

Anti-Inflammatory Effect of *Allium ursinum*

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Abstract

The aim of the present study was to evaluate *Allium ursinum* leaves and flowers extract anti-inflammatory effect. Plant extract 1:1 (w:v) was prepared from *A. ursinum* leaves by a modified Squibb repercolation method. The *in vivo* anti-inflammatory effects were evaluated on a rat turpentine oil-induced inflammation (i.m. 6 mL/kg BW). The animals were randomly assigned to nine groups (n=8): negative control, inflammation, *A. ursinum* flower extract (AUF), *A. ursinum* leaves extract (AUL), indomethacin (INDO) (20 mg/kg BW), aminoguanidine (AG) (50 mg/kg b.w./d i.p.) as a selective NOS2 inhibitor, NG-nitro-L-arginine methyl ester (NAME) (5 mg/kg b.w./d i.p.) as a nonselective NOS inhibitor, L-arginine (ARG) (100 mg/kg b.w./d i.p.), NO synthesis substrate, and Trolox (20 mg/kg b.w./d i.p) as an antioxidant. At 24h from inflammation induction total oxidantive status (TOS), oxidative stress index (OSI), nitric oxide (NOx) and *in vitro* phagocytosis test were reduced and the total antioxidative reactivity (TAR) was increased by the testes plant extracts. AUF had a better inhibitory effect than AUL. In conclusion, we provided evidence for the hypothesis that *A. ursinum* leaves and flowers extract exerts anti-inflammatory activity by inhibiting the phagocytosis through the reduction of the nitro-oxidative stress.

Keywords: *Allium ursinum*, antioxidant, inflammation, phagocytosis, nitric oxide, oxidative stress

Introduction

Inflammation is a nonspecific defence response that protects organisms against physical, chemical and infective insults. Dysregulation of this response leads to damage of normal tissues. One of the early cellular events in inflammation is the infiltration of polymorphonuclear leukocytes (PMN) (Hrabák *et al.*, 2008). Overwhelming activation of these phagocytic cells can elicit tissue damage through oxidative stress (Brown *et al.*, 2006). Oxidative stress occurs when reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed in amounts that exceed the capacity of the antioxidant defence system to remove them. The detrimental effects of ROS typically are assessed by the presence of oxidatively altered biomolecules like proteins, lipids, sugars and DNA (Staruchova *et al.*, 2008).

Allium species were found to have antioxidant activity (Nencini *et al.*, 2001). These plants have been used for many centuries for their flavouring value, for their medicinal properties, and in some parts of the world, their use also has religious connotations (Fenwick *et al.*, 1985). There were described more than 300 *Allium*

species (Brullo *et al.*, 2003, 2009; Ledezma and Apitz-Castro, 2006). The Romanian ethnobotanical data record 32 among wild and cultivated species of *Allium* L. (Ciocârlan, 2009). One of these is *Allium ursinum* L. ("ramson", "wild garlic"), a wild-growing species found in Europe and Northern Asia forests (Schmitt *et al.*, 2005). Several biological activities of *A. ursinum* plants, such as antioxidative (Stajner *et al.*, 2008), cytostatic (Sobolewska *et al.*, 2006, 2013), antimicrobial (Ivanova *et al.*, 2009; Sobolewska *et al.*, 2006, 2013), and antifungal (Bagiu *et al.*, 2012) were reported. According to epidemiologic evidence, low cancer risks are associated to a high intake of alliaceae (Putnoky *et al.*, 2013).

The antioxidant activity of *Allium* species was found to be due to a variety of sulphur-containing compounds and their precursors, but it is also related to other bioactive compounds such as polyphenols, dietary fiber and microelements (Nencini *et al.*, 2001; Gîtin *et al.*, 2012). Sulfur-containing compounds of ramson are responsible for its traditional use in terms of culinary and medicinal purposes (Schmitt *et al.*, 2005).

