Food Bioscience 36 (2020) 100616

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

# Phenolic profiling, biological activities and *in silico* studies of *Acacia tortilis* (Forssk.) Hayne ssp. *raddiana* extracts

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ARTICLE INFO

*Keywords:*
*Acacia tortilis*
Phenolic compounds
Cytotoxicity
Anti-inflammatory
Antimicrobial
Molecular docking

ABSTRACT

To evaluate the phenolic profile and biological activity of the Algerian Sahara plant *Acacia tortilis* (Forssk.) Hayne ssp. *raddiana* decoction and 80% ethanolic extracts were studied. Chromatographic profiling indicated the presence of 36 phenolic compounds, including gallic acid esterified derivatives, galloylquinic derivatives and flavan-3-ols galloyl derivatives. Both extracts showed significant cytotoxic activity and a potent anti-inflammatory activity. They were effective against several multi-drug resistant bacteria, namely methicillin-resistant *Staphylococcus aureus* (MRSA). To understand the possible mechanism of action of MRSA inhibition activity, an *in silico* docking analysis was done using a virtual library of the 36 determined phenolic compounds against penicillin-binding protein (PBP2a), a protein known to be involved in MRSA resistance to β-lactam antibiotics. *A. tortilis* extracts showed interesting biological activities and the phenolic compounds found could be an interesting starting point for the development of cytotoxic and anti-inflammatory drugs and especially anti-MRSA antibiotics.

## 1. Introduction

Natural compounds obtained from plants provide research opportunities due to their significant pharmacological and toxicological properties (Khoddami, Wilkes, & Roberts, 2013), being frequently considered as potential new drugs against drug-resistant pathogens (Farha & Brown, 2016; Rempe, Burris, Lenaghan, & Stewart, 2017) and in the treatment of tumor and inflammatory diseases, among other conditions (Padmaharish & Lakshmi, 2017). Traditional herbal preparations in Algeria are still used by rural Saharan communities as a remedy for various infectious diseases or even to treat cancer and inflammatory diseases (Ramdane et al., 2015; Ziani et al., 2019a, 2019b). A variety of medicinal species belonging to the genus Acacia (Fabaceae – Mimosoideae), including *Acacia tortilis* (Forssk.) Hayne ssp *raddiana*, are drought resistant plants growing in desertic areas (Noumi, Dhaou, Abdallah, Touzard, & Chaieb, 2013). The survival of this plant in the desert is due to its ability to endure harsh conditions of seasonal waterlogging and climatic variations of temperature (Malakootian, Mahvi, Mansoorian, & Khanjani, 2018; Noumi et al., 2013), which suggest the potential presence of bioactive metabolites. The most characteristic types of phenolic compounds of this genus are flavonoids and tannins (Seigler, 2003). Many flavonol and flavone glycosides, aglycones, flavan-3-ols, flavan-3,4-diols and (epi)-chatechins containing gallolyl moieties and hydroxyl functional groups have been reported in various Acacia species (Prakash, Basavaraj, & Murthy, 2019; Rather, Shahid-ul-Islam, & Mohammad, 2015). Moreover, aqueous preparations of various *A. tortilis* parts (leaves, pods, gum exudates and bark) showed valuable pharmacological properties for the treatment of various ailments including allergy, cough, gastric irritation, inflammatory reactions and diarrhea (Jaouadi et al., 2016; Verma, 2016), by forming protective layers on the mucous membranes, due to the presence of tannins (Embaby & Rayan, 2016). Also, the gum exudate has been shown to have hypotensive and diuretic properties (Deshmukh, Shrivastava, & Bhajipale, 2018) and has been used to treat infectious diseases, colds, pharyngitis, diarrhea, dysentery, pulmonary tuberculosis and scalds of the mouth (Jaouadi et al., 2016). Previously, Bisht, Kant, and Kumar (2013) showed that a polysaccharide isolated from *A. tortilis* gum exudates had mammalian α-D-glucosidase inhibitory activity by reducing postprandial blood glucose level and the ability to decrease total cholesterol and low density lipoprotein levels. Studies on

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https://doi.org/10.1016/j.fbio.2020.100616
Received 21 September 2019; Received in revised form 17 April 2020; Accepted 19 April 2020
Available online 24 April 2020

*A. tortilis* ethanolic extracts showed significant inhibition of inflammatory cyclooxygenase enzymes (COX): COX-1 and COX-2, which was related to rutin and catechin contents in the extracts (Gabr et al., 2018).

The use of *in silico* technology to help understand the relationships between proteins and ligands has been successfully used. These tools can be used for predicting the ligand-target (protein) behavior, giving results in a less expensive and quicker manner. Furthermore, they help to understand the mechanisms of action, binding mode, conformational changes and may predict *in vitro* and *in vivo* behaviors (Carradori et al., 2016; Mocan et al., 20147).

The current study aims at profiling the phenolic compounds of the decoction and ethanolic extracts of *A. tortilis* using high performance liquid chromatography (HPLC) coupled to a diode array detector (DAD) and to a mass spectrometer (MS) with electrospray ionization (ESI). The *in vitro* cytotoxicity against 4 human tumor cell lines and a normal porcine cell line was also investigated, alongside the anti-inflammatory and antimicrobial potential against some multi-drug resistant bacteria. Good results were obtained against a methicillin-resistant *Staphylococcus aureus* (MRSA) strain, therefore, an *in silico* molecular docking analysis was also done using the found phenolic compounds against penicillin binding protein (PBP2a), a known protein target for potential anti-MRSA antibiotics, to further understand the possible molecular mechanism of *A. tortilis* extract's anti-MRSA activity.

## 2. Material and methods

### 2.1. Plants samples and extract preparation

Fresh leaves of *Acacia tortilis* (Forssk.) Hayne ssp *raddiana* (5–10 different plants) were collected from the extreme south of the Algerian Saharan desert in the Tamanrasset region (Coordinates: 22°47′13″ N, 5°31′38″ E), during September of 2017, following standard guidelines for the collection of wild specimens (WHO, 2003). Plant identification was carried out following the botanical criteria of Quezel and Santa (1963) and further authenticated by taxonomists from the Department of Botany of the National Superior School of Agronomy (ENSA) in Algiers, Algeria. The biomass was shade-dried for one month in a well-ventilated room and further ground to a fine powder (~2 mm mesh size) before analysis using a Bel-Art Micro-Mill Grinder (Bel-Art Co., Wayne, NJ, USA). The initial moisture content was approximately ~73.5%, calculated using the AOAC, 2005 method 930.15 in which a weighed portion of the sample was placed in an oven for 2 h at 135 °C and weighed afterwards to obtain the moisture quantity.

The aqueous extract, a decoction, was prepared following the described procedure of traditional healers, form for the administration of their medicinal prescriptions. Thus, 1 g of the powder was weighed and 200 mL of hot distilled water was added, boil and boiled for 5 min, in a closed beaker to prevent water evaporation, and then filtered through a Whatman No. 2 filter paper using a side-arm flask under reduced pressure. The decoction was frozen at −20 °C and freeze-dried (FreeZone 4.5, Labconco, Kansas City, MO, USA).

Concomitantly, the ethanolic extracts were prepared by maceration of the plant powder at room temperature, using 1 g of ground samples and 30 mL of an ethanol/water 80% (v/v) ethanol obtained from absolute ethanol (Thermo Fisher Scientific Co., Waltham, MA, USA), for two successive extractions of 1 h each with continuous stirring (150 rpm). The filtered extracts were combined and, after ethanol evaporation at ~40 °C in a rotary evaporator (Büchi R-210, Flawil, Switzerland), were lyophilized.

