**A comprehensive characterization of rosemary tea obtained from *Rosmarinus officinalis*
L. collected in a sub-humid area of Tunisia**

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ABSTRACT

Introduction - Rosemary (*Rosmarinus officinalis* L.) is aromatic plant common in Tunisia and it is widely consumed as a tea in traditional cuisine and in folk medicine to treat various illnesses. Currently, most research efforts have been focused on rosemary essential oil, alcoholic and aqueous extracts, however, little is reported on rosemary infusion composition.

Objective - To investigate compounds present in rosemary tea obtained from *Rosmarinus officinalis* L. collected in Sub-humid area of Tunisia in order to assess whether the traditional rosemary tea preparation method could be considered as a reference method for rosemary's compounds extraction.

Methodology - Qualitative characterization of *Rosmarinus officinalis* tea obtained after rosemary infusion in boiled water was determined by high performance liquid chromatography coupled with electrospray quadrupole time of flight mass spectrometry (HPLC-ESI-QTOF-MS). Quantitative analysis relies on a high performance liquid chromatography with diode array detector (HPLC-DAD).

Results - Forty-nine compounds belonging to six families, namely flavonoids, phenolic acids, phenolic terpenes, jasmonate, phenolic glycosides, and lignans were identified. To the best of the author's knowledge eucommin A is characterized for the first time in rosemary. Rosmarinic acid (158.13 µg/g dried rosemary) was the main compound followed then by feruloylnepitrin (100.87 µg/g) and luteolin-3'-O-(2"-O-acetyl)-β-D-glucuronide (44.04 µg/g). Among quantified compounds, luteolin-7-O-rutinoside was the compound with the lowest concentration.

Conclusion - Infusion method permit to extract several polyphenols present in rosemary tea, then it could be a reference method for rosemary's compounds extraction. Moreover, traditional Tunisian *Rosmarinus officinalis* tea consumption is of interest for its rich phenolic content.

KEYWORDS

Rosmarinus officinalis; rosemary tea; infusion, HPLC-DAD; HPLC-ESI-QTOF-MS

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Rosmarinus officinalis tea is traditionally consumed to treat several diseases, however, little is reported on its composition. We studied rosemary tea composition to assess whether the

infusion could be a reference method for its compounds extraction. HPLC-ESI-QTOF-MS analysis identified forty-nine compounds. Rosmarinic acid was the main compound and eucommin A was characterized for the first time in rosemary. Thus, infusion method permit to extract several polyphenols and then it could be a reference method for rosemary's compounds extraction.

INTRODUCTION

Rosemary (Order: Lamiales, Family: Lamiaceae, Genus: *Rosmarinus*, Species: *officinalis*) is a shrubby aromatic plant that grows wild in the Mediterranean basin (Ali, et al. 2000) and it is particularly widespread in Central and North-Western Tunisia on sites belonging to sub-humid, semi-arid superior and arid superior bioclimatic levels (Zaouali, et al. 2003). Rosemary has been traditionally used for both culinary and medical purposes (Ribeiro-Santos, et al. 2015). In fact, the whole fresh plant or its dried milled powder is used for cooking as flavoring and in the preservation of foods (Sasikumar 2012). Similarly, according to traditional Tunisian medicine, rosemary is known as a medicinal herb with significant activities against many illnesses especially against headaches, respiratory diseases and several neuropsychiatric disorders. These traditional medicinal herb benefits are essentially reached through fresh or dried rosemary tea consumption.

Currently, most research efforts have been focused on rosemary essential oil, alcoholic and aqueous extracts. However, to the best of the author's knowledge, only a brief study on *Rosmarinus officinalis* leaf infusion composition was reported by Ferlemi et al. when investigating the neurobiological effects of this tea on mice (Ferlemi, et al. 2015). Thus, the aim of the present work was to carry out an extensive characterization of compounds present in the whole rosemary plant tea by reversed-phase high-performance liquid chromatography (RP-HPLC), coupled to different detection systems, namely diode array detector (DAD) and quadrupole time of flight (QTOF) mass analyzer in order to assess whether the traditional rosemary tea preparation method (infusion procedure) could be considered as a reference method for rosemary's compounds extraction.

EXPERIMENTAL

Reagents and materials

Acetonitrile, dimethyl sulfoxide (DMSO) and formic acid were acquired from Panreac (Barcelona, Spain). Hydroxycinnamic acids (caffeic and rosmarinic acids) and the flavonoid (luteolin) were purchased from Sigma-Aldrich Chemical Co. St. Louis, USA).

R. officinalis, growing wild in Tunisia, was collected from Hammam Jedidi-Mount Zaghouen (a sub-humid area) during the period April-May 2015 and identified by Pr. Harzallah Skhiri Fethia (Higher Biotechnology Institute of Monastir, University of Monastir, Tunisia) according to the flora of Tunisia (Pottier –Alapetite 1981).

Sample preparation

Fresh branches and leaves were kept at ambient temperature in a dark ventilated room until dryness. For infusion preparation, 50 g of rosemary (leaves and branches) were placed into 100 mL of boiled water. The leaves and branches steeped for 15 min were then filtered and made to 100 mL. The infusion was kept at -20°C for a month until chromatographic analysis.

HPLC-ESI-QTOF-MS conditions

The chromatographic analyses were carried out using an Agilent 1200 Series LC system coupled to an Agilent G6530A accurate-mass quadrupole time-of-flight (Q-ToF) with ESI-Jet Stream Technology (Agilent Technologies). Separation was performed on a Kinetex 100 C18 (4.6 × 250 mm, 5 µm, Phenomenex) column preceded by a guard column in a thermostatic oven at 30 °C. Each sample (20 µL) was injected and separated by using a mobile phase consisting of water (solvent A) and acetonitrile (solvent B), both containing 1% formic acid (all MS quality), at a flow rate of 1 mL/min. The solvent gradient changed from 10% to 20% solvent B over 20 min, 20%-25% solvent B over 10 min, 25%-35% solvent B over 10 min, isocratically for 10 min, returning to the initial conditions over 10 min and maintaining isocratically over 5min. Chromatograms were recorded at 324 nm according to the expected maxima spectra of phenolic compounds. QTOF acquisition conditions were as follows: 2.5 GHz, mass range between 100 and 1000 m/z, drying gas volume and temperature 10 L/min and 350 °C, sheath gas volume and temperature 11 L/min and 350 °C, nebulizer pressure 45 psi, cap voltage 3500 V for negative polarity, nozzle voltage 0 V, and fragmentor voltage 100 V. Collision induced fragmentation (CID) was achieved using 20 eV.

HPLC-DAD conditions

Phenolic composition of rosemary infusion was analyzed using an Agilent 1200 liquid chromatographic system equipped with a thermostatic autosampler, quaternary pump and diode-array detector (DAD). The column used for chromatographic separation was a Superspher 100 RP18 column (250 mm x 4.6 mm, 4 µm i.d., Agilent Technologies) preceded by an ODS RP18 guard column in a thermostatic oven at 30 °C. All chromatographic conditions (eluent (HPLC grade), flow rate, inject volume, gradient, etc.) were as described above. Chromatograms were acquired at 324 nm. Quantitative analysis used the external standard method. Pure standard compounds were dissolved in 10% DMSO in deionized water and injected into the HPLC system.

Data analysis

Mass spectrometry data acquisition as well as qualitative analysis were performed by using Agilent Mass Hunter Workstation Software. The accurate mass data for the molecular ions were processed using the Agilent MassHunter Qualitative Analysis B.6.00 software (Agilent Technologies, Inc. 2012). This software provides a list of possible elemental formulae by using the Generate Molecular Formula from peak spectrum.

HPLC-DAD data acquisition and quantitative analysis were performed by using Agilent Chemstation Software (Agilent Technologies, Inc 2012). Limits of detection (LOD) and quantification (LOQ) for individual compounds in standard solutions were calculated as $LOD = (3.3 \times A) / B$ and $LOQ = (10 \times A) / B$, where A is the standard deviation (SD) for the lowest concentration, and B is the slope.

