First-described recently discovered non-toxic vegetal-derived furocoumarin preclinical efficacy against SARS-CoV-2: a promising antiviral herbal drug.

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the aetiology of coronavirus disease 2019 (COVID19) pandemic. ICEP4 purified compound (ICEP4) is a recently discovered furocoumarin-related purified compound coming from roots and seeds of *Angelica archangelica* (herbal drug). ICEP4-related herbal preparations have been extensively used as active herbal ingredient in traditional medicine treatments in several European countries. Extraction method of patent pending ICEP4 (patent application no. GB2017123.7) has showed previously strong manufacturing robustness, long-lasting stability, and repeated chemical consistency. Here we show that ICEP4 presents a significant *in vitro* cytoprotective effect in highly virulent-SARS-CoV-2 challenged Vero E6 cellular cultures by using 34.5 and 69 μM doses. No dose related ICEP4 toxicity was seen on Vero E6 cells, M0 macrophages, B, CD4+ T and CD8+ T lymphocytes, Natural Killer (NK) and Natural Killer T (NKT) cells. No dose related ICEP4 inflammatory response was observed in M0 macrophages quantified by IL6 and TNFα release in cell supernatant. No survival rate decrease was observed neither on 24-hour acute nor 21-days chronic *in vivo* toxicity studies performed in *C. elegans*. Therefore, ICEP4 toxicological profile has demonstrated marked differences compared to others vegetal furocoumarins. Successful ICEP4 doses against SARS-CoV-2-challenged cells are within the maximum threshold of toxicity concern (TTC) of furocoumarins as herbal preparation, stated by European Medicines Agency (EMA). Characteristic ICEP4 chemical compounding and its safe TTC let us to assume that an antiviral first-observed natural compound has been discovered. Potential druggability of a new synthetic ICEP4-related compound remains to be elucidated. However, well-established historical use of ICEP4-related compounds as herbal preparations may point towards an already-safe widely extended remedy, which may be ready-to-go for large-scale clinical trials under EMA emergency regulatory pathway. To the best of authors´ knowledge, ICEP4-related herbal drug can be postulated as a promising therapeutic treatment for COVID19.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped non-segmented positive sense single-strand RNA virus (genus Betacoronavirus, subfamily *Orthocoronavirinae*). SARS-CoV-2 is the aetiology of coronavirus disease 2019 (COVID19) pandemic (1). Social distancing, cases identification, contact tracing, quarantine and isolation are postulated as main strategies to reduce spreading. Despite worldwide research efforts and some really promising advances, no effective antiviral drugs or mitigant sanitary products exist nowadays against SARS-CoV-2 infections, so, current pharmacological therapy given is mainly restricted to mitigate associated symptoms (2, 3).

There is continuous interest in searching for alternative antiviral drugs between phytochemical extracts, medicinal plants, and aromatic herbs. Discovery and production of novel antiviral drugs comes frequently from spices, herbal medicines, essential oils (EOs), and distilled natural products (4). Coumarins comprise a large class of compounds found within medicine herbal preparations (5-7). They are found at high levels in some EOs, particularly cinnamon bark oil, cassia leaf oil and lavender oil. Coumarin is also found in fruits (e.g. bilberry, cloudberry), green tea and other foods such as chicory (8). Most coumarins occur in higher plants, with the richest sources being the *Rutaceae* and *Umbelliferae*. Although distributed throughout all parts of the plant, the coumarins occur at the highest levels in the fruits, followed by the roots, stems and leaves. Environmental conditions and seasonal changes can influence the occurrence in diverse parts of the plant (9). Psoralens are natural products, linear furanocoumarins (most furanocoumarins can be regarded as derivatives of psoralen or angelicin), that are extremely toxic to a wide variety of prokaryotic and eukaryotic organism. Some important psoralen derivatives are xanthotoxin, imperatorin, bergapten and nodekenetin (8, 9). Demonstrated activities of coumarins are anticoagulant, anticancer, antioxidant, antiviral, anti-diabetics, anti-inflammatory, antibacterial, antifungal and anti-neurodegerative agents as drugs, and, additionally, as fluorescent sensors for biological systems (10).

The genus *Angelica litoralis* is comprised of over 90 species spread throughout most areas of the globe (11). More than half of these species are used in traditional therapies, while some of them are included in several national and European pharmacopoeias (12-15). Bioactive constituents in different *Angelica* species include coumarins, EOs, polysaccharides, organic acids and acetylenic compounds (16). *In vitro* testing confirmed cytotoxic (17, 18), anti-inflammatory (19), antibacterial (20), antifungal (21), neuroprotective (22) and serotonergic (23) activities for extracts obtained from a range of *Angelica* species.

Reducing viral replication at the beginning of SARS-CoV-2 infection and, subsequently, the associated degree of immunopathological damage, is a critical step to mitigate and cure COVID19 (2). ICEP4 (patent pending, application nº GB2017123.7) is an *Angelica archangelica*-based purified compound with previous evidence of antiviral and oncolytic *in vitro* effects (ICE-P Life, data not shown). ICEP4-related herbal preparations have been extensively used as active herbal ingredient in traditional medicine treatments in several countries, including EU and US (12-15). The main objective of this research work was to evaluate the possible cytoprotective effect of the ICEP4 extract against SARS-CoV-2 challenge by means of Crystal Violet staining, a technique used as an indirect quantification method for cell death. In parallel, ICEP4 *in vitro* and *in vivo* potential cytotoxic effects were assessed by using standard EMA-accepted methods. Both objectives should support ICEP4 using as a new antiviral safe herbal drug for COVID19 treatment by stopping viral spreading at targeted-SARS-CoV-2 epithelium without compromising the host immune response.

2. Material and Methods

2.1. Study design

Efficacy assays were performed in biosafety level 3 (BSL3) facilities at Zaragoza (Spain) (WGUSA, laboratory reference 747735/2014). Cytotoxic studies and replication of *in vitro* efficacy studies were independently repeated in biosafety level 2 (BSL2) facilities at UNATI (IISA, Zaragoza, Spain) and BSL3 facilities at the University of Zaragoza in order to demonstrate experimental repeatability and inter-laboratory consistency of obtained results.

