



Effects of a new nutraceutical ingredient on allergen-induced sulphidoleukotrienes production and CD63 expression in allergic subjects

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ABSTRACT

Allergic diseases represent conditions affecting millions of individuals across the world. The objective of this study was to investigate the potential anti-allergic effects of a new nutraceutical ingredient, Pantescal® (Bionap, Italy), contained in different food supplements. Pantescal is a mixture of plant extracts, such as *Capparis spinosa*, *Olea europaea*, *Panax Ginseng* and *Ribes nigrum*.

The study was a randomized, double-blind, placebo controlled design. 60 patients allergic to common aeroallergens were chosen. Allergic patients were divided into two groups: one group was supplemented by Pantescal and the other, using a placebo formulation. Two in vitro tests were performed on blood samples taken from patients before and at 2 h, 2, 3 and 10 days after supplementation: cellular antigen stimulation test (CAST) was used to analyze the amount of sulphidoleukotrienes (SLT) production and flow-cytometric antigen stimulation test (FAST) to measure expression of basophil degranulation marker (CD63) was also performed. CAST showed that after 2 and 3 days, a slight decrease of SLT production was evident but only after 10 days did it become significant with a percentage of inhibition (P.I.)=43.3%. FAST revealed that there were no statistical differences for the first 2 days after supplementation although there was an inhibitory trend in the supplemented patients. CD63 expression was significantly reduced after 10 days (P.I.=64.8%).

This study suggests that Pantescal is effective in reducing allergic biomarkers such as CD63 protein and SLT in atopic subjects. The higher inhibitory effect on CD63 expression compared to SLT production allows us to hypothesize cell membrane stabilization as the main potential mechanism to explain the observed Pantescal protective effects.

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1. Introduction

Allergy can be defined as an immune-mediated hypersensitivity reaction to common environmental antigens that are otherwise harmless.

In allergic subjects, exposure to allergens leads to the formation of complexes between antigens and IgE bound to specific receptors on the surfaces of mast cells and basophils [1,2]. This leads to cell degranulation with subsequent release of histamine and *de novo* synthesis of several inflammatory mediators derived from arachidonic acid metabolism. Allergic rhinitis, urticaria, eczema and asthma are inflammatory disorders induced by IgE-mediated reactions following allergen exposure.

The main therapeutic approaches to allergy are aimed at inhibiting and controlling the mediators of the inflammatory response. Drugs currently used are histamine antagonists for the relief of symptoms, and corticosteroids for the suppression of allergic inflammation. However, long term therapy with anti-allergic drugs produces several side effects. For anti-histamines, typical adverse effects are sedation, psychomotor

impairment, cardiac toxicity and peripheral anti-cholinergic effects, such as dry mouth and urinary retention [3]. The major side effects associated with the use of corticosteroids are osteoporosis, decreased growth in children and metabolic disorders [4].

Given the central position the mast cell assumes in allergy, stabilization of the mast cell would be expected to be of therapeutic value, since the preservation of physiological cell membrane integrity prevents degranulation and subsequent release of histamine and other inflammatory mediators by mast cells and basophils. [5].

Among the substances able to prevent mast cell degranulation, chromones (nedocromil sodium and sodium cromoglicate) are used for the prevention and treatment of asthma, allergic rhinitis and conjunctivitis [6]. Chromones are frequently referred as “mast cells stabilizers” but their exact molecular target has not been identified yet. Moreover, in spite of positive actions in animal models, their effects on human mast cells are modest and require high concentrations [7].

In recent years there has been increasing interest in studying alternative approaches effective in the control of allergic symptoms, while minimizing the undesirable effects frequently associated with the current pharmacological therapies.

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Herbal medicines have been suggested to be of potential value for treating allergic diseases; the low incidence of side effects together with inevitable socio-economic advantages have prompted scientists to study the potential use of such remedies as new therapies against allergy.

Side effects associated with pharmacological therapies lead to a loss of social resources and increases in medical costs. The frequent use of natural products capable of decreasing allergic symptoms and improving the quality of life could prove advantageous for allergic patients when compared to conventional drug treatment.

