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ORIGINAL ARTICLE

Effects of a new nutraceutical substance on clinical and molecular parameters in patients with chronic venous ulceration

Raffaele Serra[1,2], Raffaele Grande[2], Lucia Butrico[2], Gianluca Buffone[2], Francesco G Caliò[3], Aida Squillace[4], Barbara A Rizzo[4], Mafalda Massara[5], Francesco Spinelli[5], Alessia G Ferrarese[6], Giovanni de Caridi[5], Luca Gallelli[4] & Stefano de Franciscis[1,2]

1 Interuniversity Center of Phlebolymphology, International Research and Educational Program in Clinical and Experimental Biotechnology, University Magna Graecia of Catanzaro, Catanzaro, Italy
2 Department of Surgical and Medical Sciences, University Magna Graecia of Catanzaro, Catanzaro, Italy
3 Unit of Vascular Surgery, S. Anna Hospital, Catanzaro, Italy
4 Department of Health Science, University of Catanzaro, Catanzaro, Italy
5 Cardiovascular and Thoracic Department, "Policlinico G. Martino" Hospital, University of Messina, Messina, Italy
6 Section of General Surgery, Teaching Hospital "San Luigi Gonzaga", School of Medicine, University of Turin, Turin, Italy

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Chronic venous ulceration; Diosmin; MMPs; Nutraceutical; Patients

Correspondence to
Prof. R Serra, MD, PhD
Department of Medical and Surgical Science
University Magna Graecia of Catanzaro
Viale Europa – Germaneto
88100 Catanzaro
Italy
E-mail: rserra@unicz.it

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Abstract

Pathophysiological events involved in the onset of chronic venous ulceration (CVU) are inflammation, activation of polymorphonucleates (PMNs) and secretion of proteases such as matrix metalloproteinases (MMPs), which degrade extracellular matrix (ECM) that is a support for vascular and tissutal wall. MMPs, neutrophil gelatinase-associated lipocalin (NGAL) and inflammatory cytokines are overexpressed in CVUs and they could play a central role in pathophysiological mechanisms of skin lesion and delayed wound healing. Bioflavonoids, such as diosmin and other compounds, appear to have several provessel function activities including anti-inflammatory, antioxidant and phlebotonic effects and are widely used in the treatment of chronic venous disease (CVD)-related problems.

In this article, we evaluated the effects of Axaven®, a new nutraceutical on both clinical and molecular parameters in patients with CVUs.

During the study period, 83 patients with CVUs of both sexes were enrolled and divided into two groups: group A (treated group): 25 females and 19 males (median age is 67·7 years) received standard treatment (compression therapy and surgical correction of superficial venous incompetence) + Axaven® once a day for 8 months as adjunctive treatment.

Group B (control group): 24 females and 15 males (median age is 65·2 years) were treated only with basic treatment according to their clinical conditions.

In our study, the administration of Axaven® in patients with CVUs was able to decrease inflammatory cytokines, MMPs and NGAL, inducing an improvement of both symptoms with an increase of the speed of wound healing.

[Correction added on 20 March 2015, after first online publication: The number of patients, 36 patients (Group A: 9 females and 7 males; Group B 11 females and 9 males) were wrong and have been changed to 83 patients (Group A: 25 females and 19 males; Group B: 24 females and 15 males) in the abstract.]

Introduction

Chronic venous ulceration (CVU) is a consequence of the pathophysiologic evolution of chronic venous disease (CVD) (1–9). Pathophysiological events involved in the onset of CVU are inflammation, activation of polymorphonucleates (PMNs) and secretion of proteases (10). The biological substrate is represented by the extracellular matrix (ECM), a complex network of macromolecules that is a support for vascular and tissutal wall and is dynamically maintained by the action of matrix metalloproteinases (MMPs; which degrade ECM proteins) and their inhibitors [(tissue inhibitors of MMPs and tissue inhibitors of metalloproteinases (TIMPs)]. MMPs are involved in many different vascular (11–13) and non-vascular diseases (14,15), which have chronic inflammation as a common denominator. MMPs are overexpressed in CVUs and they could play a central role in pathophysiological mechanisms of skin lesion and delayed wound healing (16–18). Moreover, we documented an association between neutrophil gelatinase-associated lipocalin (NGAL) and MMP-9 (18). Moreover, we have also reported that doxycycline and minocycline are able to improve CVU through the action on MMPs (19,20), as according to guidelines (21), the standard of care for CVUs is local wound care and the application of compression therapy. Recently, Belczak *et al.* (22) documented that some nutraceuticals, such as diosmin + hesperidin, aminaphthone and coumarin + troxerutin are able to improve the quality of life in patients with CVD.

