

Metal Complexes Derived of Diosmin with Biological Activities *in vitro*

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Abstract

The coordination of metal ions with flavonoids is applied to improve its pharmacological properties. To evaluate the role of ions on diosmin new complexes with Fe(II), Cu(II) and Co(II) ions were synthesized and characterized by UV, FT-IR and XRD techniques and surface morphology by SEM. The biological activity of coordination complexes *in vitro*, the antioxidant (ABTS), antibacterial (disc diffusion and MIC) and antitumoral activities (MTT) were analyzed. Diosmin when reacting with Fe(II) at 50°C loses the sugar molecule becoming diosmetin (D) coordinated at 1D:1Fe ratio. In presence of Cu(II) and Co(II) at the same conditions besides losing the sugar, diosmin loses the methyl group at C4' and H at C3', producing a new ligand and complexes at 1D:2Cu or Co ratio, to produce DCu and DCo, respectively. The coordination of Cu and Fe improve the antioxidant activity of diosmin. DCo was the only presented antibacterial activity. Additionally, a specific antitumor effect of diosmin and metal complexes upon human leukemia cells was demonstrated, suggesting an immune regulatory action. The anti-melanoma activity of DCo is 10 times better than diosmin. Metal coordination could be used to improve drug activity and to give direction to a new possibility of clinical use for diosmin.

Keywords: Diosmin, Complex, Antitumor, Antibacterial

1. Introduction

Diosmin is a glycoside flavone found in higher vascular citrus plants such as Meyer Lemons (Oesterle & Wander, 1925). It is one of the polyphenolic complexes known as flavonoids. Flavonoids occur as aglycones, glycosides and methylated derivatives, and have an important role in the growth and development of plants, protecting them from ultraviolet-B (UV-B) radiation, fungal infection and microbial attack (Skaltsa, Vrykoki, Harvala, Karabourniotis, & Maneta, 1994; Grayer & Harborne, 1994; Harborne & Williams, 2000). The basic structure of the flavonoids consists of two aromatic rings (noted A and B) linked through an oxygenated heterocyclic (C) ring. The heterocyclic ring condensed with the benzene ring, is either an alpha-pyrone (flavonols and flavones) or dihydroderivative (flavanols and flavanones) (Corcoran, McKay, & Blumberg, 2012). The position of the substituent in the aromatic rings of diosmin is in C-5, C-4' and C-5' and a double bond in the C2-C3 position, characterizing then as a flavone (Middleton, 1984; Pietta, 2000).

The flavone diosmin presents pharmacological properties including antioxidant, anti-diabetic and anti-inflammatory properties (Tanaka, Kohno, & Mori, 2001; Browning, Walle, & Walle, 2005; Tahir et al., 2013). The antimicrobial effect was also observed for diverse flavonoids against different strains of bacteria, such as *H. pylori*, *S. aureus*, *C. botulinum*, *E. Coli*, *P. aeruginosa* and others (Moon et al., 2013; Ansari et al., 2015). However, neither of these studies demonstrated an antimicrobial effect of diosmin. Its mutagenic property was demonstrated by studies that investigated the effect of diosmin on prostate cancer (Lewinska, Siwak, Rzeszutek, & Wnuk, 2015) and other tumor lines. It was demonstrated that diosmin, at lower concentrations, induces senescence, apoptosis and autophagy in breast cancer cell lines (Lewinska, Adamczyk-Grochala, Kwasniewicz, Deregowska, & Wnuk,

2017). The study of diosmin, rutin and tangeritin activity against melanoma pulmonary B16F10 cells in vivo shows that diosmin presents better activity when compared with the other flavonoids, showing a reduction in the number of metastatic nodules (9.09%), implantation percentage (26.8%), and invasion index (32.28%) compared with the control (Conesa et al., 2005). Others studies observed an anti-proliferative action of diosmin in tumors such as those in the esophagus (Tanaka et al., 1997), colon (Tanaka et al., 1997b), oral (Tanaka et al., 1997c) and bladder (Yang et al. 1997), which were not similar among tumor tissue type and cell lines (Xie, Yuan, Yang, Wang, & Wu, 2009; Naso et al., 2016). Some authors suggest the antitumor mechanism of diosmin action could be associated with its capacity of diminished vein distensibility at a microcirculation level that may control angiogenesis (Lyseng-Williamson & Perry, 2003). The endothelial regulation effect of diosmin has been established by its clinical use (Daflon®) to treat chronic venous insufficiency in legs, hemorrhoids and varices (Jean & Bodinier, 1994). Although diosmin showed low cytotoxic activity against some kinds of cancer cells, these data raised the possibility of using diosmin-based anticancer therapy as an alternative treatment for specific types of tumors.

It was already known metal ions have an important role in biological systems. Further, they also exhibited and potentiated the efficacy of antibacterial, antiparasitic and antitumoral drugs (Fernández et al., 2015; Alcantara, Lozano, Velosa, dos Santos, & Pereira, 2015; Rogolino et al., 2017; Khosravi & Mansouri-Torshizi, 2018). Studies demonstrated flavonoid metal coordination has significantly improved the pharmacological properties of this class of compounds (Gopalakrishnan, Pillai, & Subramanian, 2015; Naso et al., 2016). Our group has, in the last ten years, studied the effect of Zn (II) and Cu(II) ions on the pharmacological properties of flavonoids. Results demonstrated that complexes of rutin with zinc(II) showed higher antioxidant activity and antitumoral activities, as well as less toxicity in vivo (BALB/c mice) compared with free rutin (Ikeda, Novak, Maria, Velosa, & Pereira, 2015). Also, rutin complexed with copper(II) produced an anti-inflammatory effect in mice by increasing the antioxidant activity and inhibition of iNOS and COX-2 gene expression (Miyashiro et al., 2014). Naringin with copper(II), when the ion coordinated via positions 4 and 5 of the flavonoid, showed higher antioxidant, anti-inflammatory and tumor cell cytotoxicity activities than free naringin without reducing cell viability (Pereira et al., 2007). Others authors also demonstrated diosmin zinc complex improved insulin sensitivity in a model of type 2 diabetes Mellitus induced in rats (Gopalakrishnan, Pillai, & Subramanian, 2015). To evaluate the role of ions on diosmin pharmacological properties, in the present study we described the synthesis of new complexes of diosmin with Fe(II), Cu(II) and Co(II) ions, followed by their characterization by FT-IR, UV, XRD, SEM, conductivity and elementary analysis. We also determined the stability of the complexes at different pH levels, and investigated their antioxidant, antibacterial and antitumoral properties in vitro in an attempt to find new possibilities for the clinical use of new complexes.

