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HPLC–DAD–ESI-MS/MS screening of phytochemical compounds and bioactive properties of different plant parts of *Zizyphus lotus* (L.) Desf.

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Abstract

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Zizyphus lotus L. (Desf.) has been widely used as a homemade ingredient to treat numerous diseases in Algerian folk medicine. In this context, decoction, infusion and hydroethanolic extracts were prepared from the branches, leaves, roots, and stem barks. The extracts were characterized regarding their phytochemical composition by HPLC-DAD-ESI/MSⁿ and were assessed for *in-vitro* bioactivity (cytotoxicity, anti-inflammatory, and antibacterial activity). A total of 29 compounds (flavonoid derivatives and secoiridoids) were identified in *Z. lotus*, being 15 detected in leaves, 10 in branches, 5 in roots and 7 in stem barks. The highest content in these compounds, especially secoiridoids, were detected in branches followed by leaves, being oleuropein the main compound detected in leaves and oleoside in branches. The aqueous preparations of the leaves and root barks revealed the highest cytotoxicity and anti-inflammatory potential. On the other hand, for the antibacterial activity, the hydroethanolic extract of the branches showed the highest potential, especially against MSSA strain. Hence, the chemical diversity found in *Z. lotus* makes it a source of bioactive ingredients, that can be applied in the formulations of different ingredients for the food and pharmaceutical industry.

Keywords: *Zizyphus lotus* L. (Desf.), HPLC–DAD-ESI/MS, phytochemical compounds, cytotoxicity, ant-inflammatory activity, antibacterial activity.

Introduction

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Several health-promoting properties have been widely attributed to plant polyphenols, turning them in biomolecules for medicinal interest.^{1,2} These molecules are chemically diversified and lead to new medical trends through their mechanism of action in exerting their anti-proliferative,³ anti-inflammatory processes⁴ and antibacterial infections.⁵ In order to corroborate the pharmacological bioactivities attributed to these compounds, they are chemically characterized using sophisticated analytical techniques, with high resolution and sensitivity to identify and quantify with high precision groups of compounds present in different samples.⁶ Therefore, there are several reports describing that in the same plants, these molecules can be biosynthesized and distinctly distributed according to the botanical structures of the plant,⁷ being possible to find an heterogeneous distribution of these molecules depending on the organ, representing sometimes qualitative and quantitative differences.⁸ Besides, several extraction methods are currently used to obtain these phenolic compounds from natural matrices, such as decoctions, infusions, and organic extractions using different solvents to maximize the recovery of these molecules according to their physicochemical properties.⁹

Due to its privileged geographical position, Algeria contains a rich and varied flora offering an asset in the discovery of alterative biomolecules for the potential use in the pharmaceutical sector.¹⁰ The diverse bioclimatic stages stimulate the growth of wild medicinal species, such as *Zizyphus lotus* (L.) Desf. (Rhamnaceae), known as jujube,¹¹ which is native in the Mediterranean basin. In these regions, *Z. lotus* appears to be an essential home ingredient responsible for health benefits observed among the local population, due to its multiple effects found mostly in its leaves, fruits, and roots preparations.^{11,12} In Algeria, *Z. lotus* is consumed as infusions and/or decoctions to treat a variety of ailments, including urinary tract infections, digestive and intestinal disorders, and can also act as an hypoglycemic, antidiarrheal,

hypotensive, and anti-ulcer agent.^{13–15} Based on literature surveys¹², different parts of the plant were found to have a wide range of compounds, such as alkaloids, flavonoids, and saponins. Moreover, bark, fruit, leaves, roots, and seeds have been reported to possess antimicrobial,¹² antioxidant,¹⁶ antispasmodic¹⁷ anti-inflammatory,¹⁸ cytotoxic properties on human T-cell activation, analgesic, antinociceptive, anti-ulcerogenic, immunosuppressive^{15,19,20} and hypoglycemic¹¹ activities.

To the author's best knowledge, there are very limited reports on the phytochemical composition of *Z. lotus*, being no studies found regarding its composition in phenolic compounds and secoiridoids obtained from the different parts of this plant, by applying liquid chromatography coupled to a mass spectrometer (LC-MS). In this perspective, the present study aims to chemically characterize *Z. lotus* leaves, branches, root barks and stem barks aqueous and hydroethanolic extracts in terms of its phenolic and secoiridoids composition using HPLC-ESI-MS/MSⁿ. Furthermore, the obtained extracts were further evaluated regarding their cytotoxicity, anti-inflammatory, and antibacterial effects.

2. Materiel and methods

2.1. Plant material

The plants of *Zizyphus lotus* L. locally named as “Sedra” were collected in April 2017 in Oran in the West of Algeria. Species identification was conducted based on the botanical criteria of the authors²¹ and further authenticated by Professors of Botany of the University of Oran 1 (Algeria). From the same plant material, the samples were separated into different parts namely leaves, branches, root bark, and stem bark, and shade-dried until a constant weight is obtained. Subsequently, all the different parts were grounded to a fine powder (~40 mesh) and stored at 4°C for further analysis.

2.2. Samples preparation

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The aqueous extracts were prepared as closely as possible to the traditional preparations. For the infusions preparation, each plant sample (1 g) was added to boiling distilled water (200 mL) and remained at 25 °C for 5 min, while in the case of the decoctions preparation, 1 g of the samples were added to 200 mL of distilled water, and were heated simultaneously on a heating plate (VELP Scientific, Usmate, Italy) and left boil for 5 min. Afterwards, the decoction mixture was left to stand at 25 °C for an additional 5 min. The obtained residues were filtered, frozen (-20°C) and lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas City, MO, USA).

The hydroethanolic extracts were prepared by maceration, by adding a mixture of ethanol/water (80:20, v/v, 30 mL) to 1 g of dry powdered plant under agitation for 60 min at 25 °C. The residue was then filtered, and the procedure was repeated. The ethanol fraction was evaporated under reduced pressure using a rotary vacuum (rotary evaporator Büchi R-210, Flawil, Switzerland) at 35 °C, and the aqueous fraction was frozen (-20 °C), and further lyophilized.

