



Research Paper

Reporting effectiveness of an extract of three traditional Cretan herbs on upper respiratory tract infection: Results from a double-blind randomized controlled trial



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ABSTRACT

Ethnopharmacological relevance: Observations from the island of Crete, Greece suggest that infusions of traditional Cretan aromatic plants, well known for their ethnopharmacological use in Eastern Mediterranean region and Near East, could be effective in the prevention and treatment of upper respiratory tract infections, including viral-induced infections. The aim of this study was to report the effectiveness of an essential-oil extract of three Cretan aromatic plants in the treatment of cases with an upper respiratory tract infection.

Materials and methods: A double blind randomized controlled trial was implemented between October 2013 and February 2014. An essential-oil extract of Cretan aromatic plants in olive oil (total volume of 15 ml of essential oil per litre of olive oil) was administered as 0.5 ml soft gel capsules, twice a day, for 7 days. Placebo treatment was 0.5 ml olive oil in soft gel capsules. Eligible patients were those presenting for clinical examination in the selected setting with signs and symptoms of upper respiratory tract infection that had begun within the previous 24 hours. Real-Time Polymerase Chain Reaction (PCR) was used for the detection of respiratory viruses. The primary outcome was the severity and duration of symptoms of upper respiratory tract infection, assessed using the Wisconsin Upper Respiratory System Survey (WURSS-21) questionnaire. A secondary outcome of interest was the change in C-reactive protein (CRP) status.

Results: One hundred and five patients completed the study: 51 in the placebo group, and 54 in the intervention (treated) group. Baseline characteristics were similar in the two groups. No statistically significant differences were found in symptom duration or severity between the two groups, although small and clinically favorable effects were observed. When the analysis was restricted to subjects with a laboratory-documented viral infection, the percentage of patients with cessation of symptoms after 6 days of treatment was 91% in the intervention group and 70% in the control group ($p=0.089$). At baseline, one third of the patients in each group had elevated CRP levels. At follow-up, the respective proportions were 0% in the intervention group and 15% in the placebo group ($p=0.121$). The data were also in a favorable direction when 50% and 80% symptom reduction points were considered for specific virus types.

Abbreviations: ml, Milliliter; PCR, Polymerase Chain Reaction; CRP, C-reactive protein; WURSS, Wisconsin Upper Respiratory Symptom Survey; AUC, area under the curve; RCT, randomized controlled trial; GP, General Practitioner; RBC, red blood cells; SBP, systolic blood pressure; DBP, diastolic blood pressure; WBC, white blood cell; ASAT, SGOT, serum glutamic oxaloacetic transaminase; ALP, alkaline phosphatase; PLT, thrombocytes; ALAT, SGPT, serum glutamic pyruvic transaminase; hRSV, human respiratory syncytial virus; hMPV, human metapneumovirus; hPIV, human parainfluenza virus; hCoV, human coronavirus; hRv, human rhinovirus; BMI, body mass index; ln, natural logarithm

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Conclusions: Compared with placebo the essential-oil extract of three Cretan aromatic plants provided no detectable statistically significant benefit or harm in the patients with upper respiratory illness, although descriptive differences were identified in favorable direction mainly in the virus-positive population.

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1. Introduction

Novel H1N1, referred also as “swine flu”, as well as other viral agents involved in common cold infections, compose a public health problem, accountable for many visits to primary and secondary health care services and hospital admissions; additionally, a considerable number of deaths is likewise documented, especially in vulnerable people. These infections are easily transmitted by exposure to infected droplets expelled by coughing or sneezing that can be inhaled, or contaminated hands or surfaces. Symptoms include fever, cough, headache, muscle and joint pain, sore throat and runny nose, and sometimes vomiting and diarrhea. The new Influenza A (H1N1) appears to be as contagious as seasonal influenza, and spreads rapidly, particularly amongst young people. The severity of the disease ranges from very mild symptoms to serious illness and occasionally even death. The majority of people who contract the virus experience mild disease and recover without antiviral treatment or medical care. Additionally, more than 50% of serious hospitalized cases have underlying health conditions or impaired immune function. Antiviral drugs may reduce the symptoms and duration of illness and may also contribute to preventing severe disease and death. However, the effectiveness and cost of administered therapy remains an interesting topic for further investigation, as a recent Cochrane meta-analysis, although contested, reported a limited effect of Oseltamivir (Tamiflu®), a classical antiviral drug (Jefferson et al., 2014), mainly related to the time-limited and usual self-healing nature of the disease.

Much discussion has been dedicated to the use of herbal medicine for the common cold. A randomised controlled trial (RCT) has reported on the effects of So-cheoug-ryong-tang (Ko et al., 2004) and Yeon-gyo-pae-dok-san on the common cold (Byun et al., 2011). The use of herbs as ailments in the island of Crete has been mentioned since the Bronze Age (Arnott, 1996). Herbal medicine also has a long history in ancient Greece. Popular medical handbooks from the Byzantine era forward incorporated material rooted in ancient medicine and routinely claimed Hippocrates and Galen (among others) as sources (Clark, 2002). In current times, the antioxidant activity of herbs in rural Crete has been investigated and it has been shown that herbal extracts decrease lipid peroxidation in cultured lung cells exposed to iron or ozone (Lionis et al., 2004).

