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

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1 **The hydro-alcoholic extract of Sardinian wild thistles species (*Onopordum***

2 **sp.) inhibits IL-8 and NF-κB in the TNF-α stimulated AGS cells**

3 Arianna Marengo[a,c], Marco Fumagalli[b], Cinzia Sanna[a], Andrea Maxia[a], Stefano Piazza[b], Cecilia Cagliero[c],

4 Patrizia Rubiolo[c], Enrico Sangiovanni[b]*, Mario Dell'Agli[b]

5 [a] Dipartimento di Scienze della Vita e dell'Ambiente, sezione di Botanica, Università di Cagliari, Viale

6 Sant'Ignazio da Laconi 13, 09123 Cagliari, Italy;

7 [b] Dipartimento di Scienze Farmacologiche e Biomolecolari; Università degli Studi di Milano, Via

8 Balzaretti, 9, 20133 Milano, Italy;

9 [c] Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Via P. Giuria 9, I-10125 Torino,

10 Italy;

11

12 *Corresponding author: Enrico Sangiovanni. Dipartimento di Scienze Farmacologiche e

13 Biomolecolari; Università degli Studi di Milano, Via Balzaretti, 9, 20133 Milano, Italy;

14 Tel.: +39-02-50318383, E-mail: enrico.sangiovanni@unimi.it

15

16 E-mail addresses: marengo.arianna@gmail.com (A. Marengo),

17 marco.fumagalli3@unimi.it (M. Fumagalli), cinziasanna@unica.it (C. Sanna),

18 a.maxia@unica.it (A. Maxia), stefano.piazza@unimi.it (S. Piazza),

19 cecilia.cagliero@unito.it (C. Cagliero), patrizia.rubiolo@unito.it (P. Rubiolo),

20 enrico.sangiovanni@unimi.it (E. Sangiovanni), mario.dellagli@unimi.it (M. Dell'Agli).

21

22 **Abstract:**

23 *Ethnopharmacological relevance:* Thistles species (Family: Compositae) are traditionally

24 used in the Mediterranean area, particularly in Sardinia. They are usually gathered from the

25 wild and used for both food and therapeutic purposes, including gastrointestinal disorders.

26 *Aim of the study:* This work aims to evaluate the anti-inflammatory activity of eight wild

27 thistles from Sardinia, in an *in vitro* model of gastric inflammation, and to identify the

28 major active compounds in the extracts.

29 *Materials and methods:* The hydro-alcoholic extract of the aerial part of each species was

30 prepared. After the induction of inflammation by the addition of tumor necrosis factor-α

31 (TNFα) (10 ng/ml), AGS cells were treated with extracts/pure compounds under study. The

32 inhibition of interleukin-8 (IL-8) release, IL-8 and NF-κB promoter activities and NF-κB

33   nuclear translocation was evaluated. Extracts main components were identified by

34   HPLC-PDA-MS/MS.

35   *Results:* Only *Onopordum horridum* Viv. and *Onopordum illyricum* L. hydro-alcoholic

36   extracts reduced, in a concentration-dependent fashion, the IL-8 release and promoter

37   activity in human gastric epithelial cells AGS. The effect was partially due to the NF-κB

38   pathway impairment. *Onopordum* hydro-alcoholic extracts were also chemically profiled,

39   and caffeoylquinic acid derivatives were the main compounds identified in the extract.

40   Further investigations showed that 3,5 dicaffeoylquinic acid highly inhibited IL-8 secretion

41   in AGS cells (IC_{50} 0.65 μM), thus suggesting that this compound contributed, at least in

42   part, to the anti-inflammatory activity elicited by *O. illyricum* extracts.

43   *Conclusions:* Our results suggest that *Onopordum* species may exert beneficial effects

44   against gastric inflammatory diseases. Thus, these wild plants deserve further investigations

45   as preventive or co-adjuvant agents in gastric diseases.

46

48

49   Chemical compounds studied in this article:

50   Neochlorogenic acid (PubChem ID: 5280633); Cryptochlorogenic acid (PubChem ID:

51   9798666); Chlorogenic acid (PubChem ID: 1794427); 1,3 Dicaffeoylquinic acid (PubChem

52   ID: 6474640); 3,5 Dicaffeoylquinic acid (PubChem ID: 6474310), 1,5 Dicaffeoylquinic

53   acid (PubChem ID: 122685); 4,5 Dicaffeoylquinic acid (PubChem ID: 6474309)

54

55   **1. Introduction**

56   The aetiopathogenesis of gastritis, an inflammatory state of gastric mucosa, is mostly due to

57   the presence of *Helicobacter pylori* (*H. pylori*), a Gram-negative pathogen affecting humans

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

59 Many pro-inflammatory molecules (e.g. TNFα, IL-8, NF-κB), released during gastritis, can

60 be considered as potential therapeutic targets to prevent or treat *H. pylori*-induced gastric

61 diseases (Bodger and Crabtree, 1998; Crabtree et al., 1993; Israel and Peek, 2001; Martin and

62 Wallace, 2006; Zaidi et al., 2012). Emerging resistance to antibiotics and adverse effects of

63 conventional drugs lead to search for new therapeutic strategies to counteract the

64 inflammatory processes exerted by *H. pylori* infection (Zaidi et al., 2012).

