

A new standardized phytoextract from red orange and lemon wastes (Red orange and Lemon Extract) reduces basophil degranulation and activation.

Supplementary Material

Massimo Caruso^{a, b*}, Simona Fabroni^c, Rosalia Emma^a, Gabriele Ballistreri^c, Margherita Amenta^c, Walter Currenti^a, Calogero Rinzivillo^d and Paolo Rapisarda^c

^a *Department of Biomedical Sciences and Biotechnology (BIOMETEC) – University of Catania, Via S. Sofia 97, 95123, Catania, Italy*

^b *Department of Clinical and Experimental Medicine (MEDCLIN) – University of Catania, Via S. Sofia 78, 95123, Catania, Italy*

^c *Council for Agricultural Research and Economics (CREA) - Research Centre for Olive, Citrus and Tree Fruit, Corso Savoia 190, 95024, Acireale, Italy*

^d *Clinical Nutrition & Integrated Supportive Therapy-Department of Medical-Surgical Sciences & Advanced Technology – Hospital University “Policlinico-Vittorio Emanuele” – Catania*

*Corresponding author

Massimo Caruso

Department of Biomedical Sciences and Biotechnology (BIOMETEC) – University of Catania, Via S. Sofia 97, 95123, Catania, Italy

mascaru@unict.it

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Abstract

Citrus fruits are rich sources of bioactive compounds and their consumption is associated to health-promoting effects. Citrus processing wastes contain bioflavonoids and other high added value compounds. The aim of this study was to evaluate the antiallergic properties of a new phytoextract obtained by citrus wastes and peels. Blood orange and lemon processing wastes were used to produce a Red orange and Lemon Extract (RLE). Blood samples from 30 allergic donors were collected and used to evaluate the basophil activation (CD203c) and degranulation (CD63) by stimulation through allergen with and without the RLE. Reduced basophil expression of CD203c and CD63 were observed in RLE+Allergen treated samples, with -20.21% of CD203c expression ($p < 0.0001$) and -54.11% of CD63 expression ($p < 0.0001$), compared to Allergen treated samples. The RLE evidenced a good antiallergic activity, mainly acting on basophils degranulation, and therefore reducing the key event of pro-inflammatory mediators release after allergic stimuli.

Keywords: Citrus fruit; Red Orange; Lemon; antiallergic; CD63; CD203c.

Experimental

Chemicals and Reagents

Antiallergic activity was tested on a new standardized powder phytoextract containing anthocyanins and other polyphenols, obtained by a patented extraction process (Italian Patent No. 102017000057761) from blood orange and lemon processing wastes (Red orange and Lemon Extract, here named RLE). The standardized extract was realized in the laboratories of CREA-OFA (Acireale, Italy) for research purposes only. The

patented process for the preparation of the extract involves an absorption phase on adsorbent polymeric resin followed by the elution with hydro-alcoholic solutions. After distillation and recovery of alcohol (ethanol), the concentrated aqueous extract was subjected to spray drying process to obtain the dry powdered extract. Cyanidin 3-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-rutinoside, cyanidin 3-sophoroside, delphinidin 3-glucoside, delphinidin 3,5-diglucoside, peonidin 3-glucoside, pelargonidin 3-glucoside, pelargonidin 3,5-diglucoside, malvidin 3-glucoside, hesperidin, narirutin, and didymin were purchased from Extrasynthèse (Genay, France). All other chemicals were of analytical grade. Solvents for chromatography were HPLC grade (Merck KGaA, Darmstadt, Germany). All the other chemicals used in the study were of analytical grade and were purchased on the market. Basophils activation/degranulation were studied by Allergenicity Kit (Beckman Coulter Inc., California, USA) and Basoflow-Ex (Exbio Praha, Czech Republic). Allergens used to stimulate basophils were obtained by BIAL (BIAL Aristegui, Bilbao, Spain).

Determination of total anthocyanins content

Total anthocyanin content of extract was assayed by the pH differential method (Rapisarda et al. 2000). The extract was diluted in water and the absorbance of diluted extract was measured at 520 nm with a UV–Vis spectrophotometer (Varian Cary 100 Scan, Palo Alto, CA, USA). Total anthocyanins content was expressed as g of cyanidin 3-glucoside equivalents (CGE)/100 g of powder extract.

