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Chemical composition, antioxidant, antimicrobial and anti-inflammatory activity of *Prunus spinosa* L. fruit ethanol extract☆,☆☆

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ABSTRACT

Prunus spinosa L. (from Italy) fruit ethanol extract (40 μg/mL) was assessed by evaluating the antioxidant, anti-inflammatory and antimicrobial activities against five bacterial and two fungi ATCC strains. Moreover, the phenolic profile was also investigated and results are indicative of an intense anthocyanin accumulation which may be responsible for the antioxidant properties revealed by the DPPH assay. MIC and MBC/MFC values (4.36–8.72 mg/mL; 8.72–17.44 mg/mL, respectively) revealed a wide antibacterial activity and yeast inhibition. No specific inhibitory action was observed against the tested Gram-negative or Gram-positive bacteria. Preliminary data on the effect on both miR-126 and miR-146a expression levels suggested a very interesting anti-inflammatory activity of the extract. A possible mechanism underpinning the observed effects was hypothesized and discussed. Finally, *P. spinosa* fruit extract could be used as supplementary source of functional additives and might be a promising antimicrobial compound of natural origin to be employed to fight microbial resistance.

1. Introduction

Prunus spinosa L. (also known as blackthorn, Rosaceae family), is a thorny shrub growing wild in uncultivated areas of Europe, West Asia and the Mediterranean. The plant, used in phytotherapy for the treatment of cough but also as a diuretic, laxative, antispasmodic and anti-inflammatory (see Fraternale et al., 2009 and references therein), has been widely known since the nineteenth century for its pharmaceutical properties. Because of a very pungent flavour, *P. spinosa* fruits (1–1.5 cm plums) are unsuitable for human direct consumption, but usable for the production of jams and various beverages. Such a tart taste is due to the high content of tannins that, together with the high content of anthocyanins, make these fruits interesting for their potential antioxidant, antibacterial and anti-inflammatory activity (Kubacey et al., 2012; Pinacho, Cavero, Astiasaran, Ansorena, & Calvo, 2015). For this reason, wild *P. spinosa* from different countries has progressively been receiving increasing attention, which is also a good opportunity to recover and maintain local and almost forgotten varieties.

In the last years, biological activity of natural products on human health, food preservation, pharmaceutical application, nutrition and therapy, has been subject of many studies (Bernardini, Tiezzi, Larghezza Masci, & Ovidi, 2018; Georgiev, 2014; Mostafa et al., 2018). In particular, attention has been focused on their potential antimicrobial activity (i.e. the effect on microorganisms growth), especially in view of increasing resistance of pathogenic microbes to common antimicrobials, showing that natural compounds could be also a promising means to fight microbial resistance (Aleksic & Knezevic, 2014; Balouiri, Sadiki, & Ibnsouda, 2016; Hayek, Gyawali, & Ibrahim, 2013). Veličković et al. (2014) reported that *P. spinosa* (from South East Serbia) ripe fruit ethanol extract showed antibacterial activity against some ATCC strains of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella abony* NCTC 6017, with the exception

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of *Bacillus subtilis*. Moreover, the antifungal activity tested against two pathogenic fungi such as *Aspergillus niger* and *Candida albicans* was significant only against *C. albicans.* Antibacterial and bactericidal activities of *P. spinosa* fruit methanol extract have been tested against Gram negative (*E. coli*, *P. aeruginosa*, *Salmonella enteritidis*, *Shigella sonnei*, *Klebsiella pneumoniae* and *Proteus vulgaris*) and Gram positive bacteria (*Clostridium perfringens*, *B. subtilis*, *S. aureus*, *Listeria innocua*, *Sarcina lutea* and *Micrococcus flavus*) by Radovanović, Milenković Anđelković, Radovanović, and Anđelković (2013) and, in that study, obtained results revealed a high antimicrobial activity of the extract against almost all the tested bacterial strains, with the exclusion of *Klebsiella pneumoniae*. According to available data, such a bacteriostatic and bactericidal properties mainly rely on the presence of polyphenols, in particular anthocyanins (Cisowska, Wojnicz, & Hendrich, 2011; Govardhan Singha, Negib, & Radhaa, 2013; Sikora, Bieniek, & Borczak, 2013; Veličković et al., 2014).

The high quantity of polyphenols reported for *P. spinosa* fruit extract makes the plant and, particularly, the fruits further interesting and worthy of attention suggesting a potential anti-inflammatory property to be tested and (eventually) quantified. In fact, polyphenols have already been described to exert an anti-inflammatory activity. For example, Angel-Morales, Noratto, and Mertens-Talcott (2012a) observed a reduction of mRNA expression of some LPS-induced inflammatory markers (NFkB; ICAM-1, VCAM-1 and PECAM) in human colon-derived CCD-18Co myofibroblast cells which - according to the authors - was mediated, at least in part, by the induction of miR-126, a molecule that plays an important role in the inflammation process since it is responsible for regulating cell adhesion molecules (ICAM-1 and VCAM-1). In another case study, grape tendril extract (40 μg/mL) was able to prevent the LPS stimulus in monocyte (U937) and endothelial (HUVEC) cells (Fraternale et al., 2016), probably by the same mechanism hypothesized by Angel-Morales et al. (2012a).

In addition, many authors (Cosmulescu, Trandafir, & Nour, 2017; Fraternale et al., 2009; Ganhao, Estevez, Kylli, Heinonen, & Morcuende, 2010; Radovanović et al., 2013; Varga et al., 2017; Veličković, Ilić, Mitić, Mitić, & Kostić, 2016; Veličković et al., 2014) have demonstrated a powerful antioxidant activity of *P. spinosa* fruit juice/extract by DPPH (2,2-diphenyl-1-picrylhydrazyl) test. These results strongly suggest that *P. spinosa* fruits show a very high potential. In fact, it is widely known that the consumption of fruits with a high capacity of radical scavenging is associated with a lower occurrence of degenerative diseases such as inflammation and arthritis (Egea, Sanchez-Bel, Romojaro, & Pretel, 2010).

Given all these considerations, it clearly emerges that *P. spinosa* has notably aroused the interest of the scientific community for its potential and partially already proven biological properties. Within this scenario, since some years ago, our attention has focused on the characterization of *P. spinosa* fruits from our country (the Marches, Central Italy) with the aim to promote its adoption as new food beneficial for consumers or as a supplementary source of additive elements (i.e. natural pigments or antioxidants) for food or pharmaceutical industries. In particular, in a former study including chemical composition and biological activities of *P. spinosa* fruit juice from Italy, Fraternale et al. (2009) reported that the juice shows high antioxidant properties which are likely due to particularly abundant anthocyanins. To further investigate on this item, present study was carried out with the purpose of assessing the properties of *P. spinosa* fruit ethanol extract by evaluating its antioxidant, anti-inflammatory and antimicrobial activities against a panel of isolates of five bacterial and two fungi strains for: (i) a potential use as new food or supplementary source of functional additives; or (ii) providing additional data for further development of research on antimicrobial products of natural origin. Moreover, the phenolic profile of the ethanol extract was also investigated and results were compared with available data on *P. spinosa* fruit methanol/ethanol extract from other (i.e. out of Italy) Countries.