65 Botanicals, from both wild or cultivated plants, are widely used all over the world, for

66 nutritional and health purposes, as different types of products, including herbal medicinal

67 products, food, food supplements, and functional foods.

68 Wild plants, traditionally used by the native populations, recently received attention for their

69 therapeutic properties and the high content of fibres, vitamins, minerals, and polyphenols

70 (Licata et al., 2016; Tuttolomondo et al., 2014). Some of them are traditionally used to treat

71 gastrointestinal disorders such as dyspepsia, constipation, diarrhoea, gastritis, colitis (Atzei,

72 2003; Tuttolomondo et al., 2014) and have shown beneficial effects against gastritis

73 (Colombo et al., 2013; Di Lorenzo et al., 2013; Sangiovanni et al., 2015).

74 Sardinia boasts a well-established culture on the traditional uses of wild plants (Atzei, 2003;

75 Lancioni et al., 2007; Maxia et al., 2013). The so-called thistles mostly refer to Compositae

76 species and are traditionally consumed and used for therapeutic purposes by Sardinian

77 inhabitants (Atzei, 2003; Guarrera and Savo, 2016; Lancioni et al., 2007; Signorini et al.,

78 2009). The aim of the present study was to investigate the anti-inflammatory activity of eight

79 wild thistles species from Sardinia in a cell model of gastric inflammation. The species under

80 study belong to the *Cardueae* Cass. Tribe (Family: Compositae) and to four genera: *Carduus*

81 L. (*C. argyroa* Biv.*, C. cephalanthus* Viv.*, C. pycnocephalus* L.*, C. nutans* subsp

82 *macrocephalus* (Desf.) Nyman), *Onopordum* L. (*O. illyricum* L.*, O. horridum* Viv.), *Silybum*

83 L. (*S. marianum* (L.) Gaertn.), and *Ptilostemon* Cass. (*P. casabonae* (L.) Greuter). All these

84 plants are traditionally used for food and medicinal purposes, also against gastrointestinal

85 disorders (Atzei, 2003; Guarrera and Savo, 2016; Lancioni et al., 2007; Licata et al., 2016;

86 Rinchen and Pant, 2014; Signorini et al., 2009).

87 The *in vivo* activity of *C. pycnocephalus* has been previously reported towards the rat paw

88 oedema inflammation, while the *in vitro* inhibition of NF-κB pathway, IL-1β, TNFα, and the

89 adhesion molecules VCAM-1, ICAM-1 and E-selectin release has been described for *S.*

90 *marianum* extracts, demonstrating that the effects are mostly due to the presence of silymarin

91 components (Al-Shammari et al., 2015; Giorgi et al., 2012; Kang et al., 2003; Manna et al.,

92 1999). *In vivo* studies have shown the ability of *S. marianum* to inhibit TNF-R1, TNFα, IL-4

93 and IFN-γ expression (He et al., 2004; Schumann et al., 2003). Moreover, the *in vitro* NF-κB,

94 STAT3 inhibitory activity and the Nrf2 activation were evaluated for six sesquiterpenes from

95 *O. illyricum* (Formisano et al., 2017). *O. acanthium* inhibited COX-2 and NF-κB gene

96 expression, NO production and 5-LOX, COX-1 and COX-2 enzymes activity in THP-1 cells

97 (Lajter et al., 2015). However, no studies investigating the *in vitro* anti-inflammatory activity

98 of the thistles species under study in human gastric epithelial cells have been reported so far.

99 A preliminary screening of the selected thistles hydro-alcoholic extracts was assessed to

100 investigate their inhibitory effect on IL-8 released by human gastric epithelial cells (AGS).

101 To elucidate the underlying molecular mechanisms, the extracts showing remarkable activity

102 were tested on the NF-κB pathway. The extracts were also chemically profiled to identify the

103 compounds responsible for the observed biological activity.

104

105 **2. Materials and Methods**

106 *2.1 Materials*

107 Dulbecco's Modified Eagle's Medium/F12 (DMEM)/F12 (1:1), penicillin, streptomycin,

108 L-glutamine, sodium pyruvate and trypsin-EDTA were from Gibco (Life Technologies

109 Italia, Monza, Italy). DMEM, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

110 bromide (MTT) were from Sigma Aldrich (Milan, Italy). All reagents used for analytical

111 determinations and biological assays were HPLC grade. Human TNFα and Human IL-8 Elisa

112 Development Kit were from Peprotech Inc. (London, UK). Foetal bovine serum (FBS), and

113 disposable material for cell culture were purchased by Euroclone (Euroclone S.p.A.,

114 Pero-Milan, Italy). Human adenocarcinoma cells (AGS, CRL-1739) were purchased from

115 LGC Standard S.r.l., Milano, Italy. 1,5 dicaffeoylquinic acid (purity >99.4%), 3,5

116 dicaffeoylquinic acid (purity >98.2%), 1,3 dicaffeoylquinic acid (purity >99.36%), were

117 purchased from Phytolab (Vestenbergsgreuth, Germany), chlorogenic acid (purity >99.6%)

118 was from Sequoia Research Products (Pangbourne, UK), epigallocatechin-3-*O*-gallate

119 (purity >99%, EGCG), and DMSO were from Sigma-Aldrich (St Louis, USA). The plasmid

120 NF-κB-LUC containing the luciferase gene under the control of three κB sites was a gift of

121 Dr N. Marx (Department of Internal Medicine-Cardiology, University of Ulm, Germany).

122 Native IL-8-LUC promoter was kindly provided by Dr T. Shimohata (Department of

123 Preventive Environment and Nutrition, University of Tokushima Graduate School, Japan).

124 Britelite™ plus was from Perkin Elmer (Monza, Italy). HPLC-grade acetonitrile and

125 methanol were purchased from Sigma (Bellefonte, USA). De-ionized water (18.2 MΩ cm)

126 was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Formic

127 acid (purity >98%) was obtained from Sigma (Bellefonte, USA).