Purification and HPLC-PDA-ESI/MSⁿ analysis of anthocyanins

The extract was diluted in water for HPLC analysis of anthocyanins. Aqueous extract was loaded onto C18 Bond Elut SPE cartridges (Varian Inc., Palo Alto, CA, USA) pre-washed in methanol, and then pre-equilibrated in water. Anthocyanins were adsorbed

onto the C18 Bond Elut column; while other soluble compounds were removed by washing the cartridges with water. Anthocyanins were eluted with methanol containing 1% formic acid. The acidified methanol solutions were evaporated to dryness, and then the dry fractions dissolved in 7% aqueous formic acid. Samples were filtered through a 0.45 μm membrane filter (Albet, Barcelona, Spain) and injected into the HPLC-MSⁿ chromatographic system for identification of individual anthocyanins.

Separation of anthocyanins was conducted on a Chromolith Performance RP-18 end-capped column (100 x 3.0 mm i.d., monolithic particle size; Merck KGaA, Darmstadt, Germany) using an Ultra-Fast HPLC system coupled to a photodiode array (PDA) detector and Finnigan LXQ ion trap equipped with an electrospray ionization (ESI) interface in series configuration (Thermo Electron, San Jose, CA, USA). Anthocyanins were determined by HPLC according to a previous method (Fabroni et al. 2016). A binary gradient of water with 7% formic acid and methanol was used, and the flow rate was 300 $\mu\text{l}/\text{min}$. The column effluent was monitored at 520 nm. MS parameters were: spray voltage 5.00 kV, capillary temperature 275 °C, capillary voltage 19 V. A sheath gas flow rate of 30 arb (arbitrary units) was applied, and auxiliary and sweep gases were set at 15 and 4 arb, respectively. Preliminary positive tunings were carried out with direct injection of dilute solutions of cyanidin 3-glucoside at a flow rate of 5 $\mu\text{l min}^{-1}$. Voltage of the optical lenses was optimized using TunePlus Xcalibur v. 2.0.7 software. MS full-scan acquisition (scan range 100-2000 m/z) was first performed in positive ion mode. Selected peaks were isolated in the ion trap and fragmented by MSⁿ scan acquisition. The MSⁿ spectra were obtained using a collision energy of 17% of maximum, with an isolation width (m/z) of 1.5. Anthocyanins were identified using retention time (t_R), MS and MSⁿ data in positive ion mode, and also by comparison of MS data with those of pure standards and/or literature values. The relative compositions

(%) of individual anthocyanins were calculated from peak areas at 520 nm, using Xcalibur v. 2.0.7 software.

HPLC-PDA-ESI/MSⁿ analysis of flavanones

Flavanone glycosides, expressed as hesperidin equivalents (g/100 g of powder extract), were determined by HPLC (Rouseff et al. 1987) using the HPLC-PDA-ESI/MSⁿ equipment described above. A sample of extract was dissolved in dimethyl sulfoxide and after diluted with the mobile phase, filtered through a 0.45 µm membrane filter (Albet, Barcelona, Spain), and then injected directly into the column. The eluent used was water:acetonitrile:acetic acid (79.5:20:0.5), and the flow rate was 800 µl/min. The column effluent was monitored at 280 nm. MS parameters were the same described for anthocyanins analysis. Preliminary negative tunings were performed with direct injection of the diluted solutions of hesperidin at a flow rate of 5 µl/min, and the voltages of the optical lenses were optimized by using TunePlus Xcalibur v. 2.0.7 software. MS full-scan acquisition (scan range 100–2000 m/z) was first performed in negative ion mode. Next, the chosen peaks were isolated in the ion trap and fragmented by an MSⁿ scan acquisition. The MSⁿ spectra were obtained by using a collision energy of 17% of instrument maximum, operating with an isolation width (m/z) of 1.5. Flavanones were identified using retention time (t_R), MS and MSⁿ data in negative ion mode, and also by comparison of MS data with those of pure standards and/or literature values.

