Aqueous Extract of *Arbutus unedo* Inhibits STAT1 Activation in Human Breast Cancer Cell Line MDA-MB-231 and Human Fibroblasts Through SHP2 Activation

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Abstract: *Arbutus unedo L.* has been for a long time employed in traditional and popular medicine as an astringent, diuretic, urinary anti-septic, and more recently, in the therapy of hypertension and diabetes. Signal transducer and activator of transcription 1 (STAT1) is a fascinating and complex protein with multiple yet contrasting transcriptional functions. Although activation of this nuclear factor is finely regulated in order to control the entire inflammatory process, its hyperactivation or time-spatially erroneous activation may lead to exacerbation of inflammation. The modulation of this nuclear factor, therefore, has recently been considered as a new strategy in the treatment of inflammatory diseases. In this study, we present data showing that the aqueous extract of *Arbutus unedo*'s leaves exerts inhibitory action on interferon-γ (IFN-γ)–elicited activation of STAT1, both in human breast cancer cell line MDA-MB-231 and in human fibroblasts. This down-regulation of STAT1 is shown to result from a reduced tyrosine phosphorylation of STAT1 protein. Evidence is also presented indicating that the inhibitory effect of this extract may be mediated through enhancement of tyrosine phosphorylation of SHP2 tyrosine phosphatase. The modulation of this nuclear factor turns out into the regulation of the expression of a number of genes involved in the inflammatory response such as inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1). Taken together, our results suggest that the employment of the *Arbutus unedo* aqueous extract is promising, at least, as an auxiliary anti-inflammatory treatment of diseases in which STAT1 plays a critical role.

Key Words: Arbutus unedo, inflammation, JAK/STAT pathways, STAT1, SHP2.

INTRODUCTION

One of the hallmarks of the inflammatory response is the massive synthesis of nitric oxide (NO) catalysed by inducible NO synthase (iNOS). The expression of iNOS mRNA is closely monitored by a number of pro-inflammatory cytokines such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), together with gram-negative bacterial membrane-derived lipopolysaccharides (LPS) [1]. These compounds exert their action by activating specific nuclear factors, such as signal transducer and activator of transcription 1 (STAT1) and the nuclear factor κB (NF-κB) [2]. While the former principally mediates the action of IFN-γ, the latter plays a critical role in the transduction signal of TNF-α, IL-1β and LPS.

STAT1 plays a pivotal role in regulating the expression not only of iNOS, but also of many other genes involved in the inflammatory response [3]. Following recognition of IFN-γ to its specific receptors, the receptors dimerize and activate by trans-phosphorylation the receptor-associated Janus tyrosine kinase (JAK) 1 and 2. Successive phosphorylation of IFN-γ-receptors' cytosolic region provides the docking sites for STAT1 monomer, which in turn becomes phosphorylated in tyrosine residues [4]. Phosphorylated STAT1 forms dimers through interaction with conserved SH2 domains. Successively, dimers translocate into the nucleus where they bind directly to the promoter region of specific target genes, thus regulating transcription of inflammation-associated genes. The activation of STAT1 pathways is strictly regulated at several points along the cascade. A number of negative regulators of JAK/STAT signals has been described, including suppressor of cytokine signaling (SOCS), protein inhibitors of activated STAT (PIAS), and the receptor-associated protein phosphatase SHP1 and SHP2 [5]. These latter catalyse the tyrosine dephosphorylation of JAKs, receptors or any other cellular protein downstream signal pathway. In particular, SHP2 is constitutively associated with IFN-γ receptors and associates with STAT1 after IFN-γ treatment.

As formerly described, STAT1 activation is normally well regulated; however, any deregulation of its activation may cause a deleterious effect on tissue integrity, turning out in inflammatory diseases.

Since an up-regulation of STAT1 plays a critical role in the pathogenesis of numerous diseases related to inflammation [6-14], the therapeutic trial triggering STAT1 as a target for a new drug generation has recently been postulated [3, 15].

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The finding of plant extracts like green tea extract (GTE), St. John's Wort extract, or pure compound(s) present therein with an anti-STAT1 activity led to the recent exploration of their potential therapeutic use in inflammatory diseases [16-18]. In the rat model of heart ischemia/reperfusion (I/R)-injury, the oral administration of GTE as well as EGCG infusion limited the extent of the infarct size and attenuated the magnitude of myocyte apoptosis in the heart, this protective effect being correlated with concomitant STAT1 inhibition [19]. A protective effect of GTE was further observed in an animal model of rat brain I/R-induced damage [20] and in a chronic inflammatory model of bowel disease (Crohn's disease) [21, 22].

With the aim of finding out other plant extract(s) with potent and specific anti-STAT1 activity, we focused our attention on Mediterranean plant species used in traditional medicine. In the present study, we chose *Arbutus unedo* L., widely employed as an astringent, diuretic and urinary antiseptic, as well as in the therapy of hypertension and diabetes [23]. We examined the effect of its aqueous extract on IFN-γ-elicited activation of STAT1 and TNF-α–induced NF-κB activation, using the human breast cancer cell line MDA-MB-231 and human fibroblasts. We found that in both cell models, the aqueous extract of *Arbutus unedo* exerts a strong inhibitory action on IFN-γ-elicited STAT1 activation, with less effect on the activation of NF-κB, and attenuates the expression of some inflammatory genes such as iNOS and intercellular adhesion molecule-1 (ICAM-1). Furthermore, we show data indicating that the action of *Arbutus unedo* aqueous extract may be exerted by the control of JAK2 tyrosine-phosphorylation and SHP2 activation.

RESULTS

Analysis of *Arbutus Unedo* Extract

Analysis of the polyphenols content by the Folin-Ciocalteau procedure showed that *Arbutus unedo* aqueous extract is composed of 43% (w/w) polyphenols. The remaining fraction of the aqueous extract is presumably composed of small molecules (sugars, acids, salts) soluble both in methanol and aqueous solution. Chromatography on Discovery HSPEG column (5 μm particle size, length × I.D. 25 cm × 10 mm, Supelco) with a polyethyleneglycol phase, confirmed that polyphenols in the extract were mainly hydrophilic molecules eluting in the first 18 minutes of the chromatogram (data not shown). Analysis by RP-HPLC and comparison with authentic marker compounds allowed identification of gallic acid, catechin and quercitrin (i.e. quercetin-3-rhamnoside) as the main polyphenols in the extract, with a great number of other not identified UV-adsorbing molecules (data not shown).

Aqueous Extract of *Arbutus unedo* Exerts a Strong Inhibitory Action on STAT1 DNA Binding Activity in MDA-MB-231 Cells

To examine whether *Arbutus unedo* aqueous extract exerts an inhibitory action on the DNA binding activity of the inflammatory transcription factors in MDA-MB-231, EMSA analyses for STAT1 and NF-κB were performed. 100 U IFN-γ plus 10 ng/ml TNF–α induced a consistent increase in the bands corresponding to STAT1 and NF-κB, Fig. (**1A**), as indicated by supershift experiments with anti-STAT1 or anti-NF-κB antibodies (data not shown). The administration of *Arbutus unedo* aqueous extract 30 min before a cytokine treatment was able to inhibit, in a concentration-dependent manner, STAT1 DNA-binding activity, Fig. (**1A**), with EC_{50} value of 0.2 μg GAE/ml, Fig. (**1B**). The same fraction showed a less marked effect on NF-κB DNA-binding activity Fig. (**1A**) with the EC_{50} value more than 14.8 μg GAE/ml, Fig. (**1B**).