128 *2.2. Plant material*

129 Aerial parts of eight wild species belonging to the *Cardueae* tribe were collected from

130 different sites in Sardinia, from May to June 2015 (Table 1). Plant material was identified at

131 the Department of Life and Environmental Science, University of Cagliari, Italy, where a

132 voucher specimen for each species was deposited. Several individuals from *Carduus argyroa*

133 (10 individuals), *Carduus cephalanthus* (6 individuals), *Carduus nutans* subsp.

134 *macrocephalus* (13 individuals), *Carduus pycnocephalus* (10 individuals), *Onopordum*

135 *illyricum* (10 individuals), *Onopordum horridum* (10 individuals), *Silybum marianum* (10

136 individuals), *Ptilostemon casabonae* (10 individuals) were collected. All individuals

137 sampled within each site were separated by about 1–50 m from each other and were collected

138 randomly. The fresh material was dried at 40°C to constant weight.

139 **Table 1.** Localities and dates of collection, local name (Atzei, 2003; Congia, 1998),

140 voucher numbers, and No. of individuals of the eight *Cardueae* species

141

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	Monte dei Sette Fratelli, 21 May 2015	39°20'43.60"N – 9°17'43.74"E	CAG-805	10

	pisciau				
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

142

2.3. Preparation of plant extracts

The aerial parts of each species were combined to obtain homogenous samples; 2 g from the dried and ground material were submitted to ultrasonic extraction with 10 mL of methanol/water (70:30, v/v) two times for 10 min. The extraction phases were then combined and centrifuged at 4000 rpm for 10 min. The extracts were then filtered, dried under vacuum, lyophilized and weighted. To test the biological activity, the extracts were 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

189 prepared using a Nuclear Extraction Kit from Cayman Chemical Company (Michigan, USA)

190 and stored at −80°C until assayed. The same amount of total nuclear proteins, measured by

191 the method of Bradford, was used to assess NF-κB nuclear translocation using the NF-κB

192 (p65) transcription factor assay kit (Cayman) followed by spectroscopy (λ: 450 nm, 0.1 s).

193 Data were expressed considering 100% of the absorbance related to the cytokine-induced

194 NF-κB nuclear translocation. EGCG (20 μM) was used as the reference inhibitor of NF-κB

195 nuclear translocation.

2.8. Cytotoxicity assays

197 The integrity of the cell morphology before and after treatment was assessed by light

198 microscope inspection. Cell viability was measured by the MTT and LDH methods. No sign

199 of cytotoxicity was observed in AGS cells treated for 6 h with the eight *Cardueae* extracts at

200 the concentrations used for testing the biological activity.

2.9. Phytochemical profile of Onopordum extracts

202 *Onopordum* extracts were analysed using a Shimadzu Nexera X2 system equipped with a

203 photodiode detector SPD-M20A in series to a triple quadrupole Shimadzu LCMS-8040

204 system provided with electrospray ionization (ESI) source (Shimadzu, Düsseldorf Germany).

205 An Ascentis Express RP-Amide column (10cmx2.1mmx2.7μm, Supelco, Bellefonte, USA)

206 and a mobile phase with water (eluent A, containing 0.1% formic acid) and acetonitrile

207 (eluent B, containing 0.1% formic acid) was used. The flow rate was 0.4 mL/min and the

208 column temperature was maintained at 30°C. The gradient program was as follow: 5-25% B

209 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

210 total pre-running and post-running time was 60 min. UV spectra were acquired in the

211 220-450 nm wavelength range. MS operative conditions were as follows: heat block

212 temperature: 200 °C; desolvation line (DL) temperature: 250 °C; nebulizer gas flow rate: 3

213 L/min drying gas flow rate: 15 L/min. Mass spectra were acquired both in positive and in

214 negative full-scan mode over the range 100–1000 m/z, event time 0.5 s. Product Ion Scan

215 mode (collision energy: - 35.0 V for ESI $^+$ and 35.0 V for ESI $^-$, event time: 0.2 s) was applied

216 to compounds for which a correspondence between the pseudomolecular ions [M+H]$^+$ in ESI

217 $^+$ and [M-H] $^-$ in ESI $^-$ had been confirmed. The identification of the compounds were   by

218 comparing their retention times, UV and MS spectra to those of authentic standards when

219 available. The other components were tentatively identified on the basis of their UV spectra

220 and mass spectral information, compared to those present in the literature. The major

221 components were quantified using the Multiple Reaction Monitoring acquisition in ESI$^+$

