



The Potentiation of Anti-inflammatory Effect and INOS and COX-2 Gene Expression Inhibition by Rut in When Complexed with Cooper

**Carla A. H. V. Miyashiro¹, Susana N. Diniz¹, Daniel A. F. de Oliveira¹,
Ivair D. Gonçalves¹, Regina M. S. Pereira¹, Renata G. Silva¹,
Niraldo Paulino¹ and Cristina E. Okuyama^{1*}**

¹Anhanguera University of São Paulo, UNIAN Professional Masters Degree Program in Pharmacy and Master's Degree and PhD in Biotechnology and Health Innovation Av Maria Candida, 1813, Vila Guilherme Sao Paulo, SP, CEP 02071-013, Brazil.

Authors' contributions

This work was carried out in collaboration between all authors. Author CEO designed the study, managed the literature searches, performed the statistical analysis, wrote the anti-inflammatory protocol, and revision of the manuscript. Authors CAHVM and IDG performed inflammatory in vivo studies. Author DAFO performed antioxidant assay and author RGS gene expression quantification. Author SND wrote cell culture and gene expression protocols and participated with results discussion and revision of the manuscript. Author RMSP synthesize the rut in complexes and author NP participated with results discussion. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The aim of this study was to evaluate both the antioxidant effect and anti-inflammatory activity of a new transition metal coordinated rut in compound, Rutin-Cu₂ complex.

Study Design: Flavonoids have proven antioxidant and anti-inflammatory activities. Moreover, recent researches demonstrate that the antioxidant activity of flavonoids is believed to increase when they are coordinated with transition metal ions. Our group has recently synthesized new compounds by the reaction of rut in (a flavonoid) with divalent

*Corresponding author: Email: cris_okuyama@yahoo.com.br;

metal salts (copper acetate, nickel acetate or iron sulfate), rendering new transitional metal coordinated rut in compounds, named R-Fe1 [(FeC₂₇H₃₂O₂₁S)₂], R-Cu2 [C₃₁H₃₂O₁₈Cu.2H₂O] and R-Ni2 (C₃₁H₄₂O₂₃Ni). In order to investigate the ability of these new compounds in modulating biological activity and to compare if metal coordinated rut in could increase anti-inflammatory activity of rut in alone, we used murine experimental model of peritonitis to measured cell migration and *In vitro* models of antioxidant activity to evaluate radical superoxide scavenging activity and of macrophage cell line culture to quantify nitric oxide production and iNOS and COX-2 gene expression.

Methodology: To characterize physical-chemical the new generated compounds we used elemental analysis, FT-IR and UV/Vis. The antioxidant effect was evaluate by radical superoxide scavenging assay, using NBT methodology. The anti-inflammatory activity of the new compounds were investigated by peritonitis models induced by carrageenan (1%, 4h), bradikynin (10nmol/cavity, 1h), histamine (100µg/cavity, 1h), substance P (20nmol/cavity, 1,5h) and PGE2 (10nmol/cavity, 1h). Total and leucocytes subtypes numbers were evaluated in harvest cells from mice peritoneum after phlogistic agents administration, in controls groups (not treated or treated with dexamethasone or rut in alone) or tested groups (treated with metal coordinated compounds). RAW 264.7 cells were stimulated with LPS on the absence or presence of rut in alone (0.01–90mM), or Rutin-Cu₂ complex (0.01–90mM). The production of NO was measured in culture supernatant after 24h of cell incubation, by Griess assay. And iNOS and COX-2 transcripts were quantified by real time PCR with SYBR GREEN, on cDNAs obtained after 24h of cell incubation, in a step one instrument (Applied bio systems).

Results: Complex formation was also verified by ¹H RNM, using DMSO-d₆ as solvent. The proton signals from Hydroxyl groups 5-OH, 7-OH, 3'-OH and 4'-OH shifted to lower and broader frequencies in coordinated complex R-Fe1, R-Cu2 and R-Ni2, compared with signals from free rut in. The results showed that R-Cu₂ complex presented a higher superoxide scavenging effect when compared with rut in alone (6.95% and 46.42%, at 10µM; and 51.80% and 71.32%, at 100µM, respectively). The results also showed that R-Cu₂ inhibited significantly (P<0.05; ANOVA) cell migration (neutrophils, lymphocytes and monocytes) in peritonitis induced by carrageenan, bradikynin and PGE2, in mice, when compared to controls ones (without treatment or Ru alone treatment). Furthermore, rutin and R-Cu₂ significantly (P<0.05, paired t test) inhibited iNOS and COX-2 gene expression in LPS-induced macrophage cells.

Conclusions: Taken together, our results show for the first time that the R-Cu₂ complex, a metal coordinated rut in compound, produces anti-inflammatory effects in mice, at least in part, by means of increasing the antioxidant activity and inhibition of iNOS and COX-2 gene expression. We suggest that cooper coordinated rut in compound can potentiates some biological properties of this flavonoid and could be more effective for therapeutic treatment of diseases related to oxidative stress.

Keywords: Rut in; rut in-copper complex; antioxidant; anti-inflammatory; gene expression.

1. INTRODUCTION

Flavonoids have high antioxidant and anti-inflammatory activities. Moreover, recent studies have demonstrated that the coordination of transition metal ions increase the antioxidant activity of flavonoids. Reactive oxygen species (ROS) are implicated in the etiology of several inflammatory processes [1]. The structure-activity of flavonoids has been extensively

studied in the attempt to understand which structural effects are more important on the several activities.

Flavonoid anti-inflammatory activity can be related to cellular mechanisms such as arachidonic acid enzyme modulation, and phospholipase A2 (PLA2), cyclooxygenase, lipoxygenase and nitric oxide synthase enzyme inhibition. The inhibition of these enzymes reduces the production of inflammatory mediators such as arachidonic acid, prostaglandins, leukotrienes and nitric oxide [2]. The first PLA2 inhibitor flavonoid found was quercetin, which inhibits PLA2 from human neutrophils [3].

As is well known, there are some metal essentials for all organisms. For example, copper is a well-studied important cofactor of a variety of enzymes that are involved in a variety of biological processes, such as cytochrome C oxidase [4]. Although metals as copper and nickel in excess could be toxic to life organisms [5,6], but when they were associated with compounds exhibiting antioxidant and anti-inflammatory activities, they could take an interesting part in the struggle against diseases related to damages caused by the increase in free radicals [7]. As demonstrated by Kostyuk et al. [8], iron coordinated flavonoid increase the flavonoid scavenging capacity, indicating that this compound could be beneficial for therapeutic treatment of diseases related to oxidative stress.

The flavonoid naringin has antioxidant and anti-inflammatory activities, and inhibits the proliferation of human mammary tumor cells *In vitro* [9]. The naringin-copper complex presents increased DPPH radical scavenging activity when compared with naringin alone. In addition, naringin complexed with copper has shown anti-inflammatory activity against carrageen-induced peritonitis in mice, and antitumoral activity in murine melanoma cell line B16F10 and human chronic myelogenous leukemia cell line K562 [7,10].