Aqueous Extract of *Arbutus unedo* Exerts an Inhibitory Action on STAT1 Tyrosine701 Phosphorylation

To further analyse whether the inhibition of the binding activity of STAT1 to DNA induced by *Arbutus unedo* aqueous extract is associated with tyrosine701(Tyr701)-phosphorylation of STAT1, Western blot analysis was performed using an antibody that specifically recognises the Tyr701-phosphorylated form of STAT1. As shown in Fig. (**2**), incubation of the MDA-MB-231 cells with IFN-γ/TNF-α for 30 min enhanced STAT1 Tyr701 phosphorylation. In agreement with an inhibitory action on the STAT1-DNA binding activity, pre-incubation of cells for 30 min with *Arbutus unedo* aqueous extract decreased STAT1 Tyr701-phosphorylation in a concentration-dependent manner, leading to complete inhibition at 7.4 μg GAE/ml, Fig. (**2**). Meanwhile, the amounts of STAT1 proteins were not modified, Fig. (**2**). These data indicate that the *Arbutus unedo* aqueous extract restricted the STAT1 DNA-binding activity that is strictly correlated to the inhibition of Tyr701 phosphorylation of STAT1.

Aqueous Extract of *Arbutus unedo* has no Effect on the Viability of MDA-MB-231 Cells

In order to further examine the effect of *Arbutus unedo* aqueous extract on STAT1-dependent gene expression which took place several hours after stimulation, cytotoxic action was first analysed. The cellular viability of MDA-MB-231 was measured using the WST-1 assay after 24 hours of incubation with different concentrations of *Arbutus unedo* aqueous extract. It is important to note that the incubation of MDA-MB-231 with *Arbutus unedo* aqueous extract produced no reduction in cell viability, Fig. (**3**).

Aqueous Extract of *Arbutus unedo* Diminishes the IFN-γ–Induced Expression of iNOS and ICAM-1 in MDA-MB-231 Cells

Since IFN-γ principally mediates the induction of some inflammatory genes through STAT1 activation, the expression profile of two inflammation-associated genes (iNOS, ICAM-1), whose promoters have STAT1 binding sequences, was analysed. The MDA-MB-231 cells were treated with IFN-γ in the presence or absence of *Arbutus unedo* aqueous extract and the total RNA was extracted for RT-PCR analysis. As shown in Fig. (**4A**) and (**4B**), 1000 U/ml IFN-γ induced the expression of ICAM-1 mRNA after 4 hours as well as increased the expression of iNOS mRNA after 16 hours of treatment. *Arbutus unedo* aqueous extract reduced dose-dependently the expression of these inflammatory genes.

In addition, iNOS and ICAM-1 proteins expression were analyzed. As shown in Fig. (**4C**), a 24 hours treatment with

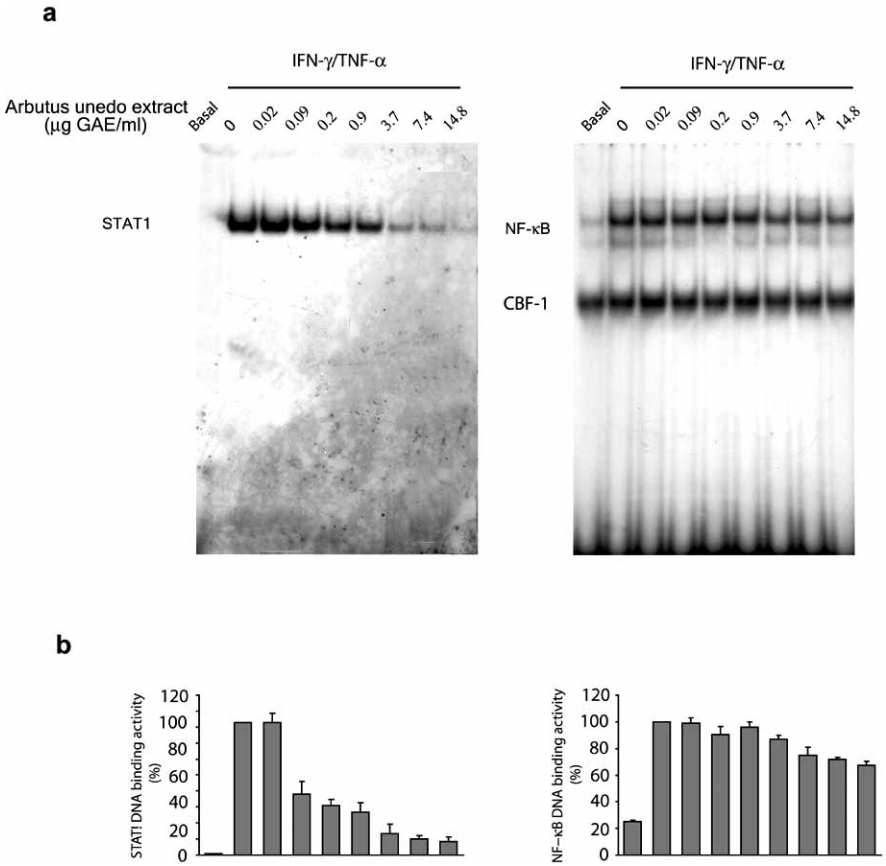

Fig. (1). Effect of *Arbutus unedo* aqueous extract on STAT1 and NF-κB DNA binding in MDA-MB-231 cell line A) The cells, pre-treated with different concentrations of *Arbutus unedo* aqueous extract for 30 min, were stimulated with IFN-γ/TNF-α for 30 min. In EMSA performed using radio-labelled oligonucleotide containing a consensus NF-κB binding site, the higher mobility shift band shows the binding of the nuclear protein CBF-1 to the interleukin-6/κB probe. The transcription factor CBF-1 is constitutively expressed and used as an internal standard. The gels are representative of four independent experiments. B) The intensity of the retarded bands were analyzed by Phosphoimager. Columns represent the average ± S.E. of the four independent experiments. Results showing STAT1 or NF-κB DNA binding activity are expressed as percentage of IFN-γ/ TNF-α treated cells (bar 2).

1000 U/ml IFN-γ induced expression of ICAM-1 protein as well as increasing the amount of iNOS protein above the basal level. In line with RT-PCR results, the expression of IFN-γ–elicited iNOS and ICAM-1 proteins was diminished by *Arbutus unedo* aqueous extract, dose-dependently. More specifically, 14.8 μg GAE/ml *Arbutus unedo* aqueous extract reduced almost completely the increase in the amount of ICAM-1 protein elicited by IFN-γ, whereas 29.6 μg GAE/ml *Arbutus unedo* aqueous extract were necessary to have the same effect on iNOS protein.

