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Flavonoids as protective agents against oxidative stress induced by gentamicin in systemic circulation. Potent protective activity and microbial synergism of luteolin

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ABSTRACT

The flavonoids effect on gentamicin (GEN)-induced oxidative stress (OS) in systemic circulation was evaluated in terms of reactive oxygen species (ROS) production, enzymatic antioxidant defenses superoxide dismutase (SOD) and catalase (CAT), and lipid peroxidation (LP) *in vitro* on human leukocytes and *in vivo* on rat whole blood. The inhibitory activity of ROS was ATS < QTS < isovitexin < vitexin < luteolin. Luteolin, the most active, showed more inhibition in ROS production than vitamin C (reference inhibitor) in mononuclear cells and a slightly lower protective behavior compared to this inhibitor in polymorphonuclear cells. In both cellular systems, luteolin tends to level SOD and CAT activities modified by GEN, reaching basal values and preventing LP. In Wistar rats, GEN plus luteolin can suppress ROS generation, collaborate with SOD and CAT and diminish LP produced by GEN at therapeutic doses. Finally, luteolin and antibiotic association was evaluated on the antimicrobial activity in *S. aureus* and *E. coli* showing a synergism between GEN and luteolin on *S. aureus* ATCC and an additive effect on *E. coli* ATCC. Therefore, simultaneous administration of luteolin and GEN could represent a potential therapeutic option capable of protecting the host against OS induced by GEN in the systemic circulation while enhancing the antibacterial activity of GEN.

1. Introduction

Gentamicin (GEN) is an aminoglycoside antibiotic used in clinical practice for the treatment of Gram-negative bacterial infections that has now regained popularity due to widespread resistance of these bacteria to other antibiotic classes (Adil et al., 2016; Denamur et al., 2011). However, its clinical use has been restricted due to important adverse effects, which would be related to oxidative stress induction (Adil et al., 2016; Noorani et al., 2011; Sweetman, 2009; Veljković et al., 2016).

In previous studies, it was demonstrated that GEN is able to produce leukotoxicity, in systemic circulation, related to an excessive production of ROS, alterations in antioxidant defense mechanisms, and an increased lipid peroxidation in human leukocytes and whole blood from rats treated with therapeutic doses of this antibiotic (Bustos et al., 2016). Leukocytes play an important role in host defense against infectious agents by producing ROS, but an exacerbated ROS production can damage the host organism and the cell that produces them, being necessary the oxidant/antioxidant balance maintenance in these cells (Mytar et al., 1999; Paiva and Bozza, 2014).

In recent years, attention has been focused on the ability of natural antioxidants to protect against the toxic effects associated with increased oxidative stress, caused by GEN (Moreira et al., 2014; Noorani et al., 2011; Veljković et al., 2016). Within the natural antioxidants, one of the chemical groups of great interest are the flavonoids, polyphenolic

Abbreviations: ATS, quercetin- 3-acetyl-7, 3′,4′-trisulphate; CAT, catalase; DMSO, dimethyl sulfoxide; GEN, gentamicin; HBSS, Hank's balanced salt solution; H_2-DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; IV, isovitexin; LT, luteolin; MDA, malondialdehyde; MIC, minimal inhibitory concentration; MN, mononuclear leukocytes; NBT, nitroblue tetrazolium; PMN, polymorphonuclear leukocytes; QTS, quercetin-3,7,3′,4′-tetrasulphate; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; V, vitexin

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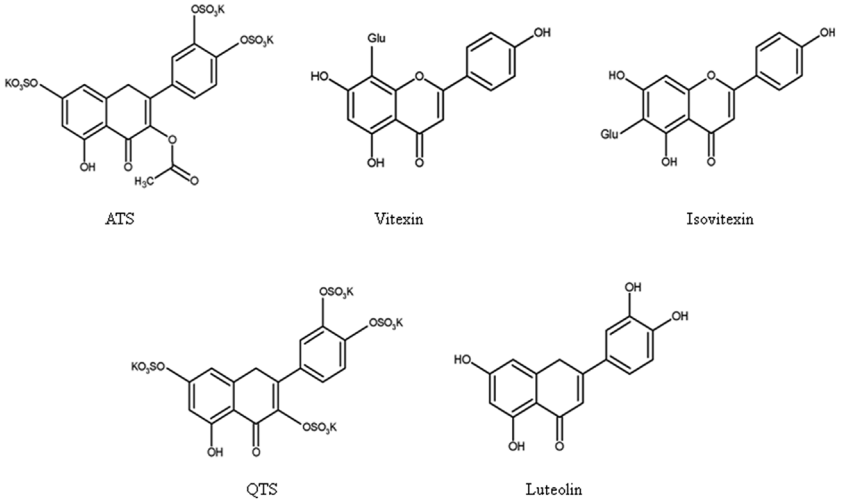

Fig. 1. Chemical structure of flavonoids isolated.

compounds found in the plant kingdom, due to its antioxidant power, i.e. its ability to reduce free radicals and chelate metals blocking their catalytic power (Mira et al., 2002; Rice-Evans et al., 1996). Recently, it was evaluated the effect of quercetin (Q), a flavonoid with strong antioxidant capacity obtained from *Flaveria bidentis*, demonstrating an important Q-protective effect on GEN-induced oxidative stress in human leukocytes *in vitro*, and in rat whole blood and plasma *in vivo* without important modification of GEN antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* strains (Bustos et al., 2016). However, due to the need to achieve therapeutic agents with potential in clinical application, it is necessary to search natural products that, in addition to avoiding GEN-induced toxic effects in systemic circulation, can enhance the antimicrobial activity of this antibiotic.