2. Materials and methods

2.1. Chemicals and reagents

Chromatographic solvents (water, acetonitrile and formic acid) were HPLC grade, purchased from Merck (Italy). HPLC-grade Standards of cyanidin 3-glucoside (≥97%), was purchased from Sigma-Aldrich (Italy); cyanidin chloride (≥96%) was purchased from Extrasynthese (Genay, France).

2.2. Preparation of P. spinosa extract

Plant species was identified by Prof. D. Fraternale (University of Urbino). Voucher samples were deposited in the herbarium of Urbino University Botanical garden: code "*Pp*125". Ripe fruits were collected in November 2017 at "La Caputa" Urbania (PU, Marche, Italy), GPS coordinates: N43°40'46.512" E12°31'42.291" (350 m above sea level) and, immediately after harvesting, frozen and stored at −20 °C until use. *P. spinosa* fruit extract was prepared as in Albertini et al. (2019). In particular, 50 g of ripe fruit pulp of *P. spinosa* were homogenized in Osterizer for 3 min in 50 mL of 70% aqueous ethanol solution (70 mL ethanol/30 mL distilled H_2O). The homogenate was subsequently filtered with a Buchner filter under vacuum and the filtrate was centrifuged at 10000*g* for 15 min. The supernatant was collected while the pellets and the residue of the first extraction were re-extracted under the same initial conditions. Subsequently, the two surnatants were combined and concentrated under vacuum up to a volume of 50 mL in a Büchi rotavapor at 37 °C. In this way, 1 mL of concentrated extract corresponds to 1 g of *P. spinosa* fruit pulp. The extract was aliquoted in eppendorf tubes, dried under vacuum using a Savant concentrator and stored at −20 °C until analysis.

2.3. Determination of phenolic compounds by HPLC-DAD and HPLC/MS analysis

The separation, identification and quantification of flavanols, hydroxycinnamic acids and benzoic acids in prune extracts was performed by High Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD) in an Agilent 1200 chromatographic system, equipped with quaternary pump, UV–VIS diode-array detector, automatic injector, and ChemStation software (Agilent Technologies, Palo Alto, USA). Prior direct injection, the samples were filtered through a 0.45 μm Nylon filter (E0034, Análisis Vínicos, Spain). Phenolic compounds were separated using a Zorbax C18 column (250 × 4.6 mm, 5 μm particle size) maintained at 40 °C following the method described in Gordillo et al. (2014). Acetonitrile/formic acid/water (3:10:87) was used as solvent A and acetonitrile/formic acid/water (50:10:40) was used as solvent B. The elution profile was 0–10 min with 6% B; 10–15 min with 11% B; 15–20 min with 20% B; 20–25 min with 23% B; 25–30 min with 26% B; 30–35 min with 40% B; 35–38 min with 50% B; 38–46 min with 60% B; and 46–47 min with 6% B. The flow rate was 0.63 mL/min, and the injection volume was 50 μL. All UV–Vis spectra were recorded from 200 to 800 nm with a bandwidth of 2.0 nm. The wavelengths of detection were 280 nm (flavanols and benzoic acids), 320 nm (hydroxycinnamic acids) and 525 nm (anthocyanins). The identification was achieved by comparison of the retention times and spectra characteristics of individual compounds with those of the available pure standards and our data library. The external calibration method was used for quantification, by comparing the areas with commercial standards of gallic acid, caffeic acid and catechin.

The determination of anthocyanin compounds in prune extract was performed by HPLC-PDA-ESI-MS. Chromatographic analysis was carried out with a Waters system equipped with an automatic injector, Alliance HT 2795 separation module, column heater, and Waters 2996 Photo Diode Array (PDA). The column used was a Merk Purospher Star RP-18 endcapped (4.0 mm × 250 mm, 5 μm particle size),

thermostated at 25 °C. The solvents used were (A) aqueous 0.1% formic acid in double distilled water and (B) Acetonitrile. The elution profile was as follows: 0–10 min, 94% A; 10–15 min, 83% A; 15–25 min, 78% A; 25–35 min, 76% A; 35–40 min, 74% A; 40–45 min, 68% A. The flow rate was 0.7 mL/min, and the injection volume was 50 µL (10 mg/mL of extract, dissolved in EtOH/H_2O 70:30 v/v). UV–Vis spectra were recorded from 220 to 800 nm with a bandwidth of 1.2 nm. Before direct injection, the samples were filtered through a 0.45 µm nylon filter. For the anthocyanin identification, the mass spectrometer was connected to the HPLC system via the PDA cell outlet. MS detection was carried out at 525 nm and performed with a Waters micromass ZQ MS system, single quadrupole, equipped with an Electrospray Ionization (ESI) interface. The instrument was operated in positive (ESI +) ion mode with a scan range from *m/z* 200–700. Capillary voltage was set at 3 kV, source temperature at 100 °C and desolvation temperature at 300 °C. The cone and desolvation nitrogen gas flows were 50 and 500 L/h, respectively. Data were processed using MassLynx 4.1 (Waters, Milford, USA). The quantification of anthocyanins was carried out by external calibration from the areas of the chromatographic peaks at 525 nm using a calibration curve of cyanidin-3-glucoside standard.

All analyses were performed in triplicate and the results were expressed as mg/100 g DW (dry weight). Total flavanols, hydroxycinnamic acids, benzoic acids and anthocyanins were estimated by summing the content of each member quantified.

2.4. Test organisms and inocula preparation

The *in vitro* activity quantification of *P. spinosa* crude extract was performed according to CLSI standard methods (CLSI, 1999, 2008; NCCLS, 2002).

Five bacterial and two fungal reference strains were used: *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteriditis* ATCC 13076, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 9642.

Starting from cultures in Tryptic Soy Agar (TSA, Liofilchem, Roseto degli Abruzzi, Italy), bacteria were grown overnight at 36 ± 1 °C in Mueller Hinton Broth (MHB, Sigma-Aldrich, Milan, Italy); each culture broth was used to obtain a standard inoculum. *C. albicans* (yeast) was grown on Sabouraud Dextrose Agar (SDA, Liofilchem) at 36 ± 1 °C for 24 h and *A. niger* (mold) on Potato Dextrose Agar (PDA, Liofilchem) at 25 °C for 7 days. The colonies were then used to prepare the fungal inoculum in sterile saline.

The microorganism suspensions were standardized to 0.5 McFarland scale (10^8 CFU/mL) using spectrophotometric measurement (600 nm for bacteria, 530 nm for yeast), and plate count method (for mold). Then the suspensions of each strains were diluted with standard culture media: MHB to obtain 10^6 CFU/mL of bacteria, RPMI 1640 (Sigma-Aldrich) to obtain 10^3 CFU/mL of yeast and 10^4 CFU/mL of mold.

