The hydro-alcoholic extracts of Sardinian wild thistles (*Onopordum* spp.) inhibit TNFα-induced IL-8 secretion and NF-κB pathway in human gastric epithelial AGS cells.

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Abstract:

Ethnopharmacological relevance: Thistles species (Family: Compositae) are traditionally used in the Mediterranean area, particularly in Sardinia. They are usually gathered from the wild and used for both food and therapeutic purposes, including gastrointestinal disorders.

Aim of the study: This work aims to evaluate the anti-inflammatory activity of eight wild thistles from Sardinia, in an *in vitro* model of gastric inflammation, and to identify the major active compounds in the extracts.

Materials and methods: The hydro-alcoholic extract of the aerial part of each species was prepared. After the induction of inflammation by the addition of tumor necrosis factor-α (TNFα) (10 ng/ml), AGS cells were treated with extracts/pure compounds under study. The

inhibition of interleukin-8 (IL-8) release, IL-8 and NF-κB promoter activities and NF-κB nuclear translocation was evaluated. Extracts main components were identified by HPLC-PDA-MS/MS.

Results: Only *Onopordum horridum* Viv. and *Onopordum illyricum* L. hydro-alcoholic extracts reduced, in a concentration-dependent fashion, the IL-8 release and promoter activity in human gastric epithelial cells AGS. The effect was partially due to the NF-κB pathway impairment. *Onopordum* hydro-alcoholic extracts were also chemically profiled, and caffeoylquinic acid derivatives were the main compounds identified in the extract. Further investigations showed that 3,5 dicaffeoylquinic acid highly inhibited IL-8 secretion in AGS cells (IC_{50} 0.65 μM), thus suggesting that this compound contributed, at least in part, to the anti-inflammatory activity elicited by *O. illyricum* extracts.

Conclusions: Our results suggest that *Onopordum* species may exert beneficial effects against gastric inflammatory diseases. Thus, these wild plants deserve further investigations as preventive or co-adjuvant agents in gastric diseases.

Keywords:, *Cardueae*, *Onopordum*, caffeoylquinic acids, anti-inflammatory, AGS, IL-8.

Chemical compounds studied in this article:

Neochlorogenic acid (PubChem ID: 5280633); Cryptochlorogenic acid (PubChem ID: 9798666); Chlorogenic acid (PubChem ID: 1794427); 1,3 Dicaffeoylquinic acid (PubChem ID: 6474640); 3,5 Dicaffeoylquinic acid (PubChem ID: 6474310), 1,5 Dicaffeoylquinic acid (PubChem ID: 122685); 4,5 Dicaffeoylquinic acid (PubChem ID: 6474309)

1. Introduction

The aetiopathogenesis of gastritis, an inflammatory state of gastric mucosa, is mostly due to the presence of *Helicobacter pylori* (*H. pylori*), a Gram-negative pathogen affecting

humans and classified as Type 1 carcinogen by WHO. (Brown, 2000; Israel and Peek, 2001).

Many pro-inflammatory molecules (e.g. TNFα, IL-8, NF-κB), released during gastritis, can be considered as potential therapeutic targets to prevent or treat *H. pylori*-induced gastric diseases (Bodger and Crabtree, 1998; Crabtree et al., 1993; Israel and Peek, 2001; Martin and Wallace, 2006; Zaidi et al., 2012). Emerging resistance to antibiotics and adverse effects of conventional drugs lead to search for new therapeutic strategies to counteract the inflammatory processes exerted by *H. pylori* infection (Zaidi et al., 2012).

Botanicals, from both wild or cultivated plants, are widely used all over the world, for nutritional and health purposes, as different types of products, including herbal medicinal products, food, food supplements, and functional foods.

Wild plants, traditionally used by the native populations, recently received attention for their therapeutic properties and the high content of fibres, vitamins, minerals, and polyphenols (Licata et al., 2016; Tuttolomondo et al., 2014). Some of them are traditionally used to treat gastrointestinal disorders such as dyspepsia, constipation, diarrhoea, gastritis, colitis (Atzei, 2003; Tuttolomondo et al., 2014) and have shown beneficial effects against gastritis (Colombo et al., 2013; Di Lorenzo et al., 2013; Sangiovanni et al., 2015).

Sardinia boasts a well-established culture on the traditional uses of wild plants (Atzei, 2003; Lancioni et al., 2007; Maxia et al., 2013). The so-called thistles mostly refer to Compositae species and are traditionally consumed and used for therapeutic purposes by Sardinian inhabitants (Atzei, 2003; Guarrera and Savo, 2016; Lancioni et al., 2007; Signorini et al., 2009). The aim of the present study was to investigate the anti-inflammatory activity of eight wild thistles species from Sardinia in a cell model of gastric inflammation. The species under study belong to the *Cardueae* Cass. Tribe (Family: Compositae) and to four genera: *Carduus* L. (*C. argyroa* Biv*., C. cephalanthus* Viv*., C. pycnocephalus* L*., C. nutans* subsp *macrocephalus* (Desf.) Nyman), *Onopordum* L. (*O. illyricum* L*., O. horridum* Viv.), *Silybum* L. (*S. marianum* (L.) Gaertn.), and *Ptilostemon* Cass. (*P. casabonae* (L.) Greuter). All these plants are traditionally used for food and medicinal purposes, also against gastrointestinal disorders (Atzei, 2003; Guarrera and Savo, 2016; Lancioni et al., 2007; Licata et al., 2016; Rinchen and Pant, 2014; Signorini et al., 2009).

The *in vivo* activity of *C. pycnocephalus* has been previously reported towards the rat paw oedema inflammation, while the *in vitro* inhibition of NF-κB pathway, IL-1β, TNFα, and the adhesion molecules VCAM-1, ICAM-1 and E-selectin release has been described for *S.*

marianum extracts, demonstrating that the effects are mostly due to the presence of silymarin components (Al-Shammari et al., 2015; Giorgi et al., 2012; Kang et al., 2003; Manna et al., 1999). *In vivo* studies have shown the ability of *S. marianum* to inhibit TNF-R1, TNFα, IL-4 and IFN-γ expression (He et al., 2004; Schumann et al., 2003). Moreover, the *in vitro* NF-κB, STAT3 inhibitory activity and the Nrf2 activation were evaluated for six sesquiterpenes from *O. illyricum* (Formisano et al., 2017). *O. acanthium* inhibited COX-2 and NF-κB gene expression, NO production and 5-LOX, COX-1 and COX-2 enzymes activity in THP-1 cells (Lajter et al., 2015). However, no studies investigating the *in vitro* anti-inflammatory activity of the thistles species under study in human gastric epithelial cells have been reported so far.