222   (collision energy: - 35.0 V for ESI+, dwell time: 20) on specific ion products derived from

223   precursor ions fragmentation. Chlorogenic acid and 1,3 dicaffeoylquinic acid were used for

224   the quantification of chlorogenic acid derivatives and the dicaffeoylquinic and succinyl

225   dicaffeoylquinic acids, respectively. Each standard solution and extracts were analysed in

226   two replicates. Calibration curves were prepared with five different concentrations, in the

227   range of 0.1-5 μg/mL, monitoring the reported transitions: ESI$^+$: m/z 355.00 →163.00, for

228   chlorogenic acid and 517.00 →163.00 for 1,3 dicaffeoylquinic acid. (dwell time: 20 msec,

229   collision energy -35 V, event time: 0.096 sec). The determination coefficients were 0.993 and

230   0.995 for chlorogenic acid and 1,3 dicaffeoylquinic acid, respectively.

231   *2.10. Statistical analysis*

232   All data are the mean ± SD of at least three experiments performed in duplicate (ELISA) or

233   triplicate (transfections). Data were analysed by unpaired one-way analysis of variance

234   (ANOVA), or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc

235   test. Statistical analyses were performed using GraphPad Prism 5.02 software (GraphPad

236   Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant. IC$_{50}$

237   was calculated using GraphPad Prism 5.02.

238

239   **3. Results**

240   *3.1. Screening of the eight Cardueae species on the TNFα-induced IL-8 release in AGS cells*

241   Preliminary screening of the eight *Cardueae* extracts on the TNFα-induced IL-8 release in

242   human epithelial gastric AGS cells was performed. As shown in Figure 1, only the extracts

243   belonging to the *Onopordum* genus inhibited the TNFα-induced IL-8 secretion at 50 μg/mL.

244   The inhibitory effect of *O. horridum* and *O. illyricum* reached 80% and 95% respectively.

245   Thus, the extracts from *O. horridum* and *O. illyricum* were selected for further studies aimed

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

247
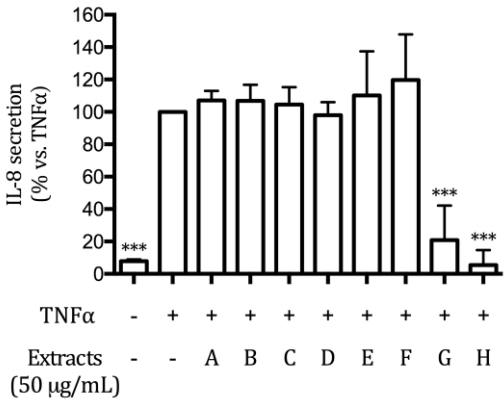
248   **Figure 1.** Effect of the eight *Cardueae* extracts on the TNFα-induced IL-8

249   secretion. AGS cells were treated for 6 h with both TNFα (10 ng/mL) and each of

250   the eight extracts at the concentration of 50 µg/mL. Secreted IL-8 was evaluated by

251   ELISA assay. **p < 0.01, ***p < 0.001 vs. TNFα alone. 20 µM EGCG was used as

252   the reference inhibitor of IL-8 secretion, according to the literature (Fumagalli et al.,

253   2016). A*: Carduus argyroa;* B: *Carduus nutans* subsp *microcephalus;* C: *Carduus*

254   *cephalanthus;* D: *Ptilostemon casabonae*; E: *Carduus pycnocephalus*; F: *Silybum*

255   *marianum*; G: *Onopordum horridum*; H: *Onopordum illyricum*.

256 *3.2. Onoopordum species inhibit TNFα-induced IL-8 release in AGS cells*

257 The extracts inhibited IL-8 release induced by TNFα in a concentration dependent fashion;

258 IC_{50} were 4.31 and 12.27 µg/mL for *O. horridum* and O. *illyricum*, respectively . Moreover,

259 *Onopordum* extracts prevented TNFα-induced IL-8 release, when added to the cells 2 h

260 before challenging with the pro-inflammatory stimulus; IC_{50} were 18.45 and 12.75 µg/mL for

261 *O. horridum* and O. *illyricum*, respectively (Figure 2, A-B).

262

263 As shown by comparison of the IC_{50}, the inhibitory effect of *O. horridum* extract under

264 co-treatment conditions was more pronounced than that observed under pre-treatment (IC_{50}:

265 4.31 vs. 18.45 µg/mL) whereas the inhibitory effect of O. *illyricum* extract was comparable.