Diosmin is a flavonoid (gamma-benzopyrone) that naturally present in citrus (23) and has vasotonic, antioedematous, lymphotropic, anti-inflammatory and antioxidant effects (24,25). Several studies documented the efficacy and the safety of diosmin in the treatment of CVD (i.e. venous disease, chronic venous insufficiency and haemorrhoidal disease) (26–30). The association of diosmin (450 mg) + hesperidin (50 mg) administered every 12 hours appears to have the greatest clinical benefits in patients with venous disease (31,32). Recently, several nutraceutical products with diosmin + hesperidin have been commercialised in Italy, and between these, a new nutraceutical is Axaven® an association between: diosmin 1000 mg, hesperidin 100 mg, rutin 300 mg, astaxanthin 5 mg, horse chestnut 50 mg, blueberry 160 mg and althea 100 mg. The effects of each component have been well-reported in literature; rutin is a flavonoid with effects on capillary permeability and oedema (swelling). It has been reported that oxerutins (derivatives of rutin) are able to improve symptoms and quality of life in patients with CVD (33,34). Similarly, in 2011 Giacalone *et al.* documented that blueberry containing polyphenols have antioxidant property (35). Astaxanthin is a potent antioxidant drug with a power that is 550-fold higher with respect to vitamin E and is able to reduce the effects reactive oxygen activation and of NF-kB (36). The primary active constituent found in horse chestnut seed extract is aescin, other constituents include bioflavonoids (quercetin and kaempferol), proanthocyanidin A2 (an antioxidant) and the coumarins fraxin and aesculin (37). Aescin from horse chestnut has been shown to have antioedematous, anti-inflammatory and venotonic properties that may be attributable to decreased vascular permeability (38). The effects of rutin have been reported in in vitro study where Li *et al.* documented its capacity to act on endothelial nitric oxide synthase. Trehalose is a non-reducing disaccharide in which two glucose units are linked by an α,α-1,1-glycosidic bond. Trehalose has protective action against reactive oxygen species and thanks to its safety, it may be used to treat chronic inflammation (39–41). Althea is a gastroprotective agent that could be used to reduce the gastrointestinal toxicity induced by the administration of previously described nutraceutics.

Therefore, the aim of this study is to evaluate the effects of Axaven® on both clinical and molecular parameters in patients with CVUs.

Key Messages

- chronic venous ulceration (CVU) is a complication of chronic venous disease (CVD). Pathophysiological events involved in CVU onset are inflammation, activation of polymorphonucleates (PMNs) and secretion of proteases, such as matrix metalloproteinases (MMPs), which, if altered, may affect the extracellular matrix (ECM), a complex network of macromolecules that is a support for vascular and tissutal wall
- bioflavonoids, such as diosmin and other compounds, appear to have several provessel function activities including anti-inflammatory, antioxidant and phlebotonic effects and are widely used in the treatment of CVD-related problems
- in this study, we evaluated the clinical and molecular effects of Axaven®, a new nutraceutical product, (an association between diosmin 1000 mg, hesperidin 100 mg, rutin 300 mg, astaxanthin 5 mg, horse chestnut 50 mg, blueberry 160 mg and althea 100 mg) in patients with CVU
- in this study, patients treated with Axaven® have shown not only an improvement in the clinical condition of the ulcers, but also a more noticeable reduction in the levels of MMPs, neutrophil gelatinase-associated lipocalin (NGAL) and inflammatory cytokines

Material and methods

We performed an open-label, parallel group study in four clinical centres, between January 2013 and December 2013. This study was approved by the Institutional Review Board – Independent Ethics Committee (IRB-IEC) of Interuniversity Center of Phlebolymphology – International Research and Educational Program in Clinical and Experimental Biotechnology – Headquarters at University Magna Graecia of Catanzaro and before the beginning of the study, all participants were informed about the aim, procedures, risks and benefits of the study and they gave an informed consent in writing.

Patients

Patients eligible for this study were of both sexes, older than 20 years with a clinical and instrumental diagnosis of venous ulcer, presence of venous reflux flow, ankle brachial pressure index (ABPI) > 0·5 and <0·8, ulcer duration > 6 weeks and

ulcer size 2·5–10 cm^2 and >50% granulation tissue on the wound bed.

Patients with diabetes mellitus, rheumatoid arthritis, malignancy, blood disorders, systemic disease, no current episode of ulceration, wound infection, ABPI < 0·5 (patients with severe arterial disease at presentation were considered for arterial imaging with a view to revascularisation) or > 0·8, an ankle pressure < 60 mm Hg, presence of necrotic tissue on the wound bed, use of medications that may impair wound healing, pain at rest, sensory loss (neuropathy), cardiac insufficiency and media calcinosis were excluded from the study.

