Molecules **2007**, *12*, 1352-1366

molecules

ISSN 1420-3049

www.mdpi.org/molecules

Full Paper

Synthesis and Characterization of a Metal Complex Containing Naringin and Cu, and its Antioxidant, Antimicrobial, Antiinflammatory and Tumor Cell Cytotoxicity

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Received: 15 May 2007; in revised form: 20 June 2007 / Accepted: 3 July 2007 / Published: 9 July 2007

Abstract: The antioxidant activity of flavonoids is believed to increase when they are coordinated with transition metal ions. However, the literature on this subject is contradictory and the outcome seems to largely depend on the experimental conditions. In order to understand the contribution of the metal coordination and the type of interaction between a flavonoid and the metal ion, in this study a new metal complex of Cu (II) with naringin was synthesized and characterized by FT-IR, UV-VIS, mass spectrometry (ESI-MS/MS), elemental analysis and ^{1}H-NMR. The results of these analyses indicate that the complex has a Cu (II) ion coordinated via positions 4 and 5 of the flavonoid. The antioxidant, anti-inflammatory and antimicrobial activities of this complex were studied and compared with the activity of free naringin. The Naringin–Cu (II) complex **1** showed higher antioxidant, anti-inflammatory and tumor cell cytoxicity activities than free naringin without reducing cell viability.

Keywords: Naringin complexes, flavonoid, anti-inflammatory, antimicrobial and antioxidant activities

Introduction

Flavonoids are a group of polyphenolic compounds found in fruits and vegetables. This class of compounds has received much attention because of their pharmacological activities in the treatment of diseases such as allergy, diabetes *mellitus*, cancer, viral infection inflammations and others [1-3]. Most of these effects are related to enzymatic inhibition, anti-cancer and antioxidant activity, and interference with reactions such as the formation of free radicals [4, 5].

Naringin [the 7-β-neohesperidoside of narigenin (4',5,7-trihydroxiflavananone)] is found in citrus plants and is most abundant in *Citrus paradisi* species [6]. It has antioxidant and anti-inflammatory activity [7-10] and is effective in the *in vitro* inhibition of proliferation of human breast tumors [11].

Transition metal complexes bearing flavonoid ligands have been investigated [12]. Although complexes like rutin-Fe (II) and rutin-Cu (II) have demonstrated higher activity as antioxidants and anti free radical agents than free rutin [12], no complex has been isolated, but rather were characterized *in situ*.

This paper describes the synthesis of a Cu (II) transition metal complex bearing a naringin ligand, and its characterization by NMR, FT-IR, UV-VIS, mass spectrometry and elemental analysis, as well as the study of its anti-inflammatory, antioxidant, antimicrobial and tumor cell cytoxicity compared with the corresponding free flavonoid, naringin.

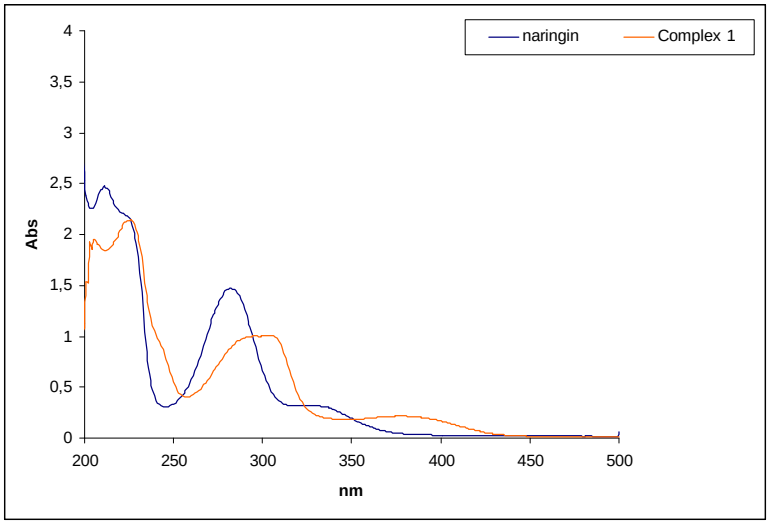
Results and Discussion

Structural Characterization

When naringin dissolved in methanol is allowed to react with a methanol solution of Cu acetate a new compound is formed, which was characterized by UV-VIS and FT-IR spectroscopy. The UV-VIS experiment was carried in pH 6.5 solution using the Femto 800XI spectrometer. Free naringin exhibits an absorption maximum in methanol solution at 282 nm, corresponding to the **A** ring portion, and a very week band at 326 nm, corresponding to the **B** ring portion. Upon binding with the Cu (II) ion, forming complex **1**, the maximum absorption band is shifted to 304 nm and the weak band shifted to 379 nm with respect to the free flavonoid (Figure 1), suggesting an interaction of the Cu (II) ion with the condensed ring of the flavanone in positions 4 and 5. These results are in agreement with the results of others [13-16] who indicate that this band shift is caused by the binding of the metal ion in this position.