Rutin is a flavonoid found in *Dimorphandra mollis*, a native plant of Brazil. Rutin (also called vitamin P) is known to exert beneficial effects such as protection against lipid oxidation and free radical scavenging. Rutin is an important potassium modulator [11] and induce vascular regulation mediated by nitric oxide [12,13]. Besides, rutin can also be used in the treatment of anemia, beta-thalassemia, cardiovascular diseases and neurodegenerative disorders [14,15], which are diseases associated with the excessive production of reactive oxygen species (ROS) [16].

Our group has recently shown that transition metal complexes bearing flavonoid ligands, such as rutin-Fe (I) and rutin-Cu (II) complexes, can be synthesized. These compounds, as well as naringin and Cu complexes, present higher antioxidant, anti-inflammatory and antitumoral activities when compared with the flavonoid alone [17,18].

Therefore, the objective of this work was to evaluate the antioxidant and anti-inflammatory activities of rutin and rutin derivatives containing transition metals: copper (II)-rutin (R-Cu2), Iron (I)-rutin (R-Fe1), and nickel (II)-rutin (R-Ni2) complexes.

2. MATERIALS AND METHODS

2.1 Antioxidant Activity

The antioxidant effects of the transition metal-Rutin complex were evaluated *In vitro* by an assay in which the activity of radical superoxide scavenging, dispersed in the environment,

was measured. The method was adapted by Nikishimi et al. [19]. The reaction consisted of the test compound (10 μ M) combined with phenazine methosulfate (PMS-20 μ M), coenzyme nicotinamide adenine dinucleotide (NADH - 156 μ M), and nitro blue tetrazolium (NBT-50 μ M) in phosphate buffer solution (0.1M; pH 7,4) at a final volume of 2.5mL. Among the evaluated composites were copper (II)-Rut in (R-Cu2), Iron (I)-Rut in (R-Fe1), nickel (II)-Rut in (R-Ni2) complexes and Rut in itself, tested at the concentrations of 1, 10 and 100 μ M. The negative control was a non-NBT run, and the flavonoid itself was used as positive control. The formazan crystals were formed by nitroblue tetrazolium (NBT) reduction in the presence of the superoxide radicals (O₂⁻), a kind of reactive oxygen species (ROS). The absorbance was measured at 560nm in a spectrophotometer (Femto[®]). The antioxidant activities of the compounds were demonstrated in superoxide radical scavenging percentage (%).

2.2 Anti-inflammatory Activity–animals

Male Swiss mice (18-35g) from CEDEME, housed at 22 \pm 2 $^{\circ}$ C under a 12h/12h light-dark cycle, were used. Food and water were freely available. The animals were acclimatized to the laboratory for at least 1h before testing and were used once throughout the experiments, which were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental plan in conscious animals from Ethical Committee UNIBAN (protocol n^o301_08) and Zimmermann [20].

Acute phlogistic-induced inflammatory reaction in the peritoneal cavity of mice. The anti-inflammatory activity was determined by analyzing the total and subtype numbers of leukocyte migration to peritoneal cavity of Swiss mice, after peritonitis induced by phlogistic agents: carrageenan (1%, 4h), bradykinin (BK, 10nmol/cavity, 1h), prostaglandin E2 (PGE2, 10nmol/cavity, 1h), histamine (100 μ g/cavity, 1h) and substance P (SP, 20nmol/cavity, 1,5h) [10], without (negative controls) or with concomitant administration of dexamethasone (5mg/kg, s.c.), rut in, R-Cu2 and R-Ni2 (16 and 160 μ mol/kg, p.o.). After the time indicated above for each treatment, the animals were sacrificed, and cells were harvested from the peritoneal cavity and stained with Panotico stain (Laborclin[®]). The results were expressed as cell number/mL.

2.3 Cell Culture

RAW 264.7 Macrophage: mouse monocytic transformed cell lines mentioned by Raschke et al. [21] were cultivated in RPMI 1640 medium supplemented with sodium bicarbonate 2g/L ("LCG biotecnologia"), streptomycin 100 μ g/mL, penicillin 100UI/mL and 10% of fetal bovine serum. Cells were maintained at 37 $^{\circ}$ C and 5% of CO₂.

2.3.1 MTT-cell viability assay

MTT (thiazolyl blue tetrazolium bromide) was used to measure macrophage (RAW 264.7) viability [22]. Cells were seeded onto 96-well plates at a density of 1x10⁴ cells/well, and incubated (37 $^{\circ}$ C, 5%CO₂) overnight. Then 2 μ L of compounds dissolved in DMSO were added and the plates were incubated for 6, 12 and 24 hours. The final concentration of rutin and R-Cu2 were: 0.01, 0.05, 0.1, 1, 5, 10, 20, 30, 40, 50, 70, 90mM. After 6, 12 or 24h, the medium was replaced with a fresh medium (200 μ L) containing MTT(0,5mg/mL). The cells were then incubated for 4h, the supernatant was collected, DMSO(200 μ L) was added, shaking for 5 min until the crystals were fully dissolved. Absorbance was measured at 540nm using a microplate reader (Labtech LT-4000MS).

2.3.2 Nitric oxide dosage

The effect of the transition metal-Rut in complex on nitric oxide (NO) production can be evaluated in macrophages (RAW 264.7) by Griess assay [23], a protocol for the detection of NO_2^- in a variety of biological liquid matrices as a measure that reflects NO production. Cells were seeded onto 96-well plates at a density of 1×10^4 cells/well and incubated overnight (37°C , $5\% \text{CO}_2$). Then the media were replaced by fresh media containing the compounds (rutin and R-Cu2) in DMSO or DMSO alone. After 1h, lipopolysaccharide (LPS-Sigma) ($1 \mu\text{g}/\text{mL}$) was added and the plate was incubated for another 6, 12 or 24h in 37°C , $5\% \text{CO}_2$. Thereafter, nitrite (NO_2^-) concentration was determined in culture supernatant incubated for 30min with Griess solution. Absorbance was measured at 540nm using a microplate reader (Labtech LT-4000 MS). Results were expressed as NO concentration ($\mu\text{mol}/\text{L}$).

2.3.3 Gene expression quantification-total RNA isolation

For RNA extraction, cells from RAW lineage were incubated with compounds (rutin and R-Cu2) at the concentration of $30 \mu\text{M}$ and stimulated with LPS($1 \mu\text{g}/\text{mL}$) for another 24h at 37°C , $5\% \text{CO}_2$. RNA was isolated from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA quantity was assessed using nanodrop (NanoDrop Products, Wilmington, DE USA).