Different species were incorporated in this study in order to obtain several flavonoids: *Prosopis strombulifera* (Lam.) Benth var. *strombulifera* a rhizomatous shrub popularly known as “retortuño” or “mastuerzo”, *Prosopis nigra* (Gris.) Hieron. var. *nigra* a spiny tree known by the common names of “algarrobo negro” or “algarrobo dulce”, both South American species grow at Central and Northern Argentina (Barboza et al., 2006), and *Flaveria bidentis* (L.) Kuntze, known as “fique” or “contrayerba”, a native species distributed in America that grows in Córdoba, Argentina (Guglielmone et al., 2002; Ortega et al., 2010). From fruits of *P. strombulifera* the flavonoid luteolin (LT) has been isolated, which has a strong, anti-inflammatory, antioxidant, antifungal and antibacterial activity (An et al., 2016; López-Lázaro, 2009). From leaves of *P. nigra,* two flavonoids isomers, vitexin (V) and isovitexin (IV), were obtained, both with wide range of pharmacological effects including antioxidant activity, anticancer, antiviral, anti-inflammatory, among others (Xiao et al., 2016). Finally, from leaves of *F. bidentis* two quercetin sulfated derivatives have been isolated: quercetin-3-acetyl-7,3′,4′-trisulphate (ATS) and quercetin-3,7,3′,4′-tetrasulphate (QTS), which although they have anticoagulant and antiplatelet effects, there are no direct studies regarding their antioxidant activity (Guglielmone et al., 2002).

Thus, the aim of this study was to search flavonoids that can counteract toxic effects of GEN related to ROS production at the systemic level evaluating ROS generation, endogenous antioxidant defenses, and lipid peroxidation in human leukocytes *in vitro* and in rat whole blood and plasma, *in vivo*. It was also evaluated the antibacterial effect of flavonoid plus GEN combination on *S. aureus* and *E. coli,* in order to determine if flavonoid can enhance the antibacterial activity of GEN.

2. Materials and methods

2.1. Plant material

Plant material was collected in Argentina. Fruits of *Prosopis strombulifera* (CORD 1285) were collected in Mendoza, leaves of *Prosopis nigra* (ACORD AMP 1285) were collected in Traslasierra and leaves of *Flaveria bidentis* (CORD 2813) were collected in Santa Rosa de Río Primero. *P. strombulifera* and *F. bidentis* were identified by experts from Instituto Multidisciplinario de Biología Vegetal (IMBIV-CONICET) and voucher specimens were deposited at the CORD (UNC Botanical Museum) as reference material. *P. nigra* was identified by experts from ACORD (Facultad de Ciencias Agropecuarias, UNC) and a voucher specimen was deposited as reference material. The plant material was dried at room temperature and powdered.

2.2. Extraction and purification of flavonoids

Fruits or leaves from *Prosopis* powdered (500 g) were extracted with ethanol (EtOH) by soxhlet and the solvent evaporated under reduced pressure to obtain the crude EtOH extract which was suspended in boiling water and, after cooling at room temperature, it was defatted with hexane and then extracted with ethyl ether (EtOEt) and ethyl acetate (AcOEt). For the *P. strombulifera* species, the EtOEt extract was selected (455 mg), this was chromatographed on preparative paper Whatman 3 MM with acetic acid 15% and the band with Rf of 0.44 was cut out and eluted with EtOH. The eluate was concentrated under reduced pressure, and carried out a column chromatography with Sephadex LH-20 and eluted with EtOH to obtain four fractions. From fraction 2 was obtained a pure compound (13 mg) identified as luteolin. For *P. nigra* species, work continued with the AcOEt extract (1.47 g), which was deposited in a column of microcrystalline cellulose and eluted with H_2O to obtain three fractions. From fraction 1 was obtained isovitexin (150 mg) and from fraction 2 was obtained vitexin (260 mg). Finally, quercetin- 3-acetyl-7, 3′,4′-trisulphate (ATS) and quercetin-3,7,3′,4′-tetrasulphate (QTS) were isolated from the leaves of *F. bidentis* as previously described (Guglielmone et al., 2002) (Fig. 1).

Flavonoids identity (Fig. 1) was confirmed by spectrophotometric UV–Vis data (Mabry et al., 1970), and chromatographic HPLC analysis against authentic sample. The HPLC analysis was performed in a Varian Pro Star chromatograph (model 210, series 4171), equipped with a reversed-phase column (Phenomenex Hipersil C18, 4.6 × 30 mm), UV–Vis detector and the conditions were as follow: wavelength of the

UV–Vis detector was at 290 nm; 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 flavonoids was higher than 95.0%.

2.3. Oxidative stress in vitro

2.3.1. Leukocytes preparation from human blood

Leukocytes were isolated from human blood of voluntary and healthy donors as previously described (Bustos et al., 2016). Mononuclear leukocytes (MN) were separated by Ficoll-Hypaque gradient. Subsequently, hypotonic lysis of the erythrocytes was carried out to obtain polymorphonuclear leukocytes (PMN). Both cells types were adjusted to 10^6 cells/ml in Hank's balanced salt solution (HBSS). Trypan blue dye exclusion was used to estimate the viability of leukocytes; it was greater than 95%.

2.3.2. Intracellular ROS measurement

Intracellular ROS were measured by H_2-DCFDA as described previously (Bustos et al., 2016; Wang and Joseph, 1999). Cells were incubated with GEN (8, 128 and 256 μg/ml in HBSS) or GEN plus flavonoids [10, 50 and 250 μM by diluting the stock solution (1 mg/ml) in HBSS, initial concentrations for screening]. The evaluation of flavonoids dose–response was performed; flavonoids concentrations tested were ranged from 0.15 to 250 μM in order 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).

2.3.3. Measurement of enzymatic antioxidants activities and lipid peroxidation

Superoxide dismutase (SOD) and catalase (CAT) activity was estimated as described previously (Bustos et al., 2016). SOD activity was determinate by the method of Beauchamp and Fridovich (1971) with riboflavin/methionine/NBT reagents, and CAT activity was measured by the protocol of Sinha (1972) modified with dichromate/acetic acid reagent. A unit of SOD was defined as the quantity of enzyme required to produce a 50% inhibition of NBT reduction and one unit of CAT was defined as the amount of enzyme able to produce a 50% inhibition of the generation of chromic acetate.

MDA content was assayed using the thiobarbituric acid test as previously described (Bustos et al., 2016). 1,1,3,3 tetraethoxypropane was used as reference standard, and MDA levels were expressed in nmol/10^6 cells.

The flavonoid concentrations used were selected according to the IC_{50} value calculated for ROS inhibition.

