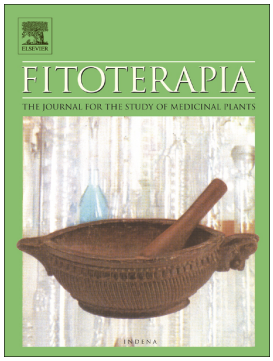

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A new cineol derivative, polyphenols and norterpenoids from Saharan myrtle tea (*Myrtus nivellei*): Isolation, structure determination, quantitative determination and antioxidant activity

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Abstract

The phytochemical profile of decoction and infusion, obtained from the dried leaves of *M. nivellei*, cosumed as tea in Saharan region, was characterized by UHPLC-PDA-HRMS. Fourteen compounds were characterized and, to confirm the proposed structures a preparative procedure followed by NMR spectroscopy was applied. Compound **3 (**2-hydroxy-1,8-cineole disaccharide) was a never reported whereas a byciclic monoterpenoid glucoside (**2**), two ionol glucosides (**1** and **12**), a tri-galloylquinic acid (**4**), two flavonol glycosides (**5** and **9**), and a tetra-galloylglucose (**7**), were reported in *Myrtus spp* for the first time. Five flavonol *O*-glycosides (**6**, **8**, **10**-**11,** and **14**) togheter a flavonol (**13**) were also identified. Quantitative determination of phenolic constituents from decoction and infusion has been performed by HPLC-UV-PDA. The phenolic content was found to be 150.5 and 102.6 mg/g in decoction and infusion corresponding to 73.8 and 23.6 mg/100 mL of a single tea cup, respectively. Myricetin 3-*O*-β-D-(6”-galloyl)glucopyranoside (**5**), isomyricitrin (**6**) and myricitrin (**8**) were the compounds present in the highest concentration. The free-radical scavenging activities of teas and isolated compounds was measured by the DPPH assay and compared with the values of other commonly used herbal teas (green and black teas). Decoction displayed higher potency in scavenging free-radicals than the infusion and green and black teas.

Keywords: *Myrtus nivellei*; NMR; UHPLC-PDA-ESI-HRMS; 2-Hydroxy-1,8-cineole disaccharide; myricetin glycoside; Free-radical scavenging activity.

1. Introduction

Myrtus nivellei Batt & Trab. (Mirtaceae), known as Saharan myrtle [1], called "Tefeltest" in Touareg language and "Raihane Essahara El Wousta" in Arabic, is used by local communities as a beverage, and for medicinal and food purposes [2]. Recently, the chemical components and antifungal activity of Saharian myrtle essential oil has been reported [1-3]. Furthermore, the antioxidant and antinflammatory properties of polar extracts have been described [4]. According to ethnobotanical investigation, the crushed *M. nivellei* leaves, added to oil or butter ointment (poultice), are used in the central Sahara traditional medicine for the treatment of dermatosis and for hair care [2]. The decoction of a handful of leaves, mixed with goat milk and heated on charcoal, is used for liver problems by nomad population of the Algerian tassili region [5]. Moreover, the leaf infusion is used locally as a common beverage, instead of green tea, and it is also consumed in traditional medicine as an anti-inflammatory and against diarrhea, diabetes, fever [2], and blennorrhea [1]. Nowadays, it is well established that the consumption of polyphenol-rich foods and beverages, such as herbal teas, may play a meaningful role in reducing risk of various diseases, which are linked with increased oxidative stress [6-7-8-9]. Nevertheless, the information about chemical composition and biological properties of Saharan myrtle teas is very limited. For this reason, the aim of this study was to assess the phenolic profile of *M. nivellei* teas (decoction and infusion) by a rapid and sensitive UHPLC-PDA-ESI-HRMS method. The proposed structures of the major constituents were confirmed by nuclear magnetic resonance (NMR) spectroscopy after isolation procedure. 1,8-Cineole glycosides (**2** and **3**), ionol glucosides (**1** and **12**), a tri-galloylquinic acid (**4**), flavonol glycosides (**5** and **9**), and a

tetra-galloylglucose (**7**), never reported in the *Myrtus nivellei* together with six constituents (**6**, **8**, **10-11**, and **13-14**) commonly present in the genus were identified.

In order to evaluate the contribution of myrtle teas to the total dietary polyphenols intake, the decoction and infusion TPC (Total Phenolic Content) was determined by the Folin-Ciocalteu and UHPLC-PDA methods. Finally, the *in vitro* free radical scavenging effects of both aqueous extracts and their major isolated phenols were evaluated by DPPH-test, in comparison with other widely consumed teas, such as green and black tea, well known as a source of compounds with putative health benefit [6-9-10].

2. Materials and methods

2.1. Chemicals

Analytical grade *n*-butanol (n-BuOH), ethyl acetate, and methanol (MeOH) employed for extraction isolation procedures were obtained from Sigma-Aldrich (Milan, Lombardia, Italy). Deutered methanol (CD_3OD), Folin-Ciocalteu phenol reagent, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), α-tocopherol, citric acid, HPLC-grade methanol (MeOH), and HPLC-grade water were purchased from Sigma-Aldrich (Milan, Lombardia, Italy). Water, MeOH and Formic acid (HCOOH) employed for the electrospray ionization ESI-MS analysis were of HPLC supergradient quality (Romil Ltd., Cambridge, UK).

2.2. General experimental procedures

Optical rotations were determined on a model DIP-1000 polarimeter (Jasco, Easton, MD, USA) equipped with a sodium lamp (589 nm) and a 10 cm microcell. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^{1}H and 150.858 MHz for ^{13}C, using the WINXNMR software package, was used for NMR experiments in CD_3OD. Chemical shifts are expressed in δ ppm referring to the solvent peaks δ_H 3.31 and δ_C 49.05 for CD_3OD, with coupling constants, *J*, in Hertz. ^{1}H-^{1}H DQF-COSY, ^{1}H-^{13}C HSQC, and HMBC experiments were obtained using conventional pulse sequences [11-12]. Chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Thin-layer chromatography (TLC) analysis was performed with Macherey-agel precoated silica gel 60 F_{254} plates (Delchimica, Naples, Italy), and the spray reagent cerium sulfate (saturated solution in dilute H_2SO4) and UV (254 and 366 nm) were used for the spot visualization. Preparative TLC were performed with precoated silica gel 60 RP-18 F_{254} aluminium sheets (20x20 cm, Merck, Darmstadt, Germany). HPLC analyses were performed on a Platin Blue UHPLC system (KNAUER GmbH, Berlin, Germany) consisting of two Ultra High-Pressure Pumps, an autosampler, a column temperature manager and a photodiode array detector, coupled to a LTQ Orbitrap XL (Thermo Scientific, San Jose, CA, USA) equipped with a electrospray ionization (ESI) probe. The data were acquired and processed with Xcalibur 2.7 software from Thermo Scientific. Preparative HPLC separations were performed with a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a Rheodyne injector (100 μL loop), using μ-Bondapak C_{18} (300 x 7.8 mm i.d., 10 μm, Waters) or Luna C_8 (250 × 10.0 mm i.d., 10 μm, Phenomenex, Torrance, CA, USA) columns.