Previous ethnobotanical field studies have revealed the existence of an indigenous knowledge system in rural Crete and certain combinations of different aromatic plants in rural areas of Crete have been used for the prevention and cure of the common cold and influenza (Lionis et al., 1998). The biological effects and bioactivity of essential oils and as well as their antibacterial properties have been reported in the literature (Cowan, 1999; Burt, 2004; Bakkali et al., 2008; Koroch et al., 2007). Similarly, the antiviral potential of medicinal plants has been discussed as well (Jassim and Naji, 2003; Mukhtar et al., 2008). It is also exciting that even though the term “virus” is a very recent term in modern medicine (Silverstein, 2009), these rural societies, without any prior knowledge of the term and in the absence of any immunologic knowledge, were protecting the population using traditional and folk-medicine. As stated above, it was interesting to study to what extent this ethnopharmacological knowledge is evidence-based and effective in virus-induced diseases. To that effort we have also taken into account certain Cretan history and archeological elements, as well as their ethnopharmacological uses in the Eastern Mediterranean and Near East regions, suggesting a beneficial action of such a combination in upper respiratory viral infections (Dafni et al., 1984;

Honda et al., 1996; Lev and Amar, 2002; Said et al., 2002; Hanlidou et al., 2004; Lardos, 2006; Hudaib et al., 2008).

As stated above, this study reports the results of the effectiveness of an extract based on three Cretan aromatic plants in the reduction of duration and severity of symptoms of patients with upper respiratory tract infections, utilizing a standardized questionnaire, physical examination measurements and the decrease of inflammation assayed by C-reactive protein [CRP] levels.

2. Subjects and methods

2.1. Plant material identification

Plant material has been identified by one of the authors (SP) and voucher specimens of the three species have been deposited at the Herbarium TAU of the Aristotle University of Thessaloniki (UOCSP101-1, UOCSP101-2, and UOCSP101-3).

2.2. Preparation of essential oil extracts

Formulation of the essential oil extracts was provided by the authors and prepared by Olvos SA, according to the patents and patent applications related to the subject (WO2010GB01836 20100929; GB20090017086 20090929; EP2482831; CN102762218) of thyme or Spanish oregano (*Coridothymus capitatus* (L.) Rchb. f. synonym of *Thymbra capitata* (L.) Cav.), dictamnus or Cretan dittany (*Origanum dictamnus* L.) and sage (*Salvia fruticosa* Mill., *Salvia pomifera* L.) extracts through steam distillation. Analysis of essential oils was performed by Gas Chromatography–Mass Spectroscopy, in a GC–MS, Shimadzu, QP 5050A apparatus. GC was equipped with MDN-5 column (length 30 m, film thickness 0.25 µm, diameter 0.25 mm, max. useable temperature 325 °C) and a Quadrupole Mass Spectrometer as detector. MDN-5 column temperature was initially 50 °C for 5 min. It was then gradually increased to 150 °C at 5 °C/min and kept for 10 min, and finally increased to 280 °C at 5 °C/min and held for 20 min. The carrier gas was helium, the flow rate 0.9 mL/min. 2 µL was used as an injection volume. The sample was measured in a split mode procedure with a split ratio 1:35. Injector and detector were maintained at 230 and 250 °C, respectively. For GS–MS detection an electron ionization system was used with ionization energy at 70 eV. The chemical and percentage composition of specific constituents in each essential oil (Adams, 2007) is shown in Supplemental Tables 1–3.

Essential oils (at a dilution of 15 mL/L) in extra virgin olive oil (used as a vehicle) were formulated as 0.5 mL soft-gel capsules, for a daily dose of two capsules. This dose has been based on anthropological reports of fieldwork work studies carried on Crete (Lionis et al., 2004) and corresponds to two cups of infusion of the aforementioned aromatic plants. Placebo capsules contained only extra-virgin oil, equally formulated as 0.5 mL soft-gel capsules. GC–MS chemical and percentage composition of the formulated mixture (Adams, 2007) are shown in Supplemental Table 4.

2.3. Subjects and setting

Participants were recruited from October 10th, 2013 to February 10th, 2014. Eligible subjects were patients aged 18 years or older, presenting at their local Health Centre of Harakas in the prefecture of Heraklion, in rural Crete, Greece, with symptoms of upper respiratory

tract infection, initiated within the previous 24 h. The Jackson criteria were used in order to identify patients suffering from common cold (Jackson et al., 1958). These criteria consist of sneezing, nasal discharge, nasal obstruction, sore throat, cough, headache, malaise and chilliness. A patient was considered as eligible for participation when the Jackson score was greater or equal than two (with at least one of the first four 'cold-specific' symptoms present). Patients on a daily dosage of acetylsalicylic acid greater than 100 mg were excluded from the study. Further criteria for exclusion were the presence of malignancy, immunosuppression and pregnancy. Harakas Health Centre serves a population of 12,284, in rural Crete. Of the 11 General Practitioners (GPs) practicing at the Centre, 10 agreed to participate. Eligible patients were informed about the study and enrolled after providing written consent.

2.4. Study design

A randomized placebo-controlled, double-blind, parallel group design was used and the CONSORT Herbal Medicinal Interventions guidelines were followed (Gagnier et al., 2006). Stratified randomization using a varying block size (2 or 4) was implemented using the statistical environment R, version 3.1 (<http://www.R-project.org>), package *blockrand*; stratification was by sex and age (cut-off taken at 65 years). The packages containing the capsules were stored at the Health Centre and were identical in appearance but with differing ID labels, corresponding to specific patients. Enrolled patients received either the Cretan aromatic plants product or the placebo. Both GPs and patients were blind to treatment group, as were the study coordinators, Clinical Chemistry and Immunology Diagnostic laboratories and other participating personnel. Patients were examined at the GP's office on the day of presentation and at follow-up (6 days later) and were contacted by telephone on a daily basis by their GPs between their initial and final visits, for the completion of the daily questionnaire (see below, Section 2.6).

2.5. Ethics

This study received approval by the Commission of Bioethics of the University General Hospital of Heraklion (No 9186, 05-09-2012). A written, fully informed consent was obtained from all patients enrolled in the study. The trial was registered in the International Standard Randomised Controlled Trial Number Register (<http://www.isrctn.org>) (ISRCTN number: ISRCTN09700238).