RESULTS AND DISCUSSION

Rosemary infusion was obtained by steeping fifty grams of rosemary leaves and branches in 100 mL of boiled water for 15 min and was analyzed by HPLC coupled to ESI-QTOF-MS in negative ionization mode as well as by HPLC-DAD in order to carry out a characterization and quantification of bioactive compounds, respectively. Peak identification was performed on the basis of their relative retention times, MS and MS/MS spectra obtained by the QTOF-MS, and by using the information previously reported in the literature.

To our knowledge, the only similar study was performed by Ferlemi et al (Ferlemi, et al. 2015) using only rosemary leaves infused during five minutes in hot water previously boiled for one minute, conditions differing from those used in the present study.

Qualitative characterization of compounds present in rosemary infusion

Fifty-one compounds were detected in the present study. Of these, forty-eight were distributed in six major categories: flavonoids (28 compounds), phenolic acids (11 compounds), phenolic terpenes (4 compounds), jasmonate (2 compounds), phenolic glycosides (2 compounds), and lignans (1 compound). Figure 1 shows the Total Ion Chromatogram (TIC) of the rosemary infusion.

Only 3 authentic standards were available (i.e. caffeic acid, rosmarinic acid and luteolin); for the remaining 48 compounds for which no standards were available, identification was based on accurate measurement of the precursor ion $[M-H]^-$ and CID fragment ions combined with data from literature and databases.

Data obtained from HPLC-ESI-DAD-QTOF-MS analysis of the rosemary infusion are summarized in Table 1, where compounds are numbered according to their retention time, and molecular weight, $[M-H]^-$ theoretical and observed values, error, molecular formulas, scores of molecular general formula, precursor ions, main fragments and literature references that have mentioned these compounds are depicted. Despite of the mass accuracy and the true isotopic pattern provided by QTOF mass analyzer in both MS and MS/MS, MS/MS data analysis was not possible when only traces of phenolic compounds were present in rosemary tea.

Flavonoids

Flavonoids constituted the largest group of polyphenols, with up to twenty-eight flavonoid derivatives found in the studied rosemary tea. Luteolin was identified by comparing its retention time and MS spectra data with an authentic standard. Accurate mass measurements and fragmentation pattern during CID confirmed its structural composition. The remaining compounds were tentatively identified according to their MS/MS spectra as well as literature.

Thus the 28 flavonoids were: 5 isomers of pterogynocide, 4 isomers of homoplantagin, 3 isomers of luteolin-3'-O-(O-acetyl)- β -D-glucuronide, 3 isomeric forms of 6-hydroxy-luteolin-7-glucoside, 2 isomers of luteolin-7-O-rutinoside, 2 isomers of luteolin-3-glucuronide, 2 isomers of feruloylnepitrin, nepitrin, hesperidin, luteolin, apigenin, diosmetin, and genkawanin.

The five isomers of pterogynocide ($C_{29}H_{26}O_{14}$) had deprotonated molecular $[M-H]^-$ ions at m/z 597.12448, 597.12526, 597.12606, 597.12303 and 597.12450, respectively. The pseudo molecular ions of these compounds produced the major fragments ions at m/z 555.11

during CID, corresponding to the loss of C_2H_2O (42 u) from the precursor ion (Cuyckens and Claeys 2004). Pterogynoside is known to be found in Fabaceae and Lamiaceae and it was isolated from fruits of *Pterogyne nitens* and rosemary leave extracts. Pterogynoside has shown to have myeloperoxidase inhibitory activity (Regasini, et al. 2008), being a potentially attractive bioactive compound in both prognostic and therapeutic interventions in the prophylaxis of cardiovascular, inflammatory, neurodegenerative, kidney and immune-mediated diseases since myeloperoxidase is a key factor in the genesis of these illnesses (Lazarević-Pasti, et al. 2015).

The second flavonoid detected in the present study was homoplantagin. The tentative mass spectrum for homoplantagin ($C_{22}H_{22}O_{12}$) showed four isomeric forms of this compound with deprotonated molecular $[M-H]^-$ ions at m/z 461.10967, 461.10978, 461.10906 and 461.10923, having respective retention times 24.10 min, 26.68 min, 37.81 min and 38.49 min. The fragmentation pattern of the most concentrated isomers (homoplantagin isomers 1 and 2) presented ions at m/z 161.02, 283.02 and 297.04, which are consistent with data found in the literature (Borrás-Linares, et al. 2014). This flavonoid has been previously reported to be found in rosemary tea (Ferlemi, et al. 2015). In vitro assays showed that homoplantagin have a protective and therapeutic effect on hepatocyte injury (Qu, et al. 2009).

The three isomers of luteolin-3'-O-(2"-O-acetyl)- β -D-glucuronide have the deprotonated molecular $[M-H]^-$ ions at m/z 503.08419, 503.08448 and 503.08442, and they eluted respectively at 32.75 min, 33.73 min and 36.31 min. The fragmentation of these isomers presented major fragment ions at m/z 285.04 and 399.07 corresponding to $[M-H-C_8H_{10}O_7]^-$ and $[M-H-C_3H_4O_4]^-$, data in agreement with other authors (Borrás-Linares, et al. 2014). Three peaks with $[M-H]^-$ at m/z 463.08844, 463.08814 and 463.08873 were detected at retention times 16.56 min, 19.92 min and 20.52 min, respectively, corresponding to isomers of 6-hydroxyluteolin-7-glucoside ($C_{21}H_{20}O_{12}$). This polyphenol was typically found in rosemary (Borrás-Linares, et al. 2014) and in Plantaginaceae. It acts as a potent inhibitor of HIV reverse transcriptase, showing higher inhibitory activity than baicalin, a well-known inhibitor (Nishibe 1994). Compounds eluting at 21.05 min and 28.30 min gave $[M-H]^-$ ions at m/z 461.07395 and 461.07364, attributed to luteolin-3-O-glucuronide ($C_{21}H_{18}O_{12}$). Their MS/MS data showed the loss of the glucuronic acid moiety (m/z 176) resulting in the fragment ions at m/z 285.04034 and 285.04124, respectively. This polyphenol was identified by Heitz et al. as the major flavonoid from *Melissa officinalis* and in rosemary leave extracts (Borrás Linares, et al. 2011) and it is known to reduce the expression of inflammatory genes (Kure, et al. 2016).

Mass accuracy of the QTOF permit to detect two isomers, having deprotonated molecular $[M-H]^-$ ions at m/z 593.15023 and 593.15163. As identified by Hossain et al, these molecules correspond to luteolin-7-O-rutinoside ($C_{27}H_{30}O_{15}$) (Hossain, et al. 2010). This flavonoid was isolated by Inoue et al. from the aerial part of *Mentha piperita* (Inoue, et al. 2002). It shows a potent inhibitory effect on histamine release induced by compound 48/80 and antigen-antibody reactions suggesting that it may be clinically useful in alleviating the symptoms of allergies (Inoue, et al. 2002). Furthermore, the analysis of the rosemary infusion showed the presence of two isomeric forms of feruloylnepitrin ($C_{32}H_{30}O_{15}$) eluting at 30.22 min and 34.50 min, with deprotonated molecular $[M-H]^-$ ions at m/z 653.15178 and 653.15165, respectively. These molecules have already been described in the literature for this vegetable matrix; *in vitro* tests have shown that they exert anti-inflammatory activity, being potent inhibitors of nitrite production in macrophages (Bai, et al. 2010, Borrás Linares, et al. 2011, Ivanović, et al. 2009).