2.3. Plant material

Leaves of *M. nivellei* Batt & Trab were collected in March 2011 at Tamanrasset area, located in central Algerian Sahara. The plant was identified by Mrs Boutamine Rabia from the laboratory of ecology INRF (National Institute of Forest Research) of Tamanrasset (Alger). A voucher specimen (MN311) has been deposited in the INRF of Tamanrasset.

2.4. M. nivellei decoction and infusion preparation

For decoction, the dried chopped leaves (3 g) were added to 100 mL of distilled water (equivalent to handful of leaves in a cup of water), heated, kept in boiling water for 10 min. Then, the mixture was removed from the heat and left stend for 5 min.

Infusion was prepared by adding 100 mL of boiling distilled water to 3 g of dried chopped leaves and the mixture was left to stand for 10 min.

Then, both decoction and infusion were filtered through filter paper and freeze-dried. The yield of the lyophilized aqueous extracts were 490.0 mg (16.3 ± 2.4 % of dried leaves) and 230.0 mg (7.6 ± 1.1 % of dried leaves), respectively. The extractions were performed in triplicate.

2.5. UHPLC-PDA-ESI-HRMS analisys

UHPLC separation was achieved with a Kinetex C_{18} (50 x 2.1 mm i.d., 1.7 µm) column protected by a C_{18} Guard Cartridge (2.1 mm i.d. 1.7 µm), both from Phenomenex (Torrance, CA, USA) held at 30 °C. The mobile phase consisted of water (A) and MeOH (B), both containing 0.1% HCOOH. The following elution gradient was used: 0-3 min, 2% B; 3-5 min, 2-13%, B; 5-9 min, 13% B; 9-12 min, 13-18% B; 12-13min, 18% B; 13-20 min, 18-30% B; 20-25 min, 30-50% B; 25-27 min, 50-98% B. After each injection, the column was washed with 100% B for 4 min and re-equilibrated (4 min). A flow rate of 0.6 mL/min and an injection volume of 5 µL (partial loop injection mode) were used.

Detection by photodiode array was performed at three wavelengths: 254, 280 and 360 nm and the UV spectra were recorded over a 200-600 nm range. The HRMS and HRMS/MS were performed with an ESI source in the negative and positive ion mode. High-purity nitrogen (N_2) was used as both drying gas and nebulizing gas, and ultra-high pure helium (He) as the collision gas. The operating parameters were optimized as follows: source voltage 4 kV, capillary voltage –33 V, tube lens voltage –41.4 V, capillary temperature 280 ºC, sheath and auxiliary gas flow (N_2) 30 e 10 (arbitrary units), respectively. The MS profile was recorded in full scan mode (scan time = 1 micro scans and maximum inject time 500 ms) with resolving power of 60000. For the HRMS/MS acquisitions, a data-dependent method, setting the normalized collision energy in the ion trap of 35%, was used.

2.6. Extraction and isolation procedure

Air-dried and coarsely powdered leaves (100g) from *M. nivellei* were macerated in MeOH-H_2O (80:20, v/v) at room temperature for 24 h and the operation was repeated 3 times. The combined hydroalcoholic solutions were concentrated in vacuum to give 14.0 g of solid residue. The residue was diluted with distilled water (40 mL) and successively extracted with ethyl acetate and *n*-BuOH to obtain 540.0 mg and 1.1 g of dried extracts, respectively. Scheme of extraction procedure is given as Supplementary material.

2.6.1. Purification of n-BuOH soluble fraction

A portion of this extract (1.0 g) was fractionated over a Sephadex LH-20 column (50 cm ×3.0 cm) using MeOH as eluent (flow rate 0.5 ml/min). Fractions (8 ml each) were collected, analyzed by TLC (Si-gel), n-BuOH–AcOH–H_2O (60:15:25), $CHCl_3$–MeOH–H_2O (7:3:0.3) and combined to five major fractions (I-V) based on TLC pattern. All fractions were further purified by RP-HPLC. Fractions I, and III were purified on C_8 column (flow rate 2.0 mL/min) with the elution solvent MeOH/H_2O 4:6 v/v. Fraction I (119.1 mg) yielded compounds **1** (7.3 mg, t_R 12.0 min), **2** (2.2 mg, t_R 21.0 min), **3** (3.4 mg, t_R 15.0 min), and **12** (3.0 mg, t_R 37.0 min), while fraction III (228.4 mg) afforded compounds **6** (6.0 mg, t_R 14.0 min) and **8** (24.6 mg, t_R 21.0 min. Faction II (205.5 mg), separated on a C_{18} column with MeOH/H_2O, 3:7 v/v, as mobile phase (flow rate 2.0 mL/min), consisted of compound **1** (0.7 mg, t_R 15.0 min). Fractions IV and V were chromatographed on a C_{18} column (flow

rate 1.8 mL/min) using as solvent system MeOH/H_2O 4:6 v/v. Fraction IV (58.8 mg) consisted of compounds **9** (1.3 mg, t_R 25.0 min) and **10** (1.0 mg, t_R 29.0 min). Fraction V (63.3 mg) gave compound **5** (5.3 mg, t_R 17.0 min). Scheme of isolation procedure is given as Supplementary material.

2.6.2. Purification of ethylacetate soluble fraction

A portion of this extract (450.0 mg) was fractionated over a Sephadex LH-20 column (50×2.0 cm) using MeOH as eluent. Fractions (8 ml each) were collected, analyzed by TLC (Si-gel, n-BuOH–AcOH–H2O (60:15:25), $CHCl_3$–MeOH–H_2O (7:3:0.3) and combined to six major fractions (I-VI) based on TLC pattern. Fractions II-V were submitted to preparative TLC-RP using as solvent a mixture of MeOH/H_2O, 8:2. Fraction II gave compounds myricetin **8** (3.6 mg) and **14** (2.0 mg). Fraction III, IV and V consisted of compounds **4** (2.5 mg), **7** (1.2 mg), and **13** (1.7 mg), respectively. Scheme of isolation procedure is given as Supplementary material.

