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Protective effect of quercetin in gentamicin-induced oxidative stress *in vitro* and *in vivo* in blood cells. Effect on gentamicin antimicrobial activity

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ABSTRACT

We have evaluated the effect of gentamicin and gentamicin plus quercetin on ROS production, endogenous antioxidant defenses (SOD and CAT) and lipid peroxidation *in vitro* on human leukocytes and *in vivo* on whole rat blood. Gentamicin generated ROS production in human leukocytes, produced a dual effect on both enzymes dosage-dependent and generated an increase in lipid peroxidation. Quercetin, in leukocytes stimulated by gentamicin, showed more inhibitory capacity in ROS production than the reference inhibitor (vitamin C) in mononuclear cells and a similar protective behavior at this inhibitor in polymorphonuclear cells. Quercetin, in both cellular systems, tend to level SOD and CAT activities, reaching basal values and could prevent lipidic peroxidation induced by gentamicin. The results in Wistar rats confirmed that therapeutic doses of gentamicin can induce oxidative stress in whole blood and that the gentamicin treatment plus quercetin can suppress ROS generation, collaborate with SOD and CAT and diminish lipid peroxidation. Finally, flavonoid and antibiotic association was evaluated on the antimicrobial activity in *S. aureus* and *E. coli*, showing that changes were not generated in the antibacterial activity of gentamicin against *E. coli* strains, while for strains of *S. aureus* a beneficial effect observes. Therefore, we have demonstrated that gentamicin could induce oxidative stress in human leukocytes and in whole blood of Wistar rats at therapeutic doses and that quercetin may to produce a protective effect on this oxidative stress generated without substantially modifying the antibacterial activity of gentamicin against *E. coli* strains, and it contributes to this activity against *S. aureus* strains.

1. Introduction

Reactive oxygen species (ROS) are a collective term that includes both oxygen radicals and certain non-radicals that are oxidizing agents and/or easily converted into radicals. The alteration of normal levels of ROS induces oxidative damage to biomolecules such as DNA, lipids and proteins producing alterations in their structure, favoring atherosclerosis, ageing, cancer, ulcer, inflammation and many degenerative disorders in human beings (Boots et al., 2008; Buonocore and Tataranno, 2010; Halliwell and Gutteridge, 1999).

Free radicals reactions have been suggested to be involved in the toxic effects of several antibiotics in the human body, with reports of damage in DNA (Hiraku and Kawanishi, 2000), leukocytes (Becerra et al., 2003; Páez et al., 2008), whole blood (Correa-

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GEN, gentamicin; Q, quercetin; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; NBT, nitroblue tetrazolium; EDTA, ethylenediaminetetraacetic acid; MDA, malondialdehide; TBA, 2-thiobarbituric acid; TCA, trichloroacetic; HBSS, Hank's balanced salt solution; PMN, polymorphonuclear leukocytes; MN, mononuclear leukocytes; MIC, minimal inhibitory concentration; DMSO, dimethyl sulfoxide; TBARS, thiobarbituric acid reactive substances.

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salde and Albesa, 2009; Ray et al., 2006) and human fibroblast (Hincal et al., 2003). Gentamicin (GEN), an aminoglycoside antibiotic, has been successfully used for decades in the treatment of Gram-negative bacterial infections and currently regains popularity because of widespread resistance to other antibiotic types (Denamur et al., 2011). However, the clinical utility of GEN is limited by the development of adverse effects, which are mostly nephrotoxicity, ototoxicity, neurotoxicity and hepatotoxicity, but also hematotoxicity with documented leukopenia, granulocytopenia and thrombocytopenia (Sweetman, 2009). Although the pathogenesis of the major side effects of GEN is still not well elucidated, many studies in rats have suggested that the toxic effects are commonly related to ROS production, alterations in antioxidant defense mechanisms, and increased lipid peroxidation in specific tissues (Al-Majed et al., 2002; Karahan et al., 2005; Noorani et al., 2011; Pedraza-Chaverrí et al., 2000; Raju et al., 2011; Yang et al., 2011). But the regulation of ROS production is also important in leukocytes, because these cells perform an important function in host defense against infectious agents by producing ROS species, hydrolytic and proteolytic enzymes, and antimicrobial polypeptides (Páez et al., 2008). However, in addition to their microbicidal activity, leukocytes can also injure the host by tissue-damaging inflammatory reactions and by disrupting tissue integrity and function (Kaneider et al., 2006; Weiss, 1989). Furthermore, due to evidence that demonstrates that antibiotics such as ciprofloxacin and chloramphenicol induce oxidative stress in human leukocytes (Becerra et al., 2003; Páez et al., 2008), and considering that leukotoxicity is an important aspect among the toxic consequences of an antibiotic is essential to determine the effect of GEN on these blood cells.

In past decades numerous pharmacological interventions have been demonstrated to prevent GEN induced oxidative stress (Balakumar et al., 2010). Using natural products as sources for new drug discovery and treatment of diseases have attracted attention due to their antioxidants capacity in order to protect against the toxic effects of GEN (Balakumar et al., 2010; Maldonado et al., 2003; Moreira et al., 2014; Noorani et al., 2011; Parlakpinar et al., 2005; Yang et al., 2011). Flavonoids are naturally occurring polyphenolic compounds found in the vegetal kingdom in flowers and in some fruits as pigments (Ortega et al., 2010), and they constitute one interesting chemical group by varied biological activities such as its antioxidant power, *i.e.* its ability to reduce RL and chelate metals blocking their catalytic ability.

Flaveria bidentis (L.) Kuntze is a native species that has a distribution in America and grows in Córdoba, Argentina. In our research group, the chemical study was carried out in the leaves of this species, obtaining quercetin (Q) and two Q sulfated derivatives quercetin- 3-acetyl-7, 3′,4′-trisulphate (ATS) and quercetin-3,7,3′,4′-tetrasulphate (QTS), among other minority flavonoids (Agnese et al., 2009; Ortega et al., 2010). Quercetin (3, 3′,4′,5,7-pentahydroxyflavone), one of the most widely distributed flavonoids in plants, has shown to possess a broad range of pharmacological properties, including protective effects against oxidative stress by its strong antioxidant capacity (Boots et al., 2008; Procházková et al., 2011; Saito et al., 2004).

In this respect, several studies of GEN have been carried out in order to evaluate the protective effect of flavonoids in nephrotoxicity and ototoxicity *in vivo*, showing an important reduction of these toxic effects. However, these studies made use high GEN concentrations (no therapeutic), with ROS analysis mainly made in specific animal tissues, and the systemic effect of this antibiotic has never been evaluated (Abdel-Raheem et al., 2009; Balakumar et al., 2010; Fouad et al., 2014).

Therefore, we set out to evaluate the effect of GEN on ROS production, endogenous antioxidant enzymatic defenses (Superoxide Dismutase and Catalase) and lipid peroxidation *in vitro*, on human mononuclear and polymorphonuclear leukocytes, and *in vivo* in rat whole blood. Furthermore, in order to find natural products with the ability to counteract GEN toxic effects related to ROS production at systemic level, we have investigated the effects of Q on GEN-ROS generation, endogenous antioxidant defenses, and lipid peroxidation in human leukocytes *in vitro* and in rat whole blood and plasma *in vivo*. Finally, since oxidative stress has been recently implicated as one of the mechanisms whereby bactericidal aminoglycoside antibiotics, such as GEN and kanamycin, kill bacteria (Kohanski et al., 2008), we have investigated the effect of the association of Q and GEN on the antimicrobial activity in *Staphylococcus aureus* and *Escherichia coli* in order to determine whether Q interferes with GEN antibacterial activity.

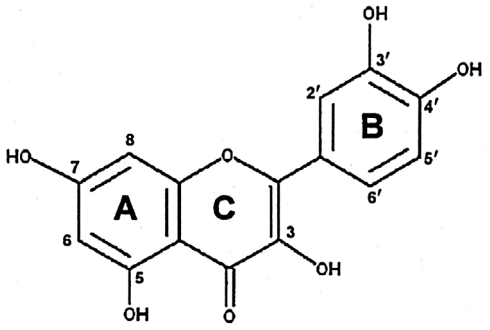

Fig. 1. Chemical structure of quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone).