The compound eluting at 22.30 min with deprotonated molecular $[M-H]^-$ ion at m/z 477.10507 was identified as nepitrin ($C_{22}H_{22}O_{12}$). The pseudo molecular ion of this compound produced the major fragment ion at m/z 315.05037 during CID, corresponding to the loss of hexose moiety (162 u) from the precursor ion in agreement with literature (Borrás Linares, et al. 2011); the product ions m/z 197.04839 for syringic acid and 179.03709 for caffeic acid revealed the constituent of nepitrin prior to condensation. Nepitrin was found to possess significant anti-inflammatory and anti-pyretic activities and a weak analgesic activity (Agarwal 1982).

Hesperidin ($C_{28}H_{34}O_{15}$) was the molecule having deprotonated molecular $[M-H]^-$ ions at m/z 609.18313. The fragmentation pattern of this compound showed the loss of deoxy-hexose moiety (m/z 148) resulting in the fragment ion at m/z 461.10967. This flavonoid is predominant in citrus fruits like sweet orange or lemon and consequently in juices made of these fruits (Kuntić Vesna, et al. 2014). Hesperidin has also been found in rosemary leave extracts (Borrás-Linares, et al. 2014). Numerous biological benefits have been attributed to this flavonoid, such as effects on the vascular system increasing capillary resistance thanks to its ability to inhibit hyaluronidase activity, as well as radioprotection, having shown powerful protective effects against DNA damage and on the decline of cell proliferation induced by α -irradiation in mice (Kuntić Vesna, et al. 2014).

A pure standard product was used to identify luteolin, which has a deprotonated molecular $[M-H]^-$ ion at m/z 285.04139 and a characteristic retention time at 35.48 min. Luteolin is known to occur in many botanical families such as Bryophyta, Pteridophyta,

Pinophyta and Magnoliophyta, and it has been found in many edible plants such as carrots, peppers, celery, olive oil, peppermint, thyme, rosemary, oregano, pomegranate, etc. (Miguel 2009). Several studies have shown that this flavone possesses a variety of pharmacological activities. Ishisaka et al. reported that luteolin has antidepressant-like effects since it attenuated the expression of endoplasmic reticulum stress-related proteins in the hippocampus of a corticosterone-treated depression mice model (Ishisaka, et al. 2011), induced apoptosis, prevented carcinogenesis in animal models, reduced tumor growth *in vivo* and sensitized tumor cells to the cytotoxic effects of some anti-cancer drugs (Miguel 2009).

The peak with $[M-H]^-$ at m/z 269.04756 eluting at 40.80 min was attributed to apigenin ($C_{15}H_{10}O_5$) in agreement with literature data (Almela, et al. 2006). It has been reported that apigenin is the major flavonoid present in the crude ethanolic extract of *Lycopodium clavatum* and in rosemary leave extracts (Borrás Linares, et al. 2011). A recent study has shown that this flavonoid stimulates nucleotide excision repair genes to protect skin keratinocytes from ultraviolet B-induced reactive oxygen species and DNA damage (Das, et al. 2013).

The compound eluting at 41.406 min with deprotonated molecular $[M-H]^-$ ion at m/z 299.05683 was identified as diosmetin ($C_{16}H_{12}O_6$). Its MS/MS data showed the characteristic fragment ion at m/z 179.05673. This fragmentation pattern was described by Brito et al. when analyzing phenolic compounds from citrus (Brito, et al. 2014). Recent *in vitro* studies have shown that diosmetin exert cytostatic effects in human hepatoma HepG2 cells via CYP1A-catalyzed metabolism, activation of JNK and ERK and P53/P21 up-regulation (Androutsopoulos and Spandidos 2013). Finally, genkwanine was the compound eluted at 42.37 min with deprotonated $[M-H]^-$ at m/z 283.06201 in agreement with literature data (Borrás Linares, et al. 2011). This flavonoid was found in rosemary leaves (Borrás-Linares, et al. 2014), in the seeds of *Alnus glutinosa* (O'Rourke, et al. 2005) and in the flowers of *Daphne genkwa*, and it is well known for its anti-oxidant and anti-tumor activities (Shu, et al. 2014).

Phenolic acids

Phenolic acids constituted the second largest number of polyphenols in the studied rosemary infusion. Eleven phenolic acid derivatives were found, including two hydroxycinnamic acids caffeic and rosmarinic acids, that were identified by comparing their retention times and MS spectral data with authentic standards. Accurate mass measurements and fragmentation pattern during CID confirmed their structural composition. Indeed, caffeic

acid ($C_9H_8O_4$) was eluted at 9.74 min with deprotonated $[M-H]^-$ at m/z 179.03619. Its MS/MS data showed the characteristic fragment ions at m/z 135.04487, while rosmarinic acid ($C_{18}H_{16}O_8$) was eluted at 25.64 min and its fragmentation pattern showed the characteristic fragment ions at m/z 135.04432, 161.02483, 179.03572, and 197.04689. Tandem mass spectrum and fragmentation pattern of this phenolic acid are shown in Figure 2. These data were in agreement with the literature (Hossain, et al. 2010). Chemical structure of this phenolic acid is shown in Figure 3. Numerous studies have reported that these hydroxycinnamic acids are the main compounds in rosemary leaves (Bai, et al. 2010). Recent *in vitro* research has shown that rosmarinic acid has antioxidant effects in view of its considerable higher activity compared to BHT and α -tocopherol (Romano, et al. 2009). In addition, Amaral et al. suggest that rosmarinic acid had antiulcerogenic properties that could be attributed to its antioxidant effect since it was able to act as gastroprotective agent against ethanol-induced damage in Wistar rats (Amaral, et al. 2013). Moreover, Sasaki et al. proved an antidepressant effect of rosmarinic acid, since proteomic analysis of PC 12 cells treated with this phenolic acid revealed its action on several neuronal pathways involved on depression physiopathology (Sasaki, et al. 2013). On the other hand, Fuhrman et al. mention that in combination with lycopene, rosmarinic acid inhibits *in vitro* low-density lipoprotein (LDL) oxidation (Fuhrman, et al. 2000).

The nine remaining compounds were tentatively identified according to their MS/MS spectra and literature. Thus, four isomers of rosmarinic acid-3-O-glucoside, three isomers of chlorogenic acid, one isomeric form of syringic acid and vanilic acid were detected. The four isomers of rosmarinic acid-3-O-glucoside were eluted at 18.49 min, 20.60 min, 21.55 min and 29.11 min. It had deprotonated molecular $[M-H]^-$ ions at m/z 521.13215, 521.13423, 521.13073 and 521.13000, respectively. This compound has been described in different plants belonging to the Lamiaceae family, especially in rosemary leave extracts (Borrás-Linares, et al. 2014) and in the seeds of Korean perilla (*Perilla frutescens*) (Zhou, et al. 2014). Three peaks with $[M-H]^-$ at m/z 353.08874, 353.08854 and 353.08931 were detected at retention times 4.97 min, 7.71 min and 7.92 min, respectively, corresponding to isomers of chlorogenic acid. The MS/MS on the precursor m/z 353.08 ions gave dominant product ion m/z 179.03592, corresponding to caffeic acid. This result was consistent with data found in the literature (Hossain, et al. 2010). This hydroxycinnamic acid is reported as the major polyphenol found in coffee (Meng, et al. 2013) and several studies has demonstrated that chlorogenic acid exhibits many biological properties, including antibacterial, antioxidant, and

antitumor activities, particularly hypoglycemic and hypolipidemic effects (Rodriguez de Sotillo and Hadley 2002).

The compound eluting at 4.037 min with deprotonated molecular $[M-H]^-$ ion at m/z 197.04560 was identified as syringic acid ($C_9H_{10}O_5$). Its pseudo molecular ion produced the major fragments ions at m/z 135.04430 and 179.03709 during CID, corresponding to the loss $[M-H-CO_2-H_2O]^-$ and $[M-H-H_2O]^-$ molecules from the precursor ion, respectively. The same fragmentation pattern was described by Hossain et al. when analyzing rosemary leave extract (Hossain, et al. 2010) and Shi et al. have shown that syringic acid has antibacterial activities (Shi, et al. 2016).