2.6. Questionnaire

After appropriate permission, the validated 21-item Wisconsin Upper Respiratory System Survey (WURSS-21) instrument, translated in Greek, was used to quantify symptoms' severity and duration (Barrett et al., 2009). The WURSS-21 is an evaluative illness-specific quality of life instrument, designed to assess the negative impact of acute upper respiratory infection. Daily WURSS summary scores are calculated by summing scores of individual items. The first and last of the 21 items are not included in the summation as they have categorically different reference domains (<http://www.fammed.wisc.edu/research/external-funded/wurss>), accessed on 10 August 2013). Higher scores indicate more severe symptoms (the theoretical maximum score being 133) whilst a score of 0 indicates the complete absence of symptoms.

The questionnaire was completed on a daily basis, seven times in total. Initially (day one) and at final follow-up (day seven) it was completed by the patient at the GP's office and in between, completion was undertaken by their GP, using telephone interviews. During the initial visit, information was also obtained on participant gender, date of birth, self-reported weight and height, smoking history (number of cigarettes per day, years of smoking, year started, year

quitted), alcohol consumption (type of drink, milligrams per day), medical history (chronic illnesses, type of drugs provided, drug dosage), vaccinations (against *Streptococcus pneumoniae* and influenza) and hospitalization history.

2.7. Clinical examination and laboratory tests

Each subject underwent a clinical examination, including the following: systolic blood pressure (SBP, mmHg), diastolic blood pressure (DBP, mmHg), O₂ saturation (%), pulse (beats per minute), body temperature (degrees Celsius) and respiratory rate (breaths per minute). These measurements were repeated at day 7, at the follow-up visit to the GP's office. Blood was drawn on days one and seven, for the following tests: erythrocyte count (RBC, 10⁶/μL), hemoglobin (g/dL), hematocrit (%), white blood cell count (WBC, 10⁹/L), neutrophil (%), lymphocytes (%), monocytes (%), thrombocytes (%), alanine aminotransferase (ALAT or SGPT, U/L), aspartate aminotransferase (ASAT or SGOT, U/L), alkaline phosphatase (ALP, U/L), creatinine (mg/dL) and C-reactive protein (CRP, mg/dL). All assays were performed at the University Hospital of Heraklion, after a specific agreement, with routine standard methods (Number of Protocol: 7420/10-10-2013).

2.8. Virology Real-Time PCR assays

2.8.1. DNA/RNA extraction

Nucleic acids were extracted from nasopharyngeal swabs, using a commercial purification kit (PureLink Viral DNA/RNA kit, Invitrogen), suitable for viral DNA/RNA extraction, according to the manufacturer's instructions. All samples were quantified spectrophotometrically and normalized aliquots were produced for each sample. DNA/RNA isolation was performed in a separate area of the laboratory distant from the Polymerase Chain Reaction (PCR) area. Meticulous care was taken to avoid cross-contamination of the DNA/RNA. One hundred swabs were taken. Six swabs were used as internal controls, resulting in 94 available samples.

2.8.2. Detection of respiratory viruses by Real-Time PCR

The detection of respiratory viruses was carried out using two commercial kits, by Real Time PCR. The first kit allowed the multiplex Real-Time PCR detection and identification of 13 human respiratory viruses, namely human respiratory syncytial virus (hRSV) RNA, human metapneumovirus (hMPV) RNA, human parainfluenza virus-1–4 (hPIV) RNA, OC43, E229, NL63 and HKU1 human coronavirus (hCoV) RNA, human rhinovirus (hRV) RNA, human B, C and E adenovirus (hAdV) DNA as well as human bocavirus (hBoV) DNA (Cat no TV57-100FRT, Sacace). In addition, a second commercial kit was also employed for the simultaneous detection of 2009 pandemic H1N1, Influenza A virus and Influenza B virus (Anyplex™ FluA/B Typing Real-time Detection (V1.1), Seegene). Real-Time PCR reactions were set up according to the manufacturers' protocols, following the amplification conditions recommended and data were collected during annealing (two measurements) and at all times during melt curve analysis. Experiments were conducted on an ABI 7500 Fast System Real-Time PCR thermal cycler, using software version v2.0.6 (Applied Biosystems). The quality control of the DNA/RNA samples was accomplished by checking for the successful amplification of *beta-2-microglobulin* gene.

2.9. Outcomes

The primary outcomes of interest were the duration of symptoms and changes in the severity of symptoms over the study period. Duration of symptoms was assessed as the number of days from enrollment, before the participant reported "Not sick" to the question

“How sick do you feel today?” i.e. a response of 0 on a Likert scale of 0–7. Symptom severity was assessed by the outcome of WURSS-21 questionnaires. A secondary outcome of interest was the normalization (CRP < 0.8 mg/dL) of CRP positive subjects, at day 7. The Number Needed to Treat (NNT) that indicates the number of patients we need to treat to benefit one was also calculated in order to measure the study's effect.

2.10. Sample size estimation

An overall sample size of 90 (45 in each group) was calculated to be necessary to detect a medium–large effect size (0.6) with power 80% at a significance level of 5% (comparison of means), using GPower 3.1.7 (Faul et al., 2007).