2.7. NMR Spectroscopic Data

(1S, 2S, 4R)-*trans*-2-hydroxy-1,8-cineole 2-*O*-α-L-arabinofuranosyl (1→6) β-D-glucopyranoside (**3**). Amorphous powder; $[\alpha]^{25}_D$ = + 2.6 (c = 0.1, MeOH); ^{1}H (CD_3OD, 600

MHz) and ^{13}C NMR (CD_3OD, 150.9 MHz) data see Table 2. HRESIMS *m/z* 463.2169 $[M-H]^-$ (calcd. $C_{21}H_{35}O_{11}$, 464.2180); for ESI-HRMS data see (Table 1).

Spectra obtained from proton and proton-carbon correlation experiments are given as Supplementary material.

NMR data of known compounds **1-2**, **4-10**, and **12-14** were consistent with those previously reported in the literature (see 3. Results and discussion). The ESI-HRMS data of all identified myrtle compounds are reported in (Table 1).

*2.8. Acid hydrolysis of compound (**3**)*

Compound **3** (0.8 mg), was heated at 60 °C with 1:1 0.5 N HCl-dioxane (3 mL) for 2 h, and then evaporated in vacuo. The solution was partitioned with CH_2Cl_2–H_2O, and the H_2O layer was neutralized with Amberlite MB-3. The upper aqueous layer containing monosaccharides was neutralized using an ion-exchange resin (Amberlite MB-3) column, and then lyophilized to give a sugar mixture. Sugar mixture was developed by TLC using as solvent system, MeCOEt-*iso*-PrOH–Me_2CO–H_2O (20:10:7:6). Monosaccharides were identified with authentic sugar samples. After preparative TLC of the sugar mixture, the optical rotation of each purified sugar was measured to afford arabinose (Rf 0.50; $[\alpha]^{20}_D$ +41) and glucose (Rf 0.45; $[\alpha]^{20}_D$ +21) [13].

2.9. UHPLC quantitative analysis

UHPLC equipment and condition were the same as used for qualitative analysis. The isomyricitrin (**6**) and myricitrin (**8**) were quantified using the calibration curves of the corresponding standards. Standard calibration curves were obtained in a concentration range of 5.0 – 200.0 µg/mL with six concentration leavels (5.0, 10.0, 25.0, 50.0, 100.0, and 200.0 µg/mL) and triplicate injections for each level. UV peak areas of the external standard (at each concentration) were plotted against the corresponding standard concentration (µg/mL) using weighed linear regression to generate standard curves. For the linear regression of external standards, for both calibration curves r^2 values was 0.9990. The extracts were injected at a concentration of 2.0 mg/mL, and 10 µL was injected for analysis. The amount of the compounds was finally expressed as milligrams per grams of extracts, as the mean of triplicate determinations.

2.10. Preparation of different types of commercial teas

Standard grade green and black teas (*Camellia sinensis*) were acquired in a local market. The aqueous extracts were obtained by pouring 100 mL of boiling distilled water on 3.0 g of dried loose leaves and steeping it for 10 min. The infusions were filtered through filter paper, and the resulting infusion was freeze-dried. The yields of the lyophilized aqueous extracts were 743.0, and 507.5 mg for green and black teas, respectively, corresponding to 24.8 ± 3.6 and 16.91 ± 2.5% of dried extracts, respectively. The extractions were performed in triplicate.

2.11. Determination of Total Phenols Content (Folin-Ciocalteu Method)

All aqueous preparations (from *M. nivellei* and commercial teas) were analyzed for their Total Phenolic Content according to the Folin-Ciocalteu colorimetric method [14]. Total phenols were expressed as gallic acid equivalents (mg/g dry extract, means ± S.D. of three determinations).

2.12. Bleaching of the free-radical 1,1-diphenyl-2-picrylhydrazyl (DPPH Test)

The antiradical activities of all aqueous extracts, and phenolic compounds (**4**-**10**, **13**-**14**) were determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), according to our procedures previously reported [15]. Briefly 1.5 mL of DPPH solution (25 mg/mL in methanol, prepared daily) was added to 3.7 μL of various concentrations of each sample under investigation in MeOH solution (ranged from 12 to 100 μg/mL). The mixtures were kept in the dark for 10 min at room temperature and the decrease in absorbance was measured at 517 nm against a blank consisting of an equal volume of methanol. α-tocopherol and L-ascorbic acid were used as positive controls. The DPPH concentration in the reaction medium was calculated from a calibration curve analyzed by linear regression, and SC_{50} (mean effective scavenging concentration) was determined by the Litchfield test as the concentration (in micrograms per milliliter) of sample necessary to decrease the initial DPPH concentration by 50%) [15]. All tests were performed in triplicate. A lower SC_{50} value indicates stronger antioxidant activity.

3. Results and discussion

The traditionally consumed decoction and infusion were prepared from *M. nivellei* leaves with the aim to investigate their phenolic profiles and biological activity.

3.1. UHPLC-PDA-ESI-HRMS analysis

UHPLC-ESI-HRMS analyses were performed in negative and positive ionization mode to obtain complementary information useful to characterize infusion and decoction. Negative ionization was selected for higher sensibility and selectivity of detection.

Fourteen (**1**-**14**) major peaks were detected, and their retention times, λ_{max} values and MS data are listed in (Table 1). Analysis of UV and MS spectra led to identification of two norterpenoids (ionol glycosides, **1** and **12**), two monoterpene glycosides (**2** and **3**), a tri galloyl quinic acid (**4**), two flavonol glycoside gallate derivatives (**5** and **9**), five flavonol O-glycosides (**6**, **8**, **10**, **11** and **14**), a tetra galloyl hexose (**7**) and a flavonol (**13**) (Figure 1).

Formic acid was added to the mobile phase not only to improve the chromatographic separation, but also to increase the ionization for several compounds, *e.g.*, monoterpene glycoside, generating adduct ion $[M\text{-}H+HCOOH]^-$. Indeed, all terpenoid compounds (**1-3**, **12**) showed formate adducts ($[M–H+HCOOH]^–$) as base peaks and their corresponding deprotonated molecular ions ($[M\text{-}H]^-$) were too weak to be observed.