2.4. Oxidative stress in vivo

2.4.1. Animals

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

2.4.2. Experimental design

At the time of the experiments the animals were randomly divided into following groups (n = 6) as follows:

Group I: Control (C): Rats received an intraperitoneal (i.p.) injection of saline plus vehicle (0.95% DMSO) in an iliac fossae and an i.p. injection of saline in the other.

Group II: flavonoid control: Rats were treated with an i.p. injection of flavonoid (7.5 mg/kg/day) in vehicle in an iliac fossae and an i.p. injection of saline in the other.

Group III: GEN control: Rats were treated with an i.p. injection of gentamicin sulfate (3–6 mg/kg/day) in an iliac fossae and an i.p. injection of saline plus vehicle in the other.

Group IV: GEN + flavonoid: Rats were treated with an i.p. injection of GEN (3–6 mg/kg/day) in an iliac fossae and an i.p. injection of flavonoid (7.5 mg/kg/day) in 0.95% DMSO in the other.

Each treatment was administered in doses separated by an 8-h interval for 5 consecutive days. GEN was dissolved in saline.

2.4.3. Sample preparation and biochemical measurements

Tail-blood was extracted from each rat before the starting the treatment and 4 h after the first dose on days 1 and 3 of treatment. On the fifth day, all animals were sacrificed and trunk blood collected. ROS levels and the antioxidant enzymes activities were determined in blood by fluorescence and colorimetry, respectively; besides MDA levels in plasma were determined by spectrophotometry as described above. Results were expressed (as applicable) as relative fluorescence units, enzymatic units or nmol MDA/mg of protein determined by Bradford assay, respectively (Bradford, 1976).

2.5. Minimun inhibitory concentration (MIC) of flavonoid and checkerboard determination in bacterial strains

The antibacterial activity of flavonoid and interaction with GEN were performed as described previously (Bustos et al., 2016) by MIC and checkerboard assays on Mueller-Hinton broth (MH, Britania) following the indications of the Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2010). Two reference strains, *S. aureus* ATCC 29213 and *E. coli* ATCC 25922, and two clinical strains: *S. aureus* resistant to GEN and *E. coli* resistant to GEN, have been evaluated. The lowest concentration of the compound that prevented bacterial growth was considered the MIC. In checkerboard assay the fractional inhibitory concentration (FIC) index of the combination of flavonoid and antibiotic was calculated (Eliopoulos and Moellering, 1996). The following formula was used for FIC index calculation: FIC index (FICI) = FIC of LT + FIC of GEN; where FIC of LT = MIC_{LT} in the combination/MIC_{LT} alone and FIC of GEN = MIC_{GEN} in the combination/MIC_{GEN} alone. When the FICI of the combination was equal to or less than 0.5, the combination is defined as synergistic; when it fell between 0.5 and 1, it was defined as an additive effect, and between 1.0 and 4.0 it was classified as “no interaction.” Finally, an FICI > 4.0 indicated antagonism between components in the combination.

2.6. Statistic analysis

Data was expressed as mean ± S.D. Data analysis was performed using Graph Pad InsStat (Graph Pad Software, San Diego, CA, USA). The results were analyzed by one-way analysis of variance (ANOVA), and Tukey tests were applied for *post-hoc* analysis. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Oxidative stress in vitro

3.1.1. Intracellular ROS in human leukocytes

In our previous studies we have demonstrated that GEN at 128 and 256 μg/ml is able to increase ROS production in MN and PMN leukocytes, but at 8 μg/ml only produces an increase in ROS content in PMN cells (Bustos et al., 2016).

When evaluating the effect of flavonoids (at the screening concentrations) on GEN-induced ROS production, in MN leukocytes, all flavonoids showed an interesting ROS inhibitory activity induced by

Table 1
Inhibitory effect of screening concentrations of flavonoids on intracellular ROS induced by 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.

% Inhibition of ROS production				
		gentamicin (μg/ml)		
		8	128	256
A. Mononuclear leucocytes				
luteolin (μM)	10	–	100.7 ± 4.7	100.7 ± 4.7
	50	–	109.7 ± 3.0	109.7 ± 3.0
	250	–	105.0 ± 6.7	105.0 ± 6.7
vitexin (μM)	10	–	34.3 ± 8.4	19.3 ± 4.5
	50	–	83.6 ± 4.1	77.3 ± 1.8
	250	–	101.1 ± 1.9	104.2 ± 3.9
isovitexin (μM)	10	–	22.4 ± 5.9	20.1 ± 2.6
	50	–	39.8 ± 5.1	37.0 ± 2.8
	250	–	57.1 ± 1.4	55.2 ± 3.0
QTS (μM)	10	–	19.0 ± 2.6	18.6 ± 7.1
	50	–	29.9 ± 1.3	40.8 ± 2.9
	250	–	91.4 ± 4.2	85.4 ± 4.0
B. Polymorphonuclear leucocytes				
luteolin (μM)	10	87.6 ± 0.6	86.7 ± 0.5	79.0 ± 0.9
	50	88.9 ± 2.6	87.4 ± 2.2	81.8 ± 1.5
	250	92.6 ± 4.4	84.4 ± 0.9	78.9 ± 0.6

128 and 256 μg/ml GEN in blood cells (Table 1, A), except ATS which did not demonstrate to have inhibitory capacity of reactive species (data no shown). From the complete group of flavonoids evaluated, the combination GEN plus LT showed the greatest decrease in ROS production in MN cells treated, compared to leukocytes exposed only to GEN, reaching 100% inhibition at the lowest flavonoid concentration evaluated (10 μM).

In order to compare the activity of flavonoids each other and with vitamin C (reference inhibitor), we evaluated five different concentration of each ones in cells exposed at 128 μg/ml GEN in order to determinate its IC_{50} values. Only LT showed a lower IC_{50} value than the reference inhibitor (Fig. 2 and Table 2), reason why it was selected to continue with assays in PMN cells, which have a greater capacity to respond to ROS stimulus whit respect MN cells (Bustos et al., 2016).

In PMN leukocytes, the cells treated with a combination of GEN and LT showed lower ROS production in comparison with leukocytes exposed only to GEN, reaching a percent inhibition greater than 85% in most cases (Table 1, B) and being the IC_{50} value slightly higher than vitamin C (Fig. 2 and Table 2).

