ANTI-INFLAMMATORY AND PRO-APOPTOTIC PROPERTIES OF THE NATURAL COMPOUND O-ORSELLINALDEHYDE THROUGH IKK-2 INHIBITION

Journal:	*Journal of Agricultural and Food Chemistry*
Manuscript ID	Draft
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Tomás-Hernández, Sarah; Universitat Rovira i Virgili, Biochemistry and Biotechnology Department Garcia-Vallvé, Santiago; Universitat Rovira i Virgili Pujadas, Gerard; Universitat Rovira i Virgili, Bioquímica i Biotecnologia Valls, Cristina; Universitat Rovira i Virgili Ojeda-Montes, María José; Universitat Rovira i Virgili Gimeno, Aleix; Universitat Rovira i Virgili Ceretó-Massagué, Adrià; Universitat Rovira i Virgili Roca-Martínez, Joel; Universitat Rovira i Virgili, Biochemistry and Biotechnology Department Recio, Manuel Suarez; Universitat Rovira i Virgili, Biochemistry and Biotechnology Department Arola, Luis; Universitat Rovira i Virgili, Dept. Bioquímica y Biotecnologia. Facultat de Química de Tarragona. Mulero, Miquel; Universitat Rovira i Virgili , Biochemistry and Biotechnology Department Beltran-Debón, Raul; Universitat Rovira i Virgili

ANTI-INFLAMMATORY AND PRO-APOPTOTIC PROPERTIES OF THE NATURAL COMPOUND *O*-ORSELLINALDEHYDE THROUGH IKK-2 INHIBITION

Sarah Tomas-Hernandez[1], *Santiago Garcia-Vallvé*[1,2], *Gerard Pujadas*[1,2], *Cristina Valls*[1], *María José Ojeda-Montes*[1], *Aleix Gimeno*[1], *Adrià Cereto-Massagué*[1], *Joel Roca-Martinez*[1], *Manuel Suárez*[3], *Lluis Arola*[2,3], *Miquel Mulero*[*1], *Raúl Beltran-Debón*[1].

[1]*Cheminformatics and Nutrition Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili (URV), Campus de Sescelades, 43007 Tarragona, Spain.*

[2] *Technological Unit of Nutrition and Health, EURECAT-Technological Center of Catalonia, Reus, Spain*

Avinguda Universitat, 1, 43204 Reus, Spain.

[3]*Nutrigenomics Research Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili , (URV), Campus de Sescelades, 43007 Tarragona, Spain*

***Corresponding author:** Miquel Mulero (fax: +34 977558232. E-mail address: miquel.mulero@urv.cat)

Abstract

Metabolic syndrome is a cluster of medical conditions that increases the risk of developing cardiovascular disease and type 2 diabetes. Numerous studies have shown that inflammation is directly involved in the onset of metabolic syndrome. In this study *in silico* techniques were applied to a natural products database containing molecules isolated from mushrooms from the Catalan forests to predict molecules that can act as human nuclear-factor kB kinase 2 (IKK-2) inhibitors. IKK-2 is the main component responsible for activating the nuclear-factor kB transcription factor (NF-kB). One of these predicted molecules was *o*-orsellinaldehyde, a molecule present in the mushroom *Grifola frondosa*. This study shows that o-orsellinaldehyde presents anti-inflammatory and pro-apoptotic properties by acting as IKK-2 inhibitor. Additionally, we suggest that the anti-inflammatory properties of *Grifola frondosa* mushroom could partially be explained by the presence of *o*-orsellinaldehyde on its composition.

Keywords:

Inflammation, apoptosis, IKK-2, NF-kB, *o*-Orsellinaldehyde, *Grifola frondosa.*

Chemical compounds studied in this article:

o-Orsellinaldehyde (PubChem CID:251690)

1. Introduction

Metabolic syndrome is a cluster of medical conditions that increase the risk of developing cardiovascular disease and type 2 diabetes. Considerable attention has been paid to this syndrome since it affects one in five people and it is a leading cause of death in developed countries. [1]

In the last few years, numerous studies have shown that the inflammation process is directly involved in the onset of metabolic syndrome [2,3]. Furthermore, nowadays it is accepted that chronic subclinical inflammation is a part of the insulin resistance syndrome [4,5]. That would explain why targeting inflammation mediators has become a new challenge for preventing or decreasing metabolic syndrome [6].

In recent years, natural products and their active metabolites have become increasingly important on pharmaceutical research [7]. Unfortunately, bioactivity screening in extracts exclusively by *in vitro* or *in vivo* approaches is a complex and expensive process that is difficult to afford [8]. However, virtual screening (VS) workflows have been successfully used to screen large natural products databases in order to identify new bioactive molecules for specific targets [9,10]. In particular, our group has wide experience on VS and has published several research projects which aimed to identify new natural compounds with undescribed bioactivity and with great interest in medicinal chemistry [11,12].

Concretely, one interesting target that has become very attractive for anti-inflammatory drugs development is the human inhibitor nuclear-factor kB kinase 2 (IKK-2) [7,13]. IKK-2 is a serine-threonine protein kinase belonging to the IKK complex and is the main component responsible for activating the nuclear-factor kB transcription factor (NF-kB) in response to inflammatory stimuli. NF-kB is an important transcription factor involved in propagating the cellular response to inflammation [14]. This universal transcription factor regulates the expression of several components of the immune system including pro-inflammatory cytokines, chemokines, adhesion molecules and inducible enzymes such as cycloxygenase-2 and inducible nitric oxide synthase [15].

Inflammatory mediators stimulate the inflammation pathway by activating the IKK-2 that phosphorylates IKβα and leads to its degradation; As a consequence, free NF-kB translocates to the nucleus and induces the transcription of all the elements that controls the inflammatory response. [16]. In this study, firstly, we applied previously validated VS workflow developed by our group [17] to a natural products database containing a total of 5134 molecules isolated from mushrooms from the Catalan forests in order to identify potential IKK-2 inhibitors. One of this predicted IKK-2 inhibitors was 2,4-dihydroxy-6-methylbenzaldehyde, most commonly known as *o*-orsellinaldehyde. It has been described that this molecule is present in some natural sources such as *Agrocybe praecox, Aspergillus rugulosus,* and *Grifola frondosa* [18–20]. Interestingly the last one, *Grifola frondosa*, has been traditionally used as a medicinal mushroom for treating pain and some inflammatory states in Asia [21], where it is commonly known as Maitake.

Secondly, we aimed to investigate and validate the anti-inflammatory properties of *o*-orsellinaldehyde by applying *in vitro* and *in vivo* techniques. Finally, Maitake mushrooms were analyzed by liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) in order to identify and quantify the amount of this compound and evaluate the feasibility of preparing extracts from this natural source that were enriched on *o*-orsellinaldehyde.

2. Materials and methods

2.1 *In silico* virtual screening

2.1.1 Building the initial database of molecules to be screened and virtual screening

The scientific name of all the mushrooms that are present at the Catalan forest were obtained from the webpage of the Catalan Society of Micology (http://www.micocat.org/). We chose the mushrooms present in the Catalan forest as source to find bioactive molecules mainly due to the easy obtention of the samples in this region. A total of 5134 molecules isolated from any mushroom that share the same genus than those found at that webpage where downloaded from the Reaxys Medicinal Chemistry database (http://www.reaxys.com). These molecules were then filtered with FaF-Drug2 [22] to keep only those that show good ADMET properties (*i.e.*, 3582 molecules) and submitted to a previously published VS workflow that has been shown to be effective to predict new IKK-2 inhibitors [11]. Details about the filters of this VS can be also found at the original publication [11].

