

Efficacy of a Nasal Spray from *Citrus limon* and *Cydonia oblonga* for the Treatment of Hay Fever Symptoms—A Randomized, Placebo Controlled Cross-Over Study

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Nasal spray from lemon and quince (LQNS) is used to treat hay fever symptoms and has been shown to inhibit histamine release from mast cells *in vitro*. Forty-three patients with grass pollen allergy (GPA) were randomized to be treated either with placebo or LQNS for one week, respectively, in a cross-over study. At baseline and after the respective treatments patients were provoked with grass pollen allergen. Outcome parameters were nasal flow measured with rhinomanometry (primary), a nasal symptom score, histamine in the nasal mucus and tolerability. In the per protocol population absolute inspiratory nasal flow 10 and 20 min after provocation was higher with LQNS compared to placebo (-37 ± 87 mL/s; $p = 0.027$ and -44 ± 85 mL/s; $p = 0.022$). The nasal symptom score showed a trend (3.3 ± 1.8 in the placebo and 2.8 ± 1.5 in the LQNS group; $p = 0.070$) in favor of LQNS; the histamine concentration was not significantly different between the groups. Tolerability of both, LQNS and placebo, was rated as very good. LQNS seems to have an anti-allergic effect in patients with GPA. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: grass pollen; allergy; lemon; quince; anthroposophic medicine.

INTRODUCTION

About 30% of patients with allergies in Europe use preparations from alternative or complementary medicine (Schäfer *et al.*, 2003; Schäfer, 2004). Preparations from lemon (*Citrus limon*) and quince (*Cydonia oblonga*) have been used to treat hay fever within the concept of anthroposophic medicine, a complementary whole medical system (Hamre *et al.*, 2014). *C. limon* contains polyphenolic compounds like eriocitrin (eriodictyol-7-O-rutinoside) and hesperidin (hesperitin-7-O-rutinoside) which have been reported as anti-allergic substances (Inoue *et al.*, 2002; Jeong *et al.*, 2011; Wei *et al.*, 2012). *C. oblonga* contains chlorogenic acids which possess anti-allergic properties as well¹. They are on the market under the trade name Gencydo[®] as registered drugs as nasal spray and as ampoules for subcutaneous injection in Germany and Switzerland. The nasal spray is also available in numerous other countries (apart from European countries e.g. in Egypt, Israel, New Zealand, USA). Recently it could be shown, that Gencydo[®] preparations have anti-allergic properties *in vitro* by inhibiting histamine release from IgE-activated mast cells and basophilic cells, inhibiting the release of interleukin (IL)-8 and tumor necrosis factor (TNF)-alpha from activated mast cells and partially blocking eotaxin release from human bronchial epithelial cells (Gründemann *et al.*, 2011). The

effective *in vitro* concentrations (0.2–0.8 mg/mL) were in a range that could be reached by topical application as nasal spray. Furthermore, the lemon and quince components had *in vitro* differential and synergistic anti-allergic effects (Baars *et al.*, 2012; Huber *et al.*, 2012). So far efficacy in humans has only been investigated in two ($n = 12$, $n = 140$) observational studies (Baars and De Bruin, 2005; Rother and Oexle, 2008) and a small ($n = 23$) randomized trial comparing the nasal and subcutaneous routes of application (Baars *et al.*, 2011). Studies with an adequate control group to show efficacy (placebo control or comparison to standard treatment) have not yet been reported. Because of the promising *in vitro* effects we wanted to determine whether the lemon/quince nasal spray (LQNS) has specific anti-allergic effects in humans.

PATIENTS AND METHODS

Study design, in- and exclusion criteria. A randomized, placebo controlled, investigator initiated cross-over trial in patients with grass pollen allergy was performed according to GCP criteria (GCP order, n.d.). Patients were recruited by announcement in Facebook for medical students of the University Medical Center Freiburg, Germany (<https://www.facebook.com/groups/medizinerfreiburg>). Eighteen to fifty-year-old females and males who had signed the written consent form and had at least in the two past years symptoms of grass pollen allergy (rhinitis, conjunctivitis, sneezing, itching in the nose), a positive Prick-test ($\geq$ score 2) for grass pollen

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and had $\geq 40\%$ reduction of the inspiratory nasal flow or $\geq 20\%$ flow reduction measured with rhinomanometry plus a symptom score of ≥ 3 after provocation with grass pollen allergen were included. Exclusion criteria were any severe acute or chronic disease, bronchial asthma, therapy with inhaled or systemic steroids, known allergy to lemon or quince, therapy with antihistamines, mast cell stabilizers or other anti-allergic preparations within one month prior to the onset of the study, abuse of alcohol (>30 g/day) or illegal drugs, pregnancy or breast feeding. The study was performed in winter time outside of the pollen season.