2. Materials and methods

2.1. Reagents and chemicals

Ficoll-Hypaque (Histopaque-1077), dextran, gentamicin sulfate, 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFDA), nitroblue tetrazolium (NBT), rivoflavin, methionine, ethylenediaminetetraacetic acid (EDTA), 1,1,3,3 tetraethoxypropane (MDA), were all obtained from Sigma-Aldrich (St. Louis, MO, USA). 2-thiobarbituric acid (TBA) was obtained from Merck (AG, Darmstadt, Germany). Trichloroacetic acid (TCA) was obtained from Fluka Biochemicals (St. Louis, MO, USA). Other chemicals used were hydrogen peroxide (Pharafarm, Buenos Aires, Argentina) and potassium dichromate (Anedra, Buenos Aires, Argentina). All solvents used were analytical grade.

2.2. Plant material

Leaves of *Flaveria bidentis* were collected in Santa Rosa de Río Primero (Córdoba, Argentina) when the plant was in bloom. The plant material was identified by Prof. Dr. Luis Ariza Espinar and voucher specimen number 2813 deposited at CORD. The plant material was dried at room temperature and powdered.

2.3. Extraction and purification of quercetin

Quercetin (Q) has been isolated from the leaves of *F. bidentis* (Cabrera and Juliani, 1979), but as it occurs in a very low concentration in relation with the other constituents, we decided to obtain the flavonoid by acid hydrolysis of the precipitate containing Q sulphated derivatives. Thus, 50 mg of precipitate was treated with HCl 2.5 M, heated by refluxing for 1 h then cooled and centrifuged. Afterwards, the precipitate was washed with distilled water until neutral pH and partitioned whit ethyl ether. The yield of Q obtained after removing the organic solvent was 40 mg.

Q identity (Fig. 1) was confirmed by comparison of the ultraviolet-visible spectroscopic data with those previously pub-

lished by our laboratory (Guglielmone et al., 2002) and by reversed-phase HPLC column (Phenomenex Hipersil C18, 4.6 × 30 mm) by comparison whit standards. A gradient event of mobile phase solvent A: water (acetic acid 1% v/v) and B: methanol (acetic acid 1% v/v) was as follows: 10–35% B (10 min), 35–42% B (15 min), 42–75% B (10 min), 75% B (5 min), 75–10% B (5 min), 10% B (5 min), at a flow rate 1.0 ml/min. The purity of Q was determined as 96.9%.

2.4. In vitro *assay*

2.4.1. *Work solutions*

For assays in leukocytes, GEN was prepared in Hank's balanced salt solution (HBSS) at concentrations of 8, 128 and 256 μg/ml. Quercetin stock solution (1.0 mg/ml) was prepared in ethanol. Different concentrations of the flavonoid were obtained by diluting the stock solution in HBSS. For the fluorescence assay, Q concentrations of 10, 50 and 250 μM were utilized. For superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation determination, the concentration of Q used were selected according to the IC_{50} value calculated for the fluorescence experiment. These concentrations were 0.06, 0.29 and 1.5 μM to mononuclear leukocytes and 0.1, 1 and 10 μM to polymorphonuclear leukocytes. For assays in bacteria, GEN was prepared in buffer solution (PBS) while Q was dissolved in PBS using ethanol 5% as vehicle to give stock solution (250 μg/ml). Fresh solutions were prepared and used immediately.

2.4.2. *Leukocytes preparation from human blood*

Polymorphonuclear (PMN) and mononuclear (MN) leukocytes were isolated by a combined dextran/Ficoll-Hypaque sedimentation procedure from voluntary and healthy donors. Sedimentation in dextran (6% solution) was performed before gradient centrifugation. A mixture of Ficoll-Hypaque (Histopaque-1077) was then used to isolate the mononuclear cell from the remaining blood cells. After sedimentation, hypotonic lysis of the erythrocytes was carried out to obtain polymorphonuclear cells. PMN and MN cells layers were washed twice and suspended in HBSS. Cell preparations were adjusted to 10^6cells/ml for the assay. The viability of cells estimated by trypan blue dye exclusion was greater than 95% (de Pablo et al., 1998).

2.4.3. *Determination of ROS by fluorescence assay*

Intracellular ROS were measured using dichlorofluorescein assay as described by Wang and Joseph (1999). H_2-DCFDA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to the non-fluorescent DCFH, which can be rapidly oxidized to the highly fluorescent DCF in the presence of ROS. For this assay, cells were incubated with 10 μM H_2-DCFDA in the dark at 37 °C for 30 min. After H_2-DCFDA was removed, the cells were washed with HBSS and incubated in 96-well plates with GEN (8, 128 and 256 μg/ml) and then GEN plus Q (10, 50 and 250 μM), for 30 min at 37 °C; HBSS was used for control. Fluorescence was measured in a Synergy HT Multi-Mode Microplate Reader (Bio-tek Instruments, Inc.). The fluorescence caused after that the cells were exposed to GEN for 60-min was measured. Wavelengths of excitation and emission were 485 and 528 nm, respectively. Background fluorescence was corrected by the inclusion of parallel blanks. In order to evaluate Q dose–response, cells were treated as described above and Q concentrations selected to cover the range of inhibition (0–100%). Values of IC_{50} (μmolar concentration inhibiting 50% of ROS production) were obtained using OriginPro® 8 (Northampton, MA).

2.4.4. *Antioxidant enzymes activity*

SOD activity was estimated according to the method developed by Beauchamp and Fridovich (1971). Briefly, riboflavin loses one electron in the presence of light and triggers $O_2^{\bullet -}$ generation, which reduces the NBT to blue formazan. The reaction mixture was obtained by adding 13 mM methionine, 75 μM NBT, 0.1 nM EDTA, and 2 μM riboflavin in HBSS. The sample (10^6 leukocytes/ml) was incubated for 1 h with GEN or GEN plus Q at different concentrations (see above, Work solutions) then, 130 μl of the reaction mixture was added. The absorbance was determined at 560 nm in Sunrise Microplate Absorbance Reader (Tecan Trading AG). A unit of SOD was defined as the quantity of enzyme required to produce a 50% inhibition of NBT reduction under the specified conditions indicated above.

CAT activity was determined by the method described by Sinha (1972) with some modifications. Briefly, 10^6 leukocytes/ml were incubated with GEN or GEN plus Q at different concentrations (see above, Work solutions) and 40 μl of 0.2 M H_2O_2. After that, the reaction mixture was rapidly mixed with 200 μl of dichromate/acetic acid reagents and the absorbance measured after 5 min at 570 nm. One unit of CAT was defined as the amount of enzyme able to produce a 50% inhibition of the generation of chromic acetate.

2.4.5. *Lipid peroxidation by TBARS assay*

Malondialdehyde (MDA) levels in leukocytes were determined spectrophotometrically as an indicator of lipid peroxidation (Aiassa et al., 2013). MDA can react with thiobarbituric acid to form a colored complex which has maximum absorbance at 535 nm. Leukocytes were incubated with GEN or GEN plus Q, at different concentrations (see above, Work solutions) for 4 h; then 200 μl of cell suspension was mixed with 350 μl of a TCA-TBA-HCl reagent (TCA 15% w/v, TBA 0.5%, HCl 0.25 N) and heated in water at 95 °C for 45 min. After cooling in an ice bath, 350 μl of *n*-butanol:pyridine (15:1 v/v) was added and mixed vigorously. The colored organic phase was separated by centrifugation and absorbance measured. The reference standard used was 1,1,3,3 tetraethoxypropane, and MDA levels were expressed in nM/10^6cells.

