

**Modulation of LPS induced inflammatory response by Lawsonyl monocyclic terpene from the marine derived *Streptomyces* sp.**

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## **Abstract**

In continuing research for compounds with immunosuppressive activity, Lawsonone (1), a novel Lawsonyl derivative isolated from marine-derived bacteria *Streptomyces* sp. was evaluated for its potent immunosuppressive activity on immune system. The effect of Lawsonone (1) was elucidated on the immune cells (splenocytes and macrophages) collected from BALB/c mice. Study was carried out to find the effect of Lawsonone (1) on Con-A and LPS stimulated splenocyte proliferation, LPS-induced NO, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  production in macrophages. Furthermore, the effect of Lawsonone (1) on T-cell subsets (CD4 and CD8) and total B-cell (CD19) population was analysed by flow cytometry. The results obtained in the present study showed that Lawsonone (1) inhibited the proliferation of both T and B splenocytes. It inhibited the nitric oxide (NO) and pro-inflammatory cytokine (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) production in LPS-stimulated macrophages in a dose-dependent manner. Moreover, flow cytometric analysis indicated the prominent inhibition of CD4, CD8 and CD19 cell populations in the spleen of mice treated with the variable doses of Lawsonone (1), with the maximum inhibition at the lowest dose (0.1  $\mu$ M). Taken together, the present results suggest that Lawsonone (1) may act as a potent molecule for immunosuppression and anti-inflammation, supporting its immunopharmacologic application to modify the immune system.

**Keywords:** Lawsonone (1); immunosuppressive; anti-inflammatory; splenocytes; macrophages; cytokines.

## 1. Introduction

Natural products remain either the source or inspiration for a significant proportion of the new small-molecule chemical entities introduced as drugs [1]. Microbial natural products are an important source of both existing and new drugs [2]. With marine species comprising approximately half of the total global biodiversity, the sea offers an enormous resource for novel compounds [3] and it has been classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity [4]. To date, most attention has been applied to marine ecosystems, especially deep sea environments, as a rich source of novel microbes and hence natural products. Workers have described antibacterial, antialgal, antifungal and antiviral activities among marine organisms [5,6]. So far several biomolecules which were isolated from sea microbes prove to be having the potent immunomodulatory and antitumor activities [7]. Among the producers of commercially important metabolites, actinomycetes have proven to be a prolific source with a surprisingly small group of taxa accounting for the vast majority of compounds. Actinomycetes are responsible for the production of about half of the discovered secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents and enzymes [8-10]. Secondary metabolites produced by actinomycetes possess a wide range of biological activities [11-13], and the vast majority of these compounds are derived from the single genus *Streptomyces* [14]. *Streptomyces* species are responsible for about two-thirds of the clinically used antibiotics, the most widely recognized secondary metabolites, including many antimicrobials such as erythromycin, streptomycin and tetracycline with unusual structures and potent biological activities [15]. That is one of the reason the isolation and chemical screening of novel *Streptomyces* recovered from unusual environments for new natural products is of great interest [16,17]. So far several distinct classes of biologically active metabolites effective against a broad spectrum of medical problems have been isolated from *Streptomyces*. Recent examples include the anticancer pladienolides [18], gutingimycin [19], new members of the mithramycin series [7], and coprolactones [20], augmented by the antifungal tokanavaenes [21] and yatakemycin [22]. All these distinct classes of natural products have been isolated from the various microbial strains of *Streptomyces*.

With increasing advancement in science and technology, there would be greater demands in future for new bioactive compounds synthesized by *Streptomyces* from various marine sources. The strong interplay of classical natural product chemistry with modern microbial genetics and bioinformatics will help to overcome supply and sustainability issues from the past and to

promote bioactive substances from marine *Streptomyces* to a well recognized alternative for future drug discovery programs.

Since the ingress of leukocytes into the site of inflammation is crucial for the pathogenesis of inflammatory conditions, the inhibition of the cellular reactions is therefore one of the targets that are generally used as an *in vitro* model for anti-inflammatory testing [23,24]. Here our ongoing research effort was to elaborate the microbial derived natural molecules showing the promising immunosuppressive potentiality, and extensively be applied for inflammation and autoimmune diseases. In this endeavor, the focus of the present investigation was to evaluate the immunosuppressive activity of Lawsonone (1) isolated from the marine-derived *Streptomyces* sp. by elucidating its effect on T and B-cell proliferation in spleen, production of pro-inflammatory mediators like NO, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in macrophages. Moreover, the effect of Lawsonone (1) was observed on CD4, CD8 and CD19 cell populations in spleen by flow cytometry.

## 2. Materials and Methods

### 2.1. Reagents

Medium RPMI 1640 (Himedia, Bombay, India), 96 V-wells micro-titration plates and micro-tissue culture plates (96 U-wells) from Tarson, trypan blue (Microlabs, Bombay), fetal calf serum (FCS), concanavalin-A (Con-A), lipopolysaccharide (LPS, Escherichia coli 055 B5), dimethylsulphoxide (DMSO), Hank's balanced salt solution (HBSS), HEPES, 2-mercaptoethanol, penicillin, streptomycin and MTT (3-[(4,5-dimethylthiazol-2yl)-2,5-diphenyl 2,5-dimethyltetrazolium bromide) were purchased from Sigma. Assay kits for all cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were purchased from R&D Systems, USA.

### 2.2. Sample Preparation

#### 2.2.1. Isolation and Taxonomy of the Producer Microorganism

UV and IR spectra were measured on a Perkin-Elmer Lambda 25 UV/vis spectrometer and a Thermo Nicolet IR 100 FT/IR spectrometer, respectively. <sup>1</sup>H, <sup>13</sup>C, and 2D NMR experiments were acquired on a Bruker, 400 & 500 MHz spectrometer. High-resolution mass spectral data were obtained from Agilent 6540 UHD Accurate Mass (Q-ToF) Mass Spectrometer, (LTQ XL/LTQ Orbitrap Discovery) coupled to a Agilent 1290 series LC system (Agilent PDA detector, autosampler, and thermostat, thermostatted column compartment, binary pump). The following conditions were used: capillary voltage 3500 V, capillary temperature 350 °C, auxiliary gas flow rate 7.0 L/min, spray voltage 4.5 kV, mass range 100-1000 amu (maximum resolution 30000). HPLC separations were made using waters spherisorb reversed-phase (C18, 250 X 10 mm, L i.d.) column connected to an Agilent 1200 series binary pump and monitored using an Agilent photodiode array detector. Detection was carried out at 220, 230, 254, 280, and 350 nm. Diaion HP-20 was obtained from Sigma Aldrich.

The microbial producer *Streptomyces* sp. was isolated from the marine ascidian *Synoicum indicum*, collected from intertidal rocky habitat of Honnavar, Karnataka, India (14.19Long, 74.45Lat). The specimens were frozen immediately after collection which was further washed in sterile seawater 3 times followed by moist heat treatment at 50°C for 30 minutes. The sample was homogenized with sterile seawater, serially diluted till 10<sup>-5</sup> dilutions and 0.1 ml aliquots of each dilution were plated on to Actinomycete Isolation Agar (AIA1). The plates were incubated at 30°C for 2 to 3 weeks. Colonies were selected based on filamentous growth and the colour of substrate and aerial mycelium. The individual colonies were purified and maintained in 20%

glycerol at -80°C. Out of several strains, NIO-10053 was selected for further bioassay-guided isolation of active molecules.

The sequences of PCR products were analyzed by using Basic Local Alignment Search Tool (BLAST). Nucleotide-nucleotide BLAST was carried out with facility of National Centre for Biotechnology Information (NCBI) database, NIO-1034 showing 99% identity with *Streptomyces* sp. FTCt21 16S, FTCt5, 219835(1), 172717-3 and *Actinobacterium* ZXY013 NCBI Accession No. [gi|343129258|JN248577.1](https://www.ncbi.nlm.nih.gov/nuclot/gi|343129258|JN248577.1), [gi|343129256|JN248575.1](https://www.ncbi.nlm.nih.gov/nuclot/gi|343129256|JN248575.1), [gi|332647051|HQ992765.1](https://www.ncbi.nlm.nih.gov/nuclot/gi|332647051|HQ992765.1), [gi|332647005|HQ992719.1](https://www.ncbi.nlm.nih.gov/nuclot/gi|332647005|HQ992719.1) and [gi|339765088|JN049462.1](https://www.ncbi.nlm.nih.gov/nuclot/gi|339765088|JN049462.1).

### 2.2.2. Fermentation

Seed inoculum of the *Streptomyces* sp. Lawsonone (1) was prepared by growing the culture in 500 ml of Modified FM 17 medium (Glucose, 20 g; yeast powder, 5 g; casein, 5 g; KNO<sub>3</sub>, 10 g; CaCO<sub>3</sub>, 2 g; sea water, 1 L; pH 7.2-7.4 for 3 days at 30 ± 2°C. This inoculum was used to seed 10 liters of FM 17 media and incubated for 10 days at 30 ± 2°C and 150 rpm.

### 2.2.3. Extraction and Isolation

The 5 L of whole broth was homogenized with EtOAc (1 L) and filtered through a pad of Celite which was further extracted with ethyl acetate (1 L X 2). The crude extract was passing through the Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure in a rotary vacuum evaporator afforded brownish, oily residue (250 mg). The 200 mg extract was subjected to flash column chromatography on silica gel (230-400 mesh), using a stepwise gradient of Hexane in EtOAc to yield eight sub-fractions Fr.1-8. Fraction 7 which was eluted 50% EtOAc afforded 42 mg with the mixture of three compounds with the very close R<sub>f</sub>, which was further purified by semi-preparative HPLC using (C-18 Column, 5μ, 10X250 mm, gradient H<sub>2</sub>O-CH<sub>3</sub>CN (5% to 50%) CH<sub>3</sub>CN in 50 min, flow rate 1.5 mL/min, UV detection at 215 nm). Lawsonone (1) as a colourless solid (8.0 mg, t<sub>R</sub> = 23.2 min), was obtained under these conditions.

The total 5 L of fermentation broth was filtered using muslin cloth and further partitioned with ethyl acetate, butanol and the water fraction was finally passed through the HP-20 which was subsequently eluted with MeOH: H<sub>2</sub>O (8:2). All these fractions were screened for immunosuppression and cytotoxic studies in distinct cancer cell lines which is mentioned in the Supporting information [Table 2]. Finally the Ethyl acetate fraction (NIO-10053-A) inhibited TNF-α (50%), IL-1β (38%) & IL-6 (18%) at concentration of 10μg/ml whereas Butanol fraction

(NIO-10053B) inhibited TNF- $\alpha$  (20%), IL-1 $\beta$  (18%) & IL-6 (10%) and water fraction (NIO-10053-C) inhibited the TNF- $\alpha$  (14%) (Data not shown), therefore the crude activity data shows that NIO-10053-A is most potent against immunosuppression. The bioassay guided ethyl acetate fraction was further separated by column chromatography followed by RP HPLC to isolate the Lawsonone (1) (8 mg) as a colorless amorphous solid.