2.2 *In vitro* experiments with RAW 264.7 cells

2.2.1 Cell culture and viability

RAW264.7 macrophages obtained from ECACC (Sigma-Aldrich Chemical, Madrid, Spain) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), L-Glutamine (2 mM), HEPES (25 mM) and Penicillin-Streptomycin (P/S) (100 U/mL of) at 37°C in a 5% CO_2 humidified atmosphere. DMEM, FBS, P/S and L-Glutamine were bought from Lonza, Cultek, (Barcelona, Spain). HEPES buffer solution was obtained from Gibco by Fisher (Madrid, Spain). For the analysis of cell viability, an MTT assay was performed. RAW264.7 cells (250.000 cells/well) were treated with different concentrations of *o*-orsellinaldehyde ranging from 1 to 50 µg/mL (ref: 657603 Sigma-Aldrich Chemical, Madrid, Spain) for 8 hours. After this incubation period, medium was replaced by 200 µl of fresh DMEM with 50 µl of 5mg/mL of MTT

solution and the plate was incubated at 37°C for 4 hours in darkness. The colored formazan product was then dissolved in DMSO and quantified using a scanning multi-well spectrophotometer (BioTek EON, Izasa, Barcelona, Spain) at a wavelength of 570 nm.

2.2.2 Cell treatment

RAW264.7 cells were seeded into a 12-well plate at a density of 250.000 cells/well. 24 hours after platting, cells were pre-incubated with *o*-orsellinaldehyde at the selected concentrations for 30 minutes and then the inflammatory stimuli was then added (LPS 1 μg/mL). As a positive control of anti-inflammatory agent we used a commercial inhibitor (IKK-2 Inhibitor IV Calbiochem, USA) which target is IKK-2. As a negative control, cells were left untreated. 8 hours after the LPS addition the culture medium was collected for IL-6 and nitrite determination. Cells were then washed with PBS and lysed in RIPA buffer (25mM Tris–HCl; pH 7.4, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a protease and phosphatase inhibitor mixture (Phenymethanesulfonyl fluoride solution), Protease inhibitor cocktail, Phosphatase inhibitor cocktail-2, and Phosphatase inhibitor cocktail-3, all from Sigma-Aldrich Chemical, Madrid, Spain). The resulting solution was collected into microcentrifuge tubes for future protein determination and Western Blot assay.

2.2.3 Nitrite and IL-6 Determination

Nitrite concentration was measured by use of Griess reaction [23]. Briefly, 50 μL of medium was added in a 96-well plate and mixed with 100 μL of 1% sulfanilamide in 0.5 M HCl. The plate was incubated at 4ºC for 10 minutes and 50 μL of NED (N-1-naftiletilendiamine) solution was then added to each well. After 30 minutes at 4°C the optical density at 540 nm was measured. The values were interpolated with a standard curve with known concentrations of nitrite oxide (NO).

Il-6 concentration in the supernatants and serum was determined by a commercial ELISA assay (IL-6 ELISA-MAX^{TM}fromBiolegend) according to the manufacturer's instructions.

134 **2.2.4 Western Blot analysis**

135 After protein quantification using BCA assay (Thermo/Pierce, Rockford,IL, USA) -proteins were
136 separated on sodium dodecyl sulfate (SDS)–polyacrylamide 10% gels and transferred to
137 polyvinylidene difluoride (PVDF) membranes using a Trans-Blot Turbo Blotting system (Bio-Rad,
138 Barcelona, Spain). Membranes were blocked with 5% nonfat milk in PBS–Tween (0.1%) for one
139 hour. Then, membranes were incubated overnight at 4ºC with the following antibodies (dilution
140 1.1000): polyclonal antibody for iNOS (Santa Cruz Biochemicals), polyclonal antibody for
141 Phospho-Ikβ-α (Ser32) (Cell Signaling Technology) or polyclonal antibody for β-actin (Sigma-
142 Aldrich Chemical, Madrid, Spain). After washing three times with PBS–Tween, membranes were
143 incubated with the secondary antibody anti-rabbit (Merck, Barcelona, Spain) (dilution 1.10000)
144 conjugated with horseradish peroxidase for 1 h and then washed three more times with PBS-Tween.
145 The immunoreactive proteins were visualized using an enhanced chemiluminescence substrate kit
146 (ECL plus; Amersham Biosciences, GE Healthcare) according to the manufacturer's instructions.
147 Digital images were obtained with a GBOX Chemi XL 1.4 system (Syngene, UK), which allows
148 quantification of the band intensity. The protein load was monitored via the immuno-detection of β-
149 actin.

150 **2.2.5 IKK-2 kinase assay**

151 The effect of *o*-orsellinaldehyde on IKK-2 kinase activity was determined using the ELISA assay
152 Cyclex IKKα and IKKβ Inhibitor Screening Kit (MBL International, Woburn, MA, USA). Briefly,
153 plates were pre-coated with a substrate corresponding to recombinant IKβα, which contains two
154 serines (Ser32 and Ser36) that are phosphorylated by IKKα and IKKβ. In order to develop the
155 assay, both test compounds and the enzyme was diluted in assay buffer and pipetted into the wells
156 allowing substrate phosphorylation. The amount of phosphorylated substrate was detected using a
157 specific anti-phospho-Ikβα (Ser32) antibody HRP-conjugate and blue color development with TMB
158 substrate. The ELISA stop solution was then used to stop the color development, and the

absorbance was read at 450 nm (with a reference wavelength of 540–600 nm) using an ELISA reader (BioTek EON, Izasa, Barcelona, Spain). The absorbance is directly related to the IKK-2 activity level. In addition, one positive and one negative control were included on the test. As a positive control we used the commercial inhibitor (1μM), mentioned before (IKK-2 Inhibitor IV, 401481 Millipore, Madrid), as a negative control, DMSO (vehicle) was added to the wells. Three repetitions were made for all the activity assay.

2.2.6 *In vitro* IKK-2 phosphorylation Assay

RAW264.7 cells were seeded into a 12-well plate at a density of 250.000 cells/well. After 24 hours of platting, cells were pre-incubated with *o*-orsellinaldehyde at the selected concentrations for 30 minutes and then the inflammatory stimuli was added (LPS 1μg/mL) for 45 additional minutes. As a positive control of IKK-2 inhibitor agent we used the same commercial drug mentioned before. Some cells were also treated just with the vehicle used to dissolve the test compound. Once the experiment finished, the medium was discarded and cells were then washed with PBS and lysed in RIPA buffer containing a protease and phosphatase inhibitor mixture. The mix result was recollected into microcentrifuge tubes for future protein determination and Western Blot assay.

2.3 *In vivo* experiments

2.3.1 Animals

Male Balb/c mice (5–6 weeks of age) were obtained from Charles River (Barcelona, Spain). Animals were housed under standard conditions with a 12-h light/dark cycle. The animals were acclimatized to the environment for ten days before starting the experiments. All procedures were performed in accordance with the European Communities Council Directive regarding the protection of experimental animals (86/609/EEC) and the procedure established by the Departament d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya.

2.3.2 Intraperitoneal administration of *o*-orsellinaldehyde

For the *in vivo* assays, an LPS endotoxic shock was induced in order to evaluate the anti-inflammatory activity of *o*-orsellinaldehyde. Mice were divided into 8 groups (n=6): groups 1 to 4 received an intraperitoneal injection of *o*-orsellinaldehyde at different concentrations (10, 40, 60 100 mg/kg body weight (b.w.)). Group 5 received dexamethasone (12 mg/kg b.w.) as an anti-inflammatory agent control. 1 hour after the molecule administration, an intraperitoneal injection of LPS at a concentration of 1 mg/kg b.w. was administered. The positive and negative groups (named group 6 and group 7) received just LPS or PBS, respectively. Finally group 8 received only *o*-orsellinaldehyde at the maximum concentration (100 mg/kg b.w) in order to discard any side effects produced by this molecule. 2 h after LPS injection, mice were anesthetized and 1ml of blood was obtained by cardiac puncture in order to measure IL-6 concentration in plasma.

2.3.3 Oral administration of *o*-orsellinaldehyde

Mice were divided into 6 groups (n=6) depending on the treatment. Thus, o-orsellinaldehyde (100 mg/kg b.w) was orally administrated 30 minutes (group 1), one hour (group 2), or two hours (group 3), before LPS stimulation.

As anti-inflammatory control, one group (group 4) received dexamethasone. The positive and negative groups (group 5 and 6 respectively) received just LPS or PBS, respectively. Finally, 2 h after LPS injection, mice were anesthetized and 1 ml of blood was obtained by cardiac puncture in order to measure IL-6 concentration in plasma.

