CHAPTER 2

Materials and methods

2.1 Study design

This was a prospective randomized, double-blind, parallel, controlled- trial study. Patients with AR who met the inclusion criteria were enrolled and randomly divided into 2 groups. The patients in the shallot group received oral capsules of shallot combined with cetirizine and the patients in placebo group received similar amounts of placebo capsules in combination with cetirizine. The treatment duration were 28 days for both groups.

2.2 Patients selection

2.2.1 Number of subjects

The number of subjects was calculated based on the G*power program that following the figure 2.1. From calculation of G*power program, setting of test family was t-test, the alpha (α) error was 0.05, and the power of test was 0.8. Mean of group 1 and mean of group 2 were estimated from the probable mean TNSS after 4 weeks of treatment with cetirizine plus shallot and cetirizine alone [58]. The standard deviation (SD) were obtained from the study of AR treatment with cetirizine [58]. As a result, the number of subjects yielded for this study was 42. The proposed drop-out rate was 15%, so 8 subjects were added. Finally, the estimated number of subjects was 50 in total (N = 50).

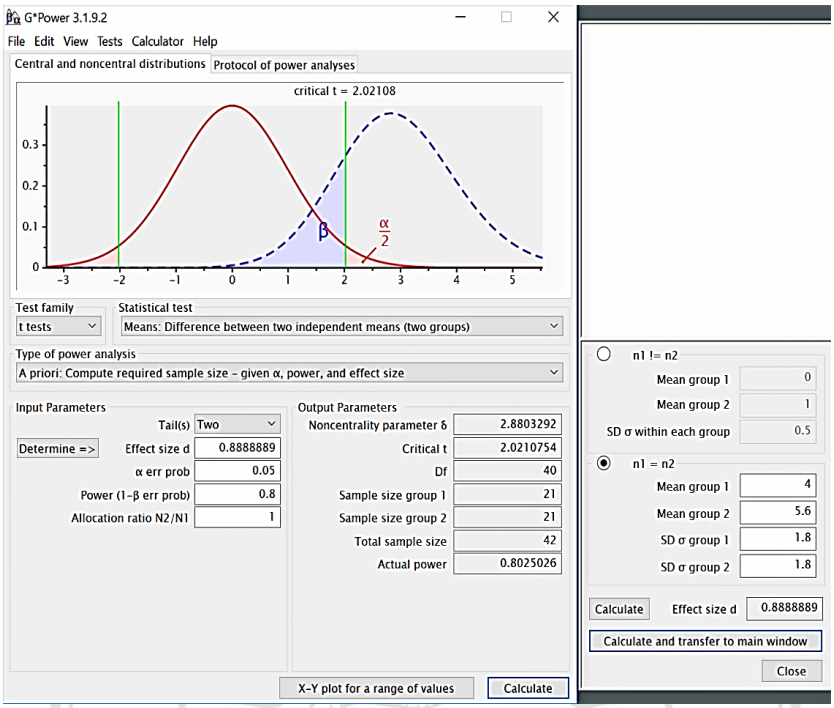

Figure 2.1 Calculation of number of subjects from G*power program

2.2.2 Inclusion criteria

1. Male and female patients, aged between 18 to 65 years old

2. Patients who were diagnosed with persistent AR, according to ARIA 2008 guidelines

3. Patients with previous history of positive skin prick test or presence of positive skin prick test to at least 1 allergen

4. Patients who had total nasal symptoms score (TNSS) ≥ 6 within prior 2 weeks and the score of nasal obstruction must not exceed 2 (≤ 2)

5. All patients were explained and clearly understood the details of this research protocol, and gave their written informed consent before entering the study

2.2.3 Exclusion criteria

1. Patients with severe nasal anatomical abnormality, for example, nasal septum deviation more than 50% and nasal polyposis