Lawsonone (1) was obtained as a colorless amorphous solid. The HRESIMS of Lawsonone (1) yielded molecular ions with peak at ( $m/z$  316.2103 [M]<sup>+</sup>) and other fragment with peak at  $m/z$  174.0325 [M-C<sub>7</sub>H<sub>11</sub>O<sub>3</sub>]<sup>+</sup> and  $m/z$  144.0791 [M-C<sub>10</sub>H<sub>5</sub>O<sub>3</sub>]<sup>+</sup>; which was further supported by NMR data in (Table 3). Molecular formula was determined as C<sub>17</sub>H<sub>16</sub>O<sub>6</sub>, accounting for ten degrees of unsaturation. The IR spectrum of Lawsonone (1) suggested the presence of hydroxyl groups and conjugated ketone and ether showing absorbance at 3510-2985 cm<sup>-1</sup> (broad), 1677 cm<sup>-1</sup>, 1210 3445 (br, OH), 1665, 1640, 1583, (quinone C=O and C=C), 1210 (C-O) cm<sup>-1</sup>, respectively, while the UV spectrum had  $\lambda_{\max}$  values at 212, 226, 274, 318, and 372 nm.

The <sup>1</sup>H NMR spectrum in (Table 3) of Lawsonone (1) displayed signals for three oxygenated methylene proton at  $\delta$  3.73 (m, 2a), 3.63 (dd, 2b), 3.57 (dd, 6a), 3.50 (d, 6b) and 5.65 (s, 8a), 5.55 (s, 8b) along with the one oxygenated methine protons at  $\delta$  4.23 (dd, 5) and hydroxyl proton  $\delta$  4.10 (t, OH, 5). Beside that there is one methine proton  $\delta$  2.77 which is linked with the carbonyl group and one methylene proton  $\delta$  1.26. In addition to that there are five aromatic signals at  $\delta$  7.72 (m, for two proton), 7.60 (m, for two proton), 6.51 (s, one proton), which was further authenticated by <sup>13</sup>C NMR (Table 3) and DEPT which indicates 17 carbon atoms containing four Methylene (CH<sub>2</sub>), seven methine (CH) and six quaternary (C) carbon atoms, which was further authenticated with the help of HSQC correlation data indicating that Lawsonone (1) has a naphthoquinone [25,26] linked with the pyran derived moiety. Other signals in <sup>13</sup>C NMR correspond to three oxygenated methylenes and one methylene, seven methines where one of which is oxygenated ( $\delta$  59.15), and seven quaternary carbons of which one is an oxygenated quaternary carbon at  $\delta$  155.90 along with three carbonyl carbons ( $\delta$  170.35, 169.82 and 169.77). All signal assignments of <sup>1</sup>H, <sup>13</sup>C, NMR and <sup>1</sup>H-<sup>1</sup>H-COSY, HSQC and HMBC spectral data (given in the supporting information) suggested that Lawsonone (1) contains 2-Hydroxy-1,4-Naphthoquinone which is linked with the pyranone derived moiety. Furthermore, the HMBC cross peak from H-2 to C-6 suggested that C-3 and C-6 were linked through an oxygen atom to form a tetrahydropyran ring. The <sup>1</sup>H-<sup>1</sup>H-COSY data of Lawsonone (1) indicated the presence of alkylated spin systems, CH<sub>2</sub>(2)-CH(3)-CH<sub>2</sub>(4)-CH (5)-CH<sub>2</sub>(6) and CH(6')-CH(7',8')-CH(7')-CH(9')-CH(8',7'). These partial structures, methines are joined together through non-protonated carbons along with carbonyl on the basis of HMBC correlations to form a naphthoquinone

framework [27] (Fig. 1). Other corresponding signals in HMBC show correlation CH<sub>2</sub>-2 ( $\delta$  3.73) to C-3 ( $\delta$  45.42), CH-3 ( $\delta$  3.73) to C-7 ( $\delta$  170.3), CH<sub>2</sub>-4 ( $\delta$  1.26) to C-3 ( $\delta$  45.4), CH-5 ( $\delta$  4.23) to C-4 ( $\delta$  36.8) and CH<sub>2</sub>-6 ( $\delta$  3.55) to C-5 ( $\delta$  59.1) a long range coupling correlation CH<sub>2</sub> ( $\delta$  5.65, 5.55) to C-2' ( $\delta$  155.9). HMBC correlations of H-5 and H-3 to C-6 and C-7 are also helped to locate the ketone ( $\delta$  170.35) hydroxyl group ( $\delta$  72.7) carbonyl carbons at C-1' and C-4', respectively. The molecular formula depicts one double bond equivalent, and the considerably deshielded nature of C-3' ( $\delta$  C 116.1). <sup>13</sup>C NMR and DEPT data with the molecular formula indicates that there must be an exchangeable proton which requires the presence of hydroxy group in pyran nucleus. The relative configuration of Lawsonone (1) was mainly elucidated from NOESY correlation as being compatible with computer modeling (Fig. 2) in which the close contacts of atoms in space calculated were consistent with NOESY correlation. In NOESY correlation, H-3 exhibited a correlation with H-2 indicating that these two protons (H-2 & H-3) were situated in a same face and assigned as  $\beta$ -protons. One proton of C-5 methylene ( $\delta$  H 4.23) exhibited the correlations with H-4 and OH-5 ( $\delta$  4.1) but not with the H-6 indicating that this proton and the hydroxyl group at C-5 were in  $\beta$ -orientation [28] in Lawsonone (1) as depicted by molecular modeling analysis. Although a correlation was found between H-5 $\alpha$  and H-4, the Z-configuration of the C-2'/3' double bond was elucidated from the correlation between H-3'  $\alpha$  and the C-8 methylene reflecting the E-geometry of the double bond at C-2'/3'. From the above evidence, the relative configuration of the chiral carbons of Lawsonone (1) were assumed to be 3S\* and 5S\*. On the basis of all considerable spectral data analysis we conclude that the structure of Lawsonone (1) was identified as 2-(2-((3S,5S)-5-hydroxytetrahydro-2H-pyran-3-yl)-2-oxoethoxy)naphthalene-1,4-dione. Lawsonone (1) as a colourless solid (8.0 mg, Retention time in HPLC  $t_R$  = 23.2 min) was obtained under these conditions.

Lawsonone (1): Colourless solid;  $[\alpha]_D^{25} +6.6$  (c 0.01, CHCl<sub>3</sub>);  $\lambda_{max}$  (log  $\epsilon$ ) 212 (2.10), 226 (2.59), 274 (3.45), 318 (3.02), 372 (2.79) nm; IR (KBr)  $\nu_{max}$  3445 (br, OH), 1656, 1630, 1581, (quinone C=O and C=C), 1210 (C-O) cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR spectra (400, 500 and 125 MHz, respectively), see Table 1; (+)-HRESIMS  $m/z$  316.0955 [M]<sup>+</sup>, 339.1004 [M+Na]<sup>+</sup>, [M-C<sub>10</sub>H<sub>6</sub>O<sub>3</sub>]<sup>+</sup>, [M-C<sub>7</sub>H<sub>12</sub>O<sub>3</sub>]<sup>+</sup>; (+)-HRESIMS  $m/z$  316.0955 (calcd for C<sub>17</sub>H<sub>16</sub>O<sub>6</sub>, 316.0947).

### 2.3. Experimental Animals

The study was conducted on male Balb/c mice (18–22 g). The ethical committee of the Indian Institute of Integrative Medicine, Jammu (CSIR, India) instituted for animal handling approved all the protocols. The animals were bred and maintained under standard laboratory conditions:

temperature ( $25 \pm 2$  °C) and a photoperiod of 12 h. Commercial pellet diet and water were given *ad libitum*.

#### **2.4. Lymphocyte proliferation studies on mice splenocytes**

Spleen collected under aseptic conditions in HBSS, was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension and the erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation ( $380 \times g$  at 4 °C for 10 min), the pelleted cells were washed three times with PBS and resuspended in complete medium [RPMI 1640 supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% FCS]. The cell number was counted with a haemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95% [29].

To evaluate the effect of the Lawsonone (1) on the proliferation of splenic lymphocytes, the spleen cell suspension ( $2 \times 10^6$  cells/mL) was pipetted into 96-well plates (200 µL/well) and cultured at 37 °C for 72 h in a humid saturated atmosphere containing 5% CO<sub>2</sub> in presence of Con-A (5 µg/mL), LPS (1µg/mL) and variable doses of Lawsonone (1) (0.1-100 µM). After 72 h, 20 µL of MTT solution (5 mg/mL) was added to each well and the plates were incubated for 4 h. The plates were centrifuged ( $1400 \times g$ , 5 min) and the untransformed MTT was removed carefully by pipetting. To each well, 100 µL of a DMSO working solution (192 µL DMSO with 8 µL 1 M HCl) was added and the absorbance was evaluated in an ELISA reader at 570 nm after 15 min.

#### **2.5. Collection of peritoneal macrophages and nitrite assay**

A volume of 10 ml of RPMI 1640 medium was injected into the mice peritoneal cavity. After 5 min, the medium was taken out and centrifuged at  $1800 \times g$  for 10 min. at 4°C. The cell pellet was resuspended in RPMI 1640 medium. Macrophages ( $3 \times 10^6$ ) were seeded in 24-well culture plate in a CO<sub>2</sub> incubator for 3 hrs. At the end of incubation period, non-adherent cells were removed and plates were further incubated for 48 h in the presence of LPS (1µg/ml) and variable doses of Lawsonone (1) (0.1-100 µM). Supernatants were collected after centrifugation and kept at -80°C for the measurement of cytokines. For the NO<sub>2</sub><sup>-</sup> assay (nitrite content), 100 µl of culture media was incubated with 100 µl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine in 2.5% phosphoric acid solution) at room temperature for 10 min in 96-well micro plate [30]. Absorbance at 540 nm was read using an ELISA plate reader. Standard calibration curves were prepared using sodium nitrite as standard.