2.4 Cytotoxic Effect against HepG2

2.4.1 Cell culture and viability

The human hepatoblastoma HepG2 cells (obtained from ECACC, Sigma-Aldrich Chemical, Madrid, Spain) were cultured in DMEM supplemented with 10% FBS, 2% P/S, 1% L-glutamine,

and 1% NEAA (non-essential amino acids) at 37°C in a 5% CO2 humidified atmosphere. In order to measure the reduction of cell viability an MTT assay was performed. For this assay cells were seeded at a density of 5×10^4 cells/well on a 96-well culture plate and were incubated overnight. Once confluence was reached, cells were treated with increasing concentrations of *o*-orsellinaldehyde and incubated for 48 hours. After this incubation period, the MTT assay was performed following the protocol mentioned above.

2.4.2 Cell treatment and analysis for cleaved caspase-3 detection

Caspases, a group of cysteine proteases, are the key mediators of apoptosis [24]. Therefore, in order to elucidate if *o*-orsellinaldehyde shows a cytotoxic effect on HepG2 cells through an apoptotic process, we treated these cells with *o*-orsellinaldehyde at increasing concentrations and we then quantified the cleaved caspase-3 protein expression.

For this purpose, HepG2 cells were seeded at a density of 3×10^5 cells/well on a 12-well culture plate and incubated overnight. Once cells reached confluence, they were treated with *o*-orsellinaldehyde at increasing concentrations for 48 hours. After this incubation period the medium was discarded and cells were washed with PBS and lysed in RIPA buffer containing a protease and phosphatase inhibitor mixture. The resulting solution was recollected into microcentrifuge tubes for future protein determination and Western Blot assay as previously explained.

After cell treatment and protein extraction and quantification, the expression of cleaved caspase-3 was determined by immunoblot following the same protocol mentioned above and using the polyclonal antibody for cleaved caspase-3 (Cell Signaling Technology. dilution 1.1000).

2.5. Extraction and quantification of *o*-orsellinaldehyde into Maitake mushrooms

Finally, once evaluated the bioactivity of the pure molecule, we decided to quantify the amount of *o*-orsellinaldehyde in Maitake mushrooms in order to evaluate its potential as natural source of this

compound. To do this, firstly we extracted the compound from the Maitake mushroom and after that we analyzed it by LC-QTOF-MS.

To perform the extractions, acetonitrile (HPLC grade), acetone, ethanol, ethyl acetate and acetic acid (all provided by ScharlauChemie; Barcelona, Spain) were used. Ortho-phosphoric acid (85%) was purchased from Panreac (Barcelona, Spain) and water was of Milli-Q quality (Millipore Corp, Bedford, MA, USA). A stock solution of *o*-orsellinaldehyde at 1000 mg/L was dissolved in Milli-Q water and stored in a dark flask at -18 ºC.

2.5.1 Preparation of the extract

To prepare an extract containing *o*-orsellinaldehyde from Maitake mushroom we followed the method described by Lee *et al.* (2012) with some modifications. Briefly, 3 grams of lyophilized Maitake mushrooms were mixed with 80 mL of pure ethanol and were continuously agitated during 5 hours at 65 ºC. Then, it was centrifuged at 3,000 rpm for 10 min at 4ºC. The supernatant was rotavaporated to dryness, setting the temperature at 30 ºC and was further dissolved in 80 mL of Milli-Qwater. Then, it was purified using 50 mL of ethyl acetate. To allow complete separation of the 2 phases it was kept overnight in darkness into the hood. Aqueous phase was separated, rotavaporated at 30 ºC to remove organic solvent and finally lyophilized. The lyophilized extract was dissolved in 10 mL of Milli-Qwater and stored at -18 ºC until analysis.

The day of analysis, extracts were further purified in order to remove components that could interfere with the quantification by using microelution plates (Waters, Milford, USA) packed with 2 mg of OASIS HLB sorbent (Waters, Milford, USA) following the method previously described with some modifications [25]. Firstly, the wells were sequentially conditioned by using 250 µL of methanol and 250 µL of Milli-Qwater:acetic acid (99.2:0.2, v/v). Then, 300 µL of extract mixed with 300 µL of phosphoric acid 4% were loaded onto the plate. After that, the clean-up of the plates was sequentially done with 200 µL of Milli-Qwater and 200 µL of Milli-Qwater:acetic acid (99.8:0.2, v/v) to eliminate any interference that the sample might contain. Finally the elution of the

retained compounds was done with 2x50 µL of acetone:Milli-Qwater:acetic acid (70:29.5:0.5, v/v/v). 5 µL of the eluted was directly injected into the LC-QTOF-MS.

2.5.2 LC-QTOF-MS analysis of extracted samples

The LC-QTOF-MS system consisted of an Agilent 1260 Series (Agilent Technologies, Palo Alto, U.S.A.) coupled to a 6540 ESI-QTOF (Agilent Technologies) operated in positive electrospray ionization mode (ESI+). Separation was carried out in a using a XBridge™ Shield RP18 column (3.5 µm, 150 mm x 2.1 mm i.d.) from Waters equipped with a Pre-Column Zorbax SB-C18 (3.5 µm, 15 mm x 2.1 mm i.d.) from Agilent. The software used was Masshunter. Drying gas temperature was 350 ºC and its flow rate was held at 12 L/min. On the other hand pressure of the gas nebulizer was 45 psi and the capillary voltage was set at 4000 V. Fragmentor was set at 120V, skimmer at 65V and OCT 1RF Vpp was set at 750V.

During the analysis, the column was kept at 25º C and the flow rate was 0.4 mL/min. The solvent composition was solvent A: Milli-Qwater/acetic acid (99.8:0.2 v/v) and solvent B: acetonitrile. Solvent B was initially 10% and was gradually increased reaching 50% at 20 minutes and 100% at 22 min. Then it was maintained isocratically 5 min and after that it was reduced to 10% in 1 minute and was held at initial conditions during 7 minutes to re-equilibrate the column.

3. Results

3.1 *In silico* virtual screening

The VS identified 20 molecules as possible IKK-2 inhibitors; one of them was *o*-orsellinaldehyde (see Figure 1A). Figure 1B shows how the docked pose of *o*-orsellinaldehyde fits at the structure-based pharmacophore that is located at the ATP binding site. This pharmacophore is formed by two hydrogen-bond donors (in blue), one hydrogen-bond acceptor (in red) and one hydrophobic region (in green), with tolerances (*i.e.*, radii) of 1.5, 1.5 and 3.0 Å, respectively. *o*-orsellinaldehyde matches three out of the four sites (*i.e.*, **A1**, **D3** and **H4**) with one of the two hydroxyls simultaneously matching **A1** and **D3** by accepting the proton from the nitrogen main chain of Cys99 and acting as a hydrogen bond donor with the main chain carbonyl oxygen of Glu97.

3.2 Anti-inflammatory effects of *o*-orsellinaldehyde in *in vitro* experiments with RAW 264.7 cells

3.2.1 Effect *o*-orsellinaldehyde on cell viability of RAW264.7 macrophage cells

To determine whether *o*-orsellinaldehyde influences the viability of RAW264.7 cells an MTT assay was performed. As shown in figure 2, after 8 hours of treatment, *o*-orsellinaldehyde did not affect cell viability at concentrations ranging from 1-50 μg/mL. These data indicates that *o*-orsellinaldehyde is not cytotoxic to RAW 264.7 cells at the tested concentrations.

3.2.2 *o*-Orsellinaldehyde inhibits nitrite production in LPS-activated macrophages

To evaluate the effect of *o*-orsellinaldehyde on NO production in RAW 264.7 cells we pretreated macrophages with or without *o*-orsellinaldehyde (20, 30, 40 or 50 μg/mL) for 30 minutes before stimulation with 1 μg/mL of LPS for 8 hours. The nitrite concentration in the medium was measured by use of Griess reaction. As it can be elucidated from figure 3A when RAW264.7 cells

were stimulated with LPS (1 μg/mL) the concentration of nitrite increased in the culture medium compared to the basal levels. However 1 hour pre-treatment of the cells with *o*-orsellinaldehyde at the assayed concentrations caused a reduction of NO production in a dose-dependent manner (see figure 3A).