2.5. Antimicrobial activity

The standard antimicrobial susceptibility test based on the determination of microorganism growth as a function of decreasing concentrations of the test product was performed by using 96-well microtiter plates. Serial doubling dilutions of *P. spinosa* extract (over the range of 69.75–0.07 mg/mL) were prepared in the standard culture media (100 µL in each well). Afterwards, extract dilutions were inoculated with the test strains (100 µL in each well); positive control (culture medium + test strains, without extract) to test viability, and negative control (only culture medium) to test sterility conditions were also added. Bacteria and yeast were incubated for 24–48 h at 36 ± 1 °C, mold for 72 h at 25 °C. Macroscopic observation of turbidity allowed the qualitative assessment of microbial growth which was, on the other hand, quantitatively determined and expressed as OD (Optical Density) units by absorbance at 600 nm (bacteria) and 530 nm (yeast) using the universal microplate reader Multiskan EX (Thermo Electron Corporation). Growth of the filamentous fungus *A. niger* was evaluated only by plate count. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of *P. spinosa* fruit ethanol extract at which microorganisms showed no visible growth. Data were reported as mg/mL of crude extract and µg/mL of total phenolic content.

In order to further evaluate the effect of the extract on microbial growth, a broth microdilution method was carried out by a series of sterile tubes containing aliquots of 5 mL of culture medium (TSB Tryptic Soy Broth, Sigma-Aldrich), different concentrations of *P. spinosa* extract (over the range of 69.75–0.07 mg/mL) and suspensions of each strain. Experimental conditions were the same as those described above.

After MIC determination, aliquots (20 µL) taken from each well showing complete growth inhibition (100% inhibition or an optically clear well), from the last positive well (showing growth similar to that of positive control well), and from the positive control well (containing extract-free medium) were subcultured on agar plates of TSA for bacteria, SDA for yeast and PDA for mold. The plates were then incubated at the specific temperature of the microbial strains. The lack of growth (i.e. the absence of colonies on the surface of the agar plates) determines the Minimum Bactericidal Concentration or Minimum Fungicidal Concentration (MBC/MFC) which is defined as the lowest concentration of the extract required to kill 99.9% of the initial inoculum (Balouiri et al., 2016; Espinel-Ingroff, Fothergill, Peter, Rinaldi, & Walsh, 2002). Every series of tests was performed in triplicate.

2.6. Quantitative real time PCR (RT-qPCR) of mature microRNAs and mRNA

The total RNA isolation kit (Norgen Biotek, Thorold, Canada) was used to isolate total RNA (including both microRNA and larger RNA species) from 1 × 10^6 U937 cells, following the manufacturer's recommended protocol; RNA was stored at −80 °C until use. Human miR-126, miR-146a and human RNU44 (reference miRNA) expressions were quantified using the TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA), as previously described (Olivieri et al., 2013).

Isolated RNA was used to synthesise cDNA using a reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative polymerase chain reaction real time (RT-qPCR) was performed with the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers (ICAM-1: Forward TGGCCCTCCATAGACATGTGT and Reverse TGGCATCCGTCAGGAAGTG; VCAM-1: Forward ACAGAAGAAGTGGCC CTCCAT and Reverse TGGCATCCGTCAGGAAGTG; IL-6: Forward AGG GCTCTTCGGCAAATGTA and Reverse GAAGGAATGCCCATTAACAA CAA; IRAK-1: Forward CAGACAGGGAAGGGAAACATTTT and Reverse CATGAAACCTGACTTGCTTCTGAA) we used were the ones designed by Angel-Morales, Noratto, and Mertens-Talcott (2012b). TATA binding protein (TBP; Forward TGCACAGGAGCCAAGAGTGAA and Reverse CACATCACAGCTCCCCACCA) was used as an endogenous control to determine relative mRNA expression. The pairs of forward and reverse primers were purchased from Sigma-Aldrich. Product specificity was examined by dissociation curve analysis. Results were calculated using the 2-ΔΔCt method and are expressed in the figures as fold change related to untreated control (CTRL) (Fraternale et al., 2016).

2.7. Monocyte-endothelial cell adhesion

HUVECs (3 × 10^5) were grown on coverslips (Sigma-Aldrich) placed into a six-well plate to reach semiconfluence. HUVECs were treated with *P. spinosa* ethanol extract during LPS (1 µg/mL) stimulation (Fraternale et al., 2016). U937 cells (1 × 10^6) were added to each well and incubated for 1 h. U937 non-adherent cells were removed by

three PBS washes and coverslips were placed on glass microscope slides (Sigma-Aldrich). Images were captured under an optical microscope (using a BX-51 Olympus microscope with a 40 × objective) and adherent cells were counted (three random fields) and expressed as the number of adhered cells per field related to the number of HUVECs (i.e. U937/HUVEC per field).

2.8. DPPH radical assay

The DPPH (DiPhenylPicrylHydrazyl) radical-scavenging method was used to evaluate the anti-oxidant capacity of *Prunus spinosa* following experimental protocol described in Fraternale et al. (2016). Briefly, the reaction solution (400 µL final volume) was mixed with 80 µL of 0.5 mM DPPH (Sigma-Aldrich) ethanol solution and 40 µL of *P. spinosa* ethanol extract at different final concentrations (ranging from 10 to 600 µg/mL). After shaking, mixtures were left for 1 h in the dark. Absorbance was measured at 517 nm using ethanol as blank. The negative control was prepared with only DPPH (80 µL of 0.5 mM) diluted in ethanol (400 µL final volume). Lower absorbance indicates high free radical scavenging ability. DPPH inhibition (I, expressed in %) was used to evaluate the antioxidant activity by the following equation:

$$I(\%) = [(A_0 - A_s)/A_0] \times 100$$

where A_0 is the absorbance of the negative control and A_s is the absorbance of the tested sample.

All analyses were performed in triplicate. L-ascorbic acid (Sigma-Aldrich) was used as standard control at a concentration of 0.040 mg/mL.

2.9. Statistical analysis

Growth curve analysis was used to analyze microbial OD in function of extract concentrations. A non-linear regression fitting of 4 parameter logistic growth curve was performed: $N_x = N_0 + \frac{N_{max}}{(1+e^{-1/b(x-c)})}$

where × is the *P. spinosa* extract concentration, N_x is the microbial OD at concentration ×, N_{max} is the maximal microbial OD expected, c is the point of inflection, and b is inversely related to the tangent slope (m) in flex point. The × value has been transformed using x* = Log (100x + 1), in order to obtain an arithmetic progression from a geometric progression. The inflection point coordinates (*Flex*) were calculated as follows: $Flex = \left(c; \frac{N_{max}}{2} + N_0\right)$ and the tangent slope (*m*) in flex point was estimated as: $m = \frac{N_{max}}{4b}$.

Non-linear regression (Bates & Watts, 2007), was performed using Levenberg-Marquardt algorithm on SPSS 22.0 software. Multivariate analysis of variance (MANOVA) was performed in order to compare the groups. The significance level was set at $\alpha = 0.05$.

The two-tailed paired Student's *t*-test was used for the anti-inflammatory (miR-126, ICAM-1, VCAM-1, miR-146a, IRAK-1, IL-6 and cell adhesion count) analyses. The results were considered significant at the level of $p < 0.05$.

