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Heterotheca inuloides (Mexican arnica) metabolites protects Caenorhabditis elegans from

oxidative damage and inhibits the nitric oxide production

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1. Introduction

Reactive oxygen and nitrogen species (RONS) such as superoxide anion ($O2^{\bullet-}$), hydroxyl radicals ($^{\bullet}OH$), singlet oxygen ($^{1}O_2$) and nitric oxide (NO) plays an important role in many physiological processes as components of intracellular signaling cascades and regulating several physiological functions thereby affecting cellular homeostasis [1]. However, when the production of highly reactive molecules with unpaired electrons overpowers the scavenging mechanisms may also lead to a physiological process known as oxidative and nitrative stress [2]. This pathological condition has been associated with deleterious effects, and oxidative damage to cellular constituents, which in turn is the major determinant of life span and aging due damage accumulates in tissues that govern many senescent functions [3,4]. Primary RONS, such as $O_2^{\bullet-}$, H_2O_2 or $NO^{\bullet}$ are precursors to the formation of highly reactive secondary species hydroxyl radical and peroxynitrite ($^{\bullet}OH$, $ONOO^-$) [5]. Both endogenous such as exogenous sources share RONS production catalyzed by transition metals, mechanism known as Fenton-catalysed Haber-Weiss reaction [6,7]. In vivo Fenton-catalysed Haber-Weiss reaction is initiated by products of aerobic respiration, such as hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$) [8]. Transition metals ions such as iron, copper and aluminium have rich coordination and redox chemistry, the ability to vary oxidation states, and their presence at high concentrations plays a significant role in the damage to biomolecules [9]. Natural products have been investigated as a promising option to acts as protective agents capable of delay oxidative damage by scavenging free radicals, inhibiting its formation or interrupting its propagation, and by chelating metal ions; this may lead to a delay in age-related diseases and an extension of lifespan. *Heterotheca inuloides* is a plant native to Mexico commonly known as árnica with a series of additional regional names [10,11]. The infusion of dried flowers has been used to treat contusions, muscular pain, wounds and other painful conditions associated with inflammatory processes [12]. This plant species is characterized by biosynthesizing sesquiterpenes with cadinene skeleton. The major sesquiterpenes isolated from this plant are 7-Hydroxy-3,4-dihydrocadalene (**1**) and 7-hydroxycadalene (**2**) [13]. The antioxidant and free radical scavenging activity using various *in vitro* antioxidant assays indicated that cadinenes **1** and **2** protect cells against peroxy radical attack and inhibit the production of lipid peroxides induced by NADPH oxidation. In addition, sesquiterpene **1** protected NADH– and succinate–cytochrome C reductase enzymes against peroxidation. Conversely, cadinenes **1** and **2** showed no inhibition against both enzymatic and non-enzymatic superoxide generation [14-16]. On the other hand, the flavonoids kaempferol and quercetin (**5**) isolated from *H. inuloides* inhibited the microsomal lipid peroxidation and showed tyrosinase inhibitory activity [16,17], and some metabolites isolated from the same source showed ability to trap radicals such as $ABTS^{\bullet+}$, $DPPH^{\bullet}$, $ONOO^-$, $O_2^{\bullet-}$, $^{1}O_2$, HOCl, H_2O_2 and $OH^{\bullet}$ [18]. Since RONS play a vital role in the oxidative damage processes, the aim of this study was to investigate whether acetone extract of *H. inuloides* and metabolites isolated from this extract could protect *Caenorhabditis elegans* against stress generated by RONS-inducing substances. In addition, the

copper sequestering ability of these metabolites and their ability to inhibit the production of nitric oxide (NO) were explored.

2. Experimental

2.1. Reagent and materials

All chemicals used, including solvents, were analytical grade. Aluminium chloride hexahydrate ($AlCl_3 \cdot 6H_2O \cdot$), aminoguanidine hydrochloride, catechin, cupric sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$), dimethyl sulfoxide (DMSO), *E. coli* serotype 055:B5 lipopolysaccharide (LPS), ethanol, Folin Ciocalteu's reagent, juglone (5-hydroxy-1,4-naphthoquinone), methanol, N-(naphtyl)-ethylene-diamine, orthophosphoric acid (H_3PO_4), phosphoric acid (H_3PO_4), 5-fluoro-2'-deoxyuridine, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydrogen phosphate ($Na2HPO4$), sodium hydroxide (NaOH), sulfanilamide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 5-fluoro-2'-deoxyuridine, and trypan blue were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant material

Plant material was kindly provided by Laboratorios Mixim (México). Flowers of *H. inuloides* were collected in 2010 from the locality of San Juan Xoconusco, municipality of Donato Guerra, State of Mexico. The authentication of the plant material was done by M. Sc. Abigail Aguilar-Contreras. A plant specimen of authenticated material was deposited at the Medicinal Plant Herbarium of the Instituto Mexicano del Seguro Social (IMSS, Mexico City) with voucher number IMSSM-16064. The compounds 7-hydroxy-3,4-dihydrocadalene (**1**), 7-hydroxycadalene (**2**), 3,7-dihydroxy-3(4H)-isocadalen-4-one (**3**), (1*R*,4*R*)-1-hydroxy-4H-1,2,3,4-tetrahydrocadalen-15-oic acid (**4**), quercetin (**5**), quercetin-3,7,3'-trimethyl ether (**6**), quercetin-3,7,3',4'-tetramethyl ether (**7**), eriodictyol-7,4'-dimethyl ether (**8**) were obtained from the acetone extract by successive fractionations using vacuum liquid chromatography (VLC), while semisynthetic compounds 7-acetoxy-3,4-dihydrocadalene (**9**), 7-benzoxy-3,4-dihydrocadalene (**10**), 7-acetoxycadalene (**11**), 7-benzoxycadalene (**12**), quercetin penta-acetate (**13**) and 7-hydroxycalamenane (**14**) (Figure 1) were obtained by conventional chemical procedures as was previously described [19]. Due to a shortage, some compounds were not tested in all assays.