2.4.6. *Minimal inhibitory concentration (MIC) determination of quercetin*

Q antimicrobial activity in *Staphylococcus aureus* ATCC 29213, *S. aureus* clinical strain resistant to GEN, *Escherichia coli* ATCC 25922 and *E. coli* clinical strain resistant to GEN was evaluated by using the standard tube dilution method on Mueller–Hinton broth (MH, Britania). Strains coming from cultures of 24 h onto Nutrient Agar (BD Difco, USA) were selected and inoculated into Mueller–Hinton broth adjusted to 10^6 colony-forming units (CFU) per ml. Two fold serial Q dilutions were made with MH to give concentrations ranging from 0.25 to 125 μg/ml. The bacterial suspension (0.5 ml) was added into each tube and another 0.5 ml of Q was placed into the tubes to give a total volume of 1 ml. Bacterial growth was observed at 18 h of incubation, following the indications of the Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2010). The lowest concentration of the compound that prevented bacterial growth was considered to be the MIC. Inoculated Q-free broths were used as negative controls. Viable bacterial counts were obtained for control samples by plating serial dilutions on MH agar plates, followed by aerobic incubation at 37 °C for 18 h for *E. coli* and *S. aureus*.

2.4.7. *Checkerboard assay with* E. coli *and* S. aureus

The interaction between GEN and Q were evaluated by the checkerboard method (Eliopoulos and Moellering, 1996). Bacterial growth inhibition resulting from the interactions was determined by the macrodilution test. The concentrations of each tested agent used in the combinations corresponded to serial 2-fold dilutions from their MIC values. The concentrations of GEN tested ranged from 2 to 512 μg/ml and for Q from 0.25 to 64 μg/ml.

2.5. In vivo *assay*

2.5.1. Animals and treatment

Adult male Wistar rats (70-day-old, weighing approximately 300 g) were maintained at 22 °C under a 12 h light/dark cycle, with free access to food and water. All procedures were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina (#564/2015).

At the time of the experiments the animals were randomly divided into 4 groups (6 rats in each) to conform the following groups: (1) Control, that received saline plus vehicle (0.95% DMSO) injection; (2) Q-treated group, animals received Q at dose of 7.5 mg/(kg day) body weight dissolved in 0.95% DMSO; (3) GEN-treated group, animals received a solution of gentamicin sulfate at increasing concentrations during the treatment ranging from 3 to 6 mg/kg/day; (4) GEN + Q-treated group, animals received gentamicin sulfate (3–6 mg/kg/day) and Q (7.5 mg/kg/day) dissolved in 0.95% DMSO.

Each treatment was administered by intraperitoneal injections twice a day for 5 day. The dose for gentamicin treatment is the recommended human therapeutic dose and the quercetin administered dose was chosen from previous studies (ranging from 5 to 30 mg/kg/day i.p) (Chi et al., 2016; González-Esquivel et al., 2015; Nabavi et al., 2012).

2.5.2. Sample preparation and biochemical measurements

Blood from a small incision in the tail of each rat was extracted before the beginning of the treatment and 4 h after the first dose on days 1 and 3 of treatment. In addition, on the fifth day of the treatment the animals were decapitated and trunk blood obtained. The fluorescence assay was used to determine ROS levels in whole blood on the first, third and fifth days of drug administration, while colorimetric assays were used for SOD and CAT activities determination (in whole blood) and MDA levels (in plasma) only on the fifth day. Results were expressed (as corresponds) as Enzimatic Units or nM MDA/mg protein determined by Bradford assay (Bradford, 1976).

2.6. Statistic analysis

All values are expressed as mean ± S.D. The results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test for multiple comparisons using GraphPad InsStat (GraphPad Software, San Diego, CA, USA) and $p < 0.05$ was selected as the criterion of significant difference.

3. Results

3.1. In vitro *assay*

3.1.1. Intracellular ROS production in human leukocytes

In the first series of experiments, we examined the concentration-dependence of GEN-induced ROS production and Q effect on GEN-induced ROS. Exposure of MN leukocytes to 8 μg/ml GEN did not produce an increase in ROS content relative to GEN-untreated control cells, whereas in PMN leukocytes ROS levels increased 2.1 times (112.8 ± 5.5%) as compared with control cells. On the other hand, GEN at 128 and 256 μg/ml increased ROS production in both cell types. In MN, ROS content significantly increased 2 times (97.9 ± 23.0% to 128 μg/ml and 106.5 ± 20.7% to 256 μg/ml of GEN) whereas in PMN this level augmented 4.0 times (296.6 ± 8.5% and 297.0 ± 24.1%, respectively) as compared with controls (Fig. 2).

In order to investigate the ability of Q to reduce intracellular oxidative stress, cells were treated simultaneously with GEN (8, 128 and 256 μg/ml) and Q (10, 50 and 250 μM) and the level of intracellular ROS was determined. The cells treated with a combination of GEN and Q diminished ROS production in comparison with cells exposed only to GEN, reaching a percent inhibition of 100% in most experimental groups (Table 1A and B).

Table 1
Effect of quercetin on intracellular ROS produced by gentamicin (GEN). Data are expressed as % inhibition of ROS production by GEN treatment compared to control. Each column represents the mean ± S.D of three independent experiments.

A. Mononuclear leukocytes

% Inhibition of ROS production			
Gentamicin (μg/ml)	Quercetin (μM)		
	10	50	250
8	–	–	–
128	100.0 ± 3.8	100.0 ± 2.4	100.0 ± 3.4
256	100.0 ± 2.0	100.0 ± 2.7	100.0 ± 1.1

B. Polymorphonuclear leukocytes

% Inhibition of ROS production			
Gentamicin (μg/ml)	Quercetin (μM)		
	10	50	250
8	100.0 ± 2.0	100.0 ± 1.4	100.0 ± 1.4
128	85.5 ± 3.8	101.9 ± 2.2	105.8 ± 0.7
256	88.6 ± 0.4	102.0 ± 0.7	108.4 ± 0.1

On the basis of this result, in the second series of experiments we evaluated five different Q concentration in cells exposed at 128 μg/ml GEN in order to determinate the IC_{50} value and compared it with the IC_{50} value of vitamin C used as reference inhibitor. The Q IC_{50} in MN was 0.28 ± 0.01 and 1.06 ± 0.04 μM for PMN; while for vitamin C the IC_{50} was 0.84 ± 0.01 and 1.05 ± 0.09 μM, in MN and PMN, respectively (Fig. 3).

3.1.2. Antioxidant enzymes activities in human leucocytes

When assessing GEN effect on the activity of the major endogenous antioxidant enzymes (SOD and CAT) we have observed an interesting behavior. At comparing with control cell, the minimum GEN concentration (8 μg/ml) increased the activity of both enzymes in MN (65.9% SOD and 46.3% CAT), whereas in PMN, SOD and CAT activity decreased when compared with the controls (74.5% SOD and 19.3% CAT). Moreover, GEN at 128 and 256 μg/ml generates a decreased in SOD (20.3 and 18.1% in MN, 63.1 and 70.4% in PMN, respectively) and CAT activity (28.8 and 26.6% in MN, 34.4 and 3539% in PMN, respectively) in both treated cells compared with control cells (Table 2).

Regarding Q effect on SOD and CAT activities in GEN-treated cells, the results showed that Q could restore the activities of these antioxidant enzymes in MN and PMN in a dose-dependent manner reaching values similar to control at concentration to 1.5 μM of Q in MN and 10 μM of Q in PMN (Figs. 4 and 5).

3.1.3. Lipid peroxidation in human leukocytes

A significant elevation of MDA levels was detected in MN and PMN treated with GEN at 8, 128 and 256 μg/ml as compared to the control leukocytes (53.3, 100.4 and 120.6% in MN, respectively; 58.3, 99.1 and 119.4% in PMN, respectively). However, leukocytes treated with GEN and Q (Q concentration: 0.06, 0.29 and 1.5 μM to MN and 0.1, 1 and 10 μM to PMN) simultaneously showed a significant reduction of MDA levels compared to the cells treated with GEN only, reaching similar values to basal level at all Q concentrations tested (Fig. 6).