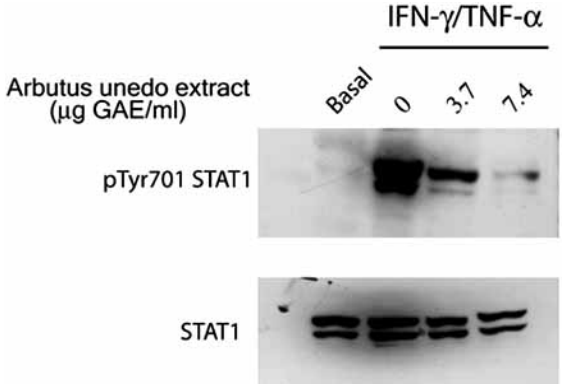

Fig. (2). Effect of *Arbutus unedo* aqueous extract on tyrosine phosphorylation of STAT1 in MDA-MB-231 cell line. The cells, pre-treated with different concentrations of *Arbutus unedo* aqueous extract for 30 min, were stimulated with IFN-γ/TNF-α for 30 min. Total extracts were analysed by Western blotting with anti phospho-STAT1 (pTyr701STAT1) and anti STAT1 antibodies. Data shown are representative of four independent experiments.

Aqueous Extract of *Arbutus unedo* Diminishes IFN-γ-Elicited Tyrosine-Phosphorylation of JAK1 and JAK2

To elucidate the molecular pathway leading to the inhibition of STAT1 DNA-binding activity by an aqueous extract of *Arbutus unedo*, its effect on tyrosine-phosphorylation of JAK1 and JAK2 was analysed. MDA-MB-231 cells, pre-treated with *Arbutus unedo* aqueous extract for 30 min, were incubated with 100 U/ml IFN-γ and examined in Western blot using an antibody that specifically recognizes tyrosine-phosphorylated JAK1 and JAK2, respectively. IFN-γ induced the tyrosine-phosphorylation of both JAK1 and JAK2 in 15 min, which was inhibited by the addition of 7.4 μg GAE/ml *Arbutus unedo* aqueous extract, Fig. (**5**).

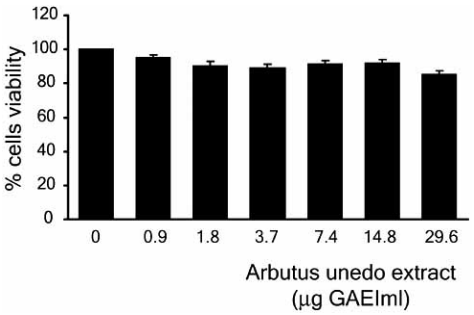

Fig. (3). Effect of *Arbutus unedo* aqueous extract on the viability of MDA-MB-231 cell line. The cells were treated with different concentrations of *Arbutus unedo* aqueous extract for 24 hours, (means ± S.E.; n=4).

Aqueous Extract of *Arbutus unedo* Enhances Tyrosine-Phosphorylation of SHP2

To study the mechanism underlying the suppression of JAKs activation by *Arbutus unedo* aqueous extract we focused our attention on protein tyrosine phosphatases (PTPs), SHP1 and SHP2, negative regulator of JAK/STAT activation [24-26].

First, the PTPs activity in the lysates of MDA-MB-231 cells treated with 100 U/ml IFN-γ for 5 or 15 min, was determined by molybdate-malachite green-phosphate complex assay. Treatment of cells with IFN-γ inhibits the PTPs activity by 40 % at 5 min. Whereas 7.4 µg GAE/ml *Arbutus unedo* aqueous extracts reverted this effect, Fig. (**6A**).

Successively, the tyrosine-phosphorylated level of SHP1 and SHP2 was analysed by the immunoprecipitation method. MDA-MB-231 cells were treated with 7.4 µg GAE/ml *Arbutus unedo* aqueous extract at various time points (5-45 min) and protein extracts were immunoprecipitated with a specific SHP1 or SHP2 antibody. Western blot analysis with an anti-phosphotyrosine specific antibody (4G10) of SHP1 and SHP2 immunoprecipitates showed that *Arbutus unedo* aqueous extract increases tyrosine-phosphorylation of SHP2, which was absent under normal conditions, starting 5 min