266 Thus, we decided to further investigate the effect of the extracts exclusively in the

267 co-treatment conditions.
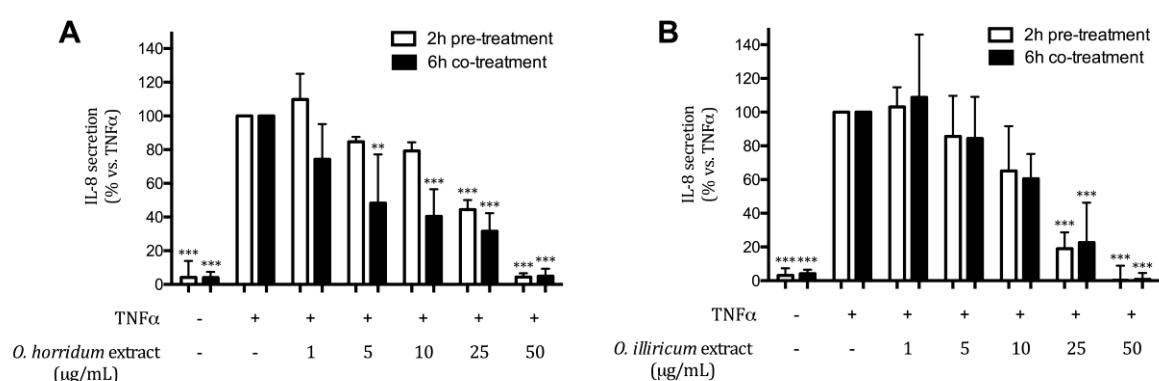
269   **Figure 2.** Effect of *O. horridum* (A) and *O. illyricum* (B) extracts on the

270   TNFα-induced IL-8 secretion. To evaluate the effect of *Onopordum* extracts (1-50

271   µg/mL) AGS cells were treated for 6 h with both TNFα (10 ng/mL) and extract

272   (black bar). Preventive effect on the TNFα-induced IL-8 secretion was assessed by

273   pre-treating AGS cells for 2 h with the two *Onopordum* extracts (1-50 µg/mL); then,

274   IL-8 release was induced by treatment with TNFα (10 ng/mL) for 6 h (white bar).

275   Secreted IL-8 was evaluated by ELISA assay. **p < 0.01, ***p < 0.001 vs. TNFα

276   alone. 20 μM EGCG was used as the reference inhibitor of IL-8 secretion, according

277   to the literature (Fumagalli et al., 2016).

278

279

280   *3.3. Onoopordum species inhibit the TNFα-induced IL-8 secretion through impairment of the*

281   *corresponding promoter activity*

282   To test if the inhibitory effect of *Onopordum* extracts on IL-8 release could be due to

283   inhibition of IL-8 promoter activity, AGS cells were transiently transfected with a plasmid

284   carrying the luciferase gene under the control of a fragment of the IL-8 promoter containing

285   several responsive sequences including a sequence responsive to NF-κB.

286   As shown in Figure 3, *Onopordum* extracts inhibited TNFα-induced IL-8 promoter activity

287   in a concentration dependent manner with comparable activity. IC_{50} for *O. horridum* and *O.*

288   *illyricum* were 17.09 and 14.8 μg/mL, respectively.

289

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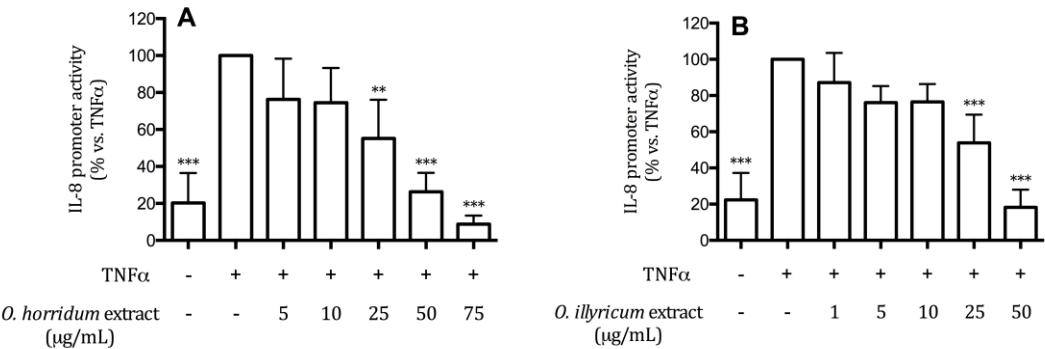
292   **Figure 3.** Effect of the *O. horridum* (A) and *O. illyricum* (B) extracts on the

293   TNFα-induced IL-8 promoter activity. AGS cells were treated for 6 h with TNFα

294   (10 ng/mL) and *O. horridum* (5-75 μg/mL) or *O. illyricum* (1-50 μg/mL) extracts.

295   IL-8 promoter activity was evaluated in transiently transfected AGS cells by the

296   luciferase assay. **p < 0.01, ***p < 0.001 vs. TNFα alone. 20 μM EGCG was used

297   as the reference inhibitor of IL-8 secretion, according to the literature (Fumagalli et

298   al., 2016).