Outcome parameter and investigations at baseline.

Main outcome parameter was the inspiratory nasal flow at 150 pascal measured with rhinomanometry 10 and 20 min after provocation with grass pollen in intraindividual comparison between placebo and LQNS. Secondary outcomes were the expiratory nasal flow, in- and expiratory nasal resistances, the nasal symptom score, histamine content in the nasal fluid, safety and tolerability. At baseline patients were randomized, and the individual grass pollen concentration which resulted in $\geq 40\%$ reduction of the inspiratory nasal flow or $\geq 20\%$ flow reduction plus a symptom score of ≥ 3 was determined stepwise after provocation with 50 standard biological units (SBE), 500 SBE and 5000 SBE 6-grasses allergen (Allergopharma, Reinbek, Germany) according to the instruction of the manufacturer. Nasal flow was measured with a master screen version 5.2 (Cardinal Health, Höchberg, Germany) with equipment from CareFusion (Merz Medizintechnik GmbH, Reutlingen, Germany) at each allergen concentration. Symptoms were measured with an established score (Riechelmann *et al.*, 2003) in which maximal 2 points were assigned for secretion (no; little; much), nasal irritation (sneezing 0–2 $\times$; 3–5 $\times$; $>5\times$) and distant symptoms (none; conjunctivitis, lacrimation, itching in the mouth; urticaria, coughing, dyspnea), respectively. The maximum total score was six points. Histamine was measured in the nasal mucus according to recommendations described by Scadding *et al.* (2011). Before and after provocation with the highest grass pollen concentration nasal lavage was performed three times with physiological saline, with the third lavage, samples for histamine were collected. Probes were kept on ice immediately after asservation and stored at -20°C until analysis at Limbach laboratories (Heidelberg, Germany) with an established radio-immuno-assay (Immunotech SAS, Marseille, France). Compliance was assessed by measuring the remains of the medication in the returned bottles.

Study medication. LQNS was Gencydo® nasal spray (Weleda AG, Schwäbisch Gmünd, Germany) which contains *C. limon* succus (lemon juice) in a concentration of 80–120 mg per 10-mL nasal spray corresponding to 6.5 mg/10 mL fruit acids calculated as citric acid, an aqueous (water for injection (Ph. Eur.)) quince extract 300 mg/10 mL (*Cydonia oblongata* fructus rec., solvent/drug ratio 1:2.1) and saline 0.9%. The origins of the lemon and quince fruits were Sicily (Italy) and Wetzgau (Germany), respectively. The fresh fruits were

kept refrigerated and sent to Weleda Schwäbisch Gmünd, Germany where the identity was analyzed by macroscopic confirmation and processed immediately upon arrival. The lemon fruits were squeezed by mechanical pressing in a roller mill to obtain *C. limon* juice. The quince fruits were homogenized and extracted with water by aid of an ultrasonic bath for 10 min with occasional swirling of the solution to obtain aqueous *C. oblonga* extract. The product has marketing authorization as drug in Germany and Switzerland and is produced according to Good Manufacturing Practice (GMP guidelines). GMP is monitored by the local German authorities (Regierungspräsidium). LQNS was provided by the manufacturer, placebo was saline 0.9% produced by the pharmacy of University Medical Center Freiburg which blinded both study medications by labeling it and filling it in spray bottles (20 mL) which were not distinguishable. The study medication was labeled with subsequent numbers and handed over to the patients in the sequence of randomization. The respective study medication was used thrice daily with two sprays ($2 \times 140 \mu\text{g}$) in each nostril for seven days and a last spray 1 hour prior to the control visits. LQNS did not smell different from placebo and looked the same, but the taste was different. In order to avoid debinding in case they accidentally swallowed the medication we explained to our patients that both placebo and LQNS had some taste.