## **2.6. Determination of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) in macrophages.**

Macrophages collected from the peritoneal cavity of mice as described above in Section 2.5 were treated with the indicated amounts of Lawsonone (1) (0.1-100  $\mu$ M) for 24 h. After subsequently stimulated with 1 $\mu$ g/mL LPS for 24 h, the supernatants were harvested. The concentrations of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in culture supernatants were measured using mouse IL-1 $\beta$ , IL-6 and TNF- $\alpha$  ELISA kits (R&D Systems, USA), as per the instructions of the manufacturer.

## **2.7. Lymphocyte immunophenotyping in spleen**

The spleen (1/3 of the organ) was placed in PBS buffer (without Mg<sup>2+</sup> and Ca<sup>2+</sup>) and stored on ice prior to preparation of single cell suspension. Splenic erythrocytes were lysed with red blood cell lysis buffer (BD Pharmingen). Cell suspensions were refrigerated (ca. 4 °C) before staining with antibodies. All reagents were purchased from BD Pharmingen. Thereafter, 2 $\times$ 10<sup>6</sup> cells were treated with variable doses of Lawsonone (1) (0.1- 100  $\mu$ M) and stained with conjugated anti-CD4 FITC, anti-CD8 PE and anti-CD19 PE antibodies. After staining with antibodies and 30 minute incubation at room temperature, cells were washed and resuspended in PBS for flow cytometric analysis which was performed on a FACS Calibur flow cytometer equipped with Cell Quest software (Becton Dickinson) [31].

## **2.8 Data analysis**

Data are expressed as mean  $\pm$  S.E.M. and statistical analysis was carried out using one-way ANOVA (Bonferroni correction multiple comparison test). Dunnett's test was used to analyze the different variables in the same subject and *P* values less than 0.05 were being taken as statistically significant.

### **3. Results**

#### **3.1. Effect of Lawsonone (1) on splenocyte proliferation assay**

To confirm the effect of the Lawsonone (1) on the cellular immune response, the proliferation of splenocytes in response to Con A (5 µg/mL) and LPS (1 µg/mL) was evaluated. Splenocyte proliferation was observed by the reduction of MTT. The results indicated that Lawsonone (1) suppressed both T and B-cell proliferation of splenocytes at the doses of 0.1, 1, 10 and 100 µM in a dose-dependent manner compared to control; maximum inhibition observed at 0.1 µM/mL (Fig. 3). Betamethasone (BMS) used in the concentration of 0.05 µM also showed a significant inhibition ( $P < 0.05$ ) of both T and B-lymphocyte proliferation compared to control.

#### **3.2. Effect of Lawsonone (1) on NO production in macrophages**

Since NO is also known as a pro-inflammatory mediator in many different acute and chronic inflammatory diseases, we then addressed whether Lawsonone (1) modulates the NO production from macrophages stimulated by LPS. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve and the results are expressed in µM. Lawsonone (1) at a dose range of 0.1-100 µM inhibited the NO production in a dose dependent manner. The maximum inhibition was observed at 0.1 µM dose compared to control. Decrease in the dose of Lawsonone (1) registered a significant inhibition in the production of nitrite content (Fig. 4). BMS also showed a significant inhibition ( $P < 0.05$ ) in nitrite content compared to control.

#### **3.3. Effect of Lawsonone (1) on IL-1 $\beta$ , IL-6 and TNF- $\alpha$ in LPS-induced macrophages**

Pro-inflammatory cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) in macrophages stimulated with LPS and variable doses of Lawsonone (1) (0.1-100 µM) were evaluated by ELISA assay using commercial kits. Results indicated that Lawsonone (1) inhibited the release of these three cytokines from macrophages in a dose-dependent manner. At a dose of 0.1 µM, inhibition in IL-1 $\beta$  (Fig. 5a), IL-6 (Fig. 5b) and TNF- $\alpha$  (Fig.5c) was found to be the most significant ( $P < 0.01$ ). BMS also registered a significant inhibition ( $P < 0.05$ ) in the release of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  from LPS stimulated macrophages compared to control.

#### **3.4. Effect of Lawsonone (1) on lymphocyte immunophenotyping in spleen**

Furthermore, the effect of Lawsonone (1) was observed on the population of T and B-cell surface markers like CD4, CD8 and CD19 in the splenocytes prepared from the spleen of mice treated with variable doses of Lawsonone A (1). Results are depicted in Table 4. Lawsonone (1) showed

a significant ( $P < 0.01$ ) dose dependent inhibition of T cell subsets (CD4 and CD8) as well as B cell (CD19) population with the maximum effect at 0.1  $\mu\text{M}$  dose compared to control. BMS, a standard immunosuppressant also showed a significant inhibition ( $P < 0.05$ ) in CD4, CD8 and CD19 cell populations compared to control.

#### **4. Discussion**

The objective of the present study was to investigate in-depth the immunomodulatory activity of Lawsonone (1) isolated from marine *Streptomyces* sp. on the immune cells. The main focus was to explore the attenuation of LPS-induced acute inflammatory response under *in vitro* conditions. Initially, the inhibitory effect of Lawsonone (1) on spleen derived T and B-cell proliferation was evaluated by MTT assay. Furthermore, to confirm the potential role of cytokines in the modulation of LPS priming effects, we evaluated LPS-dependent pro-inflammatory mediators like NO, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in macrophages treated with variable doses of Lawsonone (1). Our data strongly suggests that an exposure of naive macrophages to Lawsonone (1) A may differentially modulate cell responsiveness to LPS. Therefore, among other regulatory pathways, a cytokine network may well represent a potential mechanism controlling the bias of specific macrophage phenotypes induced by LPS priming.

Macrophages are important inflammatory cells implicated in the initiation of inflammatory responses, and play critical roles in the development of acute lung injury by secreting various pro-inflammatory mediators including tumour necrosis factor-alpha (TNF- $\alpha$ ), interleukin-6 (IL-6), interleukin-1 $\beta$  and nitric oxide (NO). By activating macrophages, numerous biologically active substances are released as important toxic substances, which participate in anti-infection, shock, fever, organ failure and other pathophysiological processes [32]. TNF- $\alpha$  is secreted by a variety of cells, including macrophages, lymphocytes, mast cells and epidermal cells, however, it mainly comes from macrophages [33]. It is a classical pro-inflammatory cytokine involved in the early phase of acute lung inflammation [34]. Excessive production of TNF- $\alpha$  is believed to underlie the progression of many serious inflammatory diseases, such as rheumatoid arthritis (RA), therefore, anti-TNF- $\alpha$  therapy would be a possible tool for treatment of acute and chronic inflammatory diseases [24]. IL-1 $\beta$  is another pro-inflammatory cytokine that has been implicated in pain, inflammation and autoimmune conditions. Overproduction of IL-1 $\beta$  is implicated in the pathophysiological changes that occur during different disease states, such as rheumatoid arthritis, neuropathic pain, inflammatory bowel disease, osteoarthritis, vascular disease, multiple sclerosis, and Alzheimer's disease [35]. Therefore, it is suggested that blockade

of IL-1 $\beta$  be considered as a therapeutic opportunity. Furthermore, IL-6 is an important pro-inflammatory cytokine involved in RA and many RA symptoms are associated with IL-6 deregulation. Thus, reducing IL-6 has long been considered a therapeutic target in RA management. In the present study, production of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in LPS-stimulated macrophages was inhibited by Lawsonone (1) at the lowest concentration (0.1  $\mu$ M) without any effect on the viability of cells.

In addition to the inhibitory effect on pro-inflammatory cytokine production, Lawsonone (1) also significantly attenuated the secretion of NO in macrophages induced by LPS. The small amount of NO produced by constitutive NOS, is an important regulator of physiological homeostasis, whereas the large amount of NO produced by inducible NOS has been closely correlated with the pathophysiology in a variety of diseases and inflammation [36]. NO is induced by LPS or immunological stimuli (e.g., IFN- $\gamma$ ) implicating as a mediator of inflammation [37]. It has been well known that excess production of NO by macrophages and other cells exposed to endotoxin may contribute to septic shock, cerebral injury, myocardical ischemia, local or systemic inflammatory disorders, diabetes and other diseases. Therefore, inhibition of NO is also potentially beneficial. The inhibition of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and NO production by Lawsonone (1) suggests the involvement in the down-regulation of late or prolonged production of pro-inflammatory mediators and may contribute to anti-inflammation and immunosuppression. Results of the present study indicate that the reduced secretion of pro-inflammatory mediators (IL-1 $\beta$ , IL-6, TNF- $\alpha$  and NO) by LPS stimulated macrophages and the inhibition of splenic T and B-cell proliferation were involved in the *in vitro* effects of Lawsonone (1) on the LPS-induced acute inflammatory responses.

To date, several important common pathways have been identified in the molecular mechanisms by which compounds and fractions inhibit the pro-inflammatory responses. Our data elucidated the targets of Lawsonone (1), because its inhibitory pattern on the production of NO was apparently consistent with the inhibition of pro-inflammatory cytokines in macrophages stimulated by LPS, thus outlining one of mechanistic explanation for the anti-inflammatory effect of Lawsonone (1).

Furthermore, to further elucidate the mechanism of Lawsonone (1) as an immunomodulator, the effects of Lawsonone (1) on CD4<sup>+</sup>, CD8<sup>+</sup> T lymphocytes and CD19 B cell populations in spleen were analyzed by flow cytometric assay. T cells differentiate into two different subsets according to their specific membrane molecule, that is CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, which play different roles in immunomodulation. It is well known that Th cells and cytotoxic T lymphocytes (CTLs)

responses are associated with the enhancement of CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes respectively. Th cells can promote proliferation, maturation, and immunologic function of other cell types, and specific lymphokines secreted by Th cells are very important for the activities of B cells, macrophages, and CTLs [38]. In contrast, CTLs are extremely important in the defence against viral infections. Cluster of differentiation (CD) 19 antigens are B-cell-specific molecules expressed on virtually all human cells of the B-lymphocyte lineage except plasma cells. CD19, total B-cell surface marker plays a regulatory role in B-cell proliferation and differentiation [39]. The importance of the CD19 marker is that it allows analysis of B lymphocytes, which are responsible for humoral immune responses. The results of present investigation revealed that the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in Lawsonone (1) treated splenocytes were significantly inhibited as depicted by flow cytometry. The decreased percentages of both CD4<sup>+</sup> and CD8<sup>+</sup>T lymphocytes in spleen indicated that both Th and CTLs were inhibited greatly by Lawsonone A (1). Moreover, Lawsonone (1) significantly inhibited the total B cell (CD19) population, thereby showing the consistency of results with the inhibition of splenic T and B-cell proliferation as discussed earlier. Betamethasone is a potent glucocorticoid steroid with anti-inflammatory and immunosuppressive properties [40], hence it has been used as a standard immunosuppressant in the present study.