299

300

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).
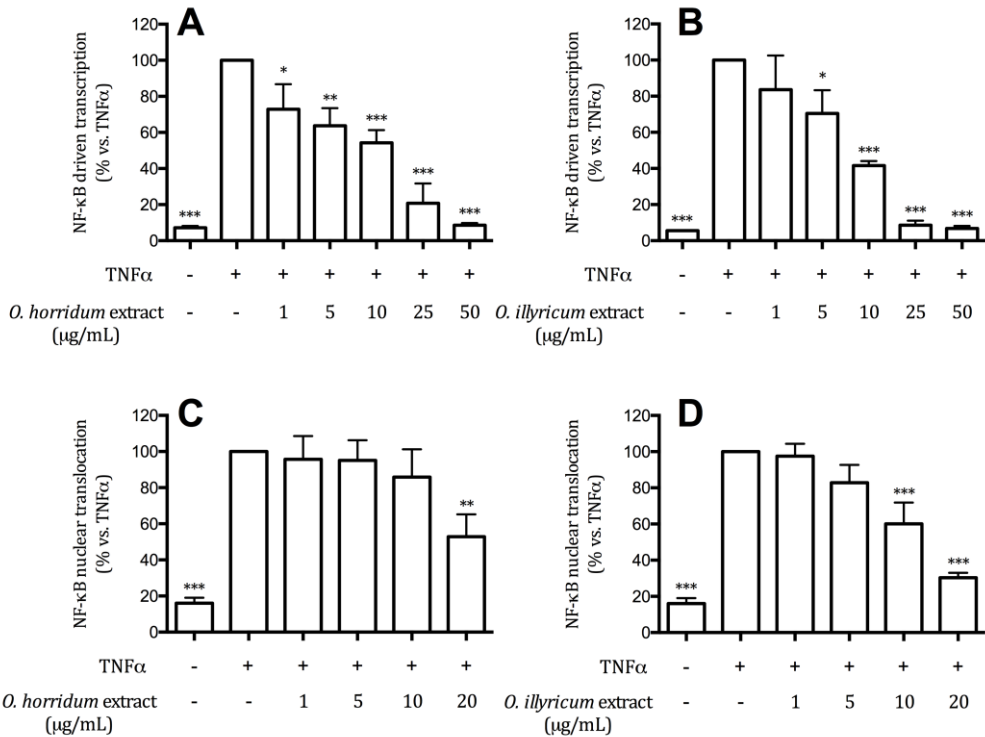
Figure 4. Effect of *O. horridum* and *O. illyricum* extracts on the TNFα-induced NF-kB driven transcription (A-B) and nuclear translocation (C-D). AGS cells were treated for 6 h (driven transcription assay) or 1 h (nuclear translocation assay) with TNFα (10 ng/mL) and *O. horridum* or *O. illyricum* extracts at 1-50 µg/mL (NF-κB driven transcription) or 1-20 µg/mL (nuclear translocation assay). **p < 0.01, ***p

321   < 0.001 vs. TNFα alone. 20 μM EGCG was used as reference inhibitor of

322   TNFα-induced NF-κB driven transcription or nuclear translocation, according to the

323   literature (Fumagalli et al., 2016).

324

325

326   *3.5 Phytochemical characterization of Onopordum extracts*

327   The literature reports several classes of metabolites as characteristics of the genus

328   *Onopordum*, including sesquiterpenoids, flavonoids, acetylenic compounds, steroids,

329   triterpenes, lipids and nitrogen containing compounds (Bruno et al., 2011; Lajter et al.,

330   2015).

331   The extracts from *O. horridum* and *O. illyricum* were chemically profiled through

332   HPLC-PDA-MS/MS analysis. Caffeoylquinic acid derivatives were identified by comparing

333   the UV, MS and MS/MS spectra to those of reference standards. Figure 5 reports the

334   chromatographic profiles of caffeoylquinic acid derivatives standard compounds and *O.*

335   *illyricum* and *O. horridum* extracts (A,B,C). 3,5 dicaffeoylquinic acid was chosen as

336   illustrative example of a tandem mass spectrometry fragmentation pattern. As shown in

337   figure 5D and 5E, the MS/MS fragmentation of both the standard compound and 3,5

338   dicaffeoylquinic acid in *O. illyricum* extract generated diagnostic fragments at *m/z* 163 and

339   191 in the positive and negative ESI mode, respectively (Marengo et al., 2017).

340   Table 2 includes the quantitative analysis of the caffeoylquinic acid derivatives identified in

341   the extracts.

342   The most abundant compounds were quantified both in the UV mode and in the MRM

343   acquisition, which provided similar results. The quantification through external calibration

344   method based on the following transitions in ESI^+: 355 → 163 for the chlorogenic acids, 517

345   → 163 for the dicaffeoylquinic acids and 617 → 163 for the succynil dicaffeoylquinic acids,

346   was chosen to obtain an accurate quantification of the compounds.

347   The most abundant components in both species were the caffeoylquinic acid derivatives.

348   Chlorogenic and dicaffeoylquinic acids are present in both species, whereas succinyl

349   dicaffeoylquinic acids were found only in *O. horridum* (Figure 5A). In our extracts,

350   chlorogenic acid, 3,5 dicaffeoylquinic acid and 1,5 dicaffeoylquinic acid are the main

351   phenolic compounds in both species, although their amount is higher in *O. illyricum* extract

352   (Table 2).