3.1.2. Activities of antioxidants enzymes in human leukocytes

Flavonoids in addition to their direct action as ROS scavengers have other antioxidant mechanisms, including their ability to activate endogenous antioxidant enzymes. On the basis of this reason, in a first series of experiments the direct effect of LT on antioxidant activity SOD and CAT in the absence of GEN was evaluated. Data revealed that in PMN leukocytes, LT would induce an increase in the activity of both enzymes at the highest concentration evaluated. SOD activity increased 21.2 ± 9.2%, whereas CAT activity increased 15.0 ± 2.8% in the presence of LT (Fig. 3).

Regarding the exposure of leukocytes to GEN, our previous studies have showed that GEN, in MN leukocytes is able to induce an increase in enzymatic activity SOD and CAT at low concentrations, and a decrease in this activity at high concentrations. While in PMN leukocytes all concentrations of GEN decreased SOD and CAT activity (Bustos et al., 2016). When assessing LT effect on endogenous antioxidant defenses SOD and CAT in MN leukocytes exposed to GEN, LT at the three concentrations evaluated tend to restore the antioxidant activity SOD and CAT in a dose-dependent manner reaching values similar to control especially at the highest concentration of LT evaluated (1.6 μM) (Fig. 4A). At the same time, in PMN leukocytes the behavior was similar given that LT again tend to restore the activity of these enzymes in a dose-dependent manner reaching values of the control cells at the highest LT concentration (6.4 μM) (Fig. 4B).

Table 2
IC_{50} values (μM) estimated for flavonoids and the reference inhibitor (vitamin C) on intracellular ROS produced by GEN (128 μg/ml) in mononuclear and polymorphonuclear leukocytes.

Compound	IC_{50} (μM)
A. Mononuclear leukocytes	
luteolin	0.51 ± 0.01
vitexin	22.7 ± 0.40
isovitexin	75.36 ± 6.13
QTS	166.75 ± 2.71
vitamin C	1.06 ± 0.04
B. Polymorphonuclear leukocytes	
luteolin	1.67 ± 0.02
vitamin C	1.05 ± 0.09

3.1.3. Lipid peroxidation in human leukocytes

MDA levels in MN and PMN treated with GEN at 8, 128 and 256 μg/ml are increased respect to control leukocytes levels (Bustos et al., 2016). However, when the leukocytes were treated with GEN and LT simultaneously, a significant reduction in a concentration-dependent manner in MDA levels was observed compared to the cells treated with GEN only. The mean and maximum LT concentrations were able to inhibit 100% lipid peroxidation increase in both cell types; whereas the

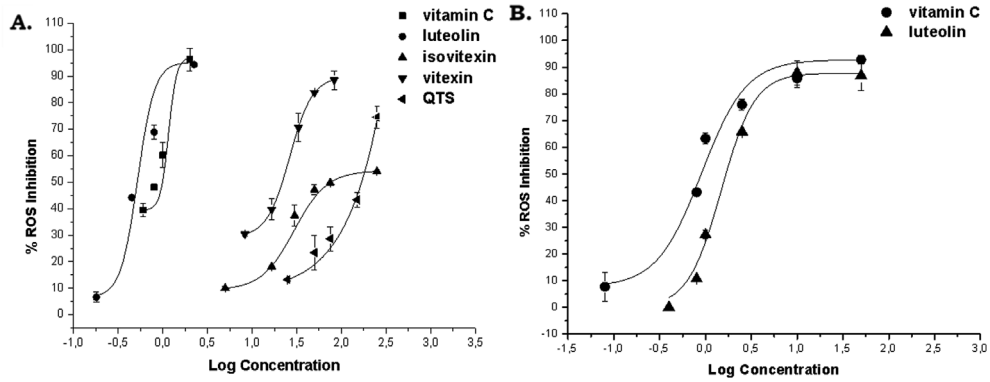

Fig. 2. Dose–response curves for flavonoids and vitamin C on intracellular ROS produced by 128 μg/ml of GEN. A. Mononuclear leukocytes, B. Polymorphonuclear leukocytes.

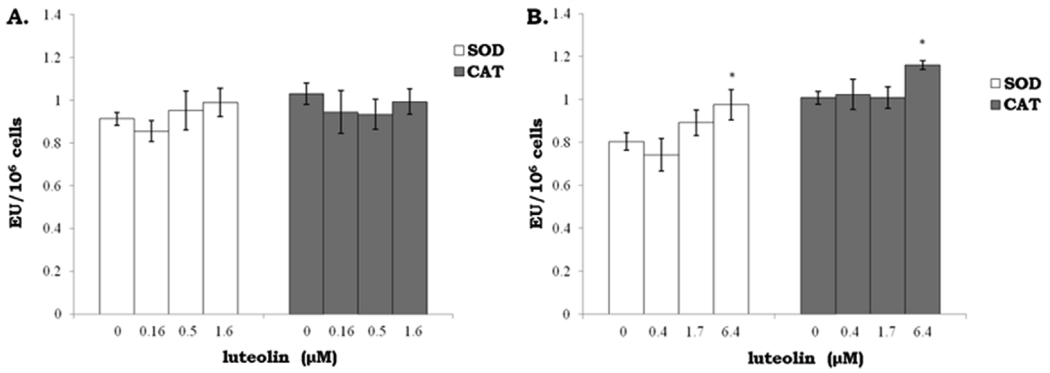

Fig. 3. Luteolin effect on superoxide dismutase (SOD) and catalase (CAT) activity in human leukocyes. A. Mononuclear leukocytes, B. Polymorphonuclear leukocytes. Data (means ± S.D) are expressed as Enzymatic Unit per 10^6 cells and values of three independent experiments.*p < 0.05 vs. control leukocytes.

minimal evaluated concentration of flavonoid (0.16 μM in MN and 0.4 μM in PMN) produced a decrease in MDA levels between 31 and 58%, depending on the type of cell and combined concentrations of LT and GEN (Fig. 5).