### 2.2. Phenolic compounds analysis using HPLC-DAD-ESI/MSn

The qualitative and quantitative phenolic profile was obtained using HPLC-DAD-ESI/MSn, following the methodology described by Bessada, Barreira, Barros, Ferreira, and Oliveira (2016). The plant extracts were prepared at a ratio of 10:1 (w/v) of dry extract/ultrapure water (Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA) for the decoction extract and in ethanol/water (80:20 v/v, HPLC-grade, Thermo Fisher Scientific Co.) for the ethanol extract and further filtered through a Whatman 0.45 μM syringe filter (Ecoject - Dispomed, Gelnhausen, Germany). The analysis was done with an UPLC Dionex Ultimate 3000 (Thermo Finnigan Co., San Jose, CA, USA) equipped with a DAD, measuring at 280, 330 and 370 nm, and a Linear Ion Trap LTQ XL mass spectrometer (Thermo Fisher Scientific Co.), working in negative ion mode. The separation was done with a gradient elution mode using a C18 reserve phase column (Waters Spherisorb S3 ODS-2 C18, 3 μm, 4.6 mm × 150 mm, Waters, Milford, MA, USA). The identification of phenolic compounds was by comparison of their retention times (RT) and mass and UV–Vis spectra to those of the available standards (Extrasynthesis Co., Genay, France) and relevant literature data, which will be cited with specific results. Compound quantification and retention time was determined using calibration curves of each available phenolic standard, by injecting known concentrations (2.5–100 μg/mL) into the HPLC. Results were processed using the Xcalibur®data (Thermo Fisher Scientific Co.) system and expressed in mg/g of extract.

### 2.3. Bioactivity

#### 2.3.1. Cytotoxicity activity

Cytotoxicity of the extracts was assessed *in vitro* according to the procedure described by Barros et al. (2013). The Sulforhodamine B colorimetric assay was used to determine *A. tortilis* extracts cell growth inhibition potential in 4 human tumor cell lines: NCI–H460 (non-small cell lung cancer) (DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Leibniz, Germany), HeLa (cervical carcinoma, HepG2 (hepatocellular carcinoma), and MCF-7 (breast carcinoma), growing under standard cell culture conditions. All standards and reagents were obtained from Thermo Fisher Scientific Co., except when stated otherwise. Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM of glutamine, 100 U/mL penicillin and 100 μg/mL of streptomycin, and kept at 37 °C in a humidified air incubator containing 5% $CO_2$ (Linde Gases Industriais Co., Lisbon, Portugal), according to the procedure described by Abreu et al. (2011). Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of $1.0 \times 10^4$ cells/well. A primary culture of non-tumor liver cells (PLP2), prepared from a freshly harvested porcine liver from a local abattoir (the animal was not sacrificed for the harvesting of the liver) and prepared using the same conditions as the cell lines described above. When the cells were ready to undergo the assay, they were plated in a 96-well micro-plate at a known density and exposed to different concentrations of *A. tortilis* extracts (3.125–400 μg/mL) which was dissolved in ultrapure water. The plates were then incubated at 37 °C for 24 h in optimal cell growth conditions, which were the same for all cell lines. Ellipticine was used as a positive control and results were expressed as $GI_{50}$ values, which represents the concentration required to inhibit 50% of cell growth. Absorbance readings (515 nm) of the adhered cell lines were obtained using a Micro-plate Reader ELX800 (Bio-Tek Instruments, Inc., Winooski, VT, USA).

#### 2.3.2. Anti-inflammatory activity

The ability of the extracts to inhibit inflammatory processes was evaluated *in vitro* using inhibition of nitric oxide (NO) production in a cell-based model of lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage-like cell line (DSMZ - German Collection of Microorganisms and Cell Cultures GmbH, Leibniz, Germany), as previously described by Sobral et al. (2016). The concentration of NO produced by the stimulated RAW 264.7 cells treated with different concentrations of *A. tortilis* extracts (dissolved in ultrapure water at a concentration of 400 μg/mL), were estimated. NO levels were

determined by measuring nitrite production spectrophotometrically (515 nm) using the Griess reagent system kit. Cells were centrifuged at 500 *g* for 10 min, resuspended in 1 mL of Dubelco's Modified Eagle's medium (DMDM) (Thermo Fisher Scientific Co.) and cultured in a t75 flask with 25 mL of the same medium for 24 h at 37 °C in 5% $CO_2$. Cells were detached with a cell scraper, and centrifuged for 10 min at 23 °C (500 *g*), and resuspended in DMEM containing 5% of fetal bovine serum (FBS) (Thermo Fisher Scientific Co.). Subsequently, cells were counted and seeded at a density of $1 \times 10^5$ cells/$cm^2$ using 300 μL of the previous medium/well in a 48-well plate. After 20 h of incubation, the macrophages were stimulated by replacing the 300 μL culturing medium with a similar volume of medium containing LPS (10 ng/mL), or LPS plus dexamethasone (1 μg/mL). Dexamethasone (Thermo Fisher Scientific Co., Waltham) was used as a positive control, and the results were expressed as $EC_{50}$ values, corresponding to the sample concentration giving 50% of NO production inhibition.

#### 2.3.3. Antimicrobial activity

The antimicrobial activity of the extracts were screened against a collection of bacterial strains, recognized as multi-drug resistant (MDR) bacteria. Strains were recovered from physiological suspensions of hospitalized patients subjected to antibiotic treatment at the hospital "Centro Hospitalar do Nordeste, EPE", in Bragança, Portugal. The specific characteristics and antibiotic resistance profiles of the strains had been previously established (Ziani et al., 2017; Ziani et al., 2019a). The *A. tortilis* extracts were serially diluted (20–0.156 mg/mL) in ultrapuredeionized water, plated in 96-well micro-plates and examined for their growth inhibition activity, following the methodology previously described by Pires et al. (2018). Four Gram-positive bacteria: *Enterococcus faecalis* (isolated from urine), *Listeria monocytogenes* (isolated from cerebrospinal fluid), methicillin-sensitive *Staphylococcus aureus* (MSSA; isolated from a wound exudate), and MRSA isolated from expectoration; and 5 Gram-negative bacteria: *Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii* (isolated from urine), *Proteus mirabilis* (isolated from wound exudate), *Pseudomonas aeruginosa* (isolated from expectoration), were used. The minimum inhibitory concentration (MIC) values were calculated after adding 40 μL of *p*-iodonitrotetrazolium chloride (Thermo Fisher Scientific Co.), dissolved in the water of the microplate wells and incubated at 37 °C for 10–30 min. To determine the minimal bactericidal concentrations (MBC), each negative well and positive control culture (10 μL) were sub-cultured into 96-well micro-plates containing culture medium and further incubated at 37 °C for 24 h. Three negative controls were prepared, including one with Mueller-Hinton broth (MHB) (Thermo Fisher Scientific Co.) medium, the extract without any other component, and the medium containing antibiotics (ampicillin (20 mg/mL), imipenem (1 mg/mL) and vancomycin (1 mg/mL)) (Thermo Fisher Scientific Co.). A positive control was prepared with MHB for each inoculum. Ampicillin and imipenem were used as positive controls for the Gram-negative bacteria, while ampicillin and vancomycin were used for Gram-positive bacteria in the concentrations described above.