The infrared spectrum of free naringin in KBr pellets showed bands at 1645 cm^{-1} (ν_{CO}) and 1298 cm^{-1} ($\nu_{C\text{-}O\text{-}C}$). Upon binding to Cu (II), and forming complex **1,** these bands shifted to 1614 cm^{-1} and 1274 cm^{-1}, respectively, suggesting an interaction of the Cu (II) ion with the condensed ring [15] *via* the carbonyl group, in position 4, and by the oxygen of the hydroxyl group in position 5 (Figure 2A), in agreement with UV-vis data.

Figure 1. UV-VIS spectra of naringin and complex **1**.



The positive ion mode ESI-MS fingerprint of complex **1** in the presence of methanol showed characteristic ions with nominal *m/z* 581, 603, 619, 641, 643, 673, 675, 705, 707, 796, 798, 889, 933, 1183 and 1222. These ions were mass selected and submitted to collision induced dissociation (CID) in order to elucidate their structures. The identified ions are listed in Table 1.

Table 1. Nominal *m/z* of the ions observed in the positive ion mode ESI-MS fingerprint of the NAR/Cu sample and the identified structures.

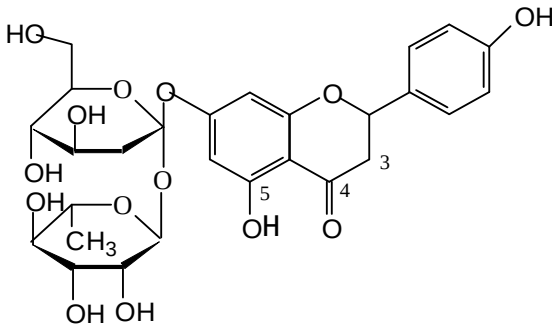
Nominal *m/z*	**Structure**
581	Naringin+H^+
603	Naringin +Na^+
619	Naringin +K^+
641	Naringin -H^++ $^{63}Cu^{2+}$
643	Naringin -H^++ $^{65}Cu^{2+}$
673	Naringin -H^++ $^{63}Cu^{2+}$+ CH_3OH
675	Naringin -H^++ $^{65}Cu^{2+}$+ CH_3OH
705	Naringin -H^++ $^{63}Cu^{2+}$+ $2CH_3OH$
707	Naringin -H^++ $^{65}Cu^{2+}$+ $2CH_3OH$
933	Doubly charged: 3 Naringin - $2H^+$+ 2 $^{63}Cu^{2+}$
1183	2 Naringin + Na^+
1222	Doubly charged: 4 Naringin - $2H^+$+ $^{63}Cu^{2+}$+ Na^+ + K^+

These results suggest that one naringin molecule interacts with one atom of Cu (II) by losing a hydrogen, coordinating preferentially with two molecules of methanol per Cu (II), and having a counter ion (lost during ionization) observed as ions of *m/z* 705 and 707. During the ionization, the methanol adducts are sometimes lost, observed as ions of *m/z* 641, 643, 673 and 675. Adducts of naringin with sodium and potassium are also observed, as these elements are commonly found in natural products, as

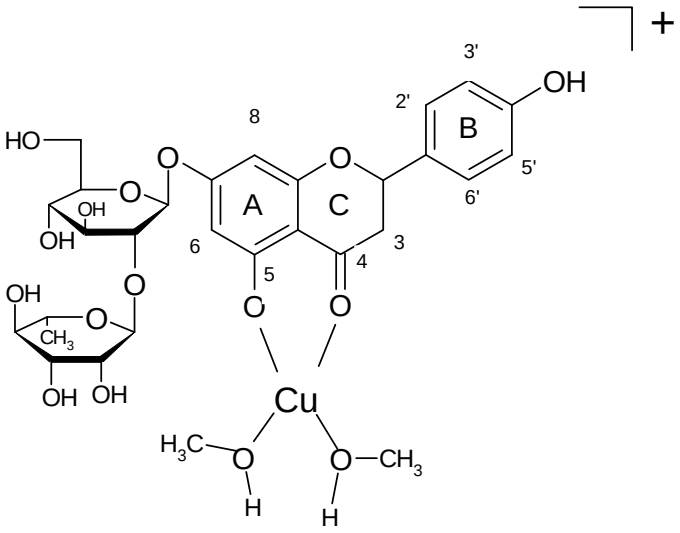
well as naringin + H^+, observed as ions *m/z* 603, 619 and 581, respectively. These are probably present in small proportions but ionize well in the positive ion mode. Clusters containing two or more molecules of naringin are also probably present in solution and can be observed as ions of *m/z* 933, 1183 and 1222.