A preliminary screening of the selected thistles hydro-alcoholic extracts was assessed to investigate their inhibitory effect on IL-8 released by human gastric epithelial cells (AGS). To elucidate the underlying molecular mechanisms, the extracts showing remarkable activity were tested on the NF-κB pathway. The extracts were also chemically profiled to identify the compounds responsible for the observed biological activity.

2. Materials and Methods

2.1 Materials

Dulbecco's Modified Eagle's Medium/F12 (DMEM)/F12 (1:1), penicillin, streptomycin, L-glutamine, sodium pyruvate and trypsin-EDTA were from Gibco (Life Technologies Italia, Monza, Italy). DMEM, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Aldrich (Milan, Italy). All reagents used for analytical determinations and biological assays were HPLC grade. Human TNFα and Human IL-8 Elisa Development Kit were from Peprotech Inc. (London, UK). Foetal bovine serum (FBS), and disposable material for cell culture were purchased by Euroclone (Euroclone S.p.A., Pero-Milan, Italy). Human adenocarcinoma cells (AGS, CRL-1739) were purchased from LGC Standard S.r.l., Milano, Italy. 1,5 dicaffeoylquinic acid (purity >99.4%), 3,5 dicaffeoylquinic acid (purity >98.2%), 1,3 dicaffeoylquinic acid (purity >99.36%), were purchased from Phytolab (Vestenbergsgreuth, Germany), chlorogenic acid (purity >99.6%) was from Sequoia Research Products (Pangbourne, UK), epigallocatechin-3-*O*-gallate (purity >99%, EGCG), and DMSO were from Sigma-Aldrich (St Louis, USA). The plasmid NF-κB-LUC containing the luciferase gene under the control of three κB sites was a gift of Dr N. Marx (Department of Internal Medicine-Cardiology, University of Ulm, Germany).

126 Native IL-8-LUC promoter was kindly provided by Dr T. Shimohata (Department of
127 Preventive Environment and Nutrition, University of Tokushima Graduate School, Japan).
128 Britelite™ plus was from Perkin Elmer (Monza, Italy). HPLC-grade acetonitrile and
129 methanol were purchased from Sigma (Bellefonte, USA). De-ionized water (18.2 MΩ cm)
130 was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Formic
131 acid (purity >98%) was obtained from Sigma (Bellefonte, USA).

132 *2.2. Plant material*

133 Aerial parts of eight wild species belonging to the *Cardueae* tribe were collected from
134 different sites in Sardinia, from May to June 2015 (Table 1). Plant material was identified
135 at the Department of Life and Environmental Science, University of Cagliari, Italy, where a
136 voucher specimen for each species was deposited. Several individuals from *Carduus*
137 *argyroa* (10 individuals), *Carduus cephalanthus* (6 individuals), *Carduus nutans* subsp.
138 *macrocephalus* (13 individuals), *Carduus pycnocephalus* (10 individuals), *Onopordum*
139 *illyricum* (10 individuals), *Onopordum horridum* (10 individuals), *Silybum marianum* (10
140 individuals), *Ptilostemon casabonae* (10 individuals) were collected. All individuals
141 sampled within each site were separated by about 1–50 m from each other and were
142 collected randomly. The fresh material was dried at 40°C to constant weight.

143 **Table 1.** Localities and dates of collection, local name (Atzei, 2003; Congia, 1998),
144 voucher numbers, and No. of individuals of the eight *Cardueae* species
145

Species	Local name	Localities and dates of collection	Coordinates	Voucher specimen	No. of individuals
Carduus argyroa	Càdru, Cardu	Decimomannu, 27 May 2015	39°17'47.96"N - 8°58'14.95"E	CAG-803	10
Carduus cephalanthus	Cardu	Capo Testa, 12 June 2015	41°14'33.80"N – 9°8'49.25"E	CAG-807	6
Carduus nutans subsp. *macrocephalus*	Gàrdu pissiaiòlu	Gennargentu, 18 June 2015	39°57'35.77"N - 9°19'12.46"E	CAG-802	13

Carduus pycnocephalus	Ardu pissiarolu, baldu aininu, cardu pisciau	Monte dei Sette Fratelli, 21 May 2015	39°20'43.60"N – 9°17'43.74"E	CAG-805	10
Onopordum illyricum L.	Ardu nieddu, cardu santu, cardu molentinu	Monte dei Sette Fratelli, 21 May 2015	39°20'43.60"N – 9°17'43.74"E	CAG-798	10
Onopordum horridum Viv.	Aldu nieddu	Gennargentu, 18 June 2015	39°53'54.9"N – 9°26'27.9"E	CAG-186/14	10
Ptilostemon casabonae (L.) Greuter	Caldu drummitu, cardu de Casteddu	Gennargentu, 18 June 2015	39°53'54.9"N – 9°26'27.9"E	CAG-796	10
Silybum marianum (L.) Gaertn	Ardu biancu, cardu tufu, cima de cardu	Uta, 27 May 2015	39°17'48.0"N – 8°58'14.9" E	CAG-801	10

146

147 *2.3. Preparation of plant extracts*

148 The aerial parts of each species were combined to obtain homogenous samples; 2 g from
149 the dried and ground material were submitted to ultrasonic extraction with 10 mL of
150 methanol/water (70:30, v/v) two times for 10 min. The extraction phases were then
151 combined and centrifuged at 4000 rpm for 10 min. The extracts were then filtered, dried
152 under vacuum, lyophilized and weighted. To test the biological activity, the extracts were
153 dissolved in sterilized distilled water and DMSO (80:20 v/v for *S. marianum*, *O. horridum*

and *C. cephalanthus;* 60:40 v/v for the other species), and immediately stored in aliquots at -80°C. The extracts were dissolved in methanol/water (70:30, v/v) and subjected to HPLC analysis.