### 2.4. In silico molecular docking

Molecular docking simulations were done using the phenolic compounds found with PBP2a as the protein target. It is a known β-lactam resistance determinant protein. The 2D structures were drawn using MarvinSketch 16.4.18.0. (ChemAxon, Budapest, Hungary) and the 3D structural representations of each compound were prepared using AutoDockTools 1.5.2. (ADT, The Scripps Research Institute, La Jolla, CA, USA) (Morris et al., 2009). Finally, each compound was saved in *.pdbqt format and visually checked for structure correctness using PyMOL software 2.2.0 (Schrödinger Co., New York, NY, USA) (DeLano, 2002) (Table A1 and Figure A1, Supplementary material). A crystalized structure of PBP2a protein was then selected and obtained from the Protein Data Bank (PDB entry: 4DKI; url: https://www.rcsb.org/structure/4dki). The next step was the preparation of the 4DKI structure for docking by removing all ligands present, so potential active sites are not blocked. Then, using ADT, polar hydrogens and Gasteiger charges (electronegativity equilibria charges of each amino acid) were added, and the structure was finally saved in *.pdbqt format. Docking simulations were done. The X,Y,Z grid center coordinates were set to values of 32, 28 and 88 values and the grid dimensions were set to 40, 40 and 40 Å. The grid center and dimensions were selected to include the catalytic center of PBP2a and to accommodate larger molecules. All docking simulations were done using AutoDock4 (AD4) with the following parameters: 2500000 evaluations, a population of 120 and 50 docking runs. Docking runs were carried out using MOLA software automation tool (Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Bragança, Portugal) (Abreu, Froufe, Queiroz, & Ferreira, 2010), using a 8-core computer cluster. Docking conformation analysis and figure preparation were done using PyMOL and GIMP 2.10.8 software (GIMP Development Team, Creative Commons Attribution-ShareAlike 4.0 International License).

### 2.5. Statistical analysis

The Statistical Package for Social Sciences v. 23.0 program (IBM Corp., Armonk, NY, USA) was used for statistical treatments using a one-way analysis of variance (ANOVA) followed by a Student's *t*-test to determine the significant differences among two different samples, with $\alpha = 0.05$. For all the extracts and tests, analyses were carried out in triplicate and the values were expressed as the mean ± standard deviation (SD).

## 3. Results and discussion

### 3.1. Phenolic profiling by HPLC-DAD-ESI/MS

A structural characterization of *A. tortilis* ssp *raddiana* leaves was done to identify and quantify phenolic compounds in the decoctions and 80% ethanolic extracts. The UV absorptions measured at 280, 330 and 370 nm, RT, wavelengths of maximum absorbance, mass spectrometry fragmentation pattern in negative mode of the deprotonated molecules $[M-H]^-$, and the major fragment ions and aglycones $[A–H]^-$ are listed in Table 1. Many phenolic compounds were found, but only 36 molecules, mainly composed of galloylquinic derivatives, gallic acid (GA) esterified derivatives, flavan-3-ols galloyl derivatives ((epi)gallocatechin-gallate (EGCG) *O*-acyl derivatives) and a flavonol were characterized. The next section describes the identification process for those who want to check the extensive work represented in Table 1. Other readers may prefer to skip this section.

### 3.2. Details of peak identification

Peaks **1–3** ($[M-H]^-$ at *m/z* 343) were identified as gallolquinic acids, the fragmentation pattern yielded an $MS^2$ base peak at *m/z* 191, related to the loss of a galloyl group $[M-H-152]^-$ and the corresponding fragment at *m/z* 169 eliminating the quinic acid with the formation of the deprotonated GA, which consisted with the fragmentation behavior of a galloylquinic acid (Clifford, Stoupi, & Kuhnert, 2007). Similarly, peaks **5** ($[M-H]^-$ at *m/z* 495) and **9** ($[M-H]^-$ at *m/z* 647) were assigned as digalloyl and trigalloylquinic acids, respectively (Clifford et al., 2007).

The following peaks were all identified as gallic acid esterified derivatives. Peak **4** ($[M-H]^-$ at *m/z* 169) was positively identified as GA in comparison with the commercial standard. Peak **10** ($[M-H]^-$ at *m/z* 197) showed a product ion at *m/z* 169, which led to the loss of $CH_2{=}CH_2$ $[M-28-H]^-$, thus confirming a tentative identification of this compound as an ethyl gallate (Kalaivani, Rajasekaran, & Mathew, 2011; Sadiq, Hanpithakpong, Tarningb, & Anal, 2015). This compound was also previously identified in the ethanolic extract (80%, v/v) of

**Table 1**
Retention time, wavelengths of maximum absorption in visible region (λmax), mass spectral data, tentative identification and quantification of phenolic compounds in *A. tortilis* ssp. *raddiana*.