Finally, the peak eluting at 4.28 min with deprotonated molecular $[M-H]^-$ ion at m/z 167.03559 was attributed to vanillic acid ($C_8H_8O_4$). This phenolic acid was also reported by Hossain et al. in an aqueous methanol extract of rosemary, however its fragmentation pattern was different (Hossain, et al. 2010). This fact could be explained by the difference in collision energy occurred during both analysis. This phenolic acid possess several biological benefits since it exerts anti-sickling activity (Ma, et al. 2008), anthelmintic activity, suppress hepatic fibrosis in chronic liver injury (Itoh, et al. 2009), inhibits snake venom 5'-nucleotidase (Dhananjaya, et al. 2009), and possess protective action to hepatotoxicity and nephrotoxicity produced by acetaminophen (Rohini Manuja, et al. 2013).

Phenolic terpenes

The phenolic terpenes found in our rosemary infusion are represented by three isomeric forms of rosmanol ($C_{20}H_{26}O_5$) and one isomeric form of carnosic acid ($C_{20}H_{28}O_4$). The rosmanol peaks with $[M-H]^-$ ions at m/z 345.17129, 345.17223 and 345.17125 eluted at 44.97 min, 47.95 min and 52.33 min, respectively. The fragmentation pattern of the first isomer had a characteristic feature in the MS/MS experiment where it showed the major fragment ions at m/z 283.17484 and 301.1798 corresponding to the loss of $[M-H-CO_2-H_2O]^-$ and $[M-H-CO_2]^-$, however, the second isomer of rosmanol named, also epirosmanol, showed only the major fragment at m/z 283.17087 corresponding to the loss of $[M-H-CO_2-H_2O]^-$ molecule. This fragmentation pattern was in agreement with literature data (Borrás-Linares, et al. 2014). Abdelhalim et al. reported that rosmanol found in rosemary did not exhibit any signs of acute toxicity in rats (50-200 mg/kg) and it elicited antinociceptive, antidepressant and anxiolytic effects (Abdelhalim, et al. 2015).

Carnosic acid ($C_{20}H_{28}O_4$) was the molecule having deprotonated molecule $[M - H]^-$ 331.19175 and eluted at 40.08 min. This phenolic terpene was reported to be found in rosemary leaves, in sage leaves and in oregano leaves, as well as in rosemary tea (Hossain, et al. 2010). When evaluating the therapeutic effects of carnosic acid in obese mice, Wang et al. suggest that this terpene acid could be a novel therapeutic agent for obesity-related non-alcoholic fatty liver disease (Wang, et al. 2011). Carnosic acid ($C_{20}H_{28}O_4$) has also shown to have neuroprotective effects on cyanide-induced brain damage in cultured rodent and human-induced pluripotent stem cell-derived neurons *in vitro* and *in vivo* in various brain areas of a mouse model (Zhang, et al. 2015).

Phenolic glycosides

Only two phenolic glycosides were found in the rosemary tea. The peak appearing at 4.84 min with $[M-H]^-$ deprotonated ion at m/z 503.14090 was attributed to the phenolic glycoside 6-O-Caffeoyl- β -D-fructofuranosyl-(2 $\rightarrow$ 1)- α -D-glucopyranoside. This polyphenol was previously found in rosemary leaves extract and in a Lamiaceae spice commonly known as sage (*Salvia officinalis*) (Wang, et al. 1999). However, two possible phenolic glycosides, primiverin/primulaverin, have been proposed for the same retention time 6.261 min, due to the fact that they cannot be discriminated with the analytical methodology used. In this sense, further studies must be carried out in order to be able to differentiate between these isomers. These phenolic glycoside isomers, primiverin and primulaverin, are characteristic compounds for the genus *Primula* (Müller, et al. 2006). They were also reported by Borrás-Linares et al. when analyzing rosemary leave extracts; these authors also reported that these isomers cannot be differentiated by using the information of DAD and TOF-MS (Borrás Linares, et al. 2011).

Lignans

Eucommin A (chemical structure showed in Figure 3) was the only lignan characterized in rosemary infusion, corresponding to the peak with $[M-H]^-$ ion at m/z 549.19784 eluted at 27.66 min. Its fragmentation pattern showed the major fragments ions at m/z 218.98664, 359.07898, and 475.11699. To the best of our knowledge this lignan is characterized for the first time in rosemary, although it has been identified in Orobanchaceae species, especially in stems of *Cistanche deserticola* (Nan, et al. 2015).

Jasmonate

The phytohormone jasmonate was found in the rosemary infusion as the 12-hydroxyjasmonic acid. In fact, two peaks with $[M-H]^-$ deprotonated ions at m/z 225.11218 and 225.11359 eluting at 11.63 min and 11.97 min, respectively, were attributed to 12-hydroxyjasmonic acid. This hormone was previously reported in several studies on species belonging to Lamiaceae family. Indeed it was isolated from an aqueous methanol extract of *Origanum vulgare* (Koukoulitsa, et al. 2006), and in rosemary leaves extracts (Borrás Linares, et al. 2011).

Others

Quinic acid ($C_7H_{12}O_6$) was the molecule having deprotonated molecular $[M-H]^-$ ion at m/z 191.05780. It had a characteristic MS/MS feature, showing major fragment ions at m/z 93.03738 and 127.04098, respectively. This compound was also found by Ferlemi et al. in rosemary tea (Ferlemi, et al. 2015) and in *Ballota nigra* infusion (Vrchovská, et al. 2007). Its MS/MS spectrum analysis showed the same fragmentation pattern reported by Borrás-Linares et al. in rosemary leave extracts (Borrás-Linares, et al. 2014)

Pseudo molecular ions at m/z 460.15808 (observed exact mass 459.15080) eluting at 8.291 min was identified as paeonolide (National Center for Biotechnology Information). However, pseudomolecular ions at m/z 683.1618 (observed exact mass 683.1643) and 287.22278 (observed exact mass 287.22286) eluting at 31.83 and 45.90 min, respectively, could not be identified.

Compared to the study by Ferlemi et al., only six compounds identified in the rosemary tea obtained by these authors were also present in the infusion analyzed in the present work, namely quinic acid, luteolin-3-O-glucuronide, homplantagin, rosmarinic acid, luteolin-3-O-(O-acetyl)- β -D-glucuronide, and rosmanol (Ferlemi, et al. 2015). However, six compounds identified by Ferlemi et al., namely cirsimaritin, caffeic acid-O-hexoside derivative, isorhamnetin-3-O-hexoside, rosmadial, notohamosin R and carnosol, have not been found in the present study. In turn, syringic acid, caffeic acid, rosmarinic acid-3-O-glucoside, nepitrin, hesperidin, luteolin, chlorogenic acid, vanillic acid, 6-O-caffeoyl- β -D-fructofuranosyl-(2 $\rightarrow$ 1)- α -D-glucopyranoside, primiverin/primulaverin, luteolin-7-O-rutinoside, 12-hydroxyjasmonic acid, 6-hydroxyluteolin-7-glucoside, nepitrin, eucommin A, pterogynoside, feruloylnepitrin, apigenin, diomestin and genkawinin were identified in rosemary tea, including the lignin eucommin A, identified in rosemary for the first time. This comparison study is summarized in Table 2.

Quantitative characterization of compounds present in rosemary infusion

In order to quantify the identified compounds, High Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD) was used. Typical HPLC-DAD chromatogram of rosemary infusion is given in Figure 4. As shown in the chromatogram, thirty-nine compounds of the fifty-one identified were detected by HPLC-DAD. The remaining compounds were below the limit of DAD detection and thus they could not be quantified.