2.11. Statistical analysis

General characteristics of the patients in each group at baseline were summarized using descriptive statistics. Univariate comparisons of patient characteristics between the two study groups were performed using Pearson's chi-square test of independence (for categorical data), the independent samples *t*-test (for normally distributed variables) and the non-parametric the Mann–Whitney test (for not

normally distributed variable). Severity of symptoms over the study period was compared between intervention and control groups, using the 19-item WURSS-21 scores, with both univariate techniques and multiple linear regression models, adjusting for age, sex, initial CRP level, body mass index (BMI) and the presence of chronic diseases. Severity was assessed in three ways: (a) firstly, the average total WURSS-21 score over the time period was compared between groups (i.e. the sum of daily WURSS-21 scores). (b) Differences between the 2 groups were assessed by calculating the area under the time–severity curve (the *y*-axis being the WURSS-21 score). The area under the curve (AUC) was calculated using a trapezoidal approximation. (c) Finally, the average maximum WURSS score (for each subject) was compared between groups. The percentage of missing values for the daily WURSS-21 scores ranged from 2.8% ($n=3$ out of 108 at baseline) to 6.5% ($n=7$ out of 108 on day three). Confidence intervals for median daily scores were calculated using a binomial method (the *centile* command in Stata 12). Multiple linear regression models were applied, with dependent variable the natural logarithm of the AUC [$\ln(\text{AUC})$] and independent variables the treatment (intervention/placebo), age (in years), BMI (kg/m^2), gender (male/female), presence of chronic illnesses (yes/no) and CRP-level upon presentation (≥ 0.8 mg/dL vs ≤ 0.8 mg/dL). Discrete-time survival analysis was used to assess possible differences in time to symptom cessation (i.e. symptom

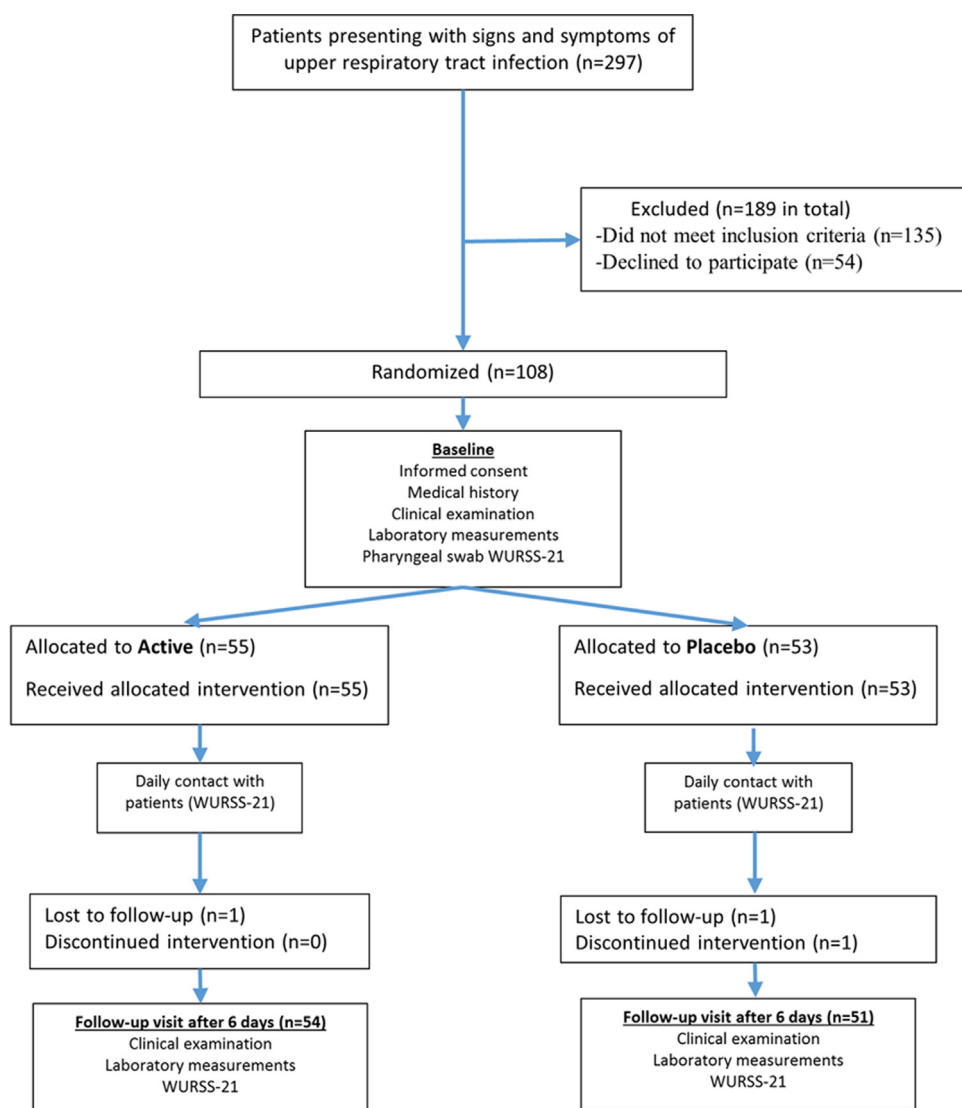


Fig. 1. Flowchart of the study. Graphical representation of the RCT study design. See *Material and methods* and *Results* for further details.

duration) between groups. Symptoms were considered absent on the first day in which the WURSS score dropped to zero. An intention-to-treat approach was used. A complementary log–log model (a discrete time proportional hazards model) was also applied to our data. The model was estimated by maximum likelihood adjusting for age and sex (Stata command *cloglog*). The proportions of initially CRP-positive subjects who were CRP-negative at the end of the study were compared between groups. In addition, within-group comparisons of CRP changes were made using McNemar's test.

At randomization, all diagnosed upper respiratory tract infections were presumed to be of viral etiology. Respiratory viruses were only detected, however, in 48 (51%) of the 94 patients tested. The statistical methods described above were initially applied to the total sample and the analyses were subsequently repeated using data only from the subsample of virus-positive patients. The significance level was set to 5% and the statistical software used was SPSS version 21 (for univariate analysis) and STATA version 12 (for the survival analysis). Results are presented below by outcome, for the complete sample and for the sample of virus-positive patients.