ICEP4 efficacy against SARS-CoV-2 challenge was evaluated *in vitro* by using the Crystal Violet staining technique. Cellular viability testing by measuring the percentage of stained cells per well after SARS-CoV-2 challenge was carried out in triplicate, on three different periods. Three (3) doses of ICEP4 were tested along with proper negative/positive controls at three different times (T1, T2 and T3).

ICEP4 *in vitro* toxicity was analysed at different concentrations in immune cells. C57BL/6 (*Mus musculus*) (B6)-mouse-derived bone marrow monocytes (BMC) were differentiated to M0 macrophages and incubated for 24h with different ICEP4 doses. Proper ICEP4-negative and highest concentration-used diluent (SHAM) controls were included for comparison. After 24 hours, cell viability was determined by using the PrestoBlue™ assay. In addition, macrophage inflammatory response was determined by measuring IL6 and TNFα expression in cell supernatants. On the other hand, B6-mouse-derived splenocytes were incubated for 24h with

ICEP4 and controls as indicated for macrophages. Subsequently cells were labelled with CD3-FITC, CD8-APC, CD4-VioBlue and NK1.1-APCVio770 or CD19-PE, CD3-FITC together with Annexin-V-PE or -APC and dead cells within T, NK and NKT or B cells was evaluated by Flow Cytometry (GALLIOS, Beckman Coulter).

In vivo acute and chronic toxicity was evaluated by using survival rate of ICEP4-challenged synchronized cultured glp-4 mutant *Caenorhabditis elegans*. Both assays were carried out at 25ºC in three different periods, and the duration of the experiments was 24 h and 21 days for acute and chronic toxicity, respectively.

2.2. Raw Material

2.2.1. ICEP4 plant-derived extract dosing

Five (5) mg of original 9-years-old certified-batch ICEP4 was submitted by Mr. Ezio Panzeri for examination. Following the previous information, ICEP4 extract comes from seeds and roots (herbal drug) of *Angelica archangelica*. Briefly, 700 g of coarsely comminated plant-derived material were successively extracted for 36 h with 15 L of methanol in a Soxhlet extractor (Quickfit™ large-scale extractor IIEX). The extracts were concentrated under reduced pressure (rotary evaporator 9200/1). ICEP4 purification was performed with high-performance liquid chromatography to reach 98% purity (UPLC High-performance Liquid Chromatographer XEVO TQD, Waters, US), showing a characteristic consistent chemical profile after a second ICEP4 manufactured batch (WGUSA, data not shown) (Acquity UPLC PDA QDA <ESI-MS>, Waters, US) (Figure 1). Standard dose for toxicology and efficacy studies of the first described natural-derived compound was determined considering the toxicologic data from previously described *Angelica*-related furocoumarin (CAS number 66-97-7) (24). Most appropriate solvent was taken from previous chemical characterization studies (WGUSA, data not shown).

Starting point was a stock dilution of original ICEP4 in ethanol whose concentration was 1 mg/mL (4.6 mM). Raw material was diluted at concentrations between 1µM and 1mM to assess preliminary ICEP4 toxicity in macrophages and immune cells. Higher concentration-used solvent was used as SHAM control of cellular viability.

Cellular viability was assessed for ICEP4 efficacy indicator after SARS-CoV-2 challenge. Three (3) doses were selected based on previous immunotoxicology data. Threshold of Toxicological Concern (TTC) for furocoumarins is 1.5 µg/Kg of daily exposure, so *in vitro* experiments were carried out taking these values into account to calculate the maximum concentration (24).

Cytotoxicity assays were performed in 96-well plates with 6.9 µM, 34.5 µM and 69 µM concentration of the ICEP4 maximum doses. ICEP4 doses were vehiculated, among others, with ethanol 0.15%, 0.75% and 1.5% respectively. Higher concentration-used solvent was included as SHAM control of cellular viability.

For *in vivo* studies 1mM dose was added for acute and chronic studies (10.000 times higher than reference dose) to a 25ºC-cultivated infertile strain of *C. elegans*.

2.2.2. SARS-CoV-2 strain

A high-pathogenic strain of SARS-CoV-2 was isolated and cultured from a 72-years old patient at University Clinical Hospital Lozano Blesa (Zaragoza, Spain). Second-passage vials with the SARS-CoV-2 strain were provided by Dr. Julian Pardos (IISA, UNATI, Zaragoza, Spain). Virus was maintained and cultured following UNATI protocols in biosafety level 3 (BSL3) facilities at Zaragoza (WGUSA, Spain). Tissue Culture Infectious Dose 50% (TCID50) was determined at $1.47 \cdot 10^6$/mL. The same strain and TCID50 was used at UNATI facilities for repeatability and inter-laboratory consistency studies.

2.3. Methods

2.3.1. SARS-CoV-2-challenged ICEP4-treated efficacy assay

Vero E6 cells were provided by Eugenia Puentes (Biofabri, Porriño, Spain) and cultured following provider´s descriptions. Cellular culture was maintained with 10^5 cell/mL of density in Vero E6 10% FBS (Sigma F7524) Dulbecco's Modified Eagle Medium (Lonza, Ref BE12-614F) along the study at 37ºC with 5% CO_2 and 90% humidity. The efficacy assays were performed in 96-well plates (Nunclon Delta Surface 167008 Thermo) with 10^4 cells/well density. Vero E6 cells were seeded a day prior to viral infection. The experiment was carried out following the described design (Table 1). Briefly, the plate contained internal growing controls of Vero E6 cells, and the described concentrations of ethanol (vehicle-related toxicity control), and ICEP4 doses (both compound toxicity control and cytoprotective effect). SARS-CoV-2 was added after one hour of incubation (37 ºC) and plates were incubated for 72 h at 37ºC and 5% CO_2 after viral challenge.

Cellular viability was observed by Crystal Violet staining. Briefly, 72 h after SARS-CoV-2 challenge cells were fixated with paraformaldehyde (Panreac 252931.1212) 4% for 1 h, room temperature. Cells were then stained with Crystal Violet solution (0.5% crystal violet and 20% methanol) (Sigma, C0775) (Panreac 131091.1212). Cellular viability was directly observed by

inverted microscope (DM IL LED Leica). Strong positive cellular staining (blue) was considered as viable cells (more than 75% of well is stained). Intermediate or weak cellular staining was considered as unviable cells (less than 75% of well is stained). Counting was performed by two different technologists per experiment.