The elemental analysis (%C 42.50 and % H 5.22) suggests a proportion of 1:1 of naringin/Cu (II) ion. Therefore, in the solid state a species like [Cu (naringin] $^+[CH_3COO]^-\cdot 5H_2O$ can be proposed. When dissolved in methanol, coordination of two molecules of solvent would lead to a square planar geometry, as shown in Figure 2B.

Figure 2. (A) Structure of Naringin; **(B)** Proposed structure for complex **1.**



A



B

Complexation of Cu (II) to naringin was also verified by ^1H NMR, but using DMSO – d_6 as a solvent (Table 2). The proton signals of the naringin shifted to lower frequencies relative to the free flavonoid: coordination with the Cu ion decreases the electron density of the flavonoid. The signal assigned to H_2, H_{3A} and H_{3B} in complex **1** becomes broader when compared to free naringin: coordination would increase the planarity of the flavonoid, therefore decreasing the mobility of the protons, broadening the signals.

When the results obtained by UV-VIS, FT-IR, mass spectrometry, elemental analysis and ^{1}H NMR are taken all together, one structure can be proposed for complex **1** in methanol solution, which is shown in Figure 2B.

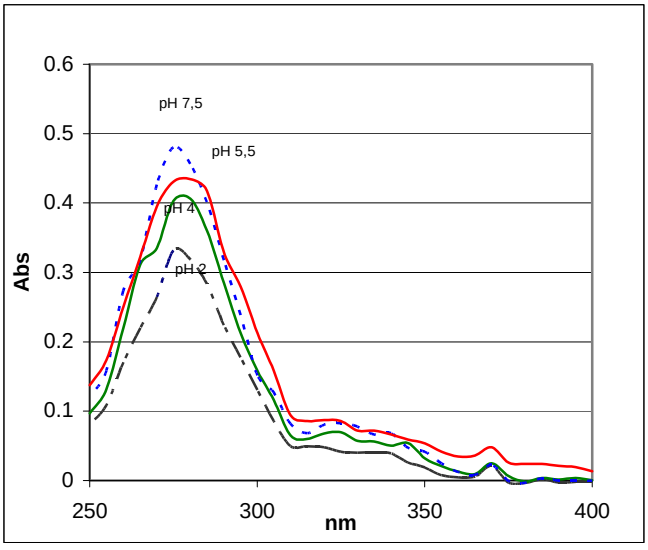
Table 2. NMR data for naringin and complex **1.**

Group	Naringin (**δ, *J***)	Complex **1** (**δ, *J***)
OH	11.88 (s, 4´-OH s,); 9.51 (s, 5-OH)	12.05 (s, 5-OH)
$H_{2'}$,$H_{6'}$	7.17 (d, J = 8.0 Hz)	7.02 (brd, J = 6.6 Hz)
$H_{3'}$,$H_{5'}$	6.64 (d, J = 8.0 Hz)	6.52 (d, J = 7.0 Hz)
H_8	5.94 (d , $J_{H6/H8}$ = 7.0 Hz)	5.78 (d, $J_{H6/H8}$ = 5.5 Hz)
H_6	4.97 (d, $J_{H6/H8}$ = 7.0 Hz)	4.79 (br s)
H_2	5.13 (dd, J_{H2-H3A} = 4.5 Hz)	5.13 (m)
H_{3A}	4.70 (dd, $J_{H3A-H3B}$ = 12.0 Hz; J_{H2-H3A} = 4.5 Hz),	4.70 (brdd, $J_{H3A-H3B}$ = 12.0 Hz; J_{H2-H3A} = 4.5 Hz),
H_{3B}	4.8 (dd, $J_{H3A-H3B}$ = 12.0 Hz; J_{H2-H3A} = 3.0 Hz)	4.8 (ddbr, $J_{H3A-H3B}$ = 12.0 Hz; J_{H2-H3A} = 3.0 Hz)

Complex stability at various pH values

In this experiment, carried in Cary spectrometer, the pH of a solution of complex **1** in methanol (8.9 x10^{-3} mol/L) is 6.5. The absorption maximum in the UV-Vis spectra of complex **1** was monitored at different pH values, from 2.0 to 12, obtained through titration with NaOH (0.01 mol/L) or HCl (0.01 mol/L) solutions. No changes in the absorption maxima were observed. This shows that the changes in the pH of the solution do not lead to the dissociation of the metal from the flavonoid (Figure 3).

Figure 3. UV-Vis spectra of complex 1 at different pH values.



