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Phenolic compounds characterization by LC-DAD- ESI/MSn and bioactive properties of *Thymus algeriensis* Boiss. & Reut. and *Ephedra alata* Decne

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ABSTRACT

Scientific research has been focused on finding natural occurring molecules from plant origin. Herein, infusion, decoction and hydroethanolic extracts of *Thymus algeriensis* Boiss. & Reut. and *Ephedra alata* Decne. from Algeria were phytochemically characterized by LC-DAD-ESI-MSn, and evaluated regarding bioactive properties (antioxidant and antibacterial). Flavonol and flavone glycoside derivatives and phenolic acids, specially rosmarinic acid and kaempferol-*O*-glucuronide were the major compounds in *T. algeriensis* extracts. Otherwise, *E. alata* presented isoflavones and flavonol derivatives as main compounds, being hydroxypuerarin isomer 1 the major molecule. Aqueous extracts had significantly higher antioxidant activity, being this activity correlated with the amount of phenolic compounds. Antimicrobial activity of the extracts was tested against multi-resistant bacteria strains from clinical isolates. The obtained MIC values indicate that the hydroethanolic extracts revealed the highest effect, especially the one of *T. algeriensis* against Gram-positive bacteria. Hence, these plant extracts could be used in the development of bioactive ingredients.

1. Introduction

Natural products from plant origin, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity (Shahidi & Ambigaipalan, 2015). These phytochemicals alone or in combination act as therapeutic agents in various disease complications (Ozcan, Akpinar-Bayizit, Yilmaz-Ersan, & Delikanli, 2014). Some of their potential different biological activities and properties have been well described, being mainly related with antioxidant (Carocho & Ferreira, 2013; Shahidi & Ambigaipalan, 2015), antimicrobial (Ozcan et al., 2014; Parsaeimehr, Sargsyan, & Javidnia, 2010) and antitumoral (Guimarães et al., 2014) properties.

Phenolic compounds are one of the most important classes of phytochemicals responsible for the possible protective role against oxidative damage diseases (coronary heart disease, stroke and cancers) (Carocho & Ferreira, 2013; Guimarães et al., 2014; Shahidi & Ambigaipalan, 2015). These molecules act against free radicals by antioxidant, redox and metal chelation capacity, acting as reducing agents, hydrogen donators or singlet oxygen quenchers (Carocho & Ferreira, 2013). However, the increase in some infectious diseases caused by bacteria and the prevalent resistance to antibiotics, urges the scientists to search for new medicinal compounds from natural origin, which are novel and more efficient (Wink, 2015). Consequently, it is essential to identify and measure all the bioactive constituents of medicinal plants in order to ensure the biological research reliability and repeatability as well as to ensure the quality control over the pharmacological benefits and/or hazardous. Numerous analytical procedures (TLC, LC–DAD, LC–FLD, LC–MS, GC and CE) have been used in herbal products detection and identification (Wu et al., 2013; Ziani et al., 2018). LC–MS plays a prominent role as an analytical tool for detecting and identifying pharmacologically active metabolites and/or reactive metabolites, due to its high selectivity, reproducibility and simplicity (Wu et al., 2013).

Traditional Algerian medicinal plants have been described as sources of valuable bioactive compounds (Ziani et al., 2018). Besides its desertic and semi-desertic areas, Algeria contains a large pool of plants with high bioactive potential that can be used for medicinal applications, and for which many of them have not been explored, wheras numerous ethnobotanical approaches indicate that the local Saharan flora has been traditionally used in many cultures as a source of medicinal agents. Many studies have focused on these plants with

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pharmacologically active compounds with a potential therapeutic interest (Benarba, 2016; Ziani et al., 2018). *Thymus algeriensis* Boiss. & Reut., belongs to the Lamiaceae family and is the most widespread North African species, endemic to Algeria and Tunisia. Fresh or dried, it is largely used only as a culinary herb, being its chemical composition already studied (Giweli, Džamić, Soković, Ristić, & Marin, 2013; Guesmi, Ben Farhat, Mejri, & Landoulsi, 2014). Although the results of its biological activity are still scarce, *T. algeriensis* is used in traditional medicine, as a fresh or dry seasoning, in respiratory and digestive tube disorders and against abortion (Giweli et al., 2013), being also used in the treatment of infectious diseases (e.g. gastrointestinal, dysentery, colds, diarrhea, prostate adenoma).

Ephedra alata Decne. (Ephedraceae family), Arabic name: Alanda, is a perennial genus of non-flowering seed with light green densely branched dioecious small and perennial stiff shrub, about 50–100 cm tall (Al-rimawi et al., 2017). The native land for this species is Iran, Algeria, Iraq, Chad, Egypt, Palestine, Lebanon, Jordan, Saudi Arabia, Morocco, Syrian Arab Republic, Libya, Mauritania, Mali, Somalia and Tunisia, where it grows wildly on the gravely rocky, sandy and clay soil in arid environments often near shifting sand dunes (Jaradat, Hussen, & Al Ali, 2015). The decoction of *E. alata* stem is used in the folk medicine as a potential stimulant, deobstruent, to treat different disorders (e.g. kidney, bronchi, circular system, digestive system disorders), to relief asthma attack and as antifungal (Al-Qarawi, Abd Allah, & Abeer, 2012). It is also used in cancer treatment and the plant stems are chewed to treat bacterial and fungal infections (Jaradat et al., 2015). Previous studies have shown that the aqueous extracts from this plant present in vitro anticancer activities, which have induced apoptosis, inhibiting proliferation with invasive behavior, causing the induction of cell cycle arrest, and the suppression of tumorangiogenesis (Shukla & Mehta, 2015). Furthermore, it has also shown antimicrobial effects (Ghanem & El-magly, 2008; Parsaeimehr et al., 2010).

