

## SUPPLEMENTARY MATERIAL

### **Chemical characterization and bioactivity of the essential oil from *Santolina insularis*, a Sardinian endemism**

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## **Chemical characterization and bioactivity of the essential oil from *Santolina insularis*, a Sardinian endemism**

*Santolina insularis* is a Sardinian endemism that is widely used in traditional medicine. The essential oil was obtained with a yield of 2.7% and is mainly characterized by  $\beta$ -phellandrene (22.6%), myrcene (11.4%) and artemisia ketone (7.6%). The oil significantly reduced NO production without affecting macrophages viability. In addition, it substantially inhibited the expression of two key pro-inflammatory enzymes, iNOS and COX-2 (71% and 25% at 0.54 mg/mL). Furthermore, the oil had a promising antifungal activity being *Cryptococcus neoformans* (MIC = 0.13 mg/mL) and the majority of dermatophytes (MIC = 0.13 mg/mL) the most susceptible fungi. Moreover, it significantly decreased the yeast-to-hypha transition (80% inhibition at 0.13 mg/mL) on *C. albicans* long before showing inhibitory effects. Overall, these results show that *S. insularis* could be applied in the treatment of fungal infections and associated inflammatory response.

**Keywords:** *Santolina*, essential oil, anti-inflammatory, pro-inflammatory mediators, antifungal, germ tube, safety profile

## **Materials and methods**

### ***Plant material***

Aerial parts of *Santolina insularis* (Gennari ex Fiori) Arrigoni was collected during flowering period in Nebida, Iglesiente subregion (South-Western Sardinia, Italy; 39°18'03" N 8°26'11" E). A voucher specimen (CAG732b) has been deposited at the Herbarium of the Botanical Garden of Cagliari, University of Cagliari, Italy. The aerial parts and flowers were air dried with forced ventilation for two days. Prior to use, the matter was ground with a Malavasi mill (Bologna, Italy). The plant name was checked on <http://www.theplantlist.org> on the 08/08/2019 and considered as accepted.

### ***Essential oil hydrodistillation and chemical characterization***

The essential oil of *S. insularis* was obtained by the hydrodistillation of the aerial parts for 3 h in a *Clevenger*-type apparatus in accordance to the European Pharmacopoeia (Europe 2010). Gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) of the essential oil was carried out as previously described (Piras et al. 2018).

### ***Anti-inflammatory assays***

#### ***Cell culture***

RAW 264.7, a mouse leukemic macrophage cell line obtained from the American Type Culture Collection (ATCC TIB-71), was cultured previously reported by our group (Zuzarte et al. 2018).

#### ***Nitric oxide production***

NO production was measured by quantifying the accumulation of nitrites in macrophages culture supernatants, using the Griess reagent (Green et al. 1982). Cells ( $0.3 \times 10^6$  cells/well) were cultured in 48-well culture plates. After an overnight stabilization, macrophages were pre-treated with varying concentrations of the essential

oil (0.07 – 1.05 mg/mL) diluted in DMSO for 1 h and subsequently activated with 1 µg/mL of LPS during 24 h. Positive and negative controls consisting of cells activated with LPS in the absence of the essential oil and untreated cells, respectively, were performed. After this time period of incubation, equal volumes of culture supernatants and Griess reagent [1:1 of 0.1% (w/v) N-(1-naphthyl) ethylenediaminedihydrochloride and 1% (w/v) sulphanilamide containing 5% (w/v) H<sub>3</sub>PO<sub>4</sub>] were mixed and further incubated during 30 min, in the dark. The absorbance at 550 nm was registered in an automated plate reader (SLT, Austria) and nitrite concentration was determined from a sodium nitrite standard curve.

#### *Nitric oxide scavenging potential*

The NO scavenging potential of the oils was evaluated by incubating varying concentrations of the essential oil (0.07 – 1.05 mg/mL) with 0.9 µL of the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) (stock solution 100 mM) in endotoxin-free DMEM, in a final volume of 300 µL, during 3 h. Then, nitrite levels were quantified as referred in the section 2.3.2.