CVUs are included in clinical–aetiology–anatomy–pathophysiology (CEAP) classification stage C6 (42); superficial and deep vein systems, severity of venous reflux were evaluated by duplex ultrasound and computed haemodynamic mapping, as previously described (8,43,44).

Experimental protocol

In all patients at the time of admission, the medical history was recorded and clinical examination, laboratory findings and duplex ultrasonography were performed. Blood samples were collected in all enrolled patients at the time of admission (T = 0) and at 1 month (T = 1), 4 months (T = 2) and 8 months (T = 3; end of the study) , in order to evaluate the plasma levels of MMPs and cytokines through enzyme-linked immunosorbent assay (ELISA) test. During the surgery, biopsies of the ulcers were taken and frozen (−80°C) for western blot evaluation of MMPs expression. Healing was assessed by means of direct ulcer tracing onto clear plastic sheet and subsequent computerised planimetry, as previously described (8,43,44). Healing was calculated by subtracting the final ulcer area from the initial area and dividing by the number of weeks that the patient had been observed to obtain the total area healed per week.

ELISA test

In order to evaluate the plasma levels of MMP-1, MMP-2, MMP-8, MMP-9, NGAL, vascular endothelial growth factor (VEGF), tumour necrosis factor (TNF)-alpha, interleukin (IL)-6 and IL-8, blood samples were collected at different times (see Experimental protocol) in agreement with our previous studies (11–13,16–20).

Western blot evaluation

Wounds were subsequently biopsied under a 1% lidocaine local anaesthesia and with full sterile precautions. The biopsy was made at a point equidistant from the centre and edge of the ulcer. Our experience with biopsies in this patient population indicates that it is well-tolerated by the subject and does not influence healing outcomes in venous ulcers. Biopsy was immediately placed into a sterile collection container and sent for quantitative (microbiology) culture.

The biopsies obtained at the time of wound bed preparation (T = 3) were lysed for Western blot analysis in 2 ml of tissue protein extraction reagent (25 mM Bicine, 150 mM sodium chloride pH = 7·6; Thermo Scientific; Cambridge, UK). Protein concentrations were determined and lysates were stored at 80°C. For Western blot, protein extracts were separated on a 12·5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes as previously described. Immunoblotting was performed using anti-MMP-1, MMP-2, MMP-8 and MMP-9 monoclonal antibodies as recently described, and results have been expressed as arbitrary units (11–13,16–20). All experiments were performed in triplicate.

Statistical analysis

All data are expressed as mean ± standard error medium (SEM). Student's *t*-test was performed in order to analyse the difference between each group with their control. Analysis of variance (ANOVA) was used to evaluate the differences between the groups. Differences identified by ANOVA were pinpointed by unpaired Student's *t*-test. The threshold of statistical significance was set at $P < 0{\cdot}05$. SPSS (SPSS Inc., Chicago, IL) software were used for the statistical analyses. We defined this study as exploratory, therefore we did not determine a power calculation. In this light, these results could only be labelled as exploratory.

Results

Patients

During the study period, 83 patients with CVUs of both sexes were enrolled and divided into two groups (Table 1):

Group A (treated group): 25 females and 19 males (median age is 67·7 years): standard treatment (compression therapy and surgical correction of superficial venous incompetence) + Axaven® once a day for 8 months (end of study) as adjunctive treatment. [Correction added on 20 March 2015, after first

Table 1 Characteristics of patients

Characteristic	Group A Patients treated with Axaven (%)	Group B Patients without Axaven (%)
Age range	48–82	48–87
Median age	67·7	65·2
Sex		
Male	19 (43·18%)	15 (38·46%)
Female	25 (56·81%)	24 (61·54%)
Familyhistory for Venous Disease	27 (61·36%)	24 (61·54%)
Venous insufficiency		
Superficial	11 (25%)	13 (33·33%)
Superficial and deep	33 (75%)	26 (66·67%)
Previous stripping or phlebectomy	18 (40·91%)	14 (35·90%)
Overweight (BMI, 25–29·9 kg/m^2)	28 (63·64%)	19 (48·72%)
Obesity (BMI, ≥30 kg/m^2)	9 (20·45%)	12 (30·77%)
Smoking	19 (43·18%)	29 (74·36%)
Arterial hypertension	27 (61·36%)	21 (53·85%)
Dyslipidemia	31 (70·45%)	23 (58·97%)
Mean length of ulcer (cm)	5·1	6·9
Ulcer area (cm^2)	10·2	10·5
Total	44 (100%)	39 (100%)

Table 2 Healing of CVUs

	Group A	Group B
Median ulcer area for each time point	9·08 cm^2 (T1); 6·34 cm^2 (T2)	9·92 cm^2 (T1); 7·18 cm^2 (T2)
Mean area healed/week (cm^2/week)	1·20 (T1); 1·4 (T2)	0·97 (T1); 1·17 (T2)

online publication: The number of patients, 36 patients (Group A: 9 females and 7 males; Group B 11 females and 9 males) were wrong and have been changed to 83 patients (Group A: 25 females and 19 males; Group B: 24 females and 15 males) in the results section.]