To the authors' best knowledge, there are no published reports describing the phenolic composition of *T. algeriensis* and *E. alata*. Therefore, the present study aims to investigate these two Algerian medicinal plants from the arid regions, by using three types of extracts (aqueous extracts obtained by infusion and decoction, and hydroethanolic extracts), in order to identify the main bioactive compounds and evaluate their antioxidant and antibacterial activity.

2. Material and methods

2.1. Plant material and samples preparation

The two medicinal plant species: *Ephedra alata* Decne ssp. *alenda* (Ephedraceae) Local name: l'Alenda, and *Thymus algeriensis* Boiss. & Reut. (Lamiaceae) Local name: Mazoukcha, were respectively harvested from Biskra (34°50′14.2"N 5°37′49.4″E) and Tebessa (35°25′56.3"N 8°01′30.6″E) arid and semi-arid Eastern arias of Algeria, during September 2016. The selected sites and gathering practices took into account local consumer's criteria for the use of these species and the optimal growth stage and gathering period of each species. Only the aerial parts of these plants were used to prepare the extracts. The voucher specimens were deposited at the Department of Botanic of the National Superior School of Agronomy (ENSA), Algiers, where the morphological key characters from the Flora of Quezel and Santa (1963) were used for plant identification. The samples were shade-dried in a dark, dry place and at room temperature for 30 days, stored into cardboard bags, and further transported to the School of Agriculture, Polytechnic Institute of Bragança, Portugal, where all the analyses were carried out. The dry plants were grinded in the laboratory scale mill (Grindomix, Retsch, Germany) to obtain homogenous samples and stored at room temperature for subsequent use.

2.2. Extracts preparation

Infusion and decoction were the chosen methods according with traditional healers in order to mimic as closely as possible the traditional 'herbal' drug administration. An hydroethanolic extraction was also prepared for extractability and bioactivity comparison.

The infusion was obtained by extracting 1 g of the plant material at a ratio of 1:100 m/v, with distillated boiling water (100 °C), allowed to infuse for 5 min at room temperature, and then filtered through Whatman No. 4 paper. However, for decoctions preparation, 1 g of the plant material was accurately added to 100 mL distilled water and boiled together, afterwards, the mixture was left boiling for 5 min and then filtered through Whatman No. 4 paper.

The obtained infusions and decoctions were frozen and further lyophilized (FreeZone 4.5 model 7,750,031, Labconco, KS, USA).

For the hydroethanolic extraction, each sample (1 g) was extracted twice by maceration in ethanol/water (30 mL, 80:20, *v*/v) at 25 °C with constant stirring rate (150 rpm for 1 h) and subsequently filtered through a Whatman No. 4 paper. The combined hydroethanolic extracts were concentrated at 40 °C by a rotary vacuum evaporator (Büchi R-210, Flawil, Switzerland) and the remaining water residue was then lyophilized.

2.3. Phenolic compounds analysis by LC-DAD-ESI/MSn

The previous mentioned extracts were re-dissolved in water and in ethanol/water (80:20, v/v) for the aqueous and hydroethanolic extracts, respectively, and analyzed using a concentration of 5 mg/mL. LC-DAD-ESI/MSn analysis was carried out using chromatographic system Dionex Ultimate 3000 UPLC with a diode array detector (DAD) and connected to a Linear Ion Trap LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA, USA), following a procedure previously performed by Bessada, Barreira, Barros, Ferreira, and Oliveira (2016). This system consists of a diode array detector coupled to an electrospray ionization mass detector (LC-DAD-ESI/MSn). Waters Spherisorb S3 ODS-2 C_{18} column (3 µm, 4.6 × 150 mm, Waters, Milford, MA, USA) allowed chromatographic separation and the gradient used consisted of the following solvents (A) 0.1% formic acid (LAB-SCAN analytical sciences, Gliwice, Poland) in water, and (B) acetonitrile (LAB-SCAN analytical sciences, Gliwice, Poland). The gradient elution applied was: 15% B (0–5 min), 15%–20% B (5–10 min), 20–25% B (10–20 min), 25–35% B (20–30 min), and 35–50% B (30–40 min); the column was then re-equilibrated using a flow rate of 0.5 mL/min. Data were collected simultaneously with a DAD (280, 330 and 370 nm) and in a mass spectrometer. Negative mode was chosen for MS detection on a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Sheath gas (nitrogen) was kept on 50 psi. Other parameters settings: 325 °C of source temperature, 5 kV of spray voltage, −20 V of capillary voltage, −66 V of tube lens offset, and 35 arbitrary units of collision energy. The full scan captured the mass between *m/z* 100 and 1500. Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA) was used for data acquisition.

Quantification of these compounds was calculated from calibration curves (2.5–200 µg/mL) of each available phenolic standard (apigenin 6-*C*-glucoside, apigenin-8-*C*-glucoside, apigenin 7-*O*-glucoside, naringenin, quercetin 3-*O*-glucoside, rosmarinic acid, kampherol-3-*O*-rutinoside; myrcetin; isorhamnetin-3-*O*-glucoside; and quercetin-3-*O*-glucoside; all with ≥99% HPLC purity; Extrasynthèse, Genay, France). constructed upon the UV signal, and the results are expressed in mg per g of extract.

2.4. Bioactive properties of the prepared extracts

2.4.1. Antioxidant activity evaluation

The antioxidant activity was evaluated by DPPH radical-scavenging, reducing power (RP), inhibition of β-carotene bleaching in the presence

of linoleic acid radicals (CBI) and inhibition of lipid peroxidation in brain cell homogenates (TBARS). For all the extracts, serial dilutions (0.039–5.00 mg/mL) were prepared from the stock solution (5 mg/mL) and further submitted to four distinct in vitro assays previously described by the authors (Bessada et al., 2016). The extract concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene bleaching and TBARS assays) or absorbance at 690 nm (reducing power assay) against extract concentrations. Trolox was used as standard.