2. Material and Methods

2.1 Materials

The inorganic salts $[\text{Cu}(\text{CH}_3\text{COO})_2] \cdot \text{H}_2\text{O}$, $[\text{Fe}(\text{SO}_4)] \cdot 7\text{H}_2\text{O}$ and $[\text{Co}(\text{CH}_3\text{COO})_2] \cdot \text{H}_2\text{O}$ and the compound diosmin were purchase with P.A. grade, purchased from Aldrich. Methanol (analytical and HPLC grade) and DMSO were purchased from Merck, while Trolox ((R)-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Aldrich, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid diammonium salt) was from Fluka, and manganese dioxide, sodium phosphate monobasic monohydrate, and sodium phosphate dibasic heptahydrate were purchased from Sigma-Aldrich.

2.2 Analytical Instruments

Melting points were determined using QUIMIS Melting Point Equipment. Infrared (IR) spectra were recorded in the solid state by diffusion reflectance, in the range $4000\text{--}400\text{cm}^{-1}$, using a Nicolet FT-IR Nexus spectrometer. Ultraviolet/visible (UV/Vis) spectra were recorded in a water solution, from 200 to 500nm, using a Femto 800-XI spectrophotometer in concentrations of $2.9\mu\text{M}$ (DCu), $8.4\mu\text{M}$ (DFe), $8.7\mu\text{M}$ (DCo) and $11.5\mu\text{M}$ (diosmin). Analysis with scanning electron micrographs (SEM) was performed with a (Supra 35-VP, Carl Zeiss, Germany). The samples were evenly distributed on SEM specimen stubs with double adhesive tape. The micrographs were obtained with an accelerating potential of 2 kV. Monochromatic Cu K α radiation (wavelength = 1.54056 \AA) was produced by Rigaku-DMAX/2500PC, Japan. The powdery samples were packed tightly in a rectangular aluminum cell prior to exposure to the X-ray beam. The scanning regions of the diffraction angle, 2θ , were $5 - 70^\circ$ and radiation was detected with a proportional detector. We measured pH using a DIGMED with a thermostated glass cell.

2.3 General Procedure for the Synthesis of Complexes

Added 126mg (0.21mmol) of diosmin in 20mL of dimethylformamid (DMF). Four equimolar quantities of each salt solution were solubilized in 10mL of DMF and dropwise added in diosmin solution, according to Pereira et al. (2007), with modifications. The mixture was left under stirring at 50°C for 5 days, until the compound precipitate. The solid was filtered in a vacuum system, washed with methanol, and dried at room temperature. The complexes DCo and DFe were solubilized in hot methanol and afterward recrystallized, while DCu was solubilized in hot water before recrystallization. Compound DCu: yellow solid; 46% yield; mp>300°C; IR (v cm^{-1}): 3030(C-H aromatic ring), 1632(C=O), 1556 and 1528 (C=C), 1421(CH₃), 1264 (C-O), 1056 (O-C-O). Compound DCo: light brown solid; 60% yield; mp>300°C; IR (v cm^{-1}): 2998 (C-H aromatic ring), 1633 (C=O), 1558 (C=C), 1419 and 1350 (CH₃), 1262 (C-O), 1048 (C-O-C). Compound DFe: dark brown solid; 46% yield; mp>300 °C; IR (v cm^{-1}): 2990 C-H aromatic ring), 1627 (C=O), 1521 (C=C), 1422 and 1350 (CH₃), 1269 (C-O), 1077 (C-O-C).

2.4 pH Stability Study of Complexes

Complex stability measurements were carried out in a methanol/PBS solution (40:20) mixture at different pH levels (pH= 3, 5, 7 and 8). The ion dissociation was demonstrated by a band shift in the spectra obtained by a UV spectrophotometer. All experiments were performed at 25°C.

2.5 ABTS Antioxidant Capacity Assay

The antioxidant activity of diosmin was evaluated by ABTS methodology according to Re et al. (1999) and Arts et al. (2004). Briefly, an aqueous ABTS²⁻ 2mM stock solution was prepared in deionized water (18 MΩ cm) and diluted in 20mM phosphate buffer (pH 7.4) to a final 100μM concentration. Aliquots of the diluted solution (5mL) were transferred to test tubes containing 0.0200g of MnO₂ and vortexed for 50s (5 repetitions, 10s each). The suspensions were centrifuged and the supernatants filtered in a 0.22μm polyvinyl difluoride (PVDF) syringe filter to remove residual manganese dioxide. Absorbance (at 734 nm) of the filtrate was adjusted to 0.90 (± 0.04), which corresponded to a final concentration of [ABTS^{•-}] = 60μM ($\epsilon_{734} = 1.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).

Kinetic traces of the reaction of ABTS^{•-} radicals with the complexes under investigation were obtained spectrophotometrically (monitoring at 734nm) using a Femto 800 XI spectrophotometer. Experiments were performed at room temperature (23°C) by rapidly adding 2mL of ABTS^{•-} (60μM), in ordinary 3mL cuvettes (path length = 1 cm) containing 10μL of methanolic complexes solutions, at different concentrations. To determine the amount (in concentration) of ABTS^{•-} radicals reduced in each experiment, the absorbance value at the plateau of each trace was subtracted from the absorbance value at time zero (i.e., 0.74 as reference absorbance), and the result was divided by the extinction coefficient of ABTS^{•-} ($\epsilon = 1.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$). The concentrations of reduced ABTS^{•-} radicals were plotted against the concentrations of each compound tested in order to determine the stoichiometric factor (n) a parameter represents the number of ABTS^{•-} radicals reduced per antioxidant molecule. For each plot, n corresponds to the slope obtained by linear regression of the data points. n values were expressed as means of duplicates \pm SD.

Trolox equivalent antioxidant capacity (TEAC) values were calculated by dividing the n values of the complexes under investigation by n stoichiometric factors for trolox ($n_{\text{trolox}} = 1.9$), which were the same values reported by others (Arts, Dallinga, Voss, Haenen, & Bast, 2004).