In *A. ursinum* plant extracts, alliin, isoalliin (Schmitt *et al.*, 2005), methiin (Fritsch and Keusgen, 2006),

flavonoid glycosides (Wu *et al.*, 2009), saponins (Sobolewska *et al.*, 2006), polyphenolic compounds (Pârnu *et al.*, 2010a), volatile oil (Godevac *et al.*, 2008) and other secondary metabolites (Ivanova *et al.*, 2009) were determined.

Therefore, the aim of the present study was to evaluate if the antioxidant action of *A. ursinum* flower and leaf extracts induce an anti-inflammatory effect. For this, an experimental acute inflammation model was used, and phagocytosis and nitro-oxidative stress were assessed.

Materials and methods

Plant material

A. ursinum aerial parts have been collected shortly after the blooming period, from the Botanical Garden of Babes-Bolyai University, Cluj-Napoca (Romania). The plant was identified at the Department of Botany, by Dr. Marcel Pârnu and a voucher specimen (CL 659750) was deposited at the Babes-Bolyai University Herbarium.

Extraction

Fragments of 0.5-1 cm of fresh flowers and respectively fresh leaves of *A. ursinum* were harvested at the same time from plants grown in the same place, and samples were extracted with 50% ethanol (Merck, Bucuresti, Romania) in the Mycology Laboratory of Babes-Bolyai University, Cluj-Napoca, Romania, by modified Squibb's repercolation method. Briefly, three successive applications of the same menstruum were repercolated to the plant material. In each percolator plant material (150 g in the first, 90 g in the second, 60 g in the third percolator) was moistened with the menstruum, macerated for two days and then percolated at a rate of about 4-6 drops per minute for each 100 g of crude material. From each percolator the first percolated fractions were reserved and the next fractions were poured upon the next percolator. Then reserved fractions (60 ml from the first, 90 ml from the second and 150 ml from the third) were mixed and the final extract of flower and leaf respectively were 1:1 (w:v) respectively (Pârnu *et al.*, 2010a).

Experimental design

The experiments were performed on adult male Wistar-Bratislava albino rats, weighing 200-250g, bred in the Animal Facility of Iuliu Hatieganu University of Medicine and Pharmacy. The animals were randomly assigned to nine groups (n=8): groups I, negative control (CONTROL), injected i.m. with 0.9% saline solution; in groups II-IX inflammation was induced by i.m. turpentine oil administration (6 mL/kg BW) (Razani-Boroujerdi *et al.* 2008). After that animals were administered i.p. as follows: groups I and II (INFLAM) received 1ml of 0.9% saline solution; to groups III was given a single injection (5 ml/kg BW) of *A. ursinum* flower extract (AUF); to groups IV was given a single injection (5 ml/kg BW) of *A. ursinum* leaves extract

(AUL); group V was injected with indomethacin (INDO) (20 mg/kg BW) (Adham *et al.*, 2012); group VI was injected with aminoguanidine (AG) (50 mg/kg b.w./d i.p.), a selective NOS2 inhibitor; group VII was injected with NG-nitro-L-arginine methyl ester (NAME) (5 mg/kg b.w./d i.p.), a nonselective NOS inhibitor; group VIII was injected with L-arginine (ARG) (100 mg/kg b.w./d i.p.), NO synthesis substrate; group IX was injected with Trolox (20 mg/kg b.w./d i.p.), an antioxidant. At 24 h from inflammation induction the rats were anesthetized by a combination of ketamine (50mg/kg BW) and xylazine (20 mg/kg BW) (Kieling *et al.*, 2012), and blood was withdrawn by retro-orbital puncture. For the phagocytosis test blood was harvested on EDTA 0.1% while for the nitro-oxidative stress tests it was collected without anticoagulant. Coagulated blood was centrifuged and the separated serum was stored in -80 °C until use. Serum was analyzed for the measurement of the total nitrites and nitrates (NOx), total oxidative status (TOS), total antioxidant response (TAR) and oxidative stress index (OSI) calculation.