HPLC analysis of the study medication. Unneeded samples of the study medication were freeze dried and analyzed by PhytoLab GmbH (Vestenbergsgreuth, Germany) on content of eriocitrin and hesperidin as main active substances of *C. limon* (Caristi *et al.*, 2003; Qiao *et al.*, 2014) and chlorogenic acid derivatives (Huber *et al.*, 2012) as characteristic active components of the *Cydonia oblongata* component.

Briefly, 90 mg of the freeze dried extract was dissolved in 10-mL methanol by ultrasonic homogenization. The clear solution was transferred to a flask and evaporated to dryness by a rotary evaporator. The residue was re-dissolved in 5-mL methanol. After centrifugation the clear solution was investigated by a reversed-phased gradient-HPLC system comprised of HPLC pumps and an UV/VIS detector, using wavelengths from 200 to 400 nm. Separations were carried out using a 5- μm Waters Symmetry C18 column (250 mm $\times$ 4.6 mm i.d.) maintained at 23°C , eluted at 1 mL/min with an 89-min gradient of 11–95% of acetonitrile (ACN) in water. Injection volume was 20 μL , and the injection temperature was 23°C . Mobile phase A: 0.1% formic acid in water; Mobile phase B: 0.1% formic acid in acetonitrile. Gradient: Initial 11% mobile phase B; 0–40 min (11–29% B), 40–70 min (29–72% B), 70–85 min (72–95% B), 85–89 min (95%), 90–120 (11% B).

At 325 nm chlorogenic acid derivatives could be detected, among others chlorogenic acid itself (Fig. 1). At 283 nm the target substances eriocitrin and hesperidin could be found (Fig. 2).

Course of the study. Day 8 after baseline side effects were documented, tolerability of the study medication

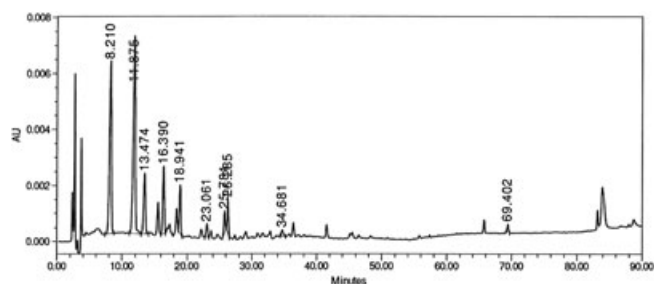


Figure 1. Detection of different chlorogenic acid derivatives in LQNS at a wavelength of 325 nm. Chlorogenic acid was proven to be the peak at 11.87 min by substance authentic reference standard (phyproof®, PhytoLab); peaks at 8.21 and 13.47 min might be neochlorogenic acid and cryptochlorogenic acid, respectively.

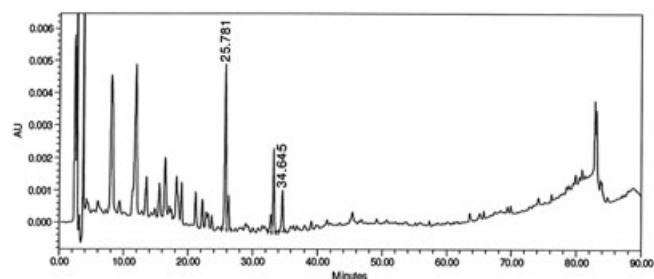


Figure 2. Detection of eriocitrin and hesperidin in LQNS at 283 nm, retention times 25.78 and 34.64 min, respectively.

was rated on a 4 point scale (1=very good, 2=good, 3=moderate, 4=bad), and compliance was checked by measuring the residual volume in the spray bottle. After measuring nasal flow and histamine in the nasal mucus before provocation patients were provoked with grass pollen allergen in the dose which reduced inspiratory nasal flow according to the criteria at baseline (5000 SBE in all patients) and nasal flow (after 10 and 20 min), histamine in nasal mucus (after 20 min) was measured again, and the symptom score (after 20 min) was determined. After 7 days washout period without medication the patients received the second study medication (cross-over design) for the same time period as the first medication. Day 22 after baseline the same measurements as day 8 after baseline were performed, and the study was finished for the individual patients. As compensation each patient received 80.- Euro. The study was approved from the Ethical Committee of University Medical Center Freiburg and the Federal Institute for Drugs and Medical Devices (BfArM), and it was registered in the German Register of Clinical Trials (DRKS00004312).