2.3.4 Reverse transcription-polymerase chain reaction

Complementary DNA (cDNA) was prepared with Reverse transcription (Fermentas), in a final volume of $50 \mu\text{L}$, using 500ng total of total RNA, from cultured cells as described above, 400U of reverse transcriptase, $10 \mu\text{L}$ of enzyme buffer, $10 \mu\text{L}$ of 10mM dNTP mixture (all from Invitrogen), $0.2 \mu\text{L}$ of oligo(dT) primer, and 50U of RNase inhibitor (Amersham Biosciences, Uppsala, Sweden). This mixture was incubated at 42°C , for 60min, and then at 70°C , for 10min.

2.3.5 Quantitative PCR for iNOS and COX-2 transcript detection

Real-time PCR reactions of cDNAs were performed with SYBR Green I on an ABI PRISM Step one Sequence Detection System (PE Applied Biosystems). Quantification of transcripts iNOS and COX-2 were calculated as delta-delta C(T) method as previously described [24] and normalized by the amount of the beta-actin gene. Results were expressed as $2^{-\Delta\Delta\text{CT}}$ from iNOS and COX-2 expression.

2.4 Rut in Complexes

The rutin complexes were produced in the chemistry laboratory of the Universidade Bandeirante de São Paulo following the patent INPI P. 018110000353 06/01/2001 [17].

2.4.1 Synthesis of complex R-Fe1

A solution of $[\text{Fe}(\text{SO}_4) \cdot 7\text{H}_2\text{O}]$ in methanol was dropped slowly to a solution of rutin in methanol. The black precipitate R-Fe1 obtained was filtered, dried and washed with methanol to remove completely the impurities. Yield: 1.0450g, 89,2%; m.p. 184°C . Experim. analysis for $[\text{FeC}_{27}\text{H}_{32}\text{O}_{21}\text{S}]_2$ (Calc.): %C, 41.59 (41.55); %H, 4.90 (4.10). IR (cm^{-1} , solid state): 3333 (OH); $\nu(\text{C}=\text{O})$ 1654; $\nu(\text{C}=\text{C})$ 1560, 1503, 1455; $\nu(\text{C}-\text{O}-\text{C})$ 1295; $\nu(\text{C}-\text{C})$ 808.

2.4.2 Synthesis of complex R-Cu₂

A solution of [Cu(CH₃COO)₂] in methanol was dropped slowly to a solution of rut in methanol. The filtrated solution was evaporated, and the obtained material purified by chromatography using Sephadex LD 20 and methanol as solvent. A pure green fraction was dried, and pure compound was obtained. Yield: 0.496g, 38%; mp. 242°C. Experim. data for [C₃₁H₃₂O₁₈Cu₂H₂O] (Calc.): %C, 46.68 (46.37); %H, 4.82 (4.40). IR (cm⁻¹, solid state): ν(OH) 3419; ν(C=O) 1655; ν(C=C) 1599, 1573, 1539, 1534; ν(C-O-C) 1295; ν(C-C)807.

2.4.3 Synthesis of complex R-Ni₂

By similar procedure, using nickel(II) acetate to metallate rutin. After drying the filtrated solution, previously purified by chromatography using Sephadex LD 20 and methanol as solvent, a pure light brown compound was obtained. Yield: 0.901g, 63%; mp. 285°C. Experim. data for C₃₁H₄₂O₂₃Ni(Calc.): %C, 44.31(44.00); %H, 5.47(4.26). IR (cm⁻¹, solid state): ν(OH) 3449; ν(C=O) 1653; 1505, 1597, 1575, 1558, ν(C=C) 1456 ; ν(C-O-C)1296; ν(C-C)807.

2.5 Statistical Analysis

Data are expressed as means±standard error of mean. Significant differences (*P<0.05) were determined by one-way ANOVA complemented with Bonferroni's test (for antioxidant activities), Dunnett's test (for anti-inflammatory effects), and paired t test (for gene expression quantification).

3. RESULTS

3.1 Antioxidant Activity

Superoxide anion radicals are produced by a number of cellular reactions, including several enzyme systems such as lipoxygenase, peroxidase, NADPH oxidase, and xanthine oxidase. The superoxide radical scavenging activities of the compounds are demonstrated (Fig. 1).

The Rut in, R-Cu₂, R-Ni₂ and R-Fe₁ exhibited 51.80%, 71.32%, 53.57% and 52.59% scavenging activity at 100µM, and 6.95%, 46.42%, 21.69%, and 11.01% at 10µM, respectively. The R-Cu₂ and R-Ni₂ complexes exhibited a statistically significant (P<0.01, ANOVA and Bonferroni post-test) higher scavenging activity when compared with Rut in alone when used at 10µM. The complex R-Fe₁ did not show a statistically significant antioxidant effect when compared with Rut in alone. However, R-Cu₂ presented the highest scavenging activity (45 and 75%) in more than one concentration (10 and 100µM, respectively).

Because R-Cu₂ and R-Ni₂ were more effective in superoxide radical anions scavenging than Rut in alone, these compounds were chosen to investigate the anti-inflammatory activity *In vivo*.

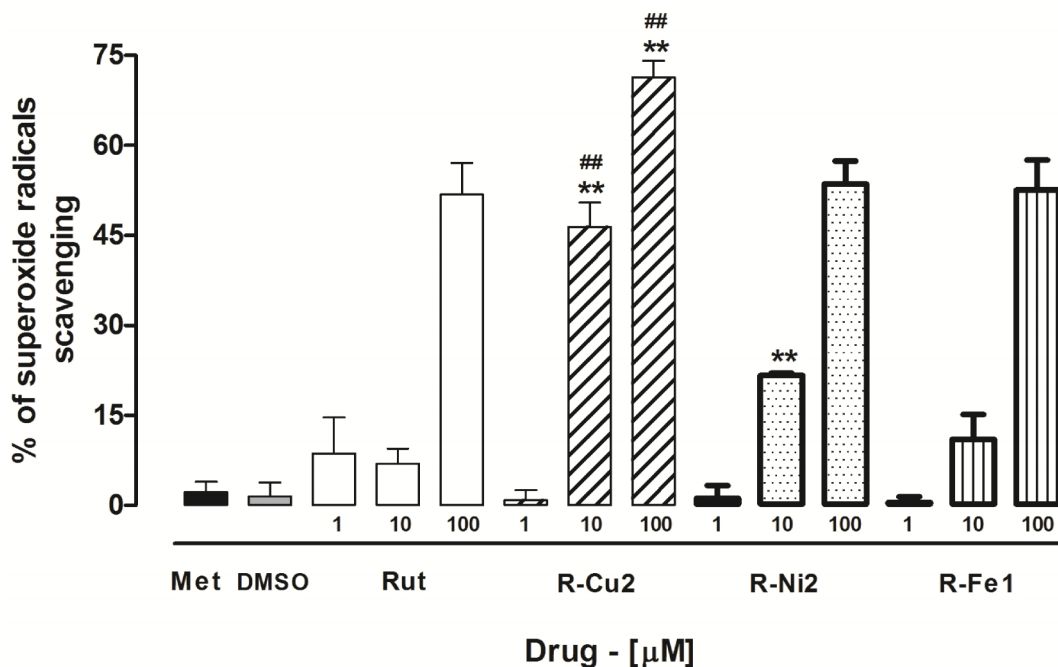


Fig. 1. Superoxide radical scavenging percentage, in different concentrations (1, 10 and 100µM) of the compounds rutin (Rut-□), R-Cu2 (///), R-Ni2 (...) and R-Fe1 (|||) in 5 minutes. Met-methanol in 30% concentration. DMSO–DMSO in 10% concentration. **P<0.01, rutin versus compounds. ##P<0.01, R-Cu2 versus R-Ni2 and R-Fe1