## **Conclusion**

In conclusion, the present results suggest that Lawsonone (1) acts as an effective immunosuppressor by specifically inhibiting the Con-A and LPS stimulated splenic T and B-cell proliferation. Its effect on LPS-induced macrophage released mediators suggest that it acts as a potent inhibitor of the LPS-induced NO and pro-inflammatory cytokine (IL-1  $\beta$ , IL-6 and TNF- $\alpha$ ) production in macrophages. In addition, the data obtained from the cytotoxicity activity against cancer cell lines which is mentioned in the Table 2 in supporting information suggests that Lawsonone (1) is a potential candidate for immunosuppressive and anti-inflammatory activity. These preliminary results exhibited may be through down regulation of anti-cancer mediators such as NF- $\kappa$ B etc. Moreover, its inhibitory effect on the splenic CD4, CD8 and CD19 cell population suggests its immunopharmacologic applications to modify the immune system during various diseases.

### **Conflicts of interest**

The authors declare that they have no conflicts of interest.

### **Acknowledgments**

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### **Supporting Information**

General experimental procedure along with the  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^1\text{H}$ - $^1\text{H}$  COSY, HSQC and HMBC correlation spectral data of Lawsonone (1) along with phylogenetic tree of *Streptomyces* sp. (Table 1) and preliminary cytotoxicity data (Table 2) are available in Supporting information.

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### Legends to Figures

1. Key COSY, HMBC, correlations of Lawsonone (1).
2. Key NOESY correlations of Lawsonone (1).
3. Effect of Lawsonone (1) on Con-A and LPS-stimulated splenocyte cell proliferation. Splenocytes were isolated and stimulated with sub-optimal amounts of mitogens; Con A (5  $\mu\text{g/ml}$ ), LPS (1  $\mu\text{g/ml}$ ) for T and B cell proliferation, respectively. Cells were incubated for 72 h and proliferation was measured by MTT reduction assay. Values (mean  $\pm$  S.E.) are represented by OD at 570 nm (n=10); \*P<0.05, \*\*P<0.01 (control vs. treated groups); one-way ANOVA followed by Bonferroni multiple comparison test. For experimental details refer to Material and Methods section.
4. Effect of Lawsonone (1) on the nitrite content in mice peritoneal macrophages. Macrophages ( $3 \times 10^6$  cells/well) were cultured and stimulated with LPS (1  $\mu\text{g/ml}$ ) for 48 h. The supernatants were used for nitrite assay using Griess reagent. Results are expressed in  $\mu\text{M}$ . Values are means  $\pm$  S.E. (n=10); \*P<0.05, \*\*P<0.01 (control vs. treated groups); one-way ANOVA followed by Bonferroni multiple comparison test. For experimental details refer to Material and Methods section.
- 5a. Effect of of Lawsonone (1) on IL-1  $\beta$  release in mice peritoneal macrophages. Macrophages ( $3 \times 10^6$  cells/well) were cultured and stimulated with LPS (1  $\mu\text{g/ml}$ ) for 48 h. IL-1  $\beta$  in the macrophage culture supernatant was assayed by ELISA following instructions of the manufacturer. Other conditions were the same as described in Materials and Methods. Values are means  $\pm$  S.E. (n=10); \*P<0.05, \*\*P<0.01 (control vs. treated groups); one-way ANOVA followed by Bonferroni multiple comparison test. For experimental details refer to Material and Methods section.
- 5b. Effect of of Lawsonone (1) on IL-6 release in mice peritoneal macrophages. Macrophages ( $3 \times 10^6$  cells/well) were cultured and stimulated with LPS (1  $\mu\text{g/ml}$ ) for 48 h.

IL-6 in the macrophage culture supernatant was assayed by ELISA following instructions of the manufacturer. Other conditions were the same as described in Materials and Methods.. Values are means  $\pm$  S.E. (n=10); \*P<0.05, \*\*P<0.01 (control vs. treated groups); one-way ANOVA followed by Bonferroni multiple comparison test. For experimental details refer to Material and Methods section.

**5c.** Effect of Lawsonone (1) on TNF- $\alpha$  release in mice peritoneal macrophages. Macrophages ( $3 \times 10^6$  cells/well) were cultured and stimulated with LPS (1  $\mu$ g/ml) for 48 h. TNF- $\alpha$  in the macrophage culture supernatant was assayed by ELISA following instructions of the manufacturer. Other conditions were the same as described in Materials and Methods.. Values are means  $\pm$  S.E. (n=10); \*P<0.05, \*\*P<0.01 (control vs. treated groups); one-way ANOVA followed by Bonferroni multiple comparison test. For experimental details refer to Material and Methods section.

Table 3. NMR Spectroscopic Data of Lawsonone (1) (400, 500 MHz, CDCl<sub>3</sub>).

No.	$\delta_c$ , mult. <sup>a</sup>	$\delta_H$ , mult. (J in Hz) <sup>b</sup>	HMBC <sup>c</sup>	COSY
1	-----	-----		
2a	68.23, CH <sub>2</sub>	3.73 m,	3, 4	3
2b		3.63 dd, (7.6, 3.6)		
3	45.42, CH	2.77 dd (10.4, 14.4)	2, 4, 7	2, 4
4	36.84, CH <sub>2</sub>	1.26 dd (4.4, 7.2)	2, 3, 5	3, 5
5	59.15, CH	4.23 dd (3.2, 2.8), 4.1 t (OH)	4, 6	4, 6
6a	72.74, CH <sub>2</sub>	3.57 dd (3.2, 4.0),	5	5
6b		3.50 d (3.6)		
7	170.35 C=O		3, 8	
8a	78.34, CH <sub>2</sub>	5.65 s,	7, 2	
8b		5.55 s		
1'	169.82, C=O		9'	
2'	155.90, C		8	
3'	116.16, CH	6.51 s	4'	
4'	169.77, C=O		6'	
5'	129.24, C			
6'	130.34, CH	7.60 m	4'	7', 8'
7'	134.79, CH	7.72 m	9'	6', 9'
8'	135.68, CH	7.72 m	10'	6'
9'	127.54, CH	7.60 m	1', 7'	8', 7'
10'	129.17, C		8'	

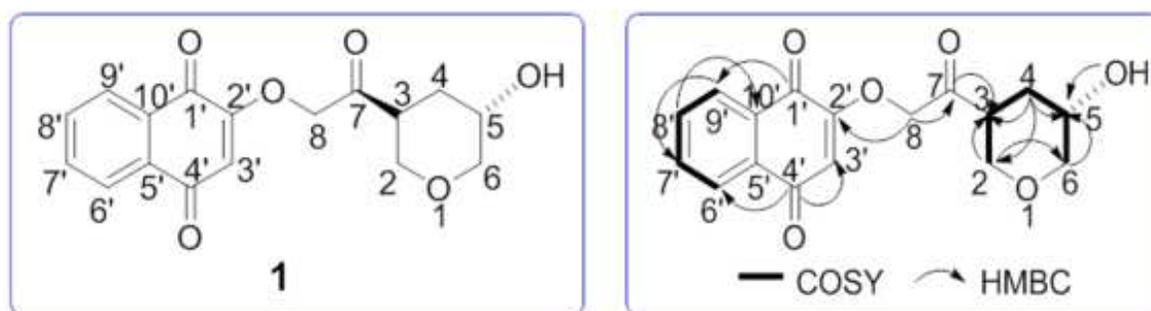
<sup>a</sup>Recorded at 125 MHz. <sup>b</sup>Recorded at 400 MHz. <sup>c</sup>From proton to the indicated carbon.

Table 4. Effect of Lawsonone (1) on CD4, CD8 and CD19 population in spleen cells.

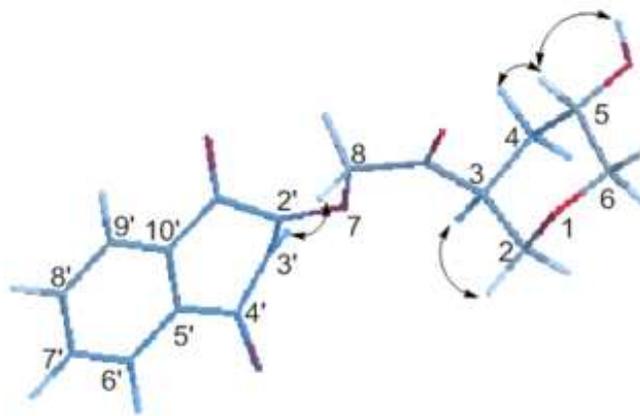
<b>Treatment</b>	<b>Doses (<math>\mu\text{M}</math>)</b>	<b>% CD4</b>	<b>% CD8</b>	<b>% CD19</b>
Control	-	24.12 $\pm$ 1.24	18.24 $\pm$ 0.23	22.12 $\pm$ 1.24
BMS	0.05	12.24 $\pm$ 0.2	9.65 $\pm$ 0.32	10.24 $\pm$ 0.22
Lawsonone (1)	100	20.16 $\pm$ 0.84*	16.18 $\pm$ 0.31*	14.11 $\pm$ 0.26*
Lawsonone (1)	10	18.36 $\pm$ 0.03*	14.48 $\pm$ 0.31*	12.46 $\pm$ 0.28*
Lawsonone (1)	1	10.28 $\pm$ 0.20*	9.36 $\pm$ 0.24**	8.35 $\pm$ 0.22**
Lawsonone (1)	0.1	8.38 $\pm$ 0.52**	7.48 $\pm$ 0.15**	6.44 $\pm$ 0.18**

Single cell suspensions of spleen were prepared and cells treated with BMS (0.05  $\mu\text{M}$ ) and variable doses of Lawsonone (1). Thereafter, cells were stained with conjugated anti-CD4 FITC, anti-CD8 PE and anti-CD19 PE antibodies as described. Values are mean  $\pm$  S.E. of six animals. \* $p < 0.05$  and \*\* $p < 0.01$  compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test). For experimental details refer to Material and Methods section.