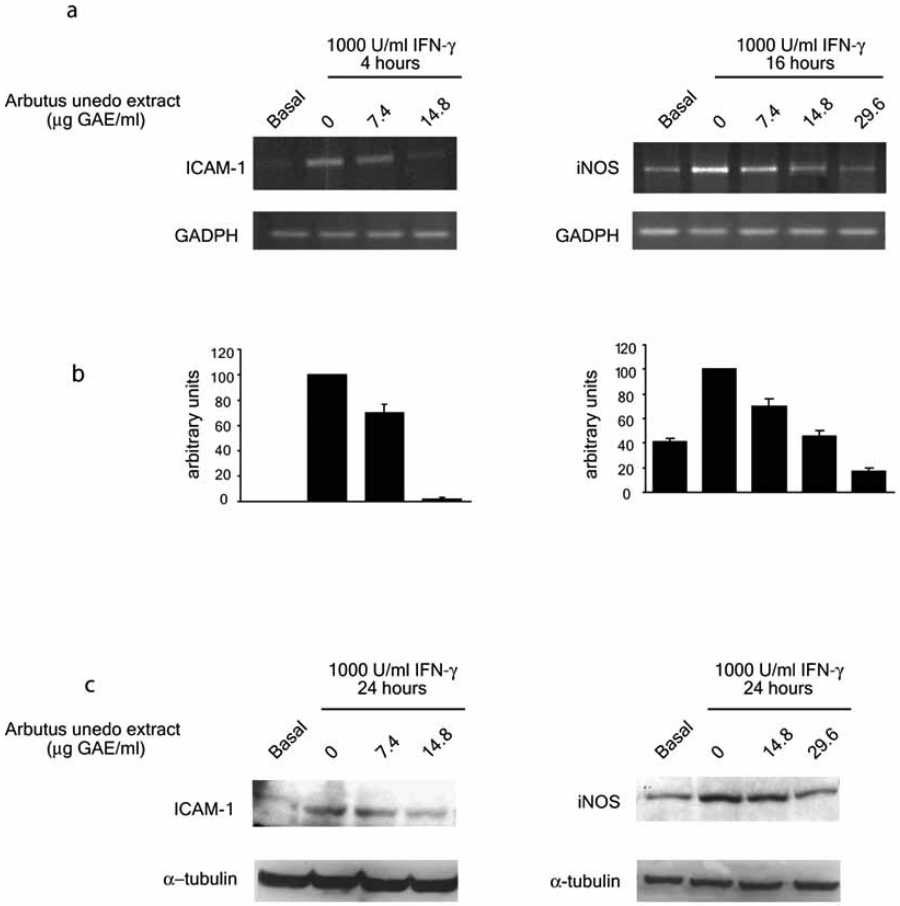

Fig. (4). Effect of *Arbutus unedo* aqueous extract on STAT1-responsive inflammatory gene expressions in MDA-MB-231 cell line. A) The cells, pre-treated with different concentrations of *Arbutus unedo* aqueous extract for 30 min, were stimulated with 1000 U/ml IFN-γ for 4 or 16 hours. Total RNA was analysed for transcript level of ICAM-1 and iNOS using an RT-PCR-based assay. The transcript of GAPDH was measured for normalization. The experiment was repeated three times and the gels show representative results. B) The histograms represent the average ± S.E. of three independent experiments. C) The cells, pre-treated with different concentrations of *Arbutus unedo* aqueous extract for 30 min, were stimulated with 1000 U/ml IFN-γ for 24 hours. Total proteins were analysed in Western Blot using an anti-iNOS and anti-ICAM-1 specific antibody. The same blots were incubated with an anti α-tubulin specific antibody to determine the amount of loaded protein. Data shown are representative of three independent experiments.

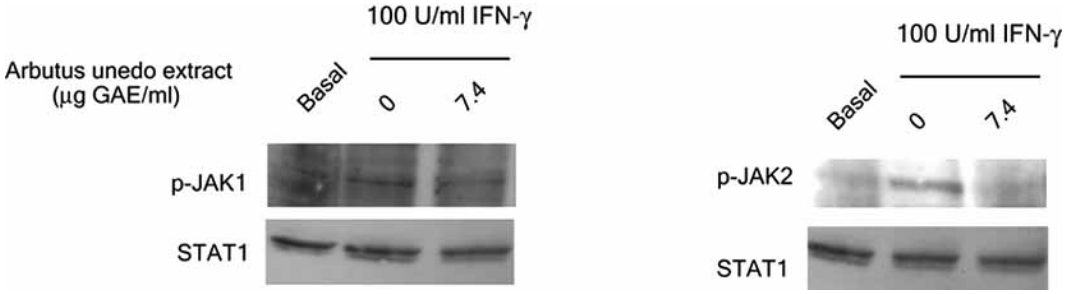

Fig. (5). Effect of *Arbutus unedo* aqueous extract on the phosphorylation of JAK1 and JAK2 in MDA-MB-231 cell line. The cells were pre-incubated with 7.4 μg GAE /ml of *Arbutus unedo* aqueous extract for 30 min and then stimulated with 100 U/ml IFN-γ for 15 min. The phosphorylation level of JAK1 and JAK2 was determined by Western Blot analysis using a specific antibody for phospho-JAK1 (Tyr $^{1022/1023}$) and phospho-JAK2 (Tyr $^{1007/1008}$). The same blot was incubated with a STAT1 specific antibody to determine the amount of loaded protein. Data shown are representative of four independent experiments.

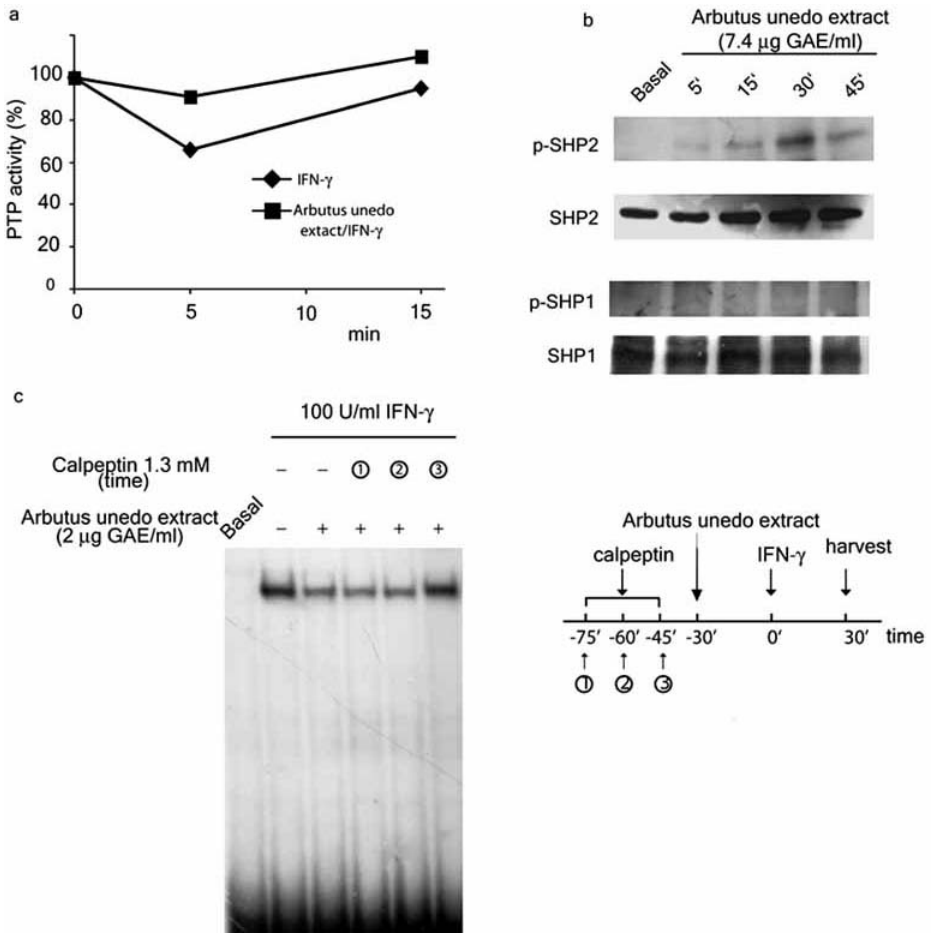

Fig. (6). Effect of *Arbutus unedo* aqueous extract on SHP2 phosphorylation in MDA-MB-231 cell line A) The cells were incubated with 7.4 μg GAE /ml of *Arbutus unedo* aqueous extract for 30 min and then stimulated with 100 U/ml IFN-γ for 5 or 15 min. The protein phospahatase activity of total cellular lysates was determined by measuring free phosphate.

B) The cells were incubated with 7.4 μg GAE /ml of *Arbutus unedo* aqueous extract for the indicated time periods and immunoprecipitated with a specific antibody for SHP1 and SHP2. Western blot analyses were performed with anti-phosphotyrosine antibody 4G10. The membranes were then stripped and analysed with an anti-SHP1 or anti-SHP2 antibody. Data shown are representative of four independent experiments.

C) The cells were treated with 1.3 mM calpeptin as time schedule shown in diagram and then with 2 μg GAE /ml of *Arbutus unedo* aqueous extract for 30 min. Thereafter the cells were stimulated with 100 U/ml IFN-γ for 30 min. Nuclear extracts were analysed by EMSA using a radio-labelled oligonucleotide containing a consensus STAT1 binding site. The gel is representative of four independent experiments.

from the treatment, with the maximum value at 30 min, Fig. (**6B**). Whereas no SHP1 tyrosine-phosphorylation was observed at any time point.