Several natural extracts, and also some food ingredients, have been shown to be effective in reducing allergic symptoms. It has been reported that some Chinese herbal remedies are able to reduce allergic responses in murine models of asthma and food allergy [8,9]. An extract of *Petasites hybridus* has been shown to be effective in treating allergic rhinitis patients by decreasing the levels of inflammatory mediators [10,11]. Recently, *Perilla frutescens* leaf extract was studied for its anti-inflammatory and anti-allergic activities [12] and its oral supplementation showed beneficial effects in patients with seasonal allergic rhinoconjunctivitis [13]. Sylibin, a naturally occurring flavonoid, was shown to possess anti-allergic activities that could be ascribed to a membrane-stabilizing activity on human basophils [14]. Dietary supplementation with fish oil rich in omega-3 polyunsaturated fatty acids seems to have a role in preventing the development of allergic sensitization and airways disease [15] and in attenuating allergen-induced asthmatic reactions [16].

Pantescal® (Bionap, Italy) is a new nutraceutical ingredient contained in different food supplements available on the European and USA markets which are recognized for their beneficial effects in relieving allergic symptoms.

Pantescal is a mixture of plant extracts, such as *Capparis spinosa*, *Olea europaea*, *Panax Ginseng* and *Ribes nigrum*. *Olea europaea* extract is obtained from olive leaves and does not include any allergenic protein typical of olive pollen. The anti-allergic and anti-inflammatory properties of each of the extracts present in the mixture are reported in the literature [17–23].

The beneficial effect of Pantescal in allergic subjects could be ascribed to a synergistic action of the four plant extracts present in this nutraceutical. However, the exact action mechanism of Pantescal has yet to be clarified.

A recent report showed that *C. spinosa* extracts were effective against antigen challenge-induced and histamine-induced bronchospasm in an animal model of allergy. Moreover, the same extract was demonstrated effective in inhibiting histamine-induced skin erythema in human volunteers (Skin Prick Test) [17].

Ginsenosides isolated from *Panax Ginseng* exhibited an anti-allergic activity more potent than that of disodium cromoglycate, as revealed by a strong inhibitory activity on β -hexosaminidase release from rat basophil. The differential scanning calorimetry revealed that these compounds also demonstrated membrane-stabilizing activity. They also inhibited nitric oxide and prostaglandin E_2 production in lipopolysaccharide (LPS)-stimulated murine macrophages, suggesting an anti-inflammatory activity of ginsenosides [20].

Anti-allergic activity of *R. nigrum* are not supported by a large amount of literature, however this plant is commonly used in folk medicine for the control of allergic symptoms [19]. Recently, a polysaccharide derived from *R. nigrum* has been demonstrated to alleviate nasal-ocular symptoms, so it appears as a useful food supplement in assisting the treatment of Japanese cedar pollinosis. [21]. A recent paper reported the anti-inflammatory property of proanthocyanidins isolated from *R. nigrum* leaves. This activity was due to the ability of proanthocyanidins in reducing pro-inflammatory cytokines and endothelial adhesion molecules [22].

Phenols, contained in olive vegetation water and leaves, demonstrated having valid health properties, such as antioxidants and anti-inflammatory activities [23,24].

Biochemical and pharmacological activities of *C. spinosa* and *Olea europaea* are mainly ascribed to the presence of bioflavonoids and phenolic compounds such as quercetin, kaempferol, luteonin, apigenin and hydrotyrosol derivatives [25–27]. Flavonoids possess inhibitory activities on some key processes involved in allergic diseases, mainly chemical mediator release and cytokine production by mast cells and basophils [28,29].

The potential effectiveness on cell membrane stabilization has been reported both on Ginseng and *C. spinosa* [17,20]. These two extracts are present in Pantescal and hence we decided to measure two biomarkers involved in cell membrane-associated allergic events: sulphidoleukotrienes and CD63.

The aim of this study was to clarify the action mechanism of Pantescal on cells involved in allergic reaction, such as basophils, by *in vitro* evaluation of allergen-induced sulphidoleukotriene (SLT) release and CD63 expression. The analysis was carried out on blood samples from allergic patients treated with a food supplement containing Pantescal.