2.3. *Caenorhabditis elegans* strain

C. elegans wild-type strain Bristol N2 was obtained from the *Caenorhabditis* Genetics Center (Minneapolis-Saint Paul, MN, USA). The nematodes were maintained at 20°C on NGM medium containing 3 g NaCl, 17 g agar, 2.5 g peptone, 1 mM $CaCl_2$, mg, 1 mM $MgSO_4$ and 25 mM KH_2PO_4, per

liter of water, as described previously [20]. Age-synchronized worms were generated in all experiments through the sodium hypochlorite method, and were allowed to hatch in Petri dishes with liquid *S*-medium (a minimal salt solution) seeded with uracil auxotroph *Escherichia coli* OP50 (*Caenorhabditis* Genetics Center) strain as food resource [21].

2.4. Cell lines

The murine monocytic macrophage cell line RAW264.7 (ATCC® TIB-71™) was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Essential Medium (DMEM), modified to contain 4 mM L-glutamine and 4.5 g/L glucose, 1mM sodium pyruvate and 1.5 g/L sodium bicarbonate (ATCC® 30-2002™), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ATCC® 30-2020™). Cells were grown at 37°C, 5 % CO_2 in fully humidified atmosphere and used for experiments between passage 5 and 18. The viability of the cells was determined by Trypan blue exclusion.

2.5. Determination of polyphenolic content

Phenolic content was determined by its reaction with Folin–Ciocalteu (FC) reagent [22]. Briefly, 1 mL of *H. inuloides* acetone extract (0.15 mg/mL in DMSO/H_2O 1:1) was mixed with 1 mL of FC reagent (diluted 10-fold with water) and incubated at room temperature for 5 min, 1 mL of 75 mg/mL Na_2CO_3 solution was added to the reaction mixture. The mixture was vortexed and incubated at room temperature in the dark for 30 min. Aliquots of the final reaction were added into each well of a 96-well plate, and absorbance was measured at 760 nm using a Synergy-HT multi-detection microplate reader (BioTec Instruments, Inc., VT, USA). Gallic acid was used as standard, and the results were expressed as µmol of gallic acid equivalents (GAE)/g of extract.

2.6. UV-Vis analysis of flavonoid content

The flavonoid type compounds present in the *H. inuloides* acetone extract was distinguished by UV-Vis spectral analysis based on their ability to form complexes with $AlCl_3$. Due to the low solubility of the extract in aqueous solution, this was dissolved in methanol. Procedure 1: 1 mL of $AlCl_3$ solution (2 %, w/v) was added to 1 mL of the test solution (0.15 mg/mL in methanol) and 0.5 mL of water. The mixture was left for 10 min at room temperature and then subjected to spectral analysis in the range of 300–600 nm against the blank, using a UV–Vis Shimadzu U160 spectrophotometer (Shimadzu Co., Kyoto, Japan). Procedure 2: 1 mL of test solution (0.15 mg/mL in methanol) was mixed with 0.3 mL of $NaNO_2$ (5 %, w/v) and after 5 min, 0.5 mL of $AlCl_3$ (2 %, w/v) was added. A sample was mixed and 6 min later was neutralized with 0.5 mL of 1 M NaOH solution. The mixture

was left for 10 min at room temperature and then subjected to spectral analysis in the range of 300–600 nm against the blank wherein the amount of $AlCl_3$ solution was substituted by the same volume of water. For quantitative analysis, the absorbance of the samples was measured at 425 or 510 nm [23], using as standards quercetin or catechin respectively to build up the calibration curve (concentration range: 30–500-μM). Aliquots of the final reaction were added into each well of a 96-well plate, and absorbance was measured at 415 and 760 nm using a Synergy-HT multi-detection microplate reader (BioTec Instruments, Inc., VT, USA).

2.7. Stress resistance

Stress resistance assays were assessed in liquid medium at 20°C in 96-well plates (Corning Life Sciences, NY, USA). Previous to the assays, the nematodes were age-synchronized in accordance with established protocols [24]. The nematodes were distributed in wells as L1 larvae (15-20 animals per well) together with *Escherichia coli* OP50 with a final concentration of 1.2 x10^9 bacteria/mL. To prevent self-fertilization, 5-fluoro-2'-deoxyuridine was added 36 h after seeding (0.12 mM final). The compounds whose effect on lifespan was to be tested were dissolved in a DMSO/methanol (1:1 v/v) solution (stocks: 100 mM). The final concentration of the DMSO was ≤ 0.6% in all conditions. After addition of compounds, sealed plates were shaking for 2-3 min and returned to the incubator MaxQ 6000 (ThermoFisher Scientific, MA., USA). The observations and counts were performed using an inverted microscope ECLIPSE TS100 (Nikon Instruments Inc., Tokyo, Japan). Worms were scored as dead when they did not respond to tactile or light stimuli. The mean percentage of live worms was calculated from three independent experiments.

2.7.1. Copper sulfate assay

Synchronized worms were dispensed into each well of a flat-bottom 96-well plate as L1 larvae (15-20 animals per well) incubated in liquid *S*-medium containing *Escherichia coli* OP50 strain as food resource and either metabolites (1 mM) or DMSO/ethanol (1:1 v/v) as the solvent control for 1 h. At the tested concentration, no precipitation in the culture medium of the compounds was observed. The pre-treated worms were subjected to oxidative stress by the addition of $CuSO_4$ (final concentration 1mM). For the experimental assay the $CuSO_4$-$5H_2O$ was prepared in sterile distilled water by diluting a stock solution of 20.025 mM. Wells with sterile distilled water and DMSO/ethanol (1:1 v/v) were included as a solvent control; a positive control treated with catechin (1 mM) was included. The final volume was 250 μl per well. The plates were incubated for 24 h with shaking at 20°C in a MaxQ 6000 incubator (ThermoFisher Scientific, MA., USA). After the incubation period with copper the worms were observed under the microscope and the fraction alive was

scored on the basis of body movement. In order to improve the counting, the 96 well plates were shaken for 2 min before counting. Comparisons between treatments and controls were performed using the two-tailed, unpaired, Student's *t*-test. Approximately 80-120 worms were scored in each experiment. Data were obtained from with at least three independent experiments.

2.7.2. Juglone assay

Synchronized L1 larvae worms (15-20 animals per well) were pretreated with 100 μM of *H. inuloides* metabolites, after 1 h, worms were treated with 250 μM of the pro-oxidant agent [25]. The plates were incubated with shaking at 20°C in a MaxQ 6000 incubator (ThermoFisher Scientific, MA., USA). The number of dead worms for stress resistance was counted and recorded every hour; juglone was applied in 92% ethanol, 8% Tween 80 (v/v). The juglone was prepared by diluting a stock solution of 3.15 mM. The curves were compared for significance using the Mantel-Cox (*log-rank*) test.