2.3. Phytochemical profiling by HPLC-DAD-ESI/MSⁿ

The chromatographic analysis was performed according to Bessada et al.²² using a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system connected to a diode array detector (DAD, recording at 280, 330 and 370 nm) and a linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) operating in the negative. The extracts were prepared at a ratio of 10:1 (w/v) of dry extract/ultrapure water for aqueous extracts and mixture of ethanol/water (80:20 v/v, HPLC-grade) for hydroethanolic extracts. Determination of the phenolic and secoiridoids compounds was performed by comparison of the standard compounds (Extrasynthesis, Genay, France) with the samples in terms of retention times (RT)

and m/z fragments and with reported literature. Calibration curves were prepared by injecting known concentrations of the standards and were used for quantification basing on the UV-Vis signal of each available phenolic and secoiridoid standards. Results were processed using the Xcalibur® data system and expressed in mg per g of extract.

2.4. Bioactive properties

2.4.1. Cytotoxicity

The cytotoxicity of the plant samples were determined using the sulforhodamine B assays described by Barros et al.²³ against the human tumor cell lines HepG2 (Human liver cancer), MCF-7 (breast cancer), NCI-H460 (non-small cell lung cancer), and HeLa (cervical cancer). The samples were dissolved in water at a final concentration of 8 mg/mL (tested concentration range from 6.5 to 400 μ g/mL). Afterwards, serial dilutions were performed in a 96-well plate containing cells at a density of 3×10^4 cells/well. Moreover, a primary culture of non-tumor liver cells (PLP2) prepared from a freshly harvested porcine liver was used to determine the hepatotoxicity. Ellipticine was employed as positive control.

In each experimental set, the cells were plated in triplicate. The results of cell proliferation were recovered by a 96-well ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA). The results of the tested extracts were shown as means \pm standard deviation and indicate the concentration inhibiting 50% of the cell growth (GI_{50}).

2.4.2. Anti-inflammatory activity

The anti-inflammatory activity was estimated on Murine macrophage (RAW 264.7) cells using the LPS-induced NO-production assay according to the method previously described by Sobral et al.⁴ A stock solution of 8 mg/mL of the aqueous and hydroethanolic extracts, dissolved in ultrapure water was prepared and concentrations ranging from 6.5 to 400 μ g/mL

were tested. The tested concentrations were selected based on the cytotoxicity evaluation on the macrophages following the procedure described in the section 2.4.1 (data not shown). The inhibitory activity of NO production in the culture medium was measured by assaying the levels of NO₂ through the Griess reaction. Dexamethasone was used as positive control. Results were expressed as EC₅₀ values corresponding to the sample concentration achieving 50% of the inhibition of NO-production.

2.4.3. Antibacterial activity

For the estimation of the antibacterial activity, clinical pathogenic bacteria were isolated from hospitalized patients under antibiotic treatment in the local health unit of Bragança and hospital centre of Trás-os-Montes and Alto-Douro-Vila Real, Northeast of Portugal.

The microorganisms included 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 *Staphylococcus aureus* (MRSA, isolated from expectoration), and seven Gram-negative bacteria: *Escherichia coli*, Extended Spectrum Beta 151 Lactamase (ESBL)-producing *E. coli*, *Klebsiella pneumoniae* and Extended-Spectrum Beta152 Lactamase (ESBL)-producing *Klebsiella pneumoniae* and *Morganella morganii* (isolated from urine), *Proteus mirabilis* (isolated from wound exudate), and *Pseudomonas aeruginosa*, (isolated from expectoration). All the bacteria were grown in TSB culture medium and further incubated for 24 hours, at 37°C.

Minimal inhibitory concentrations (MICs): The obtained extracts were dissolved in TSB culture medium at a final concentration of 20 mg/mL as described by Pires et al.²⁴ The MICs were determined by the microdilution method and the colorimetric assay using INT (*p*-iodonitrotetrazolium chlorite, 0.2 mg/mL). From the stock solution of 20 mg/mL, 190 µL were pipetted to the first well of the 96-well microplates. Afterwards, successive dilutions

were performed over the wells containing 90 μL of culture medium. Afterwards, 10 μL of inoculum (1.5×10^8 CFU/mL) were pipetted to all the wells containing the tested concentrations (0.156 to 20 mg/mL), and the microplates were further incubated in an oven (Jouan, Berlin, Germany) at 37°C for 24 h. Three negative controls were prepared (one with medium, another one with the extracts, and a third one with medium and antibiotic); and a positive control with medium and inoculum. Ampicillin, imipenem and vancomycin were the used antibiotics.

The MICs were determined after the addition of the colorant INT (0.2 mg/mL, 40 μL) and incubation at 37°C for 30 min. The present viable microorganisms can reduce the yellow dye to a pink color,²⁴ being the MIC defined as the lowest concentration that prevented this change. The assays were carried out in triplicate. The MBC (minimal bactericidal concentrations) were determined from a sub cultured negative well without any bacterial growth and from the positive control, and further incubated at 37°C for 24 h.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's HSD test with $p = 0.05$ was conducted using the SPSS v. 23.0 program (IBM Corp., Armonk, New York, USA). Experimental results outcome in this study were presented as mean value \pm SD of three samples per plant part and three determinations ($n=9$).

3. Results and discussion

3.1. Phytochemical profiling by HPLC-DAD-ESI/MS

The different phytochemical compounds identified in the hydroethanolic, infusion and decoction extracts of the different plant parts of *Z. lotus* are shown in **Table 1**, as also the extraction yields. Among the twenty-nine reported molecules, two classes of compounds were

identified, namely flavonoids glycosides and secoiridoids. MS spectra were acquired in negative ionization mode and the UV–Vis spectra allowed a more precision assignment of the tentative identification of the compounds. The majority of the compounds have been previously identified in *Zizyphus* species, such as compounds 2-7, 9, 10, 11, 13-16, 18-20, 22, 23, 25, and 28.^{25–31} Thus, the remaining compounds (peaks 1, 8, 12, 17, 21, 24, 26, 27, and 29) were identified considering MS fragmentation pattern, UV-Vis spectra and information reported in literature^{32,33} and to the best of our knowledge have not been previously reported in *Z. lotus*. Moreover, (+)-catechin (peak 3), (-)-epicatechin (peak 5), myricetin-3-*O*-rutinoside (peak 8), quercetin-3-*O*-rutinoside (peak 14), kaempferol-3-*O*-rutinoside (peak 22), and oleuropein (peak 26), were positively identified with commercial standards.