2.4.2. Antibacterial activity evaluation

2.4.2.1. Bacteria Stains. All the bacterial stains used were clinical isolates obtained from infected patients hospitalized in the Local Health Unit of Bragança and Hospital Centre of Trás-os-Montes and Alto-Douro-Vila Real, Northeast of Portugal. Six Gram-negative bacteria: *Morganella morganii* and *Pseudomonas aeruginosa* isolated from expectoration, *Escherichia coli*, *E. coli* extended producer of β-lactamases (ESBL), *Klebsiella pneumoniae* and *K. pneumoniae*, spectrum extended producer of β-lactamases (ESBL), isolated from urine; and four Gram-positive bacteria: *Enterococcus faecalis* isolated from urine, *Listeria monocytogenes* isolated from cerebrospinal fluid, Methicillin-sensitive *Staphylococcus aureus* (MSSA) isolated from wound exudate, and methicillin-resistant *S. aureus* (MRSA) were tested.

MicroScan panels (MicroScan®; SiemensMedical Solutions Diagnostics, West Sacramento, CA, USA) and microdilution plate method was used for the evaluation of the bacterial susceptibility to different antibiotics, using a methodology previously described by Dias et al. (2016). The determination of the MIC was performed according to the microdilution method, as described by Dias et al. (2016) using a rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay. Different antibiotics were used as standards for Gram-negative bacteria, such as amikacin for *K. pneumoniae* ESBL and *P. aeruginosa*, amoxicillin/clavulanic acid for *E. coli* and *K. pneumoniae* and gentamicin for *E. coli* ESBL.

2.5. Statistical analysis

Three repetitions of the samples were used and triplicates for each concentration were carried out in all the assays. Results are expressed as mean values and standard deviations (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $p = .05$ (SPSS v. 23.0 program). Furthermore, a Pearson's correlation analysis between the antioxidant activity and all the analyzed compounds was carried out, with a 95% significance level.

3. Results and discussion

3.1. Phenolic compounds profile

The phenolic compounds characterization of *Ephedra alata* and *Thymus algeriensis* was performed by applying LC-DAD-ESI/MSn to three different extracts obtained from infusion, decoction and maceration in hydroethanolic mixtures. Data regarding retention time, λ_{max}, pseudomolecular ion, main fragment ions in MS^2, tentative identification, and quantification of the individual compounds are summarized in Tables 1 and 2.

The UV–Vis and mass spectra obtained by LC-DAD-ESI/MS analysis showed that the phenolic composition of *T. algeriensis* was characterized by the presence of seventeen compounds where six were phenolic acid derivatives (compounds 7^{TH}, 11^{TH}, 12^{TH}, 15^{TH}-17^{TH}) and eleven were flavonoids linked to glycosyl groups (compounds 1^{TH}-6^{TH}, 8^{TH}-10^{TH}, 13^{TH}, 14^{TH}). As phenolic acids, compound 12^{TH} ($[M-H]^-$ at *m/z* 359, RT = 22.27 min) was positively identified according to the dissociation of the molecular ion which generated two fragment ions, that may correspond to the two main constituents of rosmarinic acid (Pacifico et al., 2016) and comparing with the characteristics of the commercial standard; it was the major phenolic compound present in the sample. Peak 7^{TH} ($[M-H]^-$ at *m/z* 521, RT = 17.58 min) presented 162 u (glycoside moiety) higher than compound 12TH, therefore being tentatively identified as rosmarinic acid hexoside. Peak 11^{TH} ($[M-H]^-$ at *m/z* 555, RT = 21.57 min) was identified as salvianolic acid K, due to a similar fragmentation pattern described by (Hauck, Gallagher, Morris, Leemans, & Winters, 2014). Compound 16^{TH} ($[M-H]^-$ at 717 *m/z*, RT = 29.97 min) was tentatively identified as salvianolic acid B (also known as lithospermic acid B), due to the fragmentation pattern with successive losses of 198 u (danshensu) or 180 u (caffeic acid) units (Zeng, Xiao, Liu, & Liang, 2006). Peaks 17^{TH} and 15^{TH}, presented the same pseudomolecular ion ([M–H]- at *m/z* 537) releasing also the same fragment ions at *m/z* 493 and 359, and taking into account the finding mention by several authors (Heleno, Martins, João, Queiroz, & Ferreira, 2015; Zeng et al., 2006); these compounds were tentatively assigned as lithospermic acid A isomer I and II.