Group B (control group): 24 females and 15 males (median age is 65·2 years) treated with basic CVUs therapy (compression therapy and surgical correction of superficial venous incompetence (4,45–47) according to their clinical conditions.

All patients completed the follow-up. Compliance with elastic stockings was optimal in both groups and no side effects related to Axaven® treatment appeared in group A.

Wound healing

For wound healing evaluation, we considered high-healing ulcers as those with a healing speed rate $\geq 1\ cm^2$/week and slow-healing ulcers as those with healing speed rate $< 1\ cm^2$/week (Table 2).

Healing evaluation

Healing rates at 12 months (Table 2) was 83·80% for group A and 60·56% for group B. The mean total ulcer area rate of healing for group A was 1·3 cm^2/week and 0·87 cm^2/week for group B. Moreover, the recurrences of ulcers were significantly higher ($P < 0·01$) in group B with respect to group A, 59·15% and 26·76%, respectively.

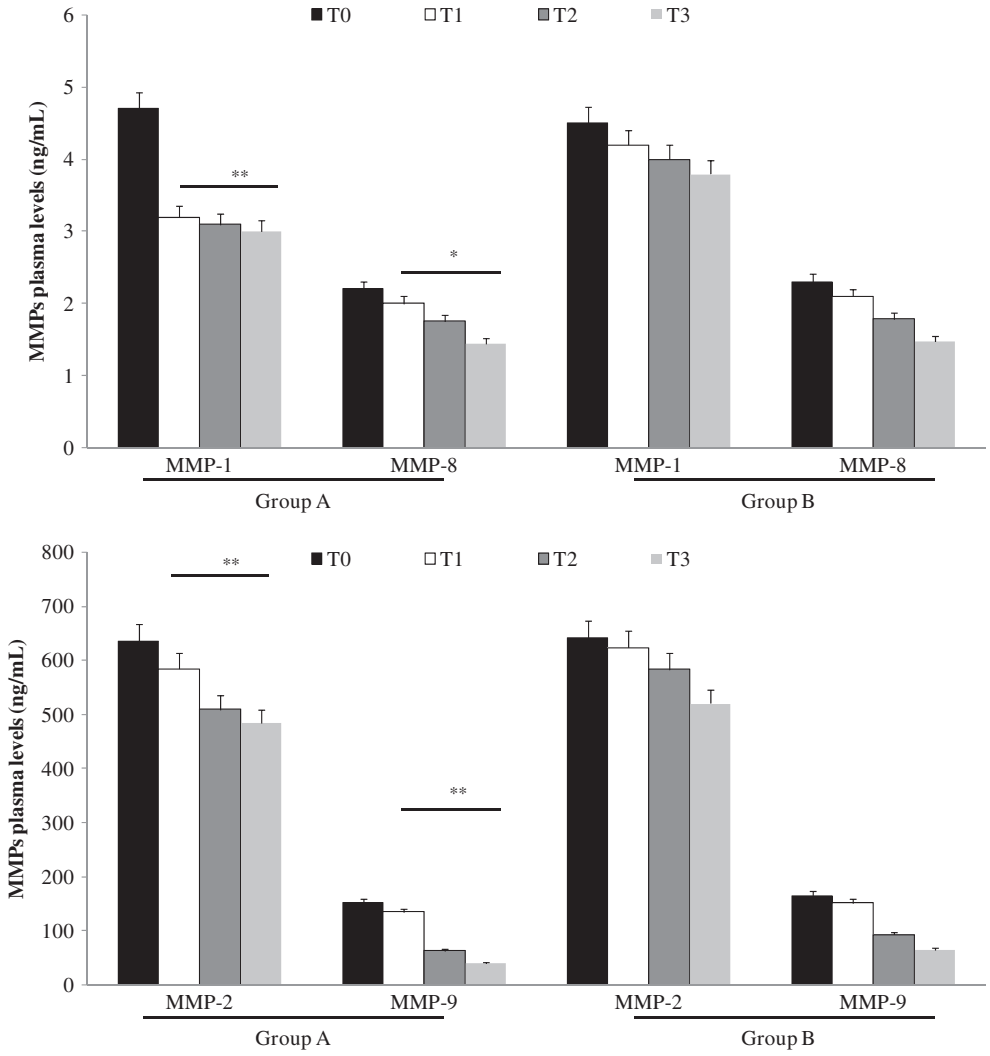

Figure 1 Enzyme-linked immunosorbent assay (ELISA) test evaluation of matrix metalloproteinase (MMP)-1, MMP-8 (up), MMP-2 and MMP-9 (down) plasma levels, at different times, in patients with chronic venous ulcerations (CVUs) treated (group A) or not (group B) with Axaven® once daily for 8 months (end of study). Data are expressed as mean ± SEM. $*P < 0·05$; $**P < 0·01$. T = 0: admission; T = 1: 1 month; T = 2: 4 months; T = 3: 8 months.

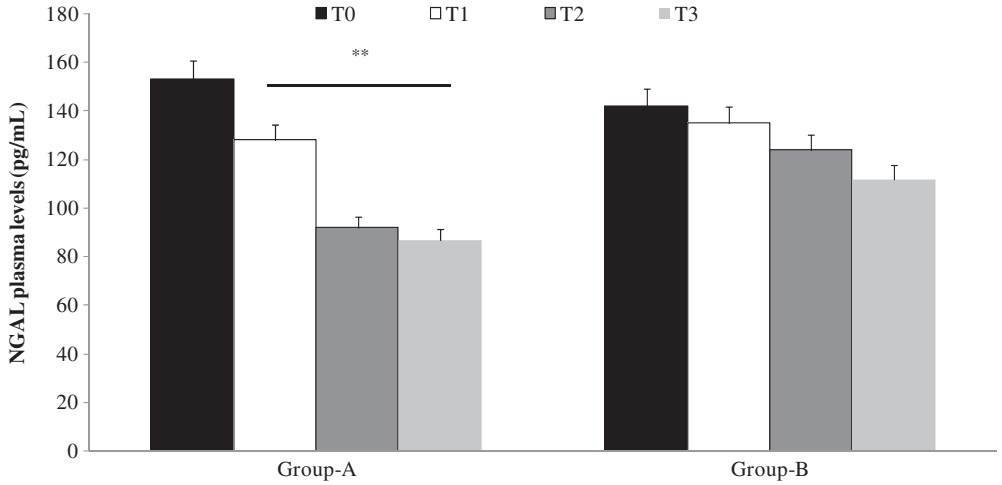

Figure 2 Enzyme-linked immunosorbent assay (ELISA) test evaluation of neutrophil gelatinase-associated lipocalin (NGAL) plasma levels, at different times, in patients with chronic venous ulcerations (CVUs) treated (group A) or not (group B) with Axaven® once daily for 8 months (end of study). Data are expressed as mean ± SEM. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$. T = 0: admission; T = 1: 1 month; T = 2: 4 months; T = 3: 8 months.