#### *Expression of the pro-inflammatory proteins iNOS and COX-2*

RAW 264.7 (0.7 x 10<sup>6</sup> cells/well) were cultured in 6-well plates and allowed to stabilize for 12 h. Then, cells were incubated with the essential oil (0.54 mg/mL) for 1 h followed by LPS (1 µg/ml) activation during 24 h. A negative control (untreated cells) and a positive control (LPS-treated cells only) was considered. Cell lysates were prepared as previously described by Zuzarte et al. (2018).

Western blot analysis was carried out to measure the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). The proteins were separated by electrophoresis on SDS-polyacrylamide 10% (v/v), at 130 V for 1.5 h, and transferred to polyvinylidene fluoride membranes (previously activated with methanol) at 400 mA for

3 h. After blocking non-specific IgGs with 5% (w/v) milk in TBS-T for 1 h at room temperature, membranes were incubated with specific antibodies against iNOS (1:500; R & D Systems) or COX-2 (1:5,000; Abcam) overnight, at 4 °C. Then, membranes were washed for 30 min with TBS-T (10 min, 3 times) and incubated at room temperature, for 1 h, with secondary antibodies (1:40,000; Santa Cruz Biotechnology) conjugated with horseradish peroxidase. Immunocomplexes were detected using a chemiluminescence scanner (Image Quant LAS 500, GE). Membranes were probed with an anti-tubulin antibody (1:20,000; Sigma) to guarantee that an equivalent amount of protein was loaded. Protein quantification was performed using TotalLab TL120.

### ***Cytotoxicity***

#### *Assessment of cell viability*

Cell viability was determined using the resazurin method described by Riss et al. (2004) with some modifications. Briefly, after the treatment described in section 2.3.2., a resazurin solution (500 µM) in DMEM was added (1:10) and cells were further incubated at 37 °C for 1 hour in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Quantification was performed using an ELISA microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm. A cell-free control was performed in order to exclude non-specific effects of the oils on resazurin (data not shown).

### ***Antifungal activity***

#### *Inhibition of fungal growth (MIC and MLC)*

Broth macrodilution methods based on the Clinical and Laboratory Standards Institute (CLSI) reference protocols M27-A3 (CLSI 2008b) and M38-A2 (CLSI 2008a) for yeasts (*Candida albicans* ATCC 10231, *C. tropicalis* ATCC 13803, *C. krusei* H9, *C. guilliermondii* MAT23, *C. parapsilosis* ATCC 90018 and *Cryptococcus neoformans* CECT 1078), dermatophytes (*Trichophyton mentagrophytes* FF7, *T. rubrum* CECT

2794, *T. mentagrophytes* var. *interdigitale* CECT 2958, *T. verrucosum* CECT 2992, *Microsporum canis* FF1, *M. gypseum* CECT 2908, *Epidermophyton floccosum* FF9) and *Aspergillus* spp. (*Aspergillus niger* ATCC 16404, *A. fumigatus* ATCC 46645, *A. flavus* F44), respectively, were used to determine the minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) of the essential oil, as reported Pinto et al. (2013).

#### *Effect on C. albicans germ tube formation*

The effect of sub-inhibitory concentrations of the essential oil on yeast-to-hypha transition, an important virulence factor of *C. albicans*, was determined as reported by Pinto et al. (2008). Germ tubes were considered when the germinating protuberance was as long as the diameter of the blastospore.

#### **Data analysis**

Results are presented as mean values  $\pm$  SEM (standard error of the mean) from at least three independent experiments performed in duplicate. Statistical significance was determined using t-test or one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA). A *P* value  $<0.05$  was considered to represent significant differences.