3.2. Oxidative stress in vivo

3.2.1. ROS production in rat blood

Blood ROS level in GEN-treated rats group at therapeutic doses (3–6 mg/kg/day), was significantly increased by 74.2 ± 10.6% respect to control group, and with a peak observed on the first day of treatment. Although a decrease in the production of these species was observed, it was not possible to recover the initial ROS level at no time during the treatment. Interestingly, a significant decrease in ROS levels was evidenced in the group treated with GEN + LT when compared to the GEN alone-injected animals, keeping these values in a similar range to the Control-group. No significant difference in ROS levels among the Control, the LT and the GEN + LT groups was observed (Fig. 6).

3.2.2. Antioxidant enzymes activity and lipid peroxidation in rats

In GEN-treated rats an increase of 103.6 ± 20.4% in SOD activity and 68.6 ± 6.5% in CAT activity in whole blood was observed, as well as an increase in MDA levels of 31.5 ± 5.6% in plasma with respect to the control group (Fig. 7). In contrast, LT administration (7.5 mg/kg/day) plus GEN caused a dramatic decrease in the antioxidant enzymes (SOD and CAT) activities reaching the control rats values, while for

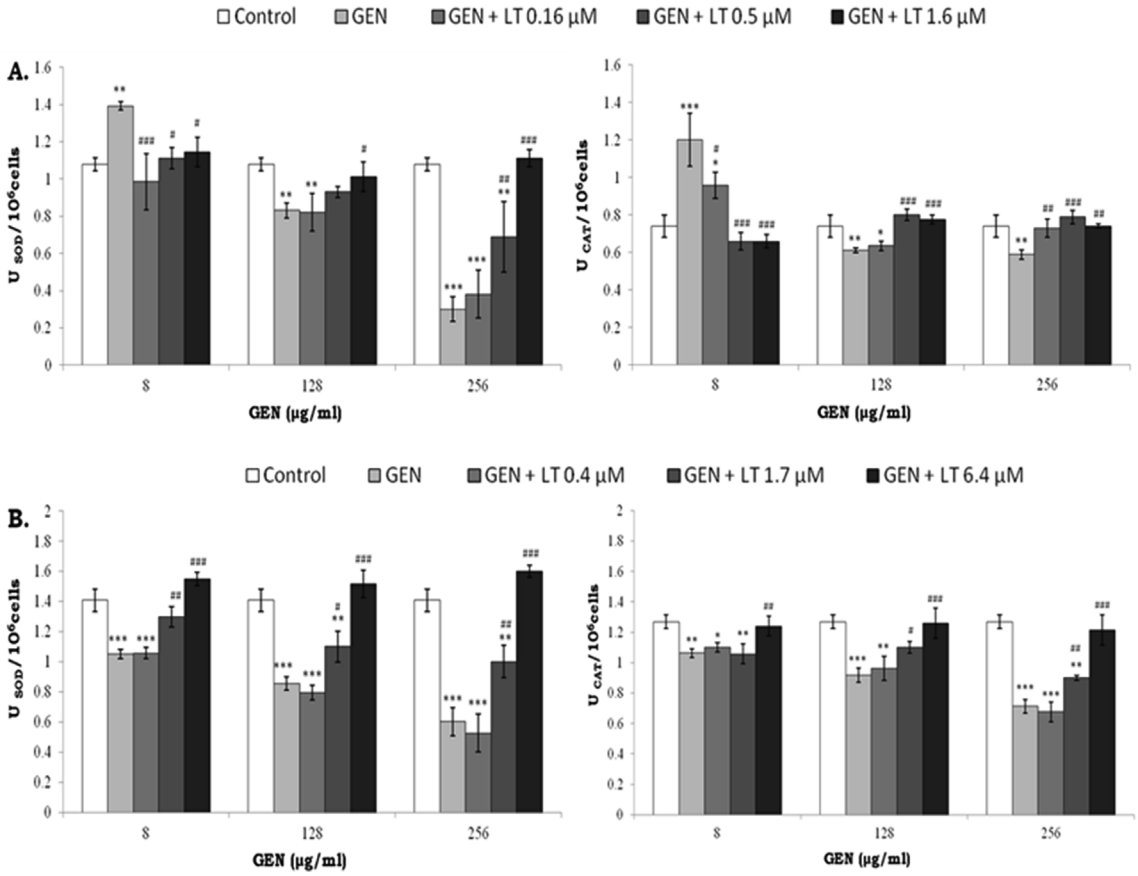

Fig. 4. Effect of luteolin (LT) on the activity of endogenous antioxidant enzymes modified by gentamicin (GEN): A. Mononuclear leukocytes, B. Polymorphonuclear leukocytes. Data (means ± S.D) are expressed as SOD or CAT unit per 10^6 cells and values of three independent experiments.*p < 0.05, **p < 0.01, ***p < 0.001, vs. control leukocytes; #p < 0.05, ##p < 0.01, ###p < 0.001, vs. GEN-treated leukocytes.

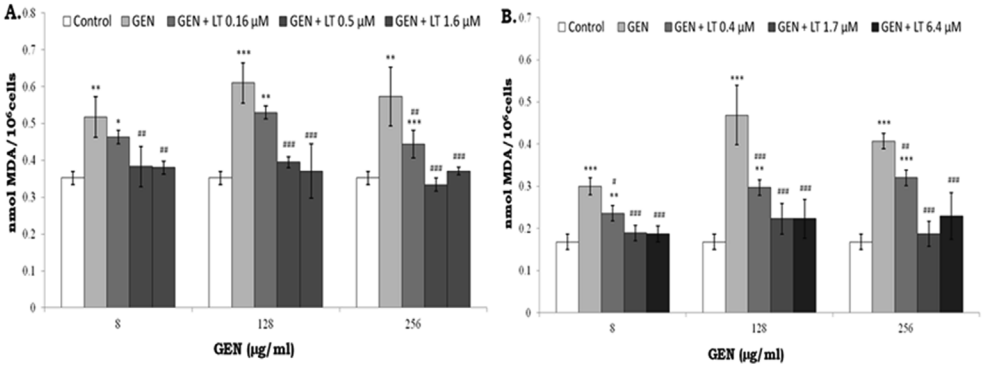

Fig. 5. Luteolin (LT) effect on lipidic peroxidation induced by gentamicin (GEN): A. Mononuclear leukocytes, B. Polymorphonuclear leukocytes. Data (means ± S.D) are expressed as nmol malondialdehyde (MDA) per 10^6 cells and values of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. control leukocytes; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. GEN-treated leukocytes.