The remaining compounds were identified as flavonoids, such as flavones (quercetin and kaempferol derivative), flavonols (apigenin and luteolin derivatives) and flavanones (eriodictyol and naringenin dervatives). Compounds 1^{TH}, 4^{TH} and 13^{TH} were identified as apigenin derivatives, being compound 4^{TH} positively identified by standard comparison with vitexin (apigenin-8-*C*-glucoside). Compound 1 ($[M-H]^-$ at *m/z* 593) released the MS^2 fragment ions at *m/z* 473 and 383 (loss of 120 and 90 mu characteristic of *C*-hexosyl flavones), and at *m/z* 353 corresponding to the apigenin aglycone (apigenin +83 mu, bearing some sugar residues), which allowed the identification of this compound as apigenin-*C*-hexoside-*C*-hexoside (Ferreres, Silva, Andrade, Seabra, & Ferreira, 2003). Thus, this compound was tentatively identified as apigenin-6,8-*C*-diglucoside. Compounds 8^{TH} ($[M-H]^-$ at *m/z* 461) and 13^{TH} ($[M-H]^-$ at *m/z* 445) released a unique fragment at *m/z* 285 and 269, respectively corresponding to luteolin and apigenin, with the loss of a glucuronyl moiety (−176 u), being tentatively assigned as luteolin-*O*-glucuronide and apigenin-*O*-glucuronide. These compounds were assigned to luteolin-7-*O*-glucuronide and apigenin-7-*O*-glucuronide, owing to the identification of this compound in other *Thymus* species (Miron, Plaza, Bahrim, Ibáñez, & Herrero, 2011; Vergara-Salinas, Pérez-Jiménez, Torres, Agosin, & Pérez-Correa, 2012). Similarly, peak 14^{TH} ($[M-H]^-$ at *m/z* 477, and MS^2 fragment ion at *m/z* 285) was tentatively assigned as kaempferol-*O*-glucuronide. Peaks 2^{TH} and 3^{TH} ($[M-H]^-$ at *m/z* 449) and 6^{TH} ($[M-H]^-$ at *m/z* 433), consistent with eridictyol-*O*-hexoside (isomer 1 and 2) and naringenin-*O*-hexoside, although the nature and position of the sugar residue could not be established. The samples presented three quercetin derivatives (λ_{max} around 354 nm, and an MS^2 fragment at *m/z* 301), being quercetin-3-*O*-glucuronide (compound 5^{TH}) and quercetin-3-*O*-glucoside (compound 9TH) positively identified by comparison with commercial standards. Peak 10^{TH} ($[M–H]^-$ at *m/z* 549) presented tree MS^2 fragments, corresponding to the losses of a malonyl (−86 u) and hexosyl (−162 u) moieties, thus being identified as quercetin-*O*-malonylglucoside.

Quercetin-3-*O*-glucoside was previously reported in *Thymus vulgaris* L. (thyme) (Vergara-Salinas et al., 2012), whereas luteolin-7-*O*-glucoside was identified in thyme infusions (Kulišić, Dragović-Uzelac, & Miloš, 2006), leaves and wild thyme (*T. serpyllum* Boiss.) (Miron et al., 2011; Vergara-Salinas et al., 2012). The presence of eriodictyol in different *Thymus* species has been reported by Boros et al. (2010) and Miron et al. (2011). Nonetheless, to the author's best knowledge the individual phenolic composition of *Thymus algeriensis* has not been previously reported.

Rosmarinic acid was the most abundant phenolic acid, while kaempferol-*O*-glucuronide was the main flavonoid present. Infusion preparations revealed the highest concentration of phenolic compounds, followed by decoctions and then hydroethanolic extracts (Table 1).

The LC-DAD-ESI/MSn profile of *E. alata* was characterized by the

presence of 10 phenolic compounds, all belonging to flavonoids class, namely five isoflavones and five flavones.

Peaks 1^{EA} and 2^{EA} ($[M-H]^-$ at *m/z* 479) and 6^{EA} ($[M-H]^-$ at *m/z* 461) were identified as myricetin 6-*C*-glucoside isomer 1 and 2 and as 5,5′-dihydroxy-3′-methoxyisoflavone-7-*O*-β-glucoside, taking into account the findings mentioned by Roriz, Barros, Carvalho, Santos-Buelga, and Ferreira (2014) in *Pterospartum tridentatum* L. flowers. Peak 3^{EA} ($[M-H]^-$ at *m/z* 445) released a fragment at *m/z* 283 ($[M-H-162]^-$, loss of a glycosyl moiety) and was assigned as sissotrin (i.e., biochanin A 7-*O*-glucoside). Peaks 4^{EA}, 5^{EA} and 7^{EA} all with the same pseudo-molecular ion $[M-H]^-$ at *m/z* 445, were identified as hydroxypuerarin isomers (hydroxydaidzein-8-*C*-glucoside isomers 1, 2, and 3), taking into account the characteristics identified by Prasain et al. (2007). Quercetin-3-*O*-rutinoside (compound 8^{EA}) and isorhamnetin-3-*O*-glucoside (compound 9^{EA}) were positively identified by comparison with commercial standards. Compound 10^{EA} ($[M-H]^-$ at *m/z* 461) released two fragments at *m/z* 431 (loss of a deoxyhexoyl moiety, −146 u) and 285 (loss of another deoxyhexoyl moiety, −146 u), corresponding to a kaempferol derivative, and was identified as kaempferol-*O*-di-deoxyhexoside.

To the authors' best knowledge, few previous works were conducted regarding the phenolic composition of *E. alata*. However, the presence of the myricetin derivatives, such as myricetin 3-rhamnoside have been also reported in the hydroethanolic extract of *E. alata* from Palestine (Al-rimawi et al., 2017). The isoflavones biochanin A and the diazein, as well as the quercitin were also previously detected in many ephedra-containing dietary supplements purchased from the market in arkansas-United states (Grippo, Capps, Rougeau, & Gurley, 2007).Whilst, kaempferol-3-*O*-rhamnoside and quercetin-3-*O*-rhamnoside were identified in *E. alata* growing in the Egyptian desert (Nawwar, El-sissi, & Barakat, 1984).

The most abundant compound was hydroxypuerarin isomer 1, and contrarily to *T. algeriensis*, the aqueous extract prepared by decoction revealed the highest amount of phenolic compounds (Table 2).