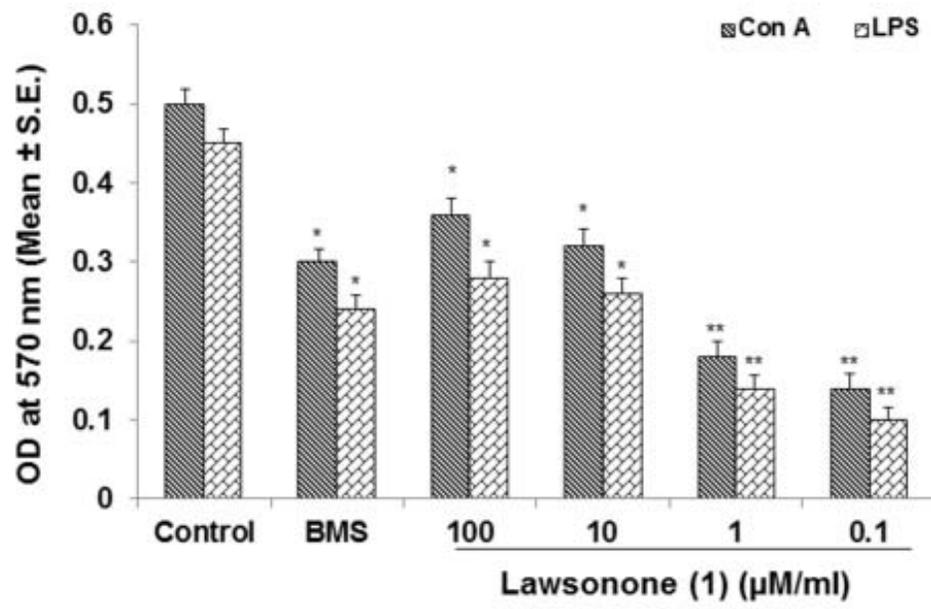
Fig.1



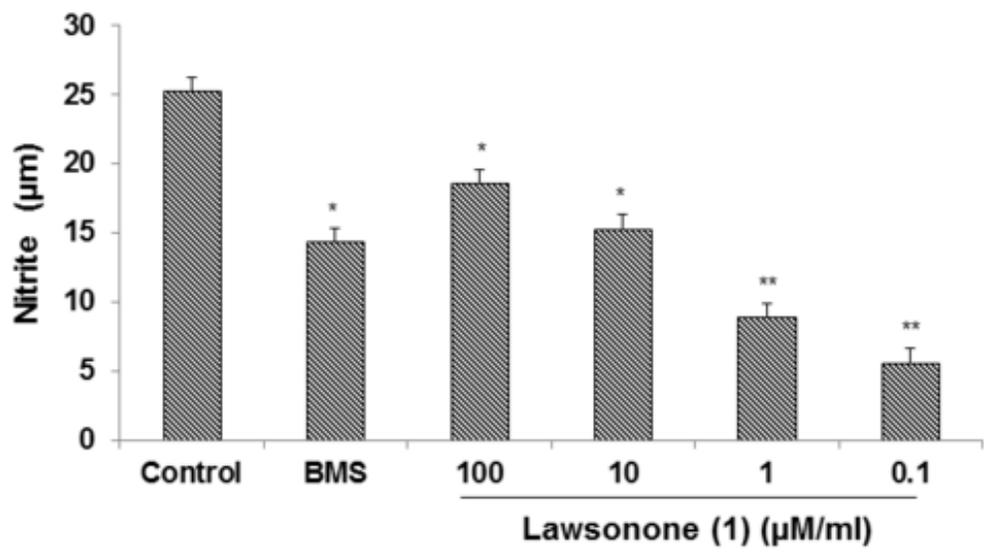
**Fig. 2**



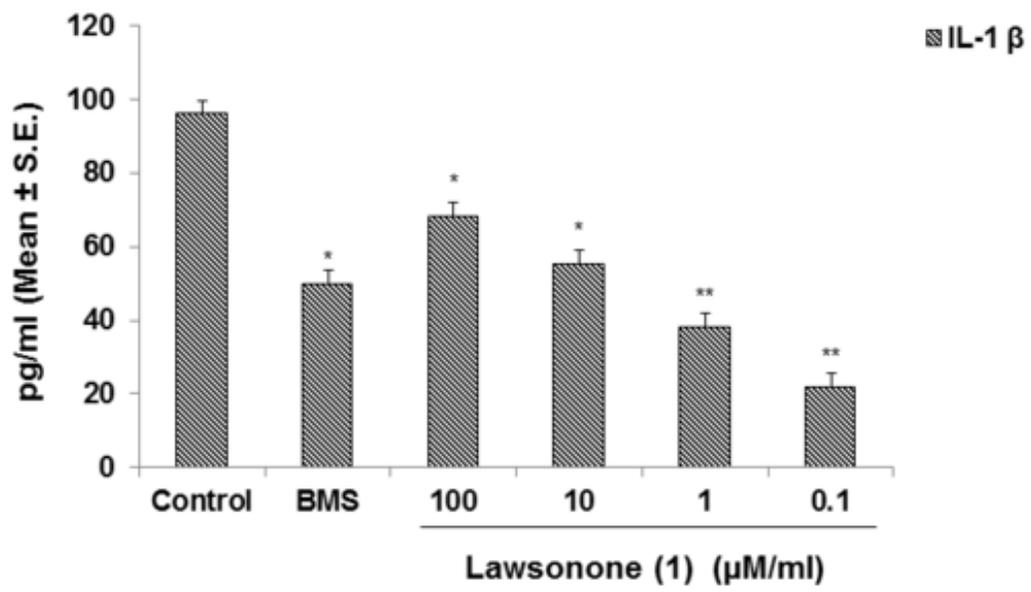
**Fig. 3**



**Fig. 4**



**Fig. 5a**



**Fig. 5b**

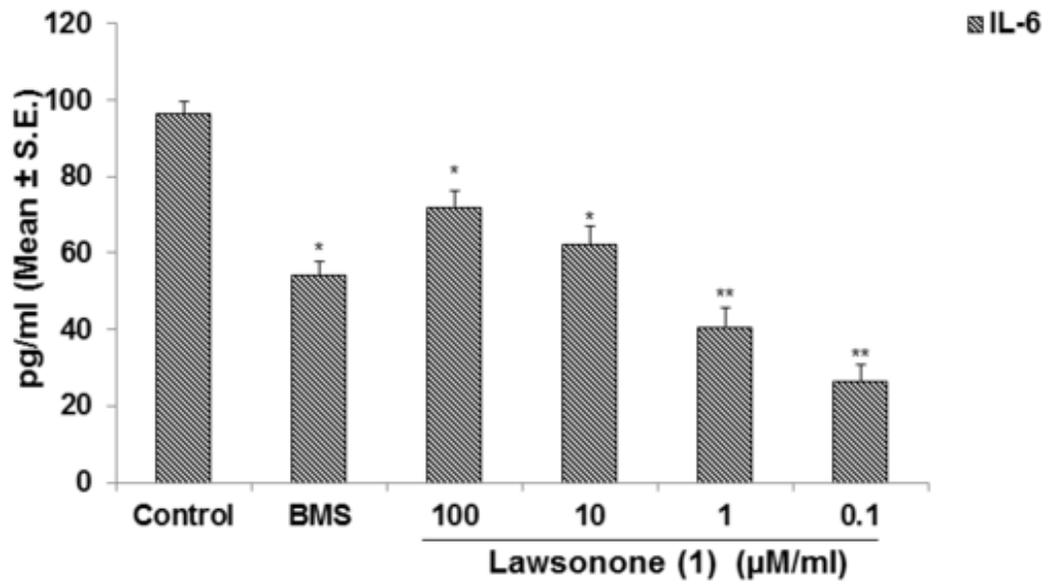
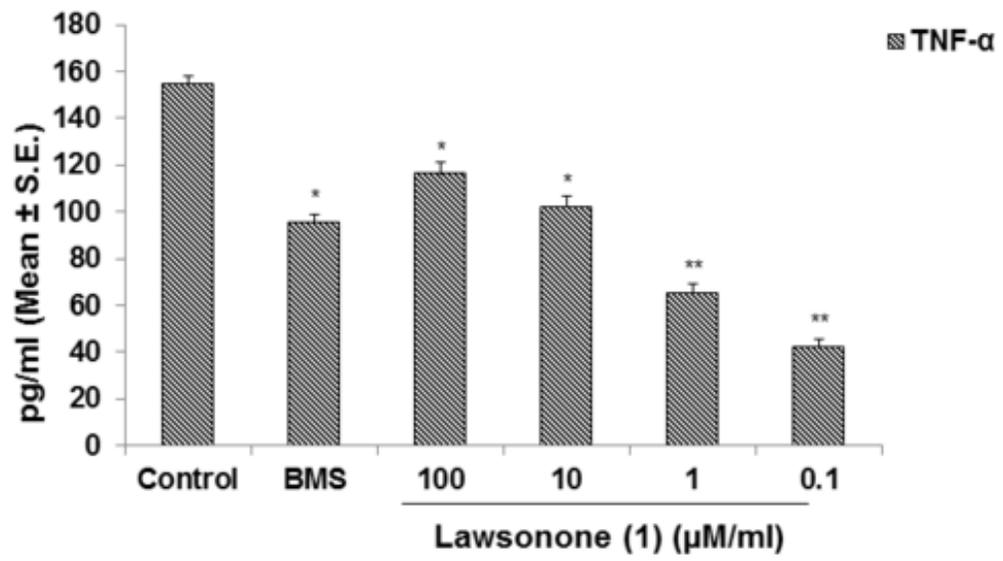
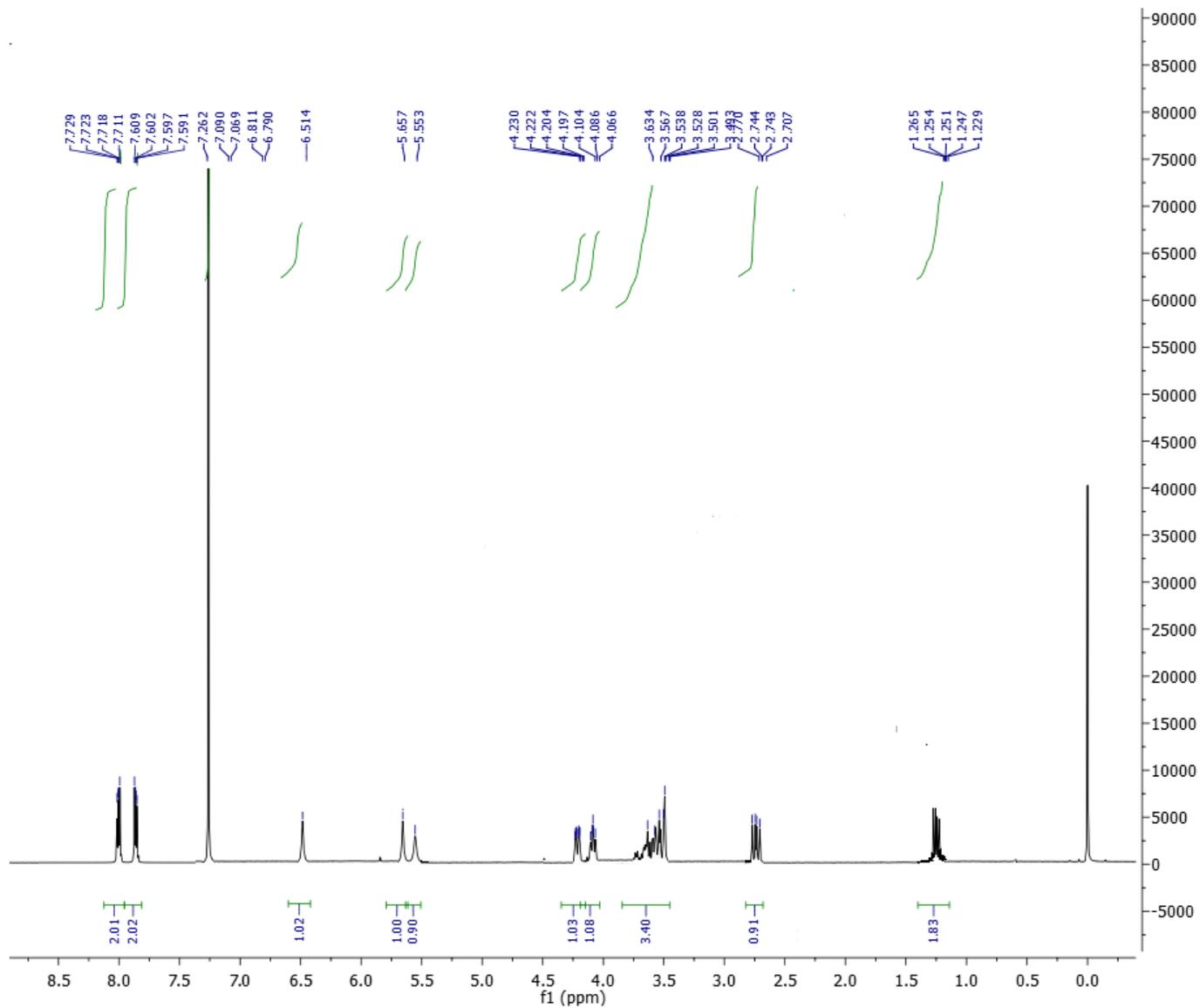