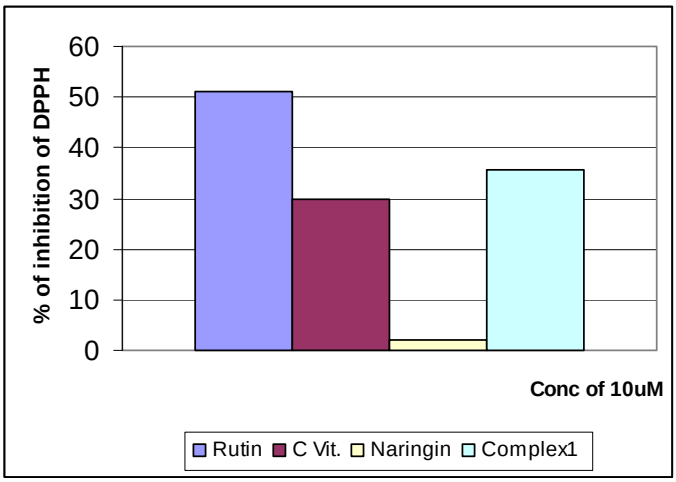
Biological Activity

The coordination of Cu (II) in the 4 and 5 positions promoted the increase of some biological properties of the free flavonoid.

DPPH Radical Scavenging Activity

The antioxidant activity of complex **1** is presented in Figure 4. At the final concentration of 10 μM, complex **1** showed a much stronger DPPH scavenging (35.5%) than free naringin (2%). The positive controls, rutin and vitamin C showed 51% and 30% DPPH scavenging, respectively. Yu et al. [17] compared the antioxidant activity of several flavonoids, resulting in a similar proportion in the activities of rutin and naringin as those found in the present study. The marked antioxidant activity of complex **1**, in comparison to free naringin and other flavonoids, could be due to the coordination of metal in the 4 and 5 positions of the condensed ring system, increasing its capacity to stabilize unpaired electrons and, thereby, to scavenge free radicals.

Figure 4. Inhibition of DPPH by naringin and complexes **1,** whit rutin and C vitamin as control. All compounds were at the final concentration of 10 μM.



Antimicrobial Tests

The plate diffusion method with paper disks gave qualitative results about antimicrobial activity of the complexes. Inhibition zones larger than 6mm indicated antimicrobial activity. The data obtained by the disk diffusion method show that the complexes have no strong antimicrobial activity.

However, in the agar dilution method (a quantitative method) complex **1** showed better efficiency than uncoordinated naringin (Table 3) for *Staphylococcus aureus* (*S. aureus*) ATCC 29213, *Escherichia coli* (*E. coli*) ATCC 25922, *Bacillus cereus* (*B. cereus*) ATCC 11778 and against *Candida albicans* (*C. albicans*). Therefore complex **1** showed an increase in activity in comparison with naringin. Studies of the antibacterial activity of grapefruit extracts and naringin against *S. aureus* [18] showed similar results.

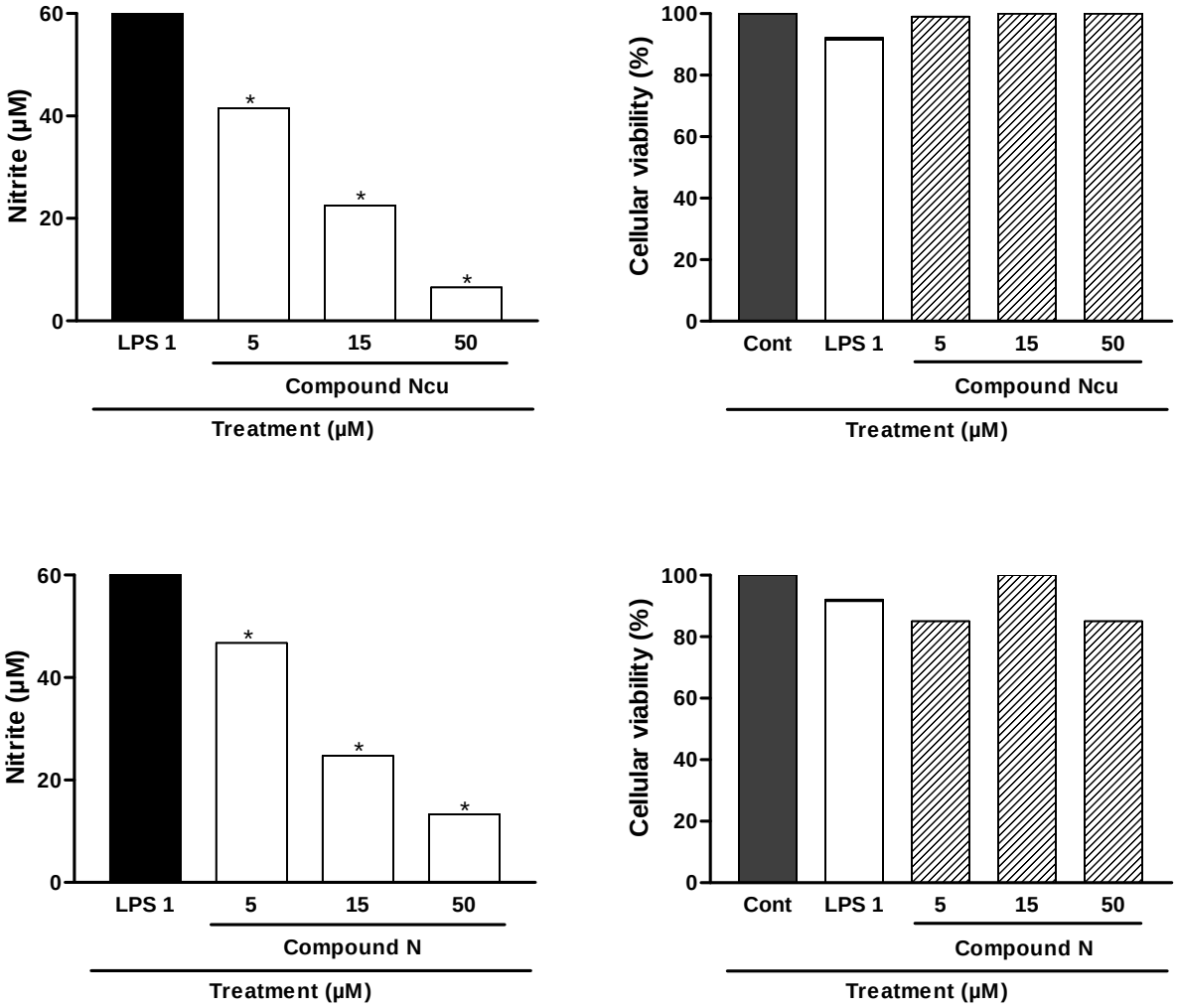
Table 3. Minimum Inhibitory Concentration (MIC) of naringin and complex **1** by the Agar Dilution method.

