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*Full Length Research Paper*

# Biological activities of extract of *Anthemis aetnensis* Schouw: *In vitro* evaluation

**Rosaria Acquaviva[1], Claudia Di Giacomo[1], Michele Malaguarnera[1]*, Salvatore Ragusa[2], Valeria Sorrenti[1], Rosa Santangelo[1], Silvana Mastrojeni[3], Ignazio Barbagallo[1] and Liliana Iauk[3]**

[1]Department of Drug Science, Biochemistry Section, University of Catania, V.le A. Doria, 6, 95125, Catania, Italy.
[2]Department of Pharmacobiological Sciences, University of Catanzaro, Catanzaro, Italy.
[3]Department of Microbiological and Gynaecological Sciences, University of Catania, Via Androne 81, 95124 Catania, Italy.

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**Although *Anthemis* spp. are commonly used as anti-inflammatory, antibacterial agents, the exact mechanism of action is still unknown. We evaluated the antioxidant effect of *Anthemis aetnensis* by testing the free radical scavenging capacity and its effect on xanthine oxidase (XO) activity. In addition, the effect of the same extract on human leukocyte elastase activity was also evaluated. Flowers of *A. aetnensis* Schouw were collected on the slopes of the volcano Mount Etna. Antioxidant activity was assessed using a method that excludes the Fenton-type reaction. Since xanthine (X)/XO system represents a source of free radicals, we also tested the hypothesis that the study's extract might inhibit the enzymatic activity of XO. In addition, the same extract of *A. aetnensis* was used to test its activity on human leukocyte elastase. Extract of *A. aetnensis* showed antioxidant activity, which was not due to inhibition of XO activity. In addition, extract of *A. aetnensis* showed a significant elastase inhibitory activity. Our data suggest that antioxidant capacity and inhibitor activity on elastase may explain the protective effects on inflammation, ulcers, irradiated skin injuries and cystitis afflictions**.

**Key words:** *Anthemis aetnensis,* reactive oxygen species, antioxidant activity, human leukocyte elastase, xanthine oxidase.

## INTRODUCTION

Medicinal components from plants play an important role in conventional western medicine. People all over the continents have long applied poultices and imbibed infusions of hundreds, if not thousands, of indigenous plants, dating back to prehistory (Barbour et al., 2004). Experience from ethnomedicine, together with extensive basic laboratory findings, have shown that natural compounds could play an important role in prevention and treatment of inflammation, cancer and other diseases. The genus *Anthemis* L. (Asteraceae) comprises about 130 species predominantly distributed around the Mediterranean, but species are also found in southwest Asia and South Africa, several of which are aromatic, herbal medicines, insecticides and dyes (Konstantinopoulou et al., 2003). The species of *Anthemis* are known to possess various biological activities and are commonly used in folk medicine. These plants prefer dry, open sites on wood-steppe hillsides and grow especially on calcareous substrates in nature. The flowers of the genus have well documented use as antiseptic and healing herbs, the main components being natural flavonoids and essential oils (Uzel et al., 2004). Baser et al. (2006), have reported that in *Anthemis aciphylla* the oxygenated-monoterpenes are the predominant group of constituents in the essential oil of aerial parts and flowers, while oxygenated monoterpenes are the predominant group of constituents in the leaves.

*Corresponding author. E-mail: michele.malaguarnera@gmail.com.

**Abbreviations: DPPH,** 1,1-Diphenyl-2-picrylhydrazyl radical; **DMSO,** dimethylsulfoxide; **HLE,** human leukocyte elastase; **MeOsucc-AAPV-pNA,** methoxysuccinyl-Ala-Ala-Pro-Val-pNitroanilide; **NF-kB,** nuclear factor-kappaB; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase; **X,** xanthine; **XO,** xanthine oxidase; **NADH,** β-Nicotinamide-adenine dinucleotide.