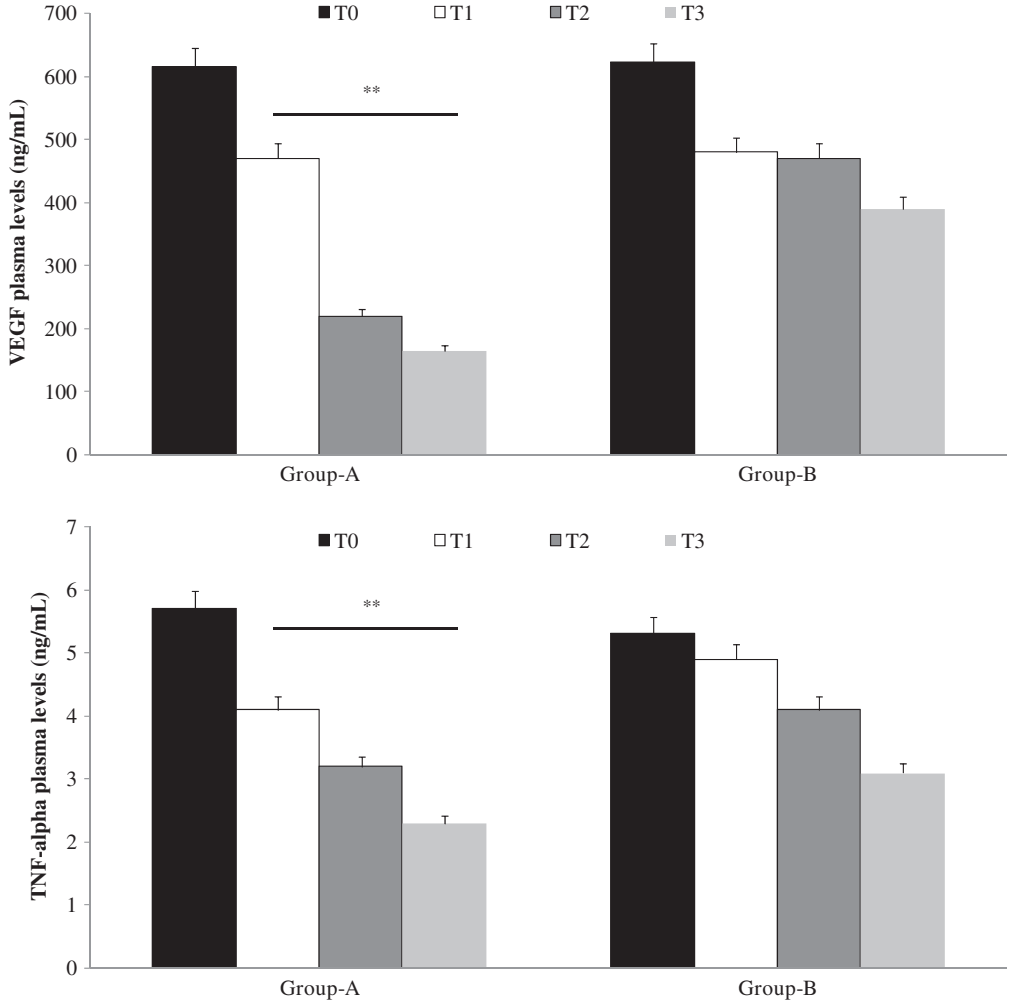

Figure 3 Enzyme-linked immunosorbent assay (ELISA) plasma evaluation of vascular endothelial growth factor (VEGF; up) and tumour necrosis factor (TNF)-alpha (down), at different times, in patients with chronic venous ulcerations (CVUs) treated (group A) or not (group B) with Axaven® once daily for 8 months (end of study). Data are expressed as mean ± SEM. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$. T = 0: admission; T = 1: 1 month; T = 2: 4 months; T = 3: 8 months.