Validation method was performed using authentic standard products (rosmarinic acid, caffeic acid, and luteolin). Each of the calibration curves was used to quantify the corresponding product and for the quantification of compounds with similar structures. Thus, quantification of rosmarinic acid-3-O-glucoside was based on the use of rosmarinic acid while luteolin calibration curve was used to quantify compounds with similar structures represented by luteolin-7-O-rutinoside isomers, 6-hydroxyluteolin-7-glucoside isomers, luteolin-3-glucuronide, nepitrin, feruloylnepitrin isomers and luteolin-3'-O-(2''-O-acetyl)- β -D-glucuronide isomers.

The main analytical parameters of the used quantification method, limits of detection (LODs) and quantification (LOQs), calibration equations, and regression coefficient (R^2) are shown in Table 3.

The calibration curves show good linearity for the analytes studied with R^2 ranged between 0.9984 and 0.9999. LODs ranged between 0.019-0.037 μ M and LOQs between 0.056 - 0.111 μ M.

Due to the lack of commercial standards, only caffeic acid, rosmarinic acid, luteolin and their derivatives were quantified. Thus, integration of the chromatogram peaks permits to quantify the corresponding compounds. Results are summarized in Table 4. Compounds are numbered according to their elution order and retention times (Table 1).

Rosmarinic acid was the main compound, with up to 158.13 μ g/g of dried rosemary, in agreement with that found by Ferlemi et al. (2015). Nevertheless, the concentration of this hydroxycinnamic acid per gram of dried rosemary was lower in the present work as compared to that of Ferlemi et al. (2015) (1.165 mg/g). In the other hand, a previous analysis of ethanol extract from *R. officinalis* grown in both sub-humid and semi-arid Tunisian areas and collected during the period from April-May showed that rosmarinic acid concentration

represent 4% of total quantified compounds (Sasaki, et al. 2013). However, in the present study, rosmarinic acid concentration represented 42.82 % of total quantified compounds. Other studies mentioned that rosmarinic acid was found only in trace amounts in methanol and acetone extracts (del Baño, et al. 2003).

The second major compound found in this work was feruloylnepitrin, with the concentration of feruloylnepitrin isomers amounting to 100.86 µg/g dried rosemary, followed by luteolin-3'-O-(2"-O-acetyl)-β-D-glucuronide, luteolin-3-glucuronide and nepitrin with concentrations ranging between 12.14 and 44.04 µg/g dried rosemary (Table 4). The amounts of the remaining quantified compounds ranged between 2.36 and 5.93 µg/g dried rosemary. In Ferlemi et al. (2015) study, only caffeic acid derivatives and luteolin-glucuronide concentrations were mentioned, and their concentrations were 0.14 and 0.075 mg/g rosemary, respectively; the concentrations of the remaining identified compounds were reported as trace amounts. In the present study, caffeic acid and luteolin glucuronide concentrations were lower than those reported by Ferlemi et al. (5.93 µg/g and 15.76 µg/dried rosemary respectively), (Table 4).

Both qualitative and quantitative results have shown differences from those reported by Ferlemi et al. (2015). This could be explained by different internal and external factors such as infusion preparation procedure (part of the plant used, infusion time), plant age, climate, season of plant recollection, soil type or stress conditions that may inhibit or trigger the synthesis of specific compounds. Indeed, in the present work the analyzed rosemary tea was prepared using leaves and branches infused for 15 min in boiled water, while Ferlemi et al. only used leaves infused for 5 min (Ferlemi et al., 2015). Thus, the compounds absent in Ferlemi's study could be contained only in branches, or alternatively they needed longer time to infuse. On the other hand, some compounds could be degraded when the infusion time was raised to fifteen minutes. Furthermore, *Rosmarinus officinalis* samples were collected at different seasons encompassing fall (September 2012) in Ferlemi's study and spring (April-May 2015) in the present work, which affects the plant age and physiology. In fact, Lemos et al. have shown that seasonality modifies rosemary's compositions and biological activities (Lemos, et al. 2015). In addition, rosemary was harvested in different geographical areas with different climates, altitude and soil type. Also, it is well known that a slight shift in soil pH and composition affects biochemical pathways in plants (Borrás-Linares, et al. 2014). Hence, it can be assumed that this factor might have influenced the composition of the infusions.

Moreover, the absence of some compounds may result from their physicochemical properties such as oxygen sensitivity, photosensitivity, and thermosensitivity.

On the other hand, by comparing the amount of rosmarinic acid in ethanol extract made from rosemary collected in Tunisian sub-humid and semi-arid climate (Sasaki et al., 2013) to that of the infusion used in the present study, we noticed that this hydroxycinnamic acid was in higher concentration in the infusion made from rosemary collected in sub-humid climate (4% versus 48.82 % of total quantified compounds). Thus, taking into account that both rosemary plants were collected on the same season, it can be assumed that climate might affect the concentration of rosmarinic acid. However, this fact may also be the result of the adopted extraction procedure, since the solvent used by Sasaki et al. (2013) might have been more effective extracting other less water-soluble compounds.

Otherwise, either when extracted by supercritical fluid, by microwave or when infused, some of the identified rosemary compounds were always present in rosemary's extracts. In fact, quinic acid, hesperidin, homoplantagin, luteolin-glucuronide, rosmarinic acid, rosmarinic acid-3-O-glucoside, luteolin 3-O-(O-acetyl)- β -D-glucuronide, cirsimaritin, and rosmanol were detected in the studied rosemary tea as well as in the microwave and supercritical fluid extracts of rosemary leaves (Borrás Linares, et al. 2011). Thus, these extraction methodologies have been proved to be useful tools for the extraction of a wide range of phenolic acids, flavonoids and diterpens among other classes of phenolic compounds present in rosemary. However, as the infusion procedure presents several advantages (mainly, it is a less time-consuming process, does not require solvents consumption, provides good recovery and, especially, it was used as a method for rosemary tea preparation in traditional medicine), this method can be considered as a reference method for rosemary's phenolic compounds extraction. Thus, traditional Tunisian *Rosmarinus officinalis* tea consumption is of interest for its rich phenolic content, having a promising use as an ingredient in complementary alternative medicine.

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TABLES

Table 1: Proposed compounds detected in rosemary infusion characterized by HPLC-DAD-ESI-Q TOF-MS in negative ionization mode