2.6 Antimicrobial Activity of Diosmin and Complex

The antibacterial activity was assayed against *Staphylococcus aureus* ATCC6538 (*S. aureus* - gram positive) and *Escherichia coli* ATCC9637 (*E. coli* - gram negative), by the standard disc diffusion method. The compounds, dissolved in dimethyl sulfoxide (10mg/mL), were used to impregnate 6 mm filter paper discs (Laborclin) with 400, 300 and 200 μg of each compound. The discs could remain at room temperature until complete diluent evaporation and placed onto the surface of Mueller-Hinton (Merck) agar plates which had been previously inoculated with each bacterium. Commercial tetracycline 30μg disc (Sensifar) was used as a control. Plates were incubated at 37°C for 24 hours. Zone sizes were measured and recorded in millimeters. Tests were performed in duplicate.

For the determination of minimal inhibitory concentration (MIC), cells (10^6 CFU/ml) were inoculated into Mueller Hinton (MH) broth and dispensed at 200 μL/well in 96-well microliter plates. MICs were determined by dilution of the test compound in MH broth. The bacterial broth was incubated for 18 h at 37°C and inhibition of bacterial growth was examined visually. Bacterial growth in each well was assayed by absorption at 620 nm using a spectrophotometer LT4000 microplate reader (LABTECH). The percentage of growth in each well was calculated as the optical density (OD) of each well divided by the OD of the drug-free well after subtracting the background OD obtained from microorganism-free microliter plates. For the tested compounds, MIC was defined as the lowest

concentration of antibacterial, which resulted in $\geq 90\%$ inhibition of growth compared with that of the drug-free control. MIC determination against *Staphylococcus aureus* (ATCC6538) and *Escherichia coli* (ATCC9637).

2.7 Antitumoral Effect of Diosmin and Complexes

2.7.1 Cell Culture

Human Leukemia (Jurkat – code: 0125), murine melanoma (B16F10 – code: 0046), human lymphoma (Raji – code: 0211) and human breast (MCF-7- code: 0162) tumor cell lines from cell collections - Rio de Janeiro (BCRJ). All lineages were cultivated in an 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°C and 5% of CO₂. Peripheral blood mononuclear cells (PBMC) were isolated from one healthy donor, by ficollpaqueTM PLUS (GE) gradient after centrifugation. The mononuclear ring was cultured with RPMI medium as described above.

2.7.2 MTT - Cell Viability Assay

MTT (thiazolyl blue tetrazolium bromide – Sigma-Aldrich) was used to measure cell viability (Mosmann, 1983). Cells were seeded onto 96-well plates at a density of 1x10⁴ cells/well (MCF-7 and B16F10) and 1x10⁵ cells/well (Jurkat and PBMC), and incubated (37°C, 5% CO₂) overnight. Then complexes dissolved in DMSO were added and the plates were incubated for 24h. The final concentration of diosmin and DCu, DCo and DFe were from 40 to 800 μ M. After 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 540 nm using a microplate reader (Labtech LT-4000 MS) and directly correlates with the number of viable cells in the culture. Results were expressed as IC₅₀ and means the concentration of an inhibitor where half reduced cell viability. All tests were made in triplicate.

3. Results and Discussion

3.1 Synthesis and Characterization and Complexes Stability at Various pH Values

The diosmin complexes DCu, DCo and DFe were synthesized by a molar ratio of 1/4 of diosmin/metal salt. The complexes were characterized by UV, FTIR, XRD, SEM, conductivity and elemental analysis. All melting points were higher than 300°C and therefore could not be determined. The molar conductance values of all complexes obtained in DMF at 25°C and analytical data are given in Table 1. The result indicated the complexes are ionic, with conductivity higher than 54 ohm⁻¹cm²mol⁻¹ (Abd El-Wahaba, Mashalyb, Salmana, El-Shetaryb, Faheima, 2004). The complexes showed different morphology and color when compared with diosmin (Figure 1).

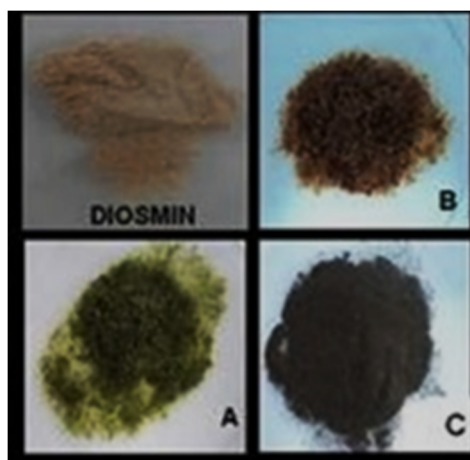


Figure 1. Photographs showing the morphologies and color change of diosmin and after coordination of metal (A) DCu, (B) DCo and (C) DFe

The results of elemental analysis indicated diosmin hydrolyses and loses the sugar molecule becoming diosmetin under reaction conditions in the presence of iron ion producing a complex in a proportion of one iron ion to one diosmetin (DFe). However, diosmin also loses methyl group at C4' and H at C3' producing a new ligand, (C₁₅H₇O₆)²⁻, when interacting with ions Cu and Co producing DCu and DCo, respectively (Table 1 and Figure 2).

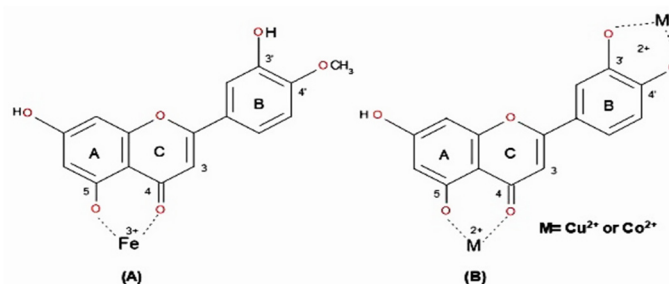


Figure 2. Coordination site of metal ions interactions in DCu, DCo and DFe complexes

Table 1. Analytical and molar conductance data for the complexes DFe, DCo and DCu

Compound	Calc (%C / %H)	Exp (%C / %H)	Molecular formula	Conductivity Ω (ohm ⁻¹ cm ² mol ⁻¹)
DFe	39.4 / 3.3	39.4 / 4.9	C ₁₆ H ₁₂ O ₆ Fe(SO ₄)(H ₂ O) ₂	109
DCu	33.3 / 4.3	32.8 / 3.8	[C ₁₅ H ₇ O ₆ Cu ₂ (H ₂ O) ₈](CH ₃ COO)	68
DCo	31.9 / 4.7	31.1 / 4.4	[C ₁₅ H ₇ O ₆ Co ₂ (H ₂ O) ₈](CH ₃ COO).2H ₂ O	62

The electronic spectra of diosmin exhibit two intensive absorption bands originated from $\pi-\pi^*$ transitions; the band at 342nm (Band I) is due to the electronic transitions on cinnamoyl system (B ring) and the band at 262nm (band II) assigned to electronic transitions on benzoyl system (A ring) (Qi, Jiang, Cui, Zhao, & Liu, 2015). UV-Vis data for ligand and complexes are presented in Table 2. Comparing the UV spectra of diosmin with the new compounds, a shift of the two characteristic bands were observed. Bathochromic shifts from 262 nm to 281 and to 268nm were observed for DCu and DCo, respectively. A hypsochromic shift to 253nm was observed for DFe and hypsochromic shift at 342nm of diosmin was observed for the three complexes, Table 2. These effects could be associated with the interaction of the metal itself, which decreased the ring conjugation, and consequently, the electronic density over B and C rings (Qi, Jiang, Cui, Zhao, & Liu, 2015).