3.2 Anti-inflammatory Activity-carrageenan-induced Peritonitis

The results of the anti-inflammatory activities analyzed by the migration of leukocytes to the peritoneal cavity of Swiss mice after carrageenan-induced peritonitis are shown in (Fig. 2).

The R-Cu2-treated (16 and 160µmol/kg) group of mice was the only one that inhibited significantly ($P<0.05$), suggesting a dose-dependent effect in the migration of total leukocyte cells to peritoneal cavity, when compared with control group. The R-Cu2 160µmol/kg group showed significant results that was similar to the positive control dexamethasone group. In addition, the rutin and R-Cu2 effect was compared, and a statistically significant difference ($P<0.05$) between both compounds was observed. The inhibition (%) of inflammatory response induced by carrageenan in the peritonitis model in mice produced by rutin and R-Cu2 (16 and 160µmol/kg) was 0 and 9.16%, 26.22 and 38.5%, respectively. The R-Ni2 did not change leukocyte migration; hence no further experiment was carried out with this compound.

In the differential leukocyte migration results the R-Cu2 significantly inhibited ($P<0.05$) neutrophils and mononuclear cells after 4 hours of carrageenan-induced peritonitis when compared with the control group (Fig. 2).

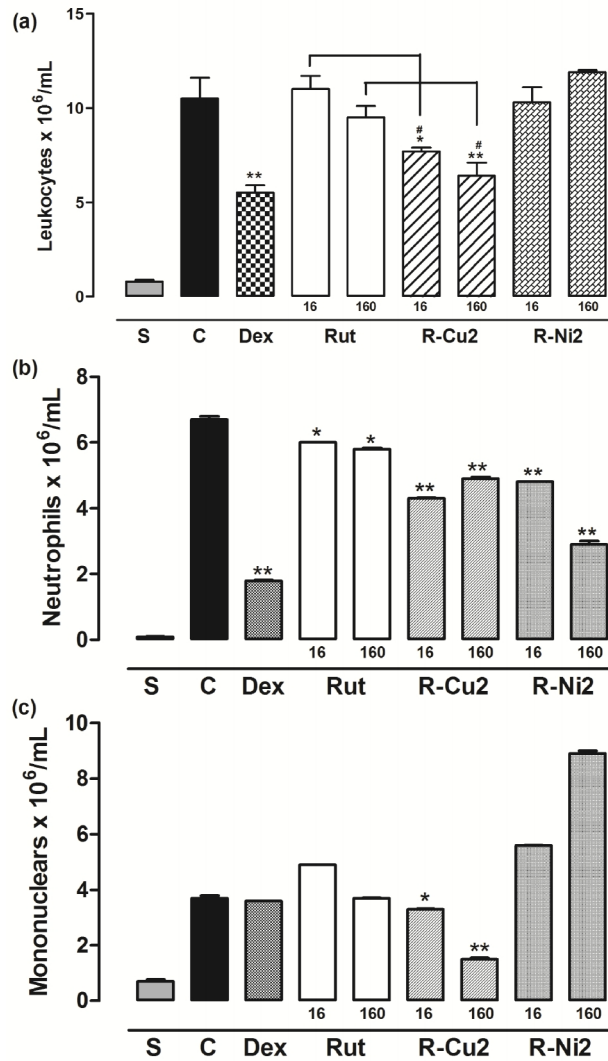
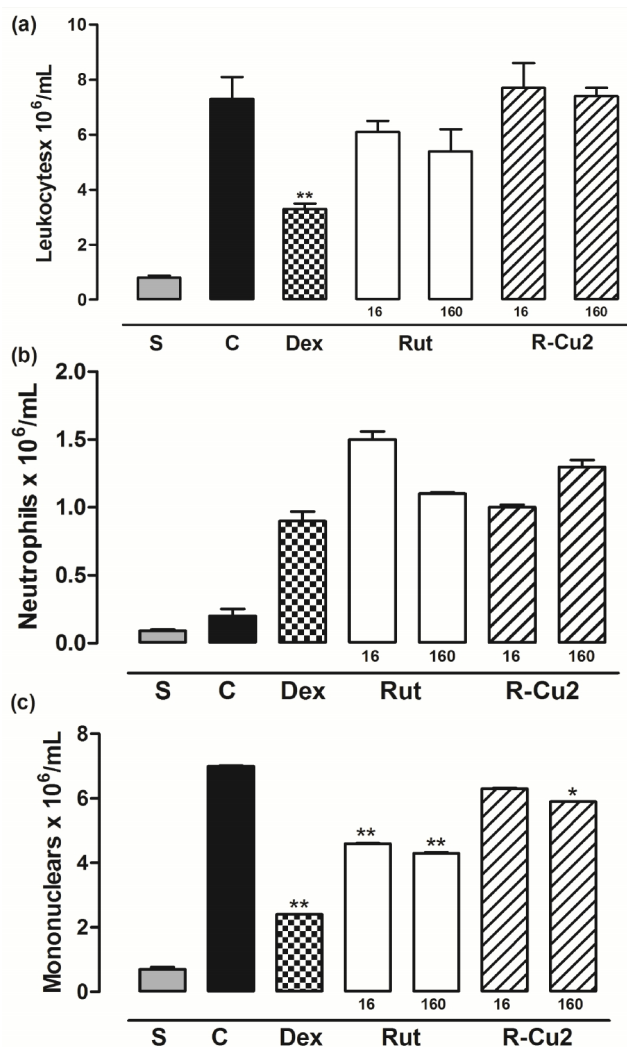


Fig. 2. Effect of rut in (Rut), R-Cu2 and R-Ni2 (16 and 160 $\mu\text{mol/kg}$, p.o.) on carrageenan-induced peritonitis in mice. Total (a) and differential (b, c) leukocyte counts were evaluated. S=animals treated only with sterile saline, C=animals treated with vehicle plus carrageenan, Dex=animal treated with dexamethasone (5mg/kg, s.c.) plus carrageenan. Each group (n=5) represents the mean \pm SEM. Significant differences determined by ANOVA complemented with Dunnett's test, *P<0.05; **P<0.01, when the control group is compared with the other groups. #P<0.05, when Rut and R-Cu2 groups are compared

3.3 Anti-inflammatory Activity-histamine-induced Peritonitis

The total leukocyte migration after histamine-induced peritonitis was not affected by rut in and R-Cu2 compounds. Though the compounds did not change the total leukocyte number, mononuclear cell migration was reduced in both compounds groups when compared with the control group (P<0.05) additional data are given in Online Resource (Material 1).