3.2. Antioxidant activity

The evaluation of the antioxidant activity of the extracts was performed by following four different in vitro protocols: 2,2-diphenyl-1-picrylhydrazyl (DPPH), reducing power, β-carotene bleaching inhibition and lipid peroxidation inhibition in brain cell homogenates. The results are summarized in Table 3. For both plants, all the preparations revealed antioxidant activity with different performances. The aqueous extracts (infusions and decoctions) showed very similar behavior with lower EC_{50} values, while the hydroethanolic extracts showed lower antioxidant activity (higher EC_{50} values). *T. algeriensis* infusion and decoction presented the lowest values (EC_{50} 26.3 µg/mL and 22.2 µg/mL, respectively) for the TBARS formation inhibition assay, meanwhile for *E. alata* infusion and decoction, RP assay revealed the most promising results. With the exception of CBI, *T. algeriensis* aqueous extract obtained by decoction demonstrated the highest antioxidant activity, whilst the infusions of *E. alata* showed the highest antioxidant activity. The mechanisms involved in the assays used to evaluate the antioxidant activity are different and, therefore, each plant preparation can have different compounds with specific capacities to participate in those mechanisms related to scavenging radical species, suppressing ROS/RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical production, thereby forming stable products, which do not initiate or propagate radical reactions (Carocho & Ferreira, 2013). Indeed, the solvent type had some influence on the antioxidant activity, as it was exemplified by the different activities measured in the hydroethanolic extracts, when compared to the remaining assayed extracts obtained through aqueous extraction.

Methanolic extract of the aerial parts of *T. hirtus sp algeriensis* Boiss. & Reut. growing in Gafsa, Tamerza and Kairouan in Tunisia (Guesmi et al., 2014), was also reported to have high antioxidant properties (in %) as free radical inhibitors and scavengers, exhibited by DPPH assay (93%), protonated radical ABTS (75%) and β-carotene bleaching inhibition (50%). To the authors' best knowledge, no reports are available on the infusion, decoction or hydroethanolic extracts of *T. algeriensis*. However, *E. alata* from Palestine (Al-rimawi et al., 2017), extracted with water, 80% ethanol, and pure ethanol has been reported as antioxidant, and the authors described higher activity, with higher contents in polyphenols, using higher polarity solvents. Thus, the highest radical scavenging activity (RSA) was reported for the hydroethanolic extract (78 µg/mL for DPPH and 57 µg/mL for ABTS); both aqueous and ethanol:water extracts presented high correlation factors between polyphenols and the antioxidant activity measured by ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC), DPPH and ABTS radical scavenging activity. Moreover, Jaradat et al. (2015) reported results for the methanolic extract of *E. alata* from Jenin, Palestine regarding RSA, also showing lower EC_{50} values (16.03 µg/mL for DPPH). Both mentioned studies described higher antioxidant activity, regarding the RSA assay, in comparison to the ones displayed by the presently studied hydroethanolic extract (540 µg/mL). This discordance noted in relation to our sample from Algeria is probably due to the effect of the region and climatic conditions affecting the biosynthesis of phenolic compounds, and to the effect of the extraction solvent used. This can generate quantitative differences on the bioactive molecules and therefore influences the antioxidant activity. Parsaeimehr et al. (2010) studied *Ephedra* wild culture species from Iran (*E. procera* Fisch. & Mey., *E. pachyclada* Boiss. and *E. strobilacea* Bunge), in which the methanolic extract of *E. strobilacea* in comparison to the other species revealed to have higher antioxidant activity evaluated on FRAP assay. The same trend was obtained with the hydroalcoholic mixture (ethanol, methanol and distilled water 7:3) of *Ephedra intermedia* Schrenk ex Meyer from Balochistan, Pakistan in DPPH free radical scavenging assay (Gul, Jan, Faridullah, Sherani, & Jahan, 2017). To the author's best knowledge there are no reports regarding *E. alata* infusions and decoctions, which are known to be the most common forms of consumption of this species.

To establish the relationship between phenolic compounds and the antioxidant activity, linear correlation coefficients were calculated. Positive significant ($p < .05$) correlations between the phenolic acids, such as rosmarinic acid, salvianolic acid K and B, lithospermic acid A isomer I, and all the antioxidant assays were established, proving the significance effect of these compounds in the radical scavenging effect, reducing power and lipid peroxidation inhibition of the studied *Thymus hirtus* extracts. Similarly, flavonoid derivatives (flavones and flavonols glycosylated), such as apigenin-*O*-glucuronide, apigenin-6,8-dihexoside and apigenin-8-*C*-glucoside, as well as kaempferol-*O*-glucuronide, luteolin-7-*O*-glucuronid, quercetin-*O*-malonyhexoside, quercetin-*O*-glucuronide and quercetin-3-*O*-glucoside showed a positive significant ($p < .05$) correlation with all the antioxidant tests. Regarding total phenolic acids and total flavonoid contents, positive significant correlations ($p < .05$) were established between total phenolic content and the antioxidant assays. A study performed by Guesmi et al. (2014), on the methanolic extract of the same plant from Tunisia, demonstrate similar positive correlations between some phenolic compounds (vanillic acid, tyrosin, vanillin, (+)-catechin hydrate, rutin) and radical scavenging assays (DPPH and ABTS). Nevertheless, these authors verified no correlation with total flavonoids. Otherwise, previous studies with several Lamiaceae species such as *Thymus vulgaris* L. (Ramkissoon, Mahomoodally, Ahmed, & Subratty, 2012), *Salvia officinalis* L., *S. verbenaca* L., *S. aegyptiaca* L. and *S. argentea* L. (Ben Farhat, Landoulsi, Chaouch-hamada, Sotomayor, & Jordán, 2013) demonstrated close correlations between polyphenolics and antioxidant activity (Guesmi et al., 2014), which could support the effectiveness of these compounds as antioxidants. Otherwise, correlation analysis established for *E. alata* extracts among the different antioxidant parameters and polyphenols exhibited no correlations with all the antioxidant assays.