SLT are important inflammatory mediators derived from arachidonic acid metabolism and released mainly by basophils and mast cells. CD63 is a protein located inside the basophilic granule membrane. After basophil degranulation, CD63 is exposed on cell surface, evidencing the fusion between granules and plasma membrane, followed by release of inflammatory mediators, such as histamine.

To carry out the investigation, we used two *in vitro* diagnostic techniques: (i) cellular antigen stimulation test (CAST) which analyzes

Table 1

Clinical characteristic of allergic patients supplemented with Pantescal

Patient no.	Age	Gender	Antigen	Disease	Stage of disease	Prior therapy	Completed protocol
1	52	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
2	12	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
3	26	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
4	28	M	<i>Parietaria judaica</i>	AR	R	Ah	Y
5	32	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
6	35	M	<i>Parietaria judaica</i>	AR, AA	R	Ah	N
7	46	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
8	72	M	<i>Parietaria judaica</i>	AR	R	Al	Y
9	62	M	<i>Parietaria judaica</i>	AR	R	Ah	Y
10	44	M	<i>Parietaria judaica</i>	AR	R	Ah	Y
11	58	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
12	74	F	<i>Parietaria judaica</i>	AR, AA	R	Ah	Y
13	61	M	<i>Parietaria judaica</i>	AR	R	Ah	Y
14	26	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
15	32	F	<i>Parietaria judaica</i>	AR	R	Ah	N
16	15	M	<i>Parietaria judaica</i>	AR	R	Ah	Y
17	19	M	<i>Parietaria judaica</i>	AR, AA	R	Ah and Al	Y
18	18	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
19	46	F	<i>Graminaceae</i> mix	AR	R	Ah	Y
20	44	F	<i>Graminaceae</i> mix	AR	R	Ah	Y
21	26	F	<i>Graminaceae</i> mix	AR	R	Ah	Y
22	35	M	<i>Graminaceae</i> mix	AR	R	Ah	Y
23	31	M	<i>Graminaceae</i> mix	AR	R	Ah	N
24	19	F	<i>Graminaceae</i> mix	AR	R	Ah	Y
25	60	M	<i>Graminaceae</i> mix	AR	R	Ah	Y
26	58	M	<i>Graminaceae</i> mix	AR	R	Ah	Y
27	46	F	<i>Graminaceae</i> mix	AR	R	Ah	Y
28	42	M	<i>Graminaceae</i> mix	AR, AA	R	Ah and Al	N
29	44	F	<i>Olea europaea</i>	AR	R	Ah	Y
30	38	F	<i>Olea europaea</i>	AR	R	Ah	Y
31	40	F	<i>Olea europaea</i>	AR	R	Ah	Y
32	39	M	<i>Olea europaea</i>	AR	R	Ah	Y
33	51	M	<i>Olea europaea</i>	AR	R	Ah	Y
34	42	F	<i>Olea europaea</i>	AR, AA	R	Ah	Y
35	24	F	<i>Olea europaea</i>	AR	R	Ah	N

AR = allergic rhinitis, AA = allergic asthma, R = Remission, Ah = Anti-histamines, Al = Anti-leukotrienes, Y = yes, N = no.

the amount of SLT released by basophil activation, and (ii) flow-cytometric cellular antigen stimulation test (FAST), which measures the expression of a basophil degranulation marker (CD63).

2. Materials and methods

2.1. Subjects

The study was carried out using a randomized, double-blind, placebo controlled design.

Clinical data of allergic subjects enrolled in this study are summarized in Table 1 and in Table 2.