Material 1. Effect of rutin (Rut), R-Cu2 (16 and 160 $\mu\text{mol/kg}$, p.o.) on histamine-induced peritonitis in mice. Total (a) and differential (b, c) leukocyte counts were evaluated.

S=animals treated only with sterile saline, C=animals treated with vehicle plus histamine, Dex = animal treated with dexamethasone (5mg/kg, s.c.) plus histamine. Each group (n=5) represents the mean \pm SEM. Significant differences determined by ANOVA complemented with Dunnett's test, *P<0.05; **P<0.01, when the control group is compared with the other groups

3.4 Anti-inflammatory Activity-bradykinin-induced Peritonitis

In peritonitis induced by bradykinin (BK), the total leukocyte migration was significantly (*P<0.05) inhibited in the R-Cu2 group (doses of 16 and 160 $\mu\text{mol/kg}$), when compared with the vehicle group. But, when compared with rutin alone, R-Cu2 was not statistically significant. The inhibition percentages of rutin and R-Cu2 activities in 16 and 160 $\mu\text{mol/kg}$ were: 27.86 and 22.21%, 54.93 and 43.43%, respectively. In differential leukocyte migration, R-Cu2 group significantly inhibited mononuclear cell migration (Fig. 3).

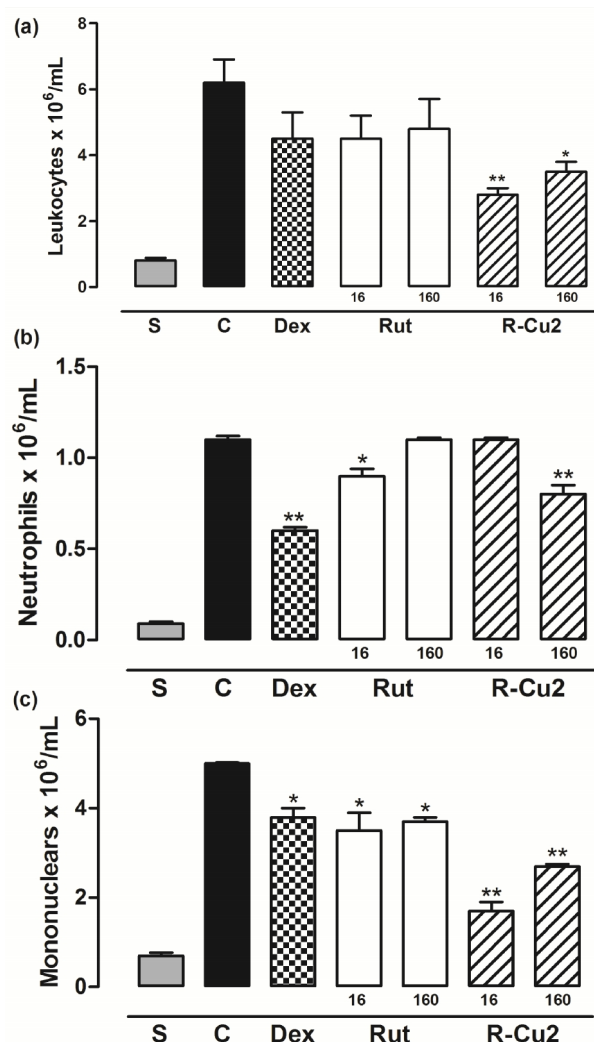


Fig. 3. Effect of rutin (Rut), R-Cu2 (16 and 160μmol/kg, p.o.) on bradykinin-induced peritonitis in mice. Total (a) and differential (b, c) leukocyte counts were evaluated. S=animals treated only with sterile saline, C=animals treated with vehicle plus BK, Dex=animals treated with dexamethasone (5mg/kg, s.c.) plus BK. Each group (n=5) represents the mean±SEM. Significant differences determined by ANOVA complemented with Dunnett's test, *P<0.05; **P<0.01, when the control group is compared with the other groups

3.5 Anti-inflammatory Activity-prostaglandin E₂-induced Peritonitis

The results of the R-Cu2 group (16 and 160μmol/kg) showed that this compound, dose dependent, significantly inhibited the total leukocyte migration (P<0.05) in PGE₂-induced peritonitis in mice (Fig. 4) when compared with the control group. In addition, the rutin and R-Cu2 effect was compared, and a significant difference can be observed between the two compounds. The inhibition percentages of rutin and R-Cu2 activities in 16 and 160μmol/kg are: 21.27 and 42.01%, 49.23 and 57.25%, respectively.