Animals were housed under controlled conditions (12h light/dark cycle, at an average temperature of 21-22 °C and humidity of 50-55%), and had free access to standard pellets as basal diet and water *ad libitum*. Experiments were performed in triplicate. Rats were euthanized by cervical dislocation at completion the study. The study protocol was approved by the Institutional Animal Ethical Committee of the Iuliu Hatieganu University of Medicine and Pharmacy Cluj-Napoca.

Serum total oxidant status determination

TOS of serum was measured using a colorimetric measurement method (Erel, 2005). In this method oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide (H₂O₂) and the results are expressed in µmol H₂O₂ Equiv./L.

Serum total antioxidant response determination

The total antioxidant status was measured in serum using a colorimetric method for the TAR (Erel, 2004). In this method the hydroxyl radical is produced by the Fenton reaction, and reacts with the colorless substrate o-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in color. Upon addition of a serum sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction medium are suppressed by the antioxidant components of the serum, preventing the color change and thereby providing an effective measure of the total antioxidant capacity of the serum.

The assay is calibrated with Trolox and results are expressed as mmol Trolox Equiv./L.

Calculation of oxidative stress index

The percent ratio of the total oxidative status to the total antioxidant response gave the oxidative stress index (OSI), an indicator of the degree of oxidative stress (Harma *et al.* 2003). To perform the calculation, the result unit of TAR, mmol Trolox equivalent/L, was changed to μmol Trolox equivalent/L and OSI was calculated with the formula: OSI (Arbitrary Unit) = TOS (μmol H₂O₂ Equiv. /L) / TAC (mmol Trolox Equiv./L).

Serum nitric oxide evaluation

The general procedure was described in our previous papers (Moldovan *et al.*, 2011). The Griess reaction was used as an indirect assay to determine the total serum nitrite (NO₂⁻) and nitrate (NO₃⁻) as a measure of the degree of NO production (NO_x). In the presence of H₂O, NO is rapidly converted into nitrite and nitrate. Total production of NO may therefore be determined by measuring the stable NO metabolite nitrite (NO₂⁻). Serum samples were passed through 10-KD filters (Sartorius AG, Goettingen, Germany) and deproteinized by methanol/diethyl ether (3/1, v/v) (sample: methanol/diethyl ether, 1:9, v/v). In brief, 100 μL of VCl₃ (8 mg/mL) was added to 100 μL of the supernatant for the reduction of nitrate to nitrite, followed by the addition of the Griess reagents, 50 μL of SULF (2%) and 50 μL of NEDD (0.1%). After 30 min of incubation at 37 °C, the absorbance was read at 540 nm. The amount of nitrite was calculated from a NaNO₂ standard curve. Serum NO_x was expressed as nitrite $\mu\text{mol/L}$ (Miranda *et al.*, 2001).

In vitro phagocytosis test

Investigation of phagocytic activity was performed as previously described with slight modifications (Moldovan *et al.*, 2011). Blood samples harvested on EDTA solution were incubated with *E.coli* suspension (4x10⁶ germs/ml, in saline solution 0,9%, in a ratio of 0.2 ml blood/20 μL *E. coli* suspension) in siliconated tube at 37°C for 30 min. Afterwards, May-Grünwald-Giemsa stained smears were prepared and the count was done by optic microscopy (Olympus microscope). The phagocytic capacity was evaluated by two parameters: the phagocytic activity (PA = the number of the *E. coli* germs phagocyted by 100 leukocytes) and the phagocytic index (PI% = percentage of leukocytes that phagocyted at least one germ).

Statistical analysis

All results were expressed as the mean \pm S.E.M. whenever data were normally distributed; otherwise, the median and [Q1; Q3] were reported (Q1=first quartile; Q3=third quartile). Statistical comparisons

between two independent groups were performed using the Student's *t*-test (with equal and unequal variances according to the results of the *F*-test) whenever data were normally distributed, whereas the Mann-Whitney test was used for parameters with non-normal distribution. Pearson's and Spearman's correlation analyses were used to calculate statistical relationships between parameters. A p-value <0.05 was considered as statistically significant. Analyses were performed using SPSS 16.0 for Windows (SPSS Inc, USA).