Table 2 reports quantitative results of the identified compounds concentration present in the hydroethanolic, infusion and decoction extracts, expressed in mg per g of extract. The two aqueous extracts, infusions and decoctions, were studied because these are the most common form of consumption and application of this species plant parts, thus a comparison to a hydroethanolic was also considered in order to compare the extraction methodologies and solvents applied, due to the fact that the hydroalcoholic solvents typically extract higher amounts of phenolic compounds.³⁴ The branch samples, especially the hydroethanolic and the infusion extracts, presented the highest contents in these type of compounds (178 ± 3 and 170 ± 5 mg/g, respectively), followed by the decoction and hydroethanolic extracts of leaf extracts (166 ± 4 and 161 ± 5 mg/g, respectively). On the other hand, the aqueous preparations of stem bark samples, were the ones revealing the lowest amounts (10.5 ± 0.3 mg/g for the decoction and 16.5 ± 0.3 mg/g for the infusion).

The main compounds found in the leaf extracts were secoiridoids (ranging from 24 to 86%) and flavonol glycosides (ranging from 14 to 76%), being the most representative compound in the hydroethanolic and decoction extracts oleuropein, thus, the infusions presented quercetin-

3-*O*-rutinoside (peak 14) and myricetin-3-*O*-rutinoside (peak 8) as the most abundant molecules. Quercetin-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside)-7-*O*-rhamnoside (peak 6), quercetin-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside) (peak 7), quercetin-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside)-7-*O*-glucuronide (peak 10), kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside) isomers 1 and 2 (peaks 11 and 19), kaempferol-*O*-hexoside (peak 18), kaempferol-3-*O*-rutinoside (peak 22), and kaempferol-3-*O*-(6-*O*-rhamnosyl-glucoside) (peak 23) were only present in leave samples, which is in agreement with a previous report that identified these compounds in *Z. spina-christi* leaves.²⁵ Quercetin-3-*O*-rutinoside (peak 14) was identified in leaves and branches and were also previously reported in *Z. jujuba* and *Z. jujuba* var. *spinosa* leaves,²⁸ while quercetin-*O*-deoxyhexoside (peak 16) was identified in branch and stem bark samples being also identified in *Z. spina-christi* leaves.²⁵ Herein, the flavonol, myricetin-3-*O*-rutinoside (peak 8) present in leaves and root bark samples and the flavone, apigenin-*O*-hexoside-*O*-deoxyhexoside (peak 24), identified in leaves and branch samples, were reported for the first time in *Zizyphus* genus. Moreover, the well-known dihydrochalcone, phloretin, has been previously identified in the *Zizyphus* genus, such as phloretin-di-*C*-hexoside (peak 9 and 13). These compounds were detected in the herein studied leaves, which is also in agreement with their previous detection in leaves of *Z. lotus*.³⁵

Flavan-3-ols (peaks 2-5) were only detected in the root bark samples and represent 94-97% of the phytochemical compounds of these extracts. (Epi)catechin-(epi)gallocatechin (peak 2) and B-type (epi)catechin dimer (peak 4) were the most abundant compounds in root bark extracts, followed by (-)-epicatechin (peak 5), these compounds were previous report in the methanol/water extracts of *Zizyphus spina-christi* leaves,²⁵ as also in fruits of *Z. jujube*.³⁰ Moreover, (+)-catechin was also identified in the root bark samples (peak 3) and were also previously identified in leaves of *Z. lotus* (south of Algeria).³⁶

The stem bark samples were characterized by the presence of flavanone glycosides (22 to 88%), especially eriodictyol glycoside derivatives. Eriodictyol glycoside derivatives were the main compounds in the aqueous samples of stem barks, while oleoside was the major molecules in the hydroethanolic extract. To the author's best knowledge, this is the first study revealing the presence of these compounds in *Z. lotus*, such as of eriodictyol-*O*-hexoside (peak 15), eriodictyol-*O*-pentoside (peak 20), and eriodictyol-*O*-deoxyhexoside (peaks 25 and 28). Thus, eriodictyol aglycone and other derivatives have been previously described in fruits of other *Zizyphus* species.^{30,31}

A cyclopentane fatty acid derivative was also tentatively identified in branches and stem bark samples and to the best of our knowledge is was reported in this samples for the first time. This compound (peak 12) exhibited a deprotonated molecule at m/z 389, suggesting its identification as (-)-11-hydroxy-9,10-dihydrojasmonic acid 11- β -D-glucoside, which is a related compound of jasmonic acid, as previously reported by Karar and Kuhnert³³ for different plant parts of *Crataegus* genus.

Secoiridoids were detected for the first time in all the aerial parts (leaves, branches and stem barks) of *Z. lotus*; namely, oleoside (peak 1), oleuropein-*O*-hexoside (peak 17 and 21) and oleuropein (peak 26, 28, and 30). This group of compounds were also the most abundant in branch samples, ranging from 92 to 94 % of the total phytochemical content, being oleoside and oleuropein the main molecules present.

The differences observed in the phytochemical composition of this species could be due to many abiotic factors, but also due to different extraction methodologies and solvents applied for the obtainment of these extracts.^{37,38}

3.2. Bioactive properties of *Z. lotus*

3.2.1. Cytotoxicity

Systemic natural drugs obtained from plant matrices are taken up by oral administration routes majorly as aqueous extracts. *In-vitro* analyses are mandatory to evaluate possible induced cytotoxicity against the cells and tissues exposed to phenolic compounds in order to understand their mechanism and to exclude potential negative, off-target effects during the treatment.³⁹ Herein, different parts of *Z. lotus* (branches, leaves, root barks and stem barks) were analyzed for their cytotoxic effects using human tumor cells lines and non-tumor porcine liver cells, as shown in **Table 3**.