We enrolled 70 patients; 60, aged 14 to 73 years with a history of allergic rhinitis and/or asthma to environmental antigens, concluded the study. Ten patients were excluded either because they consumed anti-allergic drugs during the period of study or because they consumed the nutraceutical ingredient in an irregular manner. Only nine subjects were diagnosed with moderate asthma in association to rhinitis. Recurrence of symptoms was seasonal for all patients, with a peak from April to June, coinciding with the period of major pollination of different endemic plants in our zone. Patients were positive to serum test for specific IgE and skin Prick Test: 36 resulted sensitive to pollen from *Parietaria judaica*, 12 to pollen from *Olea europaea* and 12 to pollen from graminaceae. At the time of recruitment all subjects were in a state of clinical remission for two months on average, and were not receiving any pharmacological treatment for at least a month. All participants were fully informed regarding the experimental procedures, and written consent was

Table 2
Clinical characteristic of allergic patients supplemented with placebo

Patient no.	Age	Gender	Antigen	Disease	Stage of disease	Prior therapy	Completed Protocol
1	24	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
2	18	F	<i>Parietaria judaica</i>	AR	R	Ah	N
3	36	M	<i>Parietaria judaica</i>	AR	R	Ah	Y
4	57	M	<i>Parietaria judaica</i>	AR	R	Al	Y
5	73	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
6	62	M	<i>Parietaria judaica</i>	AR	R	Ah	Y
7	44	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
8	35	M	<i>Parietaria judaica</i>	AR	R	Ah	Y
9	19	M	<i>Parietaria judaica</i>	AR	R	Ah	Y
10	15	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
11	26	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
12	32	F	<i>Parietaria judaica</i>	AR, AA	R	Ah and Al	Y
13	34	F	<i>Parietaria judaica</i>	AR	R	Ah	Y
14	51	M	Graminaceae mix	AR	R	Ah	Y
15	59	M	Graminaceae mix	AR	R	Ah	Y
16	70	F	Graminaceae mix	AR	R	Ah	Y
17	65	F	Graminaceae mix	AR	R	Al	Y
18	22	F	Graminaceae mix	AR, AA	R	Ah	N
19	59	M	Graminaceae mix	AR	R	Ah	N
20	14	F	Graminaceae mix	AR	R	Ah	Y
21	21	M	Graminaceae mix	AR	R	Ah	Y
22	26	F	Graminaceae mix	AR, AA	R	Ah	Y
23	51	F	Graminaceae mix	AR	R	Ah	Y
24	64	M	Graminaceae mix	AR	R	Ah and Al	Y
25	25	M	Graminaceae mix	AA	R	Ah	Y
26	22	F	<i>Olea europaea</i>	AR	R	Ah	N
27	36	F	<i>Olea europaea</i>	AR	R	Ah	Y
28	34	F	<i>Olea europaea</i>	AR	R	Ah	Y
29	41	M	<i>Olea europaea</i>	AR	R	Ah	Y
30	38	M	<i>Olea europaea</i>	AR	R	Ah	Y
31	54	M	<i>Olea europaea</i>	AR	R	Ah	Y
32	25	M	<i>Olea europaea</i>	AR	R	Al	Y
33	38	F	<i>Olea europaea</i>	AR, AA	R	Ah	N
34	49	F	<i>Olea europaea</i>	AR	R	Ah	Y
35	62	F	<i>Olea europaea</i>	AR	R	Ah	Y

AR = allergic rhinitis, AA = allergic asthma, R = Remission, Ah = Anti-histamines, Al = Anti-leukotrienes, Y = yes, N = no.

Table 3

Basophil SLT release and CD63 expression in treated allergic subjects (TAS) and placebo group at different supplementation times

Efficacy parameter	Time	TAS (n=30)	Placebo (n=30)	% of inhibition
SLT release (pg/ml)	T0	874.7±40.1	848.1±43.4	–
	2 h	872.8±41.4	844±44.8	0.21
	2 days	743.3±31.3	843.2±42.6	16.1
	3 days	690±26.1	843.6±41.8	21.1
	10 days	499.1±23.6§	850.4±43.4	43.3
CD63 expression (% activated basophils)	T0	32.8±2.4	27.1±1.9	–
	2 h	32.4±2.4	27.7±1.9	1.2
	2 days	28.1±2	27.3±2	14.3
	3 days	21.9±1.4*	27.7±2	33.2
	10 days	11.5±1.5**	28.6±2	64.8

Data are expressed as the mean±S.E.M. n=number of subjects.

*p<0.05 as compared with placebo, T0, T2h and T10d, **p<0.01 as compared with placebo, T0, T2h and T2d.