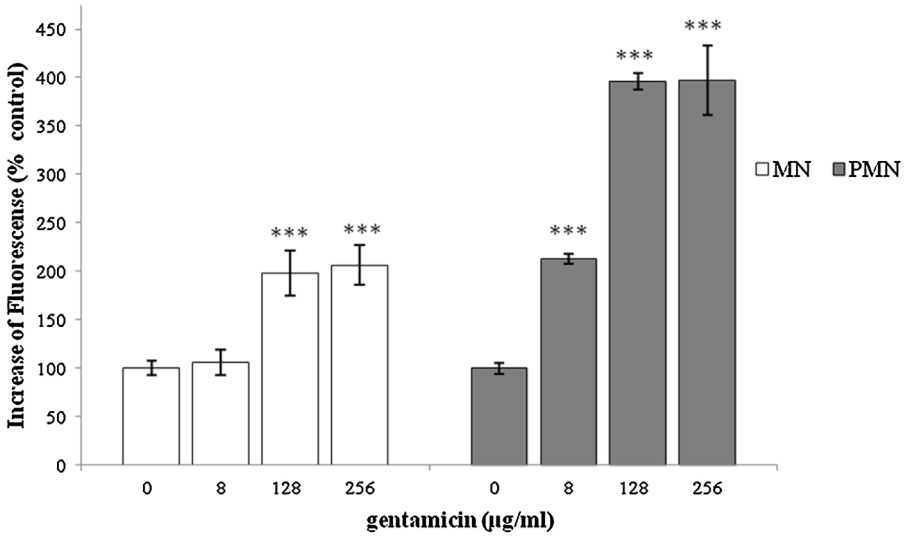

Fig. 2. Effect of gentamicin (GEN) on intracellular ROS production in mononuclear (MN) and polymorphonuclear (PMN) leukocytes. Data (means ± S.D) are expressed as percent of values in untreated cells (no GEN and no antioxidant) of three independent experiments. $^{***}p < 0.001$ *vs.* control leukocytes.

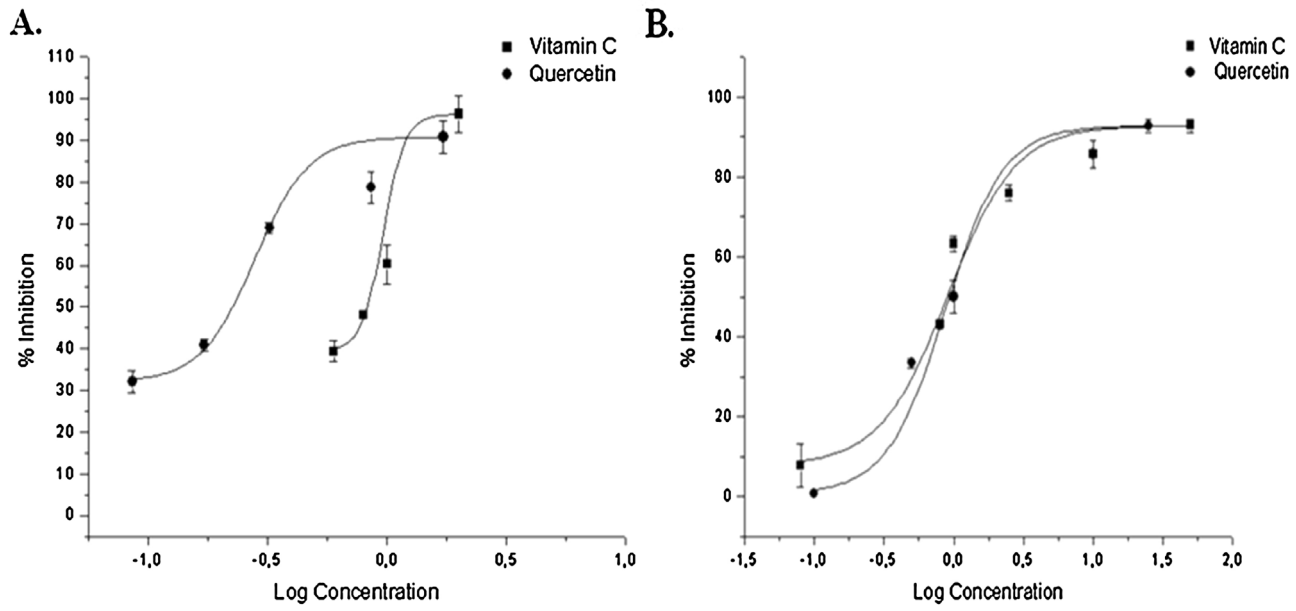

Fig. 3. IC_{50} obtained for quercetin and vitamin C on intracellular ROS produced by 128 µg/ml of gentamicin: A. Mononuclear leukocytes, B. Polymorphonuclear leukocytes.

3.1.4. Determination of quercetin minimal inhibitory concentration (MIC)

Data revealed that quercetin has not demonstrated antimicrobial activity at neither concentration tested against the two bacteria, the Gram-positive bacterium *S. aureus* (ATCC and clinical resistant to GEN) and the Gram-negative bacterium *E. coli* (ATCC and clinical resistant to GEN) (Table 3).

Table 2
Effect of gentamicin (GEN) on enzymatic antioxidant status in mononuclear (MN) and polymorphonuclear (PMN) leukocytes. Data are provided as means ± S.D of three independent experiments. $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$, *vs.* control leukocytes.

GEN (µg/ml)	SOD*		CAT#	
	MN	PMN	MN	PMN
0	0.913 ± 0.019	0.805 ± 0.020	1.029 ± 0.050	1.559 ± 0.100
8	1.515 ± 0.058^{c}	0.205 ± 0.052^{c}	1.505 ± 0.057^{c}	1.258 ± 0.090^{a}
128	0.728 ± 0.001^{c}	0.297 ± 0.001^{c}	0.733 ± 0.010^{c}	1.022 ± 0.129^{b}
256	0.748 ± 0.029^{b}	0.238 ± 0.079^{c}	0.755 ± 0.029^{c}	1.000 ± 0.129^{c}

* Superoxide dismutase (SOD) –one unit of enzyme activity was considered as the quantity of enzyme which produces 50% inhibition of NBT reduction/10^6 cells.
\# Catalase (CAT)-one unit of enzyme activity was considered as the amount of enzyme able to produce a 50% inhibition of the generation of chromic acetate/10^6 cells.