Table 1
Retention time (Rt), waveleghts of maximum absorption in visible region (λ_{max}), mass spectral data, tentative identification and quantification of phenolic compounds in *Thymus algeriensis*.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (m/z)	Tentative identification	Quantification (mg/g extract)		
						Infusion	Decoction	EtOH/H_2O
1^{TH}	10.11	329	593	473(100),383(2),353(35),311(2),297(3)	Apigenin-6,8-*C*-dihexoside[A]	20.7 ± 0.1[a]	18.8 ± 0.1[b]	10.0 ± 0.5[c]
2^{TH}	11.30	284	449	287(100)	Erydictiol-*O*-hexoside isomer 1[B]	tr	tr	tr
3^{TH}	12.74	284	449	287(100)	Erydictiol-*O*-hexoside isomer 2 [B]	tr	tr	tr
4^{TH}	14.91	338	431	413(5),341(4),311(311)	Apigenin-8-*C*-glucoside[A]	7.6 ± 0.2[a]	6.8 ± 0.1[b]	3.99 ± 0.02[c]
5^{TH}	15.33	341	477	301(100)	Quercetin-3-*O*-glucuronide[C]	4.59 ± 0.01[a]	4.4 ± 0.1[b]	1.44 ± 0.02[c]
6^{TH}	17.01	283,326	433	271(100)	Naringenin-*O*-hexoside [B]	tr	tr	tr
7^{TH}	17.58	322	521	359(50),197(2),179(37),161(100),135(3)	Rosmarinic acid hexoside[D]	6.6 ± 0.1[b]	7.06 ± 0.05[a]	2.8 ± 0.1[c]
8^{TH}	18.72	262,274,346	461	285(100)	Luteolin-7-*O*-glucuronide[E]	8.9 ± 0.1[a]	7.8 ± 0.3[b]	3.06 ± 0.04[c]
9^{TH}	19.08	350	463	301(100)	Quercetin-3-*O*-glucoside[C]	4.6 ± 0.2[a]	4.3 ± 0.2[b]	1.73 ± 0.01[c]
10^{TH}	20.35	341	549	505(10),463(25),301(100)	Quercetin-*O*-malonyhexoside[C]	3.44 ± 0.02[a]	3.23 ± 0.04[b]	1.20 ± 0.02[c]
11^{TH}	21.57	288,323	555	493(12),359(10),179(36),161(100)	Salvianolic acid K[D]	27.2 ± 0.1[b]	28.6 ± 0.4[a]	13.3 ± 0.3[c]
12^{TH}	22.27	327	359	197(25),179(36),161(100)	Rosmarinic acid[D]	58.2 ± 0.3[a]	54.4 ± 0.9[b]	29.7 ± 0.7[c]
13^{TH}	23.19	335	445	269(100)	Apigenin-7-*O*-glucuronide[E]	12.6 ± 0.5[a]	11.6 ± 0.4[b]	5.75 ± 0.03[c]
14^{TH}	24.90	263,340	461	285(100)	Kaempferol-*O*-glucuronide[C]	65.0 ± 0.4[a]	62.2 ± 0.9[b]	16.7 ± 0.2[c]
15^{TH}	25.57	324	537	493(48),359(12),313(20),295(100),197(36),179(30),161(18)	Lithospermic acid A isomer I[D]	15.8 ± 0.2[a]	16.3 ± 0.5[a]	8.0 ± 0.3[b]
16^{TH}	29.97	278,334	717	519(100), 493(7),339(84), 321(5), 295(12), 197(9), 179(16)	Salvianolic acid B[D]	7.7 ± 0.2	7.1 ± 0.3	nd
17^{TH}	30.40	329	537	493(52),359(10),331(18),295(100),197(30),179(35),161(13)	Lithospermic acid A isomer II[D]	12.9 ± 0.2[a]	12.1 ± 0.1[b]	4.54 ± 0.02[c]
					Total phenolic acids	128.5 ± 0.2[a]	126 ± 2[b]	58.5 ± 0.3[c]
					Total flavonoids	127.5 ± 0.4[a]	119 ± 2[b]	43.8 ± 0.8[c]
					Total phenolic compounds	256.0 ± 0.2[a]	245 ± 4[b]	102.3 ± 0.5[c]

Phenolic compounds used for quantification: A- apigenin 6-*C*-glucoside (y = 107,025× - 61,531; R^2 = 0.9989; LOD = 0.19 µg/mL; LOQ = 0.63 µg/mL); B- naringenin (y = 18,433× + 78,903; R^2 = 0.9998; LOD = 0.17 µg/mL; LOQ = 0.81 µg/mL); C– quercetin 3-*O*-glucoside (y = 34,843× – 160,173; R^2 = 0.9998; LOD = 0.21 µg/mL; LOQ = 0.71 µg/mL); D- rosmarinic acid (y = 191,291× – 652,903; R^2 = 0.999; LOD = 0.15 µg/mL; LOQ = 0.68 µg/mL); *E*- apigenin 7-*O*-glucoside (y = 10,683× - 45,794; R^2 = 0.999; LOD = 0.10 µg/mL; LOQ = 0.53 µg/mL);. tr – traces (below LOQ), nd- not detected (below LOD).