§p<0.01 as compared with placebo, T0, T2h and T2d.

obtained. This study was approved by our Institutional Ethical Committee in accordance with Helsinki Declaration. Experimental protocols were conducted under GMP regulations in force.

2.2. Supplementation

To evaluate the effects of Pantescal, on CD63 expression and SLT release, we used a commercial product (Xineall™, Novartis) available on the Italian market. This food supplement contains 1 g of Pantescal ingredient in each sachet. Prior to giving the product to subjects, we transferred the powder of Xineall into unlabelled sachets similar to sachets that contained only a placebo formulation made up of sweeteners and additives but deprived of the active ingredient Pantescal. The sachets were coded numerically and alphabetically, and then distributed to patients.

After the initial diagnostic tests, allergic subjects were divided at random into 2 groups (placebo and treated) of 35 patients; treated subjects received Xineall and control subjects received the placebo formulation. Supplementation was performed by administration of 2 sachets/day as indicated by producer, upon awakening, for ten days. Five patients from each group dropped out for the above mentioned criteria, hence we completed the protocol on only 60 subjects.

2.3. Analysis

Peripheral blood samples were collected in Vacutainer® tube pre-treated by EDTA-K₃. Samples from each subject (treated and placebo) were taken before the first supplementation (T0), 2 h after first

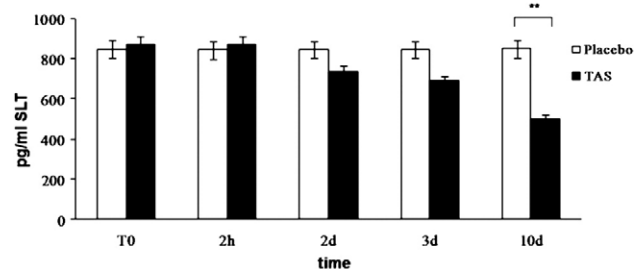


Fig. 1. Comparison of time-dependent SLT release after allergen stimulation of basophils between placebo and treated allergic subjects (TAS) at different times of Pantescal supplementation: 2 h (2 h) after supplementation it shows no effect on SLT release compared to starting time before supplementation (T0). Two days (2d), 3 days (3d) and 10 days (10d) after supplementation a gradual decrease in SLT production with respect to T0, and in TAS versus placebo was observed. A significant reduction of SLT release was observed after 10 days. Data are expressed as the mean±S.E.M. of 30 patients from each group. **p<0.01.

supplementation (T2h), and subsequently two days (T2d), three days (T3d) and ten days (T10d) after daily Pantescal and placebo supplementation. CAST and FAST were performed on all samples. All reagents were obtained from CAST®-2000 ELISA and FLOW-CAST® analysis kits (Bühlmann Laboratories-AG, Schönenbuch, Switzerland). The assays were performed strictly in accordance with manufacturer's procedural notes.

2.4. Antigen specific sulphidoleukotriene production (CAST)

Whole blood in EDTA was mixed with a dextran solution for 90 min at room temperature (RT) to obtain erythrocyte sedimentation. The plasma fraction, containing leukocytes and thrombocytes, was transferred into another tube, centrifuged (130 g, 15 min, RT) and the supernatant was discarded. The pellet was resuspended in a stimulation buffer (SB) containing IL-3. A fraction of this suspension of cells was used to evaluate background levels of SLT released from leukocytes, a second fraction was added to a solution of anti-IgE receptor antibody (anti-FcεRI), mimicking the cross-linking of receptors mediated by allergen binding, as a positive control for cell viability and functionality. Finally, a fraction of the suspension was added to the solution of allergen extract to which the subject was responsive, to evaluate the specific SLT production response. Allergen extracts, *Parietaria officinalis*, *Olea europaea*, graminaceae, were supplied by Bühlmann Laboratories-AG (Schönenbuch, Switzerland). After an incubation of 40 min, at 37 °C, the tubes were centrifuged (1000 ×g, 3 min, 2–8 °C) and the supernatant harvested for immediate analysis or stored at –20 °C. SLT concentrations in cell-free supernatants were then determined by an ELISA procedure and were expressed as pg SLT/ml.