Table 2. Electronic Spectra at 25°C of diosmin, DCu, DCo and Dfe

Compounds	λ (cm ⁻¹)	
	ring A	ring B
Diosmin	262 nm	342 nm
DCu	281 nm	331 nm
DFe	253 nm	333 nm
DCo	268 nm	327 nm

The study of DCu, DCo and DFe stability in different pH ranges (3.0 to 8.0) was monitored spectrophotometrically. The UV spectra were done in a methanol/PBS (40:20) solution at 25°C (Figure 3). In this experiment, no change in the absorption maxima were observed. This shows changes in the pH of the solution do not lead to the dissociation of the metal from the flavone for all complexes.

IR spectra of the ligand and the complexes bring evidences of coordination of metal ions. The spectra signal of complexes DCu, DCo and DFe are very broad indicating the presence of metal ions (see supplementary material) and showed significant shift of principal bands (Table 3). The $\nu_{C=O}$ shifted to lower frequencies, approximately 30 cm⁻¹, indicating coordination of metal ions at positions 4 and 5 of C ring. Usually the most acidic hydroxyl group in a flavonoid molecule is at positions 3 or 5. The hydroxyl at position 5 is the most acid, so it is the best site for the coordination of metal ions (Gopalakrishnan & Pillai, 2015). A significant shift is also observed for $\nu_{C=C}$ of C2=C3 bond (1610cm⁻¹). However, for the band of C-O-C stretching model, only a small shift was observed for all complexes, indicating that this group did not participate of the coordination with the metals. A very broad band between 1100 and 1000cm⁻¹ characteristic of SO₄²⁻ ion presence was also observed to DFe (Table 3). To acetate ion the signals in the IR analysis are observed as three principal bands: ν_{C-H} (3320cm⁻¹), $\nu_{C=O}$ (1600cm⁻¹) and ν_{C-C} (1500cm⁻¹). However, in the complexes DCu and DCo the acetate signals could not be observed separately from ligand signals because the molecule contains the same kind of bond (C=O, C-H, C-C).

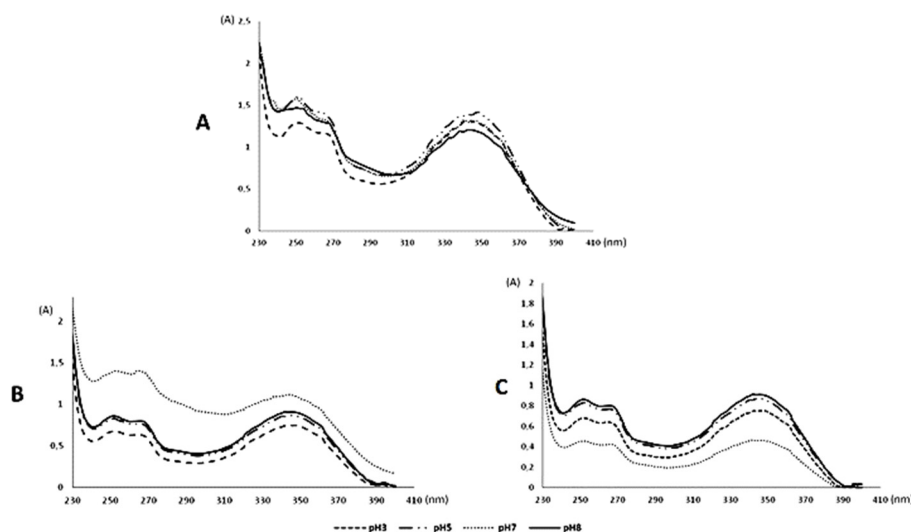


Figure 3. Study of stability by UV-vis spectrum of complexes DCu (A), DFe (B), DCo (C) in different pH solutions

Table 3. Data of IR spectra of diosmin, DCu, DFe and DCo

Compounds	$\nu_{C=O}$ cm^{-1} (A)	$\nu_{C=C}$ cm^{-1} (A)	ν_{C-O-C} cm^{-1} (A) Asymmetric	$\nu_{SO_4^{2-}}$ cm^{-1} (A) stretch
Diosmin	1660	1610;1592,1567	1262	-----
DCu	1632 (28)	1556; 1528 (54)	1264 (02)	-----
DFe	1627 (33)	1521 (88)	1268 (06)	1096
DCo	1638 (22)	1558 (52)	1258 (04)	-----

Several attempts have been made to obtain spectra of ^1H NMR of DCu, DCo, DFe in DMSO, at room temperature. However, because of low solubility in DMSO or others organic solvents and the paramagnetic characteristics due unpaired electrons of Fe(III), Co(II) and Cu(II) octahedral complexes configurations (d_5 , d_7 and d_9), respectively, the NMR signals were very broad, and no reliable data could be obtained from these spectra.

The powder X-ray diffraction patterns of diosmin, DCu, DCo and DFe complex are shown in Figure 4. The powder diffraction pattern of diosmin displayed sharp crystalline peaks, characteristic of a crystalline organic molecule (Rice-Evans, Miller, & Paganga, 1996). The DCu, DCo and DFe complexes showed amorphous property lacking crystalline peaks compared with Diosmin. This suggests the new ligands in the DCu, DCo and DFe complexes were molecularly dispersed (Rice-Evans, Miller, & Paganga, 1996; Wang, Cao, Sun, & Wang, 2011).