Compounds	MIC (μg/mL) *S. aureus*	MIC (μg/mL) *E. coli*	MIC (μg/mL) *B. cereus*	MIC (μg/mL) *C. albicans*
Naringin	3000	> 2000	> 1800	1250
Complex **1**	> 1500	> 1500	> 1500	> 100

Anti-Inflammatory test

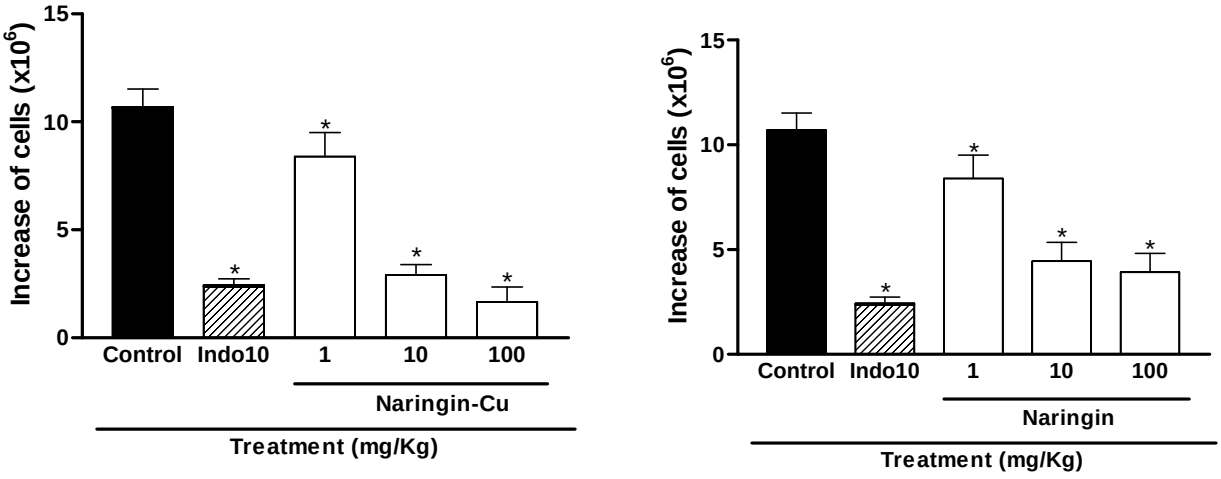
In RAW 264.7 seeded in 96-well plates cultured for two days, and incubated with or without lipopolysacharidide (LPS) in absence or presence of the compounds for 20 hours. The NO concentration decreased in the supernatant of RAW 264.7 macrophages without change the cell viability as shown in Figure 5 in the presence of Complex **1** (NCu) or Naringin (N) respectively.

Figure 5. Effect of treatment with Complex **1** (NCu) or Naringin (N) (5, 15 or 50 μM) on nitrite production by RAW 264.7 cells stimulated with 1 μg/mL LPS for 20 h or on cell viability on MTT assay. Bars represents the mean ± S.E.M. of three independent experiments performed in triplicate and asterisks indicate significant inhibition of enzyme activity in relation to the untreated group, $P < 0.05$.