2.5. Flow-cytometric cellular antigen stimulation test (FAST)

Leukocytes were collected as described for CAST analysis. Similarly to CAST, we prepared a tube with SB to evaluate background levels of the gp53 expression on basophils, a tube containing anti-FcεRI Ab as a positive control and one tube for the antigen to which the subject was responsive, to evaluate the specific degranulation response of basophils to allergens. Leukocyte suspensions were incubated at 37 °C for 40 min, then the degranulation process was stopped with a blocking buffer (BB).

A mix of anti-human-CD63 and anti-human-IgE monoclonal antibodies, labelled with phycoerythrin (PE) and fluorescein isothiocyanate (FITC) respectively, was added to each tube and cells were allowed to rest at 2–8 °C for 30 min. Erythrocytes were destroyed by adding a lysing solution for 5 min at room temperature. Samples were then centrifuged (1000–1200 ×g, 5 min), and the leukocyte pellet was resuspended in BB and analyzed within 2 h by flow-cytometry performed at 488 nm on a Epics XL-MCL equipped with a 15 mW argon-ion laser (Beckman Coulter, Miami, Florida) and analysed by Expo32 software. The percentage of CD63-expressing cells was determined by

evaluating the percentage of cells that exhibit on surface both fluorochromes (PE and FITC) that indicate the coexpression of adherent IgE and gp53 protein. The percentage of cells expressing CD63 obtained by stimulation with allergens was compared to the cells expressing this marker after incubation only with SB serving as negative control.

3. Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) followed by Tukey–Kramer test for statistical significance. All data in the table and figures are reported as mean ± SEM.

P-values ≤ 0.05 were considered statistically significant.

4. Results

4.1. Effects of Pantescal administration on SLT release

In the CAST assay, results of allergen stimulation were considered positive for values of 250 pg SLT/ml above background. As shown in Table 3 and Fig. 1, both treated subjects and placebo showed a positive CAST after stimulation of basophils with all allergens tested. The mean Patient Background (PB) level and S.E.M. of SLT concentration in treated group and placebo group were respectively 319.94 ± 35.57 pg/ml and 322.18 ± 37.42 pg/ml. The mean Positive Control (PC) level of SLT concentration observed in both treated and placebo groups, was superior to 2000 pg/ml, that is above the absorbance limit able to be measured by our instrument. At starting time (T_0), the mean and S.E.M. of SLT values in treated and in placebo group were 874.7 ± 40.1 pg/ml and 848.1 ± 43.4 pg/ml, respectively. Two hours after food supplement administration, SLT levels were not significantly different from T_0 values, indicating a lack of effect after a short time. After 2 and 3 days, a trend towards decreased SLT production was already evident, with a percentage of inhibition (P.I.) of 16.1% and 21.1%, respectively, but only after 10 days was a significant reduction of SLT observed (P.I. = 43.3%) (Table 3). The placebo group did not show any notable variation in SLT release at any time after supplementation (Fig. 1, Table 3).

4.2. Effects of Pantescal administration on basophil CD63 expression

In the FAST assay, a percentage of CD63 expressing cells equal to or greater than 10% was considered positive. A typical plot obtained from one of the subjects examined is shown in Fig. 2.

The mean Patient Background (PB) percentage and S.E.M. of CD63 expression in treated group and in placebo group were respectively $3.27 \pm 0.36\%$ and $2.98 \pm 1.02\%$, whereas the mean Positive Control (PC) percentage and S.E.M. of CD63 expression observed in treated and placebo groups was respectively $29.59 \pm 2.57\%$ and $32.40 \pm 2.89\%$. At starting time (T_0), the mean and S.E.M. of CD63-expressing cells in treated and placebo group were $32.8 \pm 2.4\%$ and $27.1 \pm 1.9\%$, respectively (Fig. 3 and Table 3), confirming the allergic status of all subjects.