Fig. 5c

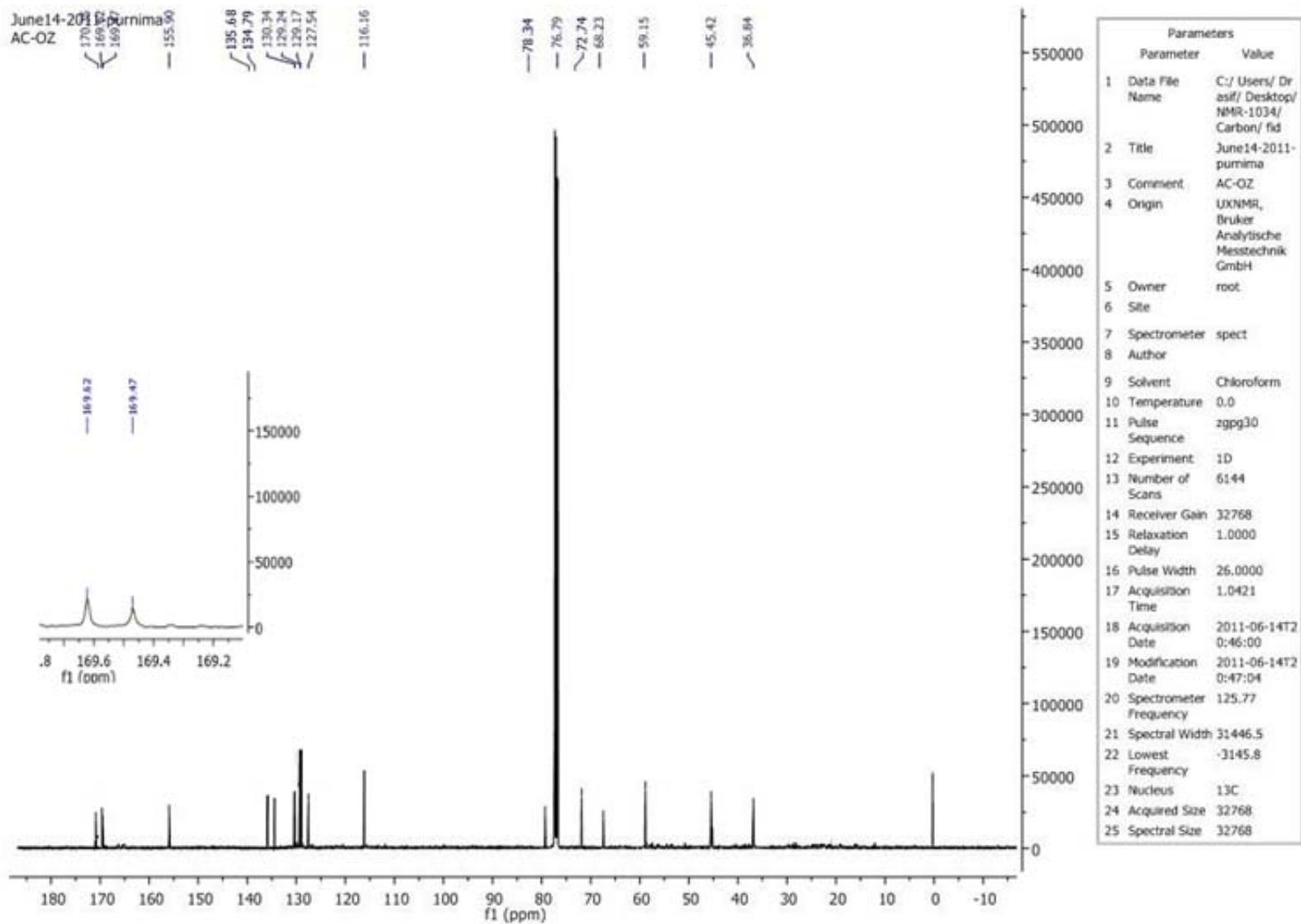


$^1\text{H}$  NMR of compound 1 ( $\text{CDCl}_3$ , 400 MHz)



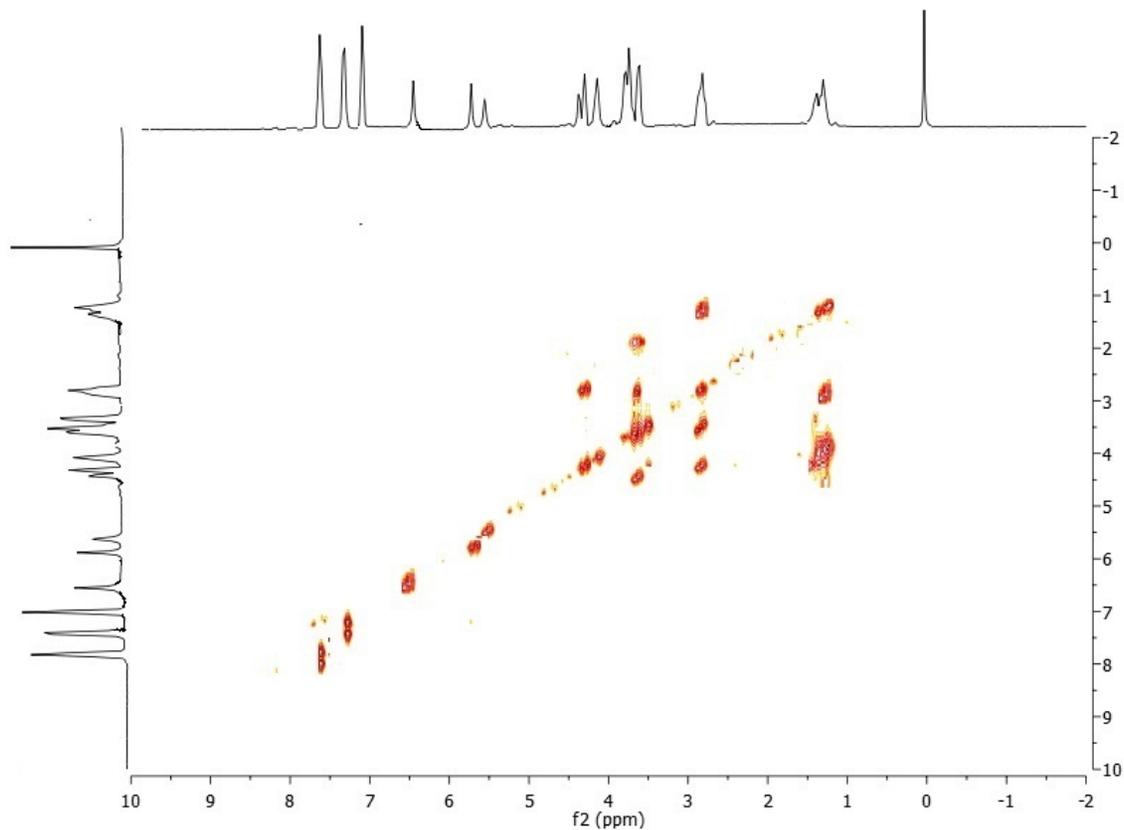
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3 Comment	Ac-01
4 Origin	Bruker BioSpin GmbH
5 Owner	IIIM
6 Site	
7 Spectrometer	spectr
8 Author	
9 Solvent	$\text{CDCl}_3$
10 Temperature	300.0
11 Pulse Sequence	zg30
12 Experiment	1D
13 Number of Scans	116
14 Receiver Gain	203
15 Relaxation Delay	1.0000
16 Pulse Width	14.0000
17 Acquisition Time	2.7263
18 Acquisition Date	2011-11-09 T14:12:00
19 Modification Date	2011-11-09 T14:13:47
20 Spectrometer Frequency	400.13
21 Spectral Width	12019.2
22 Lowest Frequency	-3104.2
23 Nucleus	$^1\text{H}$
24 Acquired Size	32768
25 Spectral Size	32768

$^{13}\text{C}$  NMR of compound 1 ( $\text{CDCl}_3$ , 125 MHz)