Table 2
Retention time (Rt), waveleghts of maximum absorption in visible region (λmax), mass spectral data, tentative identification and quantification of phenolic compounds in *Ephedra alata*.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g extract)		
						Infusion	Decoction	EtOH/H_2O
1 [EA]	7.05	291,340	479	359 (6),341(36),221(23),167(28)	Myricetin-*C*-hexoside isomer 1 [A]	47.0 ± 0.1[b]	61 ± 2[a]	47.2 ± 0.7[b]
2 [EA]	9.95	290,340	479	359(5),341(36),221(25),167(26)	Myricetin-*C*-hexoside isomer 2 [A]	28.3 ± 0.2[b]	29.7 ± 0.4[a]	18.8 ± 0.2[c]
3 [EA]	13.78	255,320	445	283(100)	Biochanin A 7-*O*-glucoside (sissotrin)[B]	6.9 ± 0.2[b]	8.9 ± 0.5[a]	5.2 ± 0.1[c]
4 [EA]	14.85	262,340	431	341(5),311(100),283(4),239(3)	Hydroxydaidzein-8-*C*-glucoside isomer 1 (hydroxypuerarin isomer 1) [B]	118.9 ± 0.4[b]	159 ± 5[a]	89 ± 4[c]
5 [EA]	15.05	262,340	431	341(4),311(100),283(3),239(2)	Hydroxydaidzein-8-*C*-glucoside isomer 2 (hydroxypuerarin isomer 2) [B]	31.8 ± 0.2[b]	41.4 ± 0.4[a]	25.0 ± 0.1[c]
6 [EA]	15.74	263,336	461	446(4),341(100),282(3),283(2)	5,5′-Dihydroxi-methoxy-isoflavone-*O*-glucoside [B]	46.3 ± 0.3[b]	57 ± 1[a]	41.0 ± 0.5[c]
7 [EA]	16.23	262,340	431	341(17),311(100),283(5),239(4)	Hydroxydaidzein-8-*C*-glucoside isomer 3 (hydroxypuerarin isomer 3) [B]	6.2 ± 0.2[b]	10.6 ± 0.6[a]	5.9 ± 0.1[b]
8 [EA]	17.89	351	609	301(100)	Quercetin-3-*O*-rutinoside [C]	3.41 ± 0.02[b]	4.266 ± 0.003[a]	2.76 ± 0.02[c]
9 [EA]	18.25	368	477	357(100),342(6),314(4)	Isorhamnetin-3-*O*-glucoside [C]	2.15 ± 0.02[c]	4.31 ± 0.07[a]	2.3 ± 0.1[b]
10 [EA]	21.79	263,348	577	431(100),285(12)	Kaempferol-*O*-di-deoxyhexoside [C]	2.60 ± 0.07[b]	3.39 ± 0.07[a]	2.16 ± 0.01[c]
					Total flavonols	83.4 ± 0.2[b]	103 ± 2[a]	73 ± 1[c]
					Total isoflavones	210 ± 1[b]	277 ± 7[a]	167 ± 4[c]
					Total phenolic compounds	294 ± 1[b]	380 ± 9[a]	240 ± 5[c]

Phenolic compounds used for quantification: A- myrcetin (y = 23,287× − 581,708; R^2 = 0.9988; LOD = 0.37 μg/mL; LOQ = 0.98 μg/mL); B- naringenin (y = 18,433× + 78,903; R^2 = 0.9998; LOD = 0.17 μg/mL; LOQ = 0.81 μg/mL); C– quercetin 3-*O*-glucoside (y = 34,843× − 160,173; R^2 = 0.9998; LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL).

Table 3
Antioxidant activity of the different extracts from the studied plant species.

Plant extract	EC_{50} values (μg/mL)			
	DPPH scavenging activity	Reducing power	β-Carotene bleaching inhibition	TBARS inhibition
Thymus algeriensis				
Infusion	64.8 ± 0.7[b]	54.0 ± 0.5[b]	139 ± 4[b]	26.3 ± 0.2[b]
Decoction	48 ± 2[c]	49.8 ± 0.4[c]	149 ± 3[a]	22.7 ± 0.3[c]
EtOH/H_2O	131 ± 3[a]	100.2 ± 0.5[a]	85 ± 3[c]	40.3 ± 0.3[a]
Homoscedasticity* (*p*-value)	0.183	0.652	0.360	0.482
1-way ANOVA** (*p*-value)	< 0.001	< 0.001	< 0.001	< 0.001
Ephedra alata				
Infusion	450 ± 7[b]	108 ± 1[b]	131 ± 1[c]	128 ± 2[a]
Decoction	455 ± 6[b]	109 ± 3[b]	173 ± 3[b]	118 ± 2[b]
EtOH/H_2O	540 ± 3[a]	377 ± 4[a]	502 ± 8[a]	118 ± 4[b]
Homoscedasticity* (*p*-value)	0.028	< 0.001	< 0.001	< 0.001
1-way ANOVA** (*p*-value)	< 0.001	< 0.001	< 0.001	< 0.001

EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC_{50} values: 41 μg/mL (reducing power), 42 μg/mL (DPPH scavenging activity), 18 μg/mL (β-carotene bleaching inhibition) and 23 μg/mL (TBARS inhibition). [1]The results are presented as the mean ± SD.*Homoscedasticity among species was tested by the Levene test: homoscedasticity, $p > .05$; heteroscedasticity, $p < .05$. **p < .05 indicates that the mean value of the evaluated parameter of at least one species differs from the others (in this case multiple comparison tests were performed). For each species condition, means within a column with different letters differ significantly ($p < .05$).