Statistics, quality assurance and data analysis. The randomization list (mixed block size randomization, random seed 99, total sample size 50) was compiled by the Department of Medical Biometry and Statistics of University Medical Center Freiburg. Randomization was concealed, and the randomization list was first disclosed to the investigators after the data file was closed. Sample size was calculated based on an expected mean difference of 40 units (approximately 17%) and a standard deviation of 75 units in the main outcome criterion in favor of LQNS. This corresponds to a Cohen's $d=0.53$ and a medium effect size. Therefore, 20 patients are required to detect a difference on a level of

significance of 5% with a power of 90%. Forty patients were planned for inclusion to get at least 20 analyzed. Intention to treat (ITT) and per protocol (PP) analyses, which included patients without major protocol violations, were predefined. For statistical analyses first, the distribution of the data was investigated by displaying them as histograms, and performing the Kolmogorov–Smirnov and Shapiro–Wilk tests. Many parameters, especially those related to expiration, showed non-normal distribution and a positive skew. Because of the cross-over design, data of the LQNS and placebo group were dependent, and the distribution of the differences for each parameter was investigated. Also here, data related to expiration showed non-normal distribution. Therefore, the Wilcoxon signed-rank test was chosen for statistical comparisons between the placebo and the LQNS group; $p < 0.05$ was considered statistically significant. Statistical analysis was performed with IBM SPSS Statistics 21.0 (Armonk, NY, USA).

RESULTS

Fig. 3 shows the flow of the study according to the consort criteria. In the ITT population there was a carry-over effect regarding the inspiratory nasal resistance after provocation ($p=0.041$). In the PP populations there was no carry-over effect. Because of this carry-over effect and because five patients developed upper respiratory tract infections during the study and other five had major protocol violations, the PP analyses were performed primarily.

Table 1 shows the patient characteristics for the ITT and PP population, which were not different.

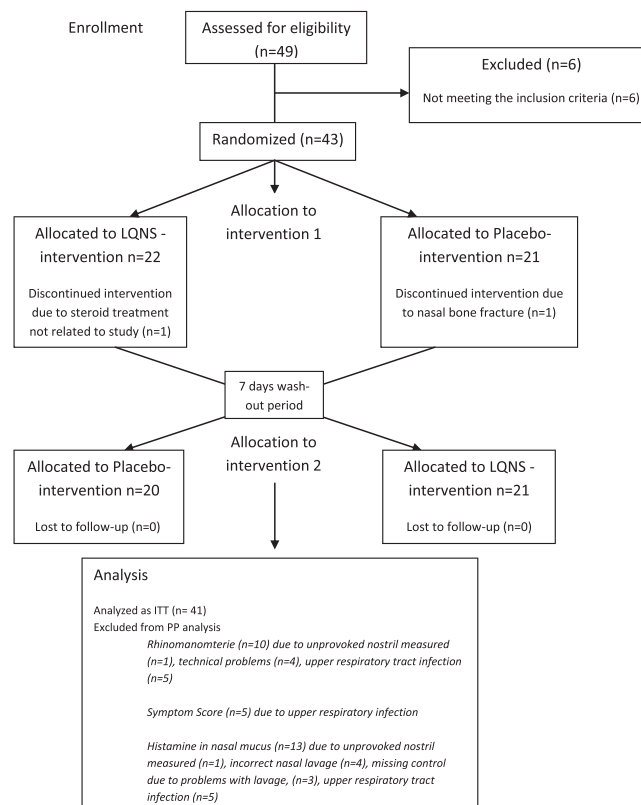


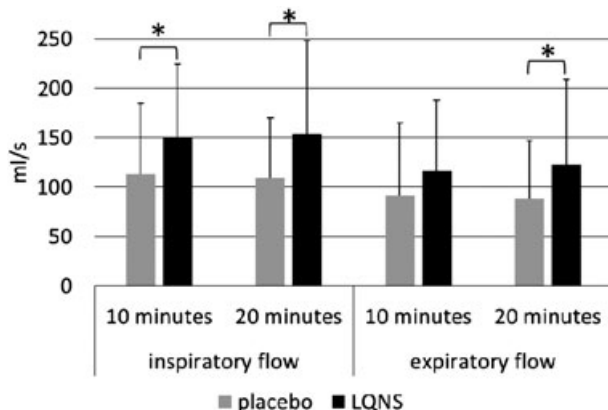
Figure 3. Flow of the study according to CONSORT criteria.