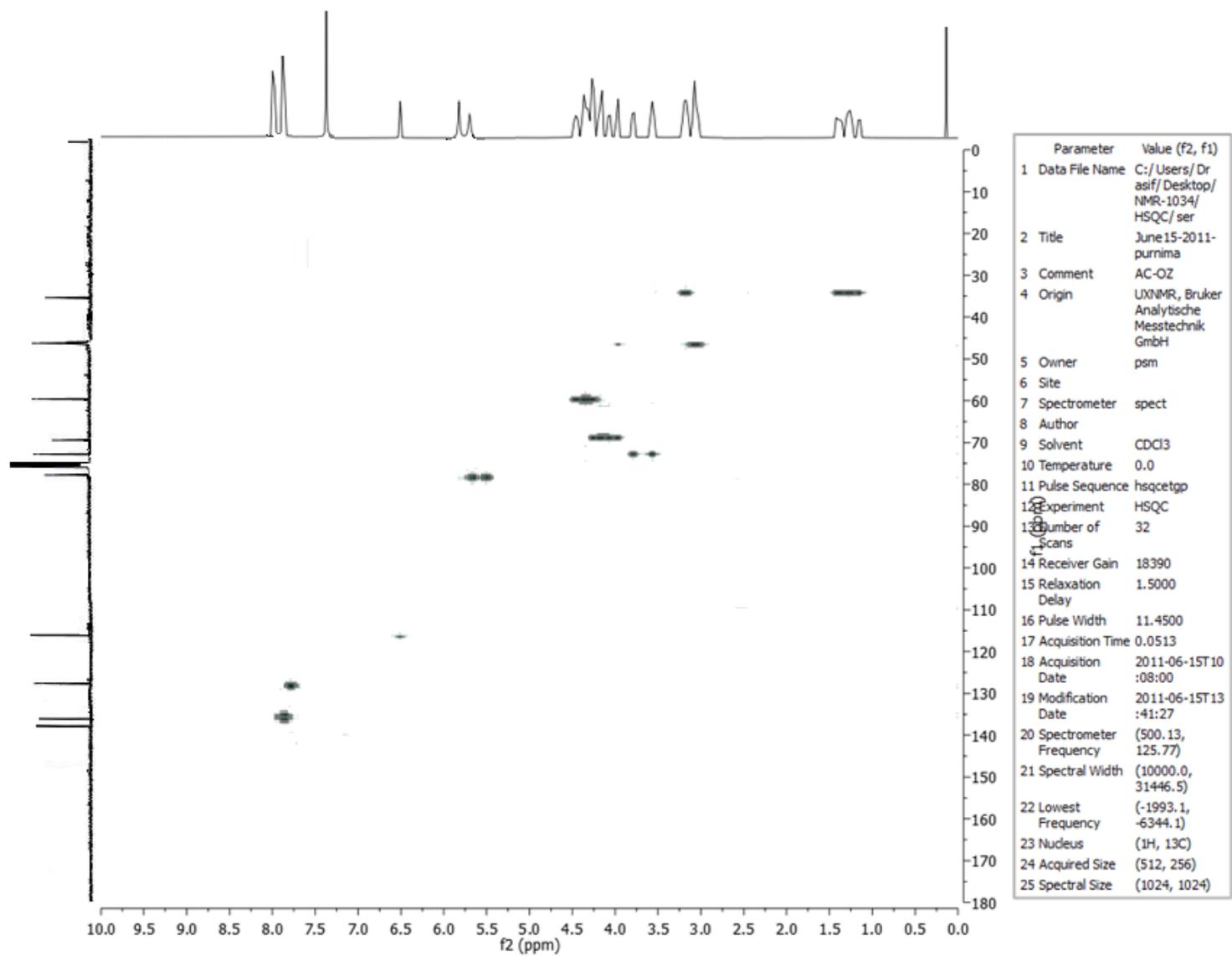


COSY of Lawsonone (1) (CDCl<sub>3</sub>, 500 MHz)

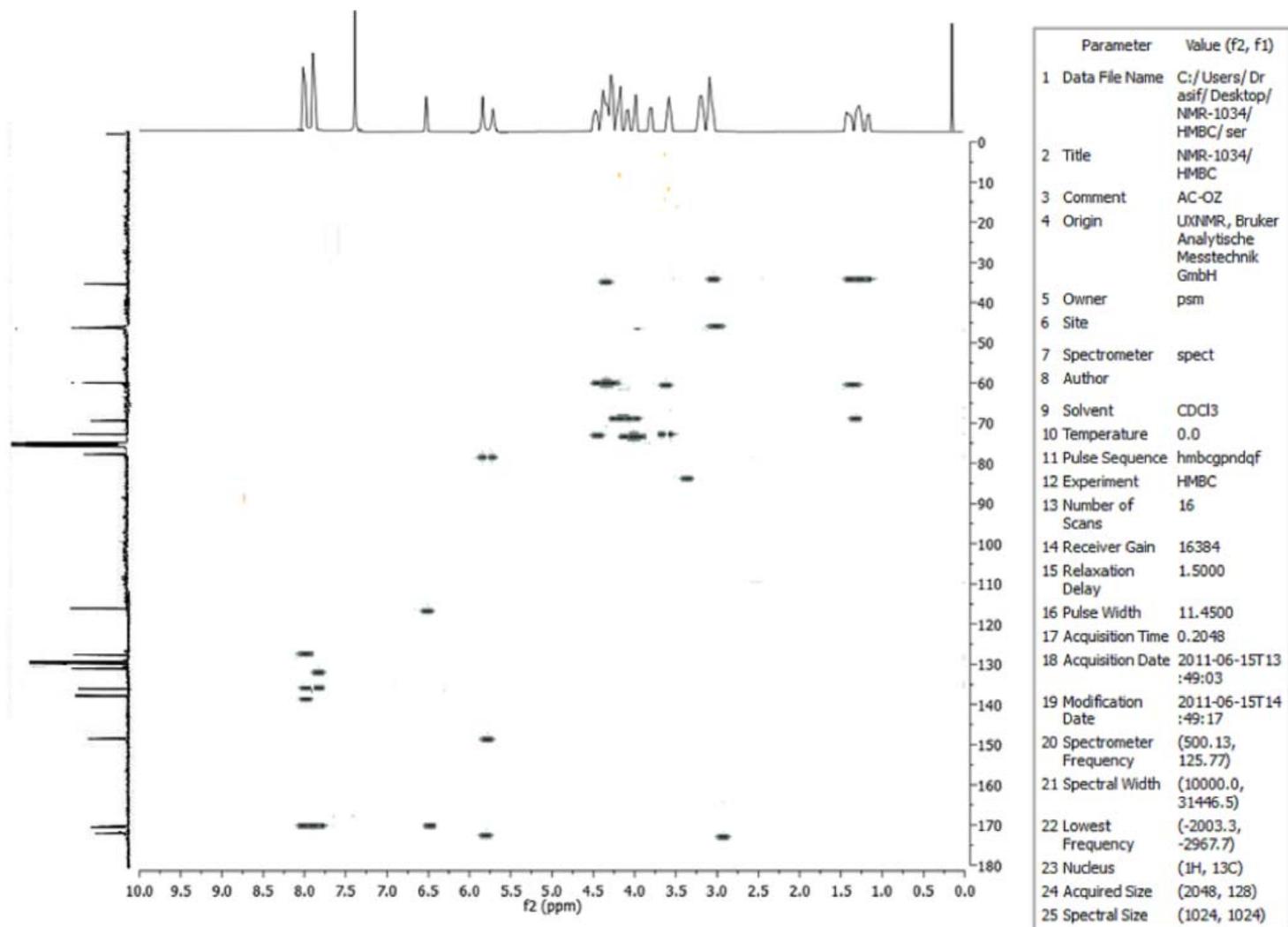


Parameter	Value (f2, f1)
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5 Owner	psm
6 Site	
7 Spectrometer	spect
8 Author	
9 Solvent	CDCl3
10 Temperature	0.0
11 Pulse Sequence	cosygpmfqc
12 Experiment	COSY
13 Number of Scans	8
14 Receiver Gain	64
15 Relaxation Delay	2.0000
16 Pulse Width	11.4500
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18 Acquisition Date	2011-06-15 T09:30:00
19 Modification Date	2011-06-15 T10:06:25
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21 Spectral Width	(10000.0, 10002.5)
22 Lowest	(-1978.6,

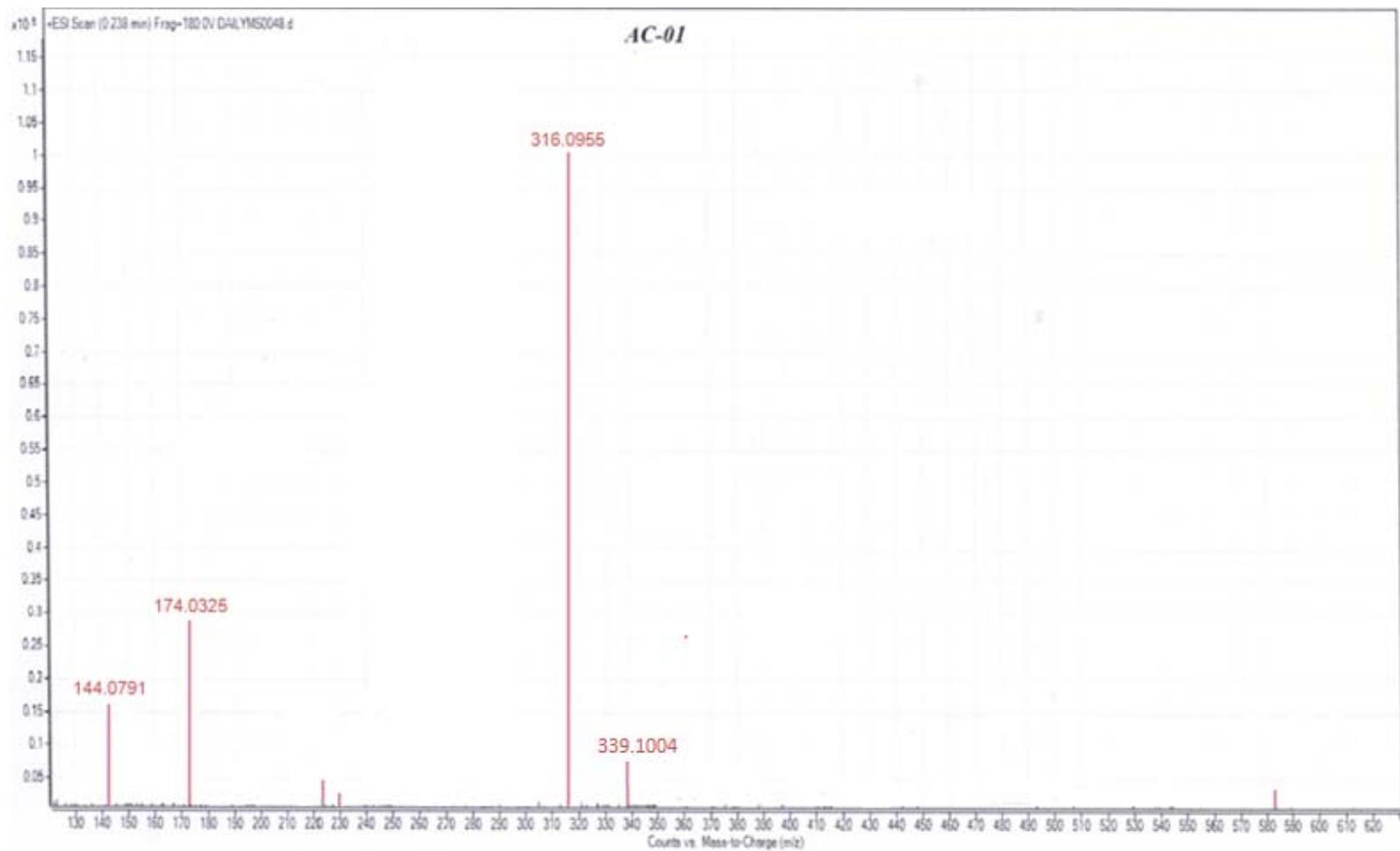
HSQC spectra of Lawsonone (1) (CDCl<sub>3</sub>, 125 MHz)