2.4. Cell culture

AGS cells were grown at 37 °C in DMEM F12 supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, and 10% heat-inactivated FBS (Euroclone S.p.A, Pero, Italy), under a humidified atmosphere containing 5% CO_2.

2.5. Measurement of IL-8 release

AGS cells were grown in 24-well plates for 48 h (30 000 cells/well); then, cells were treated with TNF-α (10 ng/ml) and extracts/pure compounds under study. IL-8 was quantified using a Human Interleukin-8 ELISA Development Kit as described below. Briefly, Corning 96 well EIA/RIA plates from Sigma-Aldrich (Milan, Italy) were coated with the antibody provided in the ELISA Kit (Peprotech Inc., London, UK) overnight at 4 °C. After blocking the reaction, each sample (200 µl) was transferred into wells at room temperature for 2 h. The amount of IL-8 was detected by spectrophotometry (λ: 450 nm, 0.1 s) using biotinylated and streptavidin–HRP conjugate antibodies, and evaluating the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate reaction. Quantification of IL-8 was done using an optimized standard curve supplied with the ELISA Kit (8-1000 pg/mL). The IL-8 release was tested after 6 h treatment in the presence of the extracts (50 µg/mL for the screening assay, 1-75 µg/mL for the extracts, 1 µM for the pure compounds and 0.1-5 µM for concentration response curves). To evaluate the ability of the extracts to prevent IL-8 release, AGS cells were pre-treated for 2 h with the extracts (1-75 µg/mL); then, IL-8 secretion was induced by 6 h treatment with TNF-α (10 ng/mL). Epigallocatechin-3-*O*-gallate (EGCG, 20 µM) was used as reference inhibitor of IL-8 release.

2.6. Transient transfection assays

AGS cells were grown in 24 well plates for 48 h (30 000 cells per well), to evaluate the NF-κB driven transcription and IL-8 promoter activity. Cells were transfected by the calcium phosphate method with native IL-8-LUC (100 ng/well) or NF-κB-LUC (50 ng/well), a plasmid containing the luciferase reporter gene under the control of the NF-κB responsive promoter. After 16 hours, cells were placed in a FBS-free medium, and treated with TNF-α (10 ng/mL) in the presence of the extracts at 1–75 µg/mL. After six hours, cells were harvested and the luciferase assay was performed using the Britelite™ Plus reagent (PerkinElmer Inc., Massachusetts, USA), according to the manufacturer's instructions. Data

were expressed considering 100% of the luciferase activity related to the cytokine induced promoter activity.

2.7. NF-κB nuclear translocation

To verify the inhibitory effect on the NF-κB (p65) nuclear translocation, AGS cells were plated for 48 h in 100 mm dishes (2×10^6 cells per dish) with fresh complete medium. Then, the medium was replaced with fresh FBS-free medium containing different concentrations of extracts (1-20 μg/mL) in the presence of TNFα (10 ng/mL) for 1 h. Nuclear extracts were prepared using a Nuclear Extraction Kit from Cayman Chemical Company (Michigan, USA) and stored at −80°C until assayed. The same amount of total nuclear proteins, measured by the method of Bradford, was used to assess NF-κB nuclear translocation using the NF-κB (p65) transcription factor assay kit (Cayman) followed by spectroscopy (λ: 450 nm, 0.1 s). Data were expressed considering 100% of the absorbance related to the cytokine-induced NF-κB nuclear translocation. EGCG (20 μM) was used as the reference inhibitor of NF-κB nuclear translocation.

2.8. Cytotoxicity assays

The integrity of the cell morphology before and after treatment was assessed by light microscope inspection. Cell viability was measured by the MTT and LDH methods. No sign of cytotoxicity was observed in AGS cells treated for 6 h with the eight *Cardueae* extracts at the concentrations used for testing the biological activity.

2.9. Phytochemical profile of Onopordum extracts

Onopordum extracts were analysed using a Shimadzu Nexera X2 system equipped with a photodiode detector SPD-M20A in series to a triple quadrupole Shimadzu LCMS-8040 system provided with electrospray ionization (ESI) source (Shimadzu, Düsseldorf Germany). An Ascentis Express RP-Amide column (10cmx2.1mmx2.7μm, Supelco, Bellefonte, USA) and a mobile phase with water (eluent A, containing 0.1% formic acid) and acetonitrile (eluent B, containing 0.1% formic acid) was used. The flow rate was 0.4 mL/min and the column temperature was maintained at 30°C. The gradient program was as follow: 5-25% B for 20 min, 25-100% B in 25 min, 100% B for 1 min, 100-5% B in 4 min, 5% for 10 min. The total pre-running and post-running time was 60 min. UV spectra were acquired in the 220-450 nm wavelength range. MS operative conditions were as follows: heat block temperature: 200 °C; desolvation line (DL) temperature: 250 °C; nebulizer gas flow rate: 3 L/min drying gas flow rate: 15 L/min. Mass spectra were acquired both in positive and in negative full-scan mode over the range 100–1000 m/z, event time 0.5 s.

Product Ion Scan mode (collision energy: - 35.0 V for ESI^+ and 35.0 V for ESI^-, event time: 0.2 s) was applied to compounds for which a correspondence between the pseudomolecular ions $[M+H]^+$ in ESI^+ and $[M-H]^-$ in ESI^- had been confirmed. The identification of the compounds was assessed by comparing their retention times, UV and MS spectra to those of authentic standards, when available. The other components were tentatively identified on the basis of their UV spectra and mass spectral information, compared to those present in the literature. The major components were quantified using the Multiple Reaction Monitoring acquisition in ESI^+ (collision energy: - 35.0 V for ESI+, dwell time: 20) on specific ion products derived from precursor ions fragmentation. Chlorogenic acid and 1,3 dicaffeoylquinic acid were used for the quantification of chlorogenic acid derivatives and the dicaffeoylquinic and succinyl dicaffeoylquinic acids, respectively. Each standard solution and extracts were analysed in two replicates. Calibration curves were prepared with five different concentrations, in the range of 0.1-5 µg/mL, monitoring the reported transitions: ESI^+: *m/z* 355.00 →163.00, for chlorogenic acid and 517.00 →163.00 for 1,3 dicaffeoylquinic acid. (dwell time: 20 msec, collision energy -35 V, event time: 0.096 sec). The determination coefficients were 0.993 and 0.995 for chlorogenic acid and 1,3 dicaffeoylquinic acid, respectively.