2.8. Spectrophotometric study of the interaction with copper

We evaluated the chelating ability and the interaction with copper of those compounds that showed ability to protect *C. elegans* using the techniques of UV-Vis spectroscopy, electron spin resonance (ESR) and electrospray ionization-mass spectrometry (ESI-MS).

2.8.1. UV-Vis analysis

25 μM solutions were prepared in phosphate buffer solution (10 mM, pH 7.5). The UV-Vis-absorbance of the samples was subjected to spectral analysis at constant temperature in the range of 300 to 600 nm using a UV-2700-CPS-240A spectrophotometer (Shimadzu Co. Kyoto, Japan), equipped with a temperature control unity. UVProbe software (Shimadzu) was used to collect and analyze the data. The same volume of 50 μM of $CuSO_4$ solution was added to the samples and 10 s later were subjected to spectral analysis and compared with compounds alone [26].

2.8.2. ESR (X-band) spectroscopy

The measurements were carried out in a quartz tube at 77 K in a 1:1 MeOH–H_2O (0.5 mM) solution, using a Jeol JES-TE300 spectrometer (JEOL Ltd. Tokyo, Japan) operating at X-Band fashions at 100 KHz modulation frequency and a cylindrical cavity in the mode TE011. The external calibration of the magnetic field was performed using a precision gaussmeter Jeol ES-FC5 (JEOL Ltd. Tokyo, Japan) with a microwave frequency counter 5350B HP (Hewlett Packard, California, USA). Spectral acquisition, manipulations and simulation were performed using the ES-IPRITS-TE softwere (JEOL

Ltd.). ESR spectra were recorded as a first derivative and the main parameter such as g- factor values were calculated.

2.8.3. Analysis by electrospray ionization mass spectrometry

The analysis was carried out using a mass spectrometer Esquire 6000 (Bruker Daltonik GmbH, Bremen, Germany) with an electrospray ionization source and ion trap analyzer. Data processing was performed using the DataAnalysis™ 3.2 software (Bruker Daltonik GmbH). The samples were diluted before measurements at 0.5 mg/mL in methanol/water and were injected in electrospray chamber by direct infusion, with a constant flow of 240 µL/h. The mass spectra were obtained in the positive and in the negative modes.

2.9. Nitric oxide production inhibition

In brief, RAW264.7 macrophage cells were seeded in 96 well microtitre plates (Corning Life Sciences, NY, USA) (2×10^5 cells/well) and incubated for 2 h. The adherent cells were incubated with *E. coli* serotype 055:B5 lipopolysaccharide (1 µg/mL) in the absence or presence of 50 µL of each test compounds dissolved in DMSO and serially diluted at a decreasing concentration of 100- 3.1 µM, or aminoguanidine hydrochloride as inducible NOS (iNOS) inhibitor. The final concentration of DMSO in the cell culture supernatant was ≤0.1% (v/v). Wells without treatment but stimulated with LPS were included as a control and received the same amount of DMSO. Cells were allowed to grow at 37 °C in 5 % CO_2 atmosphere. After 24 h, the inhibitory activity of test compounds on NO production was determined by measuring the nitrite levels in the supernatants of cultured RAW 264.7 cells. Briefly, 100 µL of cell culture supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-(-naphtyl)-ethylene-diamine dihydrochloride in water), absorbance of the mixtures was determined at 550 nm [27], using a Synergy-HT multi-detection microplate reader (BioTek Instruments Inc., VT, U.S). The nitrite concentration (µM) was determined by interpolation with the standard curve constructed with known concentrations of $NaNO_2$. Percentage of inhibition was calculated against cells that were not treated but were induced with LPS. DMEM medium alone was used as blank in all experiments. Experiments were performed 3 times in triplicates.

2.10. MTT assay for cell viability

The viability of cells was determined by reduction MTT to formazan in active mitochondria of viable cells [28]. Briefly, after 24 h incubation with the test compounds, 10 µL of MTT was added to the remaining cell in 96-well plates and incubated for 4 h at 37° C. The formazan products resulting from

dye reduction was dissolved in DMSO (100 μL). Absorbance was measured on a microplate reader (BioTek Instruments Inc., VT, USA) at 570 nm. The cell viability of control wells was considered as 100%. The test compounds were considered to be cytotoxic when the absorbance of the compounds-treated wells was less than 80% of that in the control wells.

2.11.   Statistical analysis

Data are expressed as mean ± SEM. The data of the stress resistance assays were processed using IBM SPSS Statistics 20, Chicago, IL, USA. Plots were processed using the GraphPad Prism 5.0 Software (GraphPad Software, Inc. San Diego, CA, USA). Data were obtained from three independent experiments, $p<0.05$ was considered for significant differences.

3. Results and discussion

The aging process is characterized as a progressive decline in biological functions with time, and a decreased resistance to multiple forms of stress, as well as an increased susceptibility to numerous diseases [29]. Several studies have suggested that antioxidants prevent the appearance of age-related diseases and can increase lifespan [30]. We evaluate the ability of the metabolites isolated from *H. inuloides* to protect *C. elegans* against the damage generated by exogenous stressors, its copper chelating ability and its potential to inhibit the NO production by LPS-stimulated macrophages.

3.1. UV-Vis analysis of phenolic and flavonoids type compounds of *H. inuloides* extract

Previous studies have reported the phenolic and flavonoid contents in acetone and methanol extracts of *H. inuloides* [18]; however, these studies do not describe the type of flavonoid components. Aluminum chloride produces a bathochromic shift in most of flavonoids [31], and this property was used to characterize the *H. inuloides* acetone extract. This extract does not exhibit the characteristic peak around 510 nm associated with the presence of compounds with aromatic ring containing a catechol group, or compounds containing catecholic moieties (Figure 2). However, both spectra show an absorption maximum around 400 nm, this peak is attributed to the flavonol or flavones-aluminum chloride [23]. This is the first time that the spectral analysis of an extract obtained from *H. inuloides* is reported regarding the use of the AlCl$_3$ reaction for determining the type and content of flavonoid compounds. Phenolic content estimated by using F-C reagent was 241.61±5.9 μmol gallic acid/g extract. The content of flavonoid expressed as quercetin equivalents was 137.85 ± 2.25 μmol quercetin/g extract.