2.3.2. *In vitro* cytotoxicity assays

2.3.2.1. M0 macrophage differentiation from mouse bone marrow-derived cells

Bone marrow derived cells (BMDCs) were obtained from a minimal-disease certified mouse (C57BL/6 -*M. musculus*- -B6-, Charles River, US). Femurs and tibias were dissected from euthanized mouse and, under sterile condition, the bone marrow was eluted by injecting DMEM or RPMI medium through the bone cavity. Erythrocytes were lysed and final BMDCs suspension was adjusted to 10^6 cells/mL. BMDM were differentiated into M0 macrophages after an incubation period of six days with BMDM medium, and finally seeded at a concentration of $5 \cdot 10^4$ cells/well in 96-well plates. After 24 hours, ICEP4 doses were added by making 10-fold serial dilutions and incubated for another 24 h.

2.3.2.2. Isolation of mouse splenocytes

A minimal-disease certified mouse (B6, Charles River, US) was killed by cervical dislocation. Its spleen was carefully extracted and mashed through a cell strainer. Splenocytes were washed with RPMI and centrifuged at 1200 rpm for 5 min. Splenocytes were counted and adjusted to 10^6 cells/mL.

2.3.2.3. Macrophage cellular viability by PrestoBlue™ assay and inflammatory response

Cell viability was analysed by PrestoBlue™ HS assay following manufacturer´s instructions. PrestoBlue™ HS (high sensitivity) contains resazurin and a propriety buffering system (#P50200, ThermoFisher, US). Absorbance was measured by using iMark™ Microplate Absorbance Reader (BioRad, Germany). Activation of the inflammatory response in macrophages was analysed by quantifying the cytokines IL6 and TNFα in cell supernatants by ELISA (Ready-Set-Go kit, eBiosciences) following manufacturer´s instructions.

2.3.2.4. Lymphocytes analysis by flow cytometry

Splenocytes were incubated with the same concentrations of the molecule ICEP4 used for

macrophages. After 24 hours they were collected, washed and cell viability was analysed by annexin V staining in T, B, NK and NKT cells as indicated in 2.1.

2.3.3. *In vivo* toxicity: *Caenorhabditis elegans* assay

2.3.3.1. *C. elegans* strain

The *C. elegans* strain used was the glp-4 mutant. *Caenorhabditis elegans* gene glp-4 was identified by the temperature-sensitive allele bn2 where mutants raised at the restrictive temperature (25°C) produce adults that are essentially germ cell deficient *C. elegans*.

2.3.3.2. Culturing and synchronization

C. elegans worms were propagated on nematode growth media (NGM) agar plates with kanamycin 50 µg/mL and streptomycin 100 µg/mL at 20°C (NGM Lite, US Biological Life Sciences, Swampscott in Massachusetts, US) using *E. coli OP50* as food source. Due to the presence of worms at different developmental stages in cultures, a synchronization process consisting on killing larvae and adult worms by using a combination of NaOH and NaClO was followed as described elsewhere (25). Eggs obtained from synchronization were resuspended and plated on a NGM agar plate without *E. coli OP50* (ISSA, Zaragoza, Spain) to allow them to hatch and reduce developmental differences in new larvae due to different eggs age. *E. coli OP50* was added to the NGM agar plate 24h later.

2.3.3.3. Acute survival assay

L1 larvae obtained from synchronization were cultured at 25°C until worms developed to L4 stage. L4 worms were harvested from plates and washed 3 times with M9. Approximately 15 worms per well were placed in a 96-well flat bottom microtiter plate and treated with different ICEP4 doses at 25ºC. A total of 45 worms (3 wells) were assessed by each ICEP4 dose. Worms without treatment served as negative controls. Three different independent survival assays were carried out.

2.3.3.4. Chronic survival assay

Worms were cultured as previously described and treated with different ICEP4 doses. Worms without treatment served as negative controls. Survival assays were carried out for 21 days at 25°C. Every 7 days ICEP4 and *E. coli* OP50 were added to the *C. elegans*. Chronic toxicity worms

were seeded and counted twice a week calculating the percentage of worms that survived with respect to the number of worms at time zero. Three independent experiments were performed.

2.4. Statistics of efficacy studies

Efficacy data were analysed by Microsoft® Excel® STATS (Microsoft 365 MSO - 16.0.13231.20110- 32 bits, ID 00265-80196-36405-AA936). Results were presented as Mean ± SD (Standard Deviation). One-way ANOVA was used to confirm statistical difference of multiple groups between treated and no treated groups. ICEP4 – TCID50 groups were analysed by two-sample t-test assuming equal variances to confirm significant differences. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. $P < 0.05$ was considered as significant. Results from UNATI were analysed together with WGUSA obtained data, in order to check robustness and repeatability.

3. Results

3.1. SARS-CoV-2-challenged ICEP4-treated efficacy assay

Descriptive statistics including UNATI results are shown in Table 1. Maximum standard deviation was observed in TCID50 group, mostly due to outlier results in the firsts replications of experiment (Figure 2). After that, more coherent and consistent TCID50 results (5±1,73) were obtained in the rest of replications.

Marked significant differences between groups were found for at least one group as stated by analysis of variance of a factor (ICEP4 treatment) (Table 2). No differences were found by analysing the effect of solvents or raw material vs Vero culture in a two-sample t-test assuming equal variances (Table 3).

Observational marked increase of cell viability comparing to TCID50 control were found in SARS-CoV-2-infected ICEP4-treated groups, corresponding to 34.5 µM (WP2) and 69 µM (WP3) doses (Table 4). Significant differences were found between such groups by analysing two-sample t-test, assuming equal variances as well, confirming preliminary descriptive results. Interpreting these results, ANOVA significance can be checked directly to ICEP4 treatment cytoprotective effect.

3.2. Cytotoxicity on B and T lymphocytes, NK and NKT cells and macrophages

No toxicity of tested ICEP4 doses on CD4+ and CD8+ T cells or on B, NK and NKT cells was

seen (Tables 5 and 6; Figure 3). However, increased Annexin-V staining meaning slight decrease of cellular viability was observed on NK cells at 100 µM doses. Such effect was also found on macrophages that showed a reduction of 30% in cell viability at 100 µM of ICEP4 (Figure 3).