Table 1. Characteristics of the patients of the ITT ($n = 41$) and PP (rhinomanometry $n = 31$) population in absolute numbers or means $\pm$ standard deviation

	ITT	PP
Female / Male (n)	25 / 16	19 / 12
Age (years)	25.4 ± 3.8	25.5 ± 4.3
Body mass index (kg/m^2)	21.6 ± 3.0	22.5 ± 2.6
Duration of symptoms (years)	14.5 ± 6.5	14.6 ± 6.3
Mean INF (inspiratory nasal flow) at baseline	255.8	241.2

In the ITT population no significant differences for nasal flow or histamine concentration between LQNS and placebo were found after provocation (data not shown). In the PP population we, however, found a significant difference in the primary outcome parameter, the absolute inspiratory flow after provocation with grass pollen after one week pre-treatment with either placebo or LQNS (Fig. 4). Expiratory flow only was improved 20 min after provocation.

Patients of the PP population had higher pre-provocation inspiratory nasal flow rates after one week treatment with LQNS compared to placebo ($p = 0.032$). Expiratory flow rates were not significantly different ($p = 0.077$). Ten minutes after provocation the relative reduction of inspiratory flow and the relative increase of inspiratory resistance were lower with LQNS ($p = 0.059$ and $p = 0.044$, respectively, Table 2). The other flow parameters were not different between placebo and LQNS ($p > 0.1$, Table 2).

**Figure 4.** Absolute inspiratory and expiratory nasal flow (mean $\pm$ standard deviation), measured with rhinomanometry 10 and 20 min after provocation with grass pollen allergen (5000 SBE) after one week pre-treatment with either placebo or LQNS ($n = 31$ patients, * $p < 0.05$).**Table 2.** Mean difference (Δ mean) and standard deviation (SD) of relative nasal flow (% of pre-provocation) after provocation with grass pollen between placebo and LQNS in the PP population ($n = 31$)

	10 min Δ mean $\pm$ SD	p -Value	20 min after provocation Δ mean $\pm$ SD	p -Value
Relative inspiratory flow (%)	-7.78 ± 24.02	0.059	-7.52 ± 26.31	0.147
Relative inspiratory resistance (%)	71.43 ± 198.49	0.044	39.81 ± 351.50	0.171
Relative expiratory flow (%)	-5.51 ± 26.24	0.339	-5.17 ± 27.25	0.447
Relative expiratory resistance (%)	157.89 ± 438.32	0.206	-30.36 ± 721.76	0.530

The symptom score in the PP population 20 min after provocation was 3.3 ± 1.8 in the placebo- and 2.8 ± 1.5 in the LQNS group ($p = 0.070$, relative difference to placebo 15%). Histamine concentration in the nasal mucus had a high variation and increased to $274 \pm 262\%$ in the placebo and $207 \pm 141\%$ in the LQNS group compared to pre-provocation (difference between the groups $p = 0.616$).

Tolerability of both placebo and LQNS was rated as very good (median 1.0, mean placebo 1.2 ± 0.4 , mean LQNS 1.3 ± 0.5). Serious adverse events did not occur. Compliance of the patients was excellent regarding punctuality and cooperation during the measurements. In the first week of treatment 10.5 ± 1.5 mL of the study medication was used, in the second week of treatment 10.2 ± 2.8 mL (placebo 10.8 ± 2.7 mL, LQNS 10.5 ± 2.6 mL). This indicates that given the fact that 11 mL equals 100% more than 90% of study medication was used in both groups.