*Anthemis aetnensis* is a Sicilian endemic plant growing in volcanic debris on the upper scopes of Mount Etna. Investigation of the aerial parts has now led to the isolation of hydruntinolides, a mixture of the two germacronolides, 1β hydroxyarbusculin and other sesquiterpene such as the isofraxidin-derived one (Bruno et al., 1997). Its extracts, tintures, tisanes and salves are widely used in Europe as anti-inflammatory, antibacterial, antispasmodic and sedative agents. Extracts are used to ally pain and irritation, clean wounds and ulcers and in prevention or therapy of irradiated skin injuries, treatment of cystitis and dental afflictions (Mann et al., 1986). Several studies have evidenced that oxidative stress is the causative agent in a number of human diseases, such as atherosclerosis, ischemic/reperfusion damage, carcinogenesis, ageing, inflammation and damage in the epidermis (Gassen and Youdim, 1997; Good et al., 1996; Maxwell, 1995).

Although there are many determinants in the development of these diseases, considerable experimental evidence links the production of reactive oxygen species (ROS) to biological damage, potentially providing a mechanistic basis for the initiation and/or progression of these pathologies (Bowen and Mobarhan, 1995; Halliwell and Chirico, 1993; Loft and Poulsen, 1996; Stahl and Sies, 1997). ROS are capable of chemically altering virtually all major classes of biomolecules (for example, lipids, proteins and nucleic acids) with concomitant changes in structures and functions. Humans, along with other aerobic organisms, have evolved a variety of mechanisms to protect themselves from the potentially deleterious effects of ROS. Thus, it is generally thought that oxidative pathology arises when the generation of ROS and ROS products exceeds the cell capacity to protect or repair itself.

There is evidence that exogenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress and there is increasing interest in the protective biochemical functions of natural compounds contained in plants, medicinal herbs and fruits, which are candidates for the prevention of oxidative damage caused by oxygen-free radical species (Osawa et al., 1995; Tsuda et al., 1997). The antioxidant activity of phenolic phytochemicals has been widely investigated in recent years (Rice-Evans et al., 1996; Russo et al., 2000, 2006) and many potential benefits are recently attributed to polyphenols in the regulation of cellular processes such as inflammation. Polyphenols, in fact, may act as inflammation modulatory agents by various mechanisms, such as down-regulation of Nuclear Factor-KappaB (NF-kB), inhibition of the release of inflammatory mediators and related enzymes or increasing cell ability to scavenge ROS (Acquaviva and Iauk, 2010). Human leukocyte elastase (HLE) is a serine protease produced by polymorphonuclear leukocytes following inflammatory or infective stimuli (Santagati et al., 2004); it degrades the structural components of connective tissue especially if ineffectively regulated by endogenous inhibitors, such as α1-antitrypsin, leading to several pathological diseases (Santagati et al., 2004).

Hyperexpression of HLE is associated with certain pulmonary, inflammatory, vascular and neoplastic pathologies (Houghton et al., 2006; Santagati et al., 2004; Wada et al., 2006). Therefore compounds capable of inhibiting HLE may have considerable therapeutic potential (Hlasta and Pagani, 1994; Siedile et al., 2002). Although species of *Anthemis* spp. are commonly used as anti-inflammatory and antibacterial agents, the exact mechanism of action is still unclear. In the present study the antioxidant ability of *A. aetnensis* was evaluated by measuring its free radical scavenging capacity and its effect on XO activity. In addition, since some natural compounds from medicinal plants of the Asteraceae family are able to inhibit the catalytic activity of elastase (Siedile et al., 2002, 2003), the present study also evaluated the inhibitory activity of extract of *A. aetnensis* on HLE.

## MATERIALS AND METHODS

### Chemicals

β-Nicotinamide-adenine dinucleotide (NADH), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), X and XO were purchased from Sigma-Aldrich (Italy). All other chemicals were from Merck (Frankfurter, Germany). Methoxysuccinyl-Ala-Ala-Pro-Val-pNitroanilide (MeOsucc-AAPV-pNA) and HLE were purchased from Calbiochem (UK).

### Plant materials

Flowers of *A. aetnensis* Schouw were collected on the slopes of the volcano Mount Etna (1800 to 1900 m) in November; leaves were collected in April of the same year. *A. aetnensis* specimens were obtained, thanks to the Regional Forest Corps Detachment of Catania-Nicolosi and authenticated by botanist Prof. S. Ragusa, Department of Pharmacobiological Sciences, University of Catanzaro, Catanzaro, Italy. A voucher specimen of the plant was deposited in the herbarium of the Pharmacobiological Department of the University of Messina (Italy). The fresh material was air-dried and powdered.