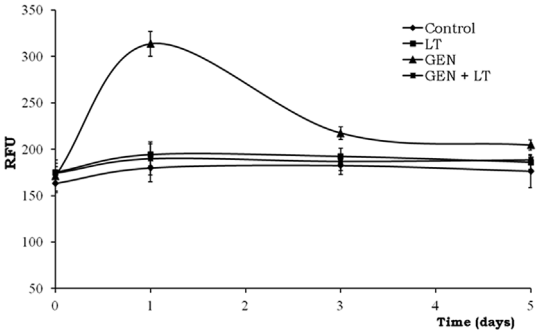

Fig. 6. Luteolin (LT) effect on ROS levels in whole blood of gentamicin (GEN)-treated rats (3–6 mg/kg/day). Data are given as Relative Fluorescence Units (RFU) ± S.D. (n = 6 each group).

MDA, a significant decrease in its levels respect to rats treated only with GEN was observed without reached the control rats values (71.1 ± 9.3% of inhibition). There were no significant differences in SOD and CAT activities between the control, L and GEN + LT groups.

3.3. Antimicrobial activity

3.3.1. Determination of luteolin minimal inhibitory concentration (MIC)

Luteolin demonstrated antimicrobial activity against the reference strains of the Gram-positive bacterium *S. aureus* and the Gram-negative bacterium *E. coli*. Data showed a MIC value of 125 μg/ml in ATCC strains, while in clinical strains LT has not demonstrated antibacterial activity (Table 3).

3.3.2. Checkerboard assay of the luteolin and gentamicin combination in E. coli and S. aureus

The combination of GEN with LT for the inhibition of clinical resistant to GEN strains of *S. aureus* and *E. coli*, did not produce changes in the antibacterial activity of GEN (Fig. 8). Moreover, when GEN and LT were combined for inhibition of ATCC strains of *S. aureus* and *E. coli*, an increase in the susceptibility of bacteria against GEN was observed since the MIC value of the antibiotic decreased two and three dilutions respect to its individual MIC (Fig. 9). A notable synergism was observed in *S. aureus* ATCC whit the FICI value of 0.258, showing that the MIC of GEN was decreased 4-fold and the MIC of LT was reduced 125-fold respect to its individual MIC. In addition, an additive effect was

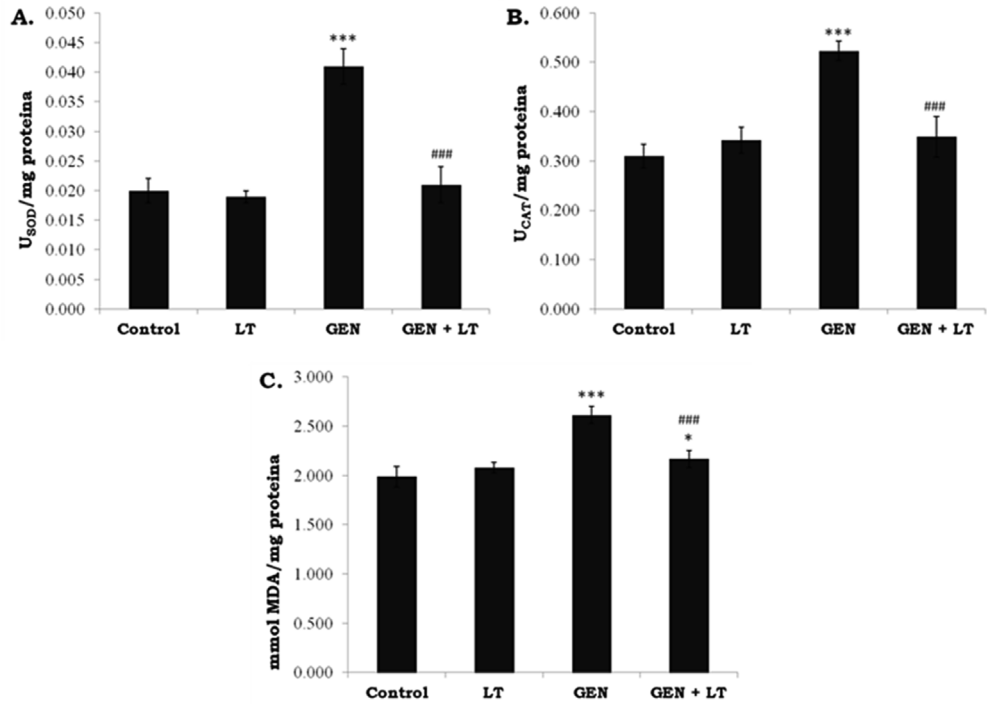

Fig. 7. Enzymatic antioxidant activities in blood and malondialdehyde (MDA) levels in plasma of control and experimental rats. All values are expressed as Enzymatic Units or nmol MDA/mg protein ± S.D. (n = 6 in each group). $^{*}p < 0.05$, $^{***}p < 0.001$, vs. control group; $^{\#\#\#}p < 0.001$, vs. GEN group.

Table 3
Minimal Inhibitory Concentration (MIC) of luteolin.

Strains	*S. aureus* ATCC	*S. aureus* clinical resistant to GEN*	*E. coli* ATCC	*E. coli* clinical resistant to GEN*
Luteolin	125	> 125	125	> 125

MIC values are expressed in μg/ml. *GEN: gentamicin.

observed in the LT + GEN combination for the inhibition of *E.coli* ATCC (Table 4).

4. Discussion

Antibiotics are important tools in modern medicine, allowing to deal with infections produced by different bacteria; however, it has been shown that these can produce several side effects on host cells, such as oxidative stress induction which has been associated with the toxicity of numerous antibiotics. Previous studies by our research group on GEN have demonstrated its ability to induce oxidative stress in leukocytes isolated *in vitro* and in rat whole blood treated with therapeutic doses of GEN *in vivo* manifesting as an increase in ROS generation, alteration of antioxidant defenses and an increase of lipid peroxidation (Bustos et al., 2016).