Peak number	Rt (min)	Molecular Weight	[M-H]-theoretical	[M-H]-observed	Error (ppm)	Empirical Formula	Score (MGF)	Precursor Ion	Major Fragments (%)	Proposal compound	Reference
1	2.345	192.06340	191.05610	191.05780	-16.04	C ₇ H ₁₂ O ₆	68.74	191.05922	93.03738 (19.27), 127.04098 (7.03)	Quinic acid	(Mahmoud, et al. 2005) (Ferlemi, et al. 2015)
2	4.037	198.05280	197.04550	197.04560	-3.33	C ₉ H ₁₀ O ₅	97.54	197.04807	135.04430 (9.9), 179.03709 (23.78)	Syringic acid	(Borrás-Linares, et al. 2014) (Hossain, et al. 2010)
3	4.279	168.04226	167.03498	167.03559	-3.01	C ₈ H ₈ O ₄	83.52	167.03536	146.9637 (11.74), 160.84215 (37.14)	Vanilic acid	(Hossain, et al. 2010)
4	4.843	504.14790	503.14060	503.14090	-4.18	C ₂₁ H ₂₈ O ₁₃	63.51	-	-	6-O-Caffeoyl-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside	(Borrás Linares, et al. 2011)
5	4.971	354.09508	353.08781	353.08874	-1.92	C ₁₆ H ₁₈ O ₉	96.57	353.08840	179.03592 (6.02)	Chlorogenic acid (Isomer 1)	(Hossain, et al. 2010)
6	6.261	476.15300	475.14640	475.14617	-0.16	C ₂₀ H ₂₈ O ₁₃	90.14	475.14679	293.12530 (10.42), 361.15117 (32.56)	Primiverin /Primulaverin,	(González, et al. 1989)
7	7.711	354.09508	353.08781	353.08854	-1.95	C ₁₆ H ₁₈ O ₉	98.42	-	-	Chlorogenic acid (Isomer 2)	(Hossain, et al. 2010)
8	7.920	354.09508	353.08781	353.08931	-3.62	C ₁₆ H ₁₈ O ₉	93.6	-	-	Chlorogenic acid (Isomer 3)	(Hossain, et al. 2010)
9	8.291	460.15808	459.15080	459.15096	0.63	C ₂₀ H ₂₈ O ₁₂	96.89	-	-	Paeonolide	(National Center for Biotechnology Information)
10	9.741	180.04230	179.03500	179.03619	-6.26	C ₉ H ₈ O ₄	93.25	179.03596	135.04487 (1.23), 160.84208 (4.92)	Caffeic acid	(Borrás Linares, et al. 2011)
11	11.401	594.15850	593.15120	593.15023	1.62	C ₂₇ H ₃₀ O ₁₅	46.02	-	-	Luteolin-7-O-rutinoside (Isomer 1)	(Borrás Linares, et al. 2011, Herrero, et al. 2010)
12	11.643	226.12050	225.11320	225.11218	5.33	C ₁₂ H ₁₈ O ₄	91.50	-	-	12-Hydroxyjasmonic acid (Isomer 1)	(Vrchovská, et al. 2007)
13	12.029	226.12050	225.11320	225.11359	-1.39	C ₁₂ H ₁₈ O ₄	99.54	225.11323	134.86551 (8.15), 146.96253 (24.94), 168.98914 (33.89), 187.86144 (47.91), 212.97963 (32.7)	12-Hydroxyjasmonic acid (Isomer 2)	(Vrchovská, et al. 2007)
14	16.558	464.09548	463.08820	463.08844	-0.83	C ₂₁ H ₂₀ O ₁₂	96.36	-	-	6-Hydroxyluteolin-7-glucoside (Isomer 1)	(Borrás-Linares, et al. 2014)
15	17.122	598.13230	597.12500	597.12448	1.04	C ₂₉ H ₂₆ O ₁₄	73.47	597.12419	373.18878 (8.15), 537.10545 (45.91), 555.11408 (100)	Pterogynoside (Isomer 1)	(Borrás Linares, et al. 2011, Müller, et al. 2006, Regasini, et al. 2008)
16	18.282	598.13230	597.12500	597.12526	0.04	C ₂₉ H ₂₆ O ₁₄	94.68	597.12436	555.11818 (41.38)	Pterogynoside (Isomer 2)	(Borrás Linares, et al. 2011, Müller, et al. 2006, Regasini, et al. 2008)
17	18.491	522.13730	521.13010	521.13215	-5.94	C ₂₄ H ₂₆ O ₁₃	77.88	-	-	Rosmarinic acid-3-D-glucoside (Isomer 1)	(Borrás-Linares, et al. 2014)
18	18.991	598.13230	597.12500	597.12606	-1.37	C ₂₉ H ₂₆ O ₁₄	97.82	597.12607	373.18906 (1.46), 555.11357 (2.09)	Pterogynoside (Isomer 3)	(Borrás Linares, et al. 2011, Müller, et al. 2006, Regasini, et al. 2008)
19	19.813	594.15850	593.15120	593.15163	-0.37	C ₂₇ H ₃₀ O ₁₅	98.53	-	-	Luteolin-7-O-rutinoside (Isomer 2)	(Borrás-Linares, et al. 2014, Herrero, et al. 2010)
20	19.925	464.09548	463.08820	463.08814	-0.63	C ₂₁ H ₂₀ O ₁₂	98.06	463.08859	-	6-hydroxyluteolin-7-glucoside (Isomer 2)	(Borrás-Linares, et al. 2014)
21	20.522	464.09548	463.08820	463.08873	-1.09	C ₂₁ H ₂₀ O ₁₂	98.33	463.0880	-	6-hydroxyluteolin-7-glucoside (Isomer 3)	(Borrás-Linares, et al. 2014)

Table 1 (Continued)

Peak number	Rt (min)	Molecular Weight	[M-H] ⁺ -theoretical	[M-H] ⁺ -observed	Error (ppm)	Empirical Formula	Score (MGF)	Precursor Ion	Major Fragments (%)	Proposal compound	Reference
22	20.602	522.13730	521.13010	521.13423	-6.33	C ₂₄ H ₂₆ O ₁₃	74.64	-	-	Rosmarinic acid-3-O-glucoside (Isomer 2)	(Borrás-Linares, et al. 2014)
23	21.053	462.07980	461.07250	461.07395	-3.21	C ₂₁ H ₁₈ O ₁₂	92.89	461.07026	285.04034 (100)	Luteolin-3-glucuronide (Isomer 1)	(Borrás-Linares, et al. 2014, Hossain, et al. 2010)
24	21.553	522.13734	521.13006	521.13073	3.36	C ₂₄ H ₂₆ O ₁₃	69.01	-	-	Rosmarinic acid-3-O-glucoside (Isomer 3)	(Borrás-Linares, et al. 2014)
25	22.300	478.11110	477.10380	477.10507	-2.07	C ₂₂ H ₂₂ O ₁₂	97.46	477.10336	135.04315 (4.22), 179.03584 (1.57), 197.04839 (4.26), 315.05037 (50.25)	Nepitrin	(Babovic, et al. 2010, Borrás-Linares, et al. 2014, Borrás Linares, et al. 2011)
26	24.099	462.11620	461.10890	461.10967	-0.81	C ₂₂ H ₂₂ O ₁₁	96.93	461.10931	161.02548 (19.37), 283.02826 (0.24), 297.04784 (2.52)	Homoplantagin (Isomer 1)	(Borrás-Linares, et al. 2014, Ferlemi, et al. 2015)
27	24.325	610.18980	609.18250	609.18302	-1.02	C ₂₈ H ₃₀ O ₁₅	96.87	609.18227	461.10967 (12.75)	Hesperidin	(Borrás-Linares, et al. 2014)
28	25.640	360.08450	359.07720	359.07850	-3.34	C ₁₈ H ₁₆ O ₈	95.78	359.07875	135.04432 (8.75), 161.02483 (100), 179.03572 (15.42), 197.04689 (25.9)	Rosmarinic acid	(Borrás-Linares, et al. 2014, Borrás Linares, et al. 2011, Chen, et al. 2005, Ferlemi, et al. 2015, Hossain, et al. 2010)
29	26.677	462.11620	461.10970	461.10978	-1.42	C ₂₂ H ₂₂ O ₁₁	98.28	461.10966	161.02451 (5.88), 283.02438 (46.26), 297.04869 (10.84)	Homoplantagin (Isomer 2)	(Borrás-Linares, et al. 2014, Ferlemi, et al. 2015)
30	27.660	550.20500	549.19780	549.19797	-0.22	C ₂₇ H ₃₄ O ₁₂	89.32	549.19784	218.98664 (8.37), 359.07898 (1.91), 475.11699 (2.06)	Eucommin A	(Nan, et al. 2015)
31	27.757	598.13230	597.12500	597.12303	2.91	C ₂₉ H ₂₆ O ₁₄	77.66	-	-	Pterogynoside (Isomer 4)	(Borrás Linares, et al. 2011, Müller, et al. 2006, Regasini, et al. 2008)
32	28.300	462.07980	461.07250	461.07364	2.03	C ₂₁ H ₁₈ O ₁₂	97.37	461.07684	285.04124(100)	Luteolin-3-glucuronide (Isomer r 2)	(Borrás-Linares, et al. 2014, Hossain, et al. 2010)
33	29.111	522.13730	521.13010	521.13000	-0.05	C ₂₄ H ₂₆ O ₁₃	81.44	-	-	Rosmarinic acid-3-O-glucoside (Isomer 4)	(Borrás-Linares, et al. 2014)
34	30.220	654.15850	653.15120	653.15178	-0.93	C ₃₂ H ₃₀ O ₁₅	70.00	-	-	Feruloylnepitritin (Isomer 1)	(Borrás Linares, et al. 2011, Ivanović, et al. 2009)
35	31.834	684.16900	683.16180	683.16221	-0.8	C ₃₃ H ₃₂ O ₁₆	99.4	-	-	Unknown	(Borrás Linares, et al. 2011)
36	32.752	504.09040	503.08310	503.08419	-1.94	C ₂₃ H ₂₀ O ₁₃	97.69	503.08439	285.04008 (39.12), 399.07188 (100)	Luteolin-3'-O-(2"-O-acetyl)-β-D-glucuronide (Isomer 1)	(Borrás-Linares, et al. 2014, Borrás Linares, et al. 2011, Ferlemi, et al. 2015)
37	33.059	598.13230	597.12500	597.12450	0.58	C ₂₉ H ₂₆ O ₁₄	97.12	597.12582	555.11515 (13.06)	Pterogynoside (Isomer 5)	(Borrás Linares, et al. 2011, Müller, et al. 2006, Regasini, et al. 2008)
38	33.735	504.09040	503.08310	503.08439	-2.14	C ₂₃ H ₂₀ O ₁₃	96.96	503.0866	285.04081 (100), 399.07458 (5.13)	Luteolin-3'-O-(2"-O-acetyl)-β-D-glucuronide (Isomer 2)	(Borrás-Linares, et al. 2014, Borrás Linares, et al. 2011, Ferlemi, et al. 2015)
39	34.500	654.15850	653.15120	653.15165	-0.92	C ₃₂ H ₃₀ O ₁₅	98.01	653.15222	331.97518 (2.4), 503.08379 (6.76), 593.12947 (6.6)	Feruloylnepitritin (Isomer 2)	(Borrás Linares, et al. 2011, Ivanović, et al. 2009)