Compounds **1** and **12** yielded a prominent $[M–H+HCOOH]^–$ ion at *m/z* 431.1915 and 415.1966 and their analysis by MS^2 fragmentation produced $[M\text{-}H]^-$ ions at *m/z* 385.1853

and 369.1904, respectively. These accurate mass data indicated $C_{19}H_{30}O_8$ and $C_{19}H_{30}O_7$ as molecular formulae of peaks **1** and **12**. The MS^3 spectra of $[M-H]^-$ ions showed the characteristic norterpenoid aglycone having 13 carbon atoms, with *m/z* 223.1324 (**1**) and 207.1375 (**12**), suggesting the presence of a hexose residue. According to MS analysis and literature data, the compounds **1** and **12** were tentatively identified as roseoside A and 3-oxo-ionol-9-*O*-glucoside [16], respectively. Likewise, compounds **2** and **3** produced adduct ions at *m/z* 377.1809 and 509.2232 $[M-H+HCOOH]^-$ in MS spectra and $[M-H]^-$ ions as base peaks at *m/z* 331.1747 and 463.2170 in MS^2 spectra, respectively. Molecular formulae of **2** and **3** were determined as $C_{16}H_{28}O_7$ and $C_{21}H_{36}O_{11}$. In MS^3 spectra these compounds yielded product ions of $[M\text{-aglycone-H-}H_2O]^-$ or fragment ions by eliminating part of glycosyl group. Compound **3** produced fragment ions at m/z 331.1744 $[M\text{-H-pentose-}H_2O]^-$ and 161.0441 $[\text{hexose-}H_2O\text{-H}]^-$, suggesting the presence of a pentose and hexose residue. While, the compound **2** showed only fragment ion at m/z 161.0440 $[\text{hexose-}H_2O\text{-H}]$. According to MS analysis and literature data these compounds were tentatively identified as 2-hydroxy-1,8-cineole-glucoside (**2**) [17] and 2-hydroxy-1,8-cineole-arabinosyl-glucoside (**3**) [18].

Compound **4** showed a $[M-H]^-$ ion at *m/z* 647.0883 with a molecular formula $C_{28}H_{24}O_{18}$. In MS^n spectra showed successive loss of three galloyl residues from quinic acid unit. It was tentatively identified as 3,4,5-tri-*O*-galloyl-quinic acid referring to the literature [19].

Compounds **5**, **6**, **8**, **9**, **10**, **11** and **14** showed characteristic UV spectra of 3-*O*-substituted flavonols with two maximum absorptions at 330-360 nm (band I) and 265-280 nm (band II) [20]. In addition, in the MS^2 spectra the abundance of the radical aglycone $(Y_0\text{-H})^{\bullet}$ was significantly higher than that of the aglycone product ion (Y_0^-). This confirms that in these

compounds the glycosydic residues were linked to phenolic hydroxyl in position 3 of the flavonol nucleus [21]. Compounds **5**, **6**, **8** and **10** produced $[M-H]^-$ ions at m/z 631.0924 ($C_{28}H_{24}O_{17}$), 479.0824 ($C_{21}H_{20}O_{13}$), 463.0867 ($C_{21}H_{20}O_{12}$) and 449,0718 ($C_{21}H_{18}O_{12}$), respectively, and similar MS^2 fragmentations, showing the same base peak with m/z 316.0218 $(Y_0-H)^{-\bullet}$ corresponding to the radical aglycon myricetin, and differing only for linked glycosidic units except for compound **5**. Compounds **6**, **8** and **10** show the loss of 162 Da (hexose), 146 Da (deoxyhexose) and 132 Da (pentose), respectively.

Compounds **9**, **11** and **14** produced $[M-H]^-$ ions at m/z 615.0982 ($C_{28}H_{24}O_{16}$), 463.0867 ($C_{21}H_{20}O_{12}$) and 447.0926 ($C_{21}H_{20}O_{11}$) and in MS^2 spectra showed a base peak at *m/z* 300.0269 $(Y_0-H)^-$ corresponding to the radical aglycon quercetin. Furthermore, compounds **11** and **14** showed the characteristic losses of hexose and deoxyhexose residues, respectively. Compound **5** and **9** showed in MS^2 spectra additional losses of 152 Da (m/z at 479.0827 and 463.0863, respectively) corresponding to the presence of a galloyl residue.

Furthermore, the aglycones myricetin and quercetin were confirmed by MS^3 spectra, they produced fragment ions at *m/z* 151.0022 ($^{1,2}A^-$–CO) and 178.9970 ($[^{1,2}A^-$–H$]^-$) via RDA (retro Diels Alder) reaction [22]. MS^3 data were very similar to those from the fragmentation of a standard solution of myricetin and quercetin. Thus, according to UV and MS analysis and literature data the compounds **5**, **6**, **8** and **10** could be tentatively identified as myricetin 3-*O*-galloyl glucoside, myricetin 3-*O*-glucoside (isomyricitrin), myricetin 3-*O*-rhamnoside (myricitrin) and myricetin 3-*O*-xyloside, respectively [23-24-25]. While, the structure of quercetin 3-*O*-galloyl glucoside, quercetin 3-*O*-glucoside (isoquercitrin) and quercetin 3-*O*-rhamnoside (quercitrin) were proposed for compounds **9**, **11** and **14**, respectively [24-25].

Compound **7** showed a $[M-H]^-$ ion at *m/z* 787.0985 with a molecular formula $C_{34}H_{27}O_{22}$. It was identified as a tetra galloyl hexose, insofar as the MS^n spectra show successive neutral losses of gallic acids (170 Da) and galloyl radicals (152 Da). However, the MS spectra information are insufficient to distinguish the link position between galloyl groups and glycosyl unit. For this reason, the compound **7** was tentatively identified as 1,2,3,6-tetra-*O*-galloyl glucose referring to the literature [26]. Compound **13** was ascertained to be myricetin by reference standard.

*3.2. Isolation and structure determination of compounds **1-14***

To confirm the chemical structures proposed by analytical investigation, isolation procedures of a hydroalcoholic extract from Saharan myrtle leaves were undertaken (see 2. Materials and methods) to yield pure compounds **1-10** and **12-14** (Figure 2). The selection of a methanol-water mixture as solvent system, followed by *n*-BuOH and ethyl acetate, had the aim to obtain extracts richer in polyphenolic compounds than the aqueous preparations, and suitable for the isolation procedure. The adapted purification procedure did not the isolation of compound **11**, probably for its low content in the extracts.