3.2. Copper sulfate assay

Copper is an essential trace element component of metalloenzymes capable to use oxygen or oxygen radicals as substrates by direct interaction with Cu^{2+} site [32]. The deficiency of this metal is associated with disorders such as neutropenia and anemia [33], bone marrow suppression [34], vascular lesions (Menkes disease) [35], hepatolenticular degeneration (Wilson disease) [36] and the inability of the organism to perform important biological processes [37]. However, exposure to high levels of free copper (no-covalently bound copper) has been associated with damage due its oxygen transferring properties, and their capability to act as a catalyst for oxidative damage by removal one electron from O_2 that results in the formation of $O_2^{\bullet-}$ and the subsequent generation of H_2O_2, $ONOO^-$ and $^{\bullet}OH$ through the Fenton-catalysed Haber-Weiss reaction [38]. Copper also participates in the oxidation of nitroxyl anion radical to biologically active nitric oxide [39]. It has been reported previously that exposure to copper reduces the feeding, and produces locomotion behavioral defects, neurodegeneration and toxic effects in *C. elegans* [40,41].

In the experiment, Cu^{2+} ions significantly decreased the *C. elegans*' survival rate (5.66%), however, pretreatment with the acetone extract and with compounds **1** (7-hydroxy-3,4-dihydrocadalene), **3** (3,7-dihydroxy-3(4H)-isocadalen-4-one) and **5** (quercetin) were able to protect the nematode from the mortality induced by copper. The survival rate of worms pre-treated with extract and compounds **1, 3** and **5** was 94.26%, 67.20%, 84.89%, and 93.06%, respectively, compared to untreated ones ($p < 0.05$). Compounds **4**, **10**, and **13** showed poor protective activity to worm. The remainder of the test compounds showed no statistically significant difference on the survival rate compared to untreated control (Table 1). The protective effects of *H. inuloides* acetone extract may be attributed to their ability to trap radicals [18]. In addition, from this plant have been isolated phenolic compounds, some of them have demonstrated ability to chelate metal ions [42].

3.3. Spectrophotometric study of the interaction with copper

Previously, interaction of biologically relevant metal ions with phenolic compounds has been studied using spectroscopic and spectrometric techniques that include photometric titration, IR, Raman, UV–Vis, 2D NMR, mass spectrometry, term gravimetric analysis, voltammetry among others [43,44, 45]. We use the ultraviolet-visible, EPR spectroscopy and electrospray ionization mass spectrometry (ESI-MS) to study the interaction between Cu^{2+} and acetone extract of *H. inuloides*, and compounds **1**, **3** and **5** which showed a positive effect in the bioassay with *C. elegans*.

All the ESR spectra in methanol-water frozen solution of copper-*H. inuloides* extract complex (Figure 3) exhibits an anisotropic ESR spectra characteristic of Cu^{2+} centers with well resolved hyperfine

lines in the $g_{\parallel}$, all signals show the dependence $g_{\parallel} > g\perp > g_e$ which is diagnostic of a d_{x2-y2} ground state for Cu^{2+}, the hyperfine structure resulted from the interaction between delocalized π electrons and the nuclear spin $I_{Cu}=3/2$. The values obtained by simulated spectra are shown in Table 2. The ESR spectra of the *H. inuloides* extract on the free copper ion, showed a significant modification of the spectrum (Figure 3) with spectral values $g_{\parallel}= 2.27$, $A_{\parallel}= 184 \times 10\text{-}4 \text{ cm}^{-1}$ showing a full chelating effect, although the spectrum shows broad signals by the overlap of signals due to the multiple components of the extract including **5**, that it is the major flavonoid component, but also contains other compounds including sesquiterpenoids, phenolic compounds, phytosterols, lipids, among others [13,46].

Compound **5** showed the greatest protective capacity against Cu^{2+}. This flavonoid compound possesses three possible chelating sites: between the 3-hydroxyl group and the 4-oxo group (a), the 5-hydroxyl group and the 4-oxo group (b), and between the *ortho*-hydroxyls groups in the B-ring (c). The chelation site involves preferably the positions "a" and/or "b" [47]. The UV-Vis spectrum for **5** showed two peaks associated with absorption due to the cinnamoyl system (B and C rings), and the benzoyl system (ring A), respectively [26]. The peak associated with the cinnamoyl system (peak I) undergoes a bathochromic shift of 371 to 434 nm ($\Delta\lambda=73nm$) due to chelation of copper (Figure 4). This amount of bathochromic shift ($\Delta\lambda$) has been associated with the formation of **5**-copper complex in the position "a" [48], implying that deprotonation of 3-OH takes place to form **5**-Cu^{2+} complex. The interactions by ESI-MS indicated that complex formed between **5** and copper has the stoichiometry copper-**5** 1:1 and 1:2. The fragments corresponding to the complex were observed at $m/z=382$ $[(M+H) + Cu^{2+} + H_2O]^+$, and $m/z=688$ $[(M+H) + Cu^{2+} + M + H_2O]^+$. The copper-**5** complex with stoichiometry of 2:3 $[(M+H) + Cu^{2+} + M + Cu^{2+}+ M]^+$ was also probably present in solution because the molecular peak at $m/z=1028$ can be observed, however, it was observed with very low intensity (Figure 5). Some studies have been conducted to investigate the products of **5** with copper ions, and complexes with a range of stoichiometries of metal:flavonoid, 1:1, 1:2, 2:2 and 2:3 have been observed [43,47,49,50,]. This range of results may be associated with the metal ion and the conditions of synthesis [43], however, the stoichiometry 1:2 is in general the preferred one [47]. For steric reasons the complexes usually include no more than two flavonoid molecules, and methanol solutions favored formation of the 1:2 metal:flavonoid complexes. The ESR spectra obtained for the pure compound **5** and copper ions showed a complete chelating effect, with $g_{\parallel}= 2.30$ and $A= 175 \times 10^{-4} \text{ cm}^{-1}$; these spectral changes are consistent with a square pyramid geometry [51]. A complex of two **5** (quercetin) molecules coordinated in position "a" with a single Cu^{2+} ion and one molecule of solvent in axial position is proposed (Figure 6). These data were supported by mass spectrum that showed a peak at $m/z=688$. The tetrahedral distortion values (*f*) calculated for the extract *f*=125 were slightly lower than the complex with **5** *f*=131, as well as an increase of $g_{\parallel}$ from 2.270 to 2.30