2.10. Statistical analysis

All data are the mean ± SD of at least three experiments performed in duplicate (ELISA) or triplicate (transfections). Data were analysed by unpaired one-way analysis of variance (ANOVA), or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Statistical analyses were performed using GraphPad Prism 5.02 software (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant. IC_{50} was calculated using GraphPad Prism 5.02.

3. Results

3.1. Screening of the eight Cardueae species on the TNFα-induced IL-8 release in AGS cells

Preliminary screening of the eight *Cardueae* extracts on the TNFα-induced IL-8 release in human epithelial gastric AGS cells was performed. As shown in Figure 1, only the extracts belonging to the *Onopordum* genus inhibited the TNFα-induced IL-8 secretion at 50 µg/mL. The inhibitory effect of *O. horridum* and *O. illyricum* reached 80% and 95% respectively. Thus, the extracts from *O. horridum* and *O. illyricum* were selected for further

studies aimed to assess the inhibitory effect on IL-8 release, under conditions of pre- or co-treatment.

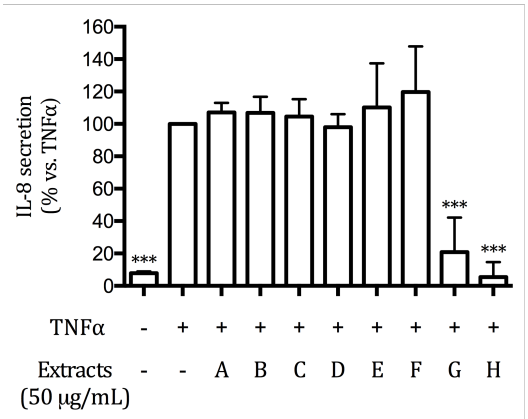
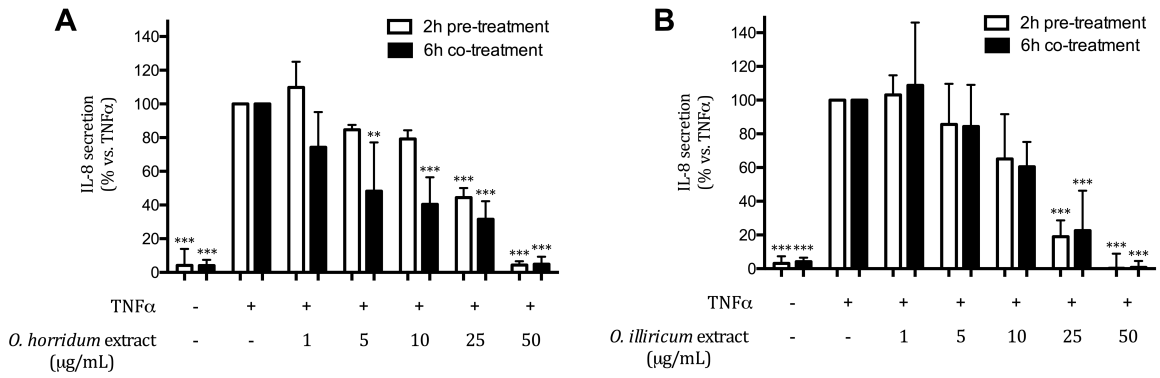


Figure 1. Effect of the eight *Cardueae* extracts on the TNFα-induced IL-8 secretion. AGS cells were treated for 6 h with both TNFα (10 ng/mL) and each of the eight extracts at the concentration of 50 μg/mL. Secreted IL-8 was evaluated by ELISA assay. **p < 0.01, ***p < 0.001 vs. TNFα alone. 20 μM EGCG was used as the reference inhibitor of IL-8 secretion, according to the literature (Fumagalli et al., 2016). A*: Carduus argyroa;* B: *Carduus nutans* subsp *microcephalus;* C: *Carduus cephalanthus;* D: *Ptilostemon casabonae*; E: *Carduus pycnocephalus*; F: *Silybum marianum*; G: *Onopordum horridum*; H: *Onopordum illyricum*.

3.2. Onopordum species inhibit TNFα-induced IL-8 release in AGS cells

The extracts inhibited IL-8 release induced by TNFα in a concentration dependent fashion; IC_{50} were 4.31 and 12.27 μg/mL for *O. horridum* and O. *illyricum*, respectively . Moreover, *Onopordum* extracts prevented TNFα-induced IL-8 release, when added to the cells 2 h before challenging with the pro-inflammatory stimulus; IC_{50} were 18.45 and 12.75 μg/mL for *O. horridum* and O. *illyricum*, respectively (Figure 2, A-B).

As shown by comparison of the IC_{50}, the inhibitory effect of *O. horridum* extract under co-treatment conditions was more pronounced than that observed under pre-treatment (IC_{50}: 4.31 vs. 18.45 μg/mL) whereas the inhibitory effect of O. *illyricum* extract was comparable. Thus, we decided to further investigate the effect of the extracts exclusively in the co-treatment conditions.



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278 **Figure 2.** Effect of *O. horridum* (A) and *O. illyricum* (B) extracts on the TNFα-
279 induced IL-8 secretion. To evaluate the effect of *Onopordum* extracts (1-50
280 μg/mL) AGS cells were treated for 6 h with both TNFα (10 ng/mL) and extract
281 (black bar). Preventive effect on the TNFα-induced IL-8 secretion was assessed by
282 pre-treating AGS cells for 2 h with the two *Onopordum* extracts (1-50 μg/mL);
283 then, IL-8 release was induced by treatment with TNFα (10 ng/mL) for 6 h (white
284 bar). Secreted IL-8 was evaluated by ELISA assay. **$p < 0.01$, ***$p < 0.001$ vs.
285 TNFα alone. 20 μM EGCG was used as the reference inhibitor of IL-8 secretion,
286 according to the literature (Fumagalli et al., 2016).