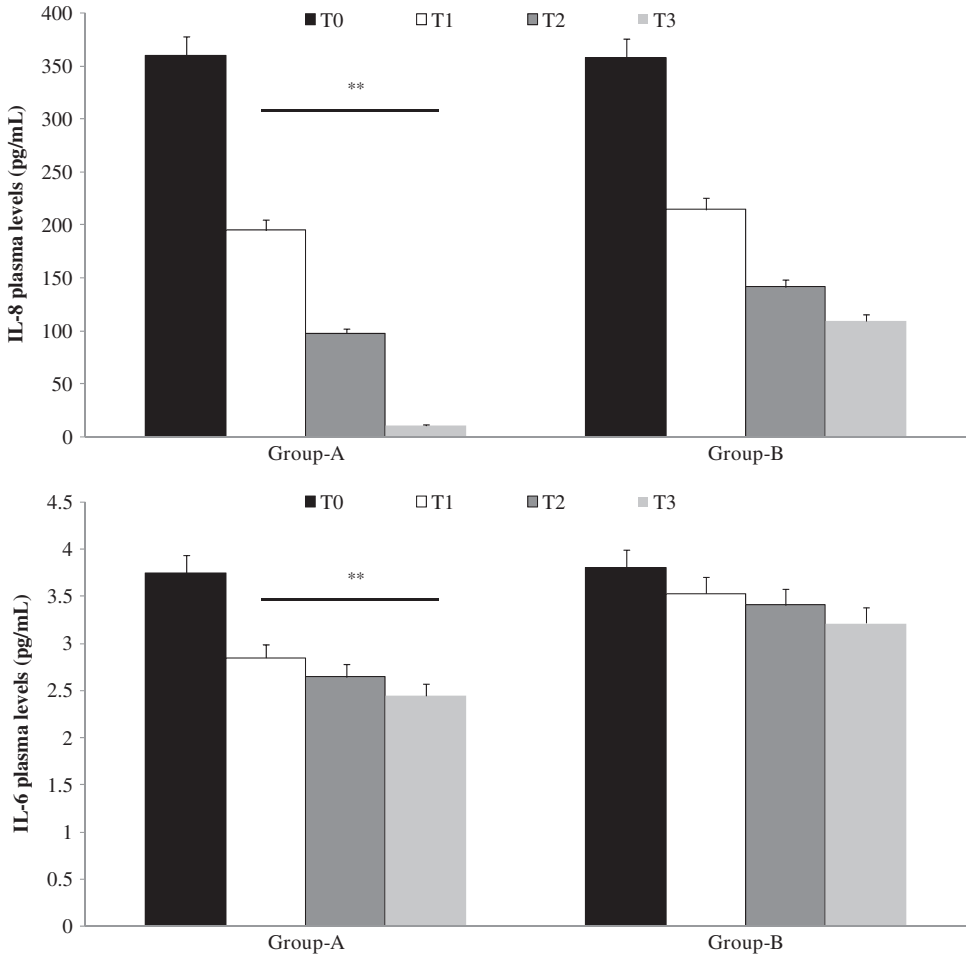

Figure 4 Enzyme-linked immunosorbent assay (ELISA) plasma evaluation of interleukin-8 (IL-8; up) and interleukin-6 (IL-6; down), at different times, in patients with chronic venous ulcerations (CVUs) treated (group A) or not (group B) with Axaven® once daily for 8 months (end of study). Data are expressed as mean ± SEM. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$. T = 0: admission; T = 1: 1 month; T = 2: 4 months; T = 3: 8 months.

ELISA test

Using ELISA test, we documented significantly lower levels of MMP-1, MMP-2, MMP-9, NGAL ($P < 0{\cdot}01$) and MMP-8 ($P < 0{\cdot}05$) in plasma fluid of patients treated with Axaven® (group A) with respect to not treated patients (group B; Figures 1 and 2) in a time-dependent pattern.

Moreover, ELISA test showed lower levels of IL-6, IL-8, VEGF and TNF-alpha in plasma fluid of patients with CVUs treated with Axaven® (group A) with respect to not treated patients (group B; Figures 3 and 4).

Western blot evaluation

Western blot analysis showed a lower expression ($P < 0{\cdot}01$) of MMP-1, MMP-2, MMP-9, NGAL ($P < 0{\cdot}01$) and MMP-8 ($P < 0{\cdot}05$) in patients treated with Axaven® (group A), with respect to not treated patients (group B; Figure 5).

Discussion

In this article, we evaluated the effects of a new nutraceutical in patients with CVUs. In agreement with literature data and with good clinical practice (48,49), all patients were treated with external compression. A previous meta-analysis of randomised prospective studies in 723 patients with venous ulcers documented that the administration of flavonoid fractions improved the effect of conventional treatment (compression and local care) (50).

Flavonoids are phenolic substances isolated from a wide range of vascular plants with antioxidant, anti-inflammatory, antimicrobial, antiviral, vasodilating and antiallergic properties (51–54). The most commonly used flavonoid is diosmin (30,5,7-trihydroxy-40-methoxyflavone-7-rutinoside), a naturally occurring O-rutinoside flavone (55), which may be obtained by dehydrogenation of the corresponding flavanone glycoside, hesperidin that is abundant in citrus (56). Both anti-inflammatory and antioxidant activity of diosmin appear to be related to the inhibition of production and release from leucocytes of chemical mediators of inflammation (histamine, bradykinin, serotonin and protease). The phlebotonic action is related to the inhibition of monoamine oxidases, the enzymes involved in the degradation of noradrenaline that increase and stimulate the alpha-1 adrenergic receptor inducing increase in blood pressure (57). Drug