Results

Serum total oxidant status determination

In order to evaluate the mechanism of the anti-inflammatory effect of *A. ursinum* nitro-oxidative stress was analyzed by serum TOS. Turpentine-induced inflammation significantly increased TOS (p<0.001) and treatment with INDO caused an important reduction (p<0.001). TOS was also lowered by all the *A. ursinum* flower and leaves extract (p<0.001). The inhibitory effects were stronger for the flower plant extract. *A. ursinum* was also a better TOS inhibitors than indomethacin, AG and NAME (p<0.001), and almost as efficient as trolox (p>0.05) (Fig. 1).

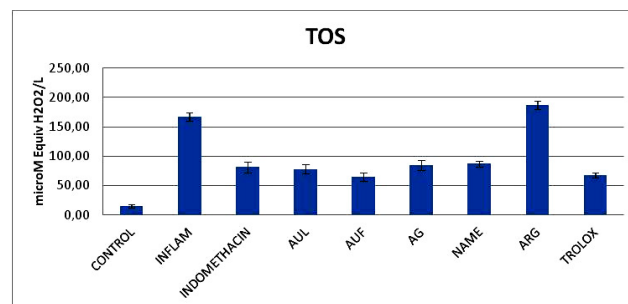


Fig. 1. Serum total oxidant status

Serum total antioxidant response determination

TAR was reduced by the experimental inflammation (p<0.001), and indomethacin significantly increased it (p<0.001). TAR decrease was prevented by *A. ursinum* treatment (p<0.001), and both flower and leaves extracts were equally effective. Compared to indomethacin, AUL had a similar antioxidant effect (p>0.05), and AUF had a better one (p<0.001).

In *A. ursinum* treated animals TAR increase was negatively correlated with NO_x and TOS decrease. *A. ursinum* plant extract was a better antioxidant than AG (p<0.01) and NAME (p<0.01).

Compared to trolox AUL had a lower antioxidant effect, and AUF had a comparable action (p>0.05). ARG lowered TAR (p<0.05) (Fig. 2).

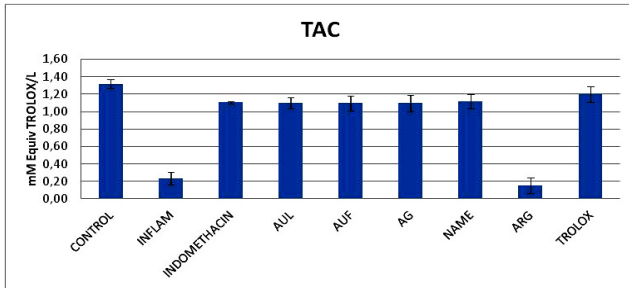


Fig. 2. Serum total antioxidant response

Calculation of oxidative stress index

In the inflammation group the OSI level was significantly elevated ($p < 0.001$), and indomethacin treatment decreased OSI ($p < 0.001$). OSI of INFLAM animals was correlated with TOS and NOx. The *A. ursinum* plant extracts induced an important decline of OSI ($p < 0.001$). Compared to indomethacin treatment, only AUF had a better inhibitory effect ($p < 0.001$). AG and NAME lowered OSI less than AUL and AUF. ARG treatment increased OSI ($p < 0.01$). Trolox administration reduced OSI ($p < 0.001$), and the effect was stronger than AUL ($p < 0.01$) and similar to that of AUF ($p > 0.05$) (Fig. 3).