To confirm the involvement of SHP2 in the negative effect of *Arbutus unedo* aqueous extract on the STAT1 DNA binding activity, a specific SHP2 inhibitor, calpeptin, was used [27-29]. MDA-MB-231 cells, pre-incubated with 1.3 mM calpeptin (Calbiochem, San Diego, CA) for 15, 30 and 45 min, were treated for 30 min with 2 μg GAE/ml *Arbutus unedo* aqueous extract and then with cytokine for an additional 30 min. The presence of calpeptin reverted, in a time dependent manner, the *Arbutus unedo* aqueous extract inhibitory effect on STAT1 activation, Fig. (**6C**).

Aqueous Extract of *Arbutus unedo* Diminishes IFN-γ-Elicited Activation of STAT1 and Expression of ICAM-1 and iNOS in Human Fibroblasts

Human fibroblasts were used in order to examine whether *Arbutus unedo* aqueous extract exerts an inhibitory action on IFN-γ-elicited STAT1 activation also in primary cells. *Arbutus unedo* aqueous extract was able to inhibit dose-dependently the STAT1 DNA-binding activity elicited by IFN-γ with EC_{50} value of 0.95 μg GAE/ml Fig. (**7A**). In agreement with the inhibitory action on the STAT1-DNA binding activity, *Arbutus unedo* aqueous extract reduced Tyr701-phosphorylated STAT1 protein in IFN-γ-treated cells without affecting the amounts of STAT1, Fig. (**7B**). Furthermore, RT-PCR showed that a 4 hours treatment with 1000 U/ml IFN-γ induced an increase in ICAM-1 mRNA, and a 16-hour treatment with 1000 U/ml IFN-γ induced iNOS mRNA. These increases were completely reverted by the addition of 14.8 μg GAE /ml *Arbutus unedo* aqueous extract to the fibroblast culture medium, Fig. (**7C**). As observed in the MDA-MB-231 cells, treatment with different concentrations of *Arbutus unedo* aqueous extract for 24 hours did not affect the viability of fibroblasts (data not shown).

DISCUSSION

Arbutus unedo L., an endemic plant of Sardegna island, Italy, is widely cultivated in the Mediterranean countries and has been used in traditional and popular medicine for a long time as an astringent, diuretic, urinary anti-septic [30] and more recently, in the therapy of hypertension and diabetes [23]. The aqueous extract of *Arbutus unedo* roots, indeed, induces endothelium-dependent relaxation of a pre-contracted aorta, this effect being mediated by activation of endothelial nitric oxide synthase (eNOS) and cGMP synthesis [31]. Legssyer *et al.*, furthermore, reported that the aqueous extract of *Arbutus unedo* leaves produced a strong relaxation of a pre-contracted aorta with an effect comparable to that of root extracts [32]. This vasorelaxant activity is assigned to polyphenolic compounds such as catechin gallate, catechin and oligomeric condensed tannins.

Although all these works suggest that polyphenols, present in aqueous extract of *Arbutus unedo* leaves, augment NO production by enhancing the catalytic activity of constitutive NOS (cNOS), so far no data has been available on the effect of *Arbutus unedo* extract on the expression of the iNOS gene. Normally the iNOS expression is finely regulated not only by modulation of the constitutive NOS activity but also by a number of nuclear factors, including NF-κB and STAT1 [1, 33]. Deregulation of the iNOS expression could occur, if these nuclear factors undergo abnormal activation. Indeed, recent vision is that the phasing out of NF-κB [34] and/or STAT1 activation may represent a new strategy in counteracting inflammation-associated tissue damage [3].

The present study shows that the aqueous extract of *Arbutus unedo* leaves exerts a remarkable inhibitory action on IFN-γ-elicited STAT1 activation and successive induction of the expression of STAT1-dependent genes such as iNOS and ICAM-1 in either MDA-MB-231 cells or human fibroblasts. Notably, a less marked effect of *Arbutus unedo* extract on TNF-α-elicited NF-κB activation in the MDA-MB-231 cells was observed. Therefore, the aqueous extract of *Arbutus unedo* is characterized by the presence of compound(s) that strongly phase out STAT1 activation. In this respect, it might be stressed that a complete inhibition of STAT1 induced by the *Arbutus unedo* extract was not fully corresponding to that of the expression of iNOS and ICAM-1. We argue that in the cell models examined in the present study, the full expression of these genes may require other nuclear factors including NF-κB. In our opinion, this point may represent an advantage in the future consideration of *Arbutus unedo* extract or compound(s) present within for therapeutic application, since down-modulation but not a complete inhibition of inflammatory genes may possibly lead to a less deleterious effect on the structural and functional integrity of the tissue, or to the maintenance of the immune response.

Following IFN-γ binding to receptors on the cell surface, STAT1 activation is mediated by a complex signal transduction pathway [3] in which the tyrosine kinases JAK1 and JAK2 play a critical role [35]. In the present study, we focused our attention on the intracellular JAK1 and JAK2 tyrosine-phosphorylation pathway, since an inhibitory action of *Arbutus unedo* extract on STAT1 activation was observed in the very early phase of STAT1 signal transduction pathway, i.e. 15 min after IFN-γ-treatment. Together, our results suggest that in the human breast cancer cell line MDA-MB-231 *Arbutus unedo* aqueous extract inhibits the initiation of the JAK-STAT signalling cascade, at least from the point of JAK phosphorylation. The increased tyrosine-phosphorylation of SHP2 observed in MDA-MB-231 cells 5 min after *Arbutus unedo* extract treatment, Fig. (**5B**) strongly suggests that SHP2 activation may be a key regulatory pathway in the phasing out of STAT1 induced by *Arbutus unedo* aqueous extract. Furthermore calpeptin, a specific SHP2 inhibitor [27-29], was able to revert *Arbutus unedo* extract inhibitory effect on the STAT1-DNA binding activity. This phenomenon was observed only when cells were treated with 2 μg/ml GAE of *Arbutus unedo* extract and not with higher amounts (data not shown), suggesting further that SHP2 is a possible molecular target of *Arbutus unedo* extract.