Allergic Donors

Allergic donors of blood samples (n=30; age 20-45) were selected on the basis of their sensitivity to environmental allergen (Parietaria and grass pollen) confirmed by a positive Skin Prick Test (wheal diameter >3 mm). Donors did not take allergen specific immunotherapy (ASIT) and/or biologic drugs (e.g. Omalizumab) and avoided to taking

antihistamines and corticosteroids within 10 days prior to the blood drawing. The study was approved by the Ethics Committee of the University Hospital “Policlinico–Vittorio Emanuele” of Catania and informed consent from each patient was obtained before proceeding with the blood sample.

RLE concentration

To establish the RLE concentration to be used on white blood cells (WBC), following lysis of red blood cells, we determined the non-cytotoxic dose for WBC by means of a morphological evaluation by flow cytometer (at 15 and 30 minutes of incubation with RLE at 37°C). After this first exploratory phase, cell viability was assessed by trypan blue exclusion assay on WBC at 15, 30 minutes to evaluate the concentration less damaging to the cells and finally we assessed the immunological effect on basophils (non-specific increase in CD63 and CD203c). By this approach decreasing concentrations of RLE extract were tested on WBC: 11.36 mg/ml (C1); 5.68 mg/ml (C2) and 2.27 mg/ml (C3). Morphological and cell viability tests provided preliminary results which indicated a strong cytotoxic activity on WBC for C1 and C2 already at incubation of 15 minutes at 37 °C. The concentration C3 seems to represent the limit at which the RLE can be used on WBC up to 30 minutes. Treating WBC with the RLE at C1 concentration, the cells maintain their morphology and the cell mortality remains below 5% at 30 minutes (data not shown). Accordingly, we exposed WBC to RLE at the concentration of 2.27 mg/ml (C1) for 15 minutes to evaluate its ability to induce non-specific activation/degranulation of basophils (increase of CD63 and CD203c expression). No significant difference in basophil activation/degranulation markers between RLE stimulated and patient background samples was observed.

Antiallergic tests by Basophil Activation Tests

In order to verify the antiallergic activity of Red orange and Lemon Extract we collected blood samples from allergic donors in EDTA and heparin pre-treated tubes and performed the basophil activation test (BATs) by a well-established method already used to study the antiallergic activity of natural products (Caruso et al. 2008; Caruso et al. 2018). The blood samples were aliquoted into two series of tubes in order to perform the BATs by two different activation markers CD63 (Basoflow-Ex) and CD203c (Allergenicity Kit) due to the different conditions required. The CD63 was tested on heparin pretreated blood samples stimulating basophils *in vitro* by an interleukin-3 (IL-3)-enriched buffer, whereas CD203c was tested on EDTA pretreated blood samples stimulating cells by an IL-3-free buffer. In both series of tubes basophils were stimulated with Red orange and Lemon Extract (at the final concentration of 2,27 mg/ml) plus 10 µl of allergen extract added at the same time, or by allergen extracts only, all dissolved in a Calcium-enriched buffer. The responsiveness of basophils was established by stimulating blood with an anti-FcεRI-mAb, one positive control (PC) for each marker. The series of tubes for the detection of CD63 were added with a mix of antibodies anti-human-CD203c/anti-human-CD63 marked respectively by Phycoerythrin (PE) and Fluorescein Isothiocyanate (FITC) (Fig. S1). The series of tubes for the detection of CD203c were added with a mix of anti-human-CD3, -CRTH2 and -CD203c marked respectively by Phycoerythrin-Cyanin 7 (PC7), Fluorescein Isothiocyanate (FITC) and Phycoerythrin (PE) (Fig. S2). In both tests, after an incubation at 37°C for 15-20 minutes, red blood cells were lysed and WBC were resuspended in phosphate buffer saline (PBS) and read by flow cytometer. Data were acquired by a flow cytometer equipped with an argon laser (Epics XL-MCL; Beckman Coulter Inc.) and analyzed by the Kaluza[®] software (Beckman Coulter Inc.).

In order to determine the relative effect of Red orange and Lemon Extract on basophils we tested Sodium Cromoglycate (450 $\mu\text{mol/L}$) (Leung et al. 1988) in addition to allergen, as a well-known antiallergic drug.