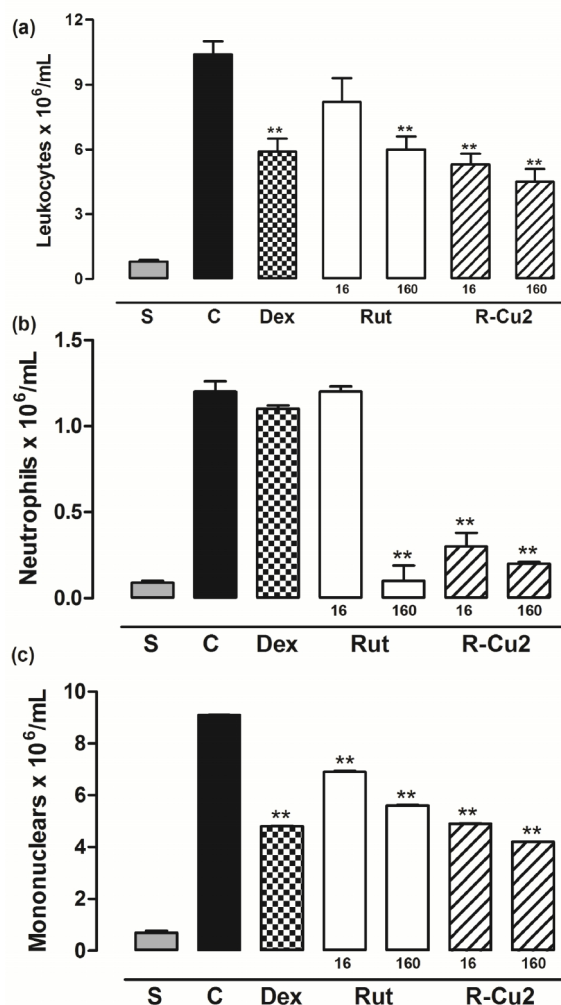


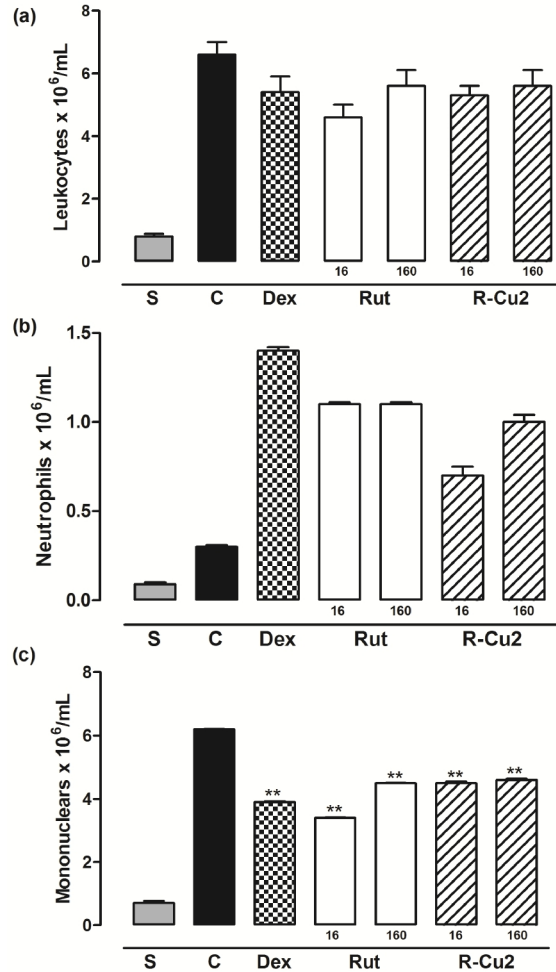
Fig. 4. Effect of rut in (Rut), R-Cu2 (16 and 160 μ mol/kg, p.o.) on PGE2-induced peritonitis in mice. Total (a) and differential (b, c) leukocyte counts were evaluated. S=animals treated only with sterile saline, C=animals treated with vehicle plus PGE2, Dex=animals treated with dexamethasone (5mg/kg, s.c.) plus PGE2. Each group (n=5) represents the mean \pm SEM. Significant differences determined by ANOVA complemented with Dunnett's test, *P<0.05; **P<0.01, when the control group is compared with the other groups

The results of differential leukocyte migration showed that rut in and R-Cu2 significantly inhibited (P<0.05) neutrophils and mononuclear cells after 1 hour of PGE2-induced peritonitis when compared with control group (Fig. 4).

3.6 Anti-inflammatory Activity-substance P-induced Peritonitis

The total leukocyte migration after substance P-induced peritonitis was not affected by rut in and R-Cu2 compounds. Though the compounds did not change the total leukocyte number,

the mononuclear cell migration was reduced by both compounds groups when compared with control group, additional data are given in Online Resource (Material 2).



Material 2. Effect of rutin (Rut), R-Cu2 (16 and 160 μ mol/kg, p.o.) on substance P-induced peritonitis in mice. Total (a) and differential (b, c) leukocyte counts were evaluated. S = animals treated only with sterile saline, C=animals treated with vehicle plus substance P, Dex=animals treated with dexamethasone (5mg/kg, s.c.) plus substance P. Each group (n=5) represents the mean \pm SEM. Significant differences determined by ANOVA complemented with Dunnett's test, **P<0.01, when the control group is compared with the other groups

3.7 Nitrite Assay in RAW 264.7 and Cell Viability by Mean MTT

The rutin and rutin complexes were assayed to determine whether they affect NO production in RAW 264.7 macrophage cell lines activated with LPS for 24 hours. When assessed *in vitro*, treatment (30mM) with rutin or R-Cu2 for 24 hours abolished the NO production in RAW 264.7 stimulated by LPS without interfering with the viability of cells, as shown by the MTT test (Fig. 5A).

In (Fig. 5B), we have investigated whether the inhibitory effect of the rut in and R-Cu₂ on NO production was dose-dependent. The pre-incubation of rut in or R-Cu₂ (0.01–10nM) on RAW 264.7 stimulated by LPS reduced dose-dependent NO production, while the 10mM of both, rut in or R-Cu₂, almost abolished the NO production in the RAW 264.7 cell line stimulated by LPS. The results also demonstrated that only a small quantity (0.01mM) of R-Cu₂ was sufficient to inhibit about 50% of NO production by RAW stimulated cells. The same percentage (50.6%) of NO production inhibition was only seen when cells were treated with higher rut in concentrations (1mM or more) (Fig. 5B).

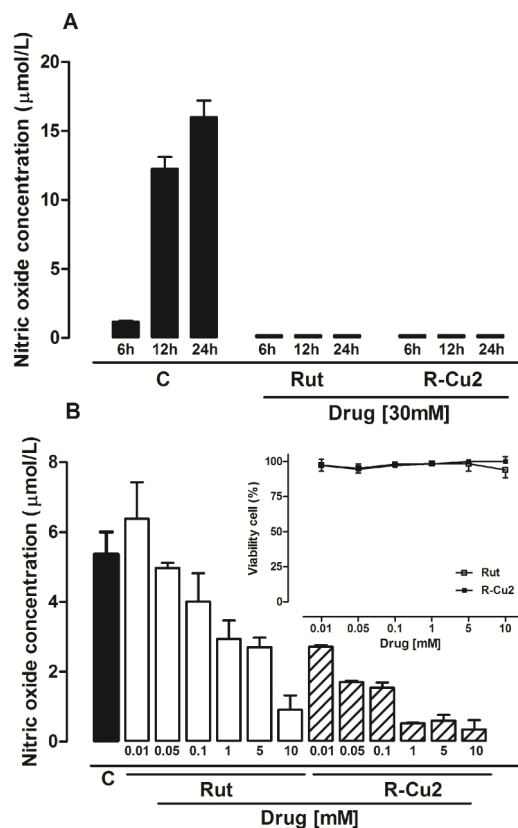


Fig. 5. Nitrite Assay in RAW 264.7 and cell viability by mean MTT. A) Nitric oxide production from LPS-induced RAW 264.7 cells in 6, 12 and 24 hours. C–control group, cells with medium; Rut (rut in) and R-Cu₂ group in 30mM concentration. B) Increase of the nitric oxide production from LPS-induced RAW 264.7 cells and cell viability in 24 hours. C–control group, cells with medium; Rut (rut in) and R-Cu₂ group in 0.01–10mM

It was also demonstrated that cells presented 100% of viability at all concentrations of the treatment compound (Fig. 5b).

3.8 INOS and COX-2 Gene Expression

To evaluate the impact of rut in and R-Cu₂ treatment on gene expression in LPS-induced RAW 264.7 cells, iNOS and COX-2 transcripts were quantified by Real Time PCR. The

results showed that LPS induced an increase (delta-delta CT values below one) in COX-2 and iNOS gene expression. However, Rut in and R-Cu₂ inhibited (delta-delta CT values above one) COX-2 and, especially, iNOS gene expression in LPS-induced cells (Fig. 6). These results corroborated those presented above, that Rut in and R-Cu₂ participated in signaling pathways activated by LPS in macrophages.