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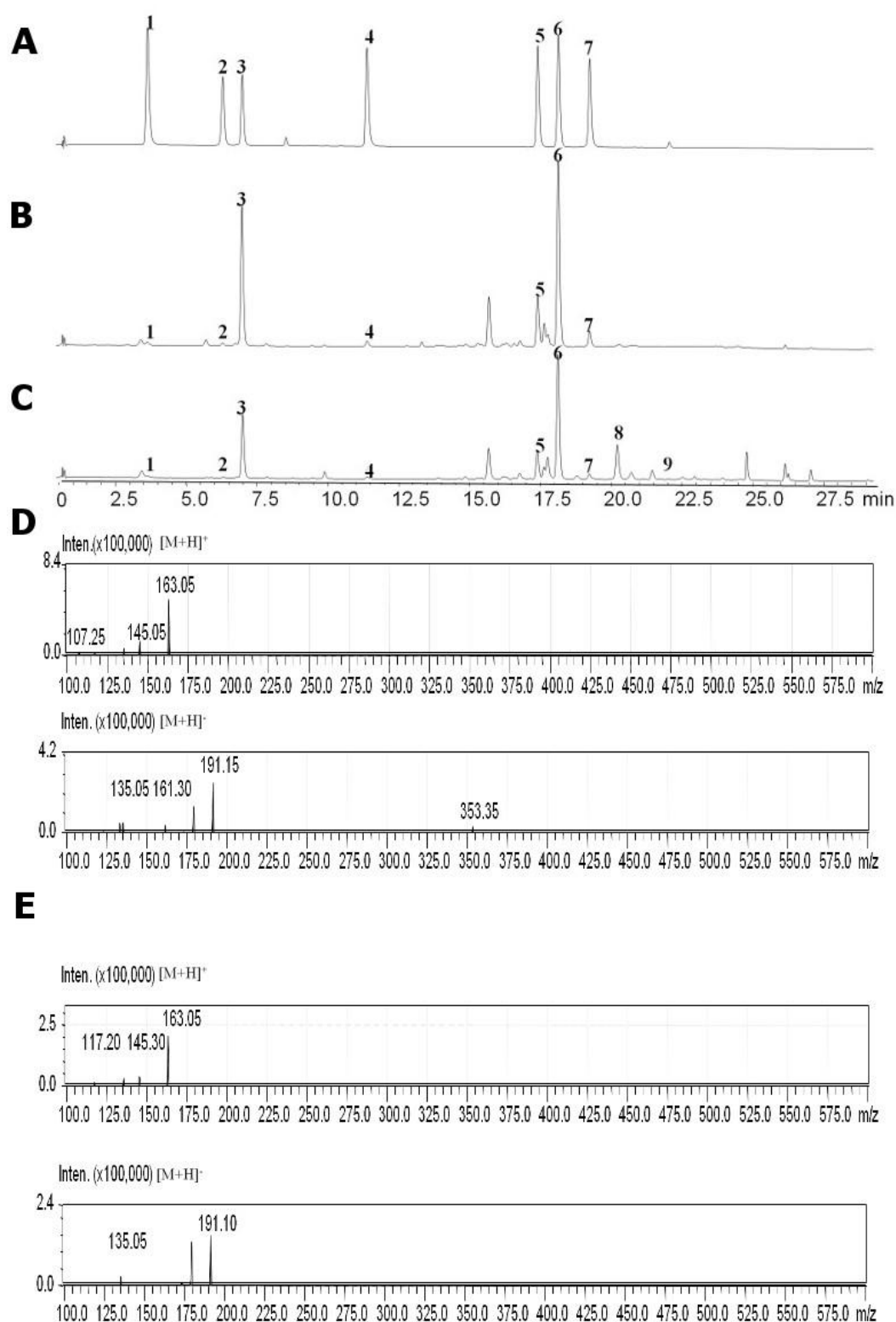
Fig. 5. Chromatographic profiles of caffeoylquinic acid derivatives standard compounds (A) and *O.illyricum* (B)) and *O. horridum* (C) extracts. Product Ion Scan spectra of the [M+H]$^+$ and [M+H]$^-$ ions of 3,5 dicaffeoylquinic acid standard compound (D) and 3,5

358   dicaffeoylquinic acid in *O. illyricum* extract (E). Compounds: 1=neochlorogenic acid;

359   2=criptochlorogenic acid; 3=chlorogenic acid; 4=1,3 dicaffeoylquinic acid; 5=3,5

360   dicaffeoylquinic acid; 6=1,5 dicaffeoylquinic acid; 7=4,5 dicaffeoylquinic acid; 8=succinyl

361   dicaffeoylquinic acid1; 9=succinyl dicaffeoylquinic acid2

362

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

364   *O. illyricum* extracts

Compound	*O. illyricum*		*O. horridum*	
	μg/mg	%	μg/mg	%
Neochlorogenic acid	1.48 ± 0.17	0.148	0.72 ± 0.19	0.072
Cryptochlorogenic acid	0.31 ± 0.08	0.031	0.04 ± 0.004	0.004
Chlorogenic acid	**23.31 ± 1.44**	**2.331**	**9.35 ± 0.67**	**0.935**
1,3 Dicaffeoylquinic acid	0.66 ± 0.12	0.066	0.28 ± 0.02	0.028
3,5 Dicaffeoylquinic acid	**15.28 ± 0.63**	**1.528**	**3.31 ± 0.36**	**0.331**
1,5 Dicaffeoylquinic acid	**38.36 ± 2.57**	**3.836**	**14.10 ± 1.44**	**1.410**
4,5 Dicaffeoylquinic acid	3.21 ± 0.67	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

365

366

367   *3.6. Caffeoylquinic acid derivatives contribute to the inhibition of IL-8 release exerted by the*

368   *extracts*

369   To connect the anti-inflammatory activity to one or more pure compounds identified in the

370   extracts, chlorogenic acid, 3,5 dicaffeoyilquinic acid and 1,5 dicaffeoylquinic acid were

371   tested at 1 μM on IL-8 release. Although 1,5 dicaffeoylquinic and chlorogenic acids showed

372   around 20% inhibition of IL-8 secretion, only the effect of 3,5 dicaffeoyliquinic acid was

373   statistically significant (Figure 6 A).