Table S1. Chemical composition of *Santolina insularis* essential oil

<b>RI<sub>EXP</sub><sup>a</sup></b>	<b>RI<sub>LIT</sub><sup>b</sup></b>	<b>Compound</b>	<b><i>Santolina insularis</i></b>
907	906	Santolina triene	3.6
925	924	$\alpha$ -Thujene	0.2
932	932	$\alpha$ -Pinene	0.3
972	969	Sabinene	4.6
976	974	$\beta$ -Pinene	5.4
991	988	Myrcene	11.4
999	999	Yomogi alcohol	1.0
1005	1002	$\alpha$ -Phellandrene	0.3
1016	1014	$\alpha$ -Terpinene	0.5
1023	1020	p-Cymene	0.5
1027	1023	$\beta$ -Phellandrene	22.6
1057	1054	$\gamma$ -Terpinene	0.8
1059	1056	Artemisia ketone	7.6
1082	1080	Artemisia alcohol	0.8
1088	1086	Terpinolene	1.7
1160	1160	cis-Chrysanthanol	2.5
1172	1169	Artemisyl acetate	0.3
1176	1174	Terpinen-4-ol	1.4
1276	1280	cis-Verbenyl acetate	0.6
1366	1373	Linalool isobutanoate	0.4
1405	1403	Methyl eugenol	0.3
1408	1409	$\alpha$ -Gurjunene	0.3
1458	1458	allo-Aromadendrene	1.2
1479	1479	ar-Curcumene	7.1
1482	1481	$\gamma$ -Curcumene	5.0
1495	1500	Bicyclogermacrene	1.2
1513	1514	$\beta$ -Curcumene	0.5
1522	1522	$\delta$ -Cadinene	0.6
1532	1536	Italicene ether	0.4
1541	1544	$\alpha$ -Calacolene	0.2

1575	1577	Spathulenol	1.6
1580	1582	Ar-Turmerol	0.8
1627	1625	trans-Isolongifolanone	2.6
1664	1667	(E)-Bisabol-11-ol	3.3
1683	1683	epi- $\alpha$ -Bisabolol	0.4
1687	1685	$\alpha$ -Bisabolol	0.5
1689	1687	Eudesma-4(15),7-dien-1 $\beta$ -ol	0.4
<b>Total identified</b>			<b>93.1</b>
<b>Monoterpenes hydrocarbon</b>			<b>51.9</b>
<b>Oxygenated monoterpenes</b>			<b>14.6</b>
<b>Sesquiterpenes hydrocarbon</b>			<b>16.1</b>
<b>Oxygenated sesquiterpenes</b>			<b>10.0</b>
<b>Phenylpropanoids</b>			<b>0.3</b>
<b>Other</b>			<b>0.2</b>

<sup>a</sup> RI<sub>EXP</sub>: Experimental retention indexes relative to two series of *n*-alkanes (C<sub>8</sub> – C<sub>20</sub> and C<sub>21</sub> and C<sub>40</sub>) in a HP-5 column

<sup>b</sup> RI<sub>LIT</sub>: Literature retention indexes as published by Adams (2007)



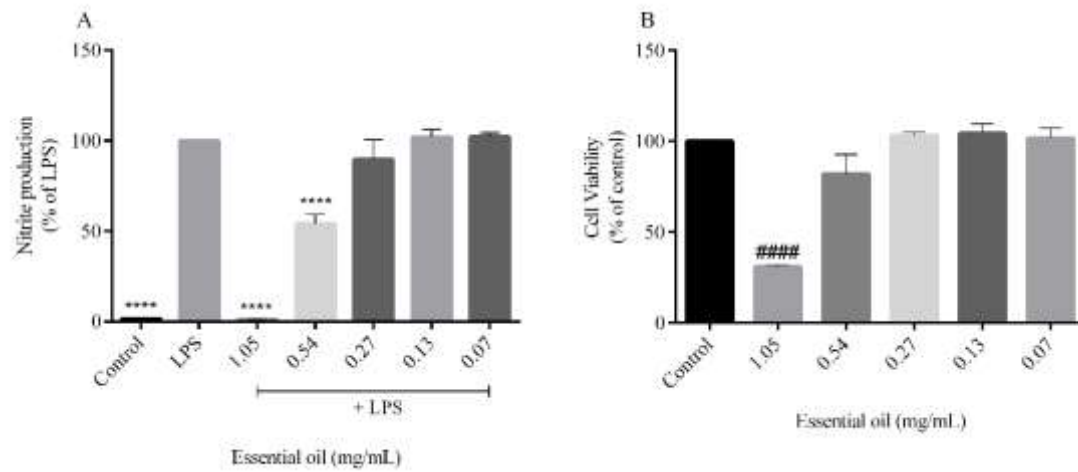


Figure S1. Effect of *Santolina insularis* essential oil on NO production (A) and cell viability (B). Each value represents the mean  $\pm$  SEM from three experiments, performed in duplicate (#### $p < 0.0001$ , compared to control; \*\*\*\* $p < 0.0001$ , compared to LPS).