The structure elucidation of **3** proceeded as follows. The molecular formula of compound **3** was determined to be $C_{21}H_{36}O_{11}$ by HRESIMS and HRMS/MS analyses (Table 1). The ^1H and ^{13}C NMR spectra (Table 2) indicated compound **3** to be a glycosilated monoterpenoid with bicyclic structure [27-17]. The ^1H NMR spectrum displayed signals for three methyl groups at δ_H 1.16, 1.21, and 1.28, in the aliphatic region, one oxygenated methine at δ_H 3.64 and one methine at δ_H 1.52. In the ^{13}C NMR spectrum the 10 signals for the aglycone were ascribable to three methyls (δ_C 24.7-29.0), three methylenes (δ_C 22.7-34.8), one methyne (δ_C

35.6), one oxymethine (δ_C 81.3), and two oxygenated quaternary carbons (δ_C 74.0 and 75.3). Protons sequences were established by DQF-COSY spectrum starting from the oxymethine signal at δ_H 3.624 (H-2) coupled with methylene signal at δ_H 1.68 (H-3a) and 2.56 (H-3b) which was further coupled with methyne signal at δ_H 1.52 attached to a quaternary carbon (C-8). A second proton sequence was established starting from the methylene signal at δ_H 1.58 (H-5a), and 1.98, (H-5b) which was coupled with another methylene signal at δ_H 1.52 (H-6a) and 2.00 (H-6b). NOE correlation observed between Me-9 and H-2 indicated that the oxygen of C-2 was bound in the *trans* position relative to the oxide bridge between C-1 and C-8. Thus, based on the 2D NMR data of DQF-COSY, HSQC, and HMBC experiments the monoterpenoid moiety of compound **3** was identified as *trans*-2-hydroxy-1,8-cineole [28-27] glycosylated at C-2 (δ_C 81.3) [17]. Considering the optical rotations and the very similar NMR data, **3** was assigned with the same absolute configuration as that of (1S, 2S, 4R)-*trans*-2-hydroxy-1,8-cineole [17].

For the sugar moiety, the ^{1}H NMR spectrum of compound **3** showed the presence of two anomeric signals at δ_H 4.32 (H-1', d, J = 7.5 Hz) and 5.03 (H-1", d, J = 2.4 Hz). The 1D TOCSY and DQF-COSY experiments allowed the assignments of all protons resonances to β-glucopyranosyl [29] and α-arabinofuranosyl [30] units in **3.** The assignments of the corresponding carbons, using the HSQC spectrum, indicated that the arabinofuranosyl is the terminal unit and glucopyranosyl is substituted at C-6. The relative positions of the glucopyranosyl and arabinofuranosyl units were established unambiguously by HMBC correlations observed between the anomeric proton signal at δ_H 4.32 (H-1′, glucopyranosyl) and C-2 (δ_C 81.3) of the aglycone and between the anomeric proton signal at δ_H 5.03 (H-1″,

arabinofuranosyl unit) and C-6′ (δ_C 68.1) of the glucopyranosyl unit. The configurations of the sugar units were determined as D-glucose and L-arabinose, after hydrolysis of **3** and confirmation by the optical rotation data of each isolated sugar. Therefore, the structure of **3** was determined as (1S, 2S, 4R)-*trans*-2-hydroxy-1,8-cineole 2-*O*-α-L-arabinofuranosyl (1→6)-β-*D*-glucopyranoside.

To the best of our knowledge, this is the first report on the isolation and structural elucidation by HRESIMS and NMR spectroscopy of compound **3**, previously reported without spectroscopic data in *Laurus nobilis* [18].

All isolated pure compounds were analyzed by 1D- and 2DNMR experiments, confirming the proposed structure of roseoside A (**1**) [12], (1S, 2S, 4R)-*trans*-2-hydroxy-1-8-cineole β-D-glucopyranoside (**2**) [17], 3,4,5 tri-*O*-galloyl quinic acid (**4**) [31], myricetin 3-*O*-β-D-(6”-galloyl)glucopyranoside (**5**), isomyricitrin (**6**) [32], 1,2,3,6-tetra-O-galloyl-D-glucopyranose (**7**) [33], myricitrin (**8**) [32], quercetin 3-*O*-β-D-(6”-galloyl)glucopyranoside (**9**) [30], myricetin 3-*O*-xyloside (**10**) [34], 3-oxo-α-ionol-9-*O*-β-D-glucopyranoside (**12**) [12], myricetin (**13**) [34], and quercitrin (**14**) [35].]. The phenolic compounds **6**, **8**, **10**-**11**, **13**-**14** have been reported as characteristic constituents of *M. communis* [23, 25]. In contrast, compounds **1-5, 7, 9** and **12,** never identified in this species, have been found in *M. nivellei*. Thus, their may be considered as markers for it. According to that reported in literature [1], the chemical composition of *M. nivellei*, found in Central Sahara, is very different from that of *M. communis* growing wild all around the Mediterranea basin.

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3.3. Total Phenolic Content (TPC) and HPLC analysis

To evaluate the share of Saharan myrtle tea to the dietary polyphenols ingestion, the TPC was determined by the Folin-Ciocalteu method [29], and expressed as gallic acid equivalents. The TPC in decoction and infusion was 150.5 and 102.6 mg/g (Table 3) corresponding to 73.8 and 23.6 mg/100 mL of a single tea cup, respectively.

Additionally, the quantitative analysis of phenolic compounds from *M. nivellei* tea was also performed by HPLC-PDA using calibration curves of corresponding standard. Myricetin 3-*O*-β-D-(6”-galloyl)glucopyranoside (**5**), isomyricitrin (**6**) and myricitrin (**8**), were found to be the main components of both aqueous dried extracts. Instead, the other compounds (**7, 9-10, 13-14**) were undetectable by our analytical methods, suggesting that in decoction and infusion, prepared according to folk information, the concentration of these compounds probably is low (Figure 1). The amounts of **5**, **6** and **8** were comparable in both aqueous dried extracts, slightly higher in decoction (7.9, 26.2 and 48.1 mg/g, respectively) than infusion (6.0, 20.5 and 38.4 mg/g, respectively), corresponding to 40.8 and 14.9 mg/100 mL of myricetin derivatives, respectively.