3.2.3 *o*-Orsellinaldehyde decreases iNOS protein expression in LPS-activated macrophages

In order to elucidate if the cause of NO reduction was due to decreased iNOS protein levels, the effect on iNOS protein expression was determined by immunoblot. As reflected in figure 3B, treatment with LPS increased iNOS protein expression in RAW264.7 cells, however the preincubation with *o*-orsellinaldehyde caused a significantly dose-dependent reduction of LPS-stimulated iNOS protein expression.

3.2.4 *o*-Orsellinaldehyde inhibits de release of IL-6 proinflammatory cytokine in murine macrophages

RAW 264.7 cells were pre-treated with or without *o*-orsellinaldehyde (20, 30, 40 or 50 μg/mL) for 30 minutes and then stimulated with LPS (1 μg/mL) for 8 h. In order to validate whether *o*-orsellinaldehyde was able to reduce IL-6 production an ELISA assay was performed. As figure 3C shows, the release of IL-6 was increased in the LPS-treated group and this effect was markedly decreased by *o*-orsellinaldehyde in a dose-dependent manner, reaching an 87% of inhibition with the higher concentration tested (50 μg/mL) which suggest that *o*-orsellinaldehyde seems to be even more effective than the commercial inhibitor.

3.2.5 *o*-Orsellinaldehyde binds IKK-2 and inhibits its kinase activity

To determine whether *o*-orsellinaldehyde directly targets IKK, the *Cyclex IKKα and IKKβ Inhibitor Screening Kit* was performed. With this assay we aimed to study the effects of *o*-orsellinaldehyde on IKK-2 kinase activity by directly applying this molecule to recombinant IKKβ protein in a cell-

free system. As figure 4A shows, *o*-orsellinaldehyde was able to reduce IKK kinase activity in a dose-response manner.

3.2.6 *o*-Orsellinaldehyde inhibits IKβα phosphorylation in LPS-activated macrophages

In order to validate that *o*-orsellinaldehyde was capable of inhibiting IKβα phosphorylation in an *in vitro* system, RAW264.7 cells were pretreated with *o*-orsellinaldehyde for 30 minutes and then, the inflammatory stimuli (LPS 1 μg/mL) was added for 45 additional minutes. As depicted in figures 4B and 4C, LPS drastically increased the phosphorylation of IKβα by IKK-2, however this phosphorylation is significantly decreased when cells are previously treated with *o*-orsellinaldehyde. Thus confirming that this molecule is interacting with IKK-2 enzyme preventing its kinase activity

3.3 *In vivo* experiments

3.3.1 *o*-Orsellinaldehyde reduced the serum IL-6 concentration in LPS-stimulated animals

To assay whether anti-inflammatory effects of *o*-orsellinaldehyde occurred *in vivo*, mice received and intraperitoneal injection of the compound at different concentrations one hour prior LPS stimulation. As depicted in figure 5A, injecting mice with LPS highly increased the concentration of IL-6 in serum compared to untreated mice. Interestingly, *o*-orsellinaldehyde significantly reduced the serum IL-6 production by 21% in the LPS-stimulated animals at the higher concentration tested. This figure also shows that pretreatment with the anti-inflammatory steroid dexamethasone caused a 33% reduction in serum IL-6 in LPS-stimulated mice. It was also evidenced that *o*-orsellinaldehyde did not produced any pro-inflammatory effect by itself.

3.3.2 Oral administration of *o*-orsellinaldehyde reduced the serum IL-6 concentration in LPS-stimulated animals.

We next examined if this reduction of the inflammation was also achieved when the *o*-orsellinaldehyde was orally administrated. In this case, 300 μl of o-orsellinaldehyde at 100 mg/kg b.w. was orally administrated 30 minutes, one hour or two hours depending on the treated group

and then the LPS stimuli was injected intraperitoneally. As can be seen in figure 5B serum IL-6 production markedly increased when mice were treated with LPS. However the IL-6 decreased when animals were also orally treated with the compound (100 mg/kg b.w), reaching a reduction of 20.5% when the tested molecule was administrated one hour previous to the LPS injection.

3.4 Cytotoxic effect against HepG2 cell

3.4.1 Cell culture and viability

As previously reported [20] *o*-orsellinaldehyde has been isolated from different strains of *Aspergillus rugulosus* as a cytotoxic compound toward Hep3B human hepatoma cells through apoptosis. In our assays, HepG2 cells were used to evaluate the tumoricidal activity of the compound. In order to evaluate this, cells were treated with *o*-orsellinaldehyde for 48 hours and then an MTT assay was carried out. As depicted in figure 6A, *o*-orsellinaldehyde exhibits a cytotoxic effect on HepG2 cells in a dose-response manner.

3.4.2 *o*-Orsellinaldehyde increased cleaved caspase-3 expression in HepG2 cells

After 48 hours treatment with *o*-orsellinaldehyde, cleaved caspase-3 expression was quantified with a western blot assay in order to elucidate if the cytotoxic effect of *o*-orsellinaldehyde shown on HepG2 cells were mediated through an apoptotic process. As shown in figures 6C and 6D, *o*-orsellinaldehyde markedly increased cleaved caspase-3 expression in a dose response manner, confirming the apoptotic process.

3.5 Quantification and detection of *o*-orsellinaldehyde in extracted samples by LC-QTOF-MS.

Quantification of *o*-orsellinaldehyde in the Maitake extract obtained following the procedure above described was carried out by using a 6-points calibration curve of the standard compound. Figure 7 confirms that *o*-orsellinaldehyde can be isolated from Maitake extracts. However the amount of this molecule contained in Maitake mushroom is very low (3.41 µg *o*-orsellinaldehyde /g lyophilized

Maitake). In that sense, larger infrastructures would be necessary in order to isolate enough amount of the anti-inflammatory molecule for treating cells directly with the enriched Maitake extracts.

4. Discussion

Natural products have attracted considerable attention on pharmaceutical research field since they are a very important source when looking for novel molecules that could be used as lead compounds during the development of new drugs. More concretely, natural products and their principles have played a very important role on the discovery of new molecules with anti-inflammatory properties [26]. However, the identification process by using exclusively *in vitro* and *in vivo* approaches is very long and expensive. In that sense, using VS workflows help to improve this identification process as it can be used to easily screen large natural product databases [9–11].

The present research project aimed to identify and validate novel molecules from natural origin that can act as IKK-2 inhibitors by using *in silico* techniques. In order to achieve this goal we first applied virtual screening workflows to a natural molecules database in order to look for natural extracts that contain IKK-2 inhibitors and then we validated the anti-inflammatory properties of the selected compound in an *in vitro* and *in vivo* context.

The VS experiments successfully identified a molecule, *o*-orsellinaldehyde, with high chance of acting as IKK-2 inhibitor. It has been described that this molecule is present in the *Grifola frondosa* mushroom specie [20]. In that sense, in order to validate the *in silico* predictions we performed a kinase assay that confirmed that *o*-orsellinaldehyde directly targets IKK-2 and reduces its IKK-2 kinase activity in a dose-response manner (Figure 4A). Additionally, we validate that *o*-orsellinaldehyde significantly inhibited IKβα phosphorylation in LPS-stimulated RAW 264.7 macrophage cells (Figures 4C and 4D). These results confirmed that *o*-orsellinaldehyde exerts its anti-inflammatory effects by modulating NF-kB activity.

Moreover, the anti-inflammatory properties of the studied molecule have also been demonstrated by the obtained results referred to the reduction of nitrites, IL-6 and iNOS expression in the *in vitro* model used as it can be shown in figure 3.

To study if this compound is also effective *in vivo* we induced an LPS endotoxic shock model in Balb/c mice. This model can greatly increase the production of serum inflammatory mediators [27]. In both accomplished studies (intraperitoneal administration and oral administration of *o*-orsellinaldehyde) the molecule significantly reduced the serum IL-6 concentration (Figure 5)

All this findings provide strong evidences that *o*-orsellinaldehyde possesses anti-inflammatory properties and that it exerts its activity by influencing NF-kB activity, more concretely, acting as IKK-2 inhibitor.