In another test, naringin showed anti-inflammatory activity against carrageen-induced inflammation (100μg/mL, i.p.) in the peritoneal cavity of mice. The results can be observed in Figure 6. However, at a concentration of 10 mg/kg, complex **1** shows better activity than naringin and the same anti-inflammatory activity than indomethacin.

Figure 6. Effect of the administration of Complex **1** (Naringin-Cu) or Naringin (1-100 mg/kg, i.p. or 10 mg/kg indomethacin, i.p.) in the acute carragenan-induced inflammatory reaction measured by the concentration of cells in the peritoneal fluid. Each value represents the mean ± S.E.M. of 6 animals, and asterisks indicate significant inhibition of total number of cells in the peritoneal cavity in relation to the untreated groups, $P < 0.05$.



The anti-inflammatory activity of complex **1** was evidenced through the decrease of inflammatory cells (neutrophill) migration to the peritoneal cavity compared with the quantity of migratory cells after inflammation induction with carrageen in the control group. The anti-inflammatory activity of complex **1** could be related to the anti-oxidant property of the compound interfering with the oxidative process of the nuclear polymorph.

Several other studies have shown an array of biological activities for flavonoids [1-3] and the results of the tests carried out in the present paper suggest that they may be linked by a common mechanism through their anti-oxidant activity. Further studies will be carried out to confirm this hypothesis.

Tumor cell cytotoxicity

Naringin is known to have antioxidant and cell cycle stimulating activities. In this study, naringin led K562 cells to a first stage of proliferating process in 24 h, followed by a decrease in cell number after 48 h of treatment (Tables 4 and 5), observed both by the MTT or PI methods. The same results were observed in experiments using B16F10 and 3T3 cells (data not shown). Complex **1** at two different concentrations (50 μM and 100 μM) impaired the cell growth in all cell lines and led to cell death after 24 h of treatment (Tables 4 and 5). PI experiments corroborate the results found with the MTT assay; complex **1** promoted three times more death than the free naringin after 24 h of treatment (Table 4). The percentage of cell death after 24 h of treatment, in all lineages, as analyzed by PI, is shown in Table 5.

Although naringin demonstrates a protective effect as well as stimulating a proliferation of all cells lines (3T3, B16F10, K562) in the first 24h; after 48 h it decreases the number of viable cells. Complex **1** led to cell death in the first 24 h in all experiments, as observed by the number of hypodiploid cells.

Table 4. Cell cycle characteristics (% cells) of K562 cells treated for 24h with naringin.

	G_0	$S/G_2/M$	Hypodiploid
Control	**53.90 ± 2.80**	**40.60 ± 3.00**	**5.50 ± 2.90**
Naringin 10μM	56.60 ± 2.80	35.28 ± 1.98	8.12 ± 1.90
Naringin 50 μM	58.10 ± 3.25	32.18 ± 2.90	9.72 ± 2.10
Naringin 100 μM	60.10 ± 3.50	26.70 ± 4.10	13.20 ± 2.10
Naringin 200 *μM*	63.40 ± 1.90	19.70 ± 3.10	16.80 ± 3.60
Complex **1** 50 μM	54.50 ± 4.70	19.05 ± 3.15	27.25 ± 1.90 *
Complex **1** *100 μM*	40.1 ± 3.15	21.5 ± 2.60	38.40 ± 3.20 *

Data are expressed as means ± SD, n = 3. * p < 0, 05 compared to control group

Table 5. Cell death (%) of several cell lines in culture after 24h exposure to naringin.

	3T3	B16F10	K562
Control	5.30 ± 3.10	1.20 ± 0.90	5.50 ± 2.90
Naringin 10μM	4.90 ± 1.30	2.60 ±1.20	8.12 ± 1.90
Naringin 50 μM	6.20 ± 2.10	5.30 ± 2.10	9.72 ± 2.10
Naringin 100 μM	12.5 ± 1.60	9.90 ± 3.80	13.20 ± 2.10
Naringin 200 *μM*	21.05 ± 3.20 *	17.60 ± 4.20 *	16.80 ± 3.60 *
Complex **1** 50 μM	21.6 ± 5.2 *	26.5 ± 4.50 *	27.25 ± 1.90 *
Complex **1** 100 μM	36.5 ± 4.5 *	34.5 ± 3.25 *	38.40 ± 3.20 *

Data are expressed as means ± SD, n = 3. * p< 0,05 compared to control group

Conclusions

A naringin-derived copper (II) complex, **1**, shows higher anti-inflammatory and anti-oxidant activity when compared with the free naringin. All data obtained from the UV-VIS, FT-IR, mass spectrometry and NMR studies suggest that the coordination of Cu (II) is in positions 4 and 5 of the condensed ring of naringin, and the increase in biological activity of complex **1** could be associated to the coordination of Cu (II) in this position.