Statistical analysis

The distribution of data was assessed by Kolmogorov-Smirnov test. Wilcoxon signed-rank test was used to evaluate differences between matched variables. All analyses were considered significant with a p-value of less than 5 %. Data analyses were performed using R version 3.4.3 (R Core Team, 2017).

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Table S1. Identification and relative amounts of anthocyanins in Red orange and Lemon Extract

Compound no.	[M] ⁺ (m/z)	MS ⁿ (m/z)	Anthocyanin	Relative composition ^a
1	611	449/287	cyanidin 3,5-diglucoside	1.29
2	465	303	delphinidin 3-glucoside	2.67
3	611	287	cyanidin 3-sophoroside	0.41
4	449	287	cyanidin 3-glucoside	39.97
5	595	287	cyanidin 3-rutinoside	1.30
6	479	317	petunidin 3-glucoside	1.59
7	551	465/303	delphinidin 3-(6"-malonyl)glucoside	1.43
8	463	301	peonidin 3-glucoside	2.98
9	565	479/317	petunidin 3-(6"-malonyl)glucoside	1.45
10	535	449/287	cyanidin 3-(6"-malonyl)glucoside	21.76
11	593	449/287	cyanidin 3-(6"-dioxalyl)glucoside	5.70
12	-	271	pelargonidin derivative	1.44
13	549	463/301	peonidin 3-(6"-malonyl)glucoside	13.80
14	-	287	cyanidin derivative	2.39
15	-	301	peonidin derivative	1.82
Total anthocyanins (g CGE/100 g) ^b				2.66 ± 0.01

^a Relative content of anthocyanins calculated from peak areas recorded at 520 nm.

^b Results are expressed as the mean ± standard deviation.

Table S2. Identification and concentration values of flavanones in Red orange and Lemon Extract

Compound no.	[M-H] ⁻	MS ⁿ	Flavanone	g/100 g ^a
1	595	287	Eriocitrin	10.79 ± 0.02
2	579	271	Narirutin	1.28 ± 0.01
3	609	301	Hesperidin	3.66 ± 0.01
4	593	285	Didymin	0.17 ± 0.02
Total flavanones (g/100 g)				15.91 ± 0.01

^a Results are expressed as the mean ± standard deviation.