Regarding intracellular ROS production, as previously demonstrated, GEN is able to induce a greater increase of ERO in PMN leukocytes respect to MN (Bustos et al., 2016). This greater responsiveness to a ROS stimulus in PMN cells could be due to the differences between MN and PMN cells in terms of the content and response of the main enzymes involved in the ROS production in leukocytes, such as NADPH oxidase and myeloperoxidase, especially considering that there are reports in renal cells that show that GEN can increase the activity of the latter (Shin et al., 2014).

Flavonoids are natural products whose potential antioxidant activity is due to, among other mechanisms, the direct scavenging of ROS and metal chelating activity, which depend on the arrangement of functional groups on its core structure. The main structural features of flavonoids required for efficient antioxidant activity are an ortho-dihydroxy (catechol) structure in the B ring, 2,3-double bond in conjugation with a 4-oxo function in the C ring and hydroxyl groups at positions 3 and 5 (Procházková et al., 2011). Concerning the inhibitory activity of studied flavonoids on GEN-induced ROS production in MN leucocytes, and taking into account their structural differences, it can be observed that the flavonoid aglycon LT, which has the majority of the structural requirements, is the most active, showing an IC_{50} two times lower than the reference inhibitor, vitamin C. The two C-glycosylated flavonoids have lower activity, which coincides with the decrease in antioxidant activity reported for glycosylated flavonoids with respect to aglycones (Xiao et al., 2016). Finally, the sulfated flavonoids of quercetin are the least active of the group studied, since they have all the OH groups substituted, which significantly alters their antioxidant activity. The highest activity demonstrated by LT led us to select it to continue the assays. In PMN leukocytes, a similar protective behavior in LT was observed. Although the estimated value for LT was slightly higher than vitamin C (1.67 ± 0.02 μM and 1.06 ± 0.04 μM, respectively), LT showed a good inhibitory capacity of GEN-induced ROS production.

Superoxide dismutase (SOD) and catalase (CAT) are two of the main endogenous antioxidant enzymes whose activities are modified by GEN in human leukocytes (Bustos et al., 2016). SOD accelerates the dismutation of superoxide anion ($O_2^{\bullet-}$) preventing further generation of free radicals and CAT collaborates with the removal of H_2O_2 formed during the reaction catalyzed by SOD (Liu et al., 2010). In MN cells, GEN induces a biphasic response, producing, at the lowest concentration (8 μg/ml), an increase in enzymatic activity, which as reported by Celik and Suzek (2009), would counteract the increase in ROS production; and at higher concentrations, a decrease in antioxidant enzymes activity, due to excessive ROS production, which as reported by Ademiluyi et al. (2013), would cause a depletion of the endogenous antioxidants. As to PMN cells, all tested GEN concentrations induce a decrease in both antioxidant enzymes activities due to excessive ROS production (Bustos et al., 2016). This particular behavior could be related whit the expression of these antioxidant enzymes. In this sense, the changes observed in the enzymatic activity of SOD and CAT by GEN have been studied *in vivo*, in order to understand the mechanism of GEN toxicity. In the conditions evaluated (GEN ranging from 100 to 200 mg/kg/day), this antibiotic was able to produce a decrease of activity SOD and CAT accompanied by a decrease in the expression of SOD2, SOD1 and CAT in kidney, liver and cochlear tissue (Arjinajarn et al., 2017; Ghaznavi et al., 2017; Park et al., 2017). Regarding the reason that GEN would alter the activity of SOD and CAT in our particular system, further studies will be necessary to explain the molecular mechanism by which the enzymatic activity of SOD and CAT is modified by GEN.

Concerning LT effect, this research has shown that when GEN increase or decrease the endogenous antioxidants activity, LT tends to restore SOD and CAT activities, in a dose-dependent manner. In turn, LT is able to prevent GEN-induced lipid peroxidation, biomarker of oxidative stress indicative of the damage of ROS in macromolecules, reaching the basal values at the highest concentration evaluated. This protective effect manifested by LT *in vitro* could be due to the scavenger activity of LT demonstrated in the ROS inhibition assay, which would be cooperating with endogenous antioxidant defenses SOD and CAT, allowing the recovery of the activity of these enzymes, modified in the presence of GEN. However, since in PMN cells LT at the maximum assay concentration is able to increase the activity of the endogenous antioxidant enzymes *per se*, in this type of leukocyte, in addition to the direct scavenger activity demonstrated in the ROS inhibition assays, the intrinsic capacity of activation of endogenous antioxidant enzymes could also be involved.

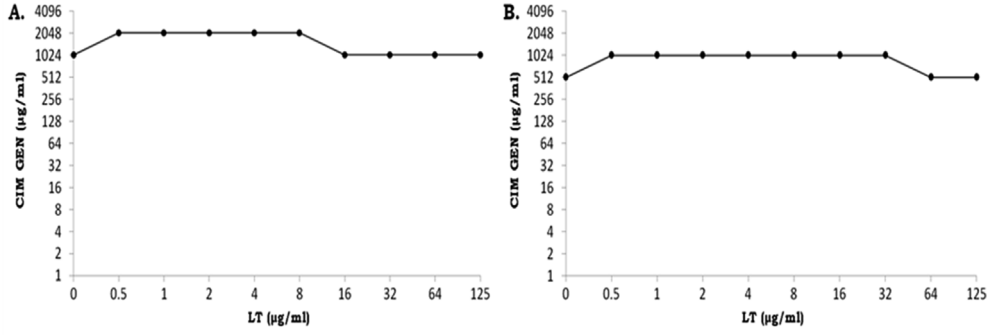

Fig. 8. Interaction between gentamicin (GEN) and luteolin (LT) by checkerboard assay. A. *Escherichia coli* clinical resistant to GEN. B. *Staphylococcus aureus* clinical resistant to GEN.