The coordination of the diosmin derivative, [diosmetin] $^-$ or $(\text{C}_{16}\text{H}_{12}\text{O}_6)^-$ and $(\text{C}_{15}\text{H}_6\text{O}_6)^{2-}$, to metal ions could be confirmed by XRD and SEM. The XRD and showed very amorphous material while the diosmin is a crystalline material. The microscopy of the complexes presented lamellar structures in plates and diosmin occurs as irregular crystal. Altogether our results showed DFe coordinated in a ratio of one [diosmetin] $^-$ to one ion Fe^{+3} , with molecular formula $\text{C}_{16}\text{H}_{12}\text{O}_6\text{Fe}$ plus two molecules of water and SO_4^{2-} ion. The coordination probably occurs at the C4-C5 position, more acid site of coordination (Figure 2). Elemental analysis suggests a ratio of one ligand to two metal ions for DCu and DCo. However, in these cases, diosmetin loses the methyl group at C4' position and deprotonates at C3' position (Figure 2).

The surface morphology of the complexes was examined by scanning electron microscope, SEM (Figure 5). SEM indicates DCu, DCo and DFe appeared as amorphous material while diosmin occurs as irregular crystals. The structure of the DFe complex presented lamellar structures in plates of different sizes. The complexes appeared as plates shapes in which the original morphology of diosmin disappeared.

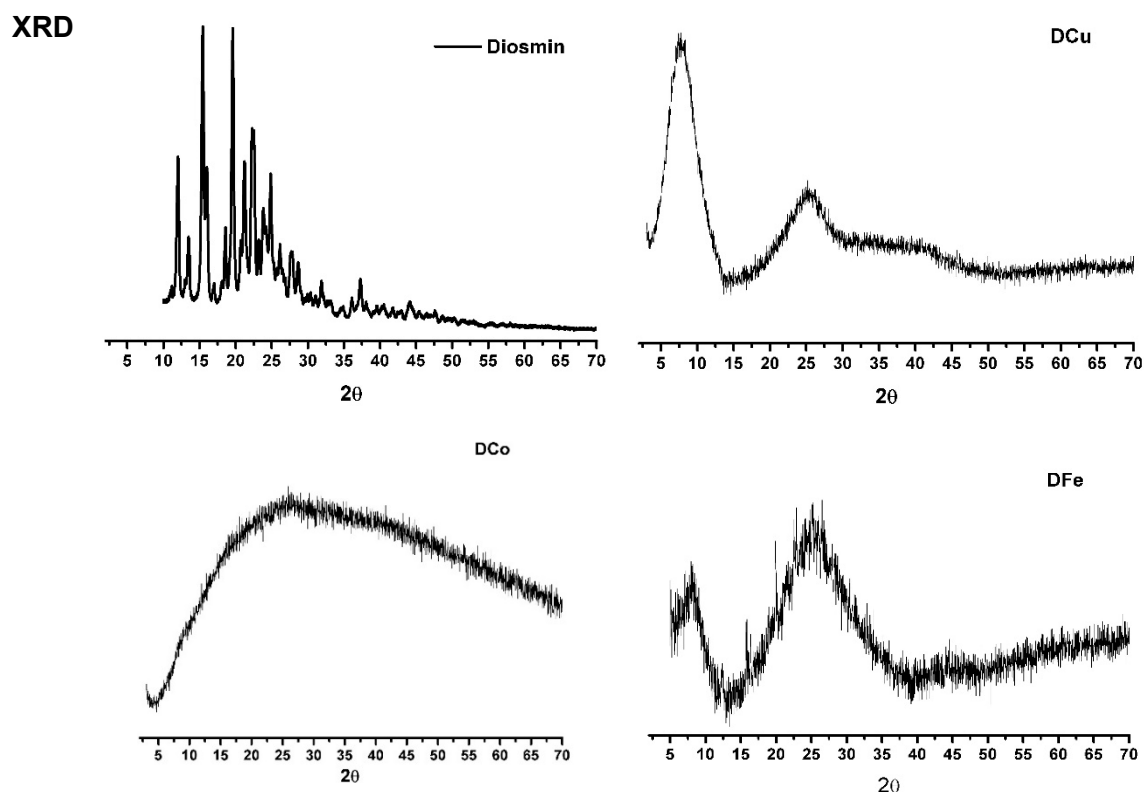


Figure 4. X-ray diffraction patterns of diosmin, DCu, DCo, DFe

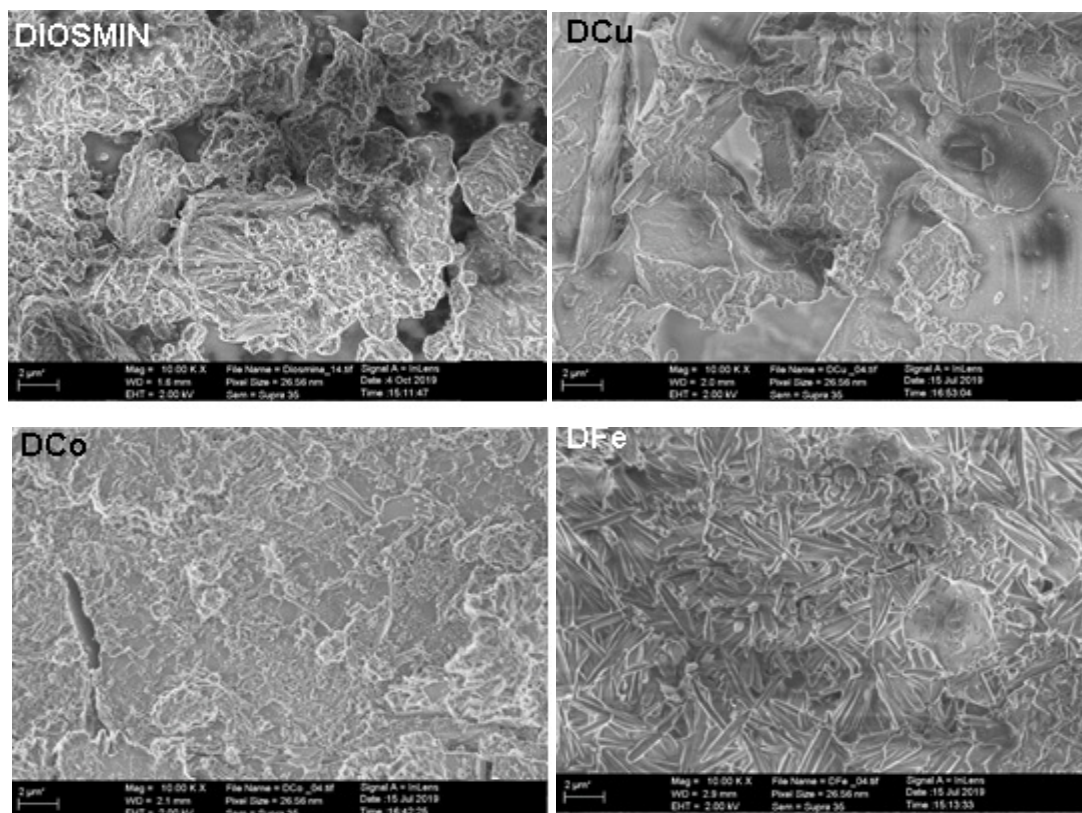


Figure 5. Scanning electron micrographs of diosmin, DCu, DCo, DFe. Magnitud: 10.000X