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289 *3.3. Onopordum species inhibit the TNFα-induced IL-8 secretion through impairment of the*
290 *corresponding promoter activity*

291 To test if the inhibitory effect of *Onopordum* extracts on IL-8 release could be due to
292 inhibition of IL-8 promoter activity, AGS cells were transiently transfected with a plasmid
293 carrying the luciferase gene under the control of a fragment of the IL-8 promoter containing
294 several responsive sequences including a sequence responsive to NF-κB.
295 As shown in Figure 3, *Onopordum* extracts inhibited TNFα-induced IL-8 promoter activity
296 in a concentration dependent manner with comparable activity. IC_{50} for *O. horridum* and *O.*
297 *illyricum* were 17.09 and 14.8 μg/mL, respectively.

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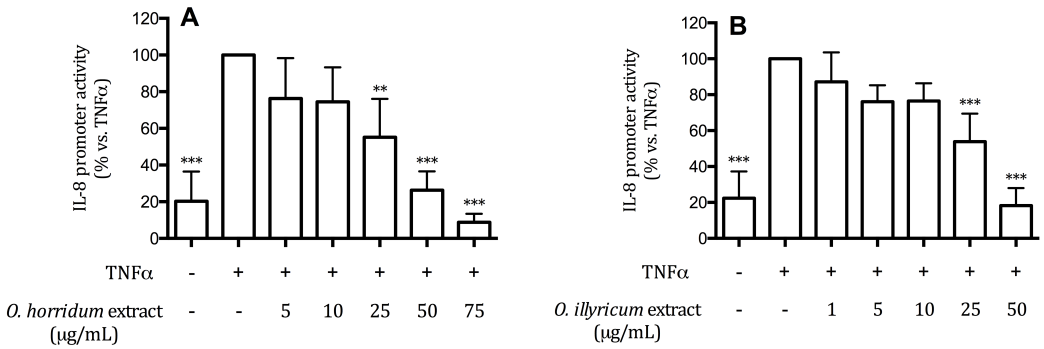

Figure 3. Effect of the *O. horridum* (A) and *O. illyricum* (B) extracts on the TNFα-induced IL-8 promoter activity. AGS cells were treated for 6 h with TNFα (10 ng/mL) and *O. horridum* (5-75 μg/mL) or *O. illyricum* (1-50 μg/mL) extracts. IL-8 promoter activity was evaluated in transiently transfected AGS cells by the luciferase assay. **p < 0.01, ***p < 0.001 vs. TNFα alone. 20 μM EGCG was used as the reference inhibitor of IL-8 secretion, according to the literature (Fumagalli et al., 2016).

3.4. Onopordum extracts inhibit the TNFα-induced IL-8 release through inhibition of NF-κB signalling

To gain further insights into the molecular mechanisms by which *Onopordum* species exert anti-inflammatory activity at gastric level, we tested the extracts on the NF-κB. In fact, it is widely reported in the literature that IL-8 expression is dependent on the NF-κB activation, contributing to exacerbate inflammation. NF-κB driven transcription was assessed in AGS cells transiently transfected with the NF-κB-LUC plasmid and treated for six hours with TNFα (10 ng/mL), in the presence of increasing concentrations of the extracts (Figure 4, A-B). The amount of p65 translocation was measured by ELISA, as indicated in the material and methods section (Figure 4, C-D). Both the extracts from *O. horridum* and *O. illyricum* inhibited the NF-κB driven transcription in a concentration dependent fashion with similar IC_{50}s (6.2 vs. 7.3 μg/ml, respectively). *O. illyricum* extract showed higher inhibition than *O. horridum* on the TNFα-induced NF-κB nuclear translocation (IC_{50}s 10.04 vs. 18.21 μg/ml, respectively).