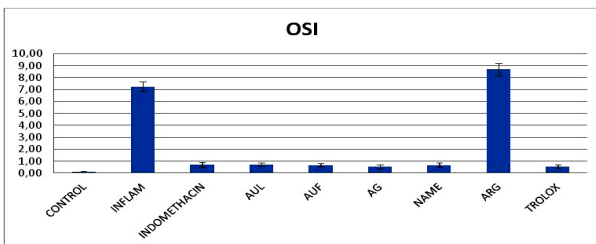


Fig. 3. Oxidative stress index

Serum nitric oxide evaluation

Compared to the control, NOx was significantly elevated in the INFLAM group ($p < 0.001$). Indomethacin lowered NOx synthesis importantly ($p < 0.001$). The treatment with *A. ursinum* plant extract caused an important reduction of NOx synthesis ($p < 0.001$) too. Comparing *A. ursinum* effect on NOx to that of indomethacin, we found that just AUL had a lower efficiency ($p < 0.001$), but AUF ($p < 0.001$) had better inhibitory activity on NOx synthesis. AG and NAME inhibitory action on NOx were stronger than the effects of tested *A. ursinum* extracts ($p < 0.01$). Compared to trolox, AUL was a weaker inhibitor ($p < 0.01$), and AUF had similar effect ($p > 0.05$) (Fig. 4).

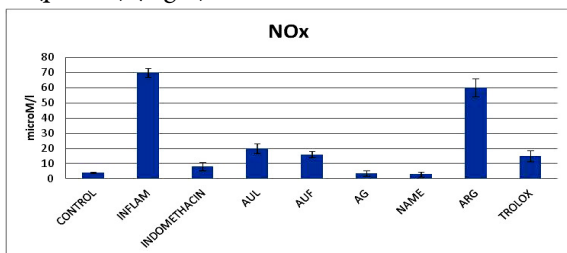


Fig. 4. Serum nitric oxide

In vitro phagocytosis test

In turpentine-induced rat inflammation there was a significant increase of both PI and PA ($p < 0.001$). Indometacin had a strong anti-inflammatory effect ($p < 0.001$) by decreasing PI and PA. In INFLAM and INDO groups PI and PA were correlated ($r = 0.91$; $r = 0.88$). *A. ursinum* showed an important inhibitory effect on phagocytosis test parameters ($p < 0.01$). AUL and AUF PI and PA were significantly smaller than in INDO group ($p < 0.01$), but correlated. In animals treated with *A. ursinum* extracts PI and PA were higher than in AG and NAME groups ($p < 0.001$), and smaller than in ARG animals ($p < 0.001$). Trolox treatment did not influenced significantly PI ($p > 0.05$), but reduced PA ($p < 0.01$). Compared to trolox, *A. ursinum* extracts had a better inhibitory effect on PI ($p < 0.01$), but on PA only AUF was better ($p < 0.05$). NOx decrease by *A. ursinum* was correlated with PA and PI (Fig. 5; Fig. 6).

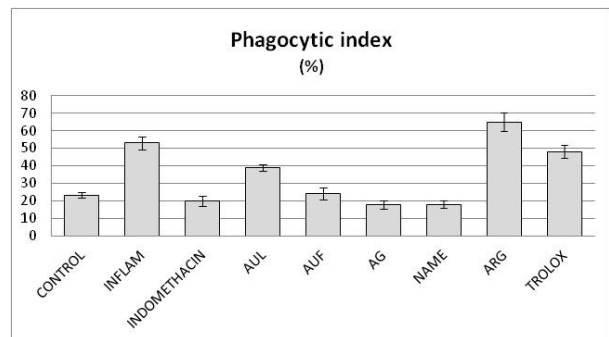


Fig. 5. Phagocytic index (%)

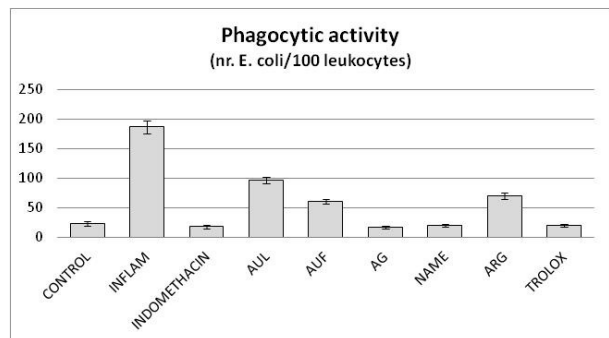


Fig. 6. Phagocytics activity (*E. coli* germs/ 100 leukocytes)

Discussion

The tested *A. ursinum* leaf and flower extracts had anti-inflammatory activity in the rat turpentine oil induced-inflammation model. The mechanism of this effect was the inhibition of the phagocytosis through the reduction of the nitro-oxidative stress.