Linear regression analysis was used to evaluate the DPPH scavenging activity of *P. spinosa* extract.

3. Results and discussion

3.1. Phenolic composition and antioxidant effect of P. spinosa extract

The phenolic profile of *P. spinosa* crude extract is shown in Fig. 1. Different classes of colourless phenolics were identified and quantified by HPLC (Fig. 1a) including hydroxycinnamic acids (caffeic, coumaric, chlorogenic acids and derivatives), hydroxybenzoic acids (gallic acid and derivatives of syringic and vainillic acids), and flavanols ((+)-catechin). Most of these compounds have already been described in fresh prune fruits (Madrau, Sanguinetti, Del Caro, Fadda, & Piga, 2010). However, the most common hydroxycinammic acid (neochlorogenic acid) was not found in *P. spinosa* extract, probably due to genetic differences between cultivars (Miletic et al., 2013). Regarding anthocyanin pigments, the chromatogram registered at 525 nm shows the presence of the two main anthocyanins (Fig. 1b). Both compounds were identified as cyanidin derivatives according to the HPLC-PDA-MS analysis (Fig. 1c and 1d). Table 1 shows the concentrations (mg/100 g DW ± SD, n = 3) of the different phenolic families described. The most abundant phenolic compounds in *P. spinosa* extract were anthocyanins (58%) and hydroxycinnamic acids (22%). Similar proportions (40–45%) of anthocyanins have been reported in literature for fresh prune fruits (Stacewicz-Sapuntzakis, Bowen, Hussain, Damayanti-Wood, & Farnsworth, 2001). Nevertheless, other studies found much higher proportions (50–70%) of hydroxycinnamic acids (Madrau et al., 2010; Piga, Del Caro, & Corda, 2003). Although in lower amounts, hydroxybenzoic acids (12%) and flavanols (8%) were also present in the extract. Amongst anthocyanins, cyanidin 3-rutinoside and cyanidin 3-glucoside have been indicated as the main ones present in prunes, especially the wild and dark varieties, being responsible for the red, or violet to black colours (Madrau et al., 2010; Mikulic-Petkovsek, Stampar, Veberic, & Sircelj, 2016). In our case, in *P. spinosa* ethanol extract, the major anthocyanin corresponded - according to its mass molecular ion ($[M]^+ = 287$) and UV–visible spectra features (Fig. 1d) - with the aglycon cyanidin (85.95 ± 1.94 mg/100 g DW). A possible explanation that could be offered for such a discrepancy with precedent findings respect to the presence of cyanidin 3-rutinoside is that, typically, the production of crude extracts with several contaminants submitted to concentration procedures can result in the hydrolysis of labile linkages of anthocyanins such as sugar residues (Rodriguez-Saona & Wrolstad, 2001) which could explain the presence of the aglycon cyanidin. Its glycoside analogue cyanidin 3-glucoside ($[M]^+ = 449$), was also detected, but in lower amount respect to what reported by Mikulic-Petkovsek et al., 2016 (about 2 mg/Kg FW vs about 1286 mg/kg FW), but - on the other hand - higher (about 13.33 mg/100 g DW vs 19.83 µg/100 g DW) than what reported by Guimarães et al. (2013). Other minor anthocyanins were also detected but their absorption or mass spectra were insufficient to allow speculation about their identity. Notably, an aspect that should not be underestimated and that perhaps could, at least, in part justify the strong discrepancy between the values observed in our study and those previously reported for *P. spinosa*, is probably related with: (i) different "environmental conditions" (i.e. fruit ripening level, temperature, insolation, pH and rainfall precipitations); (ii) different extraction techniques (i.e. methanol: water; BHT: formic acid: methanol; ethanol: water) and (iii) different concentrations of the solvent (Guimarães et al., 2013; Mikulic-Petkovsek et al., 2016), given that the conditions of each methodology may interfere with the results.

P. spinosa extract showed a concentration-dependent antioxidant activity (Fig. 2) when analysed by DPPH assay ($R^2 = 0.955$). The phenolic compounds (see Table 1) may be responsible for the antioxidant activity observed, as hypothesized in other studies (Radovanović et al., 2013).

3.2. Antimicrobial effect of P. spinosa ethanol extract on target microorganisms

The antimicrobial effects of *P. spinosa* extract against target microorganisms, as determined by evaluating the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) values (in mg/mL) of *P. spinosa* extract, are shown in Table 2. In particular, the extract appeared to exert a wide antibacterial activity and yeast inhibition, with the exception of *A. niger* (MIC 34.78 mg/mL). No specific inhibitory action was observed against the tested Gram-negative or Gram-positive bacteria, as suggested by comparable MIC values ranging from 4.36 to 8.72 mg/mL. *P. spinosa* extract concentration necessary to kill bacteria or fungi (MBC/MFC) mostly resulted to be nearly twofold the observed correspondent MIC