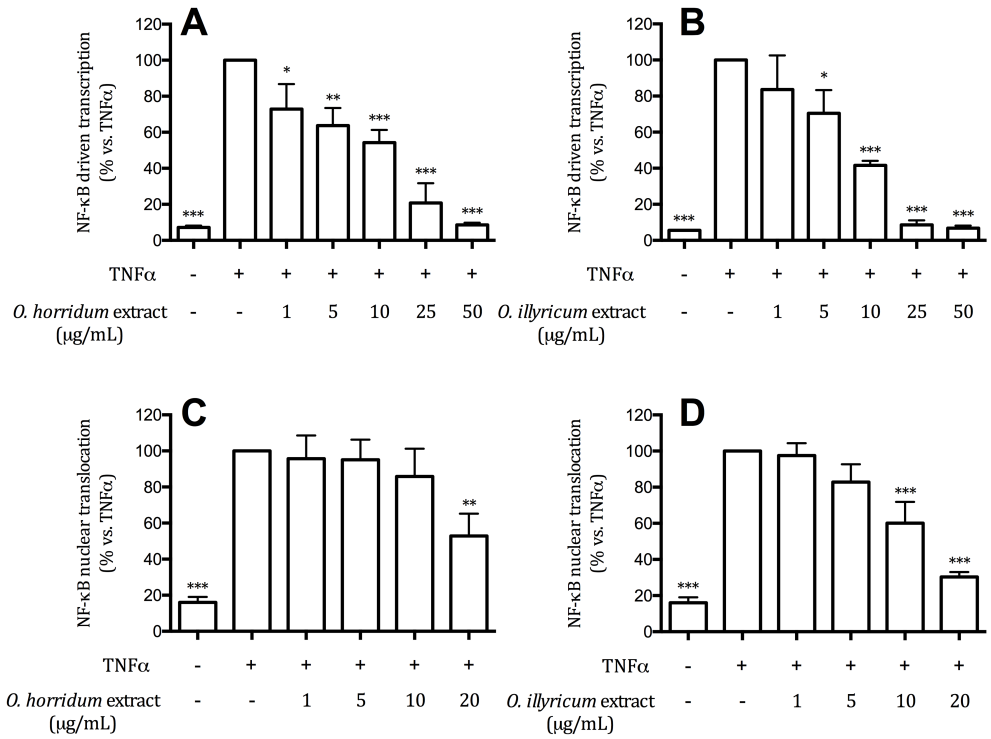

Figure 4. Effect of *O. horridum* and *O. illyricum* extracts on the TNFα-induced NF-κB driven transcription (A-B) and nuclear translocation (C-D). AGS cells were treated for 6 h (driven transcription assay) or 1 h (nuclear translocation assay) with TNFα (10 ng/mL) and *O. horridum* or *O. illyricum* extracts at 1-50 μg/mL (NF-κB driven transcription) or 1-20 μg/mL (nuclear translocation assay). **p < 0.01, ***p < 0.001 vs. TNFα alone. 20 μM EGCG was used as reference inhibitor of TNFα-induced NF-κB driven transcription or nuclear translocation, according to the literature (Fumagalli et al., 2016).

3.5 Phytochemical characterization of Onopordum extracts

The literature reports several classes of metabolites as characteristics of the genus *Onopordum*, including sesquiterpenoids, flavonoids, acetylenic compounds, steroids, triterpenes, lipids and nitrogen containing compounds (Bruno et al., 2011; Lajter et al., 2015).

The extracts from *O. horridum* and *O. illyricum* were chemically profiled through HPLC-PDA-MS/MS analysis. Caffeoylquinic acid derivatives were identified by comparing the UV, MS and MS/MS spectra to those of reference standards. Figure 5 reports the chromatographic profiles of caffeoylquinic acid derivatives standard compounds and *O. illyricum* and *O. horridum* extracts (A,B,C). 3,5 dicaffeoylquinic acid was chosen as

345 illustrative example of a tandem mass spectrometry fragmentation pattern. As shown in
346 figure 5D and 5E, the MS/MS fragmentation of both the standard compound and 3,5
347 dicaffeoylquinic acid in *O. illyricum* extract generated diagnostic fragments at *m/z* 163 and
348 191 in the positive and negative ESI mode, respectively (Marengo et al., 2017).
349 Table 2 includes the quantitative analysis of the caffeoylquinic acid derivatives identified in
350 the extracts.
351 The most abundant compounds were quantified both in the UV mode and in the MRM
352 acquisition, which provided similar results. The quantification through external calibration
353 method based on the following transitions in ESI^+: 355 → 163 for the chlorogenic acids,
354 517 → 163 for the dicaffeoylquinic acids and 617 → 163 for the succynil dicaffeoylquinic
355 acids, was chosen to obtain an accurate quantification of the compounds.
356 The most abundant components in both species were the caffeoylquinic acid derivatives.
357 Chlorogenic and dicaffeoylquinic acids are present in both species, whereas succinyl
358 dicaffeoylquinic acids were found only in *O. horridum* (Figure 5A). In our extracts,
359 chlorogenic acid, 3,5 dicaffeoylquinic acid and 1,5 dicaffeoylquinic acid are the main
360 phenolic compounds in both species, although their amount is higher in *O. illyricum* extract
361 (Table 2).