These results suggested that polyphenols intake, offered by drinking one cup of *M. nivellei* tea, obtained according to folk information, seems to be very remarkable considering their numerous biological and pharmacological effects [32-36]. The consumption of these compounds is associated with a proportional decline in the relative risk of coronary heart disease, stroke, and cancer mainly due to their antioxidant properties and it seems to exert a vasodilator effect as well as improving immune response of our body

[36]. Particurarly, myricetin derivatives possess antioxidant, antinflammatory, anti-thrombotic [32], antihyperglicemic [37] and antimutagenic [25] properties.

3.4. Free radical scavenging activity (DPPH assay)

The free radical scavenging activity of decoction and infusion was evaluated and compared with the values of commonly used herbal teas (green and black) by the DPPH assay [15]. In addition, the TPC in green and black teas was also measured by Folin-Ciocalteu method, to enable a comparison between the measured values of antioxidant activity with the content of the samples in polyphenols , according to the literature [38]. In fact, both parameters have been regarded as a quality of the tea [39].

Decoction exhibited a strong concentration-dependent free radical scavenging activity in the DPPH test (SC_{50}= 10.21 μg/mL) higher than green and black teas and similar to that of well-known antioxidant α-tocopherol (SC_{50}= 10.10 μg/mL), used as positive control. Infusion showed less potent activity in the DPPH test (SC_{50}= 18.60 μg/ml), correlated with a lower TPC value with respect to decoction (Table 3). Nevertheless, it elicited radical scavenging capacity and a TPC comparable to black and green teas obtained with the same procedure (Table 3). The relationship between total phenolic content and antioxidant activity of myrtle teas was calculated. A positive linear correlation between TPC and antioxidant activity values (r^2 = 0.9933) suggested that the antioxidant activity was essentially due to phenolic compounds present in the teas.

In order to identify the compounds responsible for the observed activities of decoction and infusion of Saharan myrtle leaves, the free-radical scavenging activity of main isolated

phenolic compounds (**4-10**, **13-14**) was evaluated and the results summarized in (Table 3). All compounds under investigation exhibited higher potency in scavenging DPPH than the positive control (α-tocopherol). Among them, myricetin (**13**) together 3,4,5 tri-O-galloyl quinic acid (**4**), and 1,2,3,6 tetra-O-galloyl-D-glucopyranose (**7**) have shown a strong free-radical scavenging activity comparable to ascorbic acid, an important and powerful natural water-soluble antioxidant, used as a reference compound. In fact, flavonol and hydrolyzable tannins with more adjacent OH groups (galloyl, pyrogalloyl, catechol groups) have higher radical scavenging activities on DPPH [40]. The glycosidation at C3 of C-ring of flavonols decreased antioxidant activity [35], as observed with compounds **5**, **6**, **8**, and **10** (all myricetin glycosides deivatives) (Table 3) as well as the lack of hydroxyl group in the B-ring, as demonstrated by SC_{50} values (Table 3) of compounds **9** and **14** (quercetin derivatives). All these considerations were in agreement with the literature data [30-35-40]. Thus, the free-radical scavenging properties of *M. nivellei* teas seem to be correlated to the structures of the isolated phenolic compounds, which are flavonoids, their glycosides, and polygalloyl derivatives.

4. Conclusions

This research provided evidence about the chemical composition of decoction and infusion from *M. nivellei* leaves showing that monoterpenoid glycosides (**2-3**), ionol derivatives (**1**, **12**) and polyphenols compounds (**4-11, 13-14**) are the main constituents. Some of them (**1-4**, **8**, **12-13**) are found for the first time in the genus *Myrtus*. Furthermore, decoction and infusion showed a powerful ability to scavenge free radicals, comparable or

higher, in the case of decoction, to that of commercial teas and related to their polyphenols content. These preliminary results suggested that a daily intake of the aqueous preparation from Saharan myrtle leaves may improve the natural endogenous antioxidant defense system, reducing the risk of chronic and degenerative ailments associated with oxidative stress.

Conflict of interest statement

All authors declare no conflict of interests

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Table 1. Retention Times and UV and ESI-HRMS data of compounds **1−14** of leaves infusion and decoction from *M. nivellei*.

No.	RtUV (min)	t_R MS(min)	[M-H]⁻ (m/z) Measured	Error (ppm)	Formula	UV (λ_{max}nm)	Fragment ions (m/z)	Compounds
1	7.58	7.62	385.1853	-1.024	$C_{19}H_{30}O_8$	-	223.1324 $[C_{13}H_{19}O_3]^-$	roseosideA[a]
2	8.88	8.91	331.1747	-1.297	$C_{16}H_{28}O_7$	-	331.1744 $[C_{16}H_{27}O_7]^-$; 161.0441 $[C_6H_9O_5]^-$	2-hydroxy-1-8-cineole β-D-glucopyranoside[a]
3	9.39	9.41	463.2169	-1.054	$C_{21}H_{36}O_{11}$	-	161.0440 $[C_6H_9O_5]^-$	2-hydroxy-1,8-cineole 2-*O*-α-L-arabinofuranosyl(1→6)-β-*D*-glucopyranoside[a]
4	10.9	10.93	647.0883	0.633	$C_{28}H_{24}O_{18}$	279	495.076 $[C_{21}H_{19}O_{14}]^-$; 343.0650 $[C_{14}H_{15}O_{10}]^-$; 191.0544 $[C_7H_{11}O_6]^-$	3,4,5-tri-*O*-galloyl-quinic acid[a]
5	13.51	13.54	631.0925	-0.753	$C_{28}H_{24}O_{17}$	266, 355	479.0827 $[C_{21}H_{19}O_{13}]^-$; 316.0219 $[C_{15}$ H8 $O_8]^-$; 317.0288 $[C_{15}H_9O_8]^-$	myricetin 3-*O*-β-D (6"galloyl)glucopyranoside[a]
6	14.51	14.54	479.0824	0.800	$C_{21}H_{20}O_{13}$	265, 360	316.0219 $[C_{15}H_8O_8]^-$; 317.0289 $[C_{15}$ H_9 $O_8]^-$	isomyricitrin[a]
7	16.08	16.11	787.0981	-0.951	$C_{34}H_{28}O_{22}$	279	635.0872 $[C_{27}H_{23}O_{18}]^-$; 483.0759 $[C_{20}H_{19}O_{14}]^-$; 331.0659 $[C_{13}H_{15}O_{10}]^-$; 161.0440 $[C_6H_9O_5]^-$	1,2,3,6-tetra-*O*-galloyl glucose
8	16.48	16.52	463.0867	-0.869	$C_{21}H_{20}O_{12}$	265, 360	316.0219 $[C_{15}H_8O8]^-$; 317.0288 $[C_{15}H_9O_8]^-$	myricitrin[a]
9	16.57	16.61	615.0985	0.714	$C_{28}H_{24}O_{16}$	265, 360	463.0863 $[C_{21}H_{19}O_{12}]^-$; 300.0269 $[C_{15}H_8O_7]^-$; 301.0346 $[C_{15}H_9O7]^-$	quercetin 3-*O*-β-D-(6''-galloyl)glucopyranoside[a]
10	16.70	16.74	449.0718	0.774	$C_{21}H_{18}O_{12}$	265, 360	316.0219 $[C_{15}H_8O_8]^-$; 317.0288 $[C_{15}H_9O_8]^-$	myricetin 3-*O*-β-xylosidea
11	17.42	17.46	463.0866	-1.085	$C_{21}H_{20}O_{12}$	265, 360	300.0270 $[C_{15}H_8O_7]^-$; 301.0347 $[C_{15}H_9O7]^-$	Isoquercitrin
12	18.11	18.15	369.1904	-1.028	$C_{19}H_{30}O_7$	-	207.1375 $[C_{13}H_{19}O_2]^-$	3-Oxo-α-ionol-9-*O*-β-D-glucopyranoside[a]
13	18.76	18.8	317.0290	-0.611	$C_{15}H_{10}O_8$	255, 376	151.022 $[C_7H_3O_4]^-$; 178.997 $[C_8H_3O_5]^-$	Myricetin[a]
14	20.21	20.25	447.0926	0.922	$C_{21}H_{20}O_{11}$		300.0270 $[C_{15}H_8O_7]^-$; 301.0347 $[C_{15}H_9O7]^-$	quercitrin[a]