### Preparation of extracts

Exhaustive ethanolic extraction of flowers of *A. aetnensis* was carried out at room temperature by maceration of 25,000 g of drug with 250 ml of ethanol 70% (drug/solvent ratio 1:10 W/V) on a rotating shaker for 24 h. The extract was then filtered and air-dried under vacum. Residue obtained from the extraction was 2,500 g. Aliquots of residue were dissolved in DMSO and diluted to give the final sample concentrations used in the experimental protocols.

### Superoxide anion scavenger activity

Superoxide anion was generated "*in vitro*" as reported by Acquaviva et al. (2002). The assay mixture contained in a total volume of 1 ml: 100 mM triethanolamine-diethanolamine buffer, pH7.4, 3 mM NADH, 25 mM/12.5 mM ethylene diamine tetraacetic

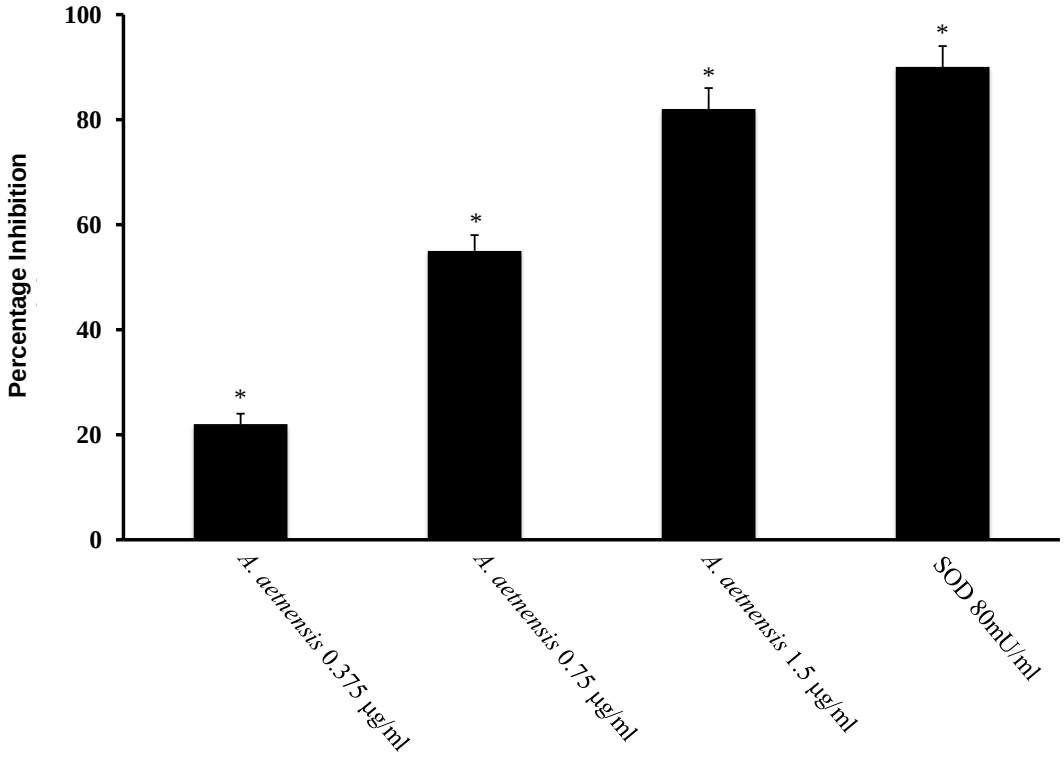


**Figure 1**. Scavenger effect of extract of *A. aetnensis* and SOD actvity on $O_2$ expressed as percentage of inhibition of NADH oxidation. Rate of $O_2$: production was 4 nmoles/min. Each value represents the mean ± SD of 5 experiments. Significance *vs.* control $p<0.005$.

acid (EDTA)/$MnCl_2$, 10 mM β-mercaptoethanol; some samples contained extract from *A. aetnensis* at different concentrations (0.375, 0.750 or 1.5 µg/ml). After 20 min incubation at 25°C, the decrease in absorbance was measured at λ = 340 nm. Superoxide dismutase (SOD) (80 mU/ml) was used as standard.