3.2 ABTS Antioxidant Capacity Assay

The antioxidant effect of diosmin complexes demonstrated that in comparison with diosmin alone (TEAC diosmin = 1.3), only DCu had higher antioxidant capacity (TEACDCu = 1.5), this complex presented only 2.5 times less antioxidant activity than quercetin, which is one of the best antioxidant flavonoids (Korbanjhon & Chungang, 2013). These results showed the coordination with Cu(II) lead to an increase activity of diosmin, like other coordinated flavonoids (Pereira et al., 2007; Gopalakrishnan, Pillai, & Subramanian, 2015), probably because of the metal capacity to stabilize oxidized ligands, and the metal ion plays the role of an additional radical scavenging center. On the other hand, the activity of DCo was similar to observed for diosmin alone, demonstrating that in this case, the coordination did not improve the antioxidant properties of diosmin (Table 4).

Table 4. Trolox equivalent antioxidant capacity (TEAC) of diosmin and DCu, DFe and DCo complexes

Compounds	N	TEAC
Trolox	1.9	1.0
Diosmin	2.5 ± 0.8	1.3 ± 0.4
DCu	2.8 ± 0.2	1.5 ± 0.1
DFe	2.0 ± 0.4	1.0 ± 0.2
DCo	2.1 ± 0.1	1.1 ± 0.1

3.3 Antibacterial Activity of Diosmin and Complexes

The antibacterial activity of DCo, DCu and DFe were evaluated against *E. coli* and *S. aureus* was detected. DCo was the only complex inhibited the growth of bacterial strains tested (Table 5). The minimum inhibitory concentration (MIC) of DCo against *S. aureus* and *E. coli* strains was determined and the analysis proved a bacteriostatic effect with a MIC value of 388µM and 274µM for *S.aureus* and for *E. coli*, respectively (Table 5).

Table 5. Antibacterial activity of diosmin and DCu, DFe and DCo complexes

	<i>S. aureus</i>				<i>E. coli</i>		
	30µg	200µg	300µg	400µg	200µg	300µg	400µg
Control*	+++						
Diosmin	-	-	-	-	-	-	-
DCu	-	-	-	-	-	-	-
DFe	-	-	-	-	-	-	-
DCo	-	++	++	-	++	++	++
Co(CH ₃ COO) ₂	-	-	-	-	-	-	-
MIC of DCo		388µM			274µM		

According to other authors, metal ions could interact with biological ligands such as protein, enzyme and membrane, and could be used as anti-microbial agents, including multi-drug resistant bacteria (Chan et al., 2013). For example, cobalt nanoparticules significantly induced cell death with the generation of reactive oxygen species and the release of TNF-alpha (Chattopadhyay et al., 2015). The quinolone, sparfloxacin, when complexed with cobalt, shows better activity than free quinolone against *X. campestris* but not against *E. coli*, *S. aureus* and *B. subtilis* (Kouris, Kalogiannis, Perdih, Turel, & Psomas, 2016).

Studies demonstrated a relationship between structure of flavonols substitution and antibacterial activity. Hydroxyl substitution at C4' position increase antibacterial activity, while substitution at C3' or C5' decrease the activity, showing that the position of the substituent group on ship of the flavonoid could determinate molecule activity (Wu, Zang, He, Pan, & Xu, 2013; Kouris, Kalogiannis, Perdih, Turel, & Psomas, 2016). Diosmetin is a flavone with 3'-OH and 5'-OH and show a MIC against *S. aureus* strains of > 426.3µM (Chan et al., 2013). Here, when diosmetin was coordinated with cobalt through C3-C4 position the new compound exhibited better antibacterial activity (Table 5).

3.4 Antitumoral Effect of Diosmin and Complexes

This study revealed diosmin and metal complexes had an antitumoral activity on human leukemia and lymphoma cells from Jurkat and Raji lines, suggesting an immune regulatory action of diosmin, not yet investigated (Table 6). It was shown diosmin alone was approximately 1.2 times more toxic to tumor cells than normal lymphocytes, demonstrating a tumor specificity effect, which was potentiated by diosmin on complexation with iron Fe(III)

(11.2 times for leukemia and 3.3 for lymphoma cells) (Table 6). On the other hand, results showed that diosmin alone had a low cytotoxicity to murine melanoma and breast tumor cell lines *in vitro*, showing a tumor cell type specific to diosmin cytotoxicity, as already suggested by others (Lewinska, Siwak, Rzeszutek, & Wnuk, 2013).

Table 6. New Antitumoral activity (IC₅₀μM) of diosmin and DCu, DCo and DFe complexes

Compounds	PBMC	Jurkat	Raji	B16F10	MCF-7
Diosmin	138± 57	112± 4	127± 4	1264± 219	1142± 292
DCu	161± 17	99± 0	115± 1	484± 80	455±15
DFe	1134± 436	101± 2	346±19	1530± 398	3354± 785
DCo	409±164	199± 38	194± 4	124 ± 7	546±40

Regarding the overall analysis of metal coordination, it was demonstrated that DCu presented significantly higher activity (5.9, 1.3 and 4.7 times more) when compared with diosmin alone, against all tested tumor cell lines B16F10, Jurkat and MCF-7. The two great differences between diosmin alone and complexes were demonstrated by the DFe treatment, which was less toxic against normal lymphocytes (PBMC) when compared to tumoral lymphocyte, showing a promising low toxicity compound to treat hematological malignances (Table 6). Additionally, diosmin alone and DCo have potentiated the anti-murine melanoma activity 10 times, showing that ion coordination could be used to improve drug activity (Table 6). Interestingly, DCo was the only compound that exhibited, significantly, no specific activity against Gram positive and negative bacteria. These results show that the coordination of diosmin with metal ions improved antitumoral and antibacterial activity of this important flavonoid. The copper, cobalt and nickel acetate and iron sulfate salts did not present anti tumoral activity against B16F10, SK-mel, MCF-7, Jurkat or Raji cells lines.