Furthermore, previous studies have shown that *o*-orsellinaldehyde is also able to inhibit growth as well as induce apoptosis in various types of cancer cell lines cases (Hep3B human hepatocellular carcinoma cells and MRC-5 human lung fibroblast cells). It is also been described that in some particular cases this molecule has cytotoxic effect [19,20]. We have also observed this apoptotic activity from *o*-orsellinaldehyde in our experiments with HepG2 cells. Figure 6 confirmed that the *o*-orsellinaldehyde exhibits a cytotoxic effect on these cells and that this effect was mediated through an apoptotic process.

At this point, it is interesting to mention that NF-kB is not only related with inflammation process, this enzyme complex is also involved in propagating the cellular response to apoptosis and carcinogenesis. The functions of NF-kB gene targets span diverse cellular processes, including adhesion, immune regulation, apoptosis, proliferation and angiogenesis [28,29]. Consequently, the NF-kB transcription factor plays an important role in cancer and related diseases.

In that sense, numerous studies have been published reporting that inhibition of NF-kB -regulated genes potentiates apoptosis and has anti-proliferative effects [30–33].

So, it is feasible that some of the cytotoxic and apoptotic effects of *o*-orsellinaldehyde observed against the cancer cells could be due to NF-kB inhibition. In agreement with our results, previous interesting studies have also described this induction of apoptosis in cancer cell lines when using IKK-2 inhibitors [34–36].

Related to the natural source *Grifola frondosa*, this mushroom is known by its Japanese name Maitake. The fungus is native to the northeastern part of Japan and North America, and it has been traditionally used as a medicinal mushroom. This mushroom has been used for treating pain and some inflammation states in Asia; however, in the past few years some groups have reported that this specie has also other properties. Briefly, in the same way that *o*-orsellinaldehyde, *Grifola frondosa* is also able to induce apoptosis in cancer cell lines and also inhibit the growth of various types of cancer cells [37–39]. Other studies revealed that Maitake have anti-cancer and anti-metastatic properties [40,41]. *Grifola frondosa* has also hypoglycemic effect, and so it is beneficial for the management and treatment of diabetes [42,43]. Finally, *Grifola frondosa* extracts also contain antioxidants and cyclooxygenase 2 inhibitors [44]. As *o*-orsellinaldehyde is one of the compounds of Maitake, it is feasible to state that at least, a part of the beneficial effects observed in the *Grifola frondosa* extracts could be explained by the IKK-2 inhibition triggered by *o*-orsellinaldehyde.

The results with the LC-QTOF-MS (Figure 7) show that *o*-orsellinaldehyde can be isolated from Maitake extracts, however the amount of this molecule contained in Maitake mushroom is very low, suggesting that further studies would be necessary in order to isolate enough amount of the anti-inflammatory molecule from the mushroom specie.

Taken together, our findings show that the molecule *o*-orsellinaldehyde was effective in suppressing the production of inflammatory mediators *in in vitro* and *in vivo* studies. The anti-inflammatory and pro-apoptotic activity of this molecules is mediated by its interaction with NF-kB complex, more concretely, we demonstrate that *o*-orsellinaldehyde is able to act as IKK-2 inhibitor, confirming the worth and importance of using bioinformatics tools when searching for new active principles.

439 Moreover, the anti-inflammatory properties of *Grifola frondosa* would partially be explained by the
440 presence of *o*-orsellinaldehyde on its composition. In summary this study presents the potential anti-
441 inflammatory activity of *o*-orsellinaldehyde, suggesting that it may be a potential preventive or
442 therapeutic candidate for the treatment of inflammatory disorders such as metabolic syndrome.

443 **Abbreviations Used**

444 NF-kB, nuclear-factor kB transcription factor; IKK-2, nuclear-factor kB kinase 2; VS, virtual
445 screening; iNOS, inducible nitric oxide synthase; LPS, Lipopolysaccharide; LC-QTOF-MS, liquid
446 chromatography/quadrupole time-of-flight mass spectrometry.

447 **Conflict of interest**

448 The authors declare no conflicts of interest.

449

450 **Funding Sources**

451 ST-H's contract was supported by grant 2012BPURV_30 from our University. AG's contract is
452 supported by grant 2015FI_B00655 from the Catalonia Government. MJO's contract was supported
453 by grants 2014PFR-URV-B2-67 and 2015PFR-URV-B2-67 from our University. MM is a Serra
454 Húnter Associate Professor.

455

456 **References**

457 (1) Zimmet, P.; Alberti, K. G. M. M.; Shaw, J. Global and societal implications of the diabetes
458 epidemic. *Nature* **2001**, *414* (December 2001), 782–787.
459 (2) Mathieu, P.; Lemieux, I.; Després, J.-P. Obesity, Inflammation, and Cardiovascular Risk.
460 *Clin. Pharmacol. Ther.* **2010**, *87* (4), 407–416.
461 (3) Roche, H. M.; Phillips, C.; Gibney, M. J. The metabolic syndrome: the crossroads of diet and
462 genetics. *Proc. Nutr. Soc.* **2005**, *64* (3), 371–377.
463 (4) Reilly, M. P.; Rader, D. J. The metabolic syndrome: more than the sum of its parts?

Circulation **2003**, *108* (13), 1546–1551.

(5) Libby, P. Inflammation and cardiovascular disease mechanisms. *Am. J. Clin. Nutr.* **2006**, *83* (2), 456S–460S.

(6) Welty, F. K.; Alfaddagh, A.; Elajami, T. K. Targeting inflammation in metabolic syndrome. *Transl. Res.* **2016**, *167* (1), 257–280.

(7) Haddad, P. S.; Azar, G. A.; Groom, S.; Boivin, M. Natural Health Products, Modulation of Immune Function and Prevention of Chronic Diseases. *eCAM* **2005**, *2* (4), 513–520.

(8) Smith, A. Screening for drug discovery: the leading question. *Nature* **2002**, *418* (6896), 453–459.

(9) Rollinger, J. M.; Stuppner, H.; Langer, T. Virtual screening for the discovery of bioactive natural products. In *Natural Compounds as Drugs Volume I*; Birkhäuser Basel: Basel, 2008; Vol. 65, pp 211–249.

(10) Rollinger, J.; Langer, T.; Stuppner, H. Strategies for Efficient Lead Structure Discovery from Natural Products. *Curr. Med. Chem.* **2006**, *13* (13), 1491–1507.

(11) Sala, E.; Guasch, L.; Iwaszkiewicz, J.; Mulero, M.; Salvadó, M.-J.; Pinent, M.; Zoete, V.; Grosdidier, A.; Garcia-Vallvé, S.; Michielin, O.; et al. Identification of human IKK-2 inhibitors of natural origin (part I): modeling of the IKK-2 kinase domain, virtual screening and activity assays. *PLoS One* **2011**, *6* (2), e16903.

(12) Guasch, L.; Ojeda, M. J.; Gonzá Lez-Abuín, N.; Sala, E.; Cereto-Massagué, A.; Mulero, M.; Valls, C.; Pinent, M.; Ardé Vol, A.; Garcia-Vallvé, S.; et al. Identification of Novel Human Dipeptidyl Peptidase-IV Inhibitors of Natural Origin (Part I): Virtual Screening and Activity Assays. *PLoS One* **2012**, *7* (9).

(13) Lewis, A. J.; Manning, A. M. New targets for anti-inflammatory drugs. *Curr. Opin. Chem. Biol.* **1999**, *3* (4), 489–494.

(14) Li, Q.; Verma, I. M. NF-κB regulation in the immune system. *Nat. Rev. Immunol.* **2002**, *2*

(10), 725–734.

(15) Kopp, E. B.; Ghosh, S. NF-kappa B and rel proteins in innate immunity. *Adv. Immunol.* **1995**, *58*, 1–27.

(16) Baldwin, A. S. The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol.* **1996**, *14* (14), 649–683.

(17) Sala, E.; Guasch, L.; Iwaszkiewicz, J.; Mulero, M.; Salvadó, M.-J. J.; Bladé, C.; Ceballos, M.; Valls, C.; Zoete, V.; Grosdidier, A.; et al. Identification of human IKK-2 inhibitors of natural origin (Part II): In Silico prediction of IKK-2 inhibitors in natural extracts with known anti-inflammatory activity. *Eur. J. Med. Chem.* **2011**, *46* (12), 6098–6103.