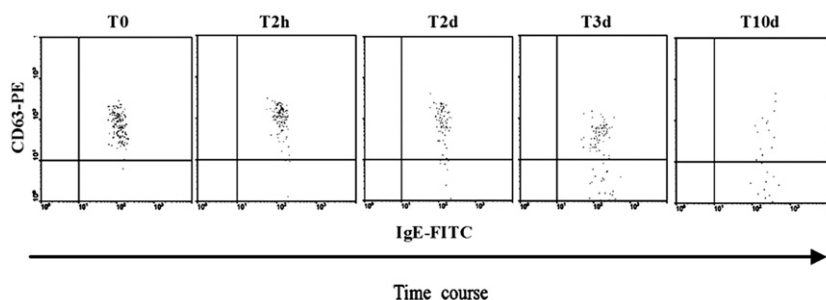


Fig. 2. Dot plot of basophil CD63 expression of a patient at different times (0, 2 h, 2, 3 and 10 days) of Pantescal supplementation (time course). Flow-cytometric analysis shows that percentage of cells marked by CD63-PE and IgE-FITC decreases in relation to Pantescal supplementation.

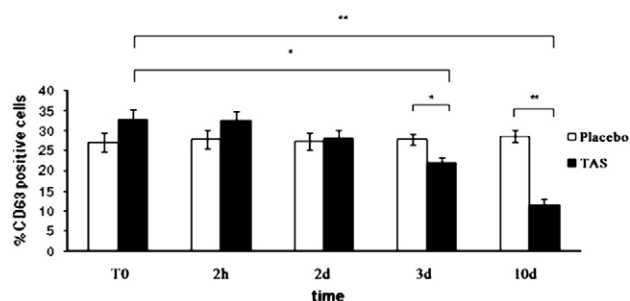


Fig. 3. Comparison of time-dependent CD63 protein expression on basophil cell membrane after allergen stimulation between placebo and treated allergic subjects (TAS) at different times of Pantescal supplementation: starting time before supplementation (T0), 2 h (2 h), 2 days (2 d), 3 days (3 d) and 10 days (10 d) after supplementation. Basophil CD63 expression did not show variation at 2 h from first supplementation, but after 2 d and 3 d, a decrease in CD63 expression was observed, and after 10 d a very significant decrease was evident compared with T0 and placebo. Data are expressed as the mean $\pm$ S.E.M. of 30 patients from each group. * $p < 0.05$, ** $p < 0.01$.

There were no statistical differences for the first 2 days after supplementation although there was an inhibitory trend in the supplemented patients. The inhibitory percentage after 2 h was 1.2% and after 2 days 14.3%. CD63 expression was significantly reduced after 3 days (P.I. = 33.2%) and further after 10 days (P.I. = 64.8%).

The control group supplemented with placebo did not show variations in CD63 expression at any time (Fig. 3, Table 3).

5. Discussion

The effects of Pantescal supplementation were monitored by CAST and FAST, two techniques commonly used to analyze basophil activation in allergic subjects. Basophil degranulation is a primary event in allergic diseases and leads to release of preformed inflammatory mediators, such as histamine and tryptase, and to the synthesis of newly generated lipid mediators, such as sulfidoleukotrienes [30,31].

CAST assay measures the amount of SLT, important inflammatory mediators produced and released by basophils and mast cells from arachidonic acid metabolism. CAST is a useful diagnostic tool for IgE and non-IgE dependent allergies [32,33].

FAST is used to monitor basophil degranulation upon allergen challenge by detecting surface expression of CD63 [34,35], a protein located inside the granule membrane in resting basophils. Following basophil degranulation, CD63 is exposed on the cell surface, showing the fusion between granules and plasma membrane and the release of inflammatory mediators. Recently, FAST has become a widely used test for the diagnosis of IgE and non-IgE-mediated reactions and it is also a reliable experimental approach for pharmacological studies [36–38].

The results obtained for the allergic subjects supplemented with placebo did not show any significant difference in both CD63 and SLT values at any time after supplementation, compared to starting values. In treated subjects, a short time after supplementation (2 h and 2 days), there was no significant variation in both tests. A notable decrease of CD63 values was observed after 3 days (P.I. = 33.2%) and it was more pronounced after 10 days, with a percentage of inhibition of 64.8% (Table 3). Regarding the values obtained in CAST, a significant reduction of allergen-induced SLT was evident after 10 days (P.I. = 43.3%). The inhibition of SLT release was lower compared to inhibition of CD63 expression and it became significant only after 10 days. These results suggest that the main effect of Pantescal could be due to a membrane-stabilizing activity rather than inflammatory mediator inhibition.