We are currently performing a number of experiments aimed at identifying anti-STAT1 compound(s) present in the aqueous extract of *Arbutus unedo*. Some studies described the isolation of several polyphenols present in *Arbutus Unedo* leaves such as quercetin, hyperoside, quercitrin, catechin gallate, and arbutoside [32]. Preliminary experiments indicated that these compounds have neither an anti-STAT1 ac-

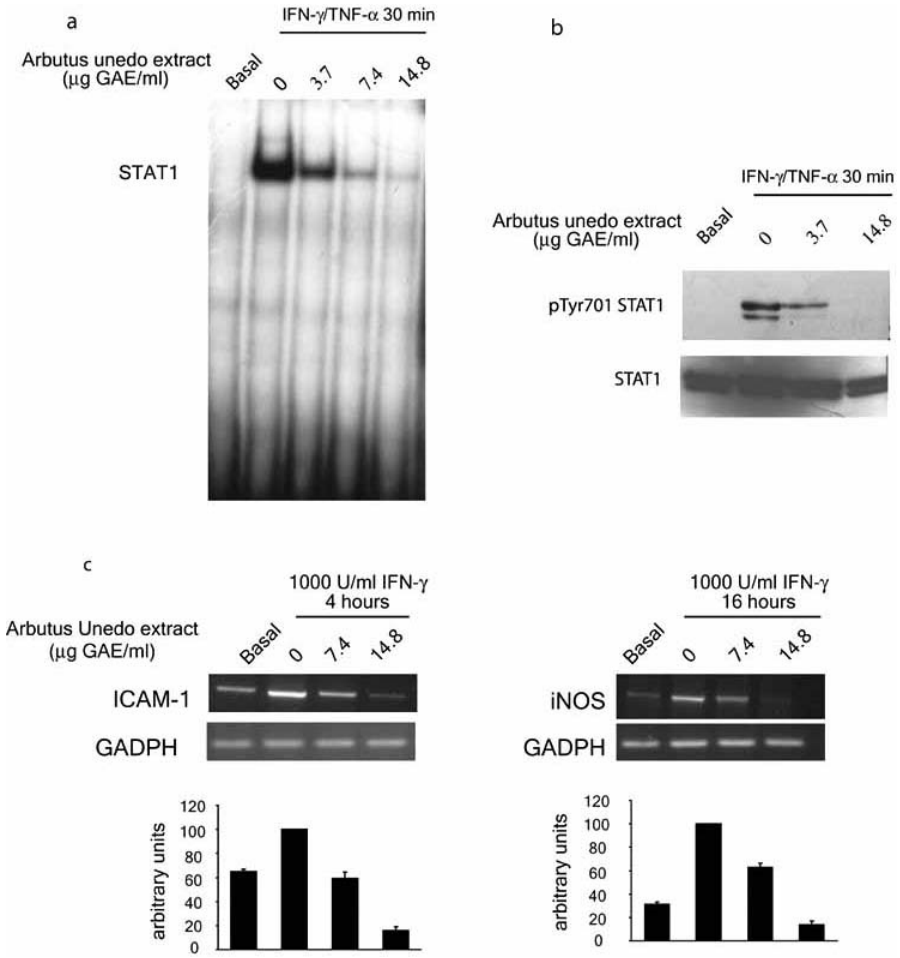

Fig. (7). Effect of *Arbutus unedo* aqueous extract on IFN-γ-elicited activation of STAT1 and on STAT1-responsive inflammatory gene expressions in human fibroblasts. A) Nuclear proteins extracted from the cells pre-treated with different concentrations of *Arbutus unedo* aqueous extract for 30 min and then stimulated with IFN-γ/TNF-α for 30 min. were analysed by EMSA. The gel is representative of four independent experiments. B) The cells, pre-treated with different concentrations of *Arbutus unedo* aqueous extract for 30 min, were stimulated with IFN-γ/TNF-α for 30 min. Total proteins were analysed by Western blotting with anti-phospho-STAT1 (pTyr701-STAT1) and anti-STAT1 antibodies. Data shown are representative of four independent experiments. C) The cells, pre-treated with different concentrations of *Arbutus unedo* aqueous extract for 30 min, were stimulated with 1000 U/ml IFN-γfor 4 or 16 hours. Total RNA was analysed for transcript level of iNOS and ICAM-1 using an RT-PCR-based assay. The transcript of GAPDH was measured for normalization. The experiment was repeated three times and the gels show representative results. The histograms represent the average ± S.E. of the three independent experiments.

tivity nor a suppressive action towards STAT1-dependent gene expression (data not shown). Since among a number of polyphenols with a strong anti-oxidant activity, only few polyphenols own a strong inhibitory action on STAT1 activation [3], the anti-STAT1 activity in *Arbutus unedo* extract may not very likely be ascribed to its claimed anti-oxidant activity.

In conclusion, *Arbutus unedo* L. aqueous extract exhibits a remarkable inhibitory action against the activation of STAT1 elicited by IFN-γ, with a less marked action on the NF-κB activation elicited by TNF-α. This effect was shown not only in MDA-MB-231 cell line but also in human fibroblasts. The compound(s) present in *Arbutus unedo* seem(s) to exert a STAT1 inhibitory action enhancing SHP2 tyrosine-phosphorylation. The expression of STAT1-dependent inflammatory genes was also diminished by *Arbutus unedo* extract, indicating a possible therapeutic employment of this extract or of the STAT1-inhibiting compound(s) present in *Arbutus unedo*.

METHODS

Reagents

All chemicals used throughout the present study were from Sigma (Sigma, Milan, Italy), unless otherwise specified. The antibodies against SHP1, SHP2 and STAT1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PhosphoSTAT1 (Tyr ^{701}) antibody came from Cell Signaling Technologies (Cambridge, MA). The rabbit anti phospho-JAK1(Tyr$^{1022/1023}$) and anti phospho JAK2 (Tyr$^{1007/1008}$) antibodies were purchased from Chemicon International Inc. (Temecula, CA). The antibody against phosphotyrosine (4G10) was from Upstate Biotechnologies (Lake Placid, NY)

and the antibodies against iNOS were obtained from BD Transduction Laboratories (Lexington, KY). The rabbit antibody against ICAM-1 was from Zymed laboratories (San Francisco, CA).

Plant Material and Extraction

Leaves of *Arbutus unedo* L. were collected during the summer in Sardegna, Italy. Leaves were air-dried (24°C in the dark), then were milled and stored at -20°C.

Material (10 g) from grounded plant leaves was extracted with 100 ml of methanol four times. Samples were centrifuged (10 min, 3000 x g), and the combined supernatants were dried under reduced pressure to afford a dark green residue (4.3 g of yield). The pellet was washed with 10 ml of water four times. After centrifugation (10 min, 10000 x g), the supernatants were lyophilised (yield of 0.95 g, referred to as *Arbutus unedo* aqueous extract).

The amount of total phenolic compounds present in the extract was determined according to the Folin-Ciocalteau procedure [36]. The content of total phenolic compounds was expressed as milligrams of gallic acid equivalents (GAE). *Arbutus unedo* aqueous extract used in the cell treatment was expressed as µg of GAE per ml of medium solution.

Cell Cultures

The MDA-MB-231 cell line and fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Cambrex Bio Science, Belgium) supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Cambrex Bio Science, Belgium), 100 UI/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 40 µg/ml gentamicin, in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. The fibroblast cells were a gentle gift from Prof. Armato research group, University of Verona, Italy.

Preparation of Cytoplasmatic and Nuclear Extracts

Nuclear extracts of MDA-MB-231 and fibroblasts were prepared according to Osborn *et al.* [38]. For the preparation of cytoplasmatic extracts, cells were homogenized at 4° C in 50 mM Tris-HCl, pH 8, containing 0.1 % NP-40, 200 mM KCl, 2 mM $MgCl_2$, 50 µM $ZnCl_2$, 2 mM DTT and proteases inhibitors (1 mM phenylmethylsulfonyl fluoride , 1 mg/ml leupeptin, 1 mg/ml antipain) and centrifuged for 20 min at 25,000 x g.