### Quenching of DPPH

The free radical-scavenging capacity of extract of *A. aetnensis* was tested by its ability to bleach the stable DPPH. The reaction mixture contained 86 µM DPPH and different concentrations of extract (37.5, 75, 150 or 300 µg/ml) in 1 ml of ethanol. After 10 min at room temperature the absorbance at λ = 517 nm was recorded. Trolox (30 µM), water-soluble derivative of vitamin E, was used as standard.

### XO (X: oxygen oxidoreductase, E.C.1.1.3.22) activity inhibition

XO activity was evaluated spectrophotometrically by following the formation of uric acid at λ = 292 nm ($\varepsilon_M = 9.2 \times 10^3$) (Russo et al., 2000). The assay mixture contained, in a final volume of 1 ml, 50 mM phosphate buffer pH 7.8, 25 µM X and 24 mU XO (specific activity 1 U/mg of protein). Different concentrations of extract of *A. aetnensis* (150, 300, 600, or 900 µg/ml) were added to samples before the enzyme and their effect on the generation of uric acid was used to calculate regression lines. The results were expressed as percentage of inhibition enzyme activity.

### HLE activity

The assay for elastase activity was carried out according to Nakajima et al. (1979) using MeOsucc-AAPV-pNA as specific chromogenic substrate. Activity was measured using a Hitachi U2000 spectrophotometer and following the increase in absorbance at λ = 410 nm, due to the release of p-nitroaniline by hydrolysis of the substrate. Measurements were made at 20°C in 1 ml of assay mixture containing: 200 µM MeOsucc-AAPV-pNA in DMSO, 5 µl of DMSO (or the same volume of extract of *A. aetnensis* to a final concentration of 0.1875, 0.375 or 75 µg/ml), 5 mU HLE in 50 mM sodium phosphate buffer containing 500 mM NaCl, pH= 7.8. Time course of product formation was tested for linearity and only the initial, linear parts were considered to calculate the rates. Ki value was determined according to Dixon, measuring HLE activity in the presence of various concentrations of extract (Dixon, 1953).

### Statistical analysis

One-way analysis of variance (ANOVA) followed by Bonferroni's *t* test was performed in order to estimate significant differences among dosages. Data were reported as mean values ± SD and differences were considered to be significant at $p<0.05$.

## RESULTS

The method used to investigate the superoxide anion scavenging capacity of extract of *A. aetnensis*, excludes