[a]Compounds were confirmed by NMR

Table 2. ^{13}C and ^{1}H NMR Spectroscopic data of compound **3** in CD_3OD[a].

Aglycone			Sugar		
position	δ_C	δ_H (*J* in Hz)[b]	position	δ_C	δ_H (*J* in Hz)[b]
1	74.0	-	[c]Glc-1’	106.3	4.32 (d, 7.5)
2	81.3	3.64, m	Glc-2’	75.5	3.18 (d, 9.0, 7.5)
3	34.8	1.68, m	Glc-3’	77.9	3.33 (t, 9.0)
		2.56, m			
4	35.6	1.52, m	Glc-4’	71.9	3,24 (t, 9.0)
5	22.7	1.58, m	Glc-5’	77.0	3.46, m
		1.98, m			
6	26.8	1.52, m	Glc-6’	68.1	3.65 (dd, 12.0, 4.5)
		2.00, m			4.02 (dd, 12.0, 4.5)
7	24.7	1.16, s	[d]Araf-1’’	110.0	5.03 (d, 2.4)
8	75.3	-	Araf-2’’	83.3	4.00, m
9	29.0	1.21, s	Araf-3’’	78.5	3.83. m
10	29.0	1.28, s	Araf-4’’	86.1	4.00, m
			Araf-5’’	63.0	3.66 (dd, 11.6, 2.0)
					3.75, m

[a]Assignments were made by 1D TOCSY, 1H-1H COSY, HSQC, HMBC, and ROESY data. [b]1H-1H coupling constants were measured from COSY spectra in Hz.[c]β-D-glucopyranoside. [d]α-L-arabinofuranosyde.

Table 3. Total Phenol Content (TPC), and free-radical scavenging activity (DPPH test) of different aqueous dried extracts and compounds **4-10** and **13-14**.

Aqueous extracts and compounds	Phenol content (mg/g extract)[a]	DPPH test SC_{50} (μg/mL)
decoction	150.5 ± 7.5[b]	10.2 ± 0.4[b]
infusion	102.6 ± 3.7	18.6 ± 0.6
green tea	111.8 ± 1.6	18.0 ± 0.5
bleak tea	86.0 ± 3.2	22.9 ± 1.4
4		3.8 ± 1.0
5		5.6 ± 0.6
6		6.7 ± 1.7
7		4.0 ± 0.9
8		4.3 ± 0.9
9		8.8 ± 1.2
10		7.0 ± 0.7
13		3.5 ± 0.6
14		5.9 ± 0.9
α-tocopherol[d]		10.1 ± 1.3
ascorbic acid[d]		3.3 ± 0.8

[a]Gallic acid equivalents

[b]Mean ± SD of three determination by the Folin-Ciocalteu method

[c]EC_{50} ± standard deviation (data from three experiments in triplicate)

[d]Positive control of the DPPH assay.

Figure 1. UHPLC profile of decoction and infusion from *M. nivellei* leaves: A) (-) HRMS chromatogram B) UV chromatogram (360 nm).