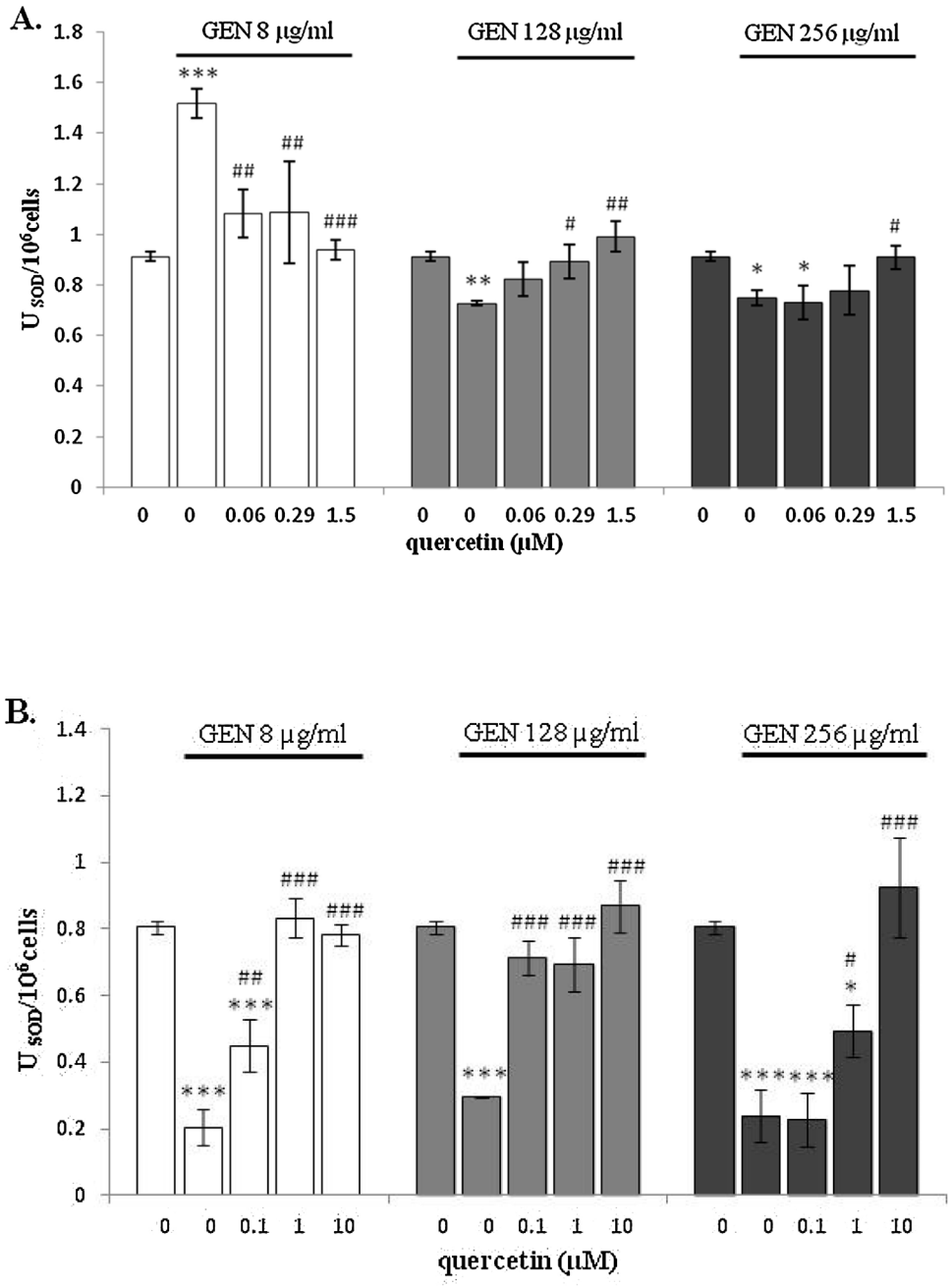

Fig. 4. Quercetin effect on superoxide dismutase (SOD) activity in presence of gentamicin (GEN): A. Mononuclear leukocytes, B. Polymorphonuclear leukocytes. Data (means ± S.D) are expressed as SOD Unit per 10^6 cells and values of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *vs.* control leucocytes; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, *vs.* GEN-treated leukocytes.

Table 3
Minimal Inhibitory Concentration (MIC) of quercetin.

Strains	*S. aureus* ATCC	*S. aureus* clinical resistant to GEN[a]	*E. coli* ATCC	*E. coli* clinical resistant to GEN
Quercetin	>125	>125	>125	>125

Values of MIC are expressed in µg/ml.
[a] Gentamicin (GEN).

3.1.5. Checkerboard assay of quercetin with E. coli and S. aureus

When GEN was combined with Q for inhibition of *S. aureus* ATCC and clinical resistant to GEN, an increase in the susceptibility of bacteria against GEN was observed when the concentration of Q was 64 µg/ml, since the MIC value of the antibiotic decreased two dilutions respect to its individual MIC (Fig. 7A and B). Moreover, when GEN and Q were combined for *E. coli* ATCC and clinical resistant to GEN inhibition, changes were not observed in the antibacterial activity of GEN (Fig. 8A and B).

3.2. In vivo *assay*

3.2.1. ROS production in rat blood

In whole blood of GEN-treated rats at therapeutic doses (3–6 mg/kg/day), ROS level significantly increased by 69% as compared with the control group, with a peak evidenced 4 h after dosing on the first day of treatment. Interestingly, the GEN + Q-group evidenced a substantial reduction in ROS levels as compared to the GEN alone-injected animals, keeping those values similar to the

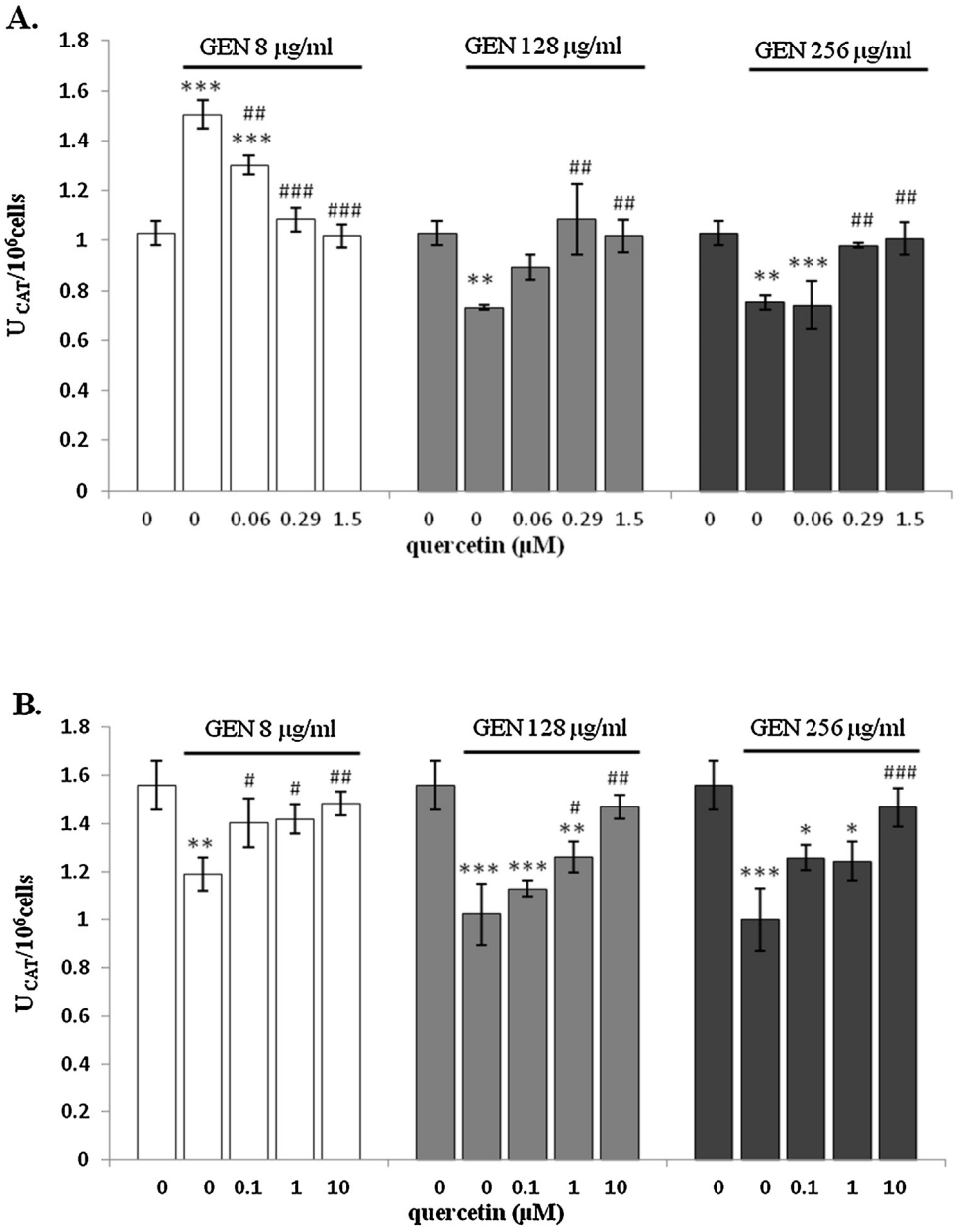

Fig. 5. Quercetin effect on catalase (CAT) activity in presence of gentamicin (GEN): A. Mononuclear leukocytes, B. Polymorphonuclear leukocytes. Data (means ± S.D) are expressed as CAT Unit per 10^6 cells and values of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *vs.* control leukocytes; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, *vs.* GEN-treated leukocytes.

Control-group. Thus, there was no significant difference in ROS levels among the Control, the Q and the GEN + Q groups (Fig. 9).

3.2.2. Antioxidant enzymes activity and lipid peroxidation in rats

SOD and CAT activities in whole blood of GEN-treated rats at therapeutic doses (3–6 mg/kg/day), was significantly increased (97% and 50%, respectively, as compared with controls). At the same time, MDA level in plasma was increased by 41% in the same GEN-treated rats as compared with the controls (Table 4). In contrast, Q administration plus GEN (7.5 mg/kg/day) caused a dramatic decrease in the antioxidant enzymes (SOD and CAT) activities and in MDA levels respect to rats treated with GEN alone, reaching the control rats values. Furthermore, there was no significant difference in SOD and CAT activities and MDA level among the Control, the Q and the GEN + Q groups.