In general, the leaves and the root barks revealed the strongest cytotoxic potential with the lowest GI₅₀ values, especially the decoction extractions. Among the tested samples, the infusion and decoction preparations of leaves parts exerted the most potent cytotoxic activity, specifically against human hepatocellular carcinoma HepG2, with GI₅₀ values of 18.6 ± 0.4 and 41.7 ± 0.9 $\mu\text{g/mL}$, respectively. The decoctions of the leaves revealed also the strongest activity on HeLa and NCI-H460 cell lines with GI₅₀ values of 44 ± 2 and 66 ± 4 $\mu\text{g/mL}$, respectively. The presence of oleuropein in high concentration in leaves and branch extracts, as well as the presence of quercetin-3-*O*-rutinoside among other glycosylated flavonoids, offer a plausible explanation of the observed cytotoxicity. Previous studies have reported the cytotoxic potential of oleuropein which is a glycosylated secoiridoid possessing several free OH groups, responsible for a wide range of pharmacological properties including cytotoxicity.⁴⁰ Han et al.⁴¹ concluded that the oleuropein found in olive oil and olive leaves is able to inhibit the cell proliferation of breast cancer cells (MCF-7) and induce apoptotic cell death by certain mechanisms through the cell growth arrest after cell shrinkage, chromatin condensation and formation of apoptotic bodies, suggesting the activation of the caspases and cysteine proteases which are of central importance in the apoptotic signaling process. The same authors found that the oleuropein induce a significant block of G1 to S phase transition. In addition, according to Imran et al.⁴⁰ the cytotoxic potential of the oleuropein is variable and

depends on the cancer type; for example, in hepatic cancer cells, oleuropein suppress the cell viability and the expression of the activated AKT and activate caspase pathway, while for cervical cancer, the oleuropein arrest the G1 and G2/M phase, increase the phosphorylated ATF-2, c-Jun NH2-terminal kinase (JNK) protein, p53, p21, Bax, and cytochrome c protein.

The root bark samples also displayed effective cytotoxicity (lower GI_{50} values) toward hepatocellular HepG2 (GI_{50} from 48.3 ± 3 to 59.8 ± 2 $\mu\text{g/mL}$), breast MCF-7 (GI_{50} from 74 ± 7 to 111 ± 4 $\mu\text{g/mL}$), and cervical HeLa (GI_{50} from 69 ± 3 to 99 ± 6 $\mu\text{g/mL}$) carcinoma. Such significant action could be linked to the presence of flavan-3-ols, such as the (epi)catechin-(epi)gallocatechin (EC-EGC) described as a strong cytotoxic agents through the inhibition of cell proliferation and inducing apoptosis in different cancer cell lines.^{42,43} According to Babich et al.⁴⁴ the epicatechin gallate (CG) and the (epi)-gallocatechin-gallate (EGCG) found as the most abundant catechin in green tea, may principally induce oxidative stress in human gingival epithelioid (S-G) cells by H_2O_2 generation and tumor cell death by caspase-independent apoptic pathway, by excision of chromatin DNA loops domains responsible of DNA cleavage.

On the other hand, the less potential was shown by the hydroethanolic extracts of stem barks on the NCI-H460 cell line, exhibiting no effects on these cells at the maximum tested concentration ($GI_{50} > 400$ $\mu\text{g/mL}$). This could be due to lowest concentration of phytochemical compounds present in this part of the plant. These results highlight the possible differences between the plant structures in terms of bioactivity against all the studied cell lines.

Regarding the effects on the normal cell line (PLP2), in general, the majority of the tested samples presented cytotoxic effects on the PLP2 cells. However, the concentrations needed to cause this effect are higher than the ones needed to exert cytotoxic activity on the tumor cell lines; except for the leaves infusion and hydroethanolic extracts, that present cytotoxic effects

for tumor and normal cells in the same range of concentrations. From this perspective, root bark samples are the ones providing the best cytotoxic effects on tumor cells, without any effect in the normal cells. Contrarily, the absence of cytotoxicity on normal cells was reported in *Z. spina christi* leaves hydrometanolic extract from Sudan.²⁵ These authors reported that short-term application of *Z. spina-christi* leaves extracts barely exhibited any cytotoxic effects on human skin keratinocytes (HaCaT) or rat small intestine epithelial cells (IEC-6). This conclusion was based on the analyses of the plasma membrane integrity as well as the intracellular cytoskeletal architecture, which remained unaffected upon short-term treatment with the leaves extracts. Moreover, the activities of the mitochondrial reductase were not affected by *Z. spinachristi* extract incubation of IEC6 cells or HaCaT keratinocyte cultures.

Additionally, some authors have reported cytotoxic effect for *Z. lotus*, such is the case of the study performed by Benammar et al.¹⁹ which reported that the decoctions of the pulp, seeds, leaves, and stems of *Z. lotus* from Algeria inhibited T-cell blastogenesis by the incorporation of ³H-thymidine. Moreover, Abdoul-Azize et al.⁴⁵ indicated that the methanolic extract and ethyl acetate fraction of the fruit of *Z. lotus* from Morocco modulate cell signaling and exert immunosuppressive effects in human Jurkat T-cells.

It is well known that the phenolic compounds play an important role in the inhibition of oxidation, preventing the free radical production caused by inflammation and oxidative stress which play an important role in tumor initiation, promotion and progression.^{46,47} These findings, suggest that the observed cytotoxicity of *Z. lotus* could be mediated by the phytochemical compounds present in each part of this plant. These molecules are also described as able to protect against the carcinogenic process, through several mechanisms resulting in a reduced level of radical-mediated DNA.⁴⁶ The cytotoxic activity presented in the different parts of *Z. lotus*, varies depending on the mechanism and function of the number of phytochemical compounds present in each plant tissue.

3.2.2. Anti-inflammatory activity

There are well-established anti-inflammatory models, conducted *in-vitro* and *in-vivo* that are currently used to evaluate the expression of the cells induced in the inflammatory process being exposed to plant extracts.⁴⁸ In the present study, the nitric oxide inhibitory activity of hydroethanolic and aqueous extracts (decoction and infusions) of different parts of *Z. lotus* was tested by measuring their effects on the pro-inflammatory mediators NO in activated RAW 264.7 cells (**Table 3**). According to the obtained results, stem barks did not reveal any anti-inflammatory potential (at the maximum tested concentration ($EC_{50} > 400 \mu\text{g/mL}$), and with the exception of root barks, only the aqueous extracts of leaves and branches showed activity. Thus, all the extracts prepared from root barks presented the highest anti-inflammatory activity (lower EC_{50} values), especially the hydroethanolic extract, revealing an EC_{50} value of $72.06 \pm 4 \mu\text{g/mL}$.