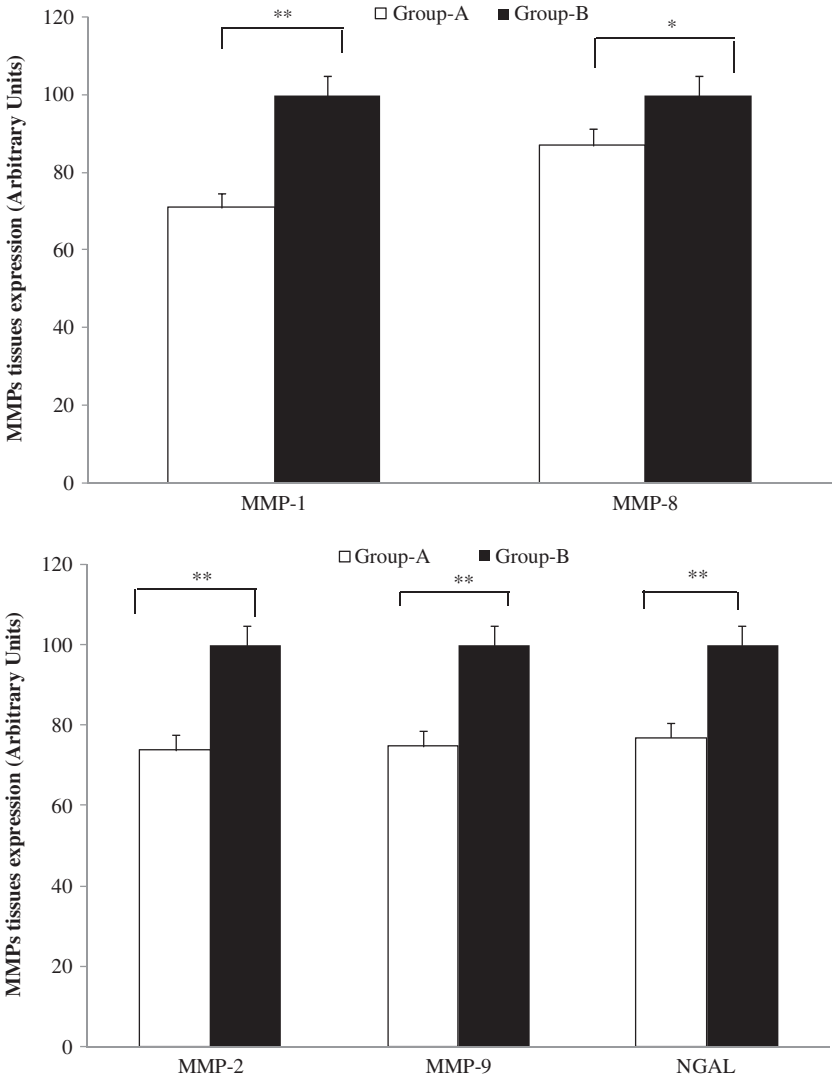

Figure 5 Western blot evaluation of matrix metalloprotease (MMP) expression in wound tissues taken at the time of surgery. MMP-1 and MMP-8 (up) and MMP-2, MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL; down) in patients with chronic venous ulcerations (CVUs) treated (group A) or not (group B) with Axaven® once daily for 8 months (end of study). Data are expressed as arbitrary unit, where the higher value has been considered as 100.

formulations currently in use are constituted by different concentrations of diosmin, which are added to other flavonoids of plant origin such as hesperidin or horse chestnut seed extract (32,50,58).

In our study, the administration of Axaven® in patients with CVUs was able to decrease inflammatory cytokines, MMPs and NGAL inducing an improvement of both symptoms with an increase of the speed of wound healing. According to literature and our previous studies, the involvement of growth factors (59,60) and leucocytes (13,16–19,61,62) in the development of venous ulceration has opened up new areas of investigation. Thus, elevated levels of ILs and VEGF in patients with CVUs in this study are attributable to the action of PMNs and the consequent inflammatory nature of the venous disease. Several studies have shown that activation of inflammatory cells alters the balance of the ECM, favouring the action of MMPs (11–13,16–20,60). Increased MMPs activity is associated with the pathophysiology of various diseases, especially in inflammatory diseases (63).

MMPs are enzymes able to degrade the basement membranes as well as the ECM, thus liberating VEFG and may be involved in wound healing. The results obtained from our study have shown elevated levels of MMPs, in particular MMP-1 and MMP-8 that are involved in delayed healing of venous leg ulcers (17) and MMP-2 and MMP-9 that are involved in the pathogenesis of the disease (11–13,16–20,60). However, patients treated with Axaven® have shown not only an improvement in the clinical condition of the ulcers, but also a more noticeable reduction in the levels of MMPs mentioned above. These effects are related to the action of single constituent other than diosmin + hesperidin. In particular, several studies have documented the effects of astaxanthin (3,30-dihydroxy-b,b-carotene-4,40-dione), present in a wide variety of plants, algae and sea foods (64), on oxidative damage and inflammation (65,66), whereas in vitro study have documented that blueberry is able to inhibit MMPs activity (67). Similarly, it has been documented that rutin derivates are able to decrease the activation of TNF-alpha and IL-8 (68). Therefore, in agreement with literature data we suppose that the effects of diosmin and classics bioflavonoids (rutin, hesperidin) increased the action of astaxanthin (69–71) and blueberry that are present in Axaven® with a greater decrease in the tissue expression of MMPs and in the levels of cytokines, MMPs and NGAL in plasma. NGAL is a protein belonging to the lipocalin family and

is expressed by activated neutrophils and has the ability to positively modulate the activity of MMP-9 in particular by forming the NGAL/MMP-9 complex, thus protecting MMP-9 from proteolytic degradation (18). Moreover, in our study we did not record any side effects that could be related to the presence of althea and the low dosage of other substance in the formulation. However, it is important to underline that the low time of observation (8 months) and the low number of patients enrolled may represent a limitation of this study.

In conclusion, we can affirm that Axaven® could represent a good choice in patients with CVUs, even if other studies in a large group of patients may be performed in order to confirm these observations.

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