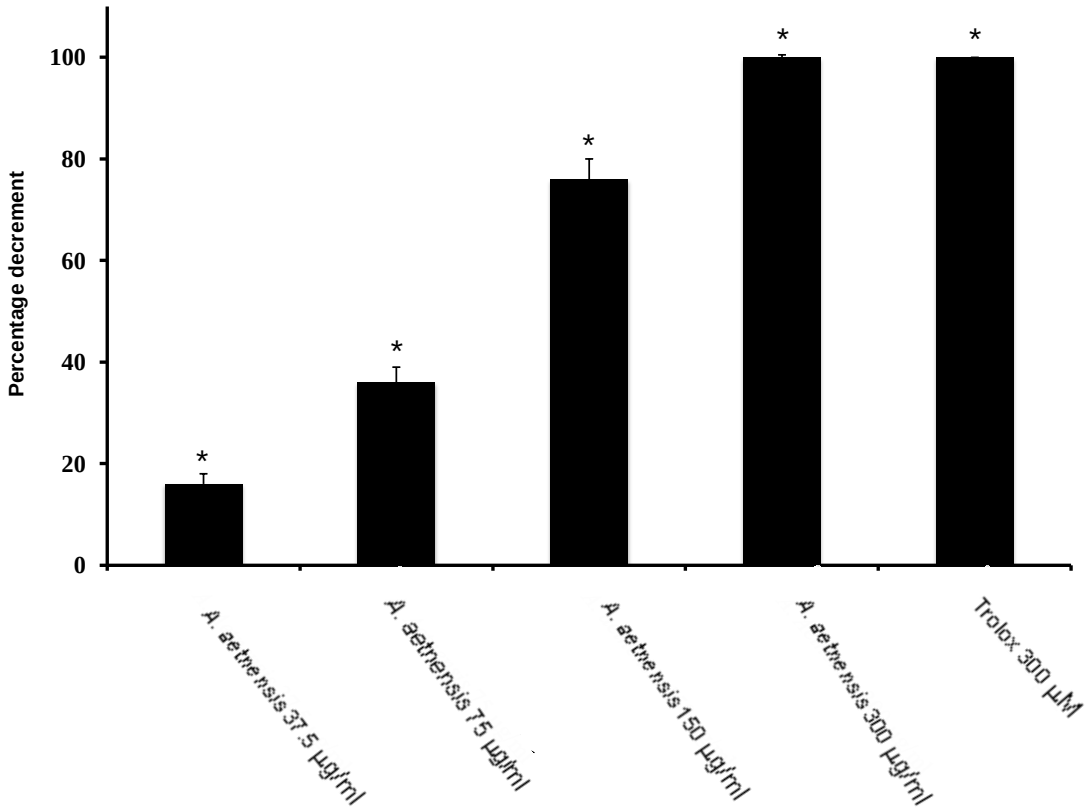


**Figure 2.** Scavenger effect of extract of *A. aetnensis* and trolox expressed as capacity to bleach the stable DPPH. Results are expressed as percentage decrease in absorbance at λ = 517 nm with respect to control. Each value represents the mean ± SD of 5 experiments. Significance *vs*. control p<0.005.

**Table 1.** Effect of extract of *A. aetnensis* on XO activity.

| *Anthemis aetnesis* (µg/ml) | Inhibition (%) |
|---|---|
| 150 | 5±0.32 |
| 300 | 5±0.5 |
| 600 | 6.3±0.9 |
| 900 | 7±0.87 |

Results are expressed as percentage of inhibition of XO activity with respect to control. Each value represents the mean ± SD of 5 experiments.

the Fenton-type reaction and the X and XO system. Extract of *A. aetnensis* inhibited superoxide anion formation in a dose-dependent manner (Figure 1); the inhibitory effect of 1.5 µg/ml was equivalent to 80 mU/ml SOD (Figure 1). In addition, the free radical scavenging activity of extract of *A. aetnensis* was also tested for its ability to bleach the stable DPPH radical (Bonina et al., 1998). This assay provides information about the reactivity of test compounds with a stable free radical. Because of its odd electron, DPPH gives a strong absorption band at λ = 517 nm in visible spectroscopy (deep violet colour). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. In this assay, 300 µg/ml of extract of *A. aetnensis* were equivalent to 30 µM of Trolox (Figure 2). Results regarding XO activity demonstrated that the antiradical effect of the extract of *A. aetnensis* is not mediated by an inhibition of this enzyme. In fact none of the tested concentrations (150, 300, 600 or 900 µg/ml) inhibited the XO activity (Table 1). As shown in Figure 3, extract of *A. aetnensis* significantly inhibited HLE activity, in a dose-dependent manner. The Dixon plot allowed us to calculate *Ki* value (0.6 µg/ml) (Figure 4).

## DISCUSSION

The vast structural diversity of natural compounds found in planta, provide unique opportunities for discovering new drugs that rationally target the abnormal molecular and biochemical signals leading to atherosclerosis, ischemic reperfusion injury, carcinogenesis, ageing, inflammation and tissue damage. In fact, compounds