Electrophoretic Mobility Shift Assay

Eight micrograms of the nuclear extract were incubated for 20 min at room temperature with 2-5 × 10^4 cpm of a ^{32}P-labeled double-stranded oligonucleotides, containing the STAT1 binding site (sis-inducible factor-binding recognition element, SIE/m67) from the c-fos promoter (5'-gtcgaCATTT CCCGTAAATCg-3') or the NF-κB binding sequence from the interleukin-6 promoter (5'-gatcCAGAGGGGACTTT CCGAGTAC-3') (Promega, Milan, Italy). Products were fractionated on a non-denaturing 5% polyacrylamide gel. The intensity of the retarded bands were measured with a Phosphorimager (Molecular Dynamic, Milan, Italy).

Supershift assay was performed by incubating the nuclear extracts in a binding buffer for 1 hour at 4°C with 1 µl of antibody before addition of labelled oligonucleotide. Polyclonal antibodies against STAT1 and NF-κB were purchased from Santa Cruz Biotecnology (Santa Cruz, CA).

Cell Viability

Cell viability was measured by a colorimetric assay based on the cleavage of the tetrazolium salt 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate WST-1 (Roche Molecular Biochemicals, Indianapolis, IN) into formazan by mitochondrial dehydrogenases [37].

RT-PCR

Cellular RNA was extracted by using TRIZOL reagent (Life Technologies, Paisley, UK) and the cDNA was synthesized by reverse transcription (Life Technologies, Paisley, UK).

For PCR reactions, 1 µl cDNA was amplified in a 25µl PCR reaction mixture. Using the thermal cycler (Perkin-Elmer) amplification was initiated by 1 min denaturation at 95°C for one cycle and then followed by three PCR temperature steps including 30 s. of denaturation at 95°C, annealing at 58°C 30 s (iNOS), 54°C 1min (ICAM-1) and 55°C 30 s (GAPDH) and 1 min of polymerization at 72°C. This process was continued for 30, 20 or 18 cycles of amplification for iNOS, ICAM-1 and GAPDH respectively. After the last cycle of amplification, the samples were incubated for 7 min at 72°C.

The PCR product of each of these genes was quantitated during the exponential phase of amplification. For this purpose, PCR amplifications were carried out after serial dilution of cDNA samples and variation of the number of amplification cycles. The mRNA for the constitutive GAPDH enzyme was examined as the reference cellular transcript. Estimates of the relative iNOS or ICAM-1 mRNA amounts were obtained dividing the area of each respective band by the area of the corresponding GAPDH band (Bio-Rad Multi-AnalystTM/PC Version 1.1).

PCR primers were as the following

iNOS: 5'-TCCTTGCATCCTCATCGGGCC-3'(forward primer), 5'-TCGTGATAGCGCTTCTGGCTCT-3' (reverse primer). The predicted size of the fragment was 450 bp.

ICAM-1: 5'AGAAATTGGCTCCATGGTGATCTC-3'(forward primer), 5'-ACATGCAGCACCTCCTGTGACCA-3' (reverse primer) The predicted size of the

fragment was 420 bp.

GAPDH: 5'-CCATGGAGAAGGCTGGGG-3'(forward primer), 5'-CAAAGTTGTCATGGATGACC-3'(reverse primer). The predicted size of the fragment was 200 bp.

Western blot analysis

For the detection of STAT1 phosphorylation, nuclear proteins were used while cytoplasmatic proteins were used for phosphoJAK1(Tyr$^{1022/1023}$), for phosphoJAK2 (Tyr$^{1007/1008}$), iNOS and ICAM-1 detection. 40 µg proteins/lane were separated by 7.5% SDS-PAGE, electro-blotted to a PVDF membrane (Millipore, S.p.A., Rome, Italy) and incubated

overnight 4°C with primary antibodies. After washing with TBS-T, membranes were developed using horseradish peroxidase-linked anti-mouse or anti-rabbit secondary antibody and chemiluminescent detection system (ECL; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Protein Tyrosine Phosphatase (PTP) Assay

The protein phosphatase activity of the total cellular lysate was determined by measuring free phosphate generated from the tyr phosphopeptide-1, END(pY)INASL using the molybdate-malachite green-phosphate complex assay as described by the manufacturer (Promega, Madison, WI)

Immunoprecipitation

The cells were lysed in ice in a RIPA buffer in the presence of a mixture of protease and phosphatase inhibitors. After centrifugation for 20 min at 25,000 x g, proteins (500 μg) were incubated overnight at 4°C with polyclonal anti-SHP1 or anti-SHP2 antibody (1μg/ml). Then the immunocomplexes were captured on protein A-sepharose beads for 1 hour at 4°C. After washing, the immunoprecipitate proteins were boiled in a SDS sample buffer and then separated by 7.5% SDS-PAGE. Immunoblot analyses were performed as described before using an antibody specific for phosphotyrosine residues. After stripping, the same blots were reprobed with a specific anti-SHP1 and anti-SHP2 antibody.

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ABBREVIATIONS

iNOS	=	Inducible nitric oxide synthase
ICAM-1	=	Intercellular adhesion molecule-1
TNF-α	=	Tumour necrosis factor-α
IFN-γ	=	Interferon-γ
IL-1β	=	Interleukin-1β
LPS	=	Lipopolysaccharides
JAK	=	Janus family kinase
STAT1	=	Signal transducer and activator of transcription 1
GAS	=	γ-activated site
NF-κB	=	Nuclear factor-κB
SOCS	=	Suppressor of cytokine signaling
PIAS	=	Protein inhibitors of activated STAT
SHP	=	Receptor-associated protein phosphatase
GTE	=	Green tea extract
EGCG	=	(-/-)-epigallocatechin-3-gallate
GAE	=	Gallic acid equivalents

REFERENCES

[1] Mariotto, S.; Menegazzi, M.; Suzuki, H. *Curr. Pharm. Des.,* **2004**, *10*, 1627.

[2] Colasanti, M.; Persichini, T.; Menegazzi, M.; Mariotto, S.; Giordano, E.; Caldarera, C. M.; Sogos, V., Lauro, G. M.; Suzuki, H. *J. Biol. Chem.,* **1995**, *270,* 26731.

[3] Carcereri De Prati, A.; Ciampa, A. R.; Cavalieri, E.; Zaffini, R.; Darra, E.; Menegazzi, M.; Suzuki, H.; Mariotto, S. *Curr. Med. Chem*., **2005**, *12,* 1623.

[4] Aaronson, D.; S.Horvath, C. M. *Science*, **2002**, *31*, 1653.

[5] Bordin, L.; Brunati, A. M.; Donella-Deana, A.; Baggio, B.; Toninello, A.; Clari, G. *Blood*, **2002**, *100*, 276.