| Peak | Rt (min) | $\lambda_{max}$ (nm) | $[M-H]^-$ (*m/z*) | $MS^2$ (m/z) | Tentative identification | Reference | Quantification (mg/g extract) | | *p*-Students *t*-test |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Decoction | Ethanolic | |
| 1 | 3.2 | 276 | 343 | 191(100),169(33),125(3) | Galloylquinic acid | Clifford et al. (2007) | 11.7 ± 0.2 | 18.7 ± 0.1 | < 0.001 |
| 2 | 3.4 | 275 | 343 | 191(100),169(15),125(3) | Galloylquinic acid | Clifford et al. (2007) | 40 ± 1 | 21 ± 1 | < 0.001 |
| 3 | 4.04 | 273 | 343 | 191(100),169(16),125(3) | Galloylquinic acid | Clifford et al. (2007) | 85 ± 2 | 14.0 ± 0,2 | < 0.001 |
| 4 | 4.1 | 273 | 169 | 125 (100) | Gallic acid | Standard compound | nd | 28.1 ± 0.2 | – |
| 5 | 5.5 | 274 | 495 | 343(100),191(5),169(3) | Digalloylquinic acid | Clifford et al. (2007) | 29 ± 1 | 27.8 ± 0.2 | 0.001 |
| 6 | 7.0 | 274 | 321 | 169 (100) | Digallic acid | (Erşan et al., 2016; Sadiq et al., 2015) | 6.3 ± 0.1 | 3.9 ± 0.1 | < 0.001 |
| 7 | 7.6 | 277 | 321 | 169 (100) | Digallic acid | (Erşan et al., 2016; Sadiq et al., 2015) | 50 ± 1 | 23 ± 1 | < 0.001 |
| 8 | 10.7 | 275 | 457 | 321(100),305(33),169(10) | (Epi)gallocatechin-3-*O*-gallate | (Kardel et al., 2013; Shen et al., 2006) | 4.4 ± 0.1 | 4.38 ± 0.03 | 0.023 |
| 9 | 12.6 | 276 | 647 | 495(100),343(34),191(23),169(8) | Trigalloylquinic acid | Clifford et al. (2007) | 23.7 ± 0.1 | 25 ± 1 | 0.001 |
| 10 | 13.7 | 275 | 197 | 169(100),125(10) | Ethyl gallate | (Kalaivani et al., 2011; Rather et al., 2015; Sadiq et al., 2015) | nd | 8.3 ± 0.3 | – |
| 11 | 14.7 | 353 | 625 | 317 (100) | Myricetin-3-*O*-rutinoside | Gabr et al. (2018) | 6.8 ± 0.1 | 2.75 ± 0.03 | < 0.001 |
| 12 | 15.4 | 271 | 473 | 321(100),169(5) | Trigallic acid | Erşan et al. (2016) | 11.7 ± 0.3 | 22 ± 1 | < 0.001 |
| 13 | 15.8 | 274 | 473 | 321(100),169(5) | Trigallic acid | Erşan et al. (2016) | 21 ± 1 | 34.5 ± 0.2 | < 0.001 |
| 14 | 16.2 | 273 | 473 | 321(100),169(5) | Trigallic acid | Erşan et al. (2016) | 12.1 ± 0.2 | 29 ± 1 | < 0.001 |
| 15 | 17.4 | 276 | 609 | 457(100),321(62),305(5),169(3) | (Epi)gallocatechin-di-*O*-gallate | (Acton, 2013; Gabr et al., 2018; Maldini et al., 2011) | 11.7 ± 0.3 | 17.1 ± 0.4 | < 0.001 |
| 16 | 17.7 | 272 | 609 | 457(100),321(71),305(5),169(3) | (Epi)gallocatechin-di-*O*-gallate | (Acton, 2013; Gabr et al., 2018; Maldini et al., 2011) | 9.3 ± 0.1 | 16 ± 1 | < 0.001 |
| 17 | 19.1 | 270 | 625 | 473(100),321(44),169(11) | Tetragallic acid | DAD, MS | 5.8 ± 0.2 | 11.7 ± 0.4 | < 0.001 |
| 18 | 19.4 | 270 | 625 | 473(100),321(57),169(15) | Tetragallic acid | DAD, MS | 5.0 ± 0.2 | 9.5 ± 0.3 | < 0.001 |
| 19 | 19.9 | 270 | 625 | 473(100),321(75),169(9) | Tetragallic acid | DAD, MS | 4.2 ± 0.1 | 10.8 ± 0.2 | < 0.001 |
| 20 | 20.3 | 276 | 761 | 609(100),457(67),321(41),305(33),169(5) | (Epi)gallocatechin-tri-*O*-gallate | (Acton, 2013; Karar et al., 2016; Karar & Kuhnert, 2015) | 9.3 ± 0.1 | 23 ± 1 | < 0.001 |
| 21 | 20.8 | 275 | 761 | 609(100),457(18),321(22),305(12),169(4) | (Epi)gallocatechin-tri-*O*-gallate | (Acton, 2013; Karar et al., 2016; Karar & Kuhnert, 2015) | 6.1 ± 0.1 | 15.7 ± 0.5 | < 0.001 |
| 22 | 21.1 | 272; 349 | 593 | 441(100),289(44) | (Epi)catechin-di-*O*-gallate | (Biswas & Roymon, 2013; Maldini et al., 2011) | 13.7 ± 0.2 | 29 ± 1 | < 0.001 |
| 23 | 22.1 | 273 | 625 | 473(100),321(38),169(9) | Tetragallic acid | DAD, MS | 6.2 ± 0.1 | 25.5 ± 0.7 | < 0.001 |
| 24 | 22.9 | 275 | 761 | 609(100),457(77),321(56),305(21),287(13),169(6) | (Epi)gallocatechin-tri-*O*-gallate | (Acton, 2013; Karar et al., 2016; Karar & Kuhnert, 2015) | 2.6 ± 0.1 | 6.3 ± 0.1 | < 0.001 |
| 25 | 23.6 | 274 | 761 | 609(100),457(66),321(43),305(17),287(18),169(8) | (Epi)gallocatechin-tri-*O*-gallate | (Acton, 2013; Karar et al., 2016; Karar & Kuhnert, 2015) | 1.13 ± 0.05 | 1.80 ± 0.03 | 0.006 |
| 26 | 24.2 | 277 | 761 | 609(100),457(68),321(41),305(14),287(12),169(7) | (Epi)gallocatechin-tri-*O*-gallate | (Acton, 2013; Karar et al., 2016; Karar & Kuhnert, 2015) | 6.3 ± 0.1 | 17.6 ± 0.4 | < 0.001 |
| 27 | 25.2 | 278 | 761 | 609(100),457(55),321(37),305(11),287(9),169(3) | (Epi)gallocatechin-tri-*O*-gallate | (Acton, 2013; Karar et al., 2016; Karar & Kuhnert, 2015) | 11.8 ± 0.2 | 25.6 ± 0.4 | < 0.001 |
| 28 | 26.0 | 275 | 777 | 625(55),473(100),321(21),169(5) | Pentagallic acid | DAD, MS | 2.6 ± 0.1 | 11.4 ± 0.3 | < 0.001 |
| 29 | 26.6 | 273 | 777 | 625(68),473(100),321(23),169(7) | Pentagallic acid | DAD, MS | nd | 12.5 ± 0.3 | – |
| 30 | 27.3 | 277 | 913 | 761(100),609(76),457(44),439(23),321(18),305(15)-,287(13),169(9) | (Epi)gallocatechin-tetra-*O*-gallate | Flamini and Traldi (2009) | 7.4 ± 0.3 | 23 ± 1 | < 0.001 |
| 31 | 28.9 | 277 | 913 | 761(100),609(71),457(47),439(28),321(15),305(12)-,287(10),169(6) | (Epi)gallocatechin-tetra-*O*-gallate | Flamini and Traldi (2009) | 3.3 ± 0.1 | 35 ± 1 | < 0.001 |
| 32 | 29.7 | 276 | 1065 | 913(100),761(67),609(33) | (Epi)gallocatechin-penta-*O*-gallate | DAD, MS | 5.3 ± 0.1 | 11.5 ± 0.3 | < 0.001 |
| 33 | 30.3 | 276 | 1065 | 913(100),761(71),609(33) | (Epi)gallocatechin-penta-*O*-gallate | DAD, MS | 2.0 ± 0.1 | 19 ± 1 | < 0.001 |
| 34 | 31.3 | 276 | 1065 | 913(100),761(73),609(33) | (Epi)gallocatechin-penta-*O*-gallate | DAD, MS | 2.5 ± 0.1 | 13.4 ± 0.4 | < 0.001 |
| 35 | 31.9 | 276 | 1217 | 1065(100),913(56),761(21),609(13) | (Epi)gallocatechin-hexa-*O*-gallate | DAD, MS | 1.62 ± 0.04 | 4.43 ± 0.04 | < 0.001 |
| 36 | 32.3 | 276 | 1217 | 1065(100),913(50),761(19),609(10) | (Epi)gallocatechin-hexa-*O*-gallate | DAD, MS | nd | 3.1 ± 0.1 | – |
| | | | | | **Total phenolic compounds** | | 420 ± 1 | 610 ± 10 | < 0.001 |

Values expressed in mean values ± standard deviation. The gallic acid (y = 1315 × $10^2$x + 2921.63 × $10^2$; $R^2$ = 0.999; Limit of detection (LOD) = 0.68 μg/mL; LOQ = 1.61 μg/mL) was used for quantification of all the phenolic compounds with the exception of compound 11 which was quantified using quercetin 3-*O*-rutinoside (y = 133.43 × $10^2$x + 767.51 × $10^2$; $R^2$ = 0.999; LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL). A Student's t-test was used to determine the significant difference between the two extraction types, with α = 0.05: "*" means a significant difference between the samples (p < 0.05).

*Acacia nilotica* (L.) Del. leaves from Pakistan (Sadiq et al., 2015) and in the ethanolic extract of *A. nilotica* leaves from India (Kalaivani et al., 2011). Peaks **6** and **7** ($[M-H]^-$ at *m/z* 321) were tentatively assigned as digallic acids, producing a major product ion at *m/z* 169, due to the loss of a GA unit (−152 u), thus the different linkages between the two galloyl moieties remains unknown (Erşan, Güçlü Üstündağ, Carle, & Schweiggert, 2016; Sadiq et al., 2015; Salem, Davidorf, & Abdel-Rahman, 2011). These compounds were also previously found in *A. nilotica* pods ethanolic extract from Pakistan (Sadiq et al., 2015) and in a crude methanolic extract of *Acacia nilotica* from Egypt (Salem et al., 2011). Taking into account similar assumptions, the pseudomolecular ions of compounds **12–14** ($[M-H]^-$ at *m/z* 473), **17–19** and **23** ($[M-H]^-$ at *m/z* 625), and **28–29** ($[M-H]^-$ at *m/z* 777) showed the presence of a trigallic, tetragallic and pentagallic acids, due to sequential loss of galloyl moieties, yielding product ions specific to GA (Erşan et al., 2016). These compounds seem to not have been previously described in Acacia species.

Peak **11** ($[M-H]^-$ at *m/z* 625), was the only flavonol derivative identified in this samples, which released a fragment at *m/z* 317 [myricetin−H]$^-$ (−308 u, loss of a deoxyhexosyl-hexoside moieties) being tentatively identified as myricetin-*O*-deoxyhexosyl-hexoside, thus the assumption as myricetin-3-*O*-rutinoside was taken into account, due to its previously identification *Acacia farnesiana* (L.) Willd., *Acacia tortilis* Hayne and *Acacia longifolia* (Andrews) Willd. leaves and barks ethanolic extracts (Gabr et al., 2018).