(18) Ballantine, J. A.; Hassall, C. H.; Jones, B. D. The biosynthesis of phenols—XVII. *Phytochemistry* **1968**, *7* (9), 1529–1534.

(19) Fushimi, K.; Anzai, K.; Tokuyama, S.; Kiriiwa, Y.; Matsumoto, N.; Sekiya, A.; Hashizume, D.; Nagasawa, K.; Hirai, H.; Kawagishi, H. Agrocybynes A-E from the culture broth of Agrocybe praecox. *Tetrahedron* **2012**, *68* (4), 1262–1265.

(20) Lin, J. T.; Liu, W. H. o-Orsellinaldehyde from the submerged culture of the edible mushroom Grifola frondosa exhibits selective cytotoxic effect against Hep 3B cells through apoptosis. *J. Agric. Food Chem.* **2006**, *54* (20), 7564–7569.

(21) Mayell, M. Maitake extracts and their therapeutic potential - A review. *Altern. Med. Rev.* **2001**, *6* (1), 48–60.

(22) Lagorce, D.; Sperandio, O.; Galons, H.; Miteva, M. A.; Villoutreix, B. O. FAF-Drugs2: free ADME/tox filtering tool to assist drug discovery and chemical biology projects. *BMC Bioinformatics* **2008**, *9*, 396.

(23) Dirsch, V.; Stuppner, H.; Vollmar, A. The Griess Assay: Suitable for a Bio-Guided Fractionation of Anti-Inflammatory Plant Extracts? *Planta Med.* **1998**, *64* (5), 423–426.

(24) Earnshaw, W. C.; Martins, L. M.; Kaufmann, S. H. Mammalian caspases: structure,

activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* **1999**, *68*, 383–424.

(25) Suárez, M.; Romero, M.-P.; Macià, A.; Valls, R. M.; Fernández, S.; Solà, R.; Motilva, M.-J. Improved method for identifying and quantifying olive oil phenolic compounds and their metabolites in human plasma by microelution solid-phase extraction plate and liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **2009**, *877* (32), 4097–4106.

(26) Welty, F. K.; Alfaddagh, A.; Elajami, T. K. Targeting inflammation in metabolic syndrome. *Transl. Res.* **2016**, *167*, 257–280.

(27) Buras, J. A.; Holzmann, B.; Sitkovsky, M. Model organisms: Animal Models of sepsis: setting the stage. *Nat. Rev. Drug Discov.* **2005**, *4* (10), 854–865.

(28) Aggarwal, B. B. Nuclear factor-kappaB: the enemy within. *Cancer Cell* **2004**, *6* (September), 203–208.

(29) Hayden, M. S.; Ghosh, S. Shared principles in NF-kappaB signaling. *Cell* **2008**, *132* (3), 344–362.

(30) Bharti, A. C.; Shishodia, S.; Reuben, J. M.; Weber, D.; Alexanian, R.; Raj-Vadhan, S.; Estrov, Z.; Talpaz, M.; Aggarwal, B. B. Nuclear factor-kappaB and STAT3 are constitutively active in CD138+ cells derived from multiple myeloma patients, and suppression of these transcription factors leads to apoptosis. *Blood* **2004**, *103* (8), 3175–3184.

(31) Anand, P.; Kunnumakkara, A. B.; Harikumar, K. B.; Ahn, K. S.; Badmaev, V.; Aggarwal, B. B. Modification of Cysteine Residue in p65 Subunit of Nuclear Factor- KappaB (NF KappaB) by Picroliv Suppresses NF-KappaB-Regulated Gene Products and Potentiates Apoptosis. *Cancer Res.* **2008**, *68* (21), 8861–8870.

(32) Bash, J.; Zong, W. X.; Gélinas, C. c-Rel arrests the proliferation of HeLa cells and affects critical regulators of the G1/S-phase transition. *Mol. Cell. Biol.* **1997**, *17* (11), 6526–6536.

(33) Aggarwal, B. B.; Ichikawa, H.; Garodia, P.; Weerasinghe, P.; Sethi, G.; Bhatt, I. D.; Pandey, M. K.; Shishodia, S.; Nair, M. G. From traditional Ayurvedic medicine to modern medicine: identification of therapeutic targets for suppression of inflammation and cancer. *Expert Opin. Ther. Targets* **2006**, *10* (1), 87–118.

(34) Schumacher, M.; Cerella, C.; Reuter, S.; Dicato, M.; Diederich, M. Anti-inflammatory, pro-apoptotic, and anti-proliferative effects of a methanolic neem (Azadirachta indica) leaf extract are mediated via modulation of the nuclear factor-κB pathway. *Genes Nutr.* **2011**, *6* (2), 149–160.

(35) Tse, A. K.-W.; Wan, C.-K.; Zhu, G.-Y.; Shen, X.-L.; Cheung, H.-Y.; Yang, M.; Fong, W.-F. Magnolol suppresses NF-kappaB activation and NF-kappaB regulated gene expression through inhibition of IkappaB kinase activation. *Mol. Immunol.* **2007**, *44* (10), 2647–2658.

(36) Jeon, J.; Park, K. A.; Lee, H.; Shin, S.; Zhang, T.; Won, M.; Yoon, H. K.; Choi, M. K.; Kim, H. G.; Son, C. G.; et al. Water extract of Cynanchi atrati Radix regulates inflammation and apoptotic cell death through suppression of IKK-mediated NF-κB signaling. *J. Ethnopharmacol.* **2011**, *137* (1), 626–634.

(37) Wang, Y.; Shen, X.; Liao, W.; Fang, J.; Chen, X.; Dong, Q.; Ding, K. A heteropolysaccharide, L-fuco-D-manno-1,6-α-D-galactan extracted from Grifola frondosa and antiangiogenic activity of its sulfated derivative. *Carbohydr. Polym.* **2014**, *101* (1), 631–641.

(38) Sanzen, I.; Imanishi, N.; Takamatsu, N.; Konosu, S.; Mantani, N.; Terasawa, K.; Tazawa, K.; Odaira, Y.; Watanabe, M.; Takeyama, M.; et al. Nitric oxide-mediated antitumor activity induced by the extract from Grifola frondosa (Maitake mushroom) in a macrophage cell line, RAW264.7. *J. Exp. Clin. Cancer Res.* **2001**, *20* (4), 591–597.

(39) Konno, S. Potential growth inhibitory effect of Maitake D-fraction on canine cancer cells. *Vet. Ther.* **2004**, *5* (4), 263–271.

(40) Masuda, Y.; Murata, Y.; Hayashi, M.; Nanba, H. Inhibitory effect of MD-Fraction on tumor metastasis: involvement of NK cell activation and suppression of intercellular adhesion molecule (ICAM)-1 expression in lung vascular endothelial cells. *Biol. Pharm. Bull.* **2008**, *31* (6), 1104–1108.

(41) Fullerton, S. A.; Samadi, A. A.; Tortorelis, D. G.; Choudhury, M. S.; Mallouh, C.; Tazaki, H.; Konno, S. Induction of apoptosis in human prostatic cancer cells with beta-glucan (Maitake mushroom polysaccharide). *Mol. Urol.* **2000**, *4* (1), 7–13.

(42) Hong, L.; Xun, M.; Wutong, W. Anti-diabetic effect of an alpha-glucan from fruit body of maitake (Grifola frondosa) on KK-Ay mice. *J Pharm Pharmacol* **2007**, *59* (4), 575–582.

(43) Xiao, C.; Wu, Q.; Xie, Y.; Zhang, J.; Tan, J. Hypoglycemic effects of Grifola frondosa (Maitake) polysaccharides F2 and F3 through improvement of insulin resistance in diabetic rats. *Food Funct.* **2015**, *6* (11), 3567–3575.

(44) Zhang, Y.; Mills, G. L.; Nair, M. G. Cyclooxygenase inhibitory and antioxidant compounds from the mycelia of the edible mushroom Grifola frondosa. *J. Agric. Food Chem.* **2002**, *50* (26), 7581–7585.

Figure captions

Figure 1. Virtual Screening of *o*-orsellinaldehyde. (A) Chemical structure of *o*-orsellinaldehyde. (B) Predicted binding pose for o-orsellinaldehyde at the binding site of human IKK-2.