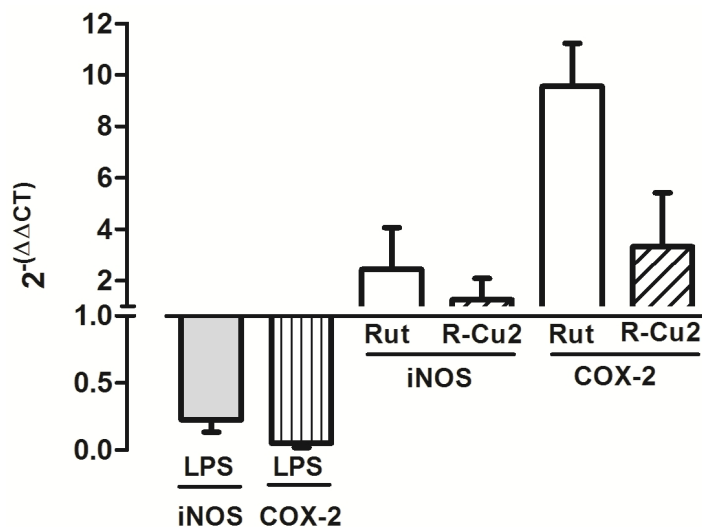


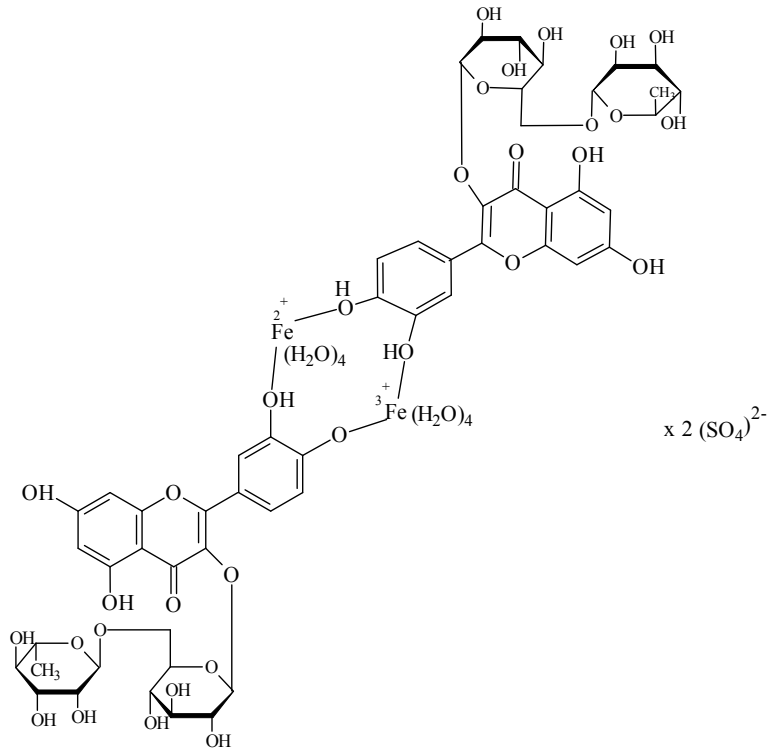
Fig. 6. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression levels modulated by LPS, and Rut in (Rut) and Rut in-Cu (R-Cu₂) compounds in RAW 264.7 cells. Results were expressed as 2^{-(ΔΔCT)} and means that values below one show an increase in gene expression while values above one demonstrate gene expression inhibition

3.9 Rut in Complexes

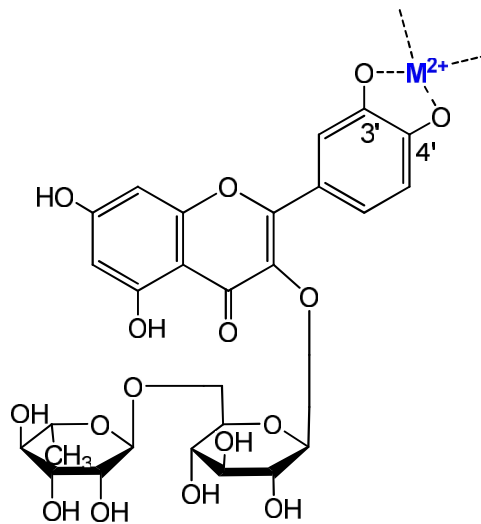
The rut in complexes (R-Fe1, R-Cu₂ and R-Ni₂) were produced following the patent INPI P. 018110000353 06/01/2001 [17]. Complex formation was also verified by ¹H RNM, using DMSO-d₆ as solvent (Table 1). The structure complexes proposal, in solid state, is given in Online Reource (Materials 3 and 4).

Table 1. NMR data for rut in and complexes R-Fe1, R-Cu₂ and R-Ni₂

Group	Rut in	R-Fe1	R-Cu ₂	R-Ni ₂
OH	12.60(s); 10.82(s); 9.67(s); 9.17(s)	12.50(br,s); 10.80(br,s); 9.60(br,s); 9.20(br,s)	12.64(s); 10.88(br,s); 9.71(br,s); 9.22(br,s)	12.15(br, s)
H ₂ ,H ₆	7.60 (d, J=8.32 Hz)	7.46(s)	7.60(d,J=8.12 Hz)	7.30 (d,J=8.33Hz)
H ₃ ,H ₅	6.83 (d, J=8.31 Hz)	6.78 (br,s)	6.9(d, J=8,13Hz)	6.98(8.40 Hz)
H ₈	6.40(s)	6.32(br,s)	6.43(br,s)	6.78
H ₆	6.20(s)	6.13(br,s)	6.24(br,s)	5.86 (s)



Material 3. Proposed structure for R-Fe1 complex, in solid state



M^{2+} = copper or nickel

Material 4. Proposed structure for R-Cu₂ or R-Ni₂ complexes, in solid state. $M=Cu^{2+}$ or Ni^{2+}

4. DISCUSSION

The present study was undertaken to investigate the antioxidant and anti-inflammatory effects of flavonoids and metal-flavonoids complexes, such as Rut in, R-Cu₂, R-Ni₂, and R-Fe, on superoxide radical scavenging activities, and murine peritonitis models induced by carrageenin, histamine, bradikynin, PGE₂, and substance P. This study also investigated the effect of Rut in, R-Cu₂, R-Ni₂, and R-Fe on the production of nitric oxide in RAW 264.7 macrophage cell lines, linked to the expression of iNOS and COX-2 protein.