and decrease of $A_{\parallel}$ from 184.3 to 175.1, respectively, was observed (Table 2); both parameters indicative of a slight increase in the distortion of the **5**-Cu complex, but both they are consistent with a square pyramid geometry [51,52]. The degree of tetrahedral distortion occurring frequently for square planar copper complexes, is reflected by the ratio of $f= g_{\parallel}/A_{\parallel}$ and can be considered as a measure of distortion and the deviation from perfect geometry depending on the nature of coordinated ligands [53, 54]. The f value in the range 110-120 is characteristic for signals of copper complex with planar geometry, whereas its increase to 150 indicates a slight or moderate distortion of planar symmetry and further rise to the values 180-250 suggest a strong deviation from square planar geometry. In general, as a complex becomes more tetrahedral, the $g_{\parallel}$ value and the g_{iso} value increase, and the A_{iso} and $A_{\parallel}$ values decrease [55]. Additionally, a good estimation of the geometry adopted by the compounds can be done employing the isotropic parameters of the ESR spectra $A_{iso}= (A_{\parallel} + 2A_{\perp})/3$ and $g_{iso}=(g_{\parallel} + 2g_{\perp})/3$, values for planar symmetry are between $A_{iso}= 75$-90 cm^{-1} and $g_{iso}=2.06$-2.11 [56,57]. Other compounds that showed a similar activity as protector against copper were **1** and **3**. Compound **3** is an α-hydroxy ketone, but unlike compound **5** (an α,β-unsaturated compound) in which the 3-hydroxyl and 4-oxo groups have been proposed as metal binding sites (site "a"), the hydroxyl group of **3** is attached to a sp^3 carbon; furthermore, the presence of isopropyl group increases steric hindrance and decreases its nucleophilicity. The UV-Vis spectral analysis for compound **1** shows three bands attributable to benzene aromaticity with a bathochromic shift due conjugation with another ring, which has double bonds. The first absorption band at 217 nm, a secondary band at 265 nm and a third band at 302 nm. Spectrum obtained for compound **1** in the presence of copper showed a hypsochromic on absorption peaks (Supplementary Figure 1). For compound **3**, the spectral analysis showed a primary absorption band at 249 nm attributable to benzene aromaticity and a secondary band at 283 nm, associated with $\eta-\pi^*$ -ketone transitions. (Supplementary Figure 2). The limited capacity of compound **1** and **3** to chelate copper was evidenced by UV-Vis, ESI-MS and ESR spectral analysis in which **1**-Cu and **3**-Cu species could not be observed. The UV-Vis spectral analysis does not show a bathochromic shift in presence of copper. The ESI-MS spectra for **1** and **3** showed the characteristic [M+H]$^+$ ions at $m/z=217$ and at $m/z=247$, respectively, we also observed clusters molecules charged with Na$^+$ and K$^+$. No copper-containing species could be observed by ESI-MS. The ESR spectra obtained for **1** and **3** showed the same parameter than the Cu^{2+} aqua free ions $g_{\parallel}=2.423$ and $A_{\parallel}=129.86$ x10^{-4} cm^{-1}, showing no effect on ion chelating copper (Table 2). It is possible that these cadinene compounds occurring in *H. inuloides* may protect *C. elegans* from oxidative damage through other mechanisms and not by chelation. Previously, it has been reported that compound **1** inhibits the lipid peroxidation, and protects against oxidative hemolysis [14,16], while compound **3** showed moderate ability to trap ROS [58], it

is important to consider that unbound copper induces lipid peroxidation [59] and RONS production [38].

3.4. Juglone assay

On the other hand, some metabolites of *H. inuloides* showed protective effects to *C. elegans* against stress generated by juglone. Agents such as juglone are cytotoxic by their capacity to produce $^1O_2$; and H_2O_2 through O_2 reduction by semiquinone intermediates and enables them to form adducts to cellular constituents, and may thereby cause oxidative stress [60,61]. Pretreatment of the *C. elegans* N2 wild-type with 100 µM of certain metabolites is indeed able to protect the organism against lethal oxidative stress generated by juglone and increased survival rates (Table 3). Compounds **5**, **3** and **6** caused an increased survival during juglone-induced oxidative stress compared to untreated controls. Compounds **4**, **7**, **10**, **12** and **13** showed no statistically significant differences compared to the control, whereas compounds **9**, **11** and **14** had a negative effect on survival of the worms.

Compound **5** showed the greatest protective effects. In this compound an *ortho*-dihydroxy and 2,3-double bond in conjugation with a 4-oxo function confer the structural characteristics necessary to be considered a good radical trapping [62]. The mean life-span of worms after treatment with 250 µM juglone was 1.91±0.042 and for the group pretreated with **5** for 1 h was 2.074±0.041 ($p< 0.001$). The *O*-methylation and *O*-acetylation of **5** resulted in a minor activity in **6**, **7** and **13**. These results are consistent with reports that indicate that the RONS scavenging activity of flavonoids is highly dependent on the presence or modification of hydroxyl substituent [63]. We found no significant effect of cadinene type sesquiterpenes against the stress caused by juglone, and this can be explained by the fact that the *H. inuloides* cadinenes are not good scavengers of $O_2^{\bullet-}$[16].

3.5. Nitric oxide production inhibition

NO participates in the control of pathogens; in the regulation of blood pressure and cardiovascular health, acts as a neurotransmitter and modulate many functions [64], however, this lipid- and water-soluble radical gas, can react with oxygen and generate NO_2, NO_2^-, NO_3^-, N_2O_3, and the strong oxidant peroxynitrite $ONOO^-$, which in turn induce inflammatory cytokines, which lead to induced cell death by apoptosis and necrosis [65,66]. Natural products represent an alternative as inhibitors of nitric oxide synthase. *H. inuloides* is a plant widely used in Mexican traditional medicine for the treatment of bruises and injuries associated with inflammatory processes [12], from this plant were isolated various metabolites with anti-inflammatory activity [13,58], however, the ability of these metabolites to inhibit nitric oxide synthesis has not been reported. We examined the