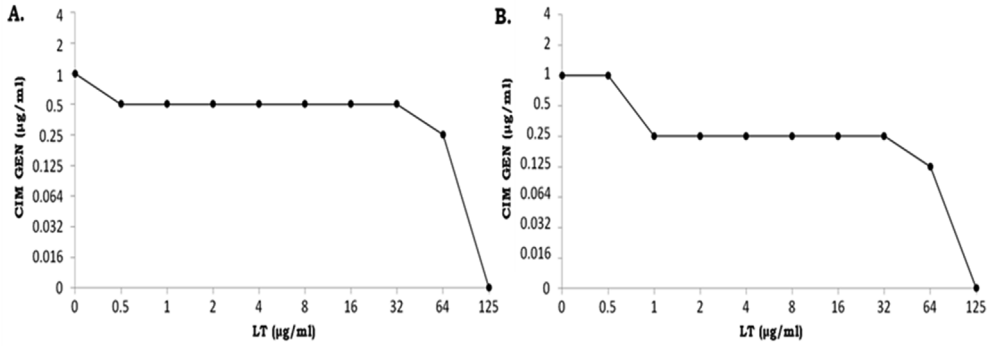

Fig. 9. Interaction between gentamicin (GEN) and luteolin (LT) by checkerboard assay. A. *Escherichia coli* ATCC 25922. B. *Staphylococcus aureus* ATCC 29213.

Table 4
Minimal Inhibitory Concentration (MIC), Fractional Inhibitory Concentration (FIC) and FIC Index (FICI) of GEN in combination with LT in ATCC strains of *E. coli* and *S. aureus*.

E. coli ATCC 25922			*S. aureus* ATCC 29213		
GEN (MIC = 1)			GEN (MIC = 1)		
MIC_G	FIC_G	$FICI_{G+LT}$	MIC_G	FIC_G	$FICI_{G+LT}$
0.5	0.5	0.504	0.25	0.25	0.258
0.25	0.125	0.762	0.125	0.125	0.637

MIC_G = MIC of gentamicin in combination with luteolin. FIC_G = FIC of gentamicin in combination with luteolin. $FICI_{G+LT}$ = F IC of gentamicin plus FIC of luteolin.

Although the protective effect of LT on human leukocytes exposed to GEN has been demonstrated, it is important to extrapolate this effect to systemic circulation. In fact, it has been demonstrated that the oxidative stress evaluated in peripheral blood reflects the redox state of the tissue at the systemic level (Margaritelis et al., 2015).

According to the results obtained *in vivo* and in relation to the ROS production curves, we could suggest that GEN administration would induce a type of stress known as Chronic Oxidative Stress at the systemic circulation level (Lushchak, 2014). Given that as a consequence of antibiotic administration, there was an increase in ROS levels and a subsequent decay in time, without the recovery of the initial steady state. This situation was also accompanied by an increase in the activity of the endogenous antioxidant enzymes as a response to counteract the ROS generated, which were evidently exceeded, since the production of ROS remained above the level observed in control rats, producing damage to biomolecules. In evaluating LT effect in Wistar rats treated with therapeutic doses of GEN, the results confirmed that LT is able to act as a protective agent against oxidative stress induced at the systemic level. This flavonoid demonstrated an important inhibitory capacity of ROS production *in vivo*, collaborated with the endogenous enzymatic antioxidant system and reduced the harmful effects of lipids oxidation.

The fact that we could confirm the protective effect of LT *in vivo* against the oxidative stress induced by GEN observed in isolated blood cells *in vitro*, is of great relevance, since it is important to consider the bioavailability of the flavonoids in an *in vivo* model. Different studies have suggested that the bioavailability of LT is sufficiently high and its metabolism sufficiently low to exert some of its biological activities in an *in vivo* environment (An et al., 2016). The results obtained allow us to confirm *in vivo* the protective effect observed in the studies with human blood cells *in vitro*.

Since one of the mechanisms of action of several antibiotics as GEN would be the oxidative stress production in bacteria (Kohanski et al., 2007; Wang and Zhao, 2009), it was imperious to determine if the antioxidant effect of LT could alter the antibacterial activity of GEN. Moreover, taking into account the aim of finding therapeutic agents that, apart from avoiding toxic effects induced by GEN, can enhance the antimicrobial activity of this antibiotic, we proposed ourselves investigating if flavonoid can enhance the antibacterial activity of GEN. The results have demonstrated that for the combination of L + GEN, the presence of flavonoid produced no changes in the sensitivity of clinical strains of *E. coli* and *S. aureus*. On the other side, in the ATCC strains of these bacterial species, in which LT exhibited antibacterial activity *per se*, the combination showed beneficial effects on antibacterial activity, demonstrating an additive effect against *E. coli* ATCC and synergistic against *S. aureus* ATCC. Gentamicin is a bactericidal antibiotic that acts by interfering in protein synthesis by irreversibly binding to the 30S ribosomal subunit preventing the formation of the initiation complex with mRNA (Balakumar et al., 2010), while studies of the mechanisms, by which flavonoids can exert antibacterial activity, have suggested three main mechanisms: damage and/or reduction of plasma membrane fluidity, inhibition of nucleic acid synthesis and inhibition of energetic metabolism in bacteria (Cushnie and Lamb, 2011). Thereby, this combination of mechanisms of action by different pathways could explain the synergistic effect observed between LT and GEN against the strain ATCC of *S. aureus*.

In conclusion, LT has showed high potential as a therapeutic agent, able to favor the antibacterial activity of GEN against the strains ATCC of *E. coli* and *S. aureus*, reinforcing the possibility of diminishing the oxidative stress generated in host cells and enhancing the activity of GEN on the bacterial cells at the same time.

5. Conclusion

Therefore, LT would demonstrate a marked protective effect against GEN-induced oxidative stress in human leukocytes *in vitro* and in whole blood *in vivo* by decreasing oxidative stress and cooperating in the restoration of the oxidant-antioxidant balance of cells, contributing also to the antibacterial activity of GEN in the ATCC strains of *E. coli* and *S. aureus* showing encouraging additive and synergistic effects. Hence, the simultaneous administration of the flavonoid LT with GEN could represent a potential therapeutic option capable of protecting the host against the oxidative stress induced by this antibiotic in the systemic circulation, preventing clinical consequences caused by the production of ROS while enhancing the antibacterial activity of GEN.

Conflicts of interest

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

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2018.05.030.

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