Pantescal is the main ingredient of some food supplements available on the Italian market which have been reported to be effective in counteracting allergic symptoms in subjects sensitive to common

aeroallergens. However, the mechanism of action of Pantescal has yet to be elucidated.

The effectiveness of Pantescal observed in this study might be explained by the anti-allergic and anti-inflammatory activities of the compounds contained in the food ingredient.

A lyophilized extract obtained from *C. spinosa*, the main constituent of Pantescal topically applied, has been reported to be effective against histamine-induced skin erythema in healthy human volunteers [17]. *C. spinosa* contains mainly flavonoids such as quercetin, kaempferol, luteolin and apigenin [17,18,25,26]. Luteolin, apigenin and quercetin are the most active flavonoids with anti-allergic properties [42]. They have been shown to inhibit histamine secretion [28], IL-4 and IL-13 synthesis [39,40] and CD40 ligand expression in basophils and mast cells [41]. These events were associated with a strong inhibition of Ca^{2+} influx and of enzymatic membrane activity [42,43]. These results suggest that the inhibition of inflammatory mediator release induced by luteolin and quercetin in human mast cells could be related to a membrane-stabilizing effect. In support of this hypothesis, it was demonstrated that flavonoids cause a modifying effect on phospholipids membranes, suggesting an interaction by flavonoids with both lipid and protein components of biological membranes and consequent modification of membrane dependent regulation mechanisms [44].

Extracts of *Panax Ginseng* and *R. nigrum*, which are known to possess anti-allergic activities, are also present in Pantescal. Ginseng extracts have been shown to exhibit anti-allergic effects originating from a potent cell membrane-stabilizing activity [20,45]. Blackcurrant (*R. nigrum* L.) is used for the control of allergic symptoms [19,21].

Data obtained in this study, along with the known properties of each extract contained in Pantescal, allow us to suggest that stabilization of basophil/mast cell membrane could be a possible mechanism underlying the action of Pantescal, leading to a lower responsiveness of cells involved in allergic processes towards aeroallergens. The physiological reinforcement of biological membranes induced by supplementation could prevent basophil/mast cell degranulation and subsequent release of inflammatory mediators. At the same time, it is reasonable to suppose that this increased cell stability might reduce availability of membrane lipids for synthesis of pro-inflammatory mediators such as SLT. Therefore, the reduced release of SLT shown by CAST test could be a secondary effect subsequent to the increased membrane stability induced by Pantescal.

Our hypothesis is supported by a large amount of evidence that points to herbal extracts, and also some foods, as useful remedies to improve natural defences against allergic diseases [46]. Other food ingredients seem to possess the same ability in increasing cell membrane stability as that demonstrated by Pantescal. A garlic extract has been shown to preserve structure and function of erythrocytes by cell membrane stabilization [47], and apple polyphenols have been suggested to prevent mast cell and basophil degranulation by promoting the stability of cell membranes in allergic subjects [48,49].

In conclusion, Pantescal was well tolerated and effective in reducing allergen-induced biomarkers such as CD63 protein and SLT in allergic subjects. The inhibition of CD63 expression was higher compared to inhibition of SLT release and both effects were time-dependent, but CD63 protein inhibition was already significant after 3 days, whereas SLT reduction became significant only 10 days after supplementation.

Recognized “mast cells stabilizers” such as chromones require a long period of time to demonstrate their beneficial effects in allergic patients compared to drugs such as histamine antagonists and corticosteroids [50,51]. Our hypothesis, therefore, is that the delayed onset of the effectiveness of Pantescal could be ascribed to the same mechanism underlying chromones action, such as the improvement of the physiological membrane stability.

However, we can not exclude that other mechanisms of action could be involved in the effectiveness of the nutraceutical ingredient. Further studies and clinical trials are needed to confirm the usefulness

of Pantescal in the treatment or in the prevention of allergic diseases, and to clarify its action mechanism.

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