3. Results

3.1. Study population

A total of 297 patients with signs and/or symptoms of upper respiratory tract infection visited the Health Centre of Charakas over the 4 month time-period, 157 (52.9%) females and 140 (47.1%) males. One hundred and thirty five (45.5%) did not meet eligibility criteria (73 females, 62 males); 113 subjects (84%) because they presented to their doctor more than 24 h after their symptoms had begun. Other reasons for exclusion included pregnancy ($n=7$, 5.3%), treatment with acetylsalicylic acid with a daily dose > 100 mg ($n=3$, 2.3%) and malignancy or immunosuppression ($n=2$, 1.5%). Fifty-four (54) out of the 162 eligible patients (33.3%) did not agree to participate in the study. The most common reason stated was lack of time/inability for follow-up ($n=23$, 42.6%). Four patients (9%) were not willing to participate in a clinical trial. Fourteen of the 46 patients (25.9%) did not provide details, while 13 (24.1%) denied for various other reasons. Comparison between patients willing to participate and those who were not did not produce significant results in terms of gender distribution. Those who were willing to participate were significantly older than those who were not (mean age 50 versus 41 years; 95% CI for the difference from 4.12 to 14.72 years). One hundred and eight (108) patients were enrolled in this study; however, three (3) patients (2.8%) did not complete it (two in the placebo group and one in the intervention group). One patient developed pneumonia, one patient reported being unable to swallow the pills and one patient was lost to follow-up after the initial examination (Fig. 1).

3.2. Demographic, clinical and laboratory evaluation at baseline

Intervention ($n=54$) and control ($n=51$) group patients had similar demographic characteristics (gender, age, BMI, smoking habit, alcohol consumption) and vaccination pattern against *Streptococcus pneumoniae* and influenza (Supplemental Table 5). In each group, about half of the patients reported suffering from at least one chronic illness. Most frequent chronic illnesses were heart diseases ($n=39$, 36%), metabolic diseases ($n=24$, 22%) followed by respiratory and musculoskeletal diseases ($n=10$, 9%). Clinical examination characteristics (which included systolic and diastolic blood pressure, oxygen saturation, pulses per minute, respiratory rate and temperature) were also similar in both groups (Supplemental Table 6). Finally, hematological (RBC, hemoglobin, hematocrit, WBC, neutrophils, lymphocytes, monocytes, thrombocytes), biochemical (SGPT, SGOT, ALP and creatinine) and immunological (CRP) parameters were sim-

ilar in both groups of patients (Supplemental Table 6). The median lymphocyte count was not increased in either group, suggestive of the very initial phase of the respiratory infection. The median WURSS-21 score at baseline was 49 (lower quartile 1 $Q_1=33$, upper quartile $Q_3=72$) in the placebo group and 48 ($Q_1=36$, $Q_3=68$) in the intervention group (Supplemental Table 6). There was one missing BMI measurement (0.9%), two missing values for pack-years of smoking (1.9%), one for pneumococcal vaccination (0.9%) and two regarding previous hospitalization of patients (1.9%). There were no missing measurements regarding presence of chronic diseases, vaccination against influenza and alcohol consumption.

Forty eight out of 94 patients that were tested (51%) were found to be positive for at least one viral strain (Fig. 2), assayed by specific RT-qPCR. The distributions of the demographic, hematological and biochemical variables appeared similar when the analysis was restricted to virus-positive patients (Supplemental Tables 7 and 8) with the possible exception of exception of lymphocyte count, which appeared somewhat higher at baseline in the intervention group ($p=0.051$). Interestingly, in 50% ($n=24$) of positive patients human rhinovirus (hRv) was detected (48% in the intervention group, 52% in the control group), while H1N1 influenza, human metapneumovirus (hMPV) and human coronavirus strain NL63 were identified in $\sim 15\%$ ($n=7$) of virus-positive patients. The severity of symptoms at baseline, as assayed by the WURSS-21 score, appeared highest in H1N1-positive patients ($p=0.190$, Supplemental Table 9).

3.3. Symptom severity over time

The severity of symptoms for the total duration of the study was assessed using the WURSS-21 score. The AUC, the maximum WURSS-21 score and the sum of the daily WURSS-21 scores were calculated per individual to estimate the severity of symptoms over the follow-up period. Using data from all participants, average AUC values and total WURSS-21 score appeared lower in the intervention compared to the placebo group but not to a statistically significant extent (with respective medians of 84 cf. 109, $p=0.795$, and 111 cf. 130, $p=0.648$). Average maximum WURSS-21 scores were 49 (min 6, max 106) and 51 (min 22 max 105) in intervention and control groups respectively ($p=0.803$). Boxplots of total WURSS-21 score by day and group are presented in Fig. 3A for all subjects and in Fig. 3B for symptomatic

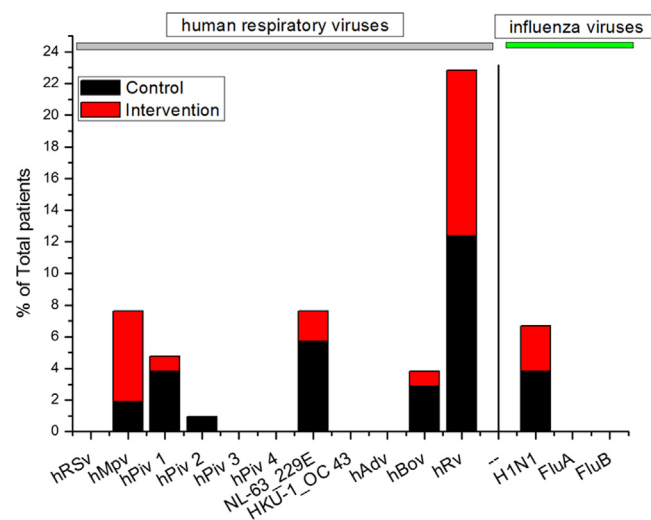


Fig. 2. Frequency charts of detected virus strains. Distribution of viruses' strains in our study population. This shows the different viruses strains while abscissa shows the percentage of positive patients in the study. The black part of the column shows virus positive patients in the control, while the red part the virus positive patients in the intervention group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

patients only. No statistically significant differences in comparisons of daily scores between placebo and intervention groups were observed.