The use of traditional medicine is widespread and many plants are important sources of natural antioxidants that might be used for the development of novel drugs. The results of many studies showed that all *Allium* species extracts, from all plant organs, exhibited antioxidant activity (Stajner et al., 2008; Putnoký et al., 2013).

Methods for quantification of oxidative damage markers are often called fingerprinting methods, as a range of specific end-products deriving from the interaction of the ROS with biomolecules, such as lipids, proteins, DNA and low-molecular-weight antioxidant, are measured. The presence of these end products serves as proof of the prior existence of ROS. TOS reflects the additive oxidant effect of different molecules (Sapunjieva *et al.*, 2012). The reduction of TOS after *A. ursinum* treatment indicated that the decrease of those molecules was part of the oxidative stress lowering process.

There are currently no golden standard methods for measuring antioxidant capacity in biology. Assays of total antioxidant capacity like TAR have been developed instead of individual antioxidants determination, because *in vivo* antioxidant systems work together and not in isolation, there are interactions between hydrophilic and lipophilic antioxidants, and undiscovered antioxidant species risk being ignored (Erel, 2004). By increasing TAR *A. ursinum* plant extracts proved to be efficient antioxidants. The present results were in accordance with experiments that studied the antioxidative properties of *A. ursinum* extracts (Stajner *et al.*, 2008; Putnoky *et al.*, 2013).

It was considered that combinations of different natural antioxidants present in medicinal plants work better than separate antioxidants alone (Sapunjieva *et al.*, 2012). The amount of natural antioxidants varies with cultivar, soil composition, climate, geographic origin, and cultivation practices or exposure to diseases, such as fungal infections (Liu *et al.*, 2013; Schmitt *et al.*, 2005; Putnoky *et al.*, 2013). Many studies have indicated that polyphenols in herbs possess anti-inflammatory activities, and the main mechanisms were antioxidant, metal chelation and enzyme modulation abilities, as well as their effects on cell signaling pathways and on gene expression (Soobrattee *et al.*, 2005). Phenolic compounds have been reported to have other biological effects too: antimutagenic, antibacterial, antiviral and antithrombotic (Bravo, 1988; Duthie *et al.*, 2000; Pratt, 1992).

The quality and quantity of the biologically active compounds from *Allium* species significantly depend on the species, the plant organ and the harvest time (Schmitt *et al.*, 2005). In *A. ursinum* leaves and bulbs, the highest amount of volatile precursors was found in March and April, shortly before flowering time (Schmitt *et al.*, 2005). We harvested *A. ursinum* plants in those specific months and p-Coumaric acid and ferulic acid were identified in the leaves and flowers. The pattern of flavonoids indicates large differences between the two samples of *A. ursinum*: the flowers contain a larger amount of kaempferol derivatives (1839.33 mg per 100 g), whereas the leaves contain a larger amount of p-coumaric acid (109.11 mg per 100 g) and ferulic acid (40.16 mg per 100 g) (Parvu *et al.*, 2010). Kaempferol derivatives were previously identified in the aerial parts of *A. ursinum* (Carotenuto *et al.*, 1996).