Table 1 (Continued)

Peak number	Rt (min)	Molecular Weight	[M-H]-theoretical	[M-H]-observed	Error (ppm)	Empirical Formula	Score (MGF)	Precursor Ion	Major Fragments (%)	Proposal compound	Reference
40	35.476	286.04774	285.04046	285.04139	-2.32	C ₁₅ H ₁₀ O ₆	93.11	285.04136	146.96406 (66.31), 190.92864 (42.64), 262.97623 (23.88)	Luteolin	(Hossain, et al. 2010)
41	36.314	504.09040	503.08310	503.08436	-2.14	C ₂₃ H ₂₀ O ₁₃	97	503.08415	285.04 (100), 399.07195 (4.43)	Luteolin-3'-O-(2"-O-acetyl)-β-D-glucuronide (Isomer 3)	(Borrás-Linares, et al. 2014, Borrás Linares, et al. 2011, Ferlemi, et al. 2015)
42	37.812	462.11620	461.10890	461.10906	1.80	C ₂₂ H ₂₂ O ₁₁	87.33	-	-	Homoplantagin (Isomer 3)	(Borrás-Linares, et al. 2014, Ferlemi, et al. 2015)
43	38.489	462.11620	461.10890	461.10923	-0.42	C ₂₂ H ₂₂ O ₁₁	95.99	-	-	Homoplantagin (Isomer 4)	(Borrás-Linares, et al. 2014, Ferlemi, et al. 2015)
44	40.800	270.05282	269.04555	269.04756	-8.14	C ₁₅ H ₁₀ O ₅	80.19	-	-	Apigenin	(Almela, et al. 2006, Borrás Linares, et al. 2011, Hossain, et al. 2010)
45	41.406	300.06340	299.05610	299.05677	-1.44	C ₁₆ H ₁₂ O ₆	89.49	299.05683	179.05673 (18.77)	Diomestine	(Brito, et al. 2014)
46	42.373	284.06847	283.06120	283.06157	-0.46	C ₁₆ H ₁₂ O ₅	81.28	-	-	Genkawinin	(Borrás-Linares, et al. 2014) (Borrás Linares, et al. 2011)
47	44.967	346.17802	345.17075	345.17176	-2.71	C ₂₀ H ₂₆ O ₅	96.4	345.17129	283.17484 (15.09) 301.1798 (100)	Rosmanol (Isomer 1)	(Ferlemi, et al. 2015) (Borrás-Linares, et al. 2014) (Borrás Linares, et al. 2011, Señoráns, et al. 2000) (Almela, et al. 2006)
48	45.902	288.23006	287.22278	287.22286	0.54	C ₁₆ H ₃₂ O ₄	97.13	-	-	Unknown	(Borrás Linares, et al. 2011)
49	47.078	332.19876	331.19148	331.19175	-0.25	C ₂₀ H ₂₈ O ₄	96.80	-	-	Carnosic acid	(Almela, et al. 2006, Borrás-Linares, et al. 2014, Borrás Linares, et al. 2011, Ferlemi, et al. 2015, Hossain, et al. 2010)
50	47.948	346.17802	345.17075	345.17127	-1.65	C ₂₀ H ₂₆ O ₅	98.68	345.17223	283.17087 (72.63)	Rosmanol (Isomer 2)	(Almela, et al. 2006, Borrás-Linares, et al. 2014, Borrás Linares, et al. 2011, Ferlemi, et al. 2015, Señoráns, et al. 2000)
51	52.331	346.17802	345.17000	345.17203	-2.53	C ₂₀ H ₂₆ O ₅	91.86	-	-	Rosmanol (Isomer 3)	(Almela, et al. 2006, Borrás-Linares, et al. 2014, Borrás Linares, et al. 2011, Ferlemi, et al. 2015)

Rt: Retention Time

Table 2. Comparison of Rosemary infusions compositions

Compounds	Present work	Ferlemi et al.
Quinic acid	+	+
Syringic acid	+	-
Caffeic acid	+	derivatives
Rosmarinic acid 3-O-Glucoside	4 isomers	-
Luteolin 3'-O- Glucuronide	2 isomers	1 isomer
Nepitrin	+	-
Homoplantagin	4 isomers	1 isomer
Hesperidin	+	-
Rosmarinic acid	+	+
Luteolin-3'-o-(2"-o-acetyl)-β-d-glucuronide	3 isomers	1 isomer
Luteolin	+	-
Rosmanol	3 isomers	3 isomers
Cirsimaritin	-	+
Caffeic acid-O-hexoside derivative	-	+
Isorhamnetin-3-O-hexoside	-	+
Rosmadial	-	+
NotohamosinR	-	+
Carnosic acid	+	+
Carnosol	-	+
Chlorogenic Acid	3 isomers	-
Vanillic acid	+	-
6-O-Caffeoyl-β-d-fructofuranosyl-(2→1)-α-d-glucopyranoside	+	-
Primiverin/Primulaverin,	+	-
Luteolin-7-O-rutinoside	2 isomers	-
12-Hydroxyjasmonic acid	2 isomers	-
6-Hydroxyluteolin-7-glucoside	3 isomers	-
Nepitrin	+	-
Eucommin a	+	-
Pterogynoside	4 isomers	-
Feruloylnepitrin	2 isomers	-
Apigenin	+	-
Diomestin	+	-
Genkawinin	+	-

+: Present, -: Absent

Table 3. Analytical parameters of the quantification method

Analyte	Calibration Equations	Slope	SD	R ²	LOD (μM)	LOQ (μM)
Rosmarinic acid	$y = 20.792x - 26.474$	20.792	0.231	0.9995	0.04	0.111
Caffeic acid	$y = 17.396x - 30.69$	17.396	0.153	0.9985	0.03	0.088
Luteolin	$y = 17.736x - 20.639$	17.736	0.100	0.9984	0.02	0.056