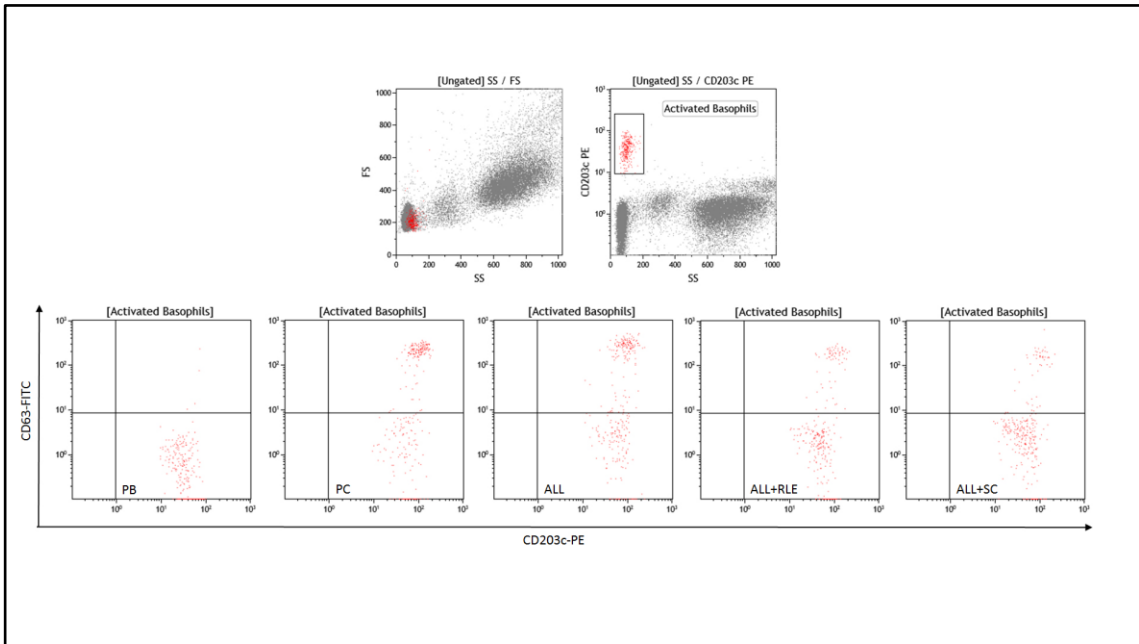


Figure S1. Dot plot of CD63 detection from a patient. Activated Basophils were selected in the first two plots as $CD203c^{pos}/SS^{low}$ cells (a, b), and then the expression of $CD203c^{pos}/CD63^{pos}$ was quantified as percentage of degranulated basophils in the third plot (c_{1-5}), that represent basophils stimulated only with PBS in patient background (PB) (c_1), with anti-IgE in positive control (PC) (c_2), with allergen (ALL) (c_3), with allergen in presence of Red orange and Lemon Extract (ALL+RLE) (c_4) and with allergen in presence of Sodium Cromoglycate (ALL+SC) (c_5).

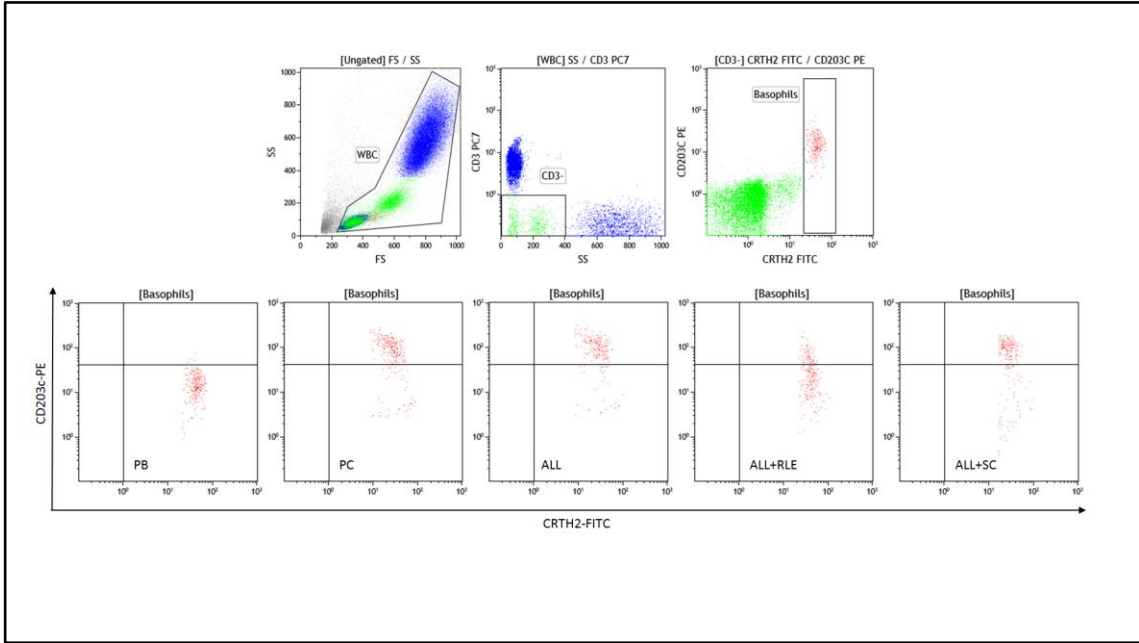


Figure S2. Dot plot of CD203c detection from a patient. Basophils were selected in the first three plots as $CD3^{\text{neg}}/SS^{\text{low}}/CRTH2^{\text{pos}}$ cells (a, b, c), and then the expression of $CRTH2^{\text{pos}}/CD203c^{\text{pos}}$ cells was quantified as percentage of activated basophils in the fourth plot (d₁₋₅), that represent basophils stimulated only with PBS in patient background (PB) (d₁), with anti-IgE in positive control (PC) (d₂), with allergen (ALL) (d₃), with allergen in presence of Red orange and Lemon Extract (ALL+RLE) (d₄) and with allergen in presence of Sodium Cromoglycate (ALL+SC) (d₅).