2. Patients who had infection of respiratory tract within 2 weeks before the study began

3. Patients who had nasal surgery within 4 weeks before study began

4. Patients who used oral or intranasal corticosteroids within 4 weeks prior to study

5. Patients who currently on immunotherapy or previously received immunotherapy within 1 year before study began

6. Patients with history of hypersensitivity to cetirizine or its ingredients

7. Patients with history of hypersensitivity of *A. ascalonicum* L. or its chemical compounds

8. Patients with history of asthma, heart disease, hepatic disease (AST or ALT more than 1.5 times of the upper limit of normal range), renal disease (eGFR less than 50 mL/min) and cancer

9. Pregnancy or breastfeeding

2.2.4 Withdrawal criteria

1. Subject who had serious adverse events (SAEs) or allergic reactions or adverse events (such as rash, nausea, vomiting) that did not improve despite appropriate treatment

2. Subject who received other antiallergic medications including decongestants

4. Subject who were lost to follow-up

5. Subject who wanted to withdraw from study

2.3 Drug and chemical substances

1. Cetirizine (Zertine film-coated, 10 mg/tablet) was purchased from Farmaline Co., Ltd.

2. Shallot and placebo capsules (250 mg/capsule) were prepared by the Medicinal Plant Innovation Center, Faculty of Pharmacy, Chiang Mai University, Thailand. The preparation was followed the quality standard control of Thai FDA guidelines for herbal products. Placebo capsules were corn starch that were packaged in similar appearance to a capsule of shallot. From the chemical assessment found in flavonoid, phenolic compound, volatile oil, and quercetin in the shallot capsule. Amount of quercetin in the shallot capsule sample was 0.0019 mg/mL (6.29 μmol/L).

3. Skin prick test kits (AllerVACtest ®) included histamine solution in distilled water (positive control), glycerinate phenol-saline (negative control), house mite *Dermatophagoides farina*, house mite *Dermatophagoides pteronyssinus*, American cockroach, careless weed, para grass, *Cladosporium spp*., dog hair, and cat hair were purchased from Greater Pharma Manufacturing Co., Ltd., produced by a partnership between Faculty of Medicine Siriraj Hospital, Mahidol University and Greater Pharma Manufacturing Co., Ltd.

2.4 Protocol outline

All patients entered a 1-day run-in period. Patients who met the inclusion criteria were randomized into 2 groups using computerized randomization (www.randomization.com). Each subject received cetirizine 10 mg once daily at bedtime (h.s.). Subjects in the shallot group received oral capsules of 3 g shallot or 12 capsules of 250 mg per capsule (equivalent to 1 ½ bulbs of fresh shallot) daily for 28 days while subjects in the placebo group received similar amounts of placebo capsules in combination with cetirizine. Patients were required to visit the study site for 3 times throughout the study period for efficacy and safety assessments. The visits included day 0 (baseline), day 14, and day 28 of treatment. Safety assessments were assessed at each visit by monitoring the adverse events and vital signs (pulse rate and blood pressure). Laboratory investigations were performed at baseline and the end of treatment (day 28) (Figure 2.2).

Subjects' compliance of medication consumption was rated and recorded by counting the number of left-over pills from previous visit up to the end of study. Medications that could affect the evaluation of AR symptoms were not allowed throughout the entire study. Subjects were firmly instructed to avoid foods or any supplements that contain shallots, onions and garlics throughout the study in order to avoid toxicity and confounding factors.