The remaining compounds (peaks **8, 15, 16, 20, 21, 24–27** and **30–36**) were characterized as flavan-3-ols galloyl derivatives (EGCG *O*-acyl derivatives), such as ester of (epi)gallocatechin linked to various units of GA. The fragmentation pattern showed the typical loss of at least one GA (M-170) or one galloyl group (M-152), indicating that these compounds were always linked to a (epi)gallocatechin-*O*-gallate, thus the different linkages between the (epi)gallocatechin and gallate moieties were not possible to be identified. Therefore, these molecules were tentatively assigned as (epi)gallocatechin-*O*-gallate ($[M-H]^-$ at *m/z* 457; peak **8**), (epi)catechin-di-*O*-gallate ($[M-H]^-$ at *m/z* 593; peak **22**), (epi)gallocatechin-di-*O*-gallate ($[M-H]^-$ at *m/z* 609; peaks **15** and **16**), (epi)gallocatechin-tri-*O*-gallate ($[M-H]^-$ at *m/z* 761; peaks **20**, **21**, and **24–27**), (epi)gallocatechin-tetra-*O*-gallate ($[M-H]^-$ at *m/z* 913; peaks **30** and **31**), (epi)gallocatechin-penta-*O*-gallate ($[M-H]^-$ at *m/z* 1065; peaks **32–34**) and (epi)gallocatechin-hexa-*O*-gallate ($[M-H]^-$ at *m/z* 1217; **35** and **36**). Peak **8** ($[M-H]^-$ at *m/z* 457) revealed a $MS^2$ fragment ion at *m/z* 321 $[M-152-H]^-$, corresponding to the loss of one galloyl moiety, being assigned as (epi)gallocatechin-*O*-gallate (Kardel, Taube, Schulz, Schu, & Gierus, 2013; Shen et al., 2006). Peaks **15** and **16** ($[M-H]^-$ at *m/z* 609) were identified as (epi)gallocatechin-di-*O*-gallate, showing two $MS^2$ fragments at *m/z* 473 $[M-152-H]^-$ and 321 $[M-152-152\text{-}H]^-$, while peaks **20, 21** and **24–27** ($[M-H]^-$ at *m/z* 761) showed three major product ions at *m/z* 609 $[M-152-H]^-$, 473 $[M-152-152-16-H]^-$ and 321 $[M-152-152-152-H]^-$, showed the consecutive loss of three galloyl units, being assigned as (epi)catechin-tri-*O*-gallate (Acton, 2013; Karar et al., 2016; Shen et al., 2006). Similarly, peaks **30** and **31** (*m/z* 913), **32, 33** and **34** (*m/z* 1065), and peaks **35** and **36** (*m/z* 1217) showed major product ions attributed to the loss of four, five and 6 GA units being tentatively assigned (epi)gallocatechin-tetra-*O*-gallate, (epi)gallocatechin-penta-*O*-gallate and (epi)gallocatechin-hexa-*O*-gallate, respectively (Flamini & Traldi, 2009). Peak **22** ($[M-H]^-$ at *m/z* 593) showed a major product ion at *m/z* 441 $[M-152-H]^-$ and 289 $[M-152-152-H]-$ due to the loss of a two galloyl moieties, being tenatively identified as (epi)catechin-di-*O*-gallate (Biswas & Roymon, 2013; Karar et al., 2016; Maldini et al., 2011). Furthermore, it was also found that as the number of galloyl moieties increased, the elution time increased.

*A. tortilis* extracts showed higher amounts of TPC in the ethanolic extract (606 ± 7 mg/g of extract) in comparison to the decoction (420 ± 1 mg/g of extract). However, the decoction had gallolyquinic acids and gallic acid esterified derivatives in higher amounts in comparison to the ethanolic preparation. Moreover, the GA and its galloyl esterified derivatives were abundant in the decoction, in comparison to the flavan-3-ols galloyl derivatives (23.4% of TPC). Thus, this last group of compounds were found in higher concentrations in the ethanolic extract, with the (epi)gallocatechin-tri-*O*-gallate as the major molecule, compared to the decoction (37.2 mg/g, 8.9% of TPC). Therefore, the remaining epigallocatechin gallate *O*-acyl derivatives (tetra, penta and hexa -gallate) were mainly present in the ethanolic extract, and at a relatively low concentration in the decoction. Decoctions were mainly characterized by the presence of galloylquinic acid, alongside with GA dimer (digallic acid 56.2 g/mg) and trimer (trigallic acid 44.8 g/mg). Whereas, the depsidic galloyl derivatives of quinic acid (digalloylquinic acid and trigalloylquinic acid), had similar quantities for both extracts.

The results were consistent with Susanti, Ratnawati, and Rudijanto (2015), where the (epi)catechin derivatives levels were obtained in higher amounts in aqueous and ethanolic extracts of green tea from Gambung, Indonesia. Other supporting data has found that some species of the genus Acacia growing in arid regions, namely *A. nilotica* (Maldini et al., 2011), *A. catechu* (Shen et al., 2006) and *A. ferruginea* (Sakthivel & Guruvayoorappan, 2013) are characterized by the occurrence of both GA and flavan-3-ols galloyl derivatives. The results were consistent with Shen et al. (2006), who reported a high concentration of catechins in a 70% methanolic extracts of *A. catechu*. Furthermore, catechin derivatives have been present in a higher quantity in *A. nilotica* from Aswan (Egypt) (Maldini et al., 2011), and the compounds were closely related to the ones found herein, in which the pods extracts (80% ethanol) contained catechin-7,3′-di-*O*-gallate (16.1 μg/g), catechin-7,4′-di-*O*-gallate (20.4 μg/g), catechin-7-*O*-gallate (26.5 μg/g), gallocatechin-7,3′-di-*O*-gallate (222 μg/g), gallocatechin-7,4′-di-*O*-gallate (203 μg/g) and gallocatechin-7-*O*-gallate (84.4 μg/g).

It seems that the gallolyation process is controlled by the enzyme 'galloytransferase' which may be present in the vegetal structures and have an important role in acylating depsidic galloly units to form more complex structures, that are linked to phenolic acids such as quinic acid or even to some flavan-3ols, such as (epi)gallocatechin structur e (Liu et al., 2012).

### 3.3. Bioactivities

Extracts were assessed for their cytotoxic activity on four human tumor cell lines and a normal primary cell line (Table 2). Both extracts showed good cell growth inhibition (lower $GI_{50}$ values) across the tumor cell lines used, with $GI_{50}$ values ranging from 33.3 to 53.0 μg/mL. The ethanolic extract was slightly more active against HepG2 cells with a $GI_{50}$ of 33 ± 1 μg/mL value compared to 52.4 ± 0.5 μg/mL for the decoction extract. Overall, there were no significant variations among the tumor cell lines used, indicating that the molecular mechanism of toxicity is probably similar across the different tumor cell lines used. On the other hand, the cytotoxicity with the normal PLP2 cell line, for both extracts, was significantly lower with a $GI_{50}$ of 253 ± 0.02 and 259 ± 0.05 μg/mL for the decoction and the ethanolic extracts, respectively. These differences in potency are significant as it indicates that both extracts are probably interfering with specific molecular targets in tumor cells that are not present, or are not activated, in non-tumor cells. The higher response of tumor cells exposed to the plant extracts, compared to the non-tumoral cells, probably reflects their higher reactivity towards the phenolic compounds present in the extracts. These phenolic compounds are probably interfering with specific mechanisms involved in tumor proliferation including cell cycle, apoptosis and cell death mechanisms (Carocho & Ferreira, 2013; Karar et al., 2016).