These results support previous cytotoxicity outcomes, showing that the aqueous and hydroethanolic extracts of the same parts (leaves and root barks) revealed higher biological activity, suggesting that the same compounds (oleuropein, catechin, and their derivatives) that have a higher number of hydroxyl groups are also responsible for the anti-inflammatory process. According to a previous work on olive leaves extracts,⁴⁹ the oleuropein abundantly present in the different parts of olive tree (leaves, fruits and stems) have enhanced nitric oxide production by a significant inhibition of tumor necrosis factor ($\text{TNF}\alpha$) secretion, from polymorphonuclear cells (PMNCs) after LPS stimulation inducing. This biphenol can be hydrolyzed to hydroxytyrosol, oleuropein aglycone, elenolic acid, and glucose. The same effects have been found in extracts containing considerable amounts of catechin and/or derivatives, that are known to exhibit potent anti-inflammatory properties. Nakanishi et al.⁵⁰ studied the anti-inflammatory effects of epigallocatechin-3-gallate (EGCG) and epicatechin gallate (ECG), the major components of green tea, on the expression of pro-inflammatory

cytokines and adhesion molecules in human dental pulp cells stimulated with bacteria-derived factors such as lipopolysaccharide (LPS) and peptidoglycan (PG). These authors reported that the presence of EGCG and ECG significantly reduced, in a concentration-dependent manner, the expression of interleukin (IL)-6 and IL-8 in dental pulp cells exposed to LPS or PG. Moreover, an increased on the expression of intercellular adhesion molecule-1 (ICAM-1) and of vascular cell adhesion molecule-1 (VCAM-1) on the dental pulp cells in response to bacterial components was also decreased by the treatment with EGCG and ECG. Ohishi et al.⁵¹ verified that green tea EGCG acts as an antioxidant to scavenge reactive oxygen species, leading to the attenuation of nuclear factor- κ B activity resulting to the gene and/or protein expression of inflammatory cytokines and inflammation-related enzymes. In another study, Fan et al.⁵² revealed that catechin and its derivatives can exert their significant anti-inflammatory properties by regulating the activation or deactivation of inflammation-related oxidative stress-related cell signaling pathways, such as nuclear factor-kappa B (NF- κ B), mitogen-activated protein kinases (MAPKs), transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), signal transducer and the activator of transcription 1/3 (STAT1/3) pathways.

Analysing other reports on the anti-inflammatory potential of *Z. lotus* from different origins, the aqueous and methanolic extracts of Tunisian *Z. lotus* root barks showed significant anti-inflammatory effects on the carrageenan-induced paw edema.¹⁵ Also, the flavonoid and saponin fractions from the leaves and root barks for the same species exhibited moderate anti-inflammatory potential on carrageenan-induced paw edema in rats and inhibitory effect on NO production in LPS activated RAW 264.7 macrophages.²⁰ The hydroethanolic extracts of *Z. lotus* leaves from Algerian Sahara Atlas also had the ability to inhibit lipoygenase.⁵³ These data highlights the ability of phenolic compounds to exert anti-inflammatory activity through NO inhibition and through the inhibition of other markers of inflammation.^{52,54,55}

3.2.2. Antibacterial activity

Phenolic compounds play also an important role regarding the bacterial inhibition. The discovery of new and effective antibacterial agents is mandatory, given the constant reports on the bacterial resistant to antibiotics.⁵⁶ The diversity of chemical structures present in the phenolic compound's family may be an effective alternative in the treatment of bacterial infections.⁵⁷ In this perspective, the aqueous and hydroethanolic extracts of the different parts of *Z. lotus* were assessed for their antibacterial action against Gram-positive and Gram-negative multi-resistant bacterial strains (**Table 4**). All the tested samples presented antibacterial activity. The strongest activity was recorded by the branch hydroethanolic extracts registering MIC values in a range of 0.3125 to 10 mg/mL. MSSA was highly susceptible to the decoction and hydroethanolic extracts of the branches (MIC= 0.3125 mg/mL), while MRSA showed higher sensitivity to the leaves infusion (MIC= 0.625 mg/mL). The branches hydroethanolic extracts, the leaves infusion and the barks decoctions, exert a significant action on *P. aeruginosa*, which was the most sensitive Gram-negative bacteria. Meanwhile, *E. faecalis*, *K. pneumoniae*, *K. pneumoniae* ESBL, and *P. mirabilis* were the most resistant strains (MIC \geq 20 mg/mL). In addition, for the majority of the bacteria, a bactericidal effect was noticed during the determination of MBC.

The observed differences between Gram-negative and Gram-positive bacteria may be attributed to cell membrane structure and permeability, due to the fact that the outer membrane (lipopolysaccharide membrane) of Gram-negative bacteria acts as a barrier to many environmental substances including antibiotics.⁵⁶

There are other reports describing that *Zizyphus* species exhibit a strong antimicrobial capacity.^{58–60} Naili et al.⁶¹ described the antibacterial potential of *Z. lotus* leave extracts from Libya, evaluated through the conventional paper disc assay, and reported promising inhibitory

effects against Gram-positive than Gram-negative bacterial species. Ait Abderrahim et al.⁶² reported an antibacterial activity of a methanolic extract obtained from *Z. lotus* stems from Algeria, against *S. aureus* (MIC= 7 mg/mL), *E. coli* (MIC= 6 mg/mL) and *P. aeruginosa* (MIC= 6 mg/mL). Moreover, Benslama et al.³⁶ also evaluated the antibacterial capacity of a methanolic extract of *Z. lotus* leaves from south of Algeria through the diffusion disc method and reported a strong effect against *S. aureus*, *Micrococcus luteus* and *Bacillus subtilis*, but no activity against *P. aeruginosa* and *K. pneumonia*. The same authors also described that the aqueous extracts of this plant did not show antibacterial activity, which is contrarily to the obtained results in this study, which could be due to the different extraction methodologies applied. Moreover, a bactericidal activity was also observed against *E.coli*, *Enterobacter aerogenes*, *Serratia liquenfaciens*, *Staphylococcus sp.*, *Streptococcus sp.*, *Lactobacillus sp.* with a MIC between 62.5 and 2000 µg/mL by the methanolic extract of Algerian *Z. lotus* root's, estimated by the microdilution method.⁶³

