37°C, bacterial growth was revealed by a 2 mg/mL solution of thiazolyl blue tetrazolium bromide (MTT) and growth inhibition zones were measured. White stains indicated where

reduction of MTT to the colored formazan did not take place due to the presence of extracts that inhibited bacterial growth.

3.5.2. MIC determination of the most active compounds against *Staphylococcus aureus* by broth microdilution.

A serial dilution technique using 96-well microliter plates was used to determine the MIC values of the most promising compounds as revealed by bioautography (Lehbili et al., 2017). For this purpose, nine concentrations of the most active fractions, from 500 μg/mL to 2 μg/mL, were tested. Two wells were represented as bacteria culture control (positive control) and medium sterility control (negative control). Then the wells were loaded with MH liquid medium and bacterial suspension (10^5 bacteria/mL) giving a final volume of 200 μL. The plates were incubated overnight at 37°C, sprayed with a 0.2 mg/mL MTT solution and incubated again at 37°C for 30 min. Bacterial growth was indicated by a violet color whatever the color intensity, while bacterial growth inhibition was admitted only for wells which remained clear. MIC values were determined as the lowest concentrations of samples having an inhibitory effect on bacteria growth (clear wells).

3.6. Antioxidant activities

3.6.1. DPPH radical scavenging activity
The antioxidant activity of our target compound was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH method (Alabdul Magid et al., 2017). Briefly, 5 μL of different concentrations of the samples (dissolved in DMSO) were added to 95 μL of DPPH solution (158 μM, dissolved in EtOH 50%). The reaction proceeded for 30 min at 37 °C on a 96-well microplate. The absorbance was then read at λ 515 nm. The percentage of inhibition was calculated using the following equation: % inhibition [($Ab_{control}$ - Ab_{sample})/$Ab_{control}$] × 100. DPPH solution in EtOH 50% was used as a control. The curve of the % scavenging activity against the concentration of sample was prepared by MSExcel based program to obtain the IC_{50} (concentration required to obtain a 50% antioxidant effect). All the tests were conducted

in triplicate. BHA, BHT and α-tocopherol were used as positive controls.

3.6.2. β-Carotene bleaching test
β-carotene bleaching assay was done according to the method of (Dapkevicius et al., 1998). BHA and BHT were used as positive controls. The IC_{50} was calculated from the graph of the % antioxidant activity against the sample concentrations. All the tests were conducted in triplicate.

3.6.3. ABTS radical cation scavenging activity
This assay was performed according to the procedure described by (Re et al., 1999). The $ABTS^{\bullet+}$ was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Before usage, the $ABTS^{\bullet+}$ solution was diluted to get an absorbance of 0.703 ± 0.020 at 734 nm with methanol. Methanol was used as negative control, while BHA and BHT were used as positive controls. All the tests were conducted in triplicate and the IC_{50} were calculated from the graph of the $ABTS^{\bullet+}$ scavenging effect percentage against the sample concentration.

3.6.4. Cupric ion reducing (CUPRAC) capacity
The cupric reducing antioxidant capacity was determined according to the method of (Apak et al., 2004). The reducing capacity of the samples was compared with those of BHT and BHA. The results were given as $A_{0.50}$, which corresponds to the concentration producing 0.500 absorbance. The sample concentration producing 0.500 absorbance ($A_{0.50}$) was calculated from the graph of the absorbance of the cupric reducing antioxidant capacity against the sample concentration. All the tests were conducted in triplicate.

3.7. Tyrosinase enzyme assay
Tyrosinase activity inhibition was determined spectrophotometrically according to the method described previously (Tadrent et al., 2017). Different concentrations of test compounds were prepared in 10% DMSO in aqueous solution and 100 μL of each concentration were added to 96-well plate and then 100 μL of 135 U/mL mushroom tyrosinase in phosphate buffer solution (PBS, pH 6.8) were added. After pre-incubation at 25 °C for 10 min, 100 μL of L-dopa (0.5 mM, PBS pH 6.8) were added into 96-well plate. The reaction mixture was incubated for another 5 min at 25 °C. The

amount of dopachrome in the mixture was determined by the measurement of the absorbance of each well at 475 nm. Kojic acid was used as positive control agent. The inhibitory percentage of tyrosinase was calculated according to the following equation: % inhibition = $\{[(A - B) - (C - D)]/(A - B)\} \times$ 100 (*A*: Ab at 475 nm without test substance; *B*: Ab at 475 nm without test substance and tyrosinase; *C*: Ab at 475 nm with test substance; *D*: Ab at 475 nm with test substance, but without tyrosinase). All the tests were conducted in triplicate and IC_{50} was determined by interpolation of concentration % inhibition curve obtained by MSExcel based program.

Supporting Information

ESI-MS and NMR data for compound **1**, experimental section and biological activities.

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