Experimental

General

Melting points ware determined using a QUIMIS Melting Point Equipment. The IR spectra were recorded in the solid state (KBr pellets) in the range 4000-400 cm^{-1} using a Nicolet FT-IR Nexus spectrometer. The UV-Vis spectra were recorded in methanol solution (pH6.5), in the range 600-200 nm, using a Varian - Cary 50 spectrometer or Femto 800XI spectrometer. The ^1H -NMR were recorded at 298K, on INOVA – 500 Mz Spectrophotometer, in DMSO-d_6. The chemical shifts are referred to DMSO signal at 3.38 ppm. The samples were analyzed by direct infusion ESI by means of a syringe pump (Harvard Apparatus) at a flow rate of 10 µL/min. Positive ion mode ESI–MS fingerprints and positive mode ESI-MS/MS with collision-induced dissociation (CID) were acquired using a hybrid high-resolution and high-accuracy (5 ppm) Micromass Q-TOF mass spectrometer. Capillary and cone voltages were set to 3000 V and 30 V, respectively, with a disolvation temperature of 100°C. Samples were added to a solution containing 70 % (v/v) chromatographic grade methanol (Tedia, Fairfield, OH, USA, 30% (v/v), deionized water and 1 µL of formic acid (Merck, Darmstadt, Germany) per mL. Fingerprint mass spectra were acquired in the range between m/z 100 and 2000 where no ions attributed to the solvents were observed.

Materials

The $Cu[CH_3COO]_2$ salt was purchased from Aldrich; naringin was a gift from Quinabra Company. Methanol (analytical and HPLC grade) were purchased from Merck. The Tript Soy Broth (TBS) was purchased from Merck and the diphenylpicrylhydrazyl radical (DPPH) was purchased from Sigma. The microrganisms tested were *S. aureus* ATCC 2923, *E. coli* ATCC 25922, *B. cereus* ATCC 11778 and *C. albicans* from clinical isolates and *S. mutans* ATCC 25175, obtained from Fundação Tropical de Pesquisas André Tosello. The Agar Nutrient and Agar Sabouraud were purchased from Merck.

Synthesis of Complex **1**

A solution of Cu (II) acetate (0.0249 g, $1.25x10^{-4}$ mol) of in distilled water (2 mL) was slowly added dropwise to a solution of naringin (0.146 g, $2.5x10^{-4}$ mol) in methanol (10 mL). The mixture was stirred for 30 min at room temperature. Complex **1** was filtered in a vacuum system, washed with water and dried by lyofilization. A pure green solid was obtained (0.0595 g, 60 %); m.p. 242 °C; %C (calc); %H (calc) for $CuC_{29}H_{46}O_{21}$: 42.50 (42.98); 5.22 (5.68).

Antimicrobial Activity

The anti-microbiological activity of the complexes was analyzed qualitatively, using the disk diffusion method, and quantitatively using the CLSI agar dilution method (NCCLS M7-A45 (2000). The bacterial and fungal cultures were maintained at –20°C in a solution of TBS (Tript Soy Broth, Merck) 15% of glycerol. The following microorganisms were reactivated in Nutrient Agar (Merck): *S. aureus*,

B. cereus and *E. coli*; Sabouraud Agar (Merck) was used for *C. albicans* and Mitis salivarius agar (DIFCO) for *S. mutans.* For the disk diffusion analysis, 5% solutions in DMSO of complex **1** were prepared.

Anti-Inflammatory Tests

Cell culture

Raw 264.7 cells obtained from the American Type Culture Collection (ATCC, Maryland, USA) were cultured in Dulbecco's Modified Essential Medium (DMEM, endotoxin level < 0.005 EU/mL, Bio Whittaker, Bioproducts, Heidelberg, Germany) with *L*-glutamine (4 mM) and glucose (4.5 g/L), supplemented with 10% heat-inactivated fetal calf serum (Gibco/BRL Life Technologies, Eggenstein, Germany). Cells were maintained at 37^{o}C, 5% CO_2 and used for experiments between passage 4 and 12. Confluent cells were stimulated with 1 µg/mL LPS (E.coli, Serotype 055:B5 Sigma, Deisenhofen, Germany). The compounds were dissolved at the time of the experiments.

Nitrite assay (Griess assay)

RAW 264.7 was seeded in 96-well plates, cultured for two days and then incubated with or without lipopolysacharidide (LPS) in absence or presence of the complex for 20 hours. As a parameter of NO synthesis, nitrite concentration was assessed in the supernatant of RAW 264.7 macrophages by the Griess reaction. Briefly, cell culture supernatant (100 µL) was removed and combined in a 96-well plate with 1% sulfanilamide in 5% H_3PO_4 (90 µL) and 0.1% N-(1-naphthyl)ethylenediamine dihydrocholoride in water (90µL), followed by spectrophotometric measurement at 550 nm using a micro plate reader. Nitrite concentration in the supernatant was determined by comparison with a sodium nitrite standard curve. Experiments were performed in triplicate.