Remarkably, ICEP4 plant-derived extract did not induce an inflammatory response in M0 macrophages, which was verifiable by released IL6 and TNFα after challenge of all doses (Table 7). IL6 and TNFα values are very low and in some samples are very close to or even below the limit of detection (detection limit IL6: 4 pg/mL, TNFα: 8 pg/mL). As expected LPS induced a high inflammatory response confirming macrophage functionality.

3.3. *In vivo* toxicity of ICEP4

A 24-hour acute and 21-days chronic *in vivo* toxicity studies were performed in *C. elegans* (Figure 4). No toxicity was observed at 1nM to 100µM in both assays. In both assays, only the highest dose showed toxicity (1 mM), which is presumably due to the higher ethanol concentration (20%) and not to active ingredient ICEP4, as confirmed when using the SHAM control (Figure 4).

4. Discussion

Antiviral herbal drugs have been widely used in clinical frontline against respiratory diseases. Traditional Chinese medicines (TCM), Ayurveda medicine (AM) or European herbal drugs (EHD) are highly encouraged as adjuvant therapies in COVID19, supported by a previous historical well documented efficacy studies against many viral infections including influenza, SARS, and MERS (16, 26, 27). Vegetal drugs are frequently used as a herbal decoction for lung clearing and detoxification in the clinical mitigant treatment of respiratory diseases and, recently, also COVID19, being the most common *Astragalus membranaceus*, *Glycyrrhizae uralensis*, *Saposhnikoviae divaricata*, *Rhizoma Atractylodis*, *Macrocephalae*, *Lonicerae Japonicae Flos*, *Fructus forsythia*, *Atractylodis Rhizoma*, *Radix platycodonis*, *Agastache rugosa*, and *Cyrtomium fortune* J. Sm., *Withania somnifera* (Ashwagandha), *Tinospora cordifolia* (Guduchi), *Asparagus racemosus* (Shatavari), *Phylanthus embelica* (Amalaki), and *Glyceriza glabra* (Yashtimadhu) (28, 29). As reviewed by Sarker and Nahal (2004), many species of *Angelica* genus, e.g. *A. acutiloba*, *A. archangelica*, *A. atropupurea*, *A. dahurica*, *A. japonica*, *A. glauca*, *A. gigas*, *A. koreana*, *A. sinensis*, *A. sylvestris*, etc., have been used for centuries as anti-inflammatory, expectorant and diaphoretic, and remedy for colds, flu, influenza, coughs, chronic bronchitis, pleurisy, headaches, fever, and diverse bacterial and fungal infections, among others (16). Active principles isolated from these plants mainly include various types of coumarins, acetylenic compounds, chalcones, sesquiterpenes and polysaccharides (9, 10, 16). Frequently, most of the

existing conventional antiviral treatments frequently lead to the development of viral resistance combined with the problem of side effects, viral re-emergence, and viral dormancy (2). Therefore, World Health Organization (WHO) also supports and welcomes innovations around the world regarding scientifically proven traditional medicine, to increase clinical alternatives of safe antiviral therapies (30).

Angelica archangelica-related ICEP4 has shown a significant *in vitro* cytoprotective effect in SARS-CoV-2-challenged Vero E6 cellular cultures by using 34.5 and 69 µM doses (0.75 and 1.5 µg/dose). Successful ICEP4 doses against SARS-CoV-2 are included within the maximum TTC of furocoumarin as herbal preparation or remedy (24). Total daily human exposure to coumarins from dietary or cosmetic sources is 0.06 mg/Kg/day, with a total daily dose of 0.2% furocoumarins (1.2 µg/Kg/day) (31). No adverse effects of coumarin have been reported in susceptible species in response to doses which are more than 100-fold the maximum human daily intake, such as it happened with successfully-used ICEP4 doses (1.5 µg in 100 µL per well) (13, 18, 31). It is worthy to mention than non-cytotoxic proof-of-concept ICEP4 dose is 4-fold lower than antiproliferative cytotoxicity threshold observed in previous *Angelica*-related studies (32-34). These promising results might open the possibility to further studies for druggability of ICEP4. Remarkably, high-virulent SARS-CoV-2 strain has been used during the experiment so, we can postulate ICEP4-related herbal drug as a promising potential treatment for COVID19.

EHD, AM and TCM either alone or in combination have been used for centuries in clinical and prophylactic antiviral treatments and have proven their efficacy when subjected to rigorous scientific investigation (35). Also, they play a major role in the discovery and development of many antiviral drugs based on their structural moiety, classical example being emetine, and quinine (36). Naturally occurring scaffolds such as coumarins display a wide spectrum of pharmacological activities including anticancer, antibiotic, antidiabetic and others, by acting on multiple targets. In this view, various coumarin-based hybrids possessing diverse medicinal attributes were synthesized in the last five years by conjugating coumarin moiety with other therapeutic pharmacophores (37). Antiviral mechanistic studies of TCM, AM or EHD have revealed how they interfere with the viral life cycle, as well as virus-specific host targets (4). Herbal drugs have demonstrated a wide range of antiviral mechanisms that includes inhibition of 3CLpro protein (Chinese *Rhubarb* extracts, *Houttuynia chordata* extract, hesperetin, etc.), blocking or inhibition of the viral RNA-dependent RNA polymerase activity, and inhibition of inflammatory cytokines like TNF-α, IL-1β, and IL-6 (*Fructus forsythiae*) (38). Psychoneuroimmune mechanism has been highlighted as possible immune-mediated pathway in COVID19 treatment support (39, 40).