Using multiple linear regression of log-transformed AUC on treatment and other possible predictor variables, treatment was not found to significantly affect $\ln(\text{AUC})$ ($b=0.01$; 95% CI from -0.28 to 0.29) adjusting for age ($b=0.01$; 95% CI from 0.00 to 0.02), BMI ($b=0.01$; 95% CI from -0.02 to 0.04), gender ($b=-0.08$; 95% CI from -0.39 to 0.23), presence of chronic illnesses ($b=-0.22$; 95% CI from -0.52 to 0.08) and baseline CRP-level ($b=0.28$; 95% CI from -0.02 to 0.58). No treatment-viral status interaction was found.

The distributions of the daily WURSS-21 scores in virus-positive intervention and control group patients are depicted in Fig. 3C. Administration of the Cretan aromatic plants extract in virus-positive

patients did not modify the total WURSS-21 score to a statistically significant extent, although lower scores were identified in the intervention group compared to placebo group: median 108 (min 36, max 359) cf. 130 (min 30 max 567), $p=0.590$. The average AUC scores were also not found to differ to a statistically significant extent, although again lower scores were seen in the intervention group (median 107) compared to the control group (median 84, $p=0.925$). Average maximum WURSS-21 scores (out of a possible 133) were 50 (min 13, max 107) and 57 (min 22, max 105) in intervention and control groups respectively ($p=0.803$).

3.4. Symptom duration

Using discrete-time hazard modeling with the complete data set, the “risk” of symptom cessation appeared 10% higher in the intervention group (hazard ratio, HR 1.10, 95% CI 0.72–1.69), adjusting for age and gender. Similar results were found when the analysis was restricted to virus-positive patients only: HR 1.42, 95% CI 0.66–3.10. The probability of cessation of symptoms per day is presented in Fig. 4A and B for the total sample and the virus-positive patients respectively. A trend towards a higher, albeit non-statistically significant, probability of symptom cessation in the intervention group can be seen.

Seventy percent of patients in the placebo group had a cessation of symptoms at the time of their final consultation, while the respective proportion in intervention group was 90.5% ($p=0.089$).

Finally, symptom amelioration was considered per virus infection (Fig. 5A–D). Fitting the curves with a logistic equation (Fig. 5E) permitted us to evaluate the time (in days) of a 50% and an 80%

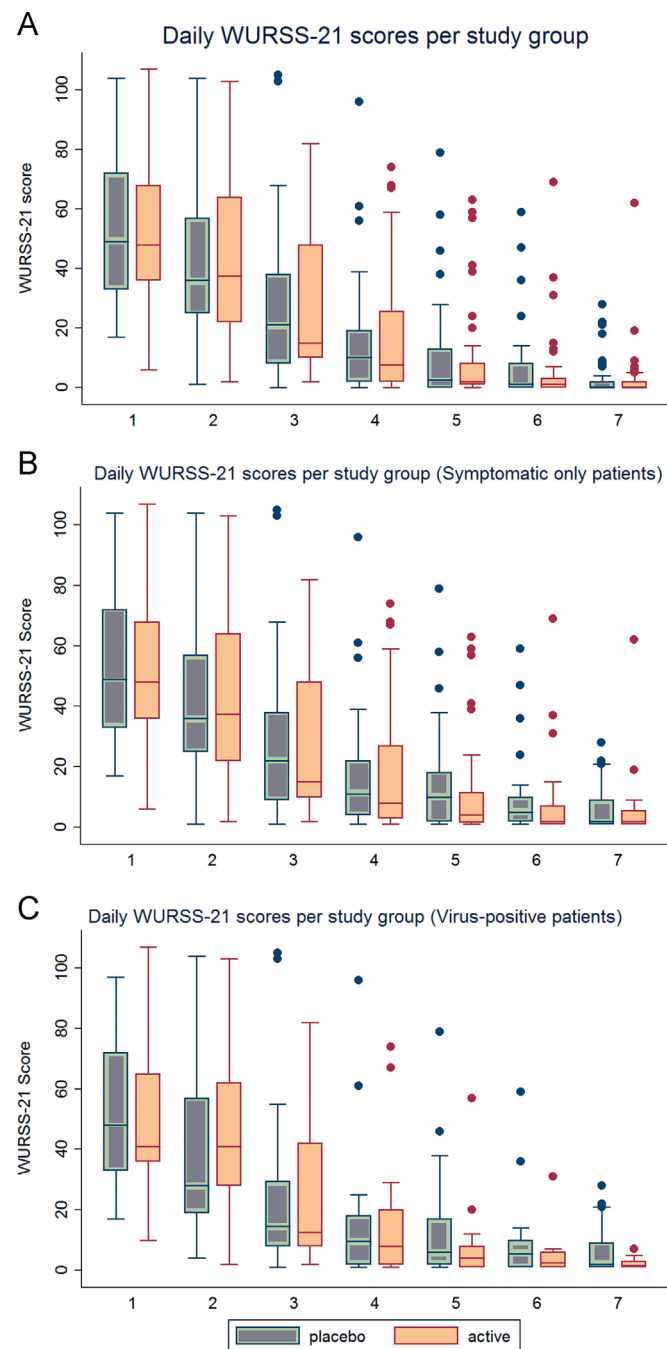


Fig. 3. Daily median WURSS-21 scores and 95% CIs per study groups. (A) Boxplots of Daily WURSS-21 scores per study group. (B) Boxplots of Daily WURSS-21 scores per study group in patients with symptoms (symptomatic patients). (C) Boxplots of Daily WURSS-21 scores per study group in virus positive patients.