4. Discussion

It has been demonstrated that some side effects of certain drugs would be related to their ability to increase intracellular production of ROS and to induce oxidative stress in different cell types including human cells, causing damage that may affect health. Some of the toxic effects produced for certain antibiotics are related to the capacity to generate free radicals reaction producing damage in the human system for example, in leukocytes (Becerra et al., 2003; Páez et al., 2008), or whole blood (Correa-salde and Albesa, 2009; Ray et al., 2006), among others.

Leukocytes and erythrocytes have been used as a model for the research of free-radical-induced oxidative stress for several reasons: they are continually exposed to high oxygen tensions, they are unable to replace damaged components, their membrane lipids are composed partly of polyunsaturated fatty acid side chains which

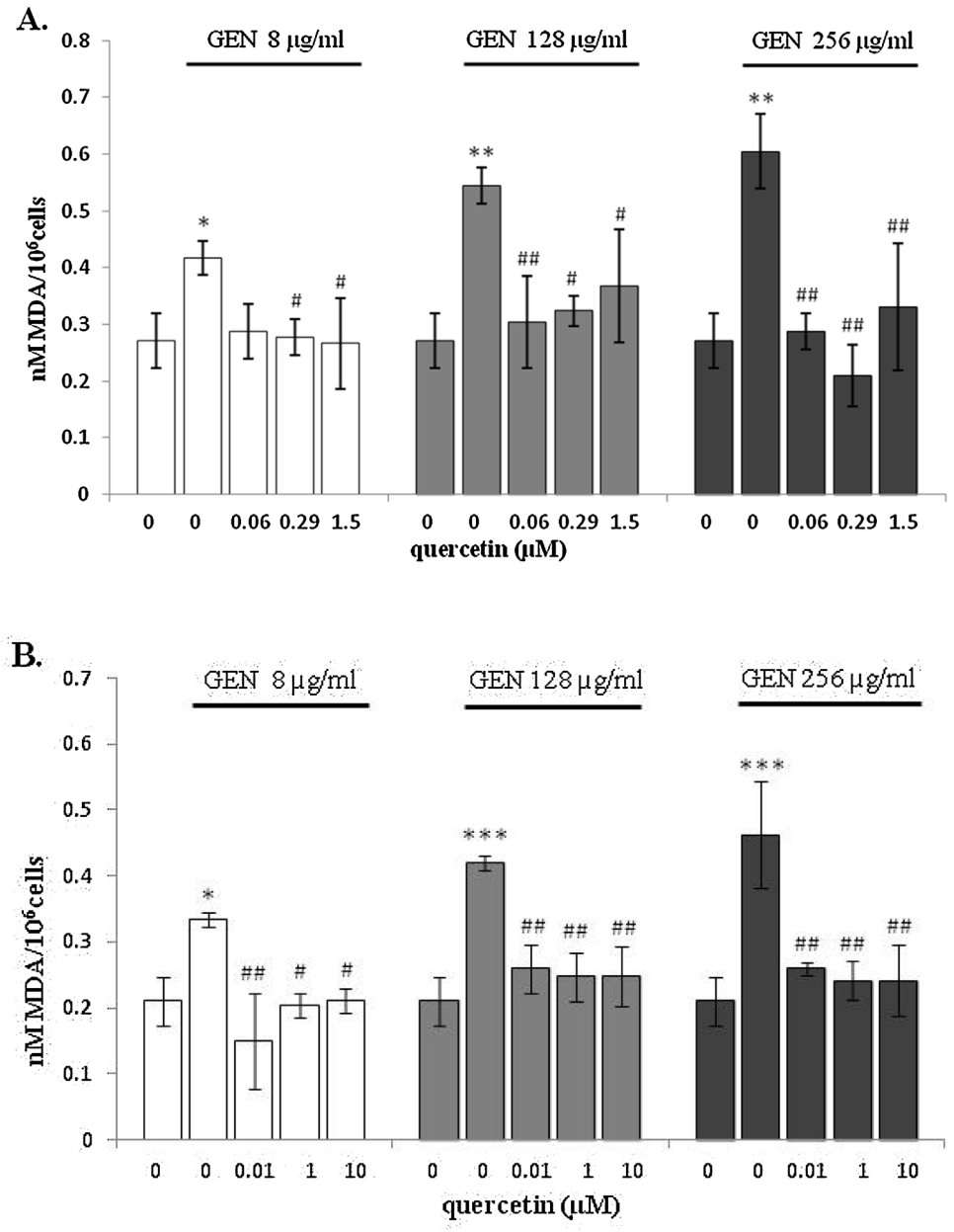

Fig. 6. Quercetin effect on lipidic peroxidation induced by gentamicin (GEN): A. Mononuclear leukocytes, B. Polymorphonuclear leukocytes. Data (means ± S.D) are expressed as nM malondialdehyde (MDA) per 10^6 cells and values of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *vs.* control leukocytes; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, *vs.* GEN-treated leukocytes.

Table 4
Changes in the enzymatic antioxidant activities status in blood and malondialdehyde (MDA) levels in plasma of control and experimental rats. All values are expressed as Enzymatic Units or nM MDA/mg protein ± S.D. (n = 6 in each group). $^{a}p < 0.01$, $^{b}p < 0.001$, *vs.* control group; $^{c}p < 0.01$, *vs.* GEN group.

Groups	Control	Quercetin	Gentamicin	Gentamicin + Quercetin
SOD*	0.039 ± 0.001	0.036 ± 0.003	0.077 ± 0.003[b]	0.049 ± 0.012[c]
CAT#	0.485 ± 0.041	0.503 ± 0.045	0.728 ± 0.027[b]	0.538 ± 0.032[c]
MDA+	3.339 ± 0.384	3.542 ± 0.038	4.712 ± 0.437[a]	3.425 ± 0.090[c]

* Superoxide dismutase (SOD)-one unit of enzyme activity was considered as the quantity of enzyme which produces 50% inhibition of NBT reduction/mg protein.
\# Catalase (CAT)-one unit of enzyme activity was considered as the amount of enzyme able to produce a 50% inhibition of the generation of chromic acetate/mg protein.
\+ Lipid peroxidation was expressed as nM MDA/mg protein.

are vulnerable to peroxidation, and they have antioxidant enzyme systems (Konyalioglu and Karamenderes, 2005).

The present study have shown that GEN induces ROS production in peripheral blood cells (human leukocytes) in a dose-depending manner, manifesting twice ROS production in PMN than in MN, which demonstrates more capacity of response to a stimulus of ROS by polymorphonuclear cells, mainly neutrophils than mononuclear cells. In turn, at 128 and 256 µg/ml of GEN concentration no significant differences in ROS production in PMN and MN were observed. This fact is consistent with reports showing that highly toxic con-