The phytochemical composition of each plant part, characterized in the previous section, may be responsible for the effect noted during the antibacterial analysis. In fact, the presence of these molecules, containing several hydroxyl groups on their molecular structure, can provide a preliminary explanation of their mechanism of action. The oleuropein has been shown to have strong antibacterial activity against both Gram-negative and Gram-positive bacteria, since it seems to produce its effect by damaging the bacterial membrane and/or disrupting cell peptidoglycans,^{64,65} although, some authors have proposed that this activity is due to the presence of the ortho-diphenolic system (catechol).⁶⁶ Saija and Uccella⁶⁷ proposed that the glycoside group modifies the ability to penetrate the cell membrane and get to the target site and modulate the activity of several extra- and intracellular enzymes. Oleuropein, as well as the galloylated catechin and the EGCG, have a quenching behavior of the chromophore group which is closer to 5-NS probe in the phospholipid membranes composed of

phosphatidylcholine.^{68,69} These features may allow an interaction with the molecular targets both in water-soluble and lipophilic environments, leading to the bacterial inactivation and loss of pathogenicity.

Indeed, the eriodictyol and quercetin glycoside derivatives, may also be able to inhibit the bacterial growth. A study on the antibacterial activity of these isolated compounds from the roots and stems of *Bauhinia sirindhorniae* revealed that (2S)-eriodictyol was found active against *B. subtilis*.⁷⁰ In another study, eriodictyol was the most active component and inhibited several bacterial strains, such as *E. coli*, *Salmonella enterica*, *Pseudomonas putida*, *B. subtilis*, *Listeria innocua*, *Lactococcus lactis*, *S. aureus*, and *Saccharomyces cerevisiae*.⁷¹ Also, the antibacterial properties of quercetin and its glucoside derivatives have already been reported in several studies as an antimicrobial agent, inhibiting the growth of a wide spectrum of bacteria strains, such as *E. coli*, *S. aureus*, *Shigella flexneri*, *Proteus vulgaris*, *P. aeruginosa*, and *Lactobacillus casei* var *shirota*.^{66,72–74}

4. Conclusions

Overall, it can be concluded that all the plant parts may be a potential source of bioactive compounds. The abundance of oleuropein and flavonoid derivatives, biosynthesized in different pathways according to the plant structure (leaves, branches, stem and root barks) are the main responsible compounds for the exhibited bioactivities. The root bark extracts revealed the strongest bioactivity among the different accessed bioassays, possibly due to the presence of flavan-3-ol derivatives, such as (epi)catechin-(epi)gallocatechin, (+)-catechin, type B (epi)catechin dimer and (-)-epicatechin, compounds that were not identified in the other plant parts. The observed differences between the different plant tissues reveal their pharmacological potential and also suggest that *Z. lotus* leaves and root barks can be envisaged as promising candidates to be used by the food and pharmaceutical industry. This study also corroborates the use of this plant for therapeutic purposes in traditional medicine.

As a future perspective, the present results provide useful insight for searching newly isolated compounds that may attract particular attention as *in-vivo* targets.

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Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{\max}), mass spectral data, identification of phytochemical compounds in *Z. lotus* extract.

Peak	Rt (min)	λ_{\max} (nm)	[M-H] ⁺ (<i>m/z</i>)	MS ² (<i>m/z</i>)	Tentative identification	References
1	5.26	288	389	345(100),209(32),165(12),121(11)	Oleoside	32
2	5.73	278	593	467(51),441(100),423(60),305(31),287(6)	(Epi)catechin-(epi)gallocatechin	25
3	7.05	280	289	245(100),205(33),179(10)	(+)-Catechin	Standard, DAD-MS; ²⁵
4	7.82	282	577	425(100),407(18),287(9)	Type B (epi)catechin dimer	²⁶
5	9.58	284	289	245(100),205(32),179(11)	(-)-Epicatechin	Standard, DAD-MS; ^{25,75}
6	14.27	355	901	755(60),609(41),301(100)	Quercetin-3- <i>O</i> -(2,6-di- <i>O</i> -rhamnosyl-glucoside)-7- <i>O</i> -rhamnoside	²⁵
7	14.62	350	755	609(45),301(100)	Quercetin-3- <i>O</i> -(2,6-di- <i>O</i> -rhamnosyl-glucoside)	²⁵
8	14.73	350	625	317(100)	Myricetin-3- <i>O</i> -rutinoside	Standard, DAD-MS
9	15.42	286,sh340	597	477(100),417(33),387(72),357(7)	Phloretin-di- <i>C</i> -hexoside	²⁵
10	15.55	353	931	755(44),301(100)	Quercetin-3- <i>O</i> -(2,6-di- <i>O</i> -rhamnosylglucoside)-7- <i>O</i> -glucuronide	²⁵
11	16.52	344	739	593(35),575(100),473(16),285(41)	Kaempferol-3- <i>O</i> -(2,6-di- <i>O</i> -rhamnosyl-glucoside) isomer 1	²⁵
12	17.32	307,sh317	389	227(100)	(-)-11-hydroxy-9,10-dihydrojasmonic acid	³³
13	17.42	286,sh340	597	477(100),417(8),387(28),357(37)	11- β -D-glucoside	²⁵
14	17.9	352	609	301(100)	Phloretin-di- <i>C</i> -hexoside	Standard, DAD-MS ; ²⁷⁻³⁰
15	18.23	292,sh340	449	287(100)	Quercetin-3- <i>O</i> -rutinoside	^{30,31}
16	18.64	350	447	301(100)	Eriodictyol- <i>O</i> -hexoside	²⁵
17	19.14	232,sh281	701	539(100), 377(10),307(3),275(3)	Quercetin- <i>O</i> -deoxyhexoside	²⁵
18	19.2	348	447	285(100)	Oleuropein hexoside	³²
19	19.63	344	739	593(100),447(47),285(10)	Kaempferol- <i>O</i> -hexoside	²⁵
20	19.68	292,sh340	419	287(100)	Kaempferol-3- <i>O</i> -(2,6-di- <i>O</i> -rhamnosyl-glucoside) isomer 2	^{30,31}
21	20.61	232,sh281	701	539(100),377(11),307(5),275(3)	Eriodictyol- <i>O</i> -pentoside	³²
22	21.21	342	593	285(100)	Oleuropein hexoside	³²
23	21.52	340	593	447(22),285(100)	Kaempferol-3- <i>O</i> -rutinoside	Standard, DAD-MS; ⁷⁵
24	21.82	336	577	415(100),269(10)	Kaempferol-3- <i>O</i> -(6- <i>O</i> -rhamnosyl-glucoside)	⁷⁵
25	23.06	292,sh338	433	287(100)	Apigenin- <i>O</i> -hexoside- <i>O</i> -deoxyhexoside	³³
26	23.84	232,sh280	539	377(100),307(42),275(28)	Eriodictyol- <i>O</i> -deoxyhexoside	^{30,31}
27	24.99	232,sh281	539	377(100),307(42),275(27)	Oleuropein	Standard, DAD-MS, ³²
28	25.01	292,sh340	433	287(100)	Oleuropein isomer 1	³²
29	26.77	232,sh280	539	377(100),307(67),275(56)	Eriodictyol- <i>O</i> -deoxyhexoside	^{30,31}
					Oleuropein isomer 2	³²
Peaks	Branches	Leaves	Root barks	Stem barks		