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Table 4. Quantitative Rosemary infusion analysis

Peak N°	Rt (min)	Compound	C (mM)	Concentration (µg/1 g-dried rosemary)	Percentage of total quantified compounds
10	9.649	Caffeic acid	0.0165	5.9281	1.6055
14	16.861	6-Hydroxyluteolin-7-glucoside (Isomer 1)	0.0057	5.3244	1.4420
17	18.421	Rosmarinic acid-3-O-glucoside (Isomer 1)	0.0023	2.4371	0.6600
19	19.821	Luteolin-7-O-rutinoside (Isomer 2)	0.0020	2.3610	0.6394
20	20.090	6-Hydroxyluteolin-7-glucoside (Isomer 2)	0.0041	3.7596	1.0182
21	20.744	6-Hydroxyluteolin-7-glucoside (Isomer 3)	0.0033	3.0793	0.8339
23	21.257	Luteolin-3-glucuronide (Isomer 1)	0.0143	13.2162	3.5793
24	21.857	Rosmarinic acid-3-O-glucoside (Isomer 2)	0.0056	5.8519	1.5849
25	22.642	Nepitrin	0.0127	12.1382	3.2873
28	26.293	Rosmarinic acid	0.2196	158.1266	42.8249
32	28.387	Luteolin-3-glucuronide (Isomer 2)	0.0027	2.5396	0.6878
34	28.803	Feruloylnepitrin (Isomer 1)	0.0448	58.6396	15.8812
33	29.338	Rosmarinic acid-3-O-glucoside (3)	0.0049	5.1668	1.3993
36	32.073	Luteolin-3'-O-(2"-O-acetyl)-β-D-glucuronide (Isomer 1)	0.0082	8.2389	2.2313
38	33.004	Luteolin-3'-O-(2"-O-acetyl)-β-D-glucuronide (Isomer 2)	0.0064	6.4312	1.7418
39	34.229	Feruloylnepitrin (Isomer 2)	0.0323	42.2266	11.4361
40	35.288	Luteolin	0.0077	4.4010	1.1919
41	36.916	Luteolin-3'-O-(2"-O-acetyl)-β-D-glucuronide (Isomer 3)	0.0291	29.3734	7.9551
TOTAL			0.4222	369.239	100

Figure 1: Total ion chromatogram (TIC) of rosemary tea

A: Total ion chromatogram (TIC) of rosemary tea, B, C, D and E: Magnifications. 1: Quinic acid, 2: Syringic acid, 3: Vanillic acid, 4: 6-O-caffeoyl- β -D-fructofuranosyl-(2 $\rightarrow$ 1)- α -D-glucopyranoside, 5: Chlorogenic acid (Isomer 1), 6: Primiverin/Primulaverin, 7: Chlorogenic acid (Isomer 2), 8: Chlorogenic acid (Isomer 3), 9: Paeonolide, 10: Caffeic acid, 11: Luteolin-7-O-rutinoside (Isomer 1), 12: 12-hydroxyjasmonic acid (Isomer 1), 13: 12-hydroxyjasmonic acid (Isomer 2), 14: 6-hydroxyluteolin-7-glucoside (Isomer 1), 15: Pterogynosome (Isomer 1), 16: Pterogynosome (Isomer 2), 17: Rosmarinic acid-3-O-glucoside (Isomer 1), 18: Pterogynosome (Isomer 3), 19: Luteolin-7-O-rutinoside (Isomer 2), 20: 6-hydroxyluteolin-7-glucoside (Isomer 2), 21: 6-hydroxyluteolin-7-glucoside (Isomer 3), 22: Rosmarinic acid-3-O-glucoside (Isomer 2), 23: Luteolin-3-glucuronide (Isomer 1), 24: Rosmarinic acid-3-O-glucoside (Isomer 3), 25: Nepittrin, 26: Homoplantagin (Isomer 1), 27: Hesperidin, 28: Rosmarinic acid, 29: Homoplantagin (Isomer 2), 30: Eucommin A, 31: Pterogynosome (Isomer 4), 32: Luteolin-3-glucuronide (Isomer 2), 33: Rosmarinic acid-3-O-glucoside (Isomer 4), 34: Feruloylnepittrin (Isomer 1), 35: Unknown, 36: Luteolin-3'-O-(2''-O-acetyl)- β -D-glucuronide (Isomer 1), 37: Pterogynosome (Isomer 5), 38: Luteolin-3'-O-(2''-O-acetyl)- β -D-glucuronide

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(Isomer 2), 39: Feruloylnepitrin (Isomer 2), 40: Luteolin, 41: Luteolin-3'-O-(2''-O-acetyl)- β -D-glucuronide (Isomer 3), 42: Homoplantagin (Isomer 3), 43: Homoplantagin (Isomer 4), 44: Apigenin, 45: Diomestin , 46: Genkawinin, 47: Rosmanol (Isomer 1), 48: Unknown, 49: Carnosic acid, 50: Rosmanol (Isomer 2), 51: Rosmanol (Isomer 3)

33x47mm (600 x 600 DPI)

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Figure 2: Tandem mass spectrum (a) and fragmentation (b) pattern of Rosmarinic Acid found in rosemary infusion

33x47mm (600 x 600 DPI)

Figure 3. Chemical structures of rosmarinic acid (a) and eucommin a (b)

33x47mm (600 x 600 DPI)

Figure 4. Typical HPLC-DAD chromatogram of rosemary infusion composition

(Wavelength: 324 nm)

A: Typical HPLC-DAD chromatogram of rosemary infusion composition, B, C and D: Magnifications. 2: Syringic acid, 3: Vanillic acid, 4: 6-O-caffeoyl- β -D-fructofuranosyl-(2 $\rightarrow$ 1)- α -D-glucopyranoside, 5: Chlorogenic acid (Isomer 1), 6: Primiverin/Primulaverin, 7: Chlorogenic acid (Isomer 2), 8: Chlorogenic acid (Isomer 3), 9: Paeonolide, 10: Caffeic acid, 12: 12-hydroxyjasmonic acid (Isomer 1), 13: 12-hydroxyjasmonic acid (Isomer 2), 14: 6-hydroxyluteolin-7-glucoside (Isomer 1), 15: Pterogynoside (Isomer 1), 16: Pterogynoside (Isomer 2), 17: Rosmarinic acid-3-O-glucoside (Isomer 1), 18: Pterogynoside (Isomer 3), 19: Luteolin-7-O-rutinoside (Isomer 2), 20: 6-hydroxyluteolin-7-glucoside (Isomer 2), 21: 6-hydroxyluteolin-7-glucoside (Isomer 3), 22: Rosmarinic acid-3-O-glucoside (Isomer 2), 23: Luteolin-3-glucuronide (Isomer 1), 24: Rosmarinic acid-3-O-glucoside (Isomer 3), 25: Nepitrin, 26: Homoplantagin (Isomer 1), 27: Hesperidin, 28: Rosmarinic acid, 29: Homoplantagin (Isomer 2), 30: Eucommin a, 31: Pterogynoside (Isomer 4), 32: Luteolin-3-glucuronide (Isomer 2), 33: Rosmarinic acid-3-O-glucoside (Isomer 4), 34: Feruloylnepitrin (Isomer 1), 35: Unknown, 36: Luteolin-3'-O-(2''-O-acetyl)- β -D-glucuronide (Isomer 1), 37: Pterogynoside (Isomer 5), 38: Luteolin-3'-O-(2''-O-acetyl)- β -D-glucuronide (Isomer 2),

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39: Feruloylnepitrin (Isomer 2), 40: Luteolin, 41: Luteolin-3'-O-(2''-O-acetyl)- β -D-glucuronide (Isomer 3),
42:
Homoplantaginin (Isomer 3), 43: Homoplantaginin (Isomer 4)
33x47mm (600 x 600 DPI)

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