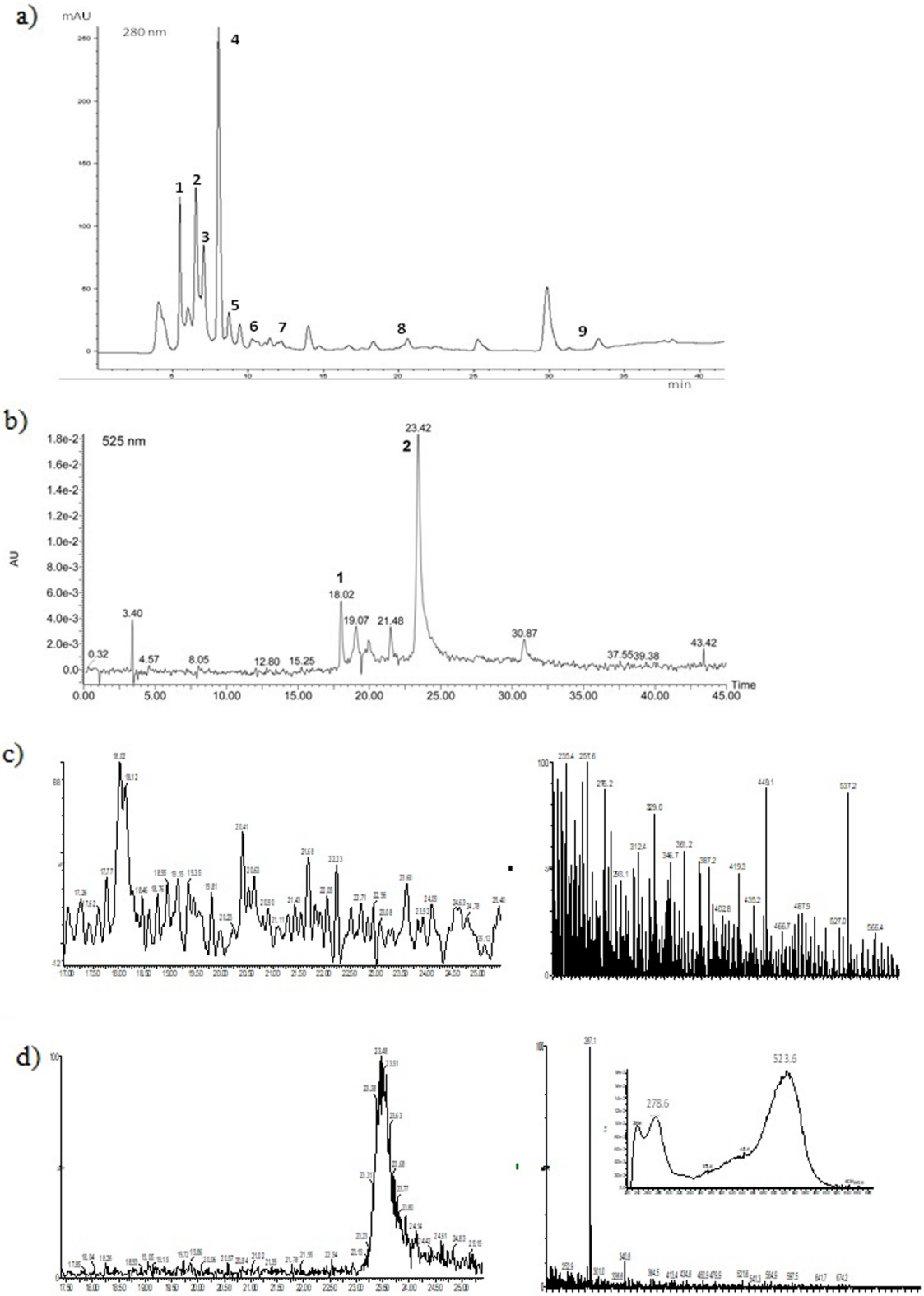

Fig. 1. (a) HPLC chromatogram of *P. spinosa* extract recorded at 280 nm, peaks: 1, Gallic acid; 2, m-coumaric derivative; 3, Vainillic acid derivative 1; 4, (+)-catechin; 5, Syringic acid derivative; 6, Vanillic acid derivative 2; 7, Caffeic acid; 8, *p*-coumaric acid; 9, Chlorogenic acid; (b) HPLC chromatogram recorded at 525 nm (0.00–45.00 min) showing the two major anthocyanins peaks: 1, cyanidin 3-glucoside; 2, cyanidin; (c) Expanded ion chromatogram at *m/z* value of 449 (TIC scan ES + 449.1 0PPM) and Mass spectra (ES^+) on peak 1 (r.t. 18.02 min); (d) Expanded ion chromatogram at *m/z* value of 287 (TIC scan ES + 287.1 0PPM) and UV–Vis and Mass spectra (ES^+) on peak 2 (r.t. 23.42 min).

Table 1
Phenolic content (mg/100 g DW ± SD) of different phenolic families in *P. spinosa* ethanol extract.

Phenolic compounds	Concentration (mg/100 g DW ± SD)
Sum of anthocyanins (525 nm)	150.11 ± 2.08
Sum of flavanols (280 nm)	30.10 ± 0.35
Sum of hydroxycinnamic acids (320 nm)	56.50 ± 1.26
Sum of benzoic acids (280 nm)	21.66 ± 0.38

value. Also in this case, no differences in antibacterial activity were evidenced between Gram-negative or Gram-positive strains.

Our findings are in accordance with other studies; for example, Veličković et al. (2014) reported the antimicrobial effect of *P. spinosa* with extract concentration ranging from 10 to 30 mg/mL; and Coccia et al. (2012) examined the antimicrobial effect of *Prunus cerasus* L. extract on different microbial strains, obtaining MICs in the range of 2.0–6.6 mg/mL. Moreover, comparing our results with available data, it was observed that MIC of *P. aeruginosa* was similar (4.36 vs 4.4 mg/mL) to that found in the study of Coccia et al. (2012). Notably, when comparing MIC values, expressed as total phenolic content, MIC for *P. spinosa* (10.50 μg/mL, present study) is lower than that of *P. cerasus* (108.24 μg/mL of GAE, Coccia et al., 2012). Taking into account that *P. spinosa* extract shows a total content of anthocyanins higher than that reported in various studies on different *Prunus* species (Coman et al., 2018), it seems reasonable to hypothesize that the greater efficacy in the antimicrobial activity demonstrated by *P. spinosa* ethanol extract may be due to anthocyanins, even if, at the moment, the hypothesis of a possible combined action of bioactive compounds cannot be ruled out.

For fungal strains, the anti-proliferative action was obtained on *C. albicans* at high concentrations, while growth of *A. niger* was inhibited only at the maximum concentration tested (69.75 mg/mL), thus confirming that the extract is not particularly active against fungi, as pharmacologically active molecules act in the microgram per millilitre (μg/mL) range, which is substantially lower than values here reported (see also Coccia et al., 2012).

In order to evaluate more accurately the minimum inhibitory concentrations (MICs), we employed a non-linear regression analysis to create microbial growth curves with OD 600 nm values vs different extract concentrations (Chorianopoulos et al., 2006). As indicated in Fig. 3, the microbial growth curves show that the antimicrobial effect of *P. spinosa* extract ranges from 2.18 mg/mL to 17.44 mg/mL.

Table 2
Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) values (mg/mL) of *P. spinosa* extract related to crude extract (mg/mL) and total phenolic content (μg/mL).

	MIC		MBC/MFC	
Target microorganisms	mg/ml	μg/ml	mg/ml	μg/ml
E. coli ATCC 25922	8.72	20.90	17.44	41.80
S. aureus ATCC 6538	8.72	20.90	17.44	41.80
P. aeruginosa ATCC 9027	4.36	10.50	8.72	20.90
E. faecalis ATCC 29212	4.36	10.50	8.72	20.90
S. enteriditis ATCC 13076	4.36	10.50	8.72	20.90
C. albicans ATCC 10231	8.72	20.90	17.44	41.80
A. niger ATCC 9642	34.87	83.70	69.75	167.40

Moreover, to compare MIC values calculated with this method to the previous MIC identified, we considered further data obtained by non-linear regression analysis indicated in Table 3. The xflex corresponds to the extract concentration (x values) where growth curve changes and microbial growth decreases. This value can be considered as the MIC value, with associated R^2 values representing the goodness of fitting. As indicated by xflex, the MIC values in the non-linear regression analysis (Table 3) were consistent to the values previously obtained (see Table 2).

Regarding the OD values (Fig. 3) and CFU mL^{-1} (data not shown), in order to evaluate the trend of microbial growth, it was observed that concentrations lower than MIC caused an increase in OD compared with untreated control (which was confirmed by CFU count), thus indicating a "paradox" effect of stimulation of both bacterial and fungal growth (Table 4).