Previous reports discussed the cytotoxic potential of many Acacia species indicating that the plants of this genus have potential against a range of tumor cell lines (Rather et al., 2015; Sakthivel & Guruvayoorappan, 2013). A study (Alajmi et al., 2017) showed that the

**Table 2**
Cytotoxic and anti-inflammatory proprieties of *A. tortilis* decoctions and ethanolic extracts.

| A. tortilis subsp. raddiana | | | | |
|---|---|---|---|---|
| **Cytotoxicity** | **Cell lines** | **Decoction** | **Ethanolic** | **Ellipticine** |
| Growth inhibition values ($GI_{50}$, μg/mL) | **Non-small cell lung cancer (NCI–H460)** | 52 ± 1 | 52 ± 1 | 1.0 ± 0.1 |
| | **Cervical carcinoma (HeLa)** | 49.4 ± 0.1 | 48.2 ± 0.1 | 1.9 ± 0.1 |
| | **Hepatocellular carcinoma (HepG2)** | 52.4 ± 0.5* | 33 ± 1* | 1.1 ± 0.2 |
| | **Breast carcinoma (MCF-7)** | 53 ± 0.1 | 52 ± 1 | 0.91 ± 0.04 |
| | **PLP2** | 253.1 ± 0.1 | 259 ± 0.1 | 3.2 ± 0.7 |
| **Anti-inflammatory** | | | | **Dexamethasone** |
| Nitric oxide NO-production ($EC_{50}$, μg/mL) | **RAW264.7** | 91 ± 1* | 88 ± 4* | 16 ± 1 |

$GI_{50}$ values (mean ± 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. $EC_{50}$ values (mean ± SD) correspond to the sample concentration achieving 50% of the inhibition of NO-production. RAW264,7: Murine macrophages. A Student's t-test was used to determine the significant difference between the two extraction types, with $\alpha = 0.05$: "*" means a significant difference between the samples ($p < 0.05$).

ethanolic extract of *A. tortilis* aerial part, from Saudi Arabia, had cytotoxic activity against HepG2 ($GI_{50}$ = 42.3 μg/mL), MCF-7 ($GI_{50}$ = 65.7 μg/mL) and kidney carcinoma cells (HEK-293, $GI_{50}$ = 49.1 μg/mL), which was consistent with the current results. According to the same study, *A. laeta* and *A. hamulosa* ethanolic extracts also presented similar levels of inhibition of tumor cell growth. Another study analyzed the methanolic and aqueous extract of *A. catechu* from India and also observed a moderate activity against several tumor cells lines including MCF-7 and HeLa cells, although a direct comparison is not possible as these reports present the inhibition percentage for only one extract concentration (Padmaharish & Lakshmi, 2017). In the same study the extracts obtained with hydrophobic solvents (acetone, chloroform and hexane) did not show any significant cell growth inhibition. According to the authors, the cytotoxic effect was probably related to the polar nature of *A. catechu* active components, especially epigallocatechin-3-*O*-gallate (EGCG), first identified in *A. catechu*, but also in the present study. In the same study, *A. catechu* methanolic extract presented more cytotoxicity than EGCG alone, indicating that there is probably a synergistic effect between different Acacia active components that surpass the EGCG effect alone (Padmaharish & Lakshmi, 2017). The LC/MS profiling of *A. tortilis* in the present study showed a considerable amount of esterified gallic acid and epicatechin galloyled with a high degree of hydroxyl ring substitutions. These compounds were shown to be involved directly on the growth inhibition of several tumor cell lines (Guimarães et al., 2014), and offer a plausible explanation for the measured cytotoxicity on human tumor cells presented in this study. *A. nilotica* also revealed antitumor potential against various types of cancer cell lines, both *in vitro* and *in vivo* assays (Padmaharish & Lakshmi, 2017; Rather et al., 2015; Sakthivel, Kannan, Angeline, & Guruvayoorappan, 2012). The non-growth effect on normal cells was previously reported by Chu, Deng, Man, and Qu (2017), who discussed the EGCG effects in normal rat osteoblasts (NRO) and human osteosarcoma (MG-63 and Saos-2). The authors reported that the growth and alkaline phosphatase activity of the studied osteosarcoma cell lines were inhibited with morphological alterations and G0/G1-phase arrest of the cell cycle under the EGCG effect at micromolar concentrations.

The anti-inflammatory activity of *A. tortilis* leaves extracts was tested *in vitro* through the ability to decrease NO production in LPS-stimulated RAW 264.7 cells. The plant extracts reduced the levels of NO considerably, in the same range of activity for both extracts, with $EC_{50}$ values of 91 ± 1 and 88 ± 4 mg/mL for the decoction and the ethanolic, respectively (Table 2). Gabr et al. (2018) reported that ethanolic extracts (70%) of *A. tortilis* leaves from Egypt showed a significant inhibition of cyclooxygenase-1 (COX-1) and COX-2 enzymes that are involved in the inflammatory response. According to the authors, the effect is related to higher concentration of rutin and catechin present in the extract. In another study, acetone extracts from Acacia (*A. leucophloea*, *A. ferruginea*, *A. dealbata*, and *A. pennata*) barks promoted a significant reduction on NO production in LPS-stimulated RAW 264.7 cells. The Acacia species showed high levels of TPC and TFC, and the extracts were shown to suppress the inducible nitric oxide synthase (iNOS), the cyclooxygenase-2 (COX-2) and the tumor necrosis factor (TNF-α) expression as a consequence of NO production reduction (Sowndhararajan, Santhanam, Hong, Jhoo, & Kim, 2016). A significant reduction of NO production was also observed using (epi)-catechin on LPS-stimulated RAW 264.7 cells, which significant release inhibition of pro-inflammatory factors including cytokines, TNF-α and mediators such as interleukin IL-1β, IL-6 and IL-12 (Khalatbary & Ahmadvand, 2011; Wu, Choi, Kang, Kim, & Shin, 2017). Other authors pointed out that a gallate ester substitution proportionally increase the anti-inflammatory proprieties of (epi)-catechins and GA (Locatelli, Monteiro, Centa, & Creczynski-Pasa, 2013; Wang & Cao, 2014). As an example, epigallocatechin-3-gallate was shown to significantly scavenge NO, peroxynitrite, and other reactive oxygen/nitrogen species (ROS/RNS) thus inhibiting transfection of nuclear factor NF-kB from cytoplasm to the nucleus, with a consequent down-regulation of expression of the inflammatory mediators iNOS and COX-2 (Chu et al., 2017). The good anti-inflammatory effects observed for both *A. tortilis* extracts were probably related to the high contents of phenolic compounds, especially (epi)-gallocatechin derivatives. The phenolic compounds in both extracts may significantly attenuate inflammatory reactions in the stimulated Raw 264.7 cells by interacting with proteins involved in reducing NO-production, inhibition of inflammatory enzymes, inhibition of cytokine factors and enhancing the production of interleukin IL-10.