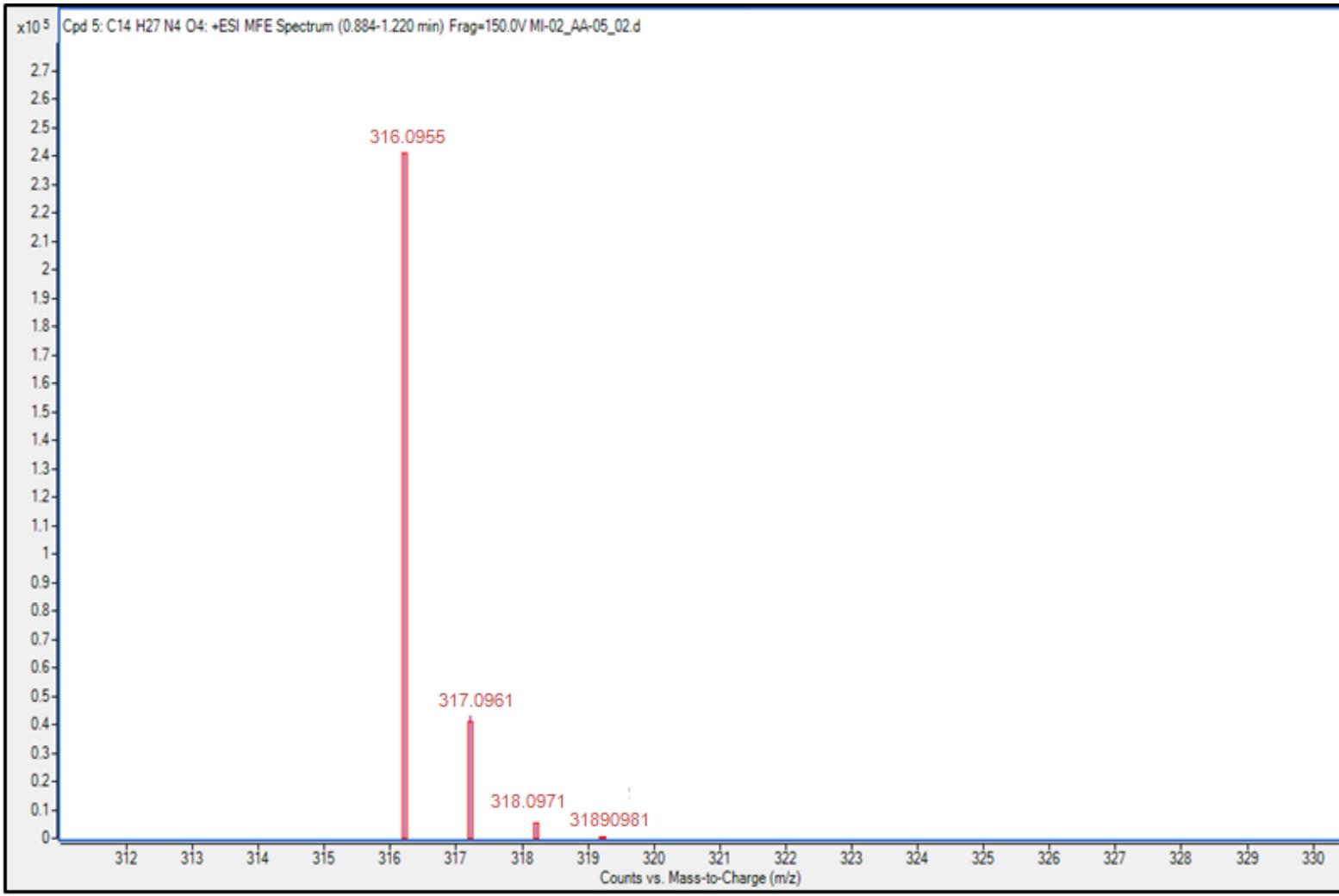


HMBC spectra of Lawsonone (1) (CDCl<sub>3</sub>, 125 MHz)



HR-ESIMS(UHD) Accurate-Mass Q-TOF LC/MS





Phylogenetic tree and table of *Streptomyces* sp.

Table 1. Phylogenetic identification of *Streptomyces* sp.

NCBI Accession No.	Nearest Phylogenetic Neighbour	Percentage similarity
<a href="#">gi 343129258 JN248577.1</a>	Streptomyces sp. FTCt21 16S	99%
<a href="#">gi 343129256 JN248575.1</a>	Streptomyces sp. FTCt5	99%
<a href="#">gi 339765088 JN049462.1</a>	Actinobacterium ZXY013	99%
<a href="#">gi 332647051 HQ992765.1</a>	Streptomyces sp. 219835(1)	99%
<a href="#">gi 332647005 HQ992719.1</a>	Streptomyces sp. 172717-3	99%

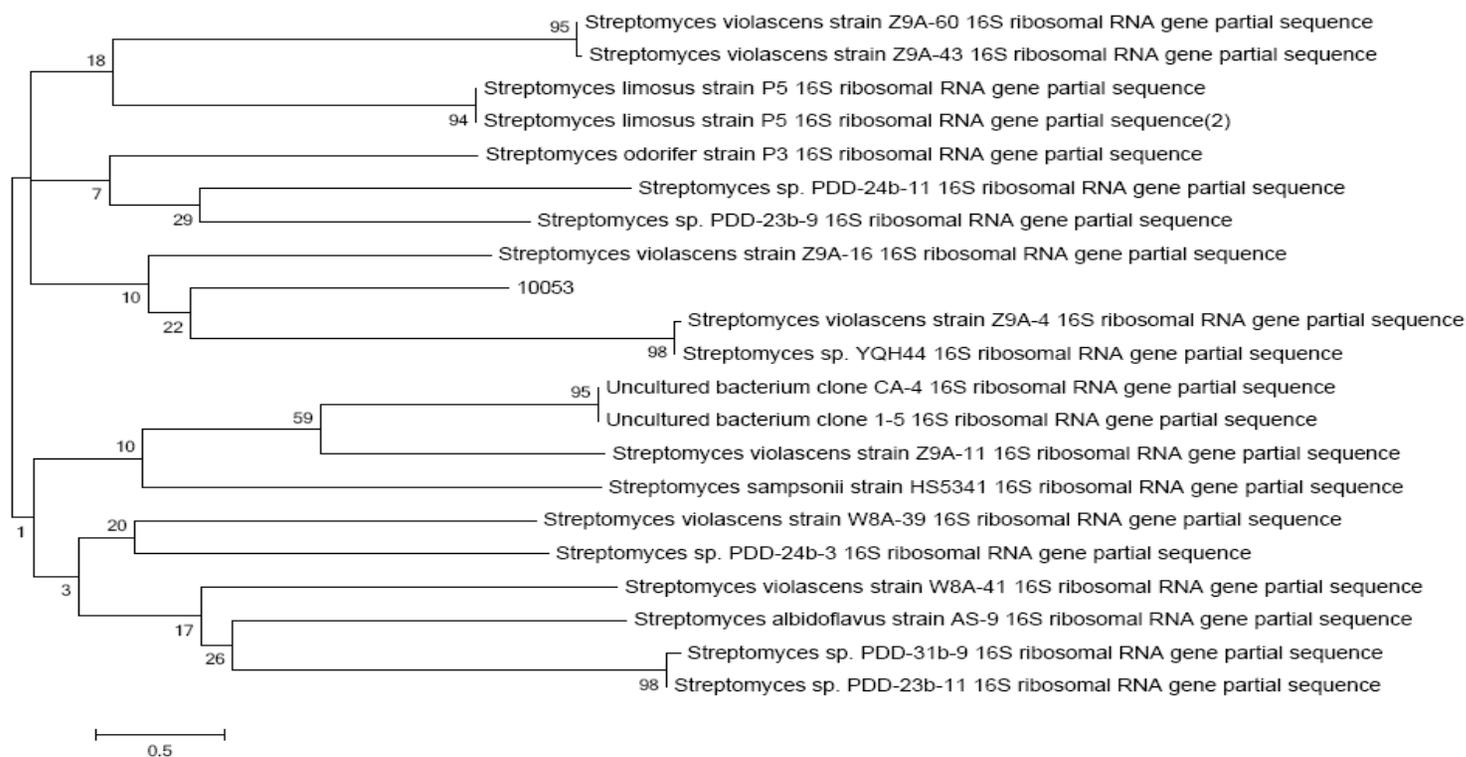


Table 2. Anticancer activity of various extracts and Lawsonone (1) on cytotoxicity of cancer cell lines.

Treatment	Doses/ $\mu\text{g/ml}$	Tissue type/cell line			
		Lung (A549)	Leukaemia (THP-1)	Breast (MCF-7)	Prostrate (PC-3)
NIOM 10134	100	73	79	80	76
NIOM 10134A	50	57	63	44	60
NIOM 10134B	50	51	56	68	85
NIOM 10134C	50	5	4	11	52
<b>Lawsonone (1)</b>	<b>50 <math>\mu\text{M}</math></b>	<b>62</b>	<b>57</b>	<b>65</b>	<b>34</b>