For *E. coli*, *S. aureus, P. aeruginosa*, *S. enteriditis* and *C. albicans* these concentrations were lower than 1 mg/mL (0.07 to 0.55 mg/mL). *E. faecalis* showed the highest growth at 1.1 mg/mL. This is in line with previous results by Silva et al. (2016) who observed that on *Acinetobacter baumannii*, *Proteus mirabilis* and two *Pseudomonas* strains the blueberry extract did not act as an antimicrobial compound, but rather promoted microbial growth. In addition, Coccia et al. (2012) verified

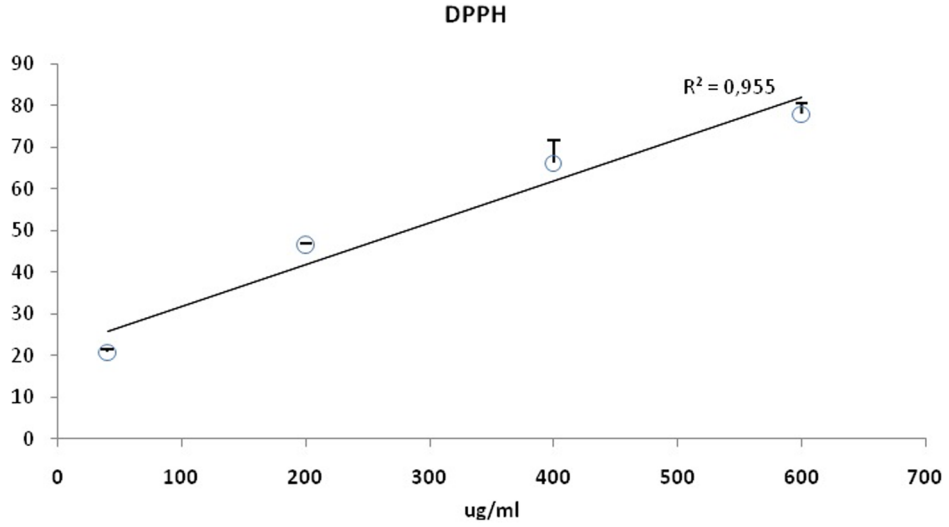

Fig. 2. DPPH analysis of *P. spinosa* extract antioxidant activity was carried out at different concentrations (from 20 μg/ml to 600 μg/ml). R^2 = 0.955 shows a concentration-dependent effect.

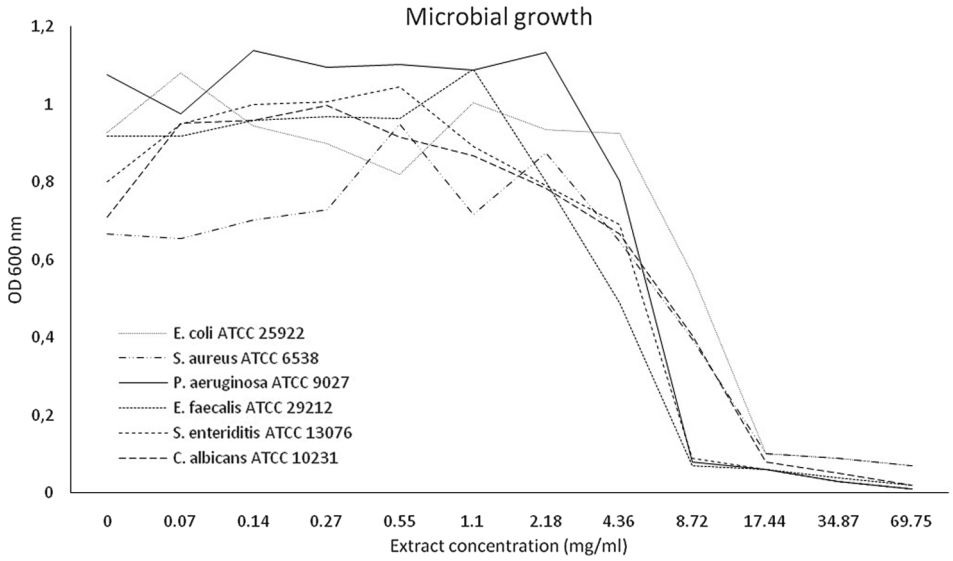

Fig. 3. Growth curve non-linear regression analysis 24 h after treatment at different extract concentrations (mg/mL). *Escherichia coli*; *Staphylococcus aureus*; *Pseudomonas aeruginosa*; *Enterococcus faecalis*; *Salmonella enteritidis*; *Candida albicans*.

Table 3
Growth parameters obtained from non-linear regression analysis.

	E. coli ATCC 25922	*S. aureus ATCC 6538*	*P. aeruginosa ATCC 9027*	*E. faecalis ATCC 29212*	*S. enteriditis ATCC 13076*	*C. albicans ATCC 10231*
y0	0.078	0.071	0.035	0.029	0.024	−0.003
ymax	0.866	0.682	1.052	0.935	0.907	0.903
xflex	9.130	8.264	5.146	4.158	5.352	7.338
yflex	0.511	0.412	0.561	0.496	0.478	0.448
mflex	−2.716	−1.364	−3.748	−1.749	−2.278	−1.037
R^2	0.975	0.925	0.993	0.986	0.971	0.962

Table 4
P. spinosa extract concentration exerting the highest microbial growth (highest OD values) obtained from non-linear regression analysis.

	P. spinosa concentration (mg/mL)	OD	MIC (mg/mL)
E. coli ATCC 25922	0.07	1.081	9.130
S. aureus ATCC 6538	0.55	0.95	8.264
P. aeruginosa ATCC 9027	0.14	1.138	5.146
E. faecalis ATCC 29212	1.1	1.09	4.158
S. enteriditis ATCC 13076	0.27	1.006	5.352
C. albicans ATCC 10231	0.27	0.997	7.338

that the sour cherry crude extract exerted a prebiotic-like effect at low concentrations against nine bacterial species and two yeast species.

3.3. Anti-inflammatory effect of *P. spinosa* ethanol extract

To investigate whether *P. spinosa* ethanol extract could have an anti-inflammatory activity, we first analyzed the expression of miR-126 and its cell adhesion molecules targets (ICAM-1 and VCAM-1). When miR-126 is down-regulated during pro-inflammatory stimulation, the expression of its targets is enhanced; while in our experimental conditions, during an acute pro-inflammatory challenge by LPS, treatment with *P. spinosa* (P. spinosa + LPS) increased miR-126 and decreased ICAM-1 and VCAM-1 expression levels (Fig. 4).

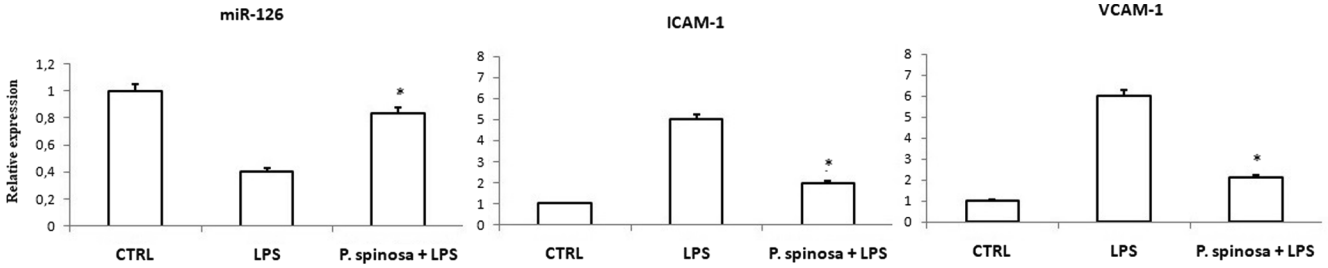

Fig. 4. Effect of *P. spinosa* extract on miR-126 and its cell adhesion molecules targets (ICAM-1 and VCAM-1) expression in LPS (lipopolysaccharide) stimulated cells. In U937, the LPS down-regulation of miR-126 (LPS) was restored during *P. spinosa* extract treatment (P. spinosa + LPS). *P. spinosa* extract treatment caused a decreased expression of both miR-126 targets, ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion protein 1). RT-qPCR values are reported as fold induction related to CTRL. Two-tailed paired Student's *t*-test: * = $p < 0.05$ LPS vs P. spinosa + LPS.