*A. tortilis* is a potential source of plant-derived antibiotics, the antimicrobial activity was also analyzed for antimicrobial activity using the p-iodonitrotetrazolium chloride assay (Table 3). The extracts were assessed against several clinical MDR pathogens including Gram-positive and Gram-negative bacteria. A good inhibitory effect was measured for both extracts and the results showed that the tested bacteria strains were significantly affected (low MIC values). The ethanolic extract showed better growth inhibition activity against all tested stains, giving MIC values between 1.25 and 2.5 mg/mL for Gram-negative and from 0.15 to 2.5 mg/mL for Gram positive. The MSSA (MIC < 0.15 mg/mL), MRSA (MIC = 0.312 mg/mL) and *L. monocytogenes* (MIC = 1.25 mg/mL) bacteria strains were especially susceptible. A MIC of 1.25 mg/mL was measured for all the Gram-negative bacteria, except for *K. pneumoniae, Proteus mirabilis*, *Pseudomonas aeruginosa* when applying the decoction extracts, which presented a MIC of 2.5 mg/mL. Moreover, the decoction preparation showed a good activity towards Gram-positive bacteria (low MIC values), especially against MSSA (MIC under 0.15 mg/mL) and MRSA (MIC = 0.625 mg/mL) which were more vulnerable. *E. faecalis* was the most resistant registering MIC values of 5 mg/mL. Regarding the MBC values, both extracts showed a uniform dose of 20 μg/mL on all studied bacteria species. For the commercial antibiotics (Ampicillin, Imipenem, and Vancomycin), the strains reacted differently to them, indicating that the extracts of *A. tortilis* and

**Table 3**
Antimicrobial activity of *A. tortilis* ssp *raddiana* extracts (decoctions and ethanolic) against various strains of Gram-positive and negative bacteria.

| | *Acacia tortilis* | | | | Controls | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Decoction | | Ethanolic | | Ampicillin (20 mg/mL) | | Imipenem (1 mg/mL) | | Vancomycin (1 mg/mL) | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| **Gram-negative bacteria** | | | | | | | | | | |
| *Escherichia coli* | 1.25 | 20 | 1.25 | 20 | < 0.15 | < 0.15 | < 0.0078 | < 0.0078 | nt | nt |
| *Klebsiella pneumoniae* | 2.5 | 20 | 1.25 | 20 | 10 | 20 | < 0.0078 | < 0.0078 | nt | nt |
| *Morganella morganii* | 1.25 | 20 | 1.25 | 20 | 20 | > 20 | < 0.0078 | < 0.0078 | nt | nt |
| *Proteus mirabilis* | 2.5 | 20 | 1.25 | 20 | < 0.15 | < 0.15 | < 0.0078 | < 0.0078 | nt | nt |
| *Pseudomonas aeruginosa* | 2.5 | 20 | 1.25 | 20 | > 20 | > 20 | 0.5 | 1 | nt | nt |
| **Gram-positive bacteria** | | | | | | | | | | |
| *Enterococcus faecalis* | 5 | 20 | 2.5 | 20 | < 0.15 | < 0.15 | nt | nt | < 0.0078 | < 0.0078 |
| *Listeria monocytogenes* | 1.25 | 20 | 1.25 | 20 | < 0.15 | < 0.15 | < 0.0078 | < 0.0078 | nt | nt |
| MRSA | 0.625 | 20 | 0.312 | 20 | < 0.15 | < 0.15 | nt | nt | < 0.0078 | < 0.0078 |
| MSSA | < 0.15 | 20 | < 0.15 | 20 | < 0.15 | < 0.15 | nt | nt | 0.25 | 0.5 |

MRSA-methicillin resistant *Staphylococcus aureus*; MSSA-methicillin susceptible *Staphylococcus aureus*; MIC- minimal inhibitory concentration; MBC- minimal bactericidal concentration, nt-not tested.

**Table 4**
Predicted Ki values and number of hydrogen bonds (H-bonds) determined for the top-ranked *A. tortilis* phenolic compounds.

| Number | Compound | Predicted Ki (nM) | H-bonds |
|---|---|---|---|
| 1 | Epigallocatechin-3,7,3′,4′,5′-penta-*O*-gallate | 0.54 | 15 |
| 2 | Epigallocatechin-3,5,4′,5′-tetra-*O*-gallate | 5.13 | 9 |
| 3 | (Epi)gallocatechin-3,5′di-*O*-gallate | 14.4 | 7 |
| 4 | Epigallocatechin-3,5,3′-tri-*O*-gallate | 19.4 | 6 |
| 5 | Trigalloylquinic acid | 21.5 | 8 |
| 6 | (Epi)gallocatechin-5,7-di-*O*-gallate | 33.9 | 7 |
| 7 | Epigallocatechin-3,5,5′-tri-*O*-gallate | 36.3 | 6 |
| 8 | Epigallocatechin-5,7,4′-tri-*O*-gallate | 36.3 | 6 |
| 9 | Epigallocatechin-3,7,5′-tri-*O*-gallate | 46.7 | 6 |
| 10 | Epigallocatechin-3,5,4′-tri-*O*-gallate | 53.53 | 8 |

the antibiotics do not share a common mode of action. *A. tortilis* ethanolic extract (70%) from Saudi Arabia, containing rutin as the major phenolic compound, showed a high potency with MIC values in the range of 0.2–3.2 mg/mL against different pathogenic bacterial strains (Alajmi et al., 2017). Eldeen, Heerden, and Staden (2010), discussed the antimicrobial potential of *A. nilotica* subsp. *kraussiana* stem bark and showed that the compounds of the ethyl acetate extract had an inhibitory activity against Gram-positive (*Bacillus subtilis* and *S. aureus*, MIC values of 4 μg/mL and 8 μg/mL respectively) and Gram-negative bacteria (*K. pneumonia* and *E. coli*, MIC values of 16 μg/mL and 33 μg/mL, respectively). The leaves, pods and bark ethanolic extracts of *A. nilotica* from Pakistan (Sadiq et al., 2015) inhibited the growth of clinical MDR and food isolates of *E. coli* and *Salmonella typhimurium* (MIC and MBC of Acacia leaves were in the range of 1.56–3.12 mg/ml and 3.12–6.25 mg/ml, respectively). These effects were related to the abundance of phenolic acids such as GA, m-digallic acid and gallolyated flavon-3-ols such as epicatechin-5-gallate and digallocatechin-5-gallate (Bansal, Vyas, Bhattacharya, & Sharma, 2013; Rempe et al., 2017). *A. tortilis* phenolic compounds may have a different pattern for the relationship of structural-antimicrobial activities, with the galloyted epistructured catechins impairing the cell membrane structure and inhibiting enzyme activity, leading to a loss of cell homeostasis (Pires et al., 2018; Rempe et al., 2017). The hydroxyl active groups found abundantly in the GA and the galloyl moieties of the flavon-3-ols may disrupt bacterial peptidoglycans in the membrane structures, causing the leakage of cellular components, leading to delocalization of electrons which then act as proton exchangers and reduce the gradient across the cytoplasmic membrane of bacterial cells (Rempe et al., 2017; Ziani et al., 2019a). A study by Stapleton, Shah, Ehlert, Hara, and Taylor (2007) demonstrated that the (−)epicatechin gallate sensitizes MRSA strain to β-lactam antibiotics, promoting staphylococcal cell aggregation and increase the thickness of the cell-wall and the quantity of autolysins associated with it.

### 3.4. Molecular docking

The MRSA inhibition ability showed by *A. tortilis* extracts is of special significance. To understand the possible molecular mechanism of the MRSA inhibition observed by the *A. tortilis* extract, an *in silico* molecular modelling study was carried out using molecular docking tools. An initial search and selection of possible protein targets was thus performed, which lead to the PBP2a, a transpeptidase protein expressed in MRSA strains, previously identified as being one of the proteins responsible for MRSA strains resistance to β-lactam antibiotics (Fishovitz, Hermoso, Chang, & Mobashery, 2014). The epigallocatechin derivatives present in the *A. tortilis* extracts were candidates as potential PBP2a inhibitors and thus a virtual library of the tentative epigallocatechins structures, discovered in the *A. tortilis* extracts, was prepared. Due to the size of some of the compounds, especially tetra-, penta- and hexa-*O*-gallate derived epigallocatechins, it was difficult to identify the exact structure of each compound so only tentative structures were determined. For this reason, a search for previously discovered compounds of each epigallocatechin group was done and a virtual library of epigallocatechin derivatives was prepared, totaling 41 compounds. The virtual library included 1 hexa-, 6 penta-, 9 tetra-, 9 tri-, 6 di-*O*-gallate epigallocatechins derivatives and 11 epigallocatechins of lower molecular weight along with other non-epigallocatechins compounds (Table A1 and Figure A1, Supplementary material). Once the protein target and the compound virtual library were established, docking studies were done using AutoDock 4.0 software, against the selected PBP2a protein. The predicted docking scores of the 10 best ranked compounds are presented in Table 4 (complete results in Table A1 and Figure A1, Supplementary material). The epigallocatechin-3,7,3′,4′,5′penta-*O*-gallate (compound 1) achieved the best PBP2a predicted inhibition constant (Ki) with a value of 0.54 nM followed by epigallocatechin-3,5,4′,5′-tetra-*O*-gallate (compound 2) with a predicted Ki value of 5.13 nM. These are low Ki predicted values and provide a good indication that *O*-gallate epigallocatechin derivatives may act as PBP2a inhibitors. The remaining top 10 compounds include 5 tri-*O*-gallate epigallocatechin derivatives, 2 di-*O*-gallate epigallocatechin derivatives