inhibitory activity of main components from the acetone extract and some semisynthetic derivatives. Table 4 shows the inhibitory activity by *H. inuloides* compounds towards NO production by LPS-activated macrophages. Compounds **3**, **5**, **6**, **8**, **10** and **12** showed greater than 25% inhibition of NO production at concentration of 25 µg/mL. Among these six compounds, **5** (quercetin) and **8** (eriodictyol-7,4'-dimethyl ether) significantly decreased to 100%, and 87.3%, respectively, the levels of NO production from LPS-stimulated RAW264.7 cells. This result is congruent with the previous report on the iNOS inhibitory effects of flavonoids [67]. The numbers of viable activated macrophages were not significantly altered, thereby indicating that the inhibition of NO synthesis by compounds **5** and **8** was not due to cytotoxic effects. The substitution of the hydroxyl groups in **5** produces a decrease in the ability to inhibit NO production in *O*-methylated derivatives **6** and **7**. Compounds **1** and **2** were found to be toxic to RAW264.7 cells as determined by MTT assay. On the other hand, benzoylated derivatives **10** and **12** showed moderate effects without affecting the viability of activated RAW264.7 macrophages. Significantly differences in NO production between **10** and **12** were not observed. The cadinene **4** showed the lowest inhibitory activity without affecting the viability of the macrophages. However, acetylated derivatives **9** and **11** showed similar toxic effects with **1** on the viability of macrophages.

4. Conclusions

In this work *C. elegans* was exposed to oxidative stressors copper and juglone. We find that some compounds of *H. inuloides* cause a significant increase in *C. elegans* life span. Particularly, quercetin (**5**) showed the highest activity. This activity is important because metal chelation is considered as other mechanism for the antioxidant effect. The spectrophotometric analyses, using the techniques of UV-Vis, ESR and ESI-MS provide evidences for complex formation between **5** and Cu^{2+}, with a metal:ligand stoichiometry of 1:2. Cu^{2+} ions are coordinated with **5** in position "a". Cadinane type compounds have a minimal protective effect against oxidative stress inductors in cultured *C. elegans*. Flavonoids of *H. inuloides* showed greater ability to inhibit nitric oxide synthesis that compounds with cadinene skeleton. Generally the modification of hydroxyl groups of flavonoids and cadinene compounds modifies their ability to inhibit the synthesis of nitric oxide.

Declaration of interest

The authors declare that there are no actual or potential conflicts of interest in relation to this article. The authors alone are responsible for the content and writing of the paper.

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Figure 1. Natural products isolated from *H. inuloides* flowers and derivatives

Figure 2. UV-Vis absorption spectra of *H. inuloides* acetone extract. Solid line was obtained without NaNO$_2$, while the dashed line was obtained with NaNO$_2$, after addition of AlCl$_3$.

Figure 3. Experimentally observed first-derivative ESR spectrum of a frozen solution (methanol/H_2O) of Cu^{2+} (a), Cu^{2+}/Acetonic extract (b) and Cu^{2+}/**5** (c).

Figure 4. UV-Vis absorption spectra of compound **5** in 10 mM buffer phosphate solution at pH 7.5. Solid line represents compound alone (25 μM), while the dashed line represents the compound (25 μM) plus Cu^{2+} ions (50μM).

Figure 5. Electrospray ionization mass spectra in the positive mode of **5**–Cu^{2+} complex. Sections showing the distribution isotope patterns for positive ion clusters of (a) $[(M+H) + Cu + H_2O]^+$, (b) $[(M+H) + Cu + M + H_2O]^+$ and (c) $[(M+H) + Cu + M + Cu+ M]^+$

Figure 6. Complex forming sites and proposed structure for the $[CuL_2]$ (L=**5**). S = solvent

Table 1: Effect of *H. inuloides* metabolites on the survival of *C. elegans* under copper (1mM CuSO$_4$) induced stress

Compound	Subjects	No. of Dead	Alive	Estimated Mean Dead	Alive
1	375	252	123	32.80	**67.20****
2	306	0	306	100	0
3	278	236	42	15.11	**84.89****
4	333	66	267	80.18	19.82
5	288	268	20	6.94	**93.06****
6	376	24	352	93.62	6.38
7	284	0	284	100	0
8	330	25	305	92.42	7.58
9	268	0	268	100	0
10	270	40	230	85.19	14.81
11	320	25	295	92.19	7.81
12	265	0	265	100	0
13	315	39	276	87.62	12.38
14	348	9	339	97.41	2.59
Acetone extract	366	345	21	5.74	**94.26****
Catechin	328	212	116	35.37	**64.63****
Control (-)	265	15	250	94.34	5.66

** $P < 0.05$. Comparisons between treatments and negative control differed significantly using the two-tailed, unpaired Student's t-test.

Table 2. Spectral parameters obtained by simulation at 77 K ESR spectra of the complexes.

Sample	$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$[§]	$A_{\perp}$[§]	g_{iso}	A_{iso}[§]	f
Cu^{2+}/ Ext.	2.272	2.058	184.36	13.48	2.1293	66.95	125.07
Cu^{2+}/**1**	2.420	2.076	131.9	9.7	2.1906	50.44	183.38
Cu^{2+}/**3**	2.420	2.081	132.8	9.71	2.194	50.53	183.07
Cu^{2+}/**5**	2.300	2.062	175.13	9.7	2.1412	64.79	131.32
Cu^{2+}	2.423	2.079	129.86	9.7	2.1936	49.75	186.58

[§]Hyperfine coupling constants (A) are expressed in $1x10^4$ cm^{-1}.

Table 3. Protective effects of *H. inuloides* metabolites on *C. elegans* under juglone-induced oxidative

stress.

Compound	Juglone 250 mM			
	Mean±SE (Hr)	N treated	Log-rank χ^2	*P*-value[a]
3	2.058±0.053	242	4.004	0.0454*
4	1.898±0.051	310	0.0496	0.8239
5	2.074±0.041	335	51.67	< 0.0001**
6	2.066±0.047	280	3.858	0.0495*
7	1.905±0.043	322	0.045	0.8312
8	2.045±0.047	301	4.373	0.0365*
9	1.248±.037	214	110.6	< 0.0001**
10	1.859±0.046	311	0.001	0.9745
11	1.481±0.034	329	95.75	< 0.0001**
12	1.791±0.051	289	0.904	0.3416
13	1.951±0.048	307	0.843	0.3586
14	0.688±0.018	255	415.6	< 0.0001**
Control	1.915±0.042	248	---	---

** *P* < 0.01, * *P* < 0.05
[a] Determined by log-rank test.

Table 4. Nitric oxide inhibition by *H. inuloides* metabolites.