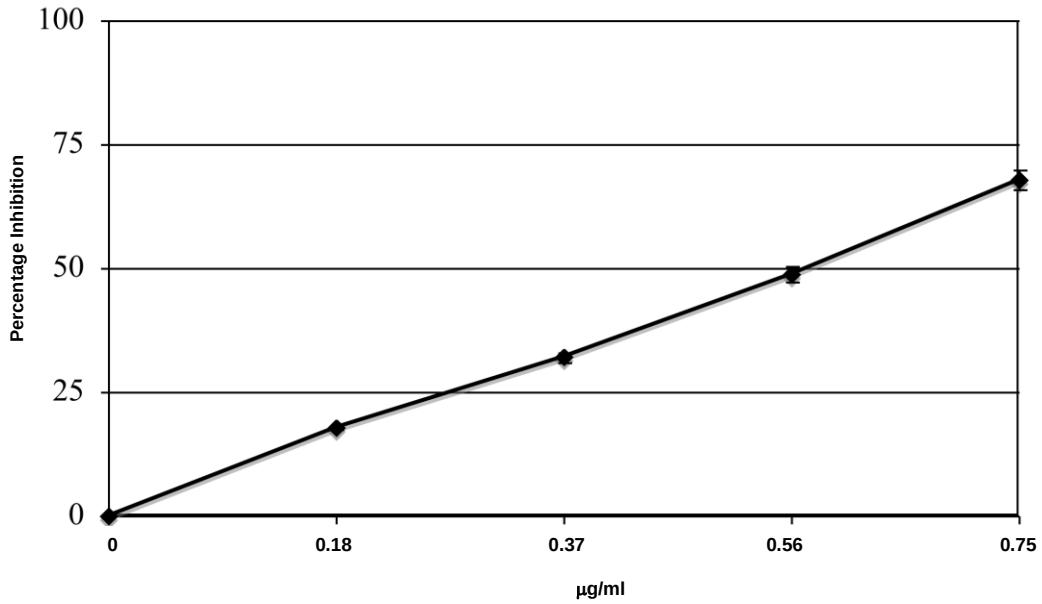


**Figure 3.** Effect of extract of *A. aetnensis* on HLE activity. Each value represents the mean ± SD of 5 experiments

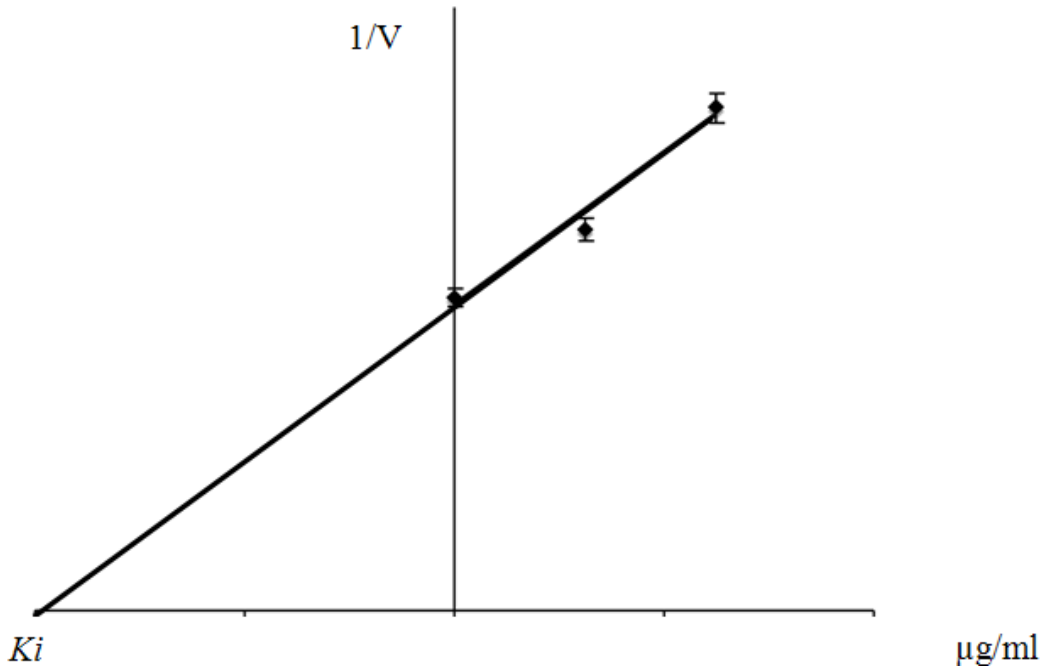


**Figure 4.** Dixon plot for the inhibition of HLE activity by extract of A. *aetnensis*. Each value represents the mean ± SD of 5 experiments.

biosynthesised in plants have been used for more than 2000 years in traditional medicine as anti-inflammatory and anti-carcinogenic agents. The species of the *Anthemis* genus are widely used in the pharmaceutics, cosmetics and food industry. Since the main components of the species of the *Anthemis* genus are flavonoids and essential oils, the antioxidant activities of the extract can be related to the presence of these compounds (Uzel et al., 2004). The antioxidant activity of phenolic phytochemicals was investigated and much attention is