Figure 2. Effect of *o*-orsellinaldehyde on the viability of RAW264.7 macrophage cells. Cells were incubated with the indicated concentrations of *o*-orsellinaldehyde for 8 hours. Cell viability was determined by the MTT assay. Results are shown as the mean ± SD of three independent experiments.

589

590 **Figure 3. Effect of *o*-orsellinaldehyde on LPS-induced NO and IL-6 production and iNOS**
591 **expression in RAW264.7 macrophage cells.** Cells were incubated with the indicated
592 concentrations of o-orsellinaldehyde for 30 minutes before treatment with LPS (1μg/ml) for 8
593 hours. **(A)** Concentration of NO were determined using Griess reaction. Results are shown as the
594 mean of the nitrite production ± SD of four independent experiments. **(B)** Whole protein was
595 extracted and then analyzed for iNOS by western blotting. One of the three experiments is shown.
596 **(C)** Concentration of IL-6 in the media were detected using a specific enzyme immunoassay.
597 Results are expressed as the mean ± SD of four independent experiments. Significant differences
598 relative to the control (vehicle) were analyzed by one-way ANOVA followed by the Bonferroni
599 post hoc test: ***p<0.001.

600

601 **Figure 4. Effect of *o*-orsellinaldehyde on IKK-2 kinase activity. Inhibition of recombinant**
602 **human IKK-2 was assayed in the presence of 10 different concentrations of *o*-orsellinaldehyde**
603 **by an *in vitro* kinase assay. (A)** The percentage of inhibition of IKK-2 activity is shown where
604 each column represents the mean ± SD from three independent experiments. Significant differences
605 relative to the control (vehicle) were analyzed by one-way ANOVA followed by the Bonferroni
606 post hoc test: ****p<0.0001, ***p<0.001. **(B)** RAW264.7 Cells were pretreated with the indicated
607 concentrations of *o*-orsellinaldehyde for 30 minutes, and then stimulated with LPS (1μg/ml) for 30
608 minutes. Whole protein was extracted and then analyzed for Phospho-Ikβα (Ser32) by western
609 blotting. One of the three experiments is shown. **(C)** Plots representing the protein levels of
610 Phospho-Ikβα normalized to the level of β-Actin. Results are expressed as the mean ± SD of three
611 independent experiments. Significant differences relative to the control (vehicle) were analyzed by
612 one-way ANOVA followed by the Bonferroni post hoc test: *** p<0.001, ** p<0.01.

613

Figure 5. Effect of intraperitoneal and oral administration of *o*-orsellinaldehyde on IL-6 concentration in the serum of LPS injected mice. (A) Mice received and intraperitoneal injection of the compound at different concentrations one hour prior LPS stimulation (1 mg/kg b.w.). Serum was collected 2 hours after LPS injection and the level of IL-6 was determined. **(B)** 100 mg/kg b.w. of *o*-orsellinaldehyde was orally administrated for half, one or two hours before LPS intraperitoneal injection. Serum was collected at the indicated times and the production of IL-6 was determined .The data are represented as the mean±SD (n=6) Significant differences relative to the control (vehicle) were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: *** $p<0.001$, ** $p<0.01$, * $p<0.1$.

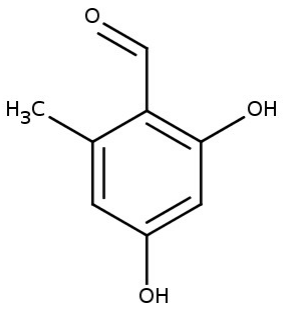
Figure 6. Cytotoxic Effect of o-orsellinaldehyde on HepG2 cells. (A) HepG2 cells were incubated with the indicated concentrations of *o*-orsellinaldehyde for 48 hours. Cell viability was determined by the MTT assay. Results are shown as the mean ± SD of three independent experiments. Significant differences relative to the control (vehicle) were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: ***$p<0.001$. **(B)** Cells were pretreated with the indicated concentrations of o-orsellinaldehyde for 48 hours. Whole protein was extracted and then analyzed for cleaved caspase-3 by western blotting. One of the three experiments is shown. **(C)** Plots representing the protein levels of cleaved caspase-3 normalized to the level of β-Actin. Results are expressed as the mean ± SD of three independent experiments. Significant differences relative to the control (vehicle) were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: **** $p<0.0001$.

Figure 7. Extraction and quantification of *o*-orsellinaldehyde into Maitake mushrooms Extraction and quantification of *o*-orsellinaldehyde into Maitake mushrooms. ESI extracted ion chromatograms acquired by LC-QTOF-MS of **(A)** the Maitake extract and **(B)** a pure standard

639 solution of *o*-orsellinaldehyde. The mass spectra scan obtained in the 10.3-10.4 interval is also
640 shown.
641
642
643

Figure 1. Virtual Screening of *o*-orsellinaldehyde

A



B

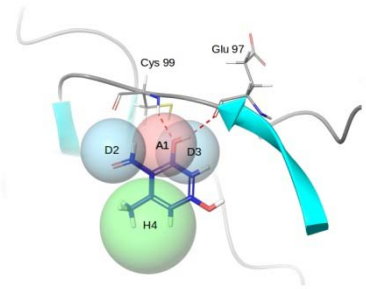


Figure 2. Effect of *o*-orsellinaldehyde on the viability of RAW264.7

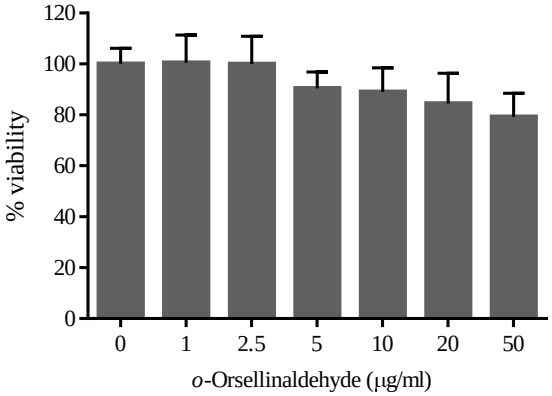
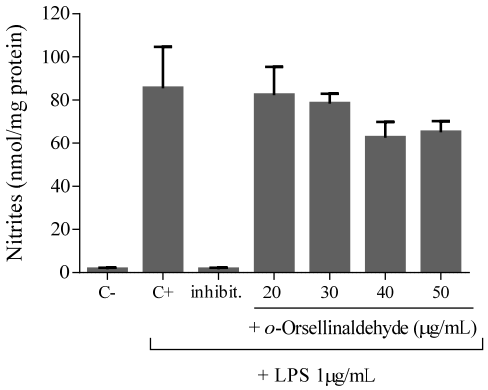
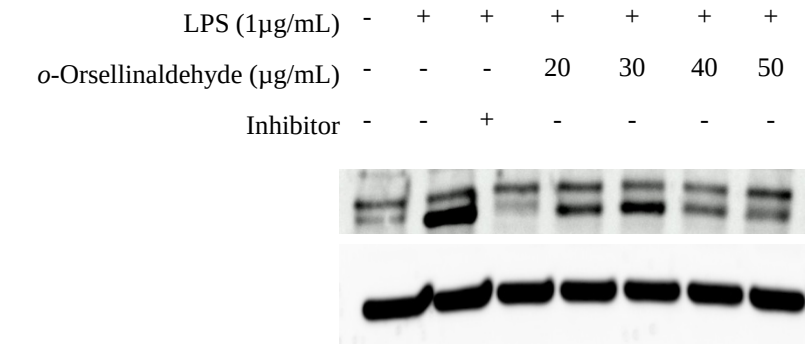


Figure 3. Effect of *o*-orsellinaldehyde on LPS-induced NO and IL-6 production and iNOS expression in RAW264.7 macrophage cells.