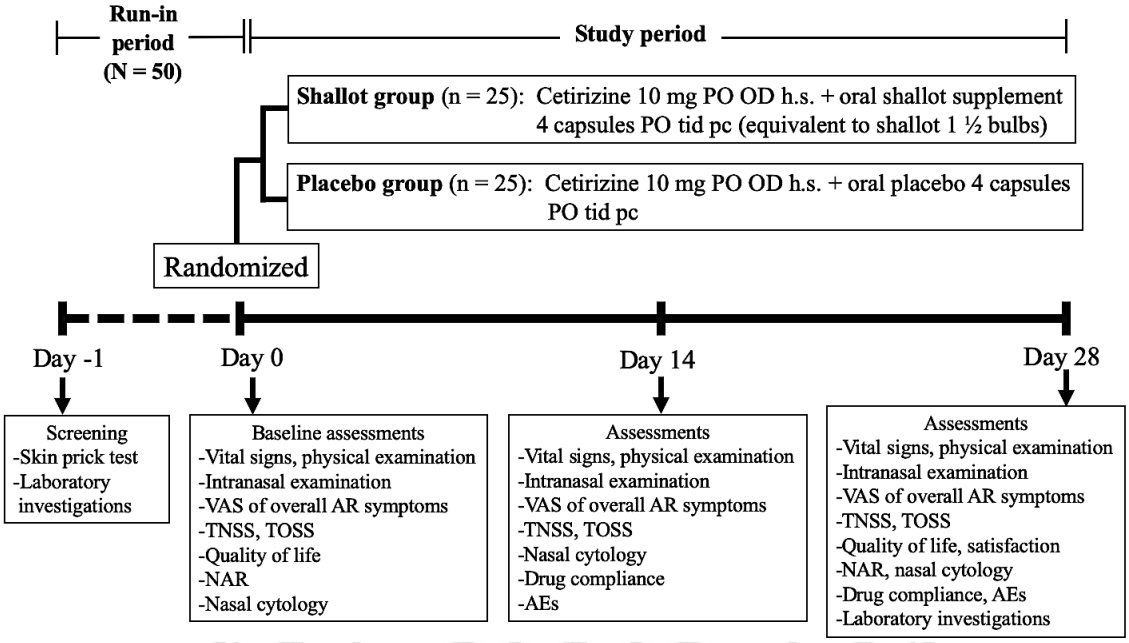

Figure 2.2 Diagram of the protocol procedure of the study

Run-in period: Screening

A nasal examination and skin prick test were performed at the otolaryngology outpatient clinic. Patients were advised to stop any antihistamines prior the skin test (7 days for the second-generation antihistamines and 3 days for the first-generation antihistamines). The following 8 common allergen solutions in Thailand were selected and tested for skin prick test: house mite *D. farina*, house mite *D. pteronyssinus*, American cockroach, careless weed, para grass, *C. spp.*, dog hair and cat hair. Histamine in distilled water was used as positive control and glycerinate phenol-saline was used as negative control. Skin tests were performed on the forearm using 1 mm. prick lancets. The skin reaction usually occurred within 15 minutes after the test which the results could be assessed. Patients were considered allergic to the allergen if at least one of allergen aroused a wheal diameter equal or more than 3 mm.

The self-rated rhinitis symptoms (see 2.5.1) and laboratory investigation (see 2.6) were assessed for enrolled the patients into study.

Visit 1: Randomization and evaluation of baseline

The eligible patients were randomized into 2 groups to received cetirizine combined with either shallot or placebo. The evaluations for baseline were performed.

1. Vital sign (pulse rate and blood pressure)
2. Physical and intranasal examination
3. TNSS and TOSS (see 2.5.1)
4. VAS (see 2.5.2)
5. Quality of life (see 2.5.4)
6. NAR (see 2.5.5)
7. Nasal cytology (see 2.5.6)

Patients were asked to rate the severity of their nasal and ocular symptoms during the past 24 hours daily by recording the scores in diary card every morning (see 2.5.1). The diary card was collected at day 14 and day 28 at the study site. Then, the researchers calculated and summarized the weekly scores.

Visit 2: Follow-up

At day 14 of treatment, patients came back to study site for

1. Vital sign (pulse rate and blood pressure)
2. Physical and intranasal examination
3. TNSS and TOSS (Patients returned the diary card and received the new diary card)
4. VAS
5. Nasal cytology
6. AEs assessment
7. Drug compliance assessment

Visit 3: Follow-up

At day 28 of treatment, patients came back to study site for

1. Vital sign (pulse rate and blood pressure)
2. Physical and intranasal examination

3. TNSS and TOSS (Patients returned the diary card and received the new diary card)

4. VAS

5. Quality of life

6. Satisfaction

7. NAR

8. Nasal cytology

9. AEs assessment

10. Drug compliance assessment

11. Laboratory investigation

2.5 Efficacy assessments

2.5.1 Self-rated allergic rhinoconjunctivits symptoms

Allergic rhinoconjunctivitis comprised with nasal and ocular symptoms.