374   Concentration response experiments revealed that 3,5 dicaffeoylquinic acid possessed a

375   strong inhibition of IL-8 secretion in AGS cells, with an IC_{50} of 0.65 μM (Figure 6 B).

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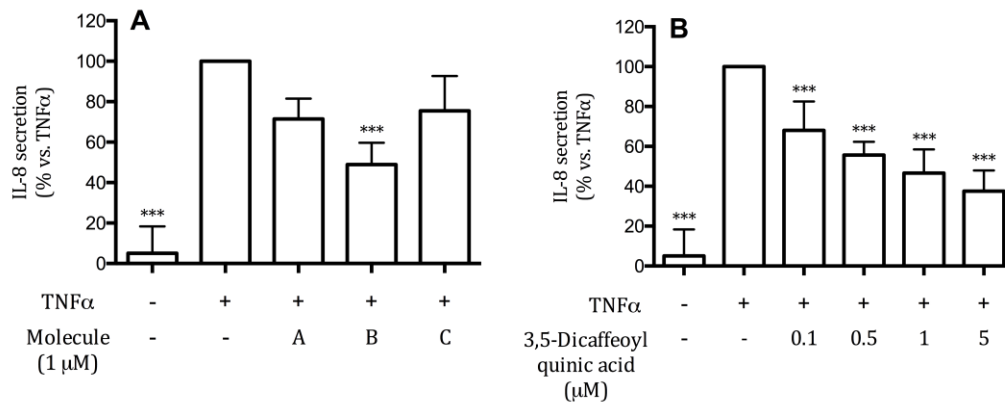
Figure 6. Effect of the most abundant pure compounds (1μM) occurring in *Onopordum* extracts on the TNFα-induced IL-8 secretion (A). AGS cells were treated for 6 h with both TNFα (10 ng/mL) and each compound at the concentration of 1 μM. Concentration dependent inhibition of 3,5 dicaffeoylquinic acid 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.

403   Both the extracts inhibited IL-8 release and expression; inhibition of IL-8 promoter activity

404   paralleled the inhibitory activity on IL-8 release for *O. illyricum* extract, whereas other

405   mechanisms seem to contribute to inhibition of IL-8 release elicited by *O. horridum* extract.

406   Additionally, both extracts inhibited the NF-κB pathway, and the efficacy resembled

407   inhibition of IL-8 release and promoter activity, thus suggesting that NF-κB is deeply

408   involved in the molecular mechanisms underlying the anti-inflammatory effect.

409   The effect appears approximately at concentrations as low as 10 μg/ml; thus, benefits could

410   be easily reached upon moderate consumption of thistles.

411   The n-hexane, chloroform and hydro-alcoholic (water/MeOH) extracts of both aerial parts

412   and roots of the *O. acanthium* (10 μg/mL) inhibited the NF-κB transcription ranging from 10

413   to 21.8 % in THP-1 cells (Lajter et al., 2015). Comparing our results obtained testing

414   *Onopordum* extracts activity with the hydro-alcoholic extract from aerial parts of *O.*

415   *acanthium*, it appears that the species investigated in the present study show higher inhibitory

416   effect.

417   The phytochemical analysis of *O. horridum* and *O. illyricum* extracts reports caffeoylquinic

418   acid derivatives as major components. Previous studies aimed to perform phytochemical

419   characterization of *O. illyricum* extracts, identified dicaffeoylquinic acids, luteolin, apigenin

420   and the corresponding glycosides, onopordopicrin and other sesquiterpene lactones, and

421   taraxasteryl acetate (Braca et al., 1999; Bruno et al., 2011; Rosselli et al., 2012; Topal et al.,

422   2016; Verotta et al., 2008). *O. horridum* chemical composition was herein investigated for

423   the first time. Caffeoylquinic acid derivatives are the most abundant compounds in both

424   extracts. Onopordopicrin, a characteristic sesquiterpene lactone found in *Onopordum* genus,

425   was not detected in our extracts. However, solvents and conditions used for extraction, in

426   addition to the plant material, could deeply affect the extraction of this compound. Our

427   findings agree with other studies occurring in the literature; as an example, onopordopicrin

428   was found in *O. illyricum* grown in Poland in the dichloromethane extract and in the ethyl

429   acetate fraction of samples from Sardinia in addition to the chloroform extract from Sicilian

430   samples. However, it was not present in the *n*-butanol extract of a Sardinian sample and in the

431   acetone extract of a sample from Sicily (Braca et al., 1999; Formisano et al., 2017; Rosselli et

432   al., 2012; Verotta et al., 2008).

433   The presence of , chlorogenic acid, 3,5 dicaffeoylquinic acid and 1,5 dicaffeoylquinic acid in

434   *Onopordum* species is confirmed by a previous study on *O. illyricum* samples from Sardinia

435   (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.

470 **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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