Table 2. HPLC-DAD quantitative (mg/g of extract) evaluation of phytochemical compounds in different parts of *Z. lotus* extract (mean \pm SD, n=9).

	Decoction	Infusion	Hydroethanolic	Decoction	Infusion	Hydroethanolic	Decoction	Infusion	Hydroethanolic	Decoction	Infusion	Hydroethanolic
Yield	21.9±0.8	27.7±0.7	12.16±0.09	32.2±0.5	27.7±0.5	25.7±0.7	19.0±0.6	14.8±0.2	16.09±0.06	21.4±0.7	15.2±0.4	18.1±0.2
1	48±1c	73±4b	89±2a	nd	nd	nd	nd	nd	nd	2.16±0.08e	1.95±0.06e	23.7±0.3d
2	nd	nd	nd	nd	nd	nd	13.7±0.2b	4.84±0.02c	24.28±0.08a	nd	nd	nd
3	nd	nd	nd	nd	nd	nd	7.5±0.4*	0.644±0.001*	nd	nd	nd	nd
4	nd	nd	nd	nd	nd	nd	13.3±0.4a	5.2±0.3b	5.2±0.1b	nd	nd	nd
5	nd	nd	nd	nd	nd	nd	7.8±0.2 ^a	4.0±0.1 ^c	5.5±0.2 ^b	nd	nd	nd
6	nd	nd	nd	1.76±0.07 ^a	1.65±0.02 ^b	1.51±0.04 ^c	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	1.9±0.1 ^a	1.98±0.04 ^a	1.60±0.04 ^b	nd	nd	nd	nd	nd	nd
8	nd	nd	nd	2.7±0.2 ^b	3.10±0.06 ^a	2.49±0.04 ^c	1.25±0.02 ^d	1.01±0.02 ^c	1.08±0.01 ^e	nd	nd	nd
9	nd	nd	nd	tr	tr	tr	nd	nd	nd	nd	nd	nd
10	nd	nd	nd	1.40±0.02 ^b	1.46±0.03 ^a	1.28±0.04 ^c	nd	nd	nd	nd	nd	nd
11	nd	nd	nd	1.44±0.01 ^b	1.47±0.03 ^a	1.16±0.01 ^c	nd	nd	nd	nd	nd	nd
12	nq	nq	nq	nd	nd	nd	nd	nd	nd	nq	nq	nq
13	nd	nd	nd	2.86±0.05 ^b	3.7±0.1 ^a	2.55±0.04 ^c	nd	nd	nd	nd	nd	nd
14	1.30±0.01 ^d	1.29±0.01 ^d	1.24±0.01 ^d	4.1±0.1 ^c	4.96±0.08 ^b	5.95±0.08 ^a	nd	nd	nd	nd	nd	nd
15	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.19±0.01*	tr	0.41±0.01*
16	1.00±0.02 ^{bc}	1.08±0.05 ^a	1.00±0.02 ^{bc}	nd	nd	nd	nd	nd	nd	1.028±0.003 ^b	0.989±0.001 ^c	1.002±0.002 ^b
17	8.13±0.4 ^c	12.0±0.5 ^a	9.0±0.1 ^b	nd	nd	nd	nd	nd	nd	nd	nd	nd
18	nd	nd	nd	1.44±0.02 ^c	1.82±0.03 ^b	2.3±0.01 ^a	nd	nd	nd	nd	nd	nd
19	nd	nd	nd	1.72±0.03 ^b	1.83±0.04 ^a	1.50±0.01 ^c	nd	nd	nd	nd	nd	nd
20	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.08±0.05 ^b	10.1±0.3 ^a	0.27±0.01 ^c
21	nd	nd	nd	3.2±0.1 ^c	4.33±0.04 ^a	3.57±0.03 ^b	nd	nd	nd	nd	nd	nd
22	nd	nd	nd	1.189±0.003 ^c	1.34±0.03 ^a	1.24±0.02 ^b	nd	nd	nd	nd	nd	nd
23	nd	nd	nd	1.13±0.03 ^c	1.50±0.03 ^a	1.073±0.004 ^b	nd	nd	nd	nd	nd	nd
24	4.51±0.03 ^c	6.3±0.1 ^a	5.45±0.1 ^b	1.51±0.07 ^c	2.46±0.01 ^d	1.55±0.02 ^c	nd	nd	nd	nd	nd	nd
25	2.37±0.06 ^d	4.3±0.2 ^a	3.23±0.1 ^b	nd	nd	nd	nd	nd	nd	4.2±0.1 ^a	2.86±0.06 ^c	3.19±0.01 ^b
26	51±2 ^c	37.6±0.2 ^c	46±1 ^d	139±4 ^a	4.4±0.1 ^f	133±5 ^b	nd	nd	nd	nd	nd	nd
27	7.7±0.4 ^c	14.6±0.4 ^a	11.1±0.2 ^b	nd	nd	nd	nd	nd	nd	nd	nd	nd
28	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.87±0.04 ^b	0.59±0.03 ^c	1.63±0.05 ^a
29	15.1±0.5 ^b	19.6±0.4 ^a	12.4±0.5 ^c	nd	nd	nd	nd	nd	nd	nd	nd	nd
SPC	140±4 ^d	170±5 ^b	178±3 ^a	166±4b ^c	36.0±0.5 ^f	161±5 ^c	43.5±0.8 ^c	15.7±0.3h ⁱ	36.1±0.2 ^f	10.5±0.3 ⁱ	16.5±0.3 ^b	30.2±0.3 ^s