1. Total nasal symptom scores (TNSS) included itchy nose, nasal obstruction, sneezing and rhinorrhea

2. Total ocular symptom scores (TOSS) included watery eyes, itchy eyes and eye redness.

The evaluation criteria were 4-points scale as follows:

0 = absent (no symptom)

1 = mild (Symptom was presented but not troublesome.)

2 = moderate (Symptom was troublesome but did not interfered normal activity.)

3 = severe (Symptom interfered normal activity.)

Additionally, the patients who had more than 50% of scores improvement after treatment was classified as “50% responder”.

2.5.2 Visual analog scores (VAS) of overall symptoms

A 100-mm. of visual analog scale was used to assess the subjects' overall symptoms as a response to treatment in both groups. The score was rated by the subjects for baseline (day 0), at day 14 and day 28 after treatment. Scoring of 0 (0 mm.) meant the subject had absolutely no AR symptoms and score 10 (100 mm.) referred to the most severe AR symptom (Figure 2.3).

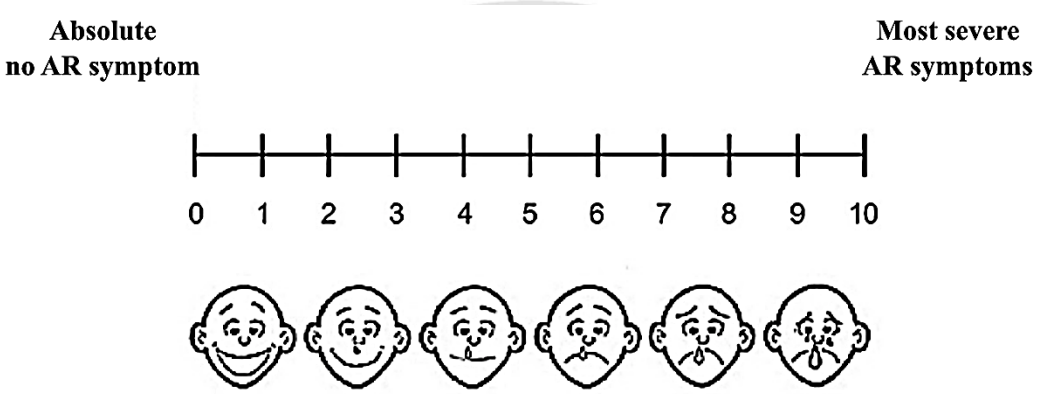

Figure 2.3 Visual analog scale for overall symptom score

2.5.3 Subject satisfaction

At the end of treatment (day 28), all subjects were asked to rate their satisfaction to the treatment of AR by the 100-mm. of visual analog scale. The subjects' satisfactory score ranges from 0 to 100% upon subjects' satisfaction to the treatment. Scoring of 0% (0 mm.) meant subject not satisfied to the treatment and score 100% (100 mm.) referred to subject very satisfied to the treatment. (Figure 2.4).

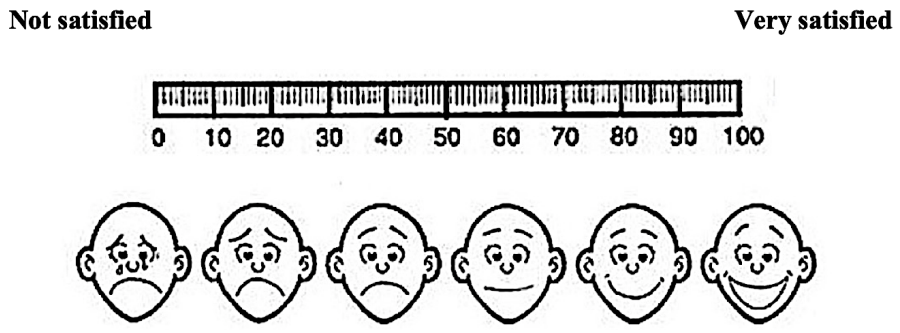

Figure 2.4 Satisfactory scale for subjects' satisfactory score (%)

2.5.4 Quality of life (QOL)

Quality of life of each subject was assessed for baseline (day 0) and at the end of the treatment (day 28) by Rhinoconjunctivitis Quality of Life Questionnaire (Rcq-36), specific questionnaire for quality of life in AR patients [59] (Appendix).