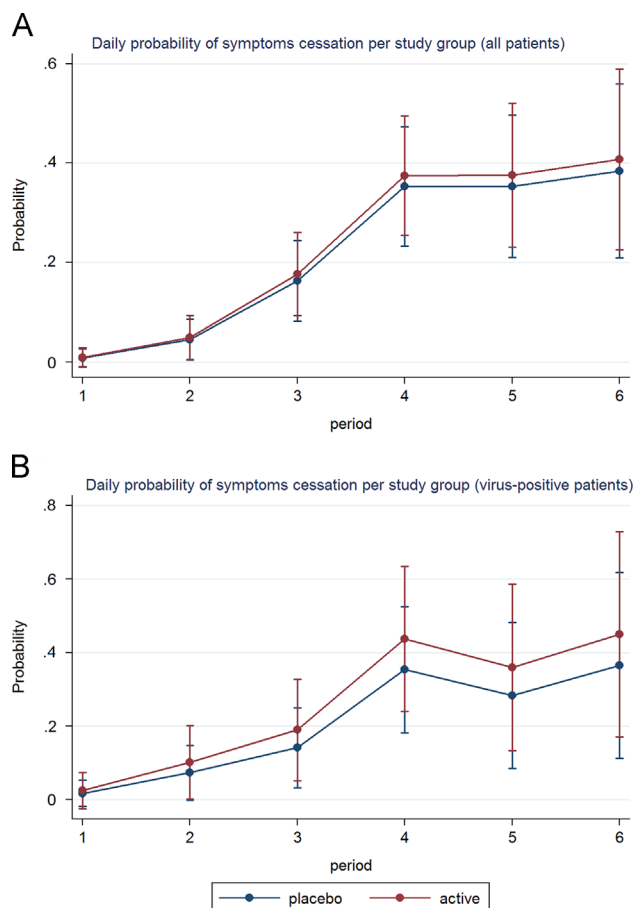


Fig. 4. Daily probability of symptom cessation. Whisker plots of the daily probabilities for symptom cessation and 95% Confidence intervals (CI's) per group in the total population of the study. (A) All patients and (B) virus-positive patients.

amelioration of symptoms, as quantified by the WURSS-21 questionnaire. Despite the small number of patients, it becomes evident that a significant amelioration (> 2 days) was observed in the 80% decrease of objective symptoms of the disease in patients with H1N1.

3.5. CRP status

At baseline, the proportion of patients with elevated CRP levels was similar in both groups (46% and 47% in intervention and placebo groups respectively). At follow-up, the respective proportions were 15% in the intervention group and 24% in the placebo group ($p=0.256$). In the intervention group, 17 out of 25 patients with initially elevated CRP levels had low CRP levels at follow-up (68%) as compared to 13 out of 24 patients in the placebo group (54%). A 95% CI for the 14 percentage unit difference in favor of the intervention treatment was calculated as -13% to 41% . Within group changes in the proportion of patients with consistently elevated CRP were statistically significant ($p < 0.0001$ and $p=0.002$ for intervention and placebo groups respectively). Details are presented in Supplemental Table 10, Fig. 6.

In the virus-positive group, normalization of CRP values also occurred preferentially in the intervention group (Fig. 6, Supplemental Table 11). At baseline, the proportion of patients with elevated CRP levels was identical in both groups. At follow-up, the respective proportions were 0% in the intervention group and 15% in the placebo group ($p=0.065$). When the analysis was restricted within each study group, a statistically significant reduction of the number and proportion of patients with consistently elevated CRP level was found only within the intervention group ($p=0.016$).

3.6. Number-Needed to Treat

From the calculation of the Number Needed to Treat (NNT), we found that we have to treat 20 subjects from the whole group to

see one with symptoms being ceased on the 7th day of the follow-up. The corresponding numbers of the NTT when we analyze the data in virus-positive subjects were 5 on the 7th day.

3.7. Adverse effects

On the final day of their participation, all patients were asked to report on any possible adverse effect. Six out of the 105 that completed the study reported adverse effects; three of them had received the Cretan aromatic plants (intervention) treatment. All three participants were females and their respective age was 39, 50 and 54 years old. One of them reported frequent urination, the second reported mild abdominal pain in the left upper quadrant (for one day), and the third participant reported rash on her arms which was present for three days. No change in hepatic or renal function was observed, as evidenced by SGOT–SGPT and creatinine levels respectively. It is therefore concluded that the administration of the herbal extract, at least for the duration of our intervention, is safe.

4. Discussion

4.1. Efforts in measuring the anti-virus effect of herbs-biobibliographic evidence

There is much research on the anti-viral and especially the anti-influenza activity of a variety of medicinal plants worldwide, either in vitro by reporting the reduction of the viral cytopathic effect or in vivo. The main country in which such in vivo research has been conducted is China, where more than 126 Chinese patented medicinal recipes have been reported in 2010 (Tang et al., 2010). An interest in the effectiveness of herbal remedies in treating or preventing influenza or influenza-like illness has been acknowledged; however, a systematic review performed in 5 databases until 2006 reported 14