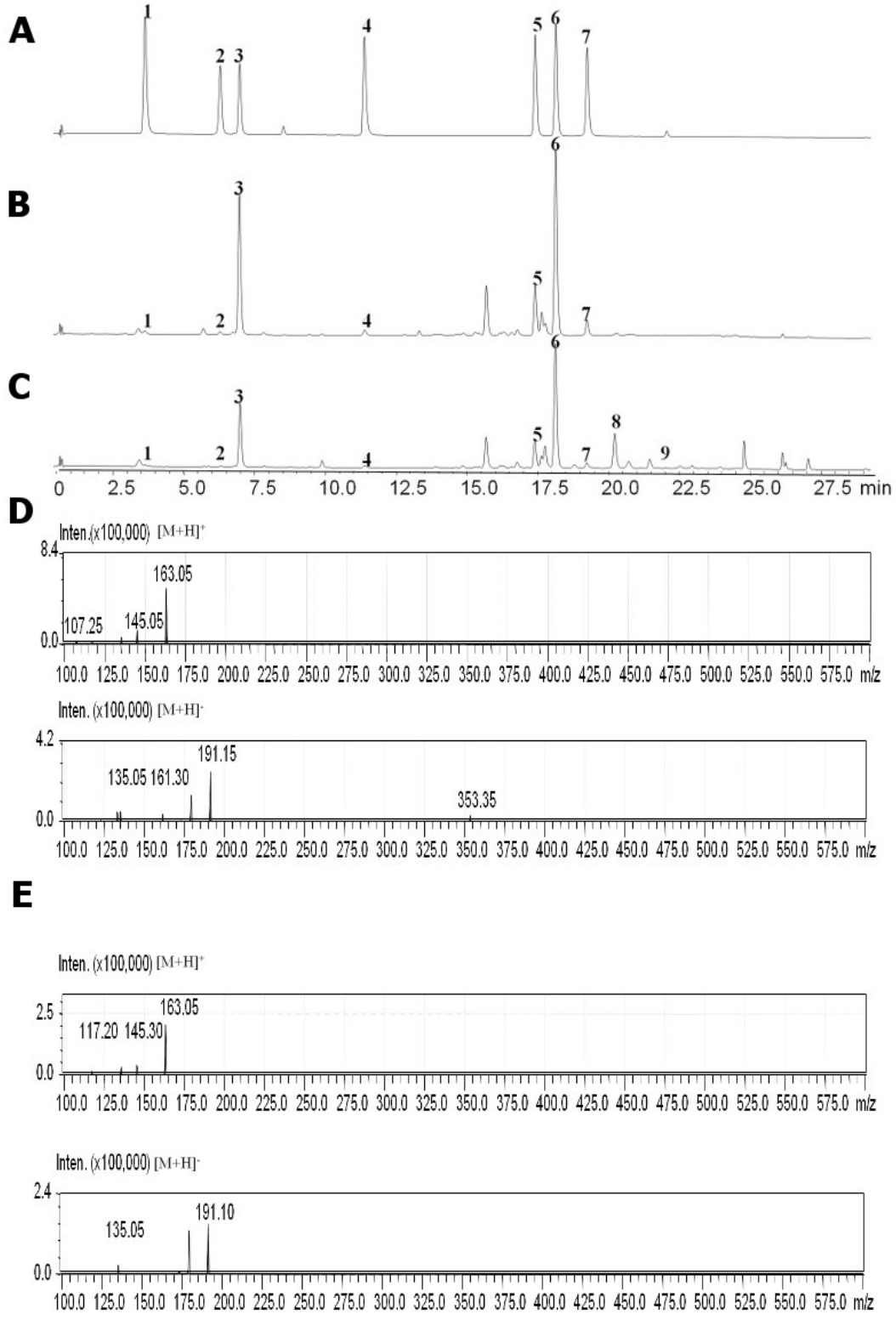

Fig. 5. Chromatographic profiles of caffeoylquinic acid derivatives standard compounds (A) and *O.illyricum* (B) and *O. horridum* (C) extracts. Product Ion Scan spectra of the $[M+H]^+$ and $[M+H]^-$ ions of 3,5 dicaffeoylquinic acid standard compound (D) and 3,5

dicaffeoylquinic acid in *O. illyricum* extract (E). Compounds: 1=neochlorogenic acid; 2=criptochlorogenic acid; 3=chlorogenic acid; 4=1,3 dicaffeoylquinic acid; 5=3,5 dicaffeoylquinic acid; 6=1,5 dicaffeoylquinic acid; 7=4,5 dicaffeoylquinic acid; 8=succinyl dicaffeoylquinic acid1; 9=succinyl dicaffeoylquinic acid2

Table 2. Quantitative analysis of caffeoylquinic acid derivatives in *O. horridum* and *O. illyricum* extracts

Compound	***O. illyricum***		***O. horridum***	
	µg/mg	**%**	**µg/mg**	**%**
Neochlorogenic acid	1.48 ±	0.148	0.72 ± 0.19	0.072
Cryptochlorogenic acid	0.31 ±	0.031	0.04 ± 0.004	0.004
Chlorogenic acid	**23.31 ±**	**2.331**	**9.35 ± 0.67**	**0.935**
1,3 Dicaffeoylquinic acid	0.66 ±	0.066	0.28 ± 0.02	0.028
3,5 Dicaffeoylquinic acid	**15.28 ±**	**1.528**	**3.31 ± 0.36**	**0.331**
1,5 Dicaffeoylquinic acid	**38.36 ±**	**3.836**	**14.10 ± 1.44**	**1.410**
4,5 Dicaffeoylquinic acid	3.21 ±	0.321	0.80 ± 0.14	0.080
Succinyl Dicaffeoylquinic acid	-	-	3.41 ± 0.41	0.341
Succinyl Dicaffeoylquinic acid	-	-	0.05 ± 0.004	0.005

3.6. Caffeoylquinic acid derivatives contribute to the inhibition of IL-8 release exerted by the extracts

To connect the anti-inflammatory activity to one or more pure compounds identified in the extracts, chlorogenic acid, 3,5 dicaffeoyilquinic acid and 1,5 dicaffeoylquinic acid were tested at 1 µM on IL-8 release. Although 1,5 dicaffeoylquinic and chlorogenic acids showed around 20% inhibition of IL-8 secretion, only the effect of 3,5 dicaffeoyliquinic acid was statistically significant (Figure 6 A).

Concentration response experiments revealed that 3,5 dicaffeoylquinic acid possessed a strong inhibition of IL-8 secretion in AGS cells, with an IC_{50} of 0.65 µM (Figure 6 B).

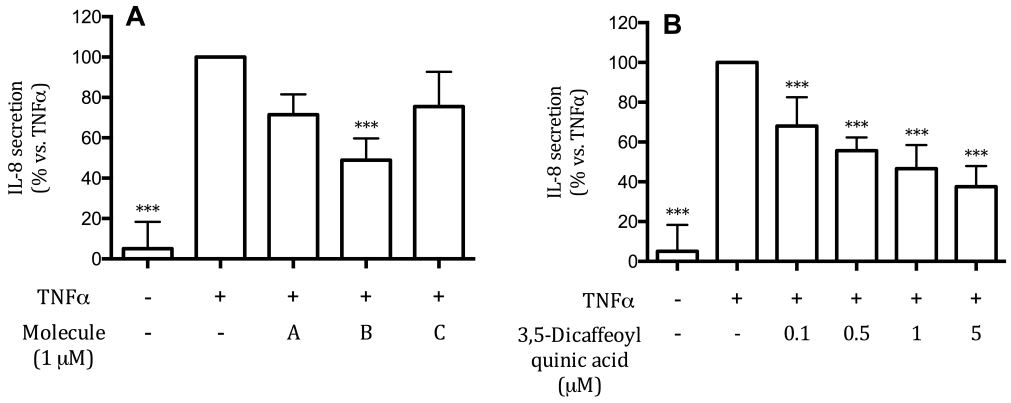

Figure 6. Effect of the most abundant pure compounds (1μM) occurring in *Onopordum* extracts on the TNFα-induced IL-8 secretion (A). AGS cells were treated for 6 h with both TNFα (10 ng/mL) and each compound at the concentration of 1 μM. Concentration dependent inhibition of 3,5 dicaffeoylquinic acid on the TNFα-induced IL-8 release (B). 3,5 dicaffeoylquinic acid was evaluated at concentrations ranging from 0.1 to 5 μM. Secreted IL-8 was evaluated by ELISA assay. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. TNFα alone. 20 μM EGCG was used as the reference inhibitor of IL-8 secretion (Fumagalli et al., 2016). A: 1,5 dicaffeoylquinic acid; B: 3,5 dicaffeoylquinic acid; C: chlorogenic acid.