2.5.5 Nasal airway resistance (NAR)

Objective measurement for nasal obstruction was nasal airway resistance (NAR) using anterior rhinomanometry (RMM). The researchers informed briefly about procedure to subjects and asked the subjects to blow their nose before the test began. Subjects sat upright position on a chair. A suitable size of nasal adapter (Endomed, Thailand) (Figure 2.5) for each subject was placed to occlude one nostril. Subjects held the mask and kept their mouth closed and breathed in and out quietly in a fitted mask for several seconds (Figure 2.6). Data of NAR ($Pa/cm^3/s$) were reported as the relationship between trans-nasal differential pressure at 75 Pascal (Pa) and nasal airflow through the left and right nostril. The anterior rhinomanometer (PC 300, ATMOS, Germany) (Figure 2.7) was used in this study. NAR measurement was performed at Clinical Pharmacology Unit, department of Pharmacology, Faculty of medicine, Chiang Mai University.

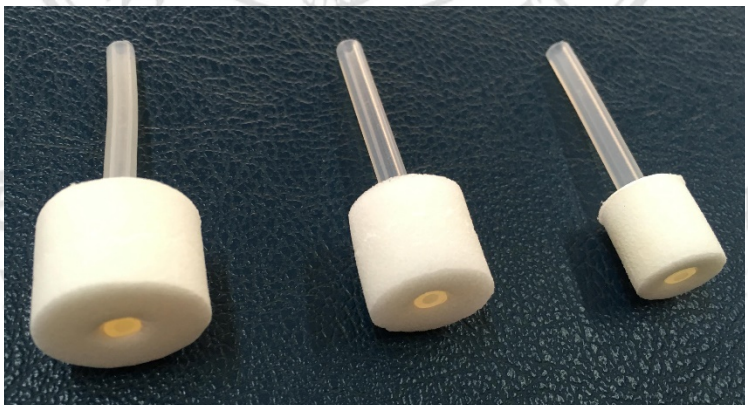

Figure 2.5 Various sizes of nasal adapter for anterior rhinomanometry procedure

Figure 2.6 Position for anterior rhinomanometry procedure

Figure 2.7 Anterior rhinomanometer (PC 300, ATMOS, Germany)

2.5.6 Nasal cytology

Under anterior rhinoscopy, a strip of epithelial mucosa from left and right inferior turbinates were took out for nasal cytology using a nasal scraper, disposable plastic scoops. The specimen was spread onto a plain slide and fixed with 95% alcohol fixative, stained with modified Wright-Giemsa stain and phosphate buffer for 3 minutes and 5 minutes respectively. After air drying, cell counting and differentiation was took under microscope, using x1000 magnification (oil-immersion lens). Cell counting was categorized as neutrophils, eosinophils, basophils, lymphocytes, macrophages, and epithelium cells. The 20 random fields were analyzed and the total number of cells in each microscopic field were counted.

2.6 Safety assessments

Any adverse event (AE) and serious AE were asked and recorded at every visit during the study by open-ended questions (non-directive questioning). The vital signs and physical examinations were performed and recorded at every visit. Laboratory investigation for complete blood count (CBC), blood urea nitrogen (BUN) level, creatinine (Cr) level, aspartate aminotransferase level (AST) and alanine aminotransferase level (ALT) were performed in screening period and at the end of treatment (day 28).

2.7 Statistical analysis

Within group

Changes from baseline and difference between time points in TNSS, TOSS, VAS and nasal cytology were analyzed by Friedman test. Wilcoxon's signed rank test was used to determine the difference in quality of life, NAR and laboratory investigation between baseline and after treatment in each group.

Between groups

Wilcoxon's rank sum test (Mann-Whitney U test) was used to determine the difference in TNSS, TOSS, VAS, satisfaction, quality of life, NAR and nasal cytology. Percentage of responders and incidences of AEs were analyzed by chi-square test.

The statistical software that used to analyze the data was SPSS version 22.0. *p*-value of less than 0.05 was considered significant.