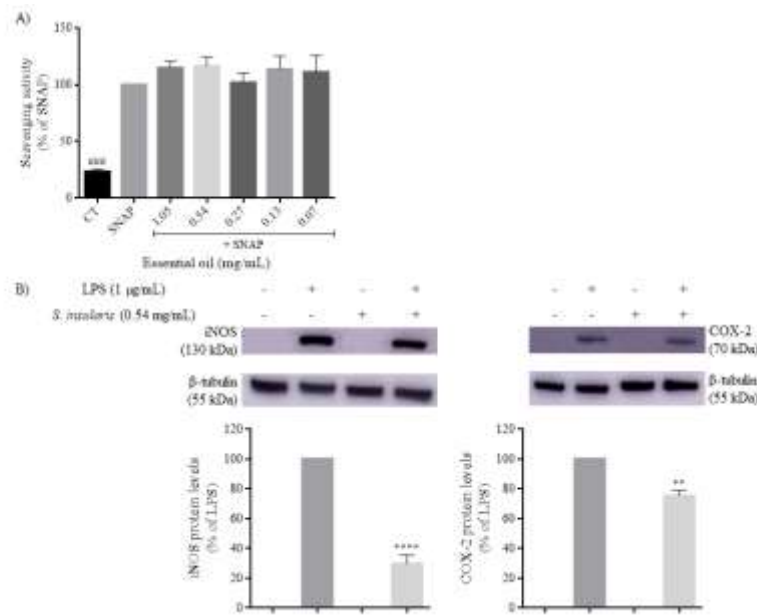


Figure S2. Underlying mechanisms of the anti-inflammatory effect of *Santolina insularis* essential oil. (A) NO scavenging activity. (B) Protein levels of iNOS and COX-2. Each value represents the mean  $\pm$  SEM from three experiments, performed in duplicate (###  $p < 0.0001$ , compared to SNAP; \*\*  $p < 0.01$ ; \*\*\*\*  $p < 0.0001$  compared to LPS).

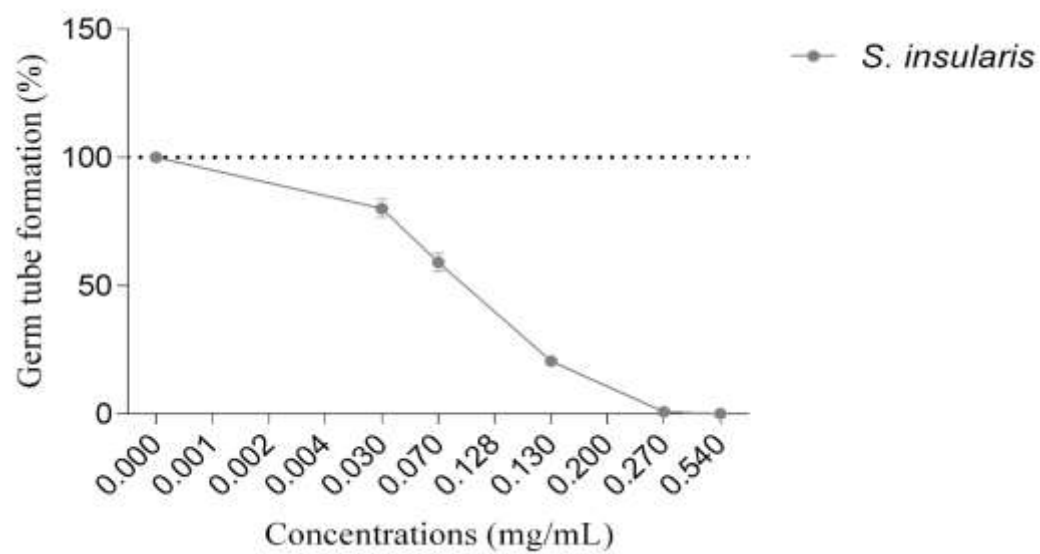


Figure S3. Influence of sub-inhibitory concentrations of *Santolina insularis* oil on the germ tube formation of *Candida albicans* ATCC 10231.

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