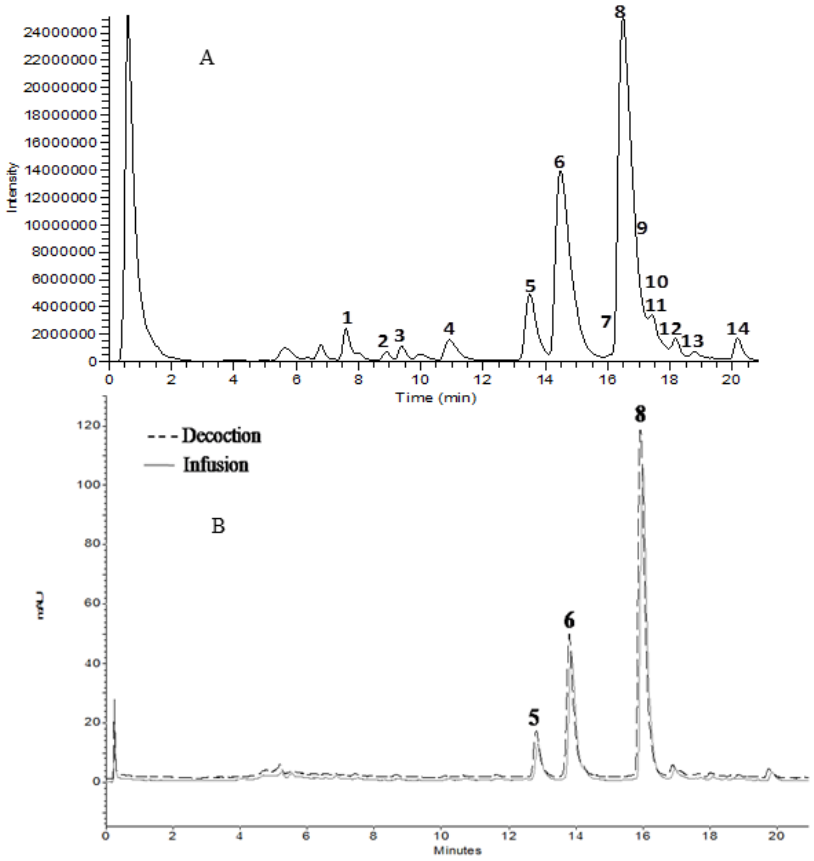
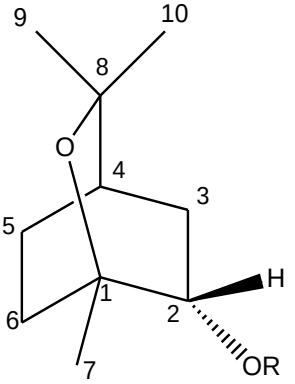
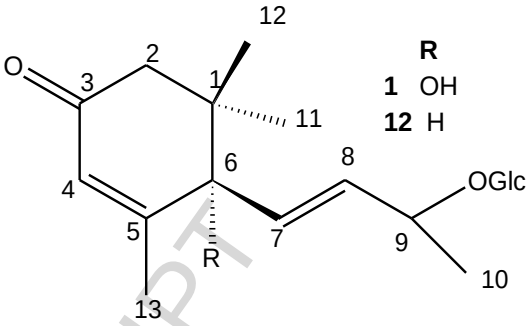


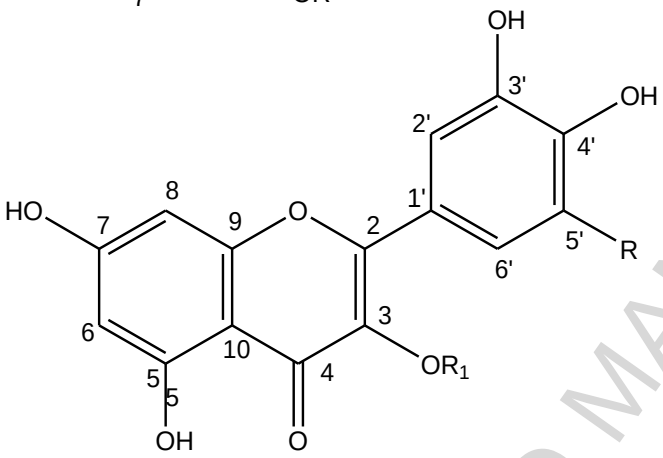
Figure 2. Structures of compounds (**1-10, 12-14**) isolated from *M. nivellei* leaves.



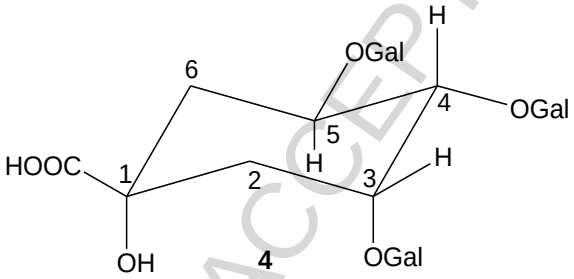
R
2 Glc
3 Araf (1→6)Glc

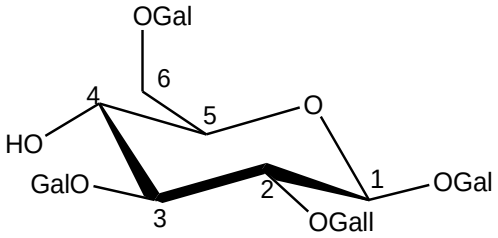


R
1 OH
12 H



	R	R1
5	OH	(6''-Gal)Glc
6	OH	Glc
8	OH	Rha
9	H	(6''-Gal)Glc
10	OH	Xyl
13	OH	H
14	H	Rha





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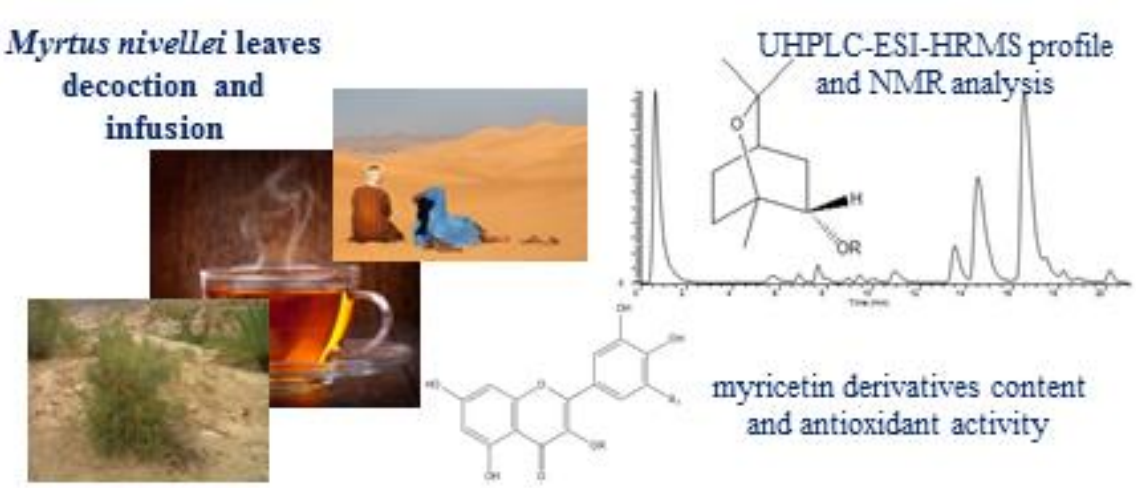
Araf(1→6)Glc: α-L-arabinofuranosyl (1→6) β-D-glucopyranoside
Glc: β-D-glucopyranoside
Rha: α-L-ramnopyranoside
Xyl: β-D-xylopyranoside
Gal: galloyl



Graphical abstract