dedicated to the biological functions of flavonoids (Hertog et al., 1993; Nardini et al., 1997; Russo et al., 2000). Studies showed that phenolic compounds may inhibit lipid peroxidation preventing formation of atherosclerotic plaques (Acquaviva and Iauk, 2010; Hertog et al., 1993). In addition, it has been reported that polyphenols inhibit the activities of several enzymes, including lipoxygenase, cyclooxygenase, monooxygenase, XO, mitochondrial succinic oxidase and NADH-oxidase, phospholipase-A2 and protein kinases (Cao et al., 1997). These biological effects are believed to come from the antioxidant properties of the related polyphenols, including their protection against iron-induced free radical reactions (Cao et al., 1997). Active oxygen species such as superoxide and hydrogen peroxide have both been implicated in the inflammation, damage in the epidermis, ageing process and in cancer. In particular, it is now recognized that the extremely reactive hydroxyl radical ($^{\cdot}OH$) derived from $O_2^{\cdot}$ and $H_2O_2$ causes deoxyribonucleic acid (DNA) strand scission in cellular damage (Halliwell and Gutteridge, 1990). The importance of removing active oxygen species from living organisms is becoming clear, while there is growing interest in the protective mechanisms whereby antioxidants that scavenge active oxygen species may have a potential therapeutic use (Rice-Evans et al., 1996). Because unrepaired or misrepaired endogenous oxidative DNA damage can lead to cancer induction, removal of these modified bases from nuclear DNA plays an important role in the prevention of mutagenesis and carcinogenesis (Cheng et al., 1992).

Since, the antioxidant activities of flavonoids could be mainly due to their free radical scavenging and/or to chelating activities (Nardini et al., 1997; Korkina and Afanas'ev, 1997; Rice-Evans et al., 1996), in this study the superoxide scavenging capacity of this natural product was investigated using a method which excludes the Fenton-type reaction (Russo et al., 2000). Extract of *A. aetnensis* inhibited superoxide anion generation in a dose-dependent manner and at 1.5 μg/ml concentration the inhibitor effect was similar to the action of 80 mU/ml SOD (Figure 1). In addition, extract of *A. aetnensis* showed a DPPH quenching capacity in a dose dependent manner and at 300 μg/ml concentration the action of this extract was equivalent to 30 μM of Trolox (Figure 2). Moreover, extract of *A. aetnensis* was also not able to inhibit XO activity, a physiological source of superoxide anion in eukaryotic cells and these effects have also been reported for other flavonoids (Gassen and Youdim, 1997). So, the inhibitory action of *A. aetnensis* on superoxide anion seems to be merely due to its scavenger activity. *In vitro* studies further supported potential of polyphenols in modulating various steps of the inflammatory process and several cellular action mechanisms have been proposed (Acquaviva and Iauk, 2010; Rice-Evans and Diplock, 1993). Polyphenolic compounds could regulate cellular activities of the inflammation-related cells such as neutrophils, which possess an anti-infectious as well as a pro-inflammatory function. This ability is due to a number of specific activities, including adherence to blood vessel walls, transmigration into tissues, chemotaxis, release of pro-inflammatory mediators, exocytosis of granules with proteolytic enzymes, for example, elastase and collagenase, phagocytosis. HLE, the major proteinase within the azurophilic granules of human neutrophils, can also modulate the function of other inflammatory cells; then accumulation of this enzyme can cause abnormal degradation of healthy tissue resulting in the development of chronic inflammatory diseases. In agreement with other authors, the present study demonstrated that extract of *A. aetnensis* exhibited HLE inhibitory activity (Figure 3) (Xu et al., 2009).

Neutrophils are known to play important roles in a host's defenses against invasion by microorganisms and in the pathogenesis of various diseases such as rheumatoid arthritis or ischemia-reperfusion injury (Hwang et al., 2006). Dysregulated neutrophil recruitment and activation results in severe damage of adjacent normal tissues, which can maintain inflammation processes. It is, therefore, of great interest to identify compounds with membrane stabilizing effects which stop the release of tissue destroying enzymes from granulocytes such as elastase (Nakajima et al., 1979). This beneficial property can be applied to flavonoids, terpenes, natural compounds being the constituents of many plants, such as *A. aetnensis,* used in traditional medicine. To this regard, equally relevant is the inhibitory effect of *A. aetnensis* extract we demonstrated on HLE activity which might contribute to anti-inflammatory action of this extract.

In conclusion, our study provides evidence that the extracts of *Anthemis* posses significative antioxidant properties and elastase inhibitory activities and further supports the use of this plant extract as adjuvant in the prevention or treatment of inflammation, ulcers, irradiated skin injuries, cystitis afflictions, which are characterized by an overproduction of free radicals, particularly the superoxide anion (Mann and Staba, 1986).

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