Prophylactic therapeutics with potential immunomodulatory activity are postulated as an add-on treatment for COVID19 (29). Many medicinal compounds and natural products have exhibited several antiviral mechanisms to prevent early stage of infection, including viral attachment and penetration (36, 41). Psoralens, main moiety of furocoumarins, may react directly with pyrimidine nucleotides forming mono and di adducts in DNA of even interstrand cross links (6). Another cause of their toxicity derives from the ability of UV-A photoactivated furanocoumarins to react with grand state oxygen generating toxic oxyradicals capable of inactivating proteins within cells (6, 7). A broad range of therapeutics applications requiring cell division inhibitors (main drug targets is the cytochrome P450 -CYPs superfamily-), as vitiligo, psoriasis and several type of cancers like T cell lymphoma have been suggested due to this reactivity (7, 32-34). Taken together cytotoxicity and *in vivo* assays data we postulate that furocoumarin-derived ICEP4 has shown very few or negligible evidence of toxicity for immune and epithelial-derived cells. NK and macrophages showed slight decrease of viability on the highest dose, probably due to the same effect of solvent stated on *in vivo* assays. It is worthy to mention that *Angelica*-based furocoumarin gold standard is phototoxic and affects cellular viability within studied doses (32, 34). Several structural changes, well-established compound concentration and composition differences are expected by meaning of geographic, stational or plant-related issues, many of them can be used as drug template design (27). Therefore, it is postulated that ICEP4, despite being extracted and purified in the same manner as another *Angelica*-related furocoumarins, presents differences in chemical or racemic compounding composition behind the extraction method that are remarkable in terms of toxicity and antiviral efficacy (SARS-CoV-2 infection model). These facts lead us to assume that an unknown different furocoumarin-related compound has been discovered, or at least, first observed. Extraction method of patent protected ICEP4 has showed previously strong manufacturing robustness, long-lasting stability, and repeated chemical consistency. Nonetheless, additional chemical-related data of ICEP4 are urgently needed, to shed light to several concerns about its use as herbal drug.

TCM, AM and EHD-prescribed herbal drugs decrease the severity and mortality rate of COVID19 (28, 35, 38, 41). Several drugs such as *Nigella sativa*, natural honey, artemisinin, curcumin, *Boswellia*, and vitamin C are in a clinical trial for COVID19 (35). Although promising clinical evidence of treatment in diverse respiratory infections are available for *Angelica archangelica* (16, 31), randomized human clinical trials are required to evaluate the efficacy and safety of ICEP4 in COVID19 patients. Historical clinical evidence of *Angelica*-related remedies in treatment of respiratory syndromes, its safe toxicity profile and appropriate range of therapeutic doses may lead to ICEP4 use as prophylactic mucosal-related non-systemic anti-SARS-CoV-2 therapy. Under this view and encouraged by WHO demands, ICEP4 herbal drug may be suitable for a complete drug development under COVID19 regulatory pathway.

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Table 1. Descriptive statistics of *in vitro* challenge test. ICEP4 doses are 6.9 (WP1), 34.5 (WP2) and 69 (WP3) µM per well. Different ethanol concentrations (0.15%, 0.50%, 0.75%) were added as SHAM control. TCID50 is considered positive control and the main infective group to be compared with treatment. TCID50-SARS-CoV-2 10-fold is considered maximum infective positive internal control (cell death). Treatment-negative Control (CT) group was composed by non-treated Vero E6 cells in 2% FBS medium (maximum cell viability).

	CT	*Et_0,15*	*Et_0,5*	*Et_0,75*	*WP1*	*WP2*	*WP2*	*TCDI50*	*SARS+ WP1*	*SARS+ WP2*	*SARS+ WP3*	*SARS 10-f*
Mean	8	8	7.33	7.67	7.33	7.33	7.67	5	7.00	7.67	7.67	0
Standard error	0	0	0.33	0.33	0.33	0.33	0.33	1	0.58	0.33	0.33	0
Median	8	8	7	8	7	7	8	4	7	8	8	0
Mode	8	8	7	8	7	7	8	4	7	8	8	0
Standard deviation	0	0	0.58	0.58	0.58	0.58	0.58	1.73	1	0.58	0.58	0
Variance	0	0	0.33	0.33	0.33	0.33	0.33	3	1	0.33	0.33	0

Table 2. Analysis of variance of a factor (ICEP4 treatment) of *in vitro* challenge test. Null hypothesis was considered as no observed differences between groups. *P < 0.05, **P < 0.01 and ***P < 0.001. P < 0.05 was considered as significant.

Origin of variations	*F*	*Probability*	*Critical value for F*
Between groups	29.03349282	0.000***	2.216308646

Table 3. Two-sample t-test assuming equal variances results of *in vitro* challenge test for solvents and raw material. Non-treated Vero E6 2% medium culture (CT) group was considered treatment-negative control (maximum cell viability) for comparison (data no shown). Ethanol 0.15%, 0.75% and 1,5% respectively were considered as solvent control. ICEP4 doses were 6.9 µM (WP1), 34.5 µM (WP2) and 69 µM (WP3) and considered as ICEP4-treated non-SARS-CoV-2-challenged groups. *P < 0.05, **P < 0.01 and ***P < 0.001. P < 0.05 was considered as significant.

	Et_0,15	*Et_0,5*	*Et_0,75*	*WP1*	*WP2*	*WP3*
Mean	8.0000	7.3333	7.6667	7.3333	7.3333	7.6667
Variance	0.0000	0.3333	0.3333	0.3333	0.3333	0.3333
t Stat	65535	-2	-1	-2	-2	-1
P(T<=t) one-tail	#¡NUM!	0.0581	0.1870	0.0581	0.0581	0.1870
t Critical one-tail	2.1318	2.1318	2.1318	2.1318	2.1318	2.1318
P(T<=t) two-tails	#¡NUM!	0.1161	0.3739	0.1161	0.1161	0.3739
t Critical two-tails	2.7764	2.7764	2.7764	2.7764	2.7764	2.7764

#¡NUM! Both groups behave equal so, no comparison could be raised up.

Table 4. Two-sample t-test assuming equal variances results of *in vitro* challenge test for SARS-CoV-2-infected ICEP4-treated groups. TCID50 SARS-CoV-2-infected cell death-positive group was considered for comparison (data no shown). *P < 0.05, **P < 0.01 and ***P < 0.001. P < 0.05 was considered as significant. ICEP4 doses were 6.9 µM (WP1), 34.5 µM (WP2) and 69 µM (WP3). Tissue Culture Infectious Dose 50% (TCID50) of SARS-CoV-2 (SARS) was determined in 1.47 x 10^6/mL.

	SARS+WP1	*SARS+WP2*	*SARS+WP3*
Mean	7	7.667	7.667
Variance	1	0.333	0.333
t Stat	1.732	2.530	2.530
P(T<=t) one-tail	0.079	0.032*	0.032*
t Critical one-tail	2.132	2.132	2.132
P(T<=t) two-tails	0.158	0.065	0.065
t Critical two-tails	2.776	2.776	2.776

Table 5. Annexin V staining (%) of B lymphocytes after ICEP4 challenge.