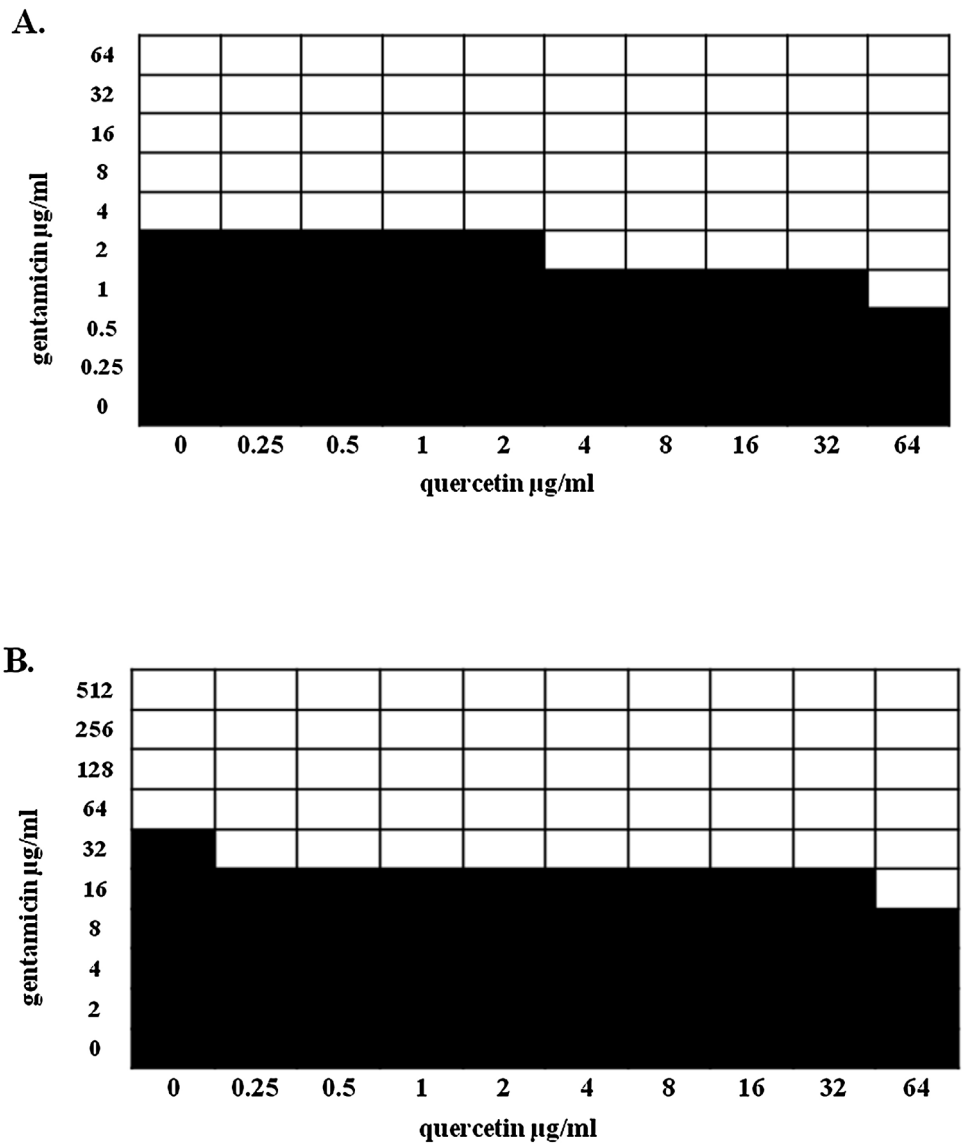

Fig. 7. Interaction between quercetin and gentamicin (GEN) using the checkerboard technique. A. *Staphylococcus aureus* ATCC 29213. B. *Staphylococcus aureus* clinical resistant to GEN.

centrations of some antibiotics produce significant alterations in the cells, causing the loss of their ability to generate free radicals with the consequent loss of linear correlation between ROS production and drug concentration (Correa-Salde and Albesa, 2009; Páez et al., 2008).

SOD and CAT are the most important enzymatic defense mechanisms against ROS toxic effects. SOD accelerates the dismutation of superoxide anion ($O_2^{\bullet -}$), and it is considered a primary defense, as it prevents further generation of free radicals. CAT collaborates with the removal of H_2O_2 formed during the reaction catalyzed by SOD (Liu et al., 2010). It has been reported that the increased activity of endogenous antioxidant enzymes served as protective responses to eliminate reactive free radicals (Celik and Suzek, 2009). Moreover, the decrease in the activity of these enzymes may be due to rapid consumption and depletion of the stored enzymes in order to eliminate the free radicals generated during the development of GEN-induced toxicity (Ademiluyi et al., 2013). In this respect, taking into account the activity of endogenous antioxidant enzymes in MN, we have seen that at the lowest GEN concentration (8 µg/ml), in which a significant increase in ROS production in the fluorescence assay was not observed, the activity of both CAT and SOD increases. This effect could be explained as a response of these antioxidant enzymes against the increment in the ROS production generated by GEN to counteract them. Contrary, at higher antibiotic concentrations we have observed, in the fluorescence assay, greater ROS production in MN, while the endogenous antioxidant enzymes activity diminishes due to excessive production of ROS, which would cause a depletion of the endogenous antioxidants. As to PMN, all GEN concentrations tested showed a significant increase in ROS production and a decrease in both antioxidant enzymes activities.

Reduced activities of one or more antioxidant endogenous systems lead to an increase in lipid peroxidation. An increase in MDA, measured as TBARS, is indicative of a decrease in the polyunsaturated fatty acid content, which serves as substrate for free radical attack (Al-Majed et al., 2002). By assessing lipid peroxidation as a biomarker of oxidative stress, after 4 h of incubation of blood cells with the antibiotic, it was observed in all cases that GEN induced an increase in lipid peroxidation in a dose-dependent manner when is compared to the controls, showing a greater increase in the lipid oxidation in the most highly toxic GEN concentrations.

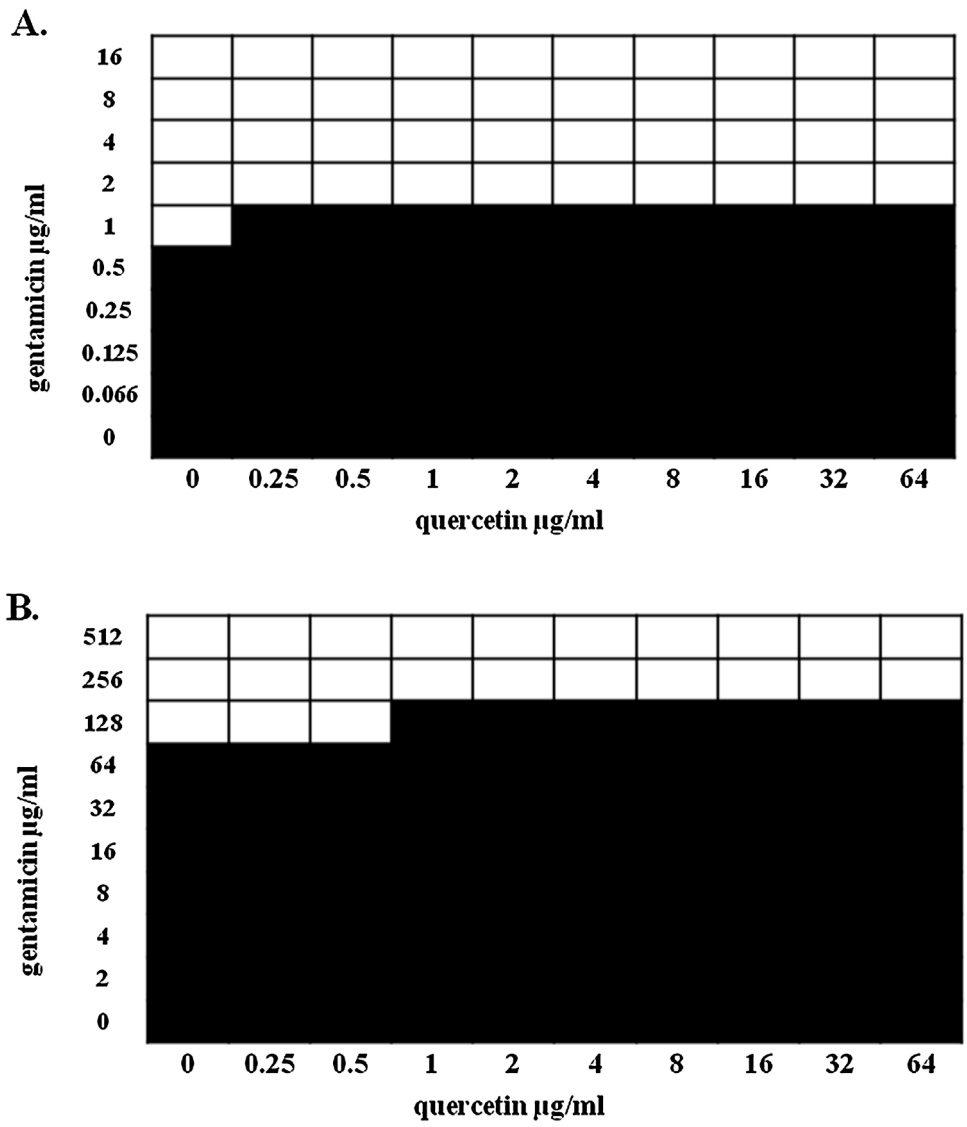

Fig. 8. Interaction between quercetin and gentamicin (GEN) using the checkerboard technique. A. *Escherichia coli* ATCC 25922. B. *Escherichia coli* clinical resistant to GEN.