Cell viability (MTT assay)

Cell respiration, an indicator of cell viability, was determined by mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, from Sigma-Aldrich, São Paulo, Brazil) to formazan [25]. After removal of supernatant for nitrite determination, the cells were incubated at 37^{o}C with MTT (0.5 mg/mL) for 45 minutes. The medium was aspirated, and the cells were dissolved in DMSO (250 µL) for 3 h in the dark. The extent of reduction of MTT was quantified by OD measurement (550 nm).

Animals

Non-fasted Swiss male mice (18-35 g) from UNISUL, housed at 22 ± 2ºC under a 12-h light-dark cycle, were used. Food and water were freely available. The animals were acclimatized to the laboratory for at least 1 h 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 pain in conscious animals from Ethical Committee UNISUL and Zimmermann [19].

Acute carrageen-induced inflammatory reaction in the peritoneal cavity of mice

The groups of animals were treated i.p. with naringin or naringin-Cu complex, **1**, (10 or 100 mg/kg), indomethacin (an anti-inflammatory drug, 10 mg/kg) 30 minutes before the inflammation induction by means of carageenan (100 μg/mL, i.p.) (all from Sigma-Aldrich, São Paulo, Brazil). After 4 hours, the peritoneal fluid was collected in sterile and heparinized PBS (1 mL) for evaluation of the total cells and the differential cellular, and for the mieloperoxidase assay (MPO). Cell migration was quantified according to the method described in the literature [20].

Antioxidant Activity

The samples were tested individually at a final concentration of 10 μM. The solution contained 1ml of DPPH (diphenylpicrylhydrazyl radical) (60 μM) and different concentrations of the anti-oxidant solutions of naringin and complex **1**, resulting in a final concentration of DPPH of 30 μM. The mixtures were vigorously mixed and allowed to stand in the dark for 30min at 25°C. The absorbance of the resulting solutions were measured at 517 nm against a blank sample containing only DPPH, the negative control; Rutin and Vitamin C served as the positive control [17,21].

Tumor cell cytotoxicity

Cell culture

Cell lines, including 3T3 (murine fibroblast), B16F10 (murine melanoma) and K562 human chronic myeloid leukemia were obtained from American Type Cell Culture (Rockville, MD,USA). Murine fibroblast cells were cultured in DMEM (Dulbecco's minimal essential medium) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL of penicilin and 100 μg/mL streptomycin sulfate. B16F10 and K562 cells were grown in RPMI 1640 medium, supplemented with 10% FBS, 2 mM glutamine, 100 U/mL of penicilin and 100 μg/mL streptomycin sulfate. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 [22].

Cell viability (MTT assay)

Cells were plated at a density of $3x10^4$ cells/well of murine fibroblast and human chronic myeloid leukemia and $2x10^4$ cells/well of murine melanoma. Ninety-six-well tissue culture plates (Corning, NY) were used. Cells were treated with naringin (10 μM to 500 μM concentrations), bis-naringin copper and nickel complex (50 μM to 200 μM concentrations) for different times intervals (24 h, 48 h, and 72 h). Cell proliferation and viability were determined by MTT (3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide) method [22]. At the end of treatment MTT (0.5 mg/mL) in fresh medium was added. After 4 h of incubation, the blue formazan crystals were dissolved with acid-isopropanol (150

μL) and the absorbance was measured at 570 nm with a ELISA reader (BioTek Instruments, Winooski, Vermont, USA). Experiments were done in triplicate. Unpaired t-test was used to compare the values of the naringin group and the control group. The level of significance was set as $p<0.05$.

Cell cycle analysis

Cell cycle was analyzed by flow cytometry. Cultured 3T3, B16F10 and K562 cells were treated with 10, 50, 100, 200 and 500 μM of naringin, or 50, 100 μM of bisnaringin copper, or nickel complexes for 24 h. Adherent cells ($10^5 mL^{-1}$) were trypsinized and washed with cold PBS and fixed in 70% ethanol at -20°C for at least 1 h. Cells were washed twice with cold PBS, incubated in 0.5 mL of 0.5% Triton X-100/PBS, 1 mg/mL of RNase A at 37°C for 30 min with, and stained with 0.5 mL of 50 mg/mL propidium iodide for 10 min [23]. Fluorescence emitted from the propidium-DNA complex after excitation of the dye was quantitated by FACScan flow cytometry [24]. For each sample, at least 10000 events were acquired and the data were analyzed using appropriate software (CELLQuest, Becton Dickinson, San Jose, CA). Cells in $S/G_2/M$ and G_0/G_1 phases, and number hypodiploid cells were analyzed. Student's *t*-test for each paired experiement was used for statistical analyzes. A *P* value < 0.05 was set for the level of significant difference.

Acknowledgements

Financial support from FAPESP, FINEP and CNPq are gratefully acknowledged.

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Sample Availability: Samples of the complex **1** are available from the author Regina M. S. Pereira.