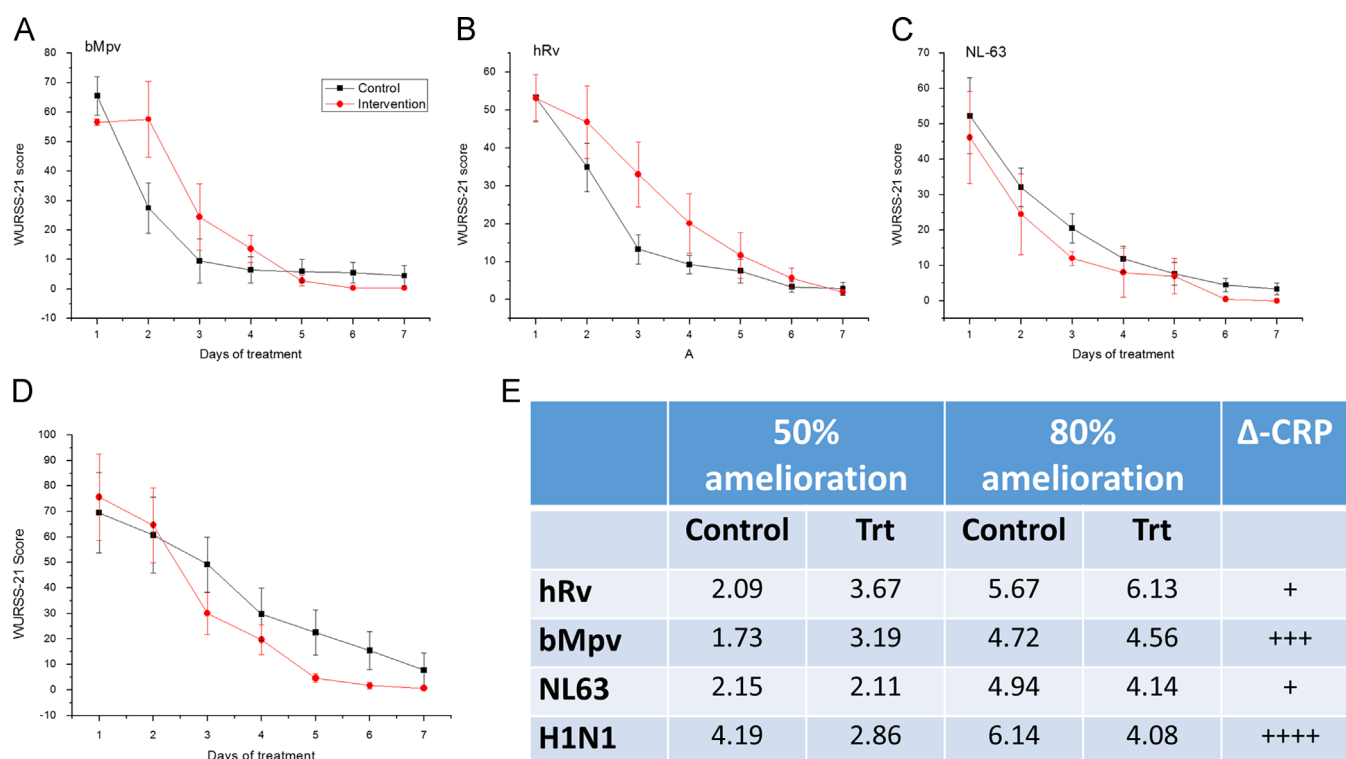


Fig. 5. Evolution of the disease in selected virus-positive groups, according to the virus strain detected. Figure depicts the WURSS-21 (mean \pm SE) values of the daily WURSS score in: (A) human metapneumovirus (hMpv), (B) human rhinovirus (hRv), (C) human parainfluenza virus-NL63, and (D) the pandemic H1, Influenza A virus (H1N1). Table presented in (E) shows the time in days at which half (50%) and 80% of WURSS-21 encoded symptoms were relieved in the control and the intervention group, according to the virus found at day 1. Data were calculated through a logistic fit of curves presented in A–D.

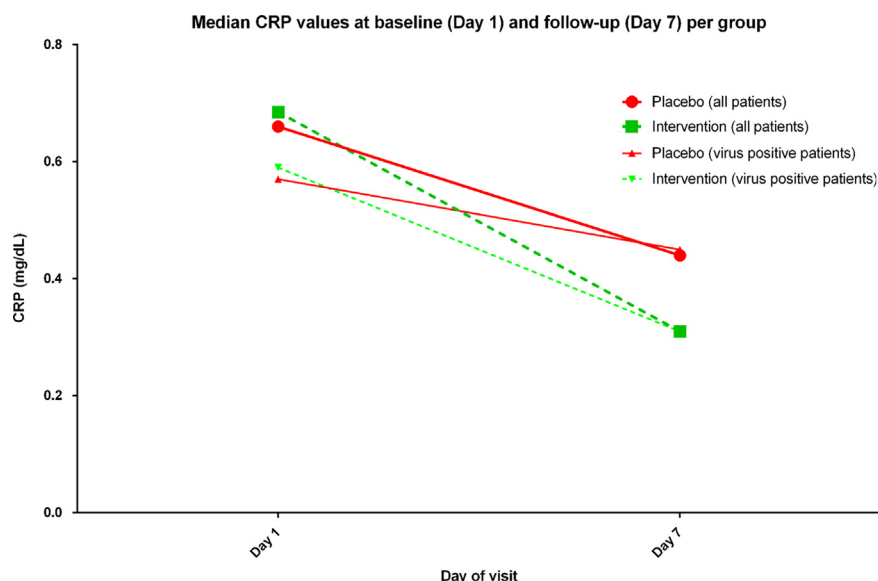


Fig. 6. CRP normalization. Line-plot representing normalization of CRP values (from day 1 to day 7) in the total and the virus-positive population.

RCTs and concludes that the majority of performed studies concern small groups, assayed with questionable methodologies or they report clinically irrelevant effects (Guo et al., 2007). Another systematic review (Wu et al., 2008), searching the evidence from Cochrane systematic reviews, about the effectiveness of traditional Chinese medicinal herbs in treating acute respiratory infections, came to similar conclusions. The authors also reported lack of quality in the researched trials and the presence of certain biases that have an impact on study validity (Wu et al., 2008). A recent Chinese RCT (Wang et al., 2010) described the mean symptom population scores, as well as the number of recovered patