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

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

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'*s 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 were dominated by sesquiterpene hydrocarbons, and the non-volatile

extract lacked hypericin, which is considered 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* hydroalcolic 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. using $CDCl_3$, CD_3OD or D_2O as deuterated solvents; the chemical shift was expressed in ppm from TMS, (the signal of HDO at 4.78 ppm is used as reference for spectra in D_2O). MS spectra were performed on a Q-TOF MICRO Spectrometer (Micromass, now Waters, Manchester, UK) equipped with an ESI source, operating in the negative and/or positive ion mode. The flow rate of sample infusion was 10 μL/min, with 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, filtered, and 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; Tab. 1).

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 $FeCl_3$). Fractions showing a very high similarity on TLC analysis were pooled together and re-fractionated in order to separate the single or the main components. Six fractions (named F1-F6) were obtained, in which mixtures of various substances were detected, 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 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 Spherisorb C18 column (5 µm, ODS2, 4.6 x 250 mm, Waters Corp., Milford, MA, USA). A calibration curve with the appropriate analytical standard at different concentrations (from 0.5 to 500 ppm) was constructed for the quantitative analysis. Peak identity and purity were confirmed by means of the PDA detector.

CA was quantified according to Schütz et al (2004). The mobile phase consisted of eluent A (2.0% acetic acid in water) and eluent B (0.5% acetic acid in water/acetonitrile, 50:50). The gradient program was set as follows: 10-18% B (20 min), 18-24% B (10 min), 24-30% B (15 min), 30% B isocratic (20 min), 30-55% B (5 min), 55-100% B (5 min), 100% B isocratic (8 min), 100-10% B (2 min). Total run time was 90 min at a flow rate of 0.4 mL/min and UV detection at 320 nm. Spectra were recorded from 200 to 600 nm and CA was detected at 320 nm.

SA was quantified according to Hertog et al. (1992), with slight modifications. An isocratic elution was performed using 25 % acetonitrile in 0.025 M KH_2PO (pH 2.4) as mobile phase. Total run time was 20 min at a flow rate of 0.8 mL/min and UV detection at 210 nm.

Q was quantified according to Hertog et al. (1992), with slight modifications. An isocratic elution was performed using 45% methanol in 0.025 M KH_2PO (pH 2.4), as mobile phase. Total run time was 30 min at a flow rate of 1.0 mL/min and UV detection at 370 nm.

2.5. Antioxidant assays

DPPH, ABTS and FRAP-Ferrozine (FZ) tests were determined according to Venditti et al. (2013). In ABTS and DPPH assays, the ethanolic extract or purified fractions were tested in a concentration range of 5-20 µg/mL, and Trolox (Tr) or pure standard compounds (CA, Q, SA) in a

concentration range of 5-50 µM. In the FRAP-FZ assay, extract or purified fractions were tested in a concentration range of 10-100 µg/mL and Tr or pure standard compounds from 10 to 100 µM. The antioxidant capacity was expressed as IC_{50} and TAC values for extract and fractions, and as IC_{50}, TAC and TEAC for pure compounds.

2.6. Collagenase activity assay

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

Total polyphenols (PF) and flavonoids detected in the ethanolic extract of the aerial parts of Sardinian *H. hircinum* samples are reported in Table 1. Total PF content was 20% on a dry weight basis, and flavonoids represent the main polyphenolic component. Total PF 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 polyphenol content 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 was carried out, in order to quantify CA, SA and Q (Table 1). 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. 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.

The phytochemical composition of the six fractions purified from the ethanolic extract, determined by NMR and MS spectra, is reported in Table 2. Following the elution order, the fractions are characterized by an increasing polarity of their phytoconstituents. The most lipophilic fraction (F1) contained waxes and BA, intermediate fractions contained flavonoid aglycons (F2-F3) and the last and most hydrophilic ones contained flavonoid glycosides, HCAs, SA and sugars (F4-F6). 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 data reported by other authors (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, as shown in Figs. 1-2. 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. (Figgs 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 resulted lower than that reported for this species (28.5 μg/ml) (Raghu et al., 2009). The FRAP-FZ test, modified with respect to the original FRAP assay as concerns the pH value (Venditti et al., 2013), was also performed in order to evaluate the reducing potential of the sample towards the redox couple $Fe^{3+/}Fe^{2+}$ (Berker et al., 2010). This test confirmed the strength of *H. hircinum* extract as antioxidant, since a TAC value of 0.91 was found (Tab. 3). 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 is shown in Figs. 1-2. F3 and F5 were the most active fractions according to both DPPH and ABTS assays, showing 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 (Tab. 1). Q and CA showed a very high antioxidant ability, which was higher than that of Tr in all tests (Fig. 1B and Table 3).

THFL is a constituent of the leaves of *Olea ferruginea* (Aiton) Steud (= *Olea europaea* L.) (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 (=Thymus japonicus (H. Hara) Kitag.) (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 our results, in particular the higher free radical scavenging activity of fraction F3 (which contained both Q and THFL) as compared to F2 (Fig. 1A), which mainly contains the flavanone derivative. 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, and 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, to a greater extent compared to 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 strong effectiveness of flavonols as collagenase inhibitors has been well documented (Sin and Kim, 2005). These authors, 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 important determinant for the inhibitory action on enzyme activity. In a subsequent work, Sim et al. (2007), studying the structure-activity relationship of several flavonoids on MMP-1 gene expression in UV-A irradiated human dermal fibroblasts, demonstrated that the inhibitory effect at both protein and mRNA level became stronger with increasing number of OH groups on the B-ring. Thus, the two hydroxyl groups present in the B ring of THFL may account for the inhibitory activity of those fractions enriched in this flavanone.

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

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

4. Conclusions

Results obtained in this work allow to conclude that *Hypericum hircinum* L. extracts show a rather strong antioxidant activity, due to its phenolic compounds. Moreover, the ethanolic extract and fractions enriched in flavonols, caffeoylquinic acids and 5,7,3',5'-tetrahydroxyflavanone, a compound recently identified in this plant, were able to inhibit MMP1 enzyme activity, through a non-competitive mechanism. This inhibitory action on MMP activity can, at least in part, explain its ethnobotanical uses. Nowadays, it is well established that the ECM not only represents the scaffold necessary for a correct tissue architecture and integrity, but that it plays a key role in a more general tissue homeostasis. Indeed, an over-production and/or inadequate endogenous inhibition of proteolytic enzymes, leading to a dysomeostasis in ECM turnover, has been demonstrated to play an important role in the pathogenesis of various multifactorial diseases and in the modulation of inflammatory processes. In this context, the discovery of new MMP inhibitors of natural origin is of great importance, taking into account the relevant role that collagenase also has in tissue engineering, due to its low antigenicity and unique biocompatibility. Moreover, it is noteworthy that the non-competitive mechanism by which *H. hircinum* extracts inhibit the enzyme makes this plant a promising source for new anti-collagenase agents. In view of the importance that the maintenance of collagen structure has in preventing the ageing and photoageing skin process, these molecules can enter as active ingredients in wrinkle-care cosmetics.

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Figure legends

Fig. 1 Antioxidant activity, assayed by the DPPH and ABTS tests, of *Hypericum 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 $P<0.001$ (***).

Fig. 2 Fe^{3+} reducing potential of *Hypericum 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 $P<0.001$ (***).

Fig. 3 Inhibition of *in vitro* collagenase activity by *Hypericum 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. Percentage of inhibition of *in vitro* collagenase activity by increasing concentrations of *Hypericum 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 ■.