A



B



C

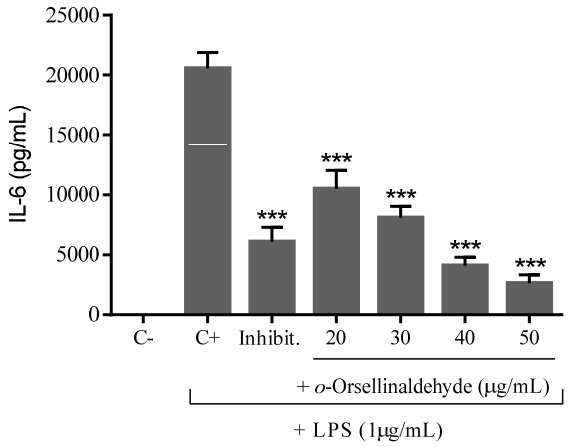


Figure 4. Effect of *o*-orsellinaldehyde on hIKK-2 kinase activity.

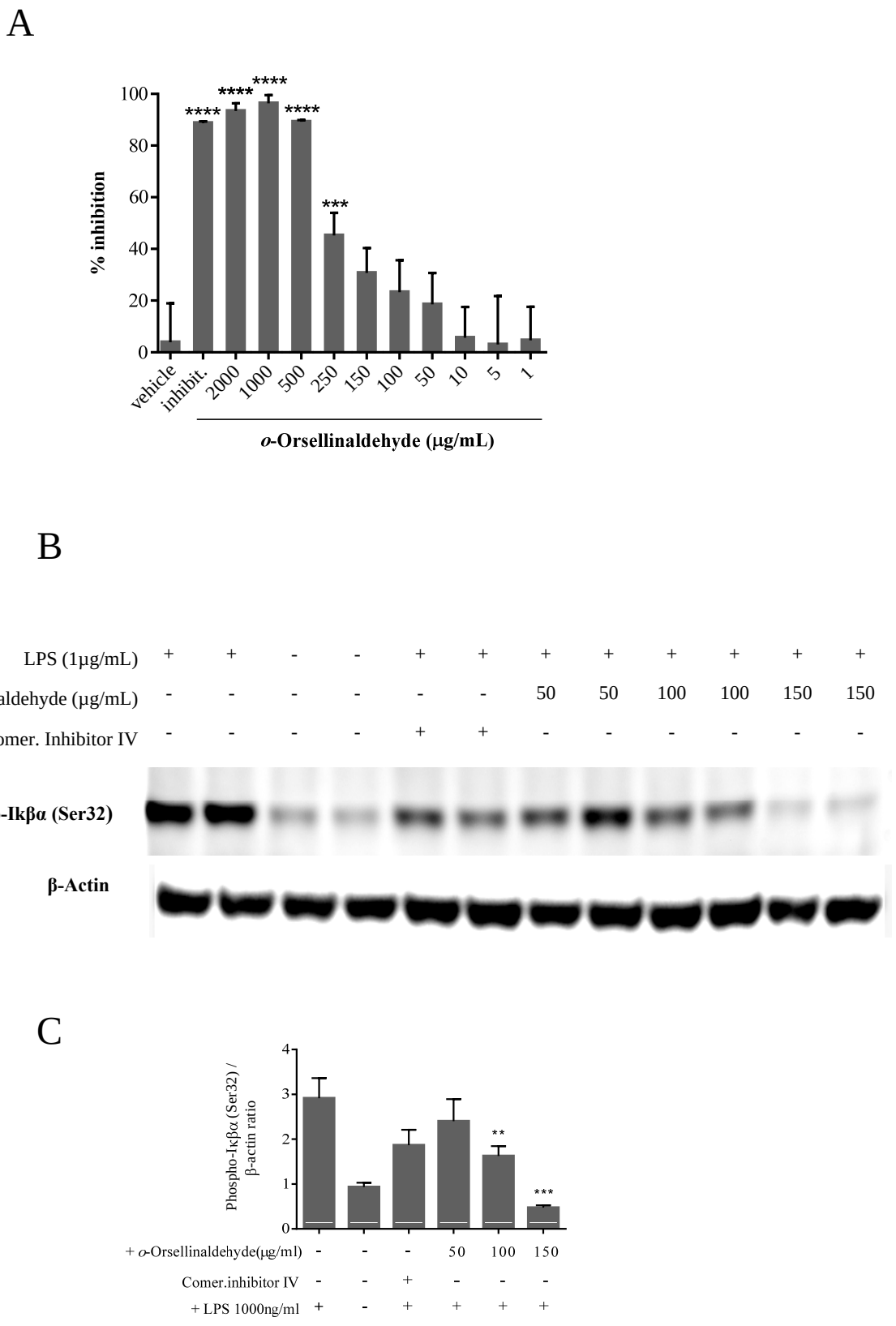
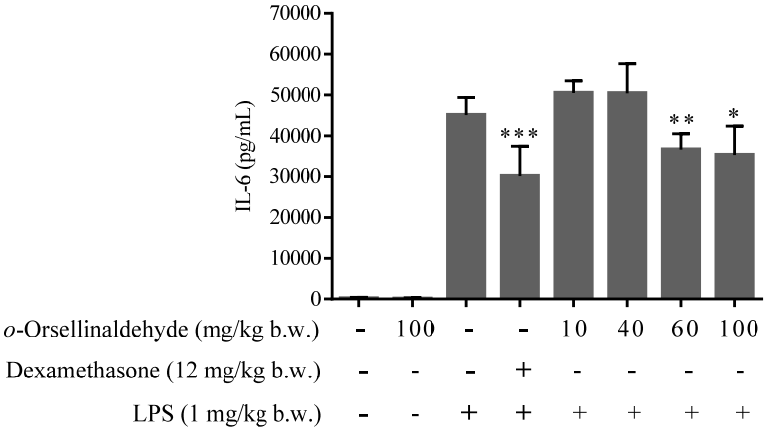


Figure 5. Effect of intraperitoneal and oral administration of o-orsellinaldehyde on IL-6 expression in the serum of LPS injected mice

A



B

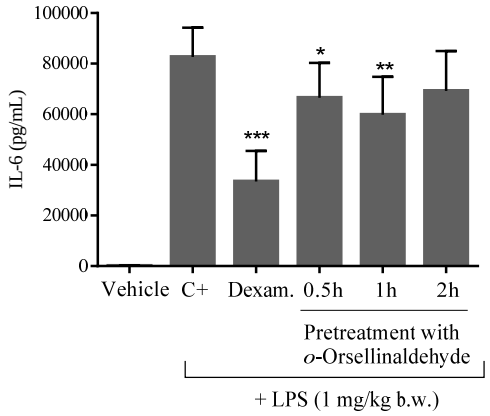
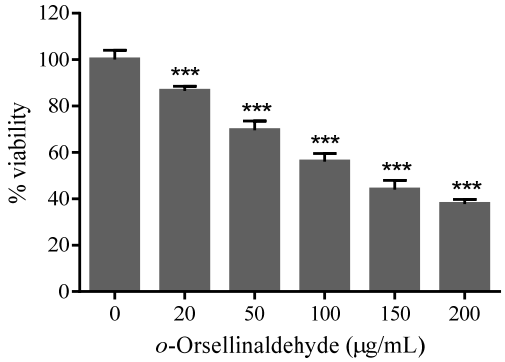
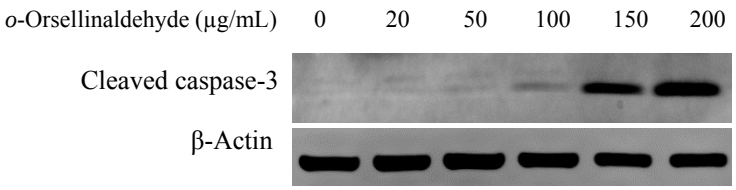


Figure 6. Effect of *o*-orsellinaldehyde on the Cleaved caspase-3 expression and toxicity in HepG2 cells.

A



B



C

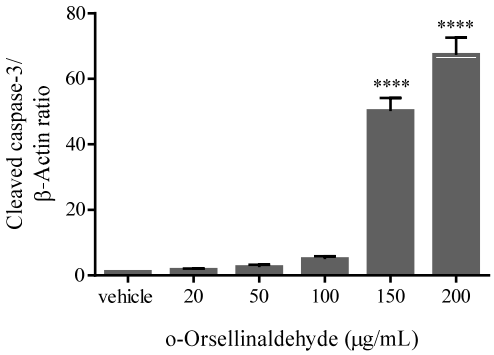


Figure 7. Extraction and quantification of o-orsellinaldehyde into maitake mushrooms