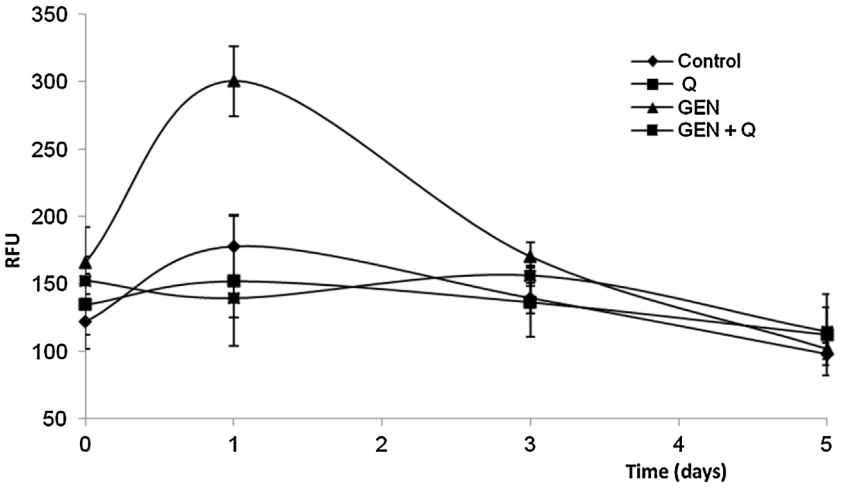

Fig. 9. Quercetin (Q) effect on ROS levels in whole blood of gentamicin (GEN)-treated rats (3–6 mg/kg/day). Data are given as Relative Fluorescence Units (RFU) ± S.D. (n = 6 each group).

Flavonoids can prevent oxidative damage as a result of their ability to scavenge ROS, such as hydroxyl radical and superoxide anion (Galati et al., 2002). Within the family of flavonoids, Q is the most potent ROS scavenger and one of the most studied (Boots et al., 2008). In fact, when we have evaluated Q protective effect in mononuclear leukocytes stimulated by GEN, a significant inhibitory capacity in ROS production was observed, showing a IC_{50} for Q (0.28 ± 0.01 μM), three times lower than the reference inhibitor, vitamin C (0.84 ± 0.01 μM). A similar protective behavior in Q was observed in polymorphonuclear leukocytes cells, with similar values in their CI_{50}: 1.06 ± 0.04 μM *vs.* 1.05 ± 0.09, for Q and vitamin C, respectively. These observed behaviors were correlated with the results obtained thereafter, in similar conditions, for the enzymatic endogenous antioxidant systems and the lipid peroxidation in leukocytes cells. Quercetin in front of GEN-ROS stimulus in both cellular systems, MN and PMN, tends to level SOD and CAT activities, in a dose-dependent manner, reaching basal values. At the same time, it is able to prevent GEN-induced lipid peroxidation at all Q concentrations tested.

Another interesting system to evaluate is whole blood. Neutrophil activation is considered to be a principal source of oxidative stress in human beings; however, both leukocytes and erythrocytes are capable of accelerating ROS generation in the presence of oxidizing agents. Consequently, oxidative stress can be evaluated either in isolated leukocytes or directly in the whole blood (Correa-Salde and Albesa, 2009). In evaluating GEN effect in Wistar rats, the results confirmed that therapeutic doses of the antibiotic (3–6 mg/kg/day) can induce oxidative stress in whole blood, which is evidenced by a significant increase in ROS production (observed by fluorescence assay), an endogenous antioxidants enzyme activation (SOD and CAT) and an increased lipid peroxidation in peripheral blood of Wistar rats. The simultaneous treatment of GEN and Q suppressed ROS generation collaborated with endogenous antioxidant enzymes and diminished lipid peroxidation, reaching values similar to control rats in the three conditions evaluated.

Our results are supported by previous research suggesting the involvement of oxidative stress in the major side effects of GEN (nephrotoxicity, ototoxicity and hepatotoxicity) and the protective effects of several antioxidants against the toxicity induced by GEN *in vivo* (Abdelsameea et al., 2016; Ademiluyi et al., 2013; Maldonado et al., 2003; Parlakpinar et al., 2005; Yang et al., 2011). However, in these previous studies a reduced activity of antioxidant enzymes was observed (SOD and CAT), fact contrary to the behavior observed in such enzymes in our studies. We suggest that these discrepancies may be due to the different concentration of GEN used in both studies. In our experiments, we have used therapeutic concentrations of the antibiotic (3–6 mg/kg/day) that allowed an increase in enzyme endogenous activity in order to counteract the ROS generated by GEN. While in the previous studies, the authors have used higher concentrations of GEN than those used in therapeutic treatments (ranging from 80 to 140 mg/kg/day), a condition that could produce the decrease in the activity of these endogenous antioxidant enzymes, that were observed in the previous studies.

When assessing whether it is possible to achieve suitable values of quercetin in blood after administration in humans, there are several studies of their bioavailability with different values in the parameters evaluated, but many of them contain Q in a food matrix which could produce a significant variability in the results obtained (Goldberg et al., 2003; Guo and Bruno, 2015; Hollman et al., 1997; Kaushik et al., 2012; Wang et al., 2016). Particularly, a pharmacokinetic study indicates that after quercetin administration of 150 mg orally, the following values were obtained, Cmax: 0.43 μM, Tmax: 360 min and AUC 0-∞: 305 μM/min, *i.e.* higher values than IC_{50} value of quercetin in our assays in mononuclear leukocytes, however, to achieve the IC_{50} value for polymorphonuclear leukocytes, higher doses of quercetin should be administered orally in human beings (Egert et al., 2008). In turn, another point to consider is that other studies have demostrated that repeated administration of quercetin increases its bioavailability compared to administration of a single dose (Guo et al., 2014; Paulke et al., 2008; Rangel-Ordóñez et al., 2010). According to the above and considering that the doses administered intraperitoneally in our *in vivo* study shown a protective effect of quercetin against oxidative stress induced by gentamicin in systemic circulation in rats, experimental parameters such as measurement of bioavailability and serum Quercetin level, should be considered in future studies.

Finally, in order to determine whether the antioxidant Q effect might alter the GEN antimicrobial activity, we have evaluated the effect of the combination of Q plus GEN upon Gram-positive and Gram-negative strains (clinical and reference strains). We have observed that, on the one hand, Q up to a concentration of 125 μg/ml showed no antimicrobial activity by itself, and, on the other hand, that the flavonoid plus antibiotic does not generate changes in the antibacterial GEN activity against *E. coli* strains, while for *S. aureus* strains a beneficial effect is observed, as the susceptibility of the bacteria to GEN increases in the presence of Q.

5. Conclusion

Therefore, we have demonstrated that GEN at therapeutic doses could induce oxidative stress in human leukocytes as well as in whole blood and plasma of Wistar rats, in which the increase in ROS production, the alteration of antioxidant defenses and the lipid peroxidation play a crucial role. At the same time, Q scavenger effect showed in its ability to inhibit ROS production could contribute to the activity of endogenous antioxidant defenses and prevent the chain reaction of GEN-induced lipid peroxidation, producing a Q-protective effect in human leukocytes *in vitro*, and in whole blood and plasma rat *in vivo*. This is evident, without substantial modification of GEN antibacterial activity against *E. coli* strains, and it contributes to this activity against *S. aureus* strains. Hence, Q could represent a potential therapeutic option for preventing GEN-induced oxidative damage in order to avoid the clinical consequences caused by the production of reactive species and free radicals.

Conflict of interest

The authors declare that there is no conflict of interest associated with this study.

Acknowledgments

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