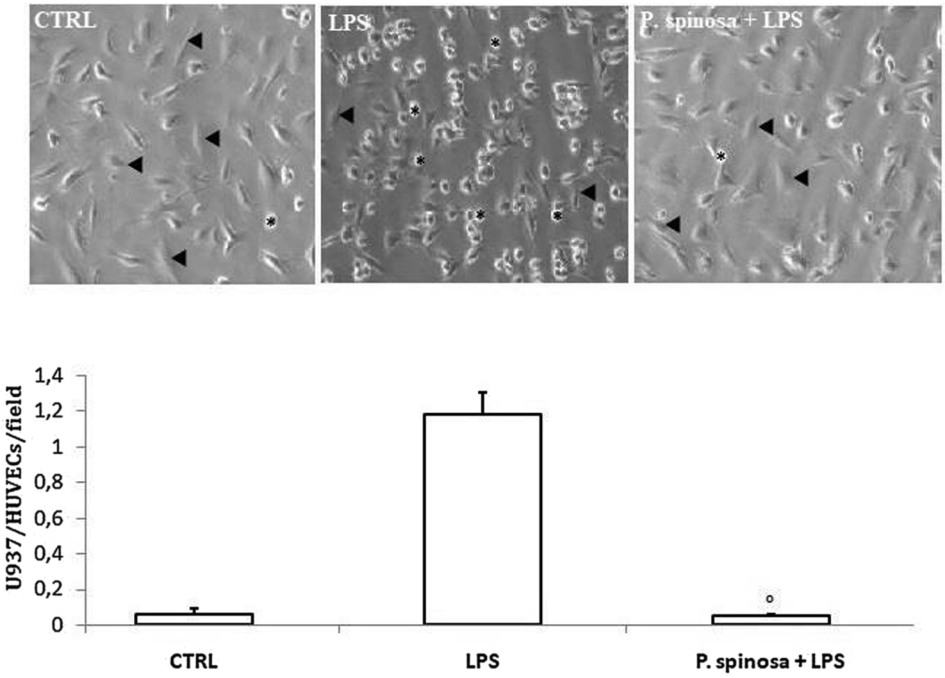

Fig. 5. U937 endothelial adhesion was evaluated adding untreated U937 cells to LPS-stimulated HUVECs without/with *P. spinosa* ethanol extract (LPS /P. spinosa + LPS, respectively). Images captured on a BX-51 Olympus microscope with a 40 × objective of U937 adherent to HUVECs were used to evaluate the number of adherent U937 (*) cells related to HUVECs (◀) present in each field analysed (3 fields for each treatment conditions). The number of adhered cells per field related to the number of HUVECs (U937/HUVECs/field) are reported in the bar graph, °= $p < 0.05$.

To confirm our results, the adherence of untreated U937 to HUVEC treated (at the same previously described experimental conditions) cells was determined. Our results identified a marked reduction of adherent U937 cells when LPS stimulated HUVECs were treated with *P. spinosa* ethanol extract, as demonstrated by both microscope images (Fig. 5) and adherent U937 cell count.

We further investigated *P. spinosa* ethanol extract anti-inflammatory activity by analyzing the TLR4 signalling through miR-146a, IRAK-1 and IL-6 expressions. As indicated in Fig. 6, when miR-146a is down-regulated during a pro-inflammatory challenge, the expression of its targets, IRAK-1 and IL-6, is enhanced. On the other hand, during an acute pro-inflammatory stimulus induced by LPS, treatment with *P. spinosa* ethanol extract (P. spinosa + LPS) increased miR-146a and decreased IRAK-1 and IL-6 expression levels.

Although the real nature of molecular interactions between TLR4 and *P. spinosa* ethanol extract are still to be investigated, our findings confirm that *P. spinosa* ethanol extract can up-regulate intracellular miR-126 and miR-146a expression levels with a consequent down-regulation of the TLR-NF-κB-mediated inflammatory response. In particular (see Fig. 7), treatment with *P. spinosa* ethanol extract can prevent the LPS stimulation by inhibiting TLR4 signalling and reducing cytokines (IL-6 and TNFα) production and cell adhesion molecules (ICAM-1 and VCAM-1).

4. Conclusions

This is the first report on the study of antimicrobial, anti-inflammatory and antioxidant properties of *P. spinosa* (from Central Italy, the Marche region) fruit ethanol extract along with its phytochemical (mostly phenolic) profile. The results are indicative of an intense anthocyanin accumulation which could explain the antioxidant activity and may be crucial for promoting consumption of *P. spinosa* fruit juice or extract. In fact, besides in case of diseases related to oxidative stress or inflammation, anthocyanins have been found to have also beneficial effects for people with obesity and metabolic syndrome (Shi, Mathai, Xu, McAinch, & Su, 2019) and, moreover, represent a source of natural pigments and antioxidants for food or pharmaceutical industries. Therefore *P. spinosa* fruits show a high potential and could be used as new food or food additives which could be considered beneficial for consumers.

As shown, the extract exhibited an interesting antimicrobial activity (higher than other *Prunus* species) against a range of bacteria and yeast, with the exception of the mold *A. niger*. As not all strains tested in the present study are pathogenic, and to assure whether there is any variability in the spectrum of the antimicrobial activity against a larger panel of pathogenic isolates, further studies in animal models and clinical trials would be essential to fill these gaps of knowledge and determine the *in vivo* benefits. Nevertheless, our findings attest that *P.*

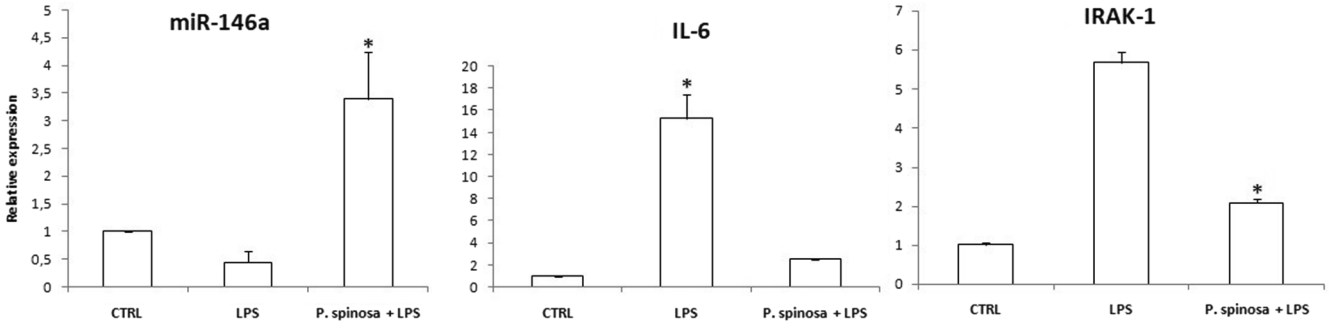

Fig. 6. Effect of *P. spinosa* extract on miR-146a, its cell IRAK-1 target and IL-6 expressions in LPS (lipopolysaccharide) stimulated cells. In U937, the LPS down-regulation of miR-146a (LPS) was increased during *P. spinosa* extract treatment (P. spinosa + LPS). *P. spinosa* extract treatment caused a decreased expression of both IRAK-1 and IL-6. RT-qPCR values are reported as fold induction related to CTRL. Two-tailed paired Student's *t*-test: *= $p < 0.05$ LPS vs P. spinosa + LPS.