Nijveldt et al. [25] showed that a main effect of flavonoids in oxygen-derived scavenging free radicals. The antioxidant activity of rut in was well defined. Noroozi et al. [26] related that rutin has a better antioxidant effect than vitamin C induced by H₂O₂ in DNA damage in human lymphocyte, and Hanasaki et al. [27] described how rutin has a better antioxidant effect than hydroxyl free radical scavenging mannitol.

Afanas'eva et al. [7] showed that flavonoid-transition metal complexes have better antioxidant and anti-inflammatory activities than flavonoid alone. Kostyuk et al. [8] also related that rut in complexed with iron (II) was an effective superoxide free radical scavenger. The potentialization of the effects that these flavonoid-metal complexes can have can thus be observed.

Most flavonoids are poorly soluble in water and, similar to lipophilic compounds, tends to accumulate in biological membranes, particularly in lipid rafts, where they can interact with different receptors and signal transducers and influence their functioning through modulation of the lipid-phase behavior [28]. The physical-chemical properties of flavonoid-metal complexes demonstrate that flavonoids carboxyl and hydroxyl groups are responsible for metal chelating ability of these compounds and also the flavonoid rutin-complex (R-Cu₂) used here. The chelation ability could be facilitated in an alkaline environment (pH 10) due to the deprotonation of hydroxyls [28]. The above-described chelation sites were observed on many flavonoid-metal complexes, such as the complex of quercetin with Cu(II) [29], suggesting that the complexation of flavonoids with metal cations can considerably change their lipophilicity and consequently improve interaction with lipid bilayer [30,31,28]. Studies have showed the capacity of flavonoids to participate in intermolecular and intercellular interactions [32,33]. Although our knowledge of transient metal cations involvement in the process is not completely elucidated, studies that shows the biological significance of these flavonoid-metal ion complexes needs to be explored.

Some flavonoids have the capacity to inhibit PLA₂, COX and iNOS enzymes, accounting for their anti-inflammatory mechanism. Also, some flavonoids modulate the expression of pro-inflammatory proteins, such as iNOS and COX-2, at least partially, via inhibition of transcriptional factor activation [34,2]. Therefore, the inhibition of iNOS activity or down-regulation of iNOS expression may be beneficial to reduce the inflammatory response [2]. Our results showed that rutin and R-Cu₂ regulated inhibition of iNOS activity in concentrations above 30mM. Moreover, it can be corroborated that this inhibition is not due to cytotoxicity concentration, since cell viability in this concentration was 90%. The results indicate that Rut in, R-Cu₂, R-Ni₂, and R-Fe are effective inhibitors of LPS-induced NO production in RAW 264.7 cells. These inhibitory effects were accompanied by a decrease in the expression of iNOS and COX-2 protein in a concentration-dependent manner. These data demonstrated that iNOS and COX-2 expression in RAW 264.7 macrophages was suppressed by R-Cu₂. This suppression can be associated with the decrease of p50NF-κB nuclear translocation, as described by Paulino et al. [34] with other related compounds.

Inflammatory mediators such as NO and pro-inflammatory cytokines are involved in host defense mechanisms: their overproduction contributes to the pathogenesis of several inflammatory diseases including bacterial sepsis, rheumatoid arthritis, chronic inflammation, and hepatitis [35- 37]. During infection and inflammation, high production of NO has shown several modulations of gene expression and in some cases to cause DNA damage [38]. There is plenty of evidence that prostaglandins also play an important role as mediators of the inflammatory response. These mediators are constitutively produced by means of cyclooxygenase 1 (COX) [39]. COX has been found in at least two isoforms: COX-1 and COX-2. COX-1 is expressed constitutively in most tissues and is responsible for the homeostatic production of prostaglandins. COX-2 is induced by several stimuli, including inflammatory stimulus, cytokines and growth factors. After expression COX-2 produces large amounts of pro-inflammatory prostaglandins at the inflammation site, and its uncontrolled activity is thought to play an important role in the pathogenesis of many chronic inflammatory diseases [40].

It has been described that in TNF- α or LPS-stimulated cells, such as RAW 264.7, results in phosphorylation and degradation of the inhibitory protein I- κ B, leading to the release of NF- κ B from I- κ B and increasing its translocation into the nucleus [41]. This is the main mechanism involved in NO, PGE₂ production and iNOS, COX-2 expression in RAW 264.7 macrophage cell lines. Our results show that R-Cu₂, R-Ni₂, and R-Fe are more efficient than rutin in decreasing the inflammatory effects induced by different phlogistic agents *In vivo* or during the evaluation of NO production in RAW 264.7. These results are in agreement with data from the expression of COX-2 and iNOS, where treatment with R-Cu₂ significantly reduced the expression of these enzymes.

Besides, studies demonstrate that macrophage express a voltage-dependent K⁺ (Kv) channels and these ionic channels are involved in immune response and antigen presentation. Using the whole-cell patch clamp technique, Wu et al. [42] analysed the electrophysiological and pharmacological properties of voltage-gated potassium channels in primary rat peritoneal macrophages, showing that peritoneal macrophages express several types of functional voltage-gated K(+) channels. Villalonga et al. [43] shown that Kv1.3 and Kv1.5 are highly expressed in activated macrophages and T-effector memory cells of autoimmune disease patients. In mononuclear phagocytes biophysically and pharmacologically response, such as NF κ B activation, NOS and COX2 expression, can be modulate by different Kv1.3/Kv1.5 ratios. In fact, the voltage-dependent potassium channels (Kv) have a crucial function in excitable cells of determining resting membrane potential and controlling action potentials. In addition, they are involved in the activation and proliferation of leukocytes. Blockade of voltage-dependent potassium channels (Kv) by specific antagonists decreases macrophage cytokine production and inhibits proliferation [44] and can be an important target for new drugs to treat inflammation processes, such as asthma and autoimmune disease [45-48]. Bohuslavizki et al. [49] demonstrated that *Ruta graveolens* extract has ability of blocking potassium channel. Also, other natural compounds like Correolide [50] or 8-prenylnaringenin [51] can blocker Kv 1.3 and modulate the response of these cells during the inflammatory response. Thus, we can suppose that rutin and its complex (R-Cu₂) modulate this channel. However further experiments are now underway to confirm this hypothesis.

5. CONCLUSION

Taken together, our results show for the first time that the R-Cu₂ complex, a metal coordinated rutin compound, produces anti-inflammatory effects in mice, at least in part, by

means of increasing the antioxidant activity and inhibition of iNOS and COX-2 gene expression. We suggest that cooper coordinated rut in compound can potentiates some biological properties of this flavonoid and could be more effective for therapeutic treatment of diseases related to oxidative stress. New experiments are now in course to identify whether the NFκB pathway is also involved in the anti-inflammatory effect of Rut in and its metal complexes.

CONSENT

Not applicable.

ETHICAL APPROVAL

The animal experiments were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals from Ethical Committee UNIBAN (protocol nº301_08) and Zimmermann (1983).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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