The previous phytochemical analysis of the tested *A. ursinum* extracts by LC/MS, has shown that the flower extract contains a higher amount of allicin (1.946 mg

allicin/ml) than the leaf extract (0.028 mg allicin/ml) (Parvu *et al.*, 2011). Allicin has antibacterial (Cai *et al.*, 2007), antiviral, antitumor, anticoagulant, antihypertensive, antiparasitic and hepatoprotective effects (Joslin, 2003). It was observed that antibacterial and antifungal activities of allicin can be attributed to its interaction with the thiol group of proteins and amino acids and that, especially with the latter, allicin forms S-allyl derivatives (Ogita *et al.*, 2007). Due to these findings the stronger antioxidant activity of the *A. ursinum* flower extract, compared to the leaf extract, may be attributed to the higher content of allicin too.

The oxidative/antioxidative balance was also evaluated by calculating OSI (Harma *et al.*, 2003). The parallel TOS decrease and TAR increase after *A. ursinum* treatments caused a better OSI reduction than indomethacin. For the inflammation point of view these antioxidant effects of *A. ursinum* were additive to the inhibition of 5-lipoxygenase, cyclooxygenase (Sendl *et al.*, 1992).

The L-arginine-NO pathway is an important mediator of the inflammatory response. Over the last couple of decades, the dual role of NO as a deleterious as well as a beneficial agent has been widely explored. Besides the essential role as signal molecule, NO reacts with oxygen as well as with superoxide to generate RNS, which inevitably subsequently react with the biological targets. This can then lead as far as cell death. On the other hand, protective effects were noted for NO, and proposed to involve antioxidant mechanisms. The balance between nitrosative and oxidative chemistry would then depend on the relative concentration of NO. Thus, at low NO fluxes these reactions would tend to lead to oxidation of substrates, whereas at higher levels of NO nitrosation reactions would predominate (Miranda *et al.*, 2003).

In turpentine-induced rat inflammation there was a high NO release. *A. ursinum* extracts reduced NOx. Furthermore, the *A. ursinum* leaves extract had comparable effect with the indomethacin, and the *A. ursinum* flower extract was better. These results have suggested that inhibition of NO synthesis is an important mechanism of the *A. ursinum* extracts effect, because pharmacological inhibition of NOS was reported to reduce the development of inflammatory response (Tracey *et al.*, 1995).

Numerous reports show that nitrosating intermediates have the greatest affinity for thiols such as glutathione (GSH) suggesting that they are a primary target under nitrosative stress conditions. Cells depleted of GSH were dramatically more susceptible to toxicity from nitrosative stress. Furthermore, in several studies the formation of S-nitrosothiol adducts with proteins has been suggested as important step in the inhibition of a variety of enzymes, including antioxidant enzymes (Thomas *et al.*, 2008). Based on these mechanisms it was concluded that decreasing the NOx the antioxidant effect will be also improved by increasing available GSH. Due to these, NOx reduction by *A. ursinum* may be considered a mechanism of the antioxidant effect.

Phagocytosis is part of the cellular acute phase response associated to inflammation. After recognizing the targets, phagocytic cells are activated to ingesting and destroying them with reactive oxidants and hydrolytic

enzymes. After proofing that *A. ursinum* has important inhibitory effect on the nitro-oxidative stress, we have investigated the consequences on the phagocytosis. As we were expecting, both *A. ursinum* extracts reduced the PI and PA significantly, and the effect was smaller than that of indomethacin.

In the inflammatory response there is also growing evidence for involvement of other blood cell populations, such as platelets (Russwurm *et al.*, 2002). It was already found that *A. ursinum* exert antiaggregatory effects by inhibition of the ADP pathway (Hiyasat *et al.* 2009; Sabha *et al.*, 2012). This mechanism may be also involved in *A. ursinum* extracts anti-inflammatory effect, but it was not evaluated in the present study.

In summary, this study provided evidence for the hypothesis that the *A. ursinum* leaf and flower extracts exert anti-inflammatory activity in the rat turpentine oil induced-inflammation model. The suggested mechanism of this action was the inhibition of the phagocytosis through the reduction of the nitro-oxidative stress. Consistent with these observations, future work will explore in inflammatory diseases adjunctive host-modulatory therapy with *A. ursinum* extracts.

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