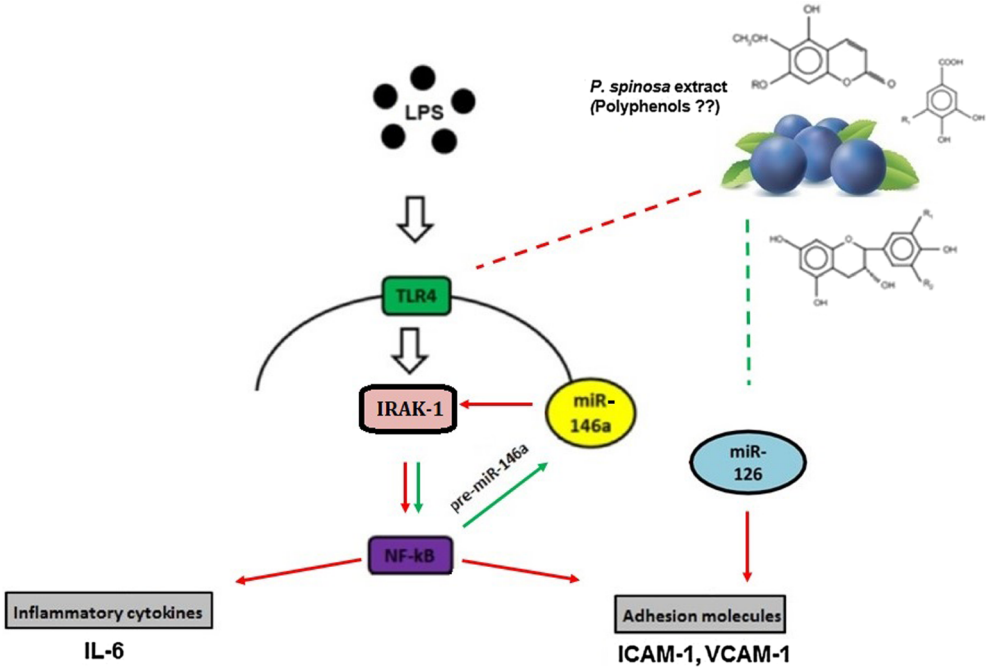

Fig. 7. Schematic representation of the possible *P. spinosa* extract action mechanism. TLR4 signalling has been analyzed to evaluate the anti-inflammatory effect of *P. spinosa* extract. During a pro-inflammatory stimulus (LPS) TLR4 and IRAK-1 activate NFkB transcription factor to express miR-146a, IL-6 and cell adhesion molecules (ICAM-1, VCAM-1). When cells are treated with *P. spinosa* extract, TLR4 signalling can be blocked by a negative feedback loop mechanism where miR-146a targets IRAK-1 (down-regulation) leading to a decreased expression of IL-6 and cell adhesion molecules (ICAM-1, VCAM-1). Furthermore, miR-126 up-regulation may also participate to decrease its cell adhesion molecules (ICAM-1, VCAM-1) targets expression. Legend: Red-dotted line: hypothesized negative interaction; Green-dotted line: hypothesized positive interaction; Red arrow: negative interaction; Green arrow: positive interaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spinosa extract is a promising candidate for the prevention of several bacterial infections, although the responsible mechanisms behind the beneficial effects require further investigation.

Finally, preliminary data seems to suggest a very interesting anti-inflammatory activity of the extract. In fact, treatment with *P. spinosa* fruit ethanol extract prevented U937 cells stimulation by LPS, inhibiting TLR4 signalling, along with pro-inflammatory cytokine production and cell adhesion. In particular, according to our experimental results the action mechanism tentatively suggested is as follows: polyphenols, (as proposed by Fraternale et al., 2016) would be capable of influencing the activation of the intracellular NF-kB signaling pathway which (along with MAPK and JAK-STAT) is responsible for the production of various inflammatory mediators. NF-kB activation increases miR-146a expression, which in turn inhibits IRAK-1 (one of the key mediators of the Toll-like receptor 4 pathway, along with TRAF-6 and MYD88) expression as a negative feedback loop. Such a process reduces NF-kB translocation into the nucleus causing a down-regulation of the transcription of target genes, including IL-6, ICAM-1 and VCAM-1. Moreover, polyphenols would increase miR-126 expression with a further down-regulation of ICAM-1 and VCAM-1. Although at the moment the molecular basis of the anti-inflammatory properties of *P. spinosa* extract needs further studies, present findings seem, however, to corroborate this hypothesis. Nevertheless, the action mechanism tentatively proposed in this report will be certainly explored in the next future to better clarify this item.

Author contributions

M.C. designed the study, wrote the main manuscript and revised the ms.; L.S. designed and performed the microbial experimental trials; D.F. prepared *P. spinosus* ethanol extract and revised the ms.; M.C.A. performed quantitative Real Time PCR, monocyte-endothelial cell adhesion and DPPH radical assays and revised the ms.; M.B.L.R. and D.S. performed the data analyses;.B.D.G., M.M. and B.G. performed HPLC-DAD and HPLC/MS analyses; All authors reviewed the ms.

Authors' declaration

The manuscript contains original unpublished work and is not being submitted for publication elsewhere at the same time.

Ethics statements

The study comprised two main items including: (i) chemical analyses by HPLC-DAD and HPLC/MS to determine the phenolic compounds; and (ii) *in vitro* experiments to assess the antioxidant, anti-inflammatory and antimicrobial properties of *P. spinosa* fruit ethanol crude extract. Neither animals nor human subjects were involved therefore no institutional or national ethical committee approval was needed.

Author contribution

M.C.: Conceptualization, Writing - original draft, Writing - review & editing; L.S.: Investigation (microbial experimental trials); D.F.: Methodology (P. spinosus ethanol extract preparation), review & editing; M.C.A.: Investigation (quantitative Real Time PCR, monocyte-endothelial cell adhesion and DPPH radical assays), review & editing; M.B.L.R. and D.S.: Data curation (statistical analysis);.B.D.G., M.M. and B.G.: Investigation (HPLC-DAD and HPLC/MS analyses); All authors reviewed the ms.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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