4. Conclusions

The synthesis and interaction of metal ions with new ligands, diosmetin and (C₁₅H₇O₆)²⁻, were suggested by the shift of IR bands and elementary analysis. DFe, DCo and DCu were formed via non-covalent bonds in the complex molecularly dispersed, as showed by XRD and SEM analysis. The interaction of metal ions leads to an increase of diosmin antioxidant and antitumoral activities. Moreover, DCo was the only complex that presented a significantly bacteriostatic activity against *E. coli* and *S. aureus* strain. The anti-melanoma activity of DCo is 10 times higher than diosmin. Metal coordination could be used to improve drug activity and to give direction to a new possibility of clinical use for diosmin.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- Abd El-Wahaba, Z. H., Mashalyb, M. M., Salmana, A. A., El-Shetaryb, B. A., & Faheima, A. A. (2004). Co(II), Ce(III) and UO₂(VI) bisalicylatothiosemicarbazide complexes binary and ternary complexes, thermal studies and antimicrobial activity. *Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy*, 60(12), 2861-2873.
- Alcantara, F. C., Lozano, V. F., Velosa, A. S. V., dos Santos, M. R. M., & Pereira, R. M. S. (2015). New Coumarin complexes of Zn, Cu, Ni and Fe with antiparasitic activity. *Polyhedron*, 101, 165-170.
- Ansari, J. A., Naz, S., Tara, O. M., Siddiqi, R., Haider, M. S., & Jamil, K. (2015). Binding effect of proline-rich proteins (PRPs) on in vitro antimicrobial activity of the flavonoids. *Brazilian Journal of Microbiology*, 46(1), 183-188.
- Arts, M. J. T. J., Dallinga, J. S., Voss, H. P., Haenen, G. R. M. M., & Bast, A. (2004). A new approach to assess the total antioxidant capacity using the TEAC assay. *Food Chemistry*, 88, 567-570.
- Browning, A. M., Walle, U. K., & Walle, T. (2005). Flavonoid glycosides inhibit oral cancer cell proliferation - role of cellular uptake and hydrolysis to the aglycones. *The Journal of Pharmacy and Pharmacology*, 57(8), 1037-1041.

- Chan, B. C., Ip, M., Gong, H., Lui, S. L., See, R. H., Jolival, C., Fung, K. P., Leung, P. C., Reiner, N. E., & Lau, C. B. (2013). Synergistic effects of diosmetin with erythromycin against ABC transporter over-expressed methicillin-resistant *Staphylococcus aureus* (MRSA) RN4220/pUL5054 and inhibition of MRSA pyruvate kinase. *Phytomedicine*, 20(7), 611-614.
- Chattopadhyay, S., Dash, S. K., Tripathy, S., Das, B., Mandal, D., Pramanik, P., & Roy, S. (2015). Toxicity of cobalt oxide nanoparticles to normal cells; an in vitro and in vivo study. *Chemico-Biological Interactions*, 226, 58-71.
- Conesa, C. M., Ortega, V. V., Gascón, M. J. Y., Baños, M. A., Jordana, M. C., Benavente-García, O., & Castillo, J. (2005). Treatment of metastatic melanoma, B16F10 by the flavonoids tangeretin, rutin and diosmin. *Journal of Agricultural and Food Chemistry*, 53(17), 6791-6797.
- Corcoran, M. M. P., McKay, D. L., & Blumberg, J. B. (2012). Flavonoid basic: Chemistry, sources, mechanisms of action, and safety. *Journal of Nutrition in Gerontology and Geriatric*, 31(3), 176-189.
- Fernández, M., Arce, E. R., Sarniguet, C., Morais, T. S., Tomaz, A. I., Azar, C. O., Figueroa, R., Diego Maya, J., Medeiros, A., Comini, M., Garcia, M. H., Otero, L., & Gambino, D. (2015). Novel ruthenium (II) cyclopentadienyl thiosemicarbazone complexes with antiproliferative activity on pathogenic trypanosomatid parasites. *Journal of Inorganic Biochemistry*, 153, 306-314.
- Gopalakrishnan, V., Pillai, S. I., & Subramanian, S. P. (2015). Synthesis, spectral characterization, and biochemical evaluation of antidiabetic properties of a new zinc-diosmin complex studied in high fat diet fed-low dose streptozotocin induced experimental type 2 diabetes in rats. *Biochemistry Research International*, 350829, 1-11.
- Grayer, R. J., & Harborne, J. B. (1994). A survey of antifungal complexes from higher plants 1982-1993. *Phytochemistry*, 37, 19-42.
- Gugala, N., Lemire, J. A., & Turner, R. J. (2017). The efficacy of different anti-microbial metals at preventing the formation of, and eradicating bacterial biofilms of pathogenic indicator strains. *Journal of Antibiotics*, 70(6), 775-780.
- Harborne, J. B., & Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry*, 55(6), 481-504.
- Ikeda, N. E., Novak, E., Maria, D. A., Velosa, A. S. V., & Pereira, R. M. S. (2015). Synthesis, characterization and biological evaluation of Rutin-zinc (II) flavonoid-metal complex. *Chemico-Biological Interactions*, 239, 184-191.
- Jean, T., & Bodinier, M. C. (1994). Mediators involved in inflammation: Effects of Daflon 500 mg on their release. *Angiology*, 45, 554-559.
- Khosravi, F., & Mansouri-Torshizi, H. (2018). Antibacterial combination therapy using Co^{3+} , Cu^{2+} , Zn^{2+} and Pd^{2+} complexes. Their Calf Thymus DNA binding studies. *Journal of Biomolecular Structure and Dynamics*, 36(2), 512-531.
- Korbanjhon, B., & Chungang, C. (2013). Physicochemical Properties of Diosmetin and Lecithin Complex. *Tropical Journal of Pharmaceutical Research*, 12(4), 453-456.
- Kouris, E., Kalogiannis, S., Perdihi, F., Turel, I., & Psomas, G. (2016). Cobalt (II) complexes of sparfloxacin: Characterization, structure, antimicrobial activity and interaction with DNA and albumins. *Journal of Inorganic Biochemistry*, 163, 18-27.
- Lewinska, A., Adamczyk-Grochala, J., Kwasniewicz, E., Deregowska, A., & Wnuk, M. (2017). Diosmin-induced senescence, apoptosis and autophagy in breast cancer cells of different p53 status and ERK activity. *Toxicology Letters*, 265, 117-130.
- Lewinska, A., Siwak, J., Rzesutek, I., & Wnuk, M. (2015). Diosmin induces genotoxicity and apoptosis in DU145 prostate cancer cell line. *Toxicology In Vitro*, 29(3), 417-425.
- Lyseng-Williamson, K. A., & Perry, C. M. (2003). Micronised purified flavonoid fraction: A review of its use in chronic venous insufficiency, venous ulcers and haemorrhoids. *Drugs*, 63(1), 71-100.
- Middleton, E. (1984). The Flavonoids. *Trends in Pharmacological Science*, 5, 335-338.
- Miyashiro, C. A. H. V., Diniz, S. N., Oliveira, D. A. F., Gonçalves, I. D., Pereira, R. M. S., Silva, R. G., Paulino, N., & Okuyama, C. E. (2014). The potentiation of anti-inflammatory effect and INOS and COX-2 gene

- expression inhibition by rutin when complexed with cooper. *British Journal Medicine & Medical Research*, 4(25), 4289-4309.
- Moon, S. H., Lee, J. H., Kim, K. T., Park, Y. S., Nah, S. Y., Ahn, D. U., & Paik, H. D. (2013). Antimicrobial effect of 7-o-butylnaringenin, a novel flavonoid, and various natural flavonoids against helicobacter pylori strains. *International Journal of Environmental Research and Public Health*, 10(11), 5459-5546.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65(1-2), 55-63.
- Naso, L., Martínez, V. R., Lezama, L., Salada, C., Valcarcecl, M., Ferrer, E. G., & Williams, P. A. M. (2016). Antioxidant, anticancer and mechanistic studies of the flavone glycoside diosmin and its oxidovanadium(IV) complex. Interactions with bovine serum albumin. *Bioorganic & Medicinal Chemistry*, 24(18), 4108-4119.
- Oesterle, O. A., & Wander, G. (1925). Naturally occurring flavonic glycoside, rhamnoglycoside of diosmetin, q. v. Isolation various plant sources. *Helvetica Chimica Acta*, 8, 519.
- Pereira, R. M. S., Andrades, N. E. D., Paulino, N., Sawaya, A. C. H. F., Eberlin, M. N., Marcucci, M. C., Favero, G. M., Novak, E. M., & Bydlowski, S. P. (2007). Synthesis and characterization of a metal complex containing naringin and Cu, and its antioxidant, antimicrobial, antiinflammatory and tumor cell cytotoxicity. *Molecules*, 12(7), 1352-1366.
- Petronini, P. G., & Carcelli, M. (2017). Anti-proliferative effects of copper (II) complexes with hydroxyquinoline-thiosemicarbazone ligands. *European Journal of Medicinal Chemistry*, 128, 140-153.
- Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of Natural Products*, 63, 1035-1042.
- Qi, Y., Jiang, M., Cui, Y., Zhao, L., & Liu, S. (2015). Novel reduction of Cr(VI) from wastewater using a naturally derived microcapsule loaded with rutin-Cr(III) complex. *Journal of Hazardous Materials*, 285, 336-345.
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine*, 26(9-10), 1231-1237.
- Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology & Medicine*, 20(7), 933-956.
- Skaltsa, H., Verykokidou, E., Harvala, C., Karabourniotis, G., & Maneta, Y. (1994). UV-B protective potential and flavonoid content of leaf hairs in *Quercus ilex*. *Phytochemistry*, 37, 987-990.
- Tahir, M., Rehman, M. U., Lateef, A., Khan, R., Khan, A. Q., Qamar, W., Ali, F., O'Hamiza, O., & Sultana, S. (2013). Diosmin protects against ethanol-induced hepatic injury via alleviation of inflammation and regulation of TNF- α and NF- κ B activation. *Alcohol*, 47, 131-139.
- Tanaka, T., Kohno, H., & Mori, H. (2001). Chemoprevention of colon carcinogenesis by dietary non-nutritive compounds. *Asian Pacific Journal of Cancer Prevention*, 2(3), 165-177.
- Tanaka, T., Makita, H., Kawabata, K., Mori, H., Kakumoto, M., Satoh, K., Hara Aumida, T., Fukutani, K., Tanaka, T., & Ogawa, H. (1997a). Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. *Carcinogenesis*, 18(5), 957-965.
- Tanaka, T., Makita, H., Kawabata, K., Mori, H., Kakumoto, M., Satoh, K., Hara Aumida, T., Fukutani, K., Tanaka, T., & Ogawa, H. (1997b). Modulation of N-methyl-N-amyl nitrosamine-induced rat oesophageal tumourigenesis by dietary feeding of diosmin and hesperidin, both alone and in combination. *Carcinogenesis*, 18(4), 761-769.
- Tanaka, T., Makita, H., Ohnishi, M., Mori, H., Satoh, K., Hara Aumida, T., Fukutani, K., Tanaka, T., & Ogawa, H. (1997). Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis in rats by flavonoids diosmin and hesperidin, each alone and in combination. *Cancer Research*, 57(2), 246-252.
- Wang, J., Cao, Y., Sun, B., & Wang, C. (2011). Characterisation of inclusion complex of trans-ferulic acid and hydroxypropyl- β -cyclodextrin. *Food Chemistry*, 124, 1069-1075.
- Wu, T., Zang, X., He, M., Pan, S., & Xu, X. (2013). Structure-activity relationship of flavonoids on their anti-Escherichia coli activity and inhibition of DNA gyrase. *Journal of Agricultural and Food Chemistry*, 61(34), 8185-90.

- Xie, Y. Y., Yuan, D., Yang, J. Y., Wang, L. H., & Wu, C. F. (2009). Cytotoxic activity of flavonoids from the flowers of *Chrysanthemum morifolium* on human colon cancer Colon205 cells. *Journal of Asian Natural Products Research*, 11(9), 771-778.
- Yang, M., Tanaka, T., Hirose, Y., Deguchi, T., Mori, H., & Kawada, Y. (1997). Chemopreventive effects of diosmin and hesperidin on N-butyl-N-(4-hydroxybutyl)nitrosamine-induced urinary-bladder carcinogenesis in male ICR mice. *International Journal of Cancer*, 73(5), 719-724.

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