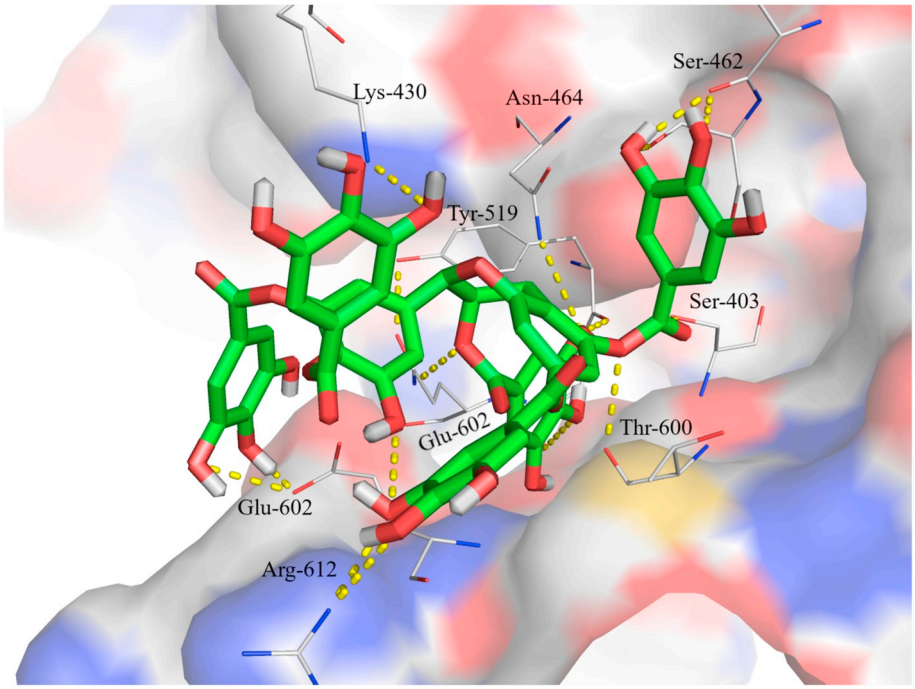


**Fig. 1.** Docking conformation of epigallocatechin-3,7,3′,4′,5′-penta-*O*-gallate (compound 1) against PBP2a catalytic site. Hydrogen bonds represented as trace yellow lines. Image prepared using PyMol software. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and trigalloylquinic acid with Ki predicted values between 14.4 and 53.5 nM values. Considering that a large number of di-, tri-, tetra-, and penta-*O*-gallate epigallocatechin derivates were identified in good amounts, it is probable that these compounds may be acting synergistically as PBP2a inhibitors. To understand the epigallocatechin derivates predicted PB2Pa inhibition ability at a molecular level, a detailed structural analysis of the predicted docking conformation pose was done for the top ranked compound (Fig. 1). PBP2a protein presents a fairly extended and elongated binding site, were the transpeptidation reaction and consequent peptidoglycan formation occurs. When analyzing the predicted docking pose of compound 1, it was possible to observe an appropriate occupation of the PBP2a transpeptidase binding site, with pockets occupied by the epigallocatechin core structure and by 2 of the *O*-gallate groups. The remaining 3 *O*-gallate groups were positioned towards the exterior of PBP2a structure, with the hydroxyl groups directed to the solvent, probably forming Hydrogen bonds (H-bonds) with water molecules. When measuring the predicted H-bonds that compound 1 was able to promote with PBP2a structure, it was observed that compound 1 docked pose was stabilized by a network of 15 H-bonds. Fig. 1 shows the amino acids that were predicted to form H-bonds with compound 1. Correct binding pocket occupation, in conjunction with the extensive network of H-bonds formed with PBP2a protein and probably with exterior water molecular, provides evidence that compound 1 is probably a good inhibitor of MRSA resistance related PBP2a protein target. When analyzing the conformations of the remaining top ranked compounds, a similar pattern was observed albeit with a lower number of H-bonds measured (Table 4). Although these results are encouraging, it is important to point out that AutoDock 4, as with any other docking software, only tries to fit the molecule within the active site without considering any external factor that could affect the binding process, including the presence of structural waters, good protein mobility and presence or other co-factors just to name a few. Therefore, although the predicted Ki values are low, the external factors are probably the reason why the observed *A. tortilis* extract MRSA inhibition activity is good. Considering the results, the authors propose that the MRSA inhibition activity observed for the *A. tortilis* compounds against PBP2a may be promoted by the epigallocatechin derivatives. Furthermore, the total inhibition activity is probably due to a synergistically effect of several of the epigallocatechin derivatives. Further experimental validation is needed to confirm the proposed mechanism of PBP2a inhibition.

## 4. Conclusion

Analyses of the decoction and ethanolic extracts from *Acacia tortilis* (Forssk.) Hayne ssp. *raddiana* showed the presence of 36 phenolic compounds, including GA esterified derivatives, galloylquinic derivatives and flavan-3-ols galloyl derivatives. Several biological activities of the extracts were determined, while recording a significant cytotoxic activity against a panel of 4 different tumor cell lines, anti-inflammatory activity in a RAW 264.7 cell model and antimicrobial activity against MDR bacteria with emphasis on a MRSA strain. The possible mechanism of MRSA inhibition activity was also investigated using molecular docking studies, using PBP2a as the protein target and virtually testing the library of previously determined phenolic compounds. PBP2a was selected because it is a protein known to be involved in MRSA resistance to β-lactams antibiotics. The epigallocatechin-3,7,3′,4′,5′penta-*O*-gallate derivative was predicted to be the most potent PBP2a inhibitor and a detailed analysis of the binding conformation was performed. In general, *A. tortilis* extracts presented interesting biological activities and the phenolic compounds discovered could be interesting starting points for the development of cytotoxic and anti-inflammatory drugs and especially anti-MRSA antibiotics. Beyond the direct usage of the plant extracts in their original forms as potential drugs, the current study suggests the possibility that these compounds can also be used as drug precursors, templates for synthetic modification and pharmacological probes.

## CRediT authorship contribution statement

**Borhane E.C. Ziani:** Methodology, Investigation, Writing - original draft. **Marcio Carocho:** Methodology, Investigation, Writing - original draft. **Rui M.V. Abreu:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Khaldoun Bachari:** Writing - review & editing. **Maria José Alves:** Conceptualization, Methodology. **Ricardo C. Calhelha:** Methodology, Investigation. **Oualid Talhi:** Writing - review & editing. **Lillian Barros:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing.

**Isabel C.F.R. Ferreira:** Methodology, Writing - original draft, Writing - review & editing.

## Declaration of competing interest

The authors declare that they have no conflicts of interest regarding this manuscript.

## Acknowledgements

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support through national funds FCT/MCTES to CIMO (UIDB/00690/2020). L. Barros and R. C. Calhelha thank the national funding by the FCT, P.I., through the institutional scientific employment program-contract for their contracts. M. Carocho also thanks the project ValorNatural for his research contract. The authors are also grateful to the FEDER-Interreg España-Portugal programme for financial support through the project 0377_Iberphenol_6_E.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2020.100616.

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