[6] Stephanou, A.; Brar, B. K.; Scarabelli, T. M.; Jonassen, A. K.; Yellow, D. M.; Marber, M. S.; Knight, R. A.; Latchman, D. S. *J. Biol. Chem.,* **2000**, *275*, 10002.

[7] Magnani, M.; Balestra, E.; Fraternale, A.; Aquaro, S.; Paiardini, M.; Cervasi, B.; Casabianca, A; Garaci, E.; Perno, C. F. *J. Leukoc. Biol.,* **2003**, *74*, 764.

[8] Sampath, D.; Castro, M.; Look, D. C.; Holtzman, M. J. *J. Clin. Invest.,* **1999**, *103*, 1353.

[9] Woods, M.; Wood, E. G.; Bardswell, S. C.; Bishop-Bailey, D.; Barker, S.; Wort, S. J.; Mitchell, J. A.; Warner, T. D. *Mol. Pharmacol.,* **2003**, *64***,** 923.

[10] Mazzarella, G.; Macdonald, T. T.; Salvati, V. M.; Mulligan, P.; Pasquale, L.; Stefanile, R.; Lionetti, P.; Auricchio, S.; Pallone, F.; Troncone, R.; Monteleone, G. *Am. J. Pathol.,* **2003**, *162*, 1845.

[11] Schreiber, S.; Rosenstiel, P.; Hampe, J.; Nikolaus, S.; Groessner, B.; Schottelius, A.; Kuhbacher, T.; Hamling, J.; Folsch, U. R.; Seegert, D. *Gut*, **2002**, *51*, 379.

[12] Banes, A. K.; Shaw, S.; Jenkins, J.; Redd, H.; Amiri, F.; Pollock, D. M.; Marrero, M. B. *Am. J. Physiol. Renal. Physiol.*, **2004**, *286*, 653.

[13] Giustizieri, M. L.; Albanesi, C.; Scarponi, C.; De Pita, O.; Girolomoni, G. *Am. J. Pathol*., **2002**, *161*, 1409.

[14] De Hooge, A. S.; Van De Loo, F. A.; Koenders, M. I.; Bennink, M.B.; Arntz, O. J.; Kolbe, T.; Van Den Berg, W. B. *Arthritis. Rheum.,* **2004**, *50*, 2014.

[15] Pfitzner, E.; Kliem, S.; Baus, D.; Litterst, C. M. *Curr. Pharm. Des*., **2004**, *10*, 2839.

[16] Tedeschi, E.; Menegazzi, M.; Margotto, D.; Suzuki, H.; Forstermann, U.; Kleinert, H. *J. Pharmacol. Exp. Ther.,* **2003**, *307*, 254.

[17] Tedeschi, E.; Menegazzi, M.; Yao, Y.; Suzuki, H.; Forstermann, U.; Kleinert, H. *Mol. Pharmacol*., **2004**, *65*, 111.

[18] Menegazzi, M.; Tedeschi, E.; Dussin, D.; De Prati, AC.; Cavalieri, E.; Mariotto, S.; Suzuki, H. *FASEB J*., **2001**, *15*, 1309.

[19] Townsend, P. A.; Scarabelli, T. M.; Pasini, E.; Gitti, G.; Menegazzi, M.; Suzuki, H.; Knight, R. A.; Latchman, D. S.; Stephanou, A. *FASEB J.,* **2004**, *18*, 1621.

[20] Hong, J. T.; Ryu, S. R.; Kim, H. J.; Lee, J. K.; Lee, S. H.; Kim, D. B.; Yun, Y. P.; Ryu, J. H.; Lee, B. M.; Kim, P. Y. *Brain Res. Bull.,* **2000**, *53*, 743.

[21] Varilek, G. W.; Yang, F.; Lee, E. Y.; Devilliers, W. J.; Zhong, J.; Oz, H. S.; Westberry, K. F.; Mcclain, C. J. *J. Nutr.,* **2001**, *131*, 2034.

[22] Mazzon, E.; Muia, C.; Paola, R. D.; Genovese, T.; Menegazzi, M.; De Sarro, A.; Suzuki, H.; Cuzzocrea, S. *Free Radic. Res*., **2005**, *39*, 1017.

[23] Ziyyat, A.; Legssyer, A.; Mekhfi, H.; Dassouli, A.; Serhrouchni, M.; Benjelloun, W. *J. Ethnopharmacol*., **1997**, *58*, 45.

[24] Hof, P.; Pluskey, S.; Dhe-Paganon, S.; Eck, M. J.; Shoelson, S. E. *Cell,* **1998**, *92*, 441.

[25] Lu, W.; Gong, D.; Bar-Sagi, D; Cole, P. A. *Mol. Cell.,* **2001**, *8*, 759.

[26] Vogel, W.; Lammers, R.; Huang, J.; Ullrich, A. *Science,* **1993**, *259*,1611.

[27] Schoenwaelder, S. M.; Petch, L. A.; Williamson, D.; Shen, R.; Feng, G. S.; Burridge, K. *Curr. Biol*., **2000**, *10*, 1523.

[28] Higashi, H.; Tsutsumi, R.; Muto, S.; Sugiyama, T.; Azuma, T.; Asaka, M.; Hatakeyama, M. *Science,* **2002**, *295*, 683.

[29] Salvi, M.; Stringaro, A.; Brunati, A. M.; Agostinelli, E.; Arancia, G.; Clari, G.; Tondello, A. *Cell. Mol. Life Sci.,* **2004**, *61*, 2393.

[30] Grieve, M. *A Modern Herbal,* Hafner: New York and London, **1967**.

[31] Ziyyat, A.; Mekhfi, H.; Bnouham, M.; Tahri, A.; Legssyer, A.; Hoerter, J.; Fischmeister, R. *Phytother. Res.,* **2002**, *16*, 572.

[32] Legssyer, A.; Ziyyat, A.; Mekh, H.; Bnouham, M.; Herrenknecht, C.; Roumy, V.; Fourneau, C.; Laurens, A.; Hoerter, J.; Fischmeister, R. *Phytother. Res*., **2004**, *18*, 889.

[33] Colasanti, M.; Suzuki, S. *Trends Pharmacol. Sci*., **2000**, *21*, 249.
[34] Yamamoto, Y.; Gaynor, R. B. *J. Clin. Invest*., **2001**, *107*, 135.
[35] Briscoe, J.; Rogers, N. C.; Witthuhn B.A.; Watling, D.; Harpur, A.G.; Wilks, A. F.; Stark, G. R.; Ihle, J. N.; Kerr, I. M. *EMBO J*., **1996**, *15*, 799.
[36] Singleton, V. L.; Rossi, J. A. *Am. J. Enol. Vitic.,* **1965**, *16*, 144.
[37] Osborn, L.; Kunkel, S.; Nabel, G. J. *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 2336.
[38] Saito, T.; Kijima, H.; Kiuchi, Y.; Isobe, Y.; Fukushima, K. *Neurosci. Lett.,* **2002**, *305*, 61.

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