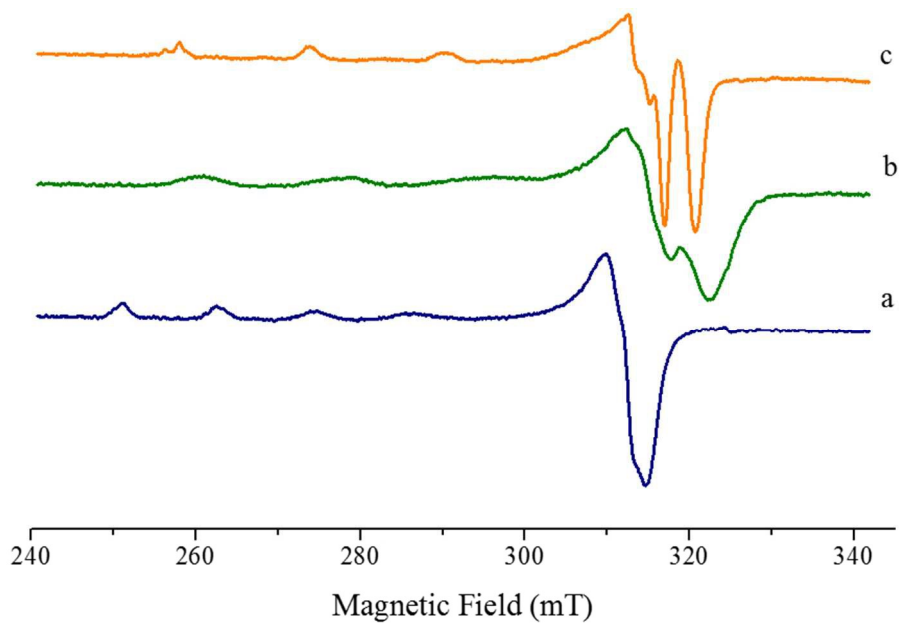| Compound (25 µg/mL)	Nitrites(µM)[a]	NO inhibition (%)
LPS 1 µg/ml	14.99±0.86	0
1	–	[b]
2	–	[b]
3	10.51±0.52	29.9
4	13.25±0.66	11.6
5	0	**100**
6	6.55±0.32	56.30
7	12.67±0.60	15.47
8	1.9±0.90	**87.3**
9	–	[b]
10	7.04±0.35	53.03
11	–	[b]
12	7.91±0.39	47.23
Oleic acid	12.0±0.60	19.94

[a] Value represents mean ± S.E.M.

[b] Cytotoxic effect in murine RAW 264.7 macrophages was observed with compounds **1**, **2**, **9** and **11**. With the remaining compounds, the observed viability was greater than 80%. (–) = not determined.

1   9   10
R   H   Ac   Bz

2   11   12
R   H   Ac   Bz

3

4

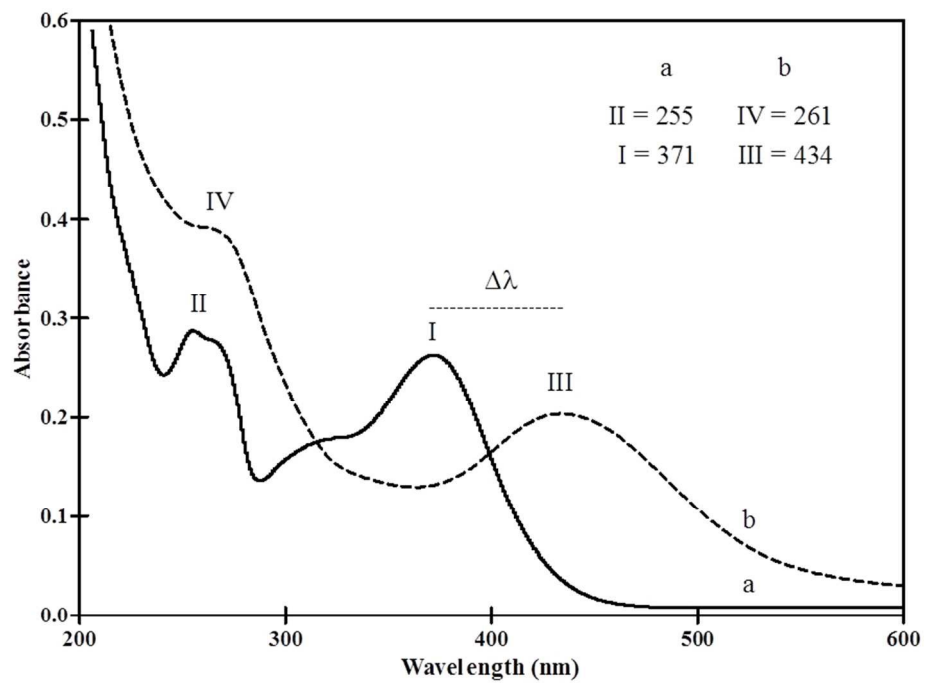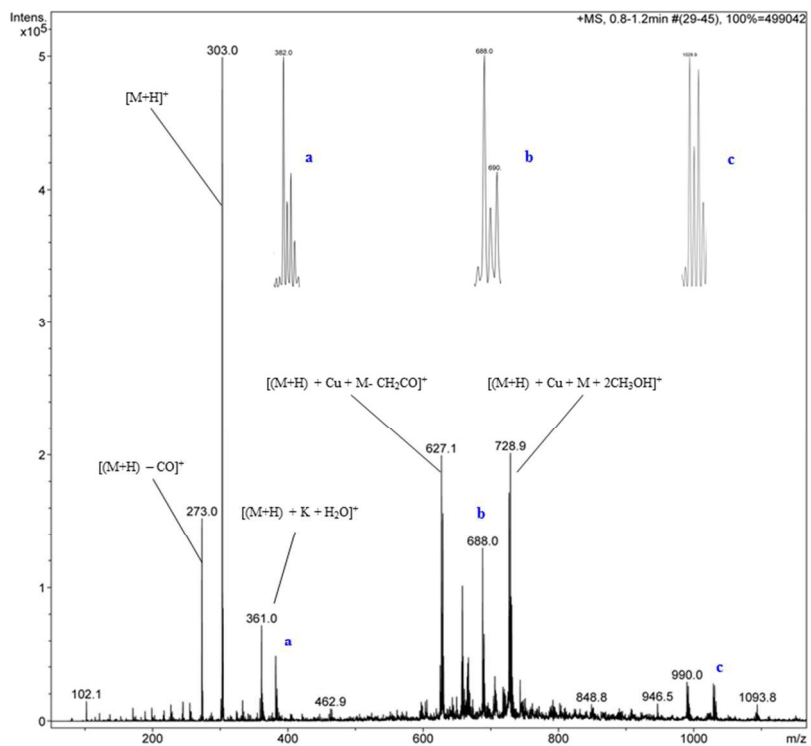|	R1	R2	R3	R4	R5
5	H	H	H	H	H
6	CH3	H	CH 3	CH3	H
7	CH3	H	CH3	CH3	CH3
13	Ac	Ac	Ac	Ac	Ac