Doses	Annexin V	B cells
B CELLS 0	7.33	63.44
B CELLS 1 nM	3.64	68.66
B CELLS 10 nM	8.64	68.23
B CELLS 100 nM	8.18	66.79
B CELLS 1 µM	8.65	68.26
B CELLS 10 µM	3.49	69.33
B CELLS 100 µM	5.07	65.71
B CELLS 0	6.84	67.28
B CELLS 1 nM	3.54	69.54
B CELLS 10 nM	8.37	68.46
B CELLS 100 nM	9.96	69.17
B CELLS 1 µM	8.62	68.57
B CELLS 10 µM	5.01	69.06
B CELLS 100 µM	4.65	65.3

Table 6. Annexin V staining (%) on immune cells (CD4+, CD8+, NK+ and NKT+ cells), and CD4+, CD8+, NK+ and NKT+ staining of lymphocytes T and NK after ICEP4 challenge.

Data set	Annexin V				Lymphocyte marker			
	CD4+	CD8+	NK Cells	NKT Cells	CD4+	CD8+	NK	NKT
T NK CELLS 000 00063130.715	17.66	7.25	5.6	10.12	4.09	9.09	7.28	18.37
T NK CELLS 001 00063137.722	17.56	2.55	6.4	4.52	5.21	7.33	10.34	16.55
T NK CELLS 010 00063136.721	14.02	2.66	4.22	4.13	4.4	8.83	8.72	17.77
T NK CELLS 100 00063135.720	13.76	2.96	3.74	4.71	4.3	9.1	8.95	17.83
T NK CELLS 001 00063134.719	14.21	2.22	3.86	4.41	4.44	8.87	8.62	17.72
T NK CELLS 010 00063133.718	14.29	1.21	6.45	2.16	6.9	6.01	7.24	16.89
T NK CELLS 100 00063132.717	20.47	0.76	11.42	0.63	4.41	6.25	8.33	18.68
T NK CELLS 000 00063139.724	15.79	5.07	5.29	7.33	5.07	8.51	6.1	16.41
T NK CELLS 001 00063146.731	15.72	0.26	5.52	1.77	6.68	6.11	8.63	16.46
T NK CELLS 010 00063145.730	12.92	2.37	4.34	3.98	4.45	8.64	8.26	17
T NK CELLS 100 00063144.729	12.47	2.46	3.79	5.24	3.87	8.69	7.35	17.54
T NK CELLS 001 00063143.728	11.45	2.44	2.65	4.72	4.24	8.38	8.44	17.63
T NK CELLS 010 00063142.727	15.04	0.67	5.82	1.85	6.31	6.27	6.23	16.65
T NK CELLS 100 00063141.726	12.08	0.19	10.51	0.65	9.85	4.86	8.18	18.51

Table 7. IL6 and TNFα analyses from M0 macrophages supernatant.

		IL6		media	TNFα		media
M1	control-	4.8	5.52	5.16 pg/ml	20.52	28.96	24.74 pg/ml
M2	0	4.48	5.28	4.88 pg/ml	17.08	28.32	22.7 pg/ml
M3	1nM	4.6	5.28	4.94 pg/ml	19.16	25.84	22.5 pg/ml
M4	10nM	4.4	5.8	5.1 pg/ml	17.48	32.52	25 pg/ml
M5	100nM	4.48	6.96	5.72 pg/ml	21	38.2	29.6 pg/ml
M6	1uM	5.04	0.12	2.58 pg/ml	24.68	0.04	12.36 pg/ml
M7	10uM	7.12	0.16	3.64 pg/ml	29.64	0.76	15.2 pg/ml
M8	100uM	5.16	0.24	2.7 pg/ml	26.44	0	13.22 pg/ml
M9	Diluyente	5.52	0.12	2.82 pg/ml	27.04	0.36	13.7 pg/ml
Control +	LPS	605.44 pg/ml			1236.12 pg/ml		

Author contributions

Original ICEP4 batch and stability data (Ezio Panzeri). Data collection, samples management and analytic ICEP4 control by HPLC, flow cytometry and staining techniques (Adriana Toledo Núñez, María Celaya Fernández, Iratxe Uranga-Murillo, Maykel Arias Cabrero and Ariel Ramírez Labrada). Study design and data interpretation (Iván José Galindo-Cardiel, Ariel Ramirez Labrada). Writing and editing tables and figures (Iván José Galindo-Cardiel, Ariel Ramírez Labrada, Ezio Panzeri). Quality assurance (Iván José Galindo-Cardiel, Julián Pardo).

Disclosure

Dr. Iván José Galindo-Cardiel and Ezio Panzeri are co-authors of ICEP4-related patent application (GB2017123.7).