nd – not detected. nq- not quantitate. SPC – sum of phytochemical compounds. tr- traces. Standard calibration curves: (1) catechin ($y = 84950x - 23200$, $R^2 = 0.9999$); (2) myricetin-3-*O*-glucoside ($y = 23287x - 581708$, $R^2 = 0.9988$); (3) quercetin-3-*O*-glucoside ($y = 34843x - 160173$, $R^2 = 0.9998$); (4) kaempferol-3-*O*-glucoside ($y = 11117x + 30861$, $R^2 = 0.9999$); (5) apigenin-7-*O*-glucoside ($y = 10683x - 45794$, $R^2 = 0.9999$); (6) naringenin ($y = 18433x + 78905$, $R^2 = 0.9999$); oleuropein ($y = 32226x + 12416$, $R^2 = 0.9999$); (7) isoliquiritigenin ($y = 42820x + 184902$, $R^2 = 0.9998$). In each row, different letters mean significant differences between extracts for each plant part ($p < 0.05$). When only two samples were present a Student's t-test was used to determine the significant difference between two different samples, with $\alpha = 0.05$. *means statistical differences between the samples ($p < 0.05$).

Table 3. Cytotoxic and anti-inflammatory properties of the different parts of *Z. lotus* decoctions, infusions and hydroethanolic extracts (mean \pm SD, n=9).

	Branches			Leaves			Root barks			Stem barks			Nitric oxide NO-production (EC ₅₀ values, µg/mL)
	Decoction	Infusion	Hydroethanolic	Decoction	Infusion	Hydroethanolic	Decoction	Infusion	Hydroethanolic	Decoction	Infusion	Hydroethanolic	
Branches	Gram-positive bacteria												
	MIC/MBC												
	<i>E. faecalis</i>	2.5/20	2.5/20	2.5/20	2.5/20	5/20	5/20	5/20	5/20	20/20	>20/20	10/20	
	<i>L. monocytogenes</i>	5/20	5/20	5/20	10/20	10/20	20/20	20/20	20/20	2.5/20	10/20	10/20	
	MRS-A	1.25/2.5	5/10	2.25/2.5	1.25/2.5	2.5/5	143 ± 6 ^a	2.5/20	2.5/20	2.5/20	2.5/20	5/10	
	MSSA	0.625/1.25	5/10	0.227/0.625 ^{bc}	5/10	2.36 ± 7 ^b	2.5/5	158 ± 6 ^a	2.5/20	2.5/20	0.625/2.5	2.5/5	
Leaves	Gram-negative bacteria												
	MIC/MBC												
	<i>E. coli</i>	5/10	5/10	5/10	5/10	10/20	44 ± 2 ^h	5/20	5/20	2.5/5	10/20	2.5/5	
	<i>E. coli</i> ESBL	5/10	5/10	6 ± 1 ^g	5/10	66 ± 4 ^g	10/20	44 ± 2 ^h	5/20	2.5/5	10/20	2.5/5	
	<i>K. pneumoniae</i>	10/20	10/20	2.5/10	20/20	2.16 ± 2 ^h	>20/20	151 ± 6 ^e	20/20	10/20	10/20	10/20	
	<i>K. pneumoniae</i> ESBL	10/20	10/20	1.8 ± 4 ^f	20/20	2.54 ± 8 ^g	>20/20	142 ± 6 ^d	20/20	10/20	10/20	10/20	
Root barks	<i>M. morganii</i>	5/10	5/10	5/10	74 ± 7 ^g	5/10	96 ± 9 ^f	10/20	10/20	5/10	10/20	5/10	
	<i>P. mirabilis</i>	2.5/5	10/20	2.5/5	>20/20	>20/20	>20/20	>20/20	>20/20	>20/20	10/20	>20/20	
	Infusion			49.8 ± 0.5 ^h		83 ± 4 ^f		99 ± 6 ^f		59.8 ± 2 ^h		226 ± 9 ^e	
Stem barks	Hydroethanolic			50 ± 2 ^h		111 ± 4 ^e		69 ± 3 ^g		48.9 ± 3 ⁱ		211 ± 4 ^e	
	Decoction			210 ± 4 ^d		227 ± 2 ^{bc}		185 ± 7 ^b		126 ± 1 ^d		375 ± 6 ^a	
	Infusion			232 ± 5 ^b		248 ± 5 ^a		154 ± 6 ^{cd}		115 ± 1 ^e		380 ± 6 ^a	
	Hydroethanolic			242 ± 5 ^a		> 400		200 ± 8 ^a		143 ± 1 ^b		> 400	

GI₅₀ values (mean \pm SD) correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. NCI-H460: Non-small cell lung carcinoma, HeLa: Cervical carcinoma, HepG2: hepatocellular carcinoma, MCF-7: Breast carcinoma. MCF-7: breast carcinoma; NCI-H460: non-small lung cancer; HeLa: cervical carcinoma; HepG2: hepatocellular carcinoma; GI₅₀ values – concentration that inhibited 50% of the net cell growth. Ellipticine (positive control) GI₅₀ values: 1.21 μ g/mL (MCF-7), 1.03 μ g/mL (NCI-H460), 0.91 μ g/mL (HeLa), 1.10 μ g/mL (HepG2) and 2.29 μ g/mL (PLP2). EC₅₀ values (mean \pm SD) correspond to the sample concentration achieving 50% of the inhibition of NO-production. RAW264,7: Murine macrophages. Dexamethasone (positive control) EC₅₀ value: 16 μ g/mL. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test, and in each column different letters mean significant differences (p < 0.05).

Table 4. Antibacterial activity of *Z. lotus* extracts (MIC and MBC values, mg/mL).

<i>P. aeruginosa</i>	2.5/5	2.5/5	0.625/1.25	5/10	2.5/5	5/10	1.25/20	5/>20	5/>20	0.625/1.25	10/20	2.5/5
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MRSA- Methicillin resistant *S. aureus*; MSSA methicillin susceptible *S. aureus*; MIC- minimal inhibitory concentration; MBC- minimal bactericidal concentration; ESBL- spectrum extended producer of β -lactamases.

Graphical abstract

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