DISCUSSION

Here we report about efficacy and tolerability of a nasal spray from *C. limon* and *C. oblonga* in a randomized placebo controlled cross-over study in patients with grass pollen allergy. The PP population was analyzed primarily, because the ITT analysis was biased by a carry-over effect and a substantial number of respiratory tract infections and protocol violations (see Fig. 1). Respiratory tract infections were an exclusion criterion but five patients developed a common cold with obstructed nose during the study period, which was in winter time. Carry-over effect means that independent of treatment the measurements at the first visit affected the result of the second visit, which should be avoided (Wellek and Blettner, 2012). After excluding dropouts and patients who had protocol violations (see Fig. 1) there was no more carry-over effect. This shows that the carry-over effect was not caused by a too short wash-out phase but by the patients with respiratory infections and protocol violations which were not equally distributed between the treatment phases. Baseline characteristics were in the PP the same as for the ITT population; the PP population was defined before debinding the study. Inspiratory nasal flow after provocation with grass pollen was selected as primary outcome because it seemed to be more objective than the symptom score and because handling was more easy and reliable than collecting nasal mucus in pre-experiments.

For the PP population the main outcome parameter was significantly improved after treatment with LQNS compared to placebo (Fig. 2). The difference between the groups was, however, small, and there was a large standard deviation. The secondary outcome parameters relative flow reduction, nasal symptom score and histamine in the nasal mucus all were better in the LQNS group, but the differences were significant only in part. Interestingly, patients treated with LQNS had already before provocation a better nasal flow than the placebo-treated patients. The difference in the main outcome criterion (nasal flow after provocation) is, therefore, a combination of two effects, the pre-provocation difference and the difference in the relative flow reduction after provocation. *In vitro*, LQNS not only inhibited the early IgE mediated phase of the allergic immune response but also inhibited the late, chronic phase, which is mediated by, e.g. IL-8 and TNF- α (Gründemann *et al.*, 2011). This inhibition of the late phase of the allergic response might have contributed to the improved nasal flow after one week pre-treatment with LQNS that was measured prior to provocation. Despite absence of grass pollen in the atmosphere during the study period October–December 2012 (Wachters, 2014) the patients might already have had a swelling of the nasal mucosa before provocation because of non-allergic chronic rhinosinusitis, grass pollen which persisted in house dust (Fahlbusch *et al.*, 2001) or concomitant allergy to house dust mites, which is a frequent allergy in Germany and Switzerland (Schmitz *et al.*, 2013; Wüthrich, 2001) and was no exclusion criterion for our study. What exactly caused this difference in pre-provocation nasal flow remains unclear. A two-fold effect on the early and late phase of an allergic reaction would be advantageous compared to antihistamines which are only effective against the early-phase allergic reaction and do not prevent the effects of cytokines which initiate the late-phase allergic reaction (Holgate and Polosao, 2008). Eriocitrin, hesperidin and chlorogenic acids, which were found in the study medication by HPLC analyses, are known to have anti-allergic properties *in vitro* (Kimura *et al.*, 1985; Inoue *et al.*, 2002; Jeong *et al.*, 2011; Wei *et al.*, 2012) Because

our study is the first of this kind the sample size had to be determined based on assumptions of expected effects, i.e. clinical relevance which was calculated as a medium effect size (Cohen's $d=0.53$). At a given effect size, a cross-over study design is much more robust than a parallel group design which in turn leads to requirement of a lower sample size for a cross-over compared to a parallel group design (Wellek and Blettner, 2012). Strength of the study was the strict standardization: Provocation of the individual patients was always performed at the same time of the day to exclude the influence of circadian changes, provocation and assessment of outcome parameters were always performed by the same investigator (AH), room temperature was controlled and kept constant and all patients rested at least 10 min before provocation. Compliance as measured by the amount of nasal spray used was excellent.

In summary, our randomized placebo controlled cross-over study demonstrates a small anti-allergic clinical effect of a nasal spray from *C. limon* and *C. oblonga* and supports preclinical results that the preparation also reduces the late phase of the allergic response. Because of the substantial number of major protocol violations and intercurrent respiratory tract infections the study needs, however, to be confirmed.

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Conflict of Interest Statements

Weleda AG Schwäbisch Gmünd, Germany has in part contributed to financially supporting preclinical research of CG. This support had no influence whatsoever on designing, performing and reporting of the study. The other authors declare that they have no conflict of interest.

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