Antioxidant and anti-collagenase activity of *Hypericum hircinum* L.

Manuela Mandrone[a], Beatrice Lorenzi[a], Alessandro Venditti[b,e], Laura Guarcini[b,f], Armandodoriano Bianco[b], Cinzia Sanna[c], Mauro Ballero[c], Ferruccio Poli[a], Fabiana Antognoni[d *]

[a] Dipartimento di Farmacia e Biotecnologie, Università di Bologna, via Irnerio, 42 , 40126 Bologna
[b] Dipartimento di Chimica, Sapienza Università di Roma, Piazzale Aldo Moro 5, 00185 Roma, Italia
[c] Dipartimento di Scienze della Vita e Ambientali, Università di Cagliari, viale Sant'Ignazio, 13, 09123 Cagliari, Italia, Italia
[d] Dipartimento di Scienze per la Qualità della Vita, Università di Bologna, sede di Rimini, Corso Augusto 237, Rimini, Italia
[e] Dipartimento di Biologia Ambientale, Sapienza Università di Roma, Piazzale Aldo Moro 5, 00185 Roma, Italia
[f] Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza Università di Roma, Piazzale Aldo Moro 5, 00185 Roma, Italia

Corresponding author: Prof. Fabiana Antognoni
Dipartimento di Scienze per la Qualità della Vita – Corso Augusto, 237 -47921 Rimini - Italia
Email : fabiana.antognoni@unibo.it
Tel . 0039 0541 434623; 0039 051 2091291

Abstract

Hypericum hircinum L. is a shrub traditionally used in some Mediterranean areas as a remedy for several diseases, mainly linked to the airway apparatus. In this work, an ethanolic extract prepared from aerial parts of plants collected in Sardinia (Italy), and six fractions isolated by a chromatographic separation of the extract, were evaluated for their antioxidant activity using three *in vitro* assays. Based on the ethnobotanical use of the plant and in view of the emerging role that enzymes belonging to matrix metalloproteinases (MMPs) play in the pathogenesis of some respiratory diseases, the effect of the crude extract and the fractions on *in vitro* collagenase activity was also evaluated. Results show that the ethanolic extract and fractions containing quercetin, chlorogenic acid, and 5,7,3',5'-tetrahydroxyflavanone, a component recently identified in this species, are endowed with the highest free radical scavenging activity. Conversely, fractions containing as a main component shikimic acid did not show this property. The crude extract was able to inhibit *in vitro* collagenase activity with an IC_{50} value of 156 µg/mL. A Lineaweaver-Burk plot, built to obtain the kinetic parameters of the enzymatic reaction, revealed that the inhibitory mechanism is non-competitive. Single fractions were also evaluated for their inhibitory activity on collagenase, and fractions mainly containing flavonols and the substituted flavanone showed the highest inhibitory effect. Thus, *H. hircinum* can be considered a new natural source of molecules able to inhibit enzymes of the MMP family, which could enter as active ingredients in wrinkle-care cosmetics.

Keywords: Anti-collagenase activity, *Hypericum hircinum* L., Matrix metalloproteinase inhibition, Mediterranean Traditional Medicine.

Abbreviations: BA: betulinic acid; CA: chlorogenic acid; HCAs: hydroxycinnamic acids; Q: quercetin; SA: shikimic acid, THFL: 5,7,3',5'-tetrahydroxyflavanone; THFL-Glc: 5,7,3',5'-tetrahydroxyflavanone-7-*O*-glucoside.

1. Introduction

Hypericum hircinum L. is a semi-evergreen shrub belonging to the Hypericaceae family. It is widely distributed in the Mediterranean area, where it is commonly known as 'goat St. John's wort' and traditionally used as a remedy for several diseases. In Italy, both the decoction and the infusion are utilized in the treatment of airway diseases; in Sardinian folk medicine, its use is reported in chronic catarrhal affections and asthma (Atzei, 2003), and in Lucanian folk medicine for treatment of cough (Pieroni et al., 2004). In Sardinia, *H. hircinum* oil is used to treat skin burns and is considered effective as antiseptic, while the hydroalcoholic extract is topically used to relieve rheumatic pains, to treat sciatica, sprains and dislocations, as well as for wound healing (Ballero et al., 1997).

Despite the numerous studies carried out on the species *H. perforatum*, which is well known for its therapeutic use in the treatment of mild to moderate depression (Butterweck, 2003; Ernst and Izzo, 2003; Rodriguez-Landa and Contreras, 2003), much less information is available on other species of this genus. As regards *H. hircinum*, studies have demonstrated antimicrobial and antifungal activities (Maggi et al., 2010a; Cecchini et al., 2007; Pistelli et al., 2000; Barbagallo and Chisari, 1987), herbicide potential, (Araniti et al., 2012; Marandino et al., 2011), a selective action as MAO-A inhibitor (Chimenti et al., 2006) and a protective effect on doxorubicin-induced cardiotoxicity in rats (Shah et al., 2013). Recently, Esposito et al. (2013) have reported for this species an inhibitory activity on HIV replication, targeted on both DNA polymerase and ribonuclease H activities.

The phytochemical composition of both the essential oil (Maggi et al., 2010b) and the non-volatile fraction obtained from different parts of *H. hircinum* subsp. *majus* (Aiton) from the Marche (Italy), has been investigated (Cecchini et al., 2007; Maggi et al., 2010a; Maggi et al., 2010b). Essential oils from aerial parts are dominated by sesquiterpene hydrocarbons, while the non-volatile leaf extract contains chloro-genic acid, quercetin, mangiferin, and biagenin (Pistelli et al., 2000), but lacks hypericin, one of the most active constituents of *H. perforatum* (Cecchini et al., 2007).

Matrix metallo proteinases (MMPs) are a family of transmembrane zinc-containing endoproteinases, which have been traditionally characterized by their collective ability to degrade, at neutral pH, all components of the extracellular matrix. They include, among others, collagenases and gelatinases, the former being metalloproteinases capable of cleaving, besides collagen, other molecules found in cells, such as aggrecan, elastin, fibronectin, gelatine and laminin (Raffetto and Khalil, 2008). A plethora of roles has been recently recognized for this class of enzymes; they are responsible for excessive cartilage degradation, which is considered the most important pathological event associated with rheumatoid arthritis and osteoarthritis (Elliott and Cawston, 2001) and an excessive degradation of newly formed extracellular matrix (ECM) has been found to be related to non-healing wounds.(Schultz et al., 2005; Vaalamo et al., 1996) An important role for MMPs has been reported in the pathological processes associated with chronic obstructive pulmonary disease (COPD), being responsible for the destruction of alveoli due to the degradation of elastin in their walls (Belvisi and Bottomley, 2003).

In view of these evidences, and based on the ethnobotanical uses of *H. hircinum*, we hypothesized that matrix protease inhibition may represent one of the mechanisms by which extracts of this plant exert their beneficial action. Thus, in this work, our attention was focused on the effect of *H. hircinum* hydro alcoholic extracts on activity of collagenase, an enzymatic target involved in ECM degradation. Moreover, considering the role that free radicals play in the pathogenesis of different diseases and in the activation of MMPs (Fu et al., 2001), an evaluation of the antioxidant potential of these extracts was carried out using different *in vitro* assays in order to obtain a more comprehensive picture. With the aim of identifying the most active components in modulating the enzyme activities, a comparison between the crude extracts and fractions obtained by chromatographic separation and pure standards of the most abundant phytochemical constituents was also carried out.

2. Material and methods

2.1. Chemicals and instruments

Reagents were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA). Solvents of analytical or HPLC grade were purchased from VWR (Darmstadt, Germany). Biological assays were performed using a microplate reader Victor X3 Perkin Elmer (Perkin Elmer Inc., Massachusetts USA) and data analysed by Work Out 2.5 software, or in a Jasco V-530 Spectrophotometer (Jasco Europe, Cremella, Italy).

HPLC analyses were carried out on a Jasco system (Jasco Corp., Tokyo, Japan) consisting of a PU-1580 pump, an LG-1580-02 ternary gradient unit, a DG-1580-53 three-line degasser and a PDA detector (MD-2018 Plus) linked to an autosampler (AS 2055 Plus).

NMR spectra were recorded on Varian Mercury 300 MHz instrument and/or on Bruker Avance III 400 MHz instruments. operating at 9.4 T at 298° K. Deuterated solvents ($CDCl_3$, CD_3OD or D_2O) were used to solubilise the samples; the chemical shift was expressed in ppm from TMS for spectra spectra in $CDCl_3$, the signal of HDO at 4.78 ppm is used as reference for spectra in D_2O, the internal solvent signal at 3.31 ppm was used as reference for spectra in CD_3OD.

HR-MS spectra were acquired on a Q-TOF MICRO Spectrometer (Micromass, now Waters, Manchester, UK) equipped with an ESI source, operating in both negative and positive ion mode. The flow rate of sample infusion was 10 µL/min, with a minimum of 100 acquisitions per spectrum. Data were analysed by using the MassLynx software developed by Waters.

2.2. Plant material

Aerial parts of *Hypericum hircinum* L. plants at flowering stage were collected in Jerzu (Sardinia, Italy) in July 2010 and authenticated by C. Sanna. A voucher specimen (Herbarium CAG 232) was deposited in the General Herbarium of the 'Dipartimento di Scienze della Vita e dell'Ambiente, Macrosezione Botanica e Orto Botanico', University of Cagliari (CAG), Sardinia, Italy.

2.3. Preparation of extract and fractions

Plant material was dried at 40°C until constant weight and finely powdered with an electric grinder. Milled material (400 g) was extracted with 96% ethanol for 24 h and extraction was repeated three times (3 × 600 mL). The extracts were gathered and filtered, then the ethanol was evaporated under reduced pressure at 40°C. The resulting water suspension was freeze dried, and the extract was stored at 4 °C until use. The final yield of the extraction was 9.25% (w/w).

The crude ethanolic extract was fractionated by repeated column chromatography (CC) on silica gel, using different solvent mixtures as eluting solutions: saturated *n*-butanol/water and chloroform/methanol at different percentages (starting with a 9.5:0.5 ratio and gradually increasing the polarity during the chromatographic run to 9.0:1.0, 8.0:2.0, 7.0:3.0, 6.0:4.0). The fractionation was monitored by TLC by using spray reagents for detection (2 N H_2SO_4, 3 % aqueous $FeCl_3$). Six fractions (named F1-F6) were obtained, and the identity of the single components was evaluated by means of spectroscopic methods (NMR, MS) in comparison with literature data, and/or by comparison with standard compounds available in our laboratory. The main components were the following: betulinic acid (BA), shikimic acid (SA), chlorogenic acid (CA), quercetin (Q), 5,7,3',5'-tetrahydroxyflavanone (THFL), and 5,7,3',5'-tetrahydroxyflavanone-7-*O*-glucoside.

2.4. Spectrophotometric and chromatographic analyses

Total polyphenol (TP) and flavonoid concentrations of the extract were determined as reported by Di Pompo et al. (2014) and expressed as mg Gallic Acid Equivalents (GAE)/mg extract and mg Rutin Equivalents (RE)/mg extract, respectively.

HPLC analysis of the extract was carried out using a C18 column (Luna C18, 5 μm, 100 Å, 4.6 × 150 mm, Phenomenex Italy, Castelmaggiore, Bologna, Italy). CA, Q and SA were identified according to Brolis et al. (1998). UV detection of CA was performed at 320 nm, SA at 210 nm and Q at 370 nm. Retention times were 2.5, 13.5 and23.7 min for SA, CA and Q, respectively.

158 A calibration curve with the appropriate analytical standard at different concentrations (from
159 0.5 to 500 ppm) was constructed for the quantitative analysis. For Q the following concentrations
160 were used: 0.5, 1.0, 5.0, 10.0, 20.0, 50.0, 100.0 ppm; for CA and SA 1.0, 5.0, 10.0, 50.0, 100.0,
161 200.0, 500.0 ppm were used. Peak identity and purity were confirmed by means of the PDA
162 detector.

163 *2.5. Antioxidant assays*

164 DPPH, ABTS and FRAP-Ferrozine (FZ) tests were performed according to Venditti et al.
165 (2013). The FRAP-FZ was modified as compared to the original FRAP assay (Benzie and Strain,
166 1996) with respect to the pH value. In the ABTS and DPPH assays, the ethanolic extract or purified
167 fractions were tested in a concentration range of 5-20 µg/mL, and Trolox (Tr) or pure standard
168 compounds (CA, Q, SA) in a concentration range of 5-50 µM. In the FRAP-FZ assay, extract or
169 purified fractions were tested in a concentration range of 10-100 µg/mL and Tr or pure standard
170 compounds from 10 to 100 µM. The antioxidant capacity is expressed as IC_{50} and TAC values for
171 extract and fractions, and as IC_{50}, TAC and TEAC for pure compounds.

172 *2.6. Collagenase activity assay*

173 Collagenase assay was performed according to Van Wart and Steinbrink (1981) with slight
174 modifications. Collagenase (E.C. 3.4.24.3) from *Clostridium histolyticum* (type IA, ChC; specific
175 activity 11.72 U/mg) was purchased from Sigma Aldrich Co (Saint Louis, MO). 20 mU enzyme,
176 prepared in Tricine buffer (0.05 M, pH 7.5), containing 0.4 M NaCl and 0.01 M $CaCl_2$, were
177 incubated for 10 min with test samples at different concentrations (from 10 to 300 µg/mL). The
178 synthetic substrate N-(3-[2-Furyl]-acryloyl)-Leu-Gly-Pro-Ala (FALGPA), prepared in the same
179 buffer solution, was added to start the reaction (final concentration 0.8 mM) in a final volume of
180 125 µL. The change in absorbance was monitored for a time interval of 5 min at 340 nm in the
181 microplate reader under a constant temperature of 30 °C. The IC_{50} value was calculated by
182 constructing a linear regression curve showing sample concentrations on the x-axis and percentage

inhibition on the y-axis. The percentage of inhibition of enzyme activity was calculated by the following formula:

$$\% \text{ Inhibition} = [1-(\Delta Abs/min_{\text{ sample}} / \Delta Abs/min_{\text{ negative control}}) \times 100]$$

Water, instead of samples, was used as negative control. ΔAbs values were calculated in the time interval and referred to 1 min. The positive control was represented by epigallocatechin gallate (EGCG), a well-known natural collagenase inhibitor (Madhan et al., 2004).

A Lineweaver-Burk (L-B) plot was constructed to calculate the kinetic parameters (Km expressed in mM and Vmax in μkat) of the enzymatic reaction without and with samples at the IC_{50} concentration (Km_{app} is the apparent Km in the presence of the inhibitor). Different FALGPA concentrations were used from 0.4 to 1 mM; the rate of the enzymatic reaction expressed in μkat was calculated from ΔAbs 0-300 s, considering FALGPA ε at 345 nm=24.7 M^{-1} cm^{-1} and a light path length of 0.4 cm.

2.7. Statistical analysis

All values are expressed as the mean ± SD of three independent experiments with samples in duplicate. Statistical analysis was performed using Graph Pad Prism 4 software (La Jolla, CA) by One-way Analysis of Variance (ANOVA), considering significant differences at $P < 0.05$.

IC_{50} values were calculated by Regression Analysis using Graph Pad Prism 4 software.

TEAC values, defined as the mM concentration of a Tr solution having an activity equivalent to a 1.0 mM solution of the substance under investigation (Rahman and MacNee, 1996), was calculated for pure standard compounds. TAC values, calculated for crude extracts and fractions, are expressed as mmol Tr equivalent/g extract.

3. Results and discussion

3.1. Chemical composition of H. hircinum extract and fractions

Total polyphenols (TP) and flavonoids detected in the ethanolic extract of the aerial parts of Sardinian *H. hircinum* samples. TP content was 20% on a dry weight basis, and flavonoids represent the main polyphenolic component (16.7%). TP content was higher, but of the same order of magnitude, as that observed by Pilepić and Males (2013) in cultivated populations of the same species. This difference can be related to the different growing conditions since plants collected in nature, such as those used in this study, are more exposed to environmental factors able to induce the production of defense compounds compared to cultivated plants. Moreover, the different weather conditions may also justify the higher content. Indeed, variations in TP were observed by these authors also within a same *Hypericum* species in two subsequent harvesting years (Pilepić and Males, 2013).

The phytochemical profile of the same *H. hircinum* extract used in this work has been reported by Esposito et al. (2013) and results revealed the presence of CA, SA, BA, Q, THFL, and THFL-Glc, the latter two compounds having been identified in this species for the first time. A quali-quantitative analysis of the extract by HPLC-DAD revealed that the SA content of the ethanolic extract was 112.39 ± 2.13 mg/g extract, which, considering the extraction yield, corresponds to 0.99% on a dry mass basis. This confirms that *Hypericum* spp. represent a good source of this metabolic intermediate (Bochov et al., 2012). SA content of the ethanolic extract was 107.51 mg/g extract, which corresponds to 0.99 % on a dry mass basis, considering the extraction yield, confirming that *Hypericum* spp. represents a good source of this metabolic intermediate (Bochkov et al., 2012). CA was also found in rather high amounts (40 mg/g extract), while Q was present at much lower levels (2.31 ± 0.30 mg/g). The amount of CA was about 10 times higher than that found by Cecchini et al. (2007), in *H. hircinum* plants collected in central Italy, and this may reflect the variability due to growth and environmental conditions.

Based on NMR and MS spectra, the phytochemical composition of the six fractions purified from the ethanolic extract, was the following: the most lipophilic fraction (F1) contained waxes and BA, intermediate fractions (F2-F3) contained flavonoid aglycons THFL and Q, and the most

hydrophilic ones (F4-F6) contained flavonoid glycosides, HCAs, SA and sugars. F2 and F4 contain as unique components THFL and its glucoside, respectively, which have been recently identified in this species (Esposito et al., 2013). F3 contains THFL and Q as main components. The presence of other constituents, such as BA, CA, SA and HCAs confirmed earlier reports (Chimenti et al., 2006; Pistelli et al., 2000; Esposito et al., 2013).

3.2 Antioxidant activity of crude extract, fractions and isolated compounds

The antioxidant activity of the *H. hircinum* ethanolic extract and the six fractions was evaluated by three different *in vitro* tests, all based on the same single electron transfer mechanism. The IC_{50} values of the crude extract obtained by the ABTS and DPPH tests were very similar to each other (Fig.1A), and reflect a rather high radical scavenging activity, since they were only twice or three times higher compared to Tr. (Figures 1-2). The radical scavenging capacity resulting from the DPPH assay was comparable to the one reported for *H. perforatum* extract (7.5 μg/ml) whereas the value obtained in the ABTS test was lower than that reported earlier for this species (28.5 μg/ml) (Raghu et al., 2009). The FRAP-FZ test, performed in order to evaluate the reducing potential of the sample towards the redox couple Fe^{3+}/Fe^{2+} (Berker et al., 2010), confirmed the strength of *H. hircinum* extract as antioxidant, since a TAC value of 0.91 was found (Tab. 1). This value was higher than that obtained by Berker et al. (2010) for *Camellia sinensis* extract, which is considered a rather powerful antioxidant.

A comparison between the antioxidant activity of the ethanolic extract and of the single fractions (Figs. 1-2) shows that, according to both the DPPH and ABTS assays, F3 and F5 were the most active fractions, with an activity that was not significantly different from that of the crude extract (Fig. 1A). Since the main components of these fractions were Q, THFL, CA, and an unknown flavonol glycoside (Tab. 2), the antioxidant activity of pure compounds Q and CA was assayed, in order to identify those mainly responsible for this property. Besides these, SA was also tested, since it was the main component in the ethanolic extract. Q and CA showed a very high antioxidant ability, which was higher than that of Tr in all tests (Fig. 1B and Table 1).

THFL is a constituent of the leaves of *Olea ferruginea* (Aiton) Steud (Hashmi et al., 2014), and *Blumea balsamifera* (L.) DC. (Nessa et al., 2004), twigs of *Broussonetia papyrifera* (L.) L'Hér. ex Vent. (Zheng et al., 2008), and of aerial parts of *Thymus quinquecostatus* var. *japonicus* H. Hara (Lee et al., 2011). A clear antioxidant capacity for this flavanone derivative has been demonstrated, even though comparatively lower than that exerted by flavones and flavonols (Nessa et al., 2004). This can explain the higher free radical scavenging activity of F3 (that contained both Q and THFL) as compared to F2, which mainly contains the flavanone derivative (Fig. 1A). Thus, it is plausible that the flavonol components, together with derivative flavanones and caffeoylquinic acids, mainly contributed to the antioxidant capacity of the *H. hircinum* crude extract (Fig.1). Conversely, no radical scavenging activity was recorded for SA (data not shown), although it was present in rather high amounts in the ethanolic extract.

3.3. Anti-collagenase activity of crude extract, fractions, and isolated compounds

H. hircinum extract was able to inhibit the *in vitro* collagenase activity, with an IC_{50} value of 156.0 µg/mL (Fig. 3A). In order to understand which components of the crude extract may account for the inhibitory effect, single fractions were investigated for their effect on enzyme activity. A fixed concentration of 60 µg/mL of each fraction was tested, and the percentage of enzymatic inhibition was compared (Fig. 3B). The inhibitory effect decreased in the order: F2>F3>F5>F6>F4. IC_{50} values were calculated for fractions showing a percentage inhibition of at least 30% at the tested concentration (Fig. 3B). All tested fractions were able to inhibit collagenase activity largely than the total extract, and, among them, the most active ones were F2 and F3. These fractions mainly contained THFL, alone and together with Q, respectively. The effectiveness of flavonols as collagenase inhibitors has been well documented (Sin and Kim, 2005). These authors, by comparing various flavonoids for their inhibitory action on collagenase activity, found that quercetin and kaempherol showed a higher inhibitory effect compared to flavones, isoflavones, and flavanones, the latter being almost ineffective. Nevertheless, by comparing different structures, they concluded that the hydroxylation pattern in the B-ring of the flavonoid structure may be an

285 important determinant for the inhibitory action on enzyme activity. Sim et al. (2007) studied the
286 structure-activity relationship of several flavonoids on MMP-1 gene expression in UV-A irradiated
287 human dermal fibroblasts and demonstrated that the inhibitory effect at both protein and mRNA
288 level, became stronger with increasing number of OH groups on the B-ring. Thus, the two hydroxyl
289 groups present in the B ring of THFL may account for the inhibitory activity of those fractions
290 enriched in this flavanone.

291 Fraction F5, containing CA and flavonol glycosides, showed a higher inhibitory action on
292 collagenase than the total extract. This can be attributed to CA more than the glycoside molecules.
293 Indeed, pure CA was tested on the enzyme activity and an IC_{50} value very close to that of the total
294 extract was obtained (fig. 4A). The positive control EGCG yielded an IC_{50} value of 9.45 µM.

295 To investigate the mechanism of enzyme inhibition, a Lineaweaver-Burk plot was built by
296 following the kinetics of collagenase in the absence and in the presence of *H. hircinum* extract. A
297 60% decrease in V_{max} occurred when the extract was added, while the K_m value remained
298 unchanged (Fig. 5), indicating that a non-competitive inhibitory mechanism was involved. This
299 suggests that components of the extract may interact with sites different from the active site of the
300 enzyme, an important aspect to consider when examining the potential of this plant as source of
301 new anticollagenase agents. Indeed, results obtained by means of both computer simulation and
302 experimental design by Westley and Westley (1996) indicated that competitive inhibitors are likely
303 to be an inappropriate basis for design of potential therapeutic agents, due to the difficulty in
304 providing a long-term inhibition, while the uncompetitive mechanism is much more likely to
305 succeed in this.

306 **4. Conclusions**

307 Present results indicate that *Hypericum hircinum* L. extracts show a rather strong antioxidant
308 activity, which is mainly due to its phenolic compounds. Moreover, both the ethanolic extract and
309 fractions containing quercetin, caffeoylquinic acids and 5,7,3',5'-tetrahydroxyflavanone, a

310 compound recently identified in this plant, were able to inhibit MMP1 enzyme activity through a
311 non-competitive mechanism. This inhibitory action on MMP activity can explain, at least in part, its
312 ethnobotanical uses. Based on theseresults, we suggest H. hircinum as a promising source for new
313 anti-collagenase agents. In view of the importance that the maintenanceof collagen structure has in
314 preventing the skin ageing and pho-toageing processes, these molecules can enter as active
315 ingredientsin wrinkle-care cosmetics..

316 **Acknowledgements:** This research was supported by the FARB Basic Research Program (Grants
317 for Basic Research, 2012; project No. FFBO123113) of the University of Bologna to F. P.

318

319 **References**

320 Araniti, F., Sorgonà, A., Lupini, A., Abenavoli, M.R., 2012. Screening of mediterranean wild plant
321 species for allelopathic activity and their use as bio-herbicides. Allelopath. J. 29, 107-124.

322 Atzei, A.D., 2003. Le piante nella tradizione popolare della Sardegna, First ed. Delfino C. Sassari.

323 Ballero, M., Floris, R., Poli, F., 1997. Le piante utilizzate nella medicina popolare nel territorio di
324 Laconi (Sardegna Centrale). Boll. Soc. Sarda. Sci. Nat. 31, 207-229.

325 Barbagallo, C., Chisari, G., 1987. Antimicrobial activity of three *Hypericum* species. Fitoterapia 58,
326 175-180.

327 Belvisi, M.G., Bottomley, K.M., 2003. The role of matrix metalloproteinases (MMPs) in the
328 pathophysiology of chronic obstructive pulmonary disease (COPD): a therapeutic role for
329 inhibitors of MMPs. Inflamm. Res. 52, 95-100.

330 Berker, K.I. Glüçü, K., Demirata, B., Apak, R., 2010. A novel antioxidant assay of ferric reducing
331 capacity measurement using ferrozine as the colour forming complexation reagent. Anal.
332 Methods 2, 1770–1778.

Bochov, D.V., Sysolyatin, S.V., Kalashnikov, A.I., Surmacheva, I. A., 2012. Shikimic acid: review of its analytical, isolation, and purification techniques from plant and microbial sources. J. Chem. Biol. 5, 5–17.

Butterweck, V., 2003. Mechanism of action of 'St John's wort' in depression: what is known? CNS Drugs 17, 539-562.

Cecchini, C., Cresci, A., Coman, M.M., Ricciutelli, M., Sagratini, G., Vittori, S., Lucarini, D., Maggi, F., 2007. Antimicrobial activity of seven *Hypericum* entities from central Italy. Planta Med. 73, 564-566.

Chimenti, F., Cottiglia, F., Bonsignore, L., Casu, L., Casu, M., Floris, C, Secci, D., Bolasco, A., Chimenti, P., Granese, A., Befani, O., Turini, P., Alcaro, S., Ortuso, F., Trombetta, G., Loizzo, A., Guarino, I., 2006. Quercetin as the active principle of *Hypericum hircinum* exerts a selective inhibitory activity against MAO-A: extraction, biological analysis, and computational study. J. Nat. Prod. 69, 945-949.

Di Pompo, G., Poli, F., Mandrone, M., Lorenzi, B., Roncuzzi, L., Baldini, N., Granchi, D., 2014. Comparative in vitro evaluation of the antiresorptive activity residing in four Ayurvedic medicinal plants. Hemidesmus indicus emerges for its potential in the treatment of bone loss diseases. J. Ethnopharmacol. 154, 462-470.

Elliott, S., Cawston, T., 2001. The clinical potential of matrix metalloproteinase inhibitors in the rheumatic disorders. Drugs & Aging 18, 87-99.

Ernst, E., Izzo, A.A., 2003. The clinical pharmacology of *Hypericum perforatum*. In: Ernst, E. (Eds.), Hypericum: The Genus Hypericum. Taylor and Francis, New York, pp. 155–172.

Esposito, F., Sanna, C., Del Vecchio, C., Cannas, V., Venditti, A., Corona, A., Bianco, A., Serrilli, A.M., Guarcini, L., Parolin, C., Ballero, M., Tramontano, E., 2013. *Hypericum hircinum* L.

components as new single molecule inhibitors of both HIV-1 reverse transcriptase-associated DNA polymerase and ribonuclease H activities. Pathogens and Dis. 68, 116-124.

Fu, X., Kassim, S.Y., Parks, W.C., Heinecke, J.W., 2001. Hypochlorous acid oxygenates the cysteine switch domain of pro-matrilysin (MMP-7). J. Biol. Chem. 276, 41279-41287.

Hashmi, M. A., Khan, A., Ayub, K., Farooq, U., 2014. Spectroscopic and density functional theory studies of 5,7,3',5'-tetrahydroxyflavanone from the leaves of *Olea ferruginea*. Spectrochim. Acta Mol. Biomol. Spectros. 128, 225–230.

Hertog, M.G.L., Hollman, P.C.H., Kahn, M.B., 1992. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. J. Agric. Food Chem. 40, 2379-2383.

Lee, I., Bae, J., Kim, T., Kwon, O. J., Kim, T.H., 2011. Polyphenolic constituents from the aerial parts of *Thymus quinquecostatus* var.*japonica* collected on ulleung Island. J. Korean Soc. Appl. Biol. Chem. 54, 811- 816.

Madhan B., Krishnamoorthy G., Rao J.R., Nair B.U., 2007. Role of green tea polyphenols in the inhibition of collagenolytic activity by collagenase. Int. J. Biol. Macromolec. 41, 16-22.

Maggi, F., Cecchini, C., Cresci, A., Coman, M.M., Tirillini, B., Sagratini, G., Papa, F., Vittori, S., 2010a. Chemical composition and antimicrobial activity of *Hypericum hircinum* L. subsp. majus essential oil. Chem. Nat.Compounds 46, 125-129.

Maggi, F., Tirillini, B., Vittori, S., Sagratini, G., Papa, F., 2010b. Chemical Composition and Seasonal Variation of *Hypericum hircinum* L. subsp. majus (Aiton) N. Robson essential oil. J. Essent. Oil Res. 22, 434-443.

Marandino, A., De Martino, L., Mancini, E., Milella, L., De Feo, V., 2011. Chemical composition and possible in vitro antigermination activity of three *Hypericum* essential oils. Nat. Prod. Commun. 6, 1735-1738.

380 Nessa, F., Ismail, Z., Mohamed, N., Haris, M.R.H.M., 2004. Free radical-scavenging activity of
381 organic extracts and of pure flavonoids of *Blumea balsamifera* DC leale. Food Chem. 88, 243–
382 252.

383 Pieroni, A, Quave, C.L, Santoro, R.F., 2004. Folk pharmaceutical knowledge in the territory of the
384 Dolomiti Lucane, inland southern Italy. J. Ethnopharmacol. 95, 373-384.

385 Pilepić, K.H., Males, Z., 2013. Quantitative analysis of polyphenols in eighteen Hypericum taxa.
386 Periodicum Biologorum 3, 459-462.

387 Pistelli, L., Bertoli, U.A., Zucconelli, S., Morelli, I., Panizzi, L., Menichini, F., 2000. Antimicrobial
388 activity of crude extracts and pure compounds of *Hypericum hircinum.* Fitoterapia 71, S138-
389 S140.

390 Raffetto, J.D., Khalil, R.A., 2008. Matrix metalloproteinases and their inhibitors in vascular
391 remodeling and vascular disease. Biochem. Pharm. 75, 346–359.

392 Raghu Chandrashekhar, H., Venkatesh, P., Arumugam, M., Vijayan, P., 2009. Estimation of total
393 phenols with special emphasis to antioxidant potentials of few *Hypericum* species.
394 Pharmacologyonline 1, 680-687.

395 Rahman, I., MacNee, W., 1996. Role of oxidants/antioxidants in smoking-induced lung diseases.
396 Free Radic. Biol. and Med. 21, 669-681.

397 Rodriguez-Landa, J.F., Contreras, C.M., 2003. A review of clinical and experimental observations
398 about antidepressant actions and side effects produced by *Hypericum perforatum* extracts.
399 Phytomed. 10, 688-699.

400 Schultz, G., Mozingo, D., Romanelli, M., Claxton, K., 2005. Wound healing and TIME; new
401 concepts and scientific applications. Wound Repair Regen. 13, S1-S11.

Schütz, K., Kammerer, D., Carle, R., Schieber, A., 2004. Identification and quantification of caffeoylquinic acids and flavonoids from Artichoke (*Cynara scolymus* L.) heads, juice, and pomace by HPLC-DAD-ESI/MS. J. Agric. Food Chem. 52, 4090-4096.

Shah, S., Mohan M., Kasture, S., Ballero, M., Maxia, A., Sanna C., 2013. Protective effect of *Hypericum hircinum* on doxorubicin-induced cardiotoxicity in rats. Nat. Prod. Res. 27, 1502-1507.

Sin, B.Y., Kim H. P., 2005. Inhibition of Collagenase by Naturally-Occurring Flavonoids. Arch. Pharmacal Res. 10, 1152-1155.

Vaalamo, M., Weckroth, M., Puolakkainen, P., Kere, J., Saarinen, P., Lauharanta, J., Saarialho-Kere, U., 1996. Patterns of matrix metalloproteinase and TIMP-1 expression in chronic and normally healing human cutaneous wounds. Br. J. Dermatol. 135, 52-59.

Van Wart, H.E., Steinbrink, D.R., 1981. A continuous spectrophotometric assay for Clostridium histoliticum collagenase. Anal. Biochem. 113, 356-365.

Venditti, A., Mandrone, M., Serrilli, A.M., Bianco, A., Iannello, C., Poli, F., Antognoni, F., 2013. Dihydroasparagusic acid: antioxidant and tyrosinase inhibitory activities and improved synthesis. J. Agric. Food Chem. 61, 6848-6855.

Figure legends

Fig. 1 Antioxidant activity, assayed by the DPPH and ABTS tests, of *H. hircinum* L. ethanolic extract and the six fractions (A), and of pure standard compounds (B). Data are expressed as IC_{50} (µg/mL in A and µM in B). Asterisks indicate significant differences within the same assay at $P<0.01$ (**) and $P<0.001$ (***) compared to the total extract (A) and Tr (B).

Fig. 2 Fe^{3+} reducing potential of *H. hircinum* ethanolic extract, fractions and pure compounds determined by the FRAP-FZ method. Results obtained for the extract and fractions are expressed as TAC units (A), while those for standard compounds are expressed as TEAC (B, insert). Asterisks indicate significant differences at $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) compared to the total extract (A) and Tr (B)..

Fig. 3 Inhibition of *in vitro* collagenase activity by *H. hircinum* L. ethanolic extract and fractions. A: IC_{50} values of total extract and fractions giving a ≥ 30% inhibition of enzyme activity at a fixed concentration of 60 µg/mL. (B): Percentage of inhibition of the six fractions shown in decreasing order. Different letters indicate statistically significant differences at $P<0.05$.

Fig. 4. Inhibition of *in vitro* collagenase activity by *H. hircinum* ethanolic extract (A), CA (B) and EGCG (C). The enzyme assay was performed using 2 mM FALGPA as substrate. The IC_{50} value for crude extract was calculated from the logarithmic curve y=a ln(x)+b, where a=40.44 and b=-121.7 and R^2=0.989. The IC_{50} value for CA was calculated by the linear regression curve y=a x +b, where a=0.5684x; b=27.956 and R^2=0.997. The IC50 value for EGCG was calculated from the logarithmic curve y=a ln(x)+b, where a=20.523, b=2.779 and R^2=0.981.

Fig. 5. Lineaweaver-Burk plot of collagenase and FALGPA without (♦, CTR) and with (■) 156 µg/mL of *H. hircinum* L. ethanolic extract. Kinetic parameters were calculated from the linear regression curves y=ax+c, where a=2.6676; c=6.4377 and R^2=0.999 for ♦, while a=4.9275; c=10.279; R^2=0.996 for ■.

Fig.1

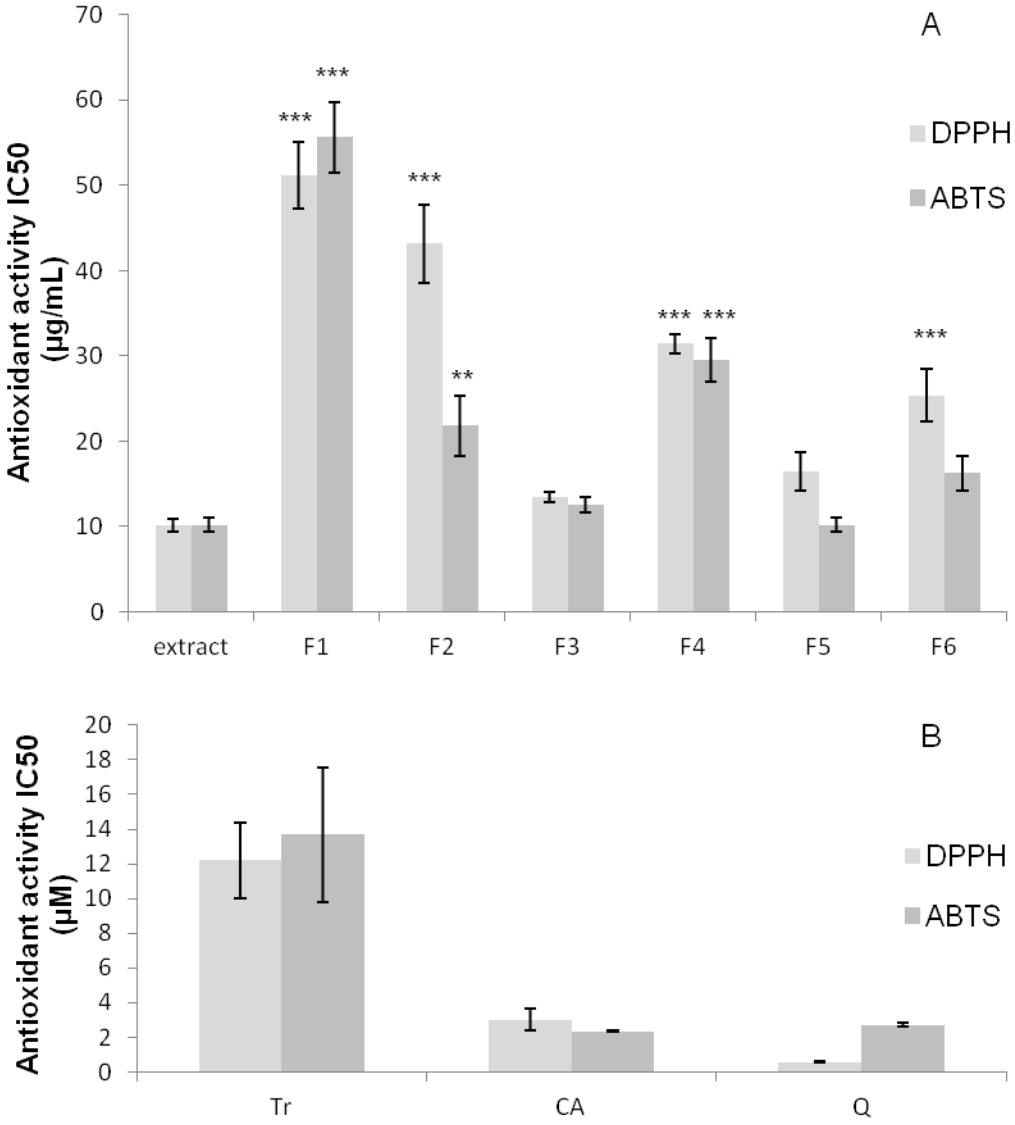
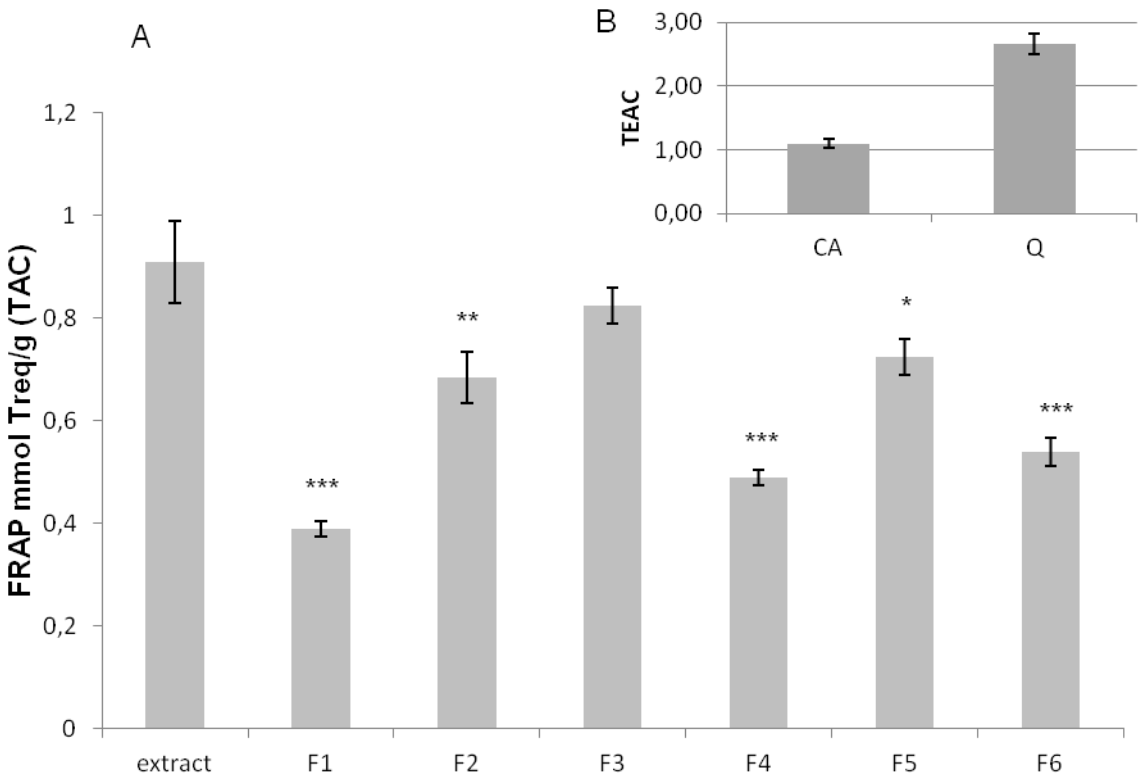
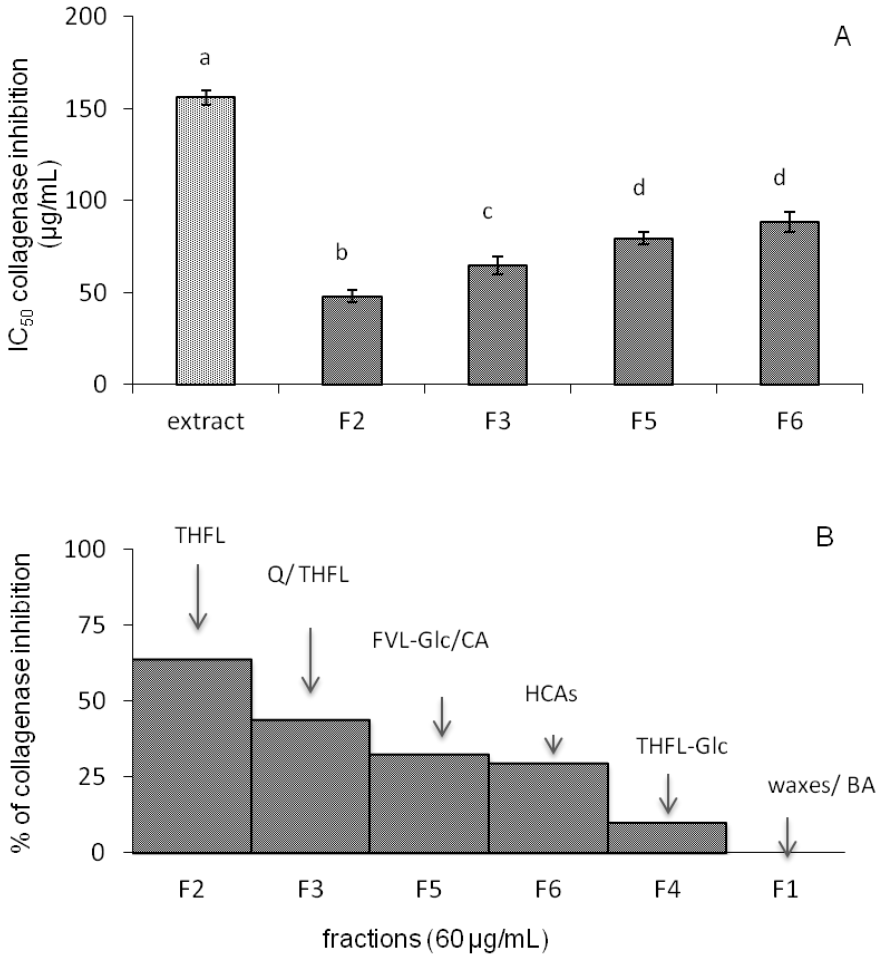

A
Antioxidant activity IC50 (µg/mL)
70
60
50
40
30
20
10
0
***
***
***
**
***
***
***
DPPH
ABTS
extract
F1
F2
F3
F4
F5
F6
B
Antioxidant activity IC50 (µM)
20
18
16
14
12
10
8
6
4
2
0
DPPH
ABTS
Tr
CA
Q

Fig. 2


A
B
FRAP mmol Treq/g (TAC)
TEAC
1,2
1
0,8
0,6
0,4
0,2
0
3,00
2,00
1,00
0,00
CA
Q
extract
F1
F2
F3
F4
F5
F6
***
**
***
*
***

485 **Fig. 3.**
486



487
488
489 **Fig. 4**

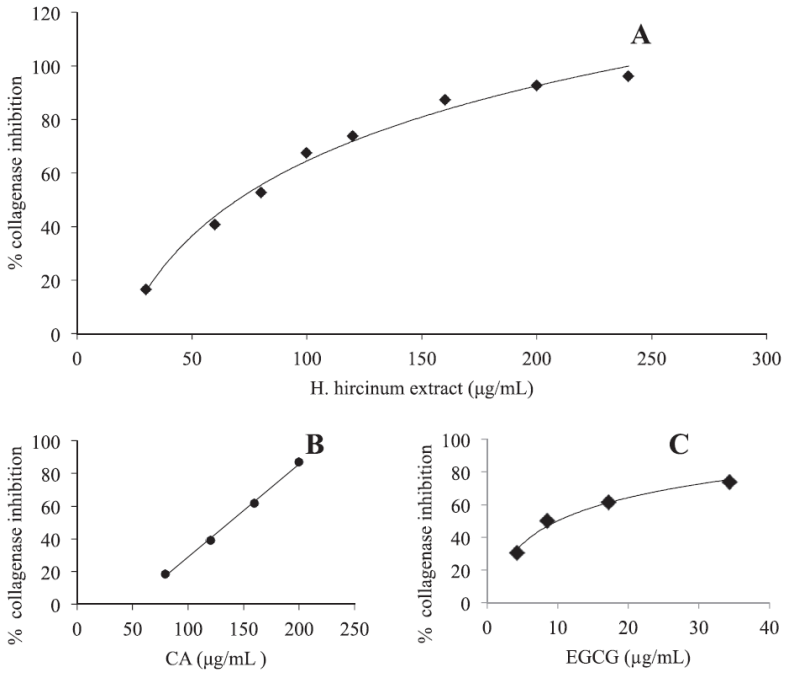


Fig. 5

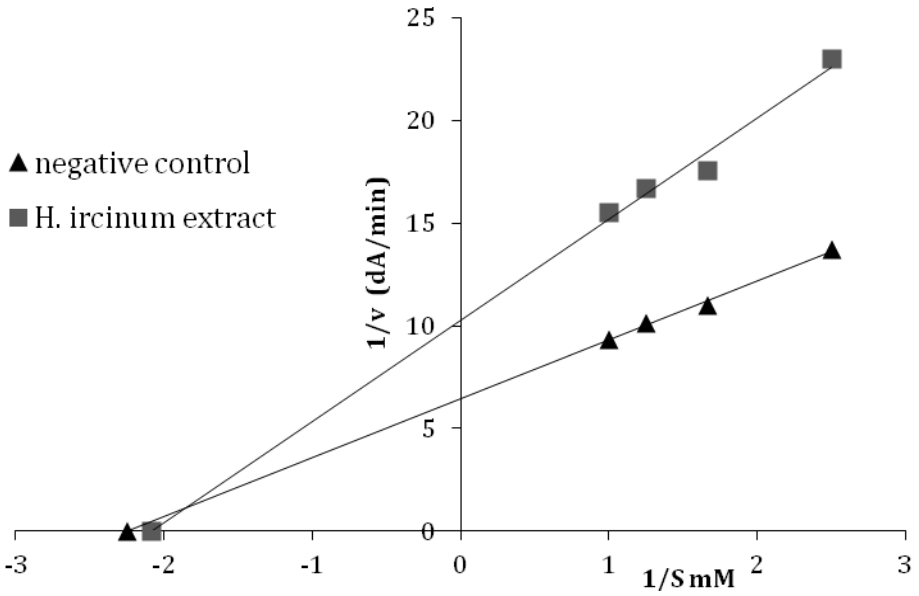


Table 1. Antioxidant activities measured by ABTS, DPPH, and FRAP-FZ. Values are expressed as IC_{50} (µg/mL or µM for pure compounds), TAC (mmol TE/g extract) or TEAC (Trolox equivalent antioxidant capacity). Data are the mean of three independent experiments. Different letters, within the same assay, indicate significant differences ($P < 0.01$) among IC_{50} values.

		DPPH	ABTS	FRAP-FZ
***H. hircinum* extract**	IC_{50} (µg/mL)	10.13±0.73[a]	10.19±0.82[A]	46.39±2.57[a]
	TAC	1.32±0.16	1.99±0.17	0.91±0.08
CA	IC_{50} (µg/mL)	1.06±0.22[b]	0.83±0.02[B]	38.47±1.33[b]
	IC_{50} (µM)	3.01±0.64	2.33±0.07	
	TAC	12.89±3.05	24.52±0.76	
	TEAC	4.57±1.07	6.92±1.48	1.10±0.08
Q	IC_{50} (µg/mL)	0.20±0.01[c]	0.92±0.03[C]	15.88±0.32[c]
	IC_{50} (µM)	0.58±0.02	2.72±0.09	
	TAC	68.09±7.19	21.95±0.77	
	TEAC	23.02±2.40	7.43±0.26	2.66±0.16
SA	IC_{50} (µg/mL)	> 100[d]	> 100[D]	423±12. [d]
	IC_{50} (µM)	> 50	> 50	
	TAC	< 1	< 1	
	TEAC	<5	<5	0.10 ± 0.01
Tr	IC_{50} (µg/mL)	3.35±0.34[e]	5.07±0.03[E]	*42.33±2.5[a]
	IC_{50} (µM)	13.38±1.34	20.28±0.12	