3.3. Antibacterial activity

The emerging antimicrobial drug resistant (ADR) pathogens is a serious problem in clinical environment and the plant-derived phenolic compounds may act as antimicrobials with generally low toxic effects (Wink, 2015). Ten clinical isolates representing both Gram-positive and Gram-negative bacteria and with a high rate of resistance to drugs were tested. The results of the obtained MIC values of the infusions, decoctions and hydroalcoholic extracts prepared from both plants are summarized in Table 4. Results clearly demonstrated different degrees of bacteria growth inhibition. Gram-positive bacteria were more sensitive to the studied extracts presenting lower MICs ranging from 2.5 to 10 mg/mL. The decoction and infusion of *T. algeriensis* showed almost similar antibacterial activity by presenting similar MIC values, while the hydroethanolic extract revealed strongest activity as well as the hydroethanolic extract of *E. alata*, which exhibited MIC values of 5 mg/mL against MRSA and MSSA. Otherwise, the lowest effect was observed for Gram-negative bacteria, revealing MICs ranging from 5 to ≥20 mg/mL. *Pseudomonas aeruginosa* was the less susceptible/most resistant bacteria (MIC ≥20 mg/mL). For both plant species, infusions and decoctions had a weak effect, except against *E. coli* strains, which was the most susceptible microorganism with a MIC value of 0.625 mg/mL. Although, there are no reports on the antibacterial effects of *T. algeriensis* aqueous and organic extracts, according to a study developed by Guesmi et al. (2014), the MICs obtained for the essential oil of *Thymus hirtus* sp. *algeriensis* from Tunisia are slightly higher than chloramphenicol, ampicillin and streptomycin. Essential oil of *T. algeriensis* from Zentan (Libya) showed also significant antibacterial activity, especially against *S. aureus*, *Listeria monocytogenes* as Gram-positive bacteria and also against *P. aeruginosa*, *E. coli* and *S. typhimurium* as Gram-negative bacteria (Giweli et al., 2013). Regarding infusion and decoction of *E. alata*, no effects were observed for the tested bacteria stains. On the contrary, high antibacterial and antifungal effects of this plant was previously reported in extracts prepared with water, methanol and acetonitrile, the latter exhibiting the most potent effect against all the microorganisms supplied by the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt (Ghanem & El-magly, 2008). The higher antimicrobial of essential oils and acetonitrile extracts of *T. algeriensis* is related to less polar compounds in comparison to the ones present in water or water: ethanol extracts; furthermore, in the present work multiresistant clinical isolated were used, being much less susceptible than ATCC bacteria used in the reported study. The results described are satisfactory, since the bacteria resistance to antibiotics is turning into a global issue. In this perspective, the exploiting of matrices with antibacterial agents are of high interest for the application in the pharmaceutical industry.

The obtained results suggest that the extracts present phytochemicals with a broad-spectrum activity against both Gram-positive and

Table 4
Antimicrobial activity (MIC values mg/mL) of the prepared extracts from the studied plant species.

Antimicrobial activity MIC values (mg/mL)	*Thymus algeriensis*			*Ephedra alata*		
	Infusion	Decoction	EtOH/H_2O	Infusion	Decoction	EtOH/H_2O
Gram-negative bacteria						
Escherichia coli ESBL	5	10	5	20	20	5
Escherichia coli	5	10	5	20	20	5
Klebsiella pneumoniae ESBL	10	10	5	20	20	10
Klebsiella pneumoniae	10	10	5	20	20	10
Morganella morganii	10	10	5	20	> 20	20
Pseudomonas aeruginosa	20	20	20	> 20	> 20	20
Gram-positive bacteria						
Enterococcus faecalis	10	10	10	20	20	10
Listeria monocytogenes	10	10	10	20	20	10
MRSA	5	10	2,5	10	20	5
MSSA	5	10	2,5	10	20	5

MIC-minimal inhibitory concentration; MSSA - methicillin susceptible *Staphylococcus aureus*; MRSA- Methicillin resistant *S. aureus*.

Gram-negative bacteria. According to the chemical characterization presented above, flavonoids (flavones and flavonols) were the major phenolic class in *T. algeriensis*. These compounds may act as antibiotics due to their ability to complex with extracellular and soluble proteins and also with bacterial cell walls, often leading to their inactivation and loss of function (Cushnie & Lamb, 2011; Wink, 2015). Many research works identified the structure of flavonoids that possess antibacterial activity. Examples of such flavonoids found in the studied plants are apigenin derivatives (Nayaka, Londonkar, Umesh, & Tukappa, 2014), luteolin derivatives (Kozyra, Biernasiuk, & Malm, 2017), various quercetin glycosides and kaempferol and its derivatives (Teffo, Aderogba, & Eloff, 2010). Other flavones, flavone glycosides (Kozyra et al., 2017; Nayaka et al., 2014), isoflavones (Mukne, Viswanathan, & Phadatare, 2011), flavanones, isoflavanones, isoflavans, and flavonols (Cushnie & Lamb, 2011; Ziani et al., 2018), flavonol glycosides (Cushnie & Lamb, 2011) with antibacterial activity have also been identified. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Cushnie & Lamb, 2011). In addition, some authors have found that more highly oxidized phenols are responsible for bacterial inhibition (Wink, 2015). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins and inhibition of cytoplasmic membrane function, as well as the inhibition of energy metabolism of the bacteria (Wink, 2015).

Among the studied plants, *T. algeriensis* hydroethanolic extract presented the strongest activity against the tested bacterial strains, presenting the lowest MIC values, and being MSSA and MRSA the most susceptible bacteria to this extract. This activity may be due to the presence of less polar compounds present in the hydroethanolic extract, since this extract presented lowest content in phenolic compounds.

Overall, *T. algeriensis* and *E. alata* infusions, decoctions and hydroethanolic extracts revealed antioxidant activity and antibacterial effects against clinical isolates, correlated with the amount of phenolic compounds (mainly in aqueous extracts). To the authors' best knowledge, this is the first report on detailed phenolic composition obtained by LC-DAD-ESI/MSn. Phenolic acids (e.g., rosmarinic acid, salvianolic acid K and lithospermic acid) and glycolysed flavonoids (e.g. kaempferol-*O*-glucuronide, apigenin-6,8-*C*-dihexoside and apigenin-7-*O*-glucuronide) were the main phenolic compounds in *T. algeriensis* extracts. Whilst, for *E. alata* extracts, flavonols (e.g. myricetin-*C*-hexoside) and isoflavones (e.g. hydroxypuerarin and 5,5′-dihydroxi-methoxy-isoflavone-*O*-glucoside) were in majority. The present manuscript highlights the importance of natural products in the discovery of new bioactive compounds that may be successfully used as herbal drugs after in-vivo validation studies.

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