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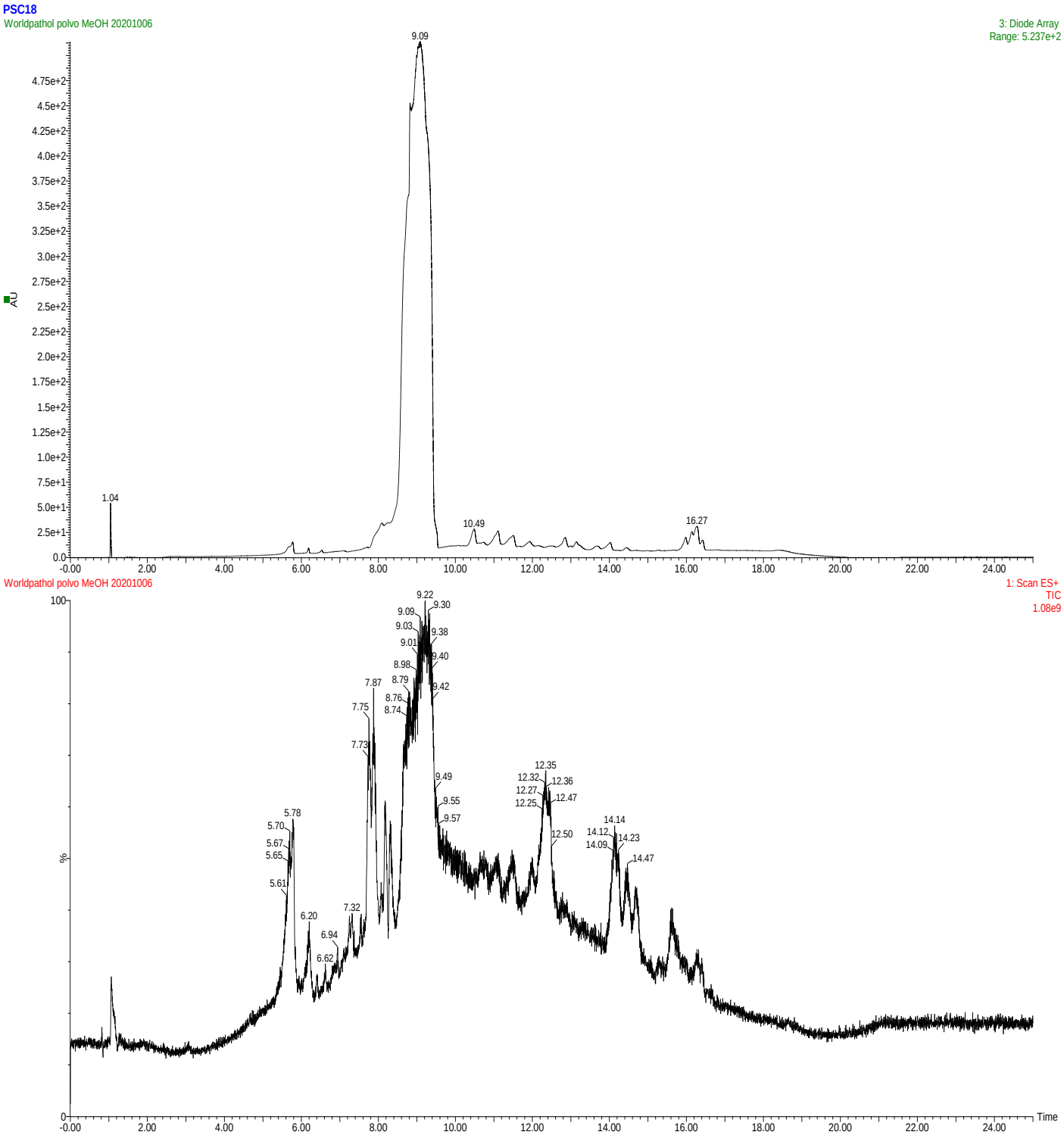


Figure 1. Units of absorbance of the original 9-years-old ICEP4 batch. Methanol was used as solvent. Note that there is saturation in expected wavelength. 320 nm at a retention time of 9 minutes (Acquity UPLC PDA QDA <ESI-MS>, Waters, US).

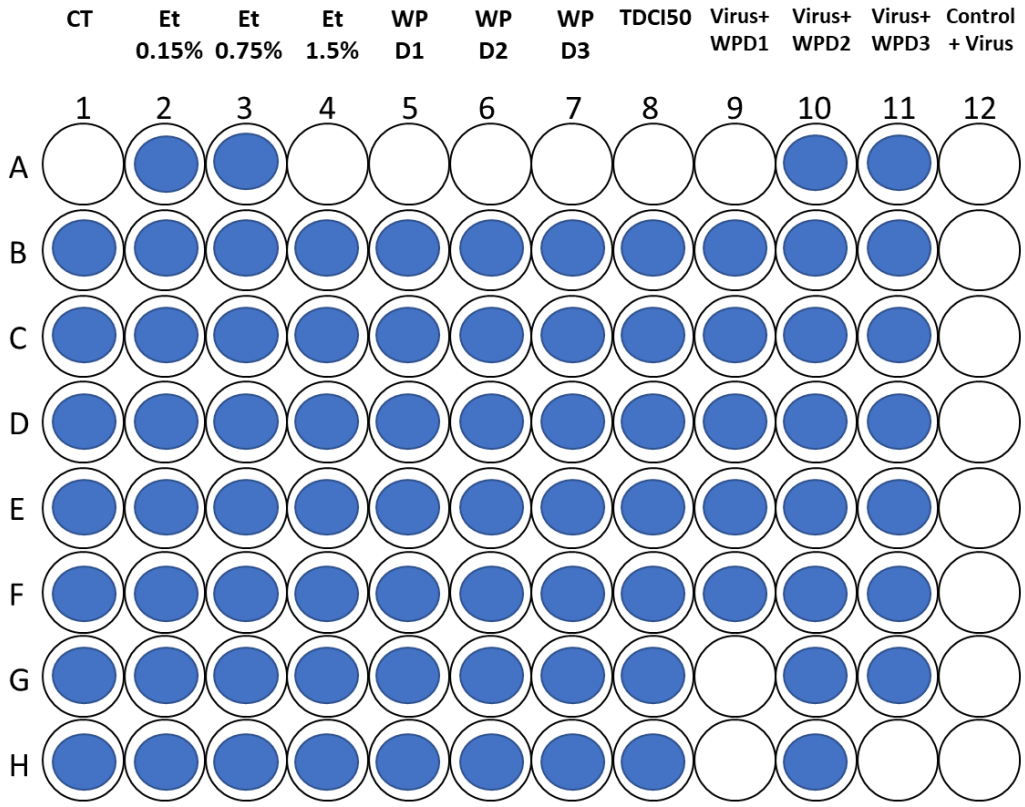


Figure 2. Cytotoxicity assay graphic representation. Shown data were collected at 27/07/2020 (first replicate). Columns are divided as indicated in experimental design. Blue-coloured dots represents viable cells. White-coloured dots represents unviable cells (dead). Note this replica had a TCID50 control weak (not kill 50% of Vero E6 cells). This example is shown to observe the marked cytoprotective effect in WPD2 and WPD3 columns comparing to 10-fold viral load positive control (100% of cellular death). No dose related WP D1, D2 and D3 cytotoxicity was observed on epithelial-derived Vero E6 cells (ICEP4 raw material, columns 5, 6 and 7).