4. Discussion

Gastric inflammation is mostly due to *H. pylori* infection. It causes the degeneration of the gastric epithelium and the infiltration of immune cells through the gastric mucosa, thus leading to release a variety of pro-inflammatory mediators (Bodger and Crabtree, 1998). This work reports, for the first time, the screening of eight wild thistles species, traditionally used in Sardinia, to test their anti-inflammatory activity in human gastric epithelial cells. Two inflammatory target molecules (IL-8, NF-κB) were evaluated in an *in vitro* model of gastric inflammation. IL-8 was chosen since it plays a pivotal role in the development of gastric inflammation during *H. pylori* infection. NF-κB is a transcription factor playing a crucial role in the development of gastro-intestinal inflammatory diseases, its activation is involved in the transcription of several pro-inflammatory mediators, including IL-8. TNFα as pro-inflammatory stimulus was chosen since it is widely released by immune cells during gastritis, thus leading to a massive production of IL-8 (Bodger and Crabtree, 1998; Crabtree, 1996; Crabtree et al., 1993; Israel and Peek, 2001).
Our results, summarized in TableS1, suggest that, among the tested samples, *O. horridum* and *O. illyricum* extracts may exert a beneficial effect against gastric inflammatory

diseases. Both the extracts inhibited IL-8 release and expression; inhibition of IL-8 promoter activity paralleled the inhibitory activity on IL-8 release for *O. illyricum* extract, whereas other mechanisms seem to contribute to inhibition of IL-8 release elicited by *O. horridum* extract. Additionally, both extracts inhibited the NF-κB pathway, and the efficacy resembled inhibition of IL-8 release and promoter activity, thus suggesting that NF-κB is deeply involved in the molecular mechanisms underlying the anti-inflammatory effect. The effect appears approximately at concentrations as low as 10 μg/ml; thus, benefits could be easily reached upon moderate consumption of thistles.

The n-hexane, chloroform and hydro-alcoholic (water/MeOH) extracts of both aerial parts and roots of the *O. acanthium* (10 μg/mL) inhibited the NF-κB transcription ranging from 10 to 21.8 % in THP-1 cells (Lajter et al., 2015). Comparing our results obtained testing *Onopordum* extracts activity with the hydro-alcoholic extract from aerial parts of *O. acanthium*, it appears that the species investigated in the present study show higher inhibitory effect.

The phytochemical analysis of *O. horridum* and *O. illyricum* extracts reports caffeoylquinic acid derivatives as major components. Previous studies aimed to perform phytochemical characterization of *O. illyricum* extracts, identified dicaffeoylquinic acids, luteolin, apigenin and the corresponding glycosides, onopordopicrin and other sesquiterpene lactones, and taraxasteryl acetate (Braca et al., 1999; Bruno et al., 2011; Rosselli et al., 2012; Topal et al., 2016; Verotta et al., 2008). *O. horridum* chemical composition was herein investigated for the first time. Caffeoylquinic acid derivatives are the most abundant compounds in both extracts. Onopordopicrin, a characteristic sesquiterpene lactone found in *Onopordum* genus, was not detected in our extracts. However, solvents and conditions used for extraction, in addition to the plant material, could deeply affect the extraction of this compound. Our findings agree with other studies occurring in the literature; as an example, onopordopicrin was found in *O. illyricum* grown in Poland in the dichloromethane extract and in the ethyl acetate fraction of samples from Sardinia in addition to the chloroform extract from Sicilian samples. However, it was not present in the *n*-butanol extract of a Sardinian sample and in the acetone extract of a sample from Sicily (Braca et al., 1999; Formisano et al., 2017; Rosselli et al., 2012; Verotta et al., 2008).

The presence of , chlorogenic acid, 3,5 dicaffeoylquinic acid and 1,5 dicaffeoylquinic acid in *Onopordum* species is confirmed by a previous study on *O. illyricum* samples from Sardinia (Verotta et al., 2008).

IL-8 inhibition by pure compounds suggests that 3,5 dicaffeoylquinic acid may contribute, at least in part, to the anti-inflammatory activity elicited by *O. illyricum*, which reports high levels of this compound. However, other compounds, still unidentified, may be responsible for the anti-inflammatory activity of *O. horridum* extract. Previous works report the anti-inflammatory activity of caffeoylquinic acids derivatives, including chlorogenic acid and 3,5 dicaffeoylquinic acid, against several pro-inflammatory molecules and in different cell models (Chen et al., 2015; Han et al., 2015; Hong et al., 2015; Liu et al., 2015; Znati et al., 2014). To our knowledge no data on the inhibitory activity of these molecules against the TNFα-induced IL-8 secretion in AGS cells are currently available.

5. Conclusion

This work reports the anti-inflammatory activity of two *Onopordum* species traditionally used in Sardinia. These findings support the traditional use of *Onopordum* species for medicinal and food purposes, and make these plants exploitable as preventive or co-adjuvant agents in gastric diseases. Since caffeoylquinic acid derivatives are commonly present in botanical supplements on the market, these extracts may be considered as new sources of compounds active against gastric inflammation.

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Abbreviations

IL-8	Interleukin 8
NF-κB	Nuclear factor κB
WHO	World Health Organization
TNFα	Tumour necrosis factor alpha
IL-1β	Interleukin 1β
VCAM-1	Vascular cell adhesion protein 1
ICAM-1	Intercellular Adhesion Molecule 1
TNF-R1	Tumor necrosis factor receptor 1
IL-4	Interleukin 4
IFN-γ	Interferon γ
STAT3	Signal transducer and activator of transcription 3
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
NO	Nitric oxide
5-LOX	5-lipoxygenase
AGS	Human gastric adenocarcinoma AGS cells
DMEM F12	Dulbecco's Modified Eagle Medium F12
MTT	3,4,5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
s.d.	Standard deviation
EGCG	Epigallocatechin-3-gallate
DMSO	Dimethyl sulfoxide
LUC	Luciferase
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
TMB	3,31 ,5,51 -tetramethylbenzidine
LDH	Lactate dehydrogenase
ANOVA	Analysis of Variance
IC_{50}	Half maximal inhibitory concentration

THP-1	Human monocytic leukaemia derived cells
PDA	Photodiode Array Detector
MS/MS	Tandem mass spectrometry
UV	Ultraviolet
MRM	Multiple reaction monitoring

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