8

14
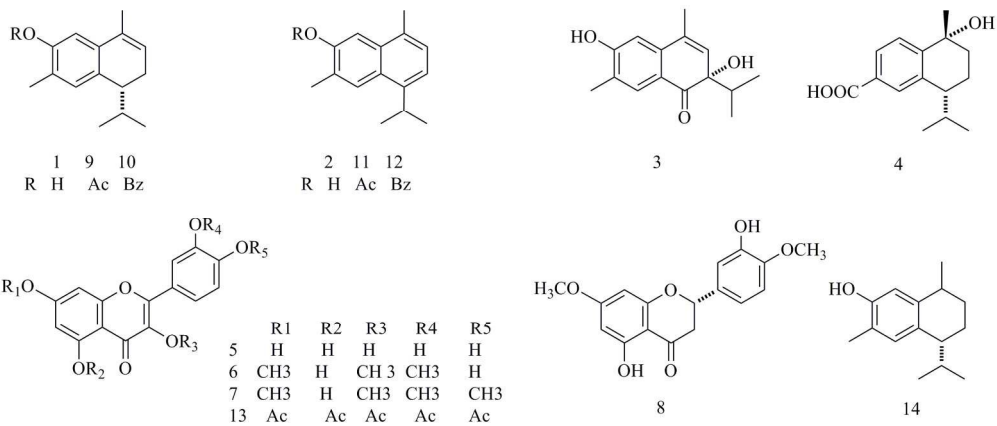
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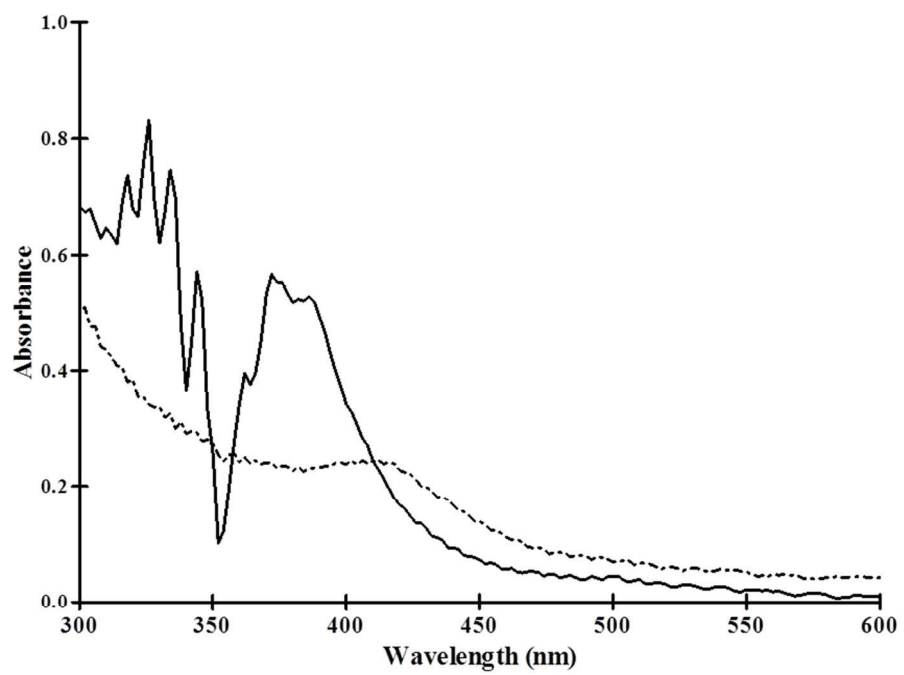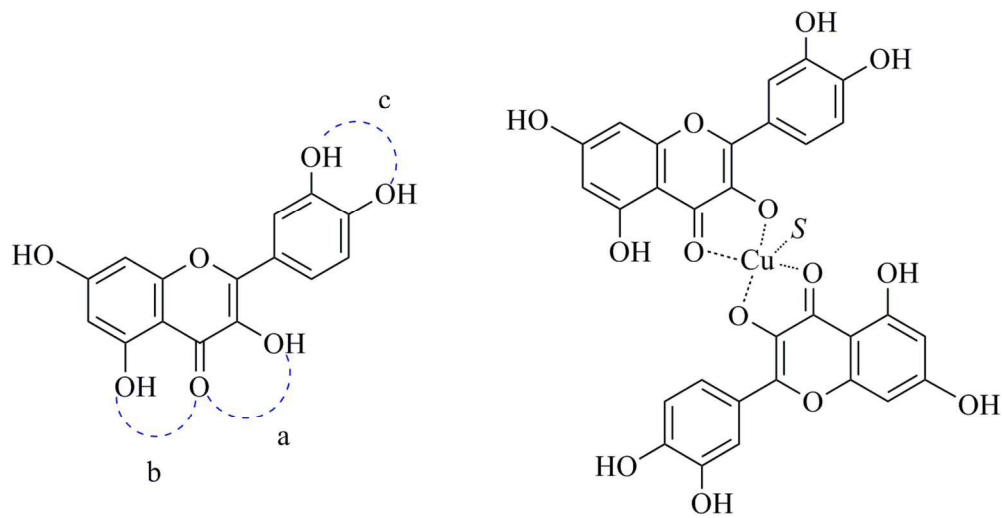
254x190mm (96 x 96 DPI)

254x190mm (96 x 96 DPI)

254x190mm (96 x 96 DPI)

254x190mm (96 x 96 DPI)

118x60mm (300 x 300 DPI)