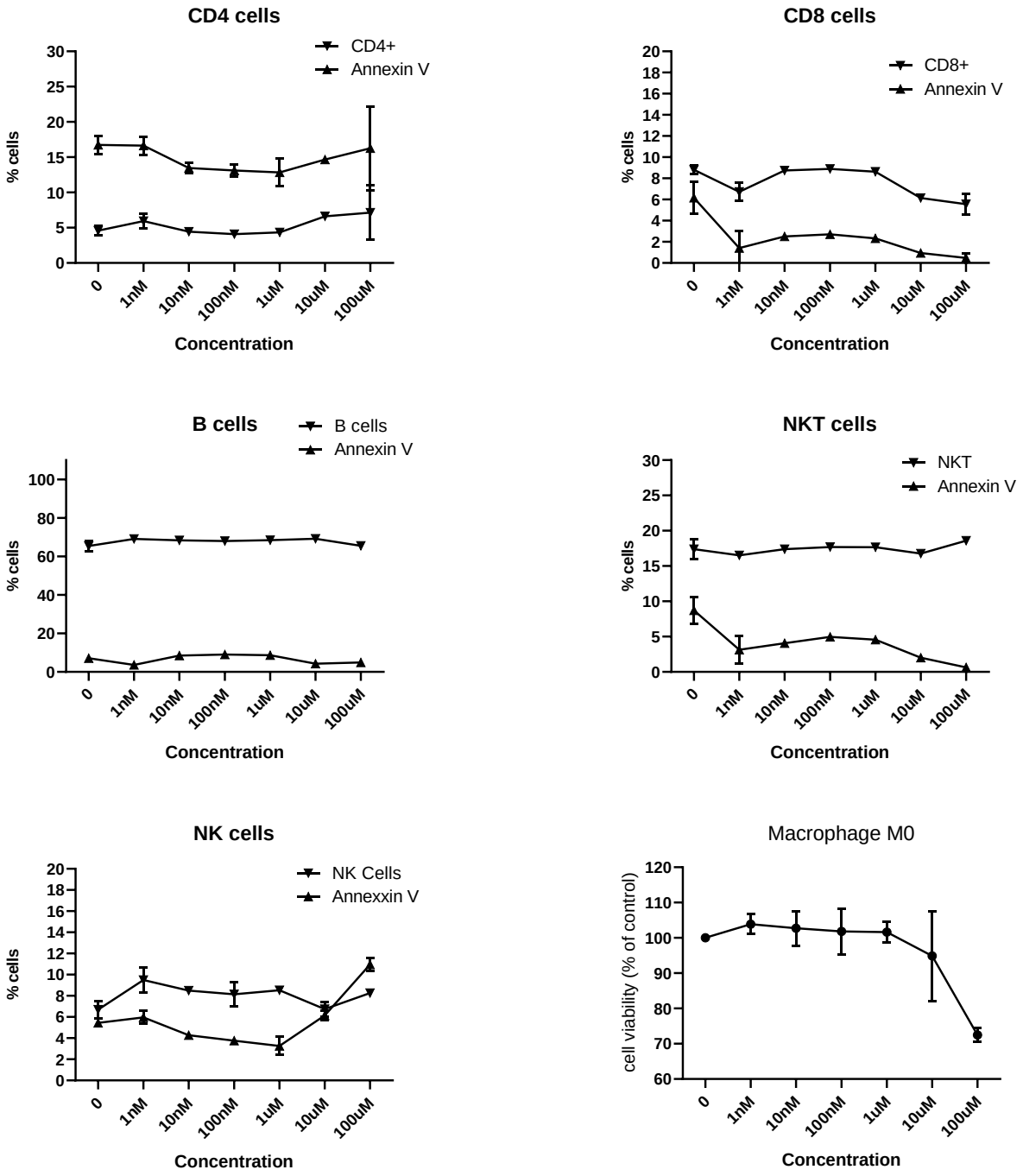
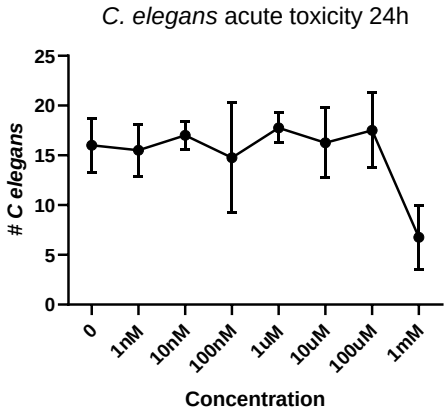


Figure 3. Effect of ICEP4 on cellular viability of M0 macrophages, B, $CD4^+$ T and $CD8^+$ T lymphocytes, Natural Killer (NK) and Natural Killer T (NKT) cells. Bone marrow derived macrophages (M0) or mouse splenocytes were incubated with different compound concentrations for 24h. Subsequently, the percentage of viable cells was determined using antibodies specific for T, B, NK and NKT cells and Annexin V staining as indicated in methods section. For M0 macrophages cell viability was measured by Presto Blue™ assay. Individual points represent the mean value ±SD of 3 independent experiments.

A)



B)

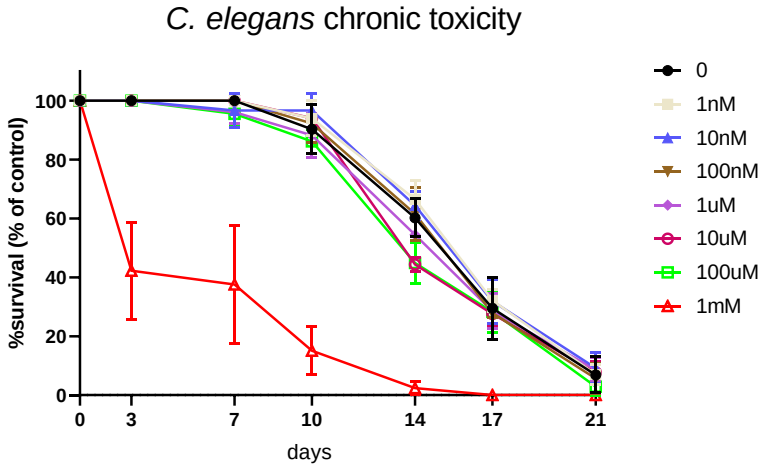


Figure 4. Acute (A) and chronic (B) toxicity of ICEP4 on *C. elegans* survival. Worms were incubated with different compound concentrations for 24h or 21 days and the percentage of viable worms was determined as indicated in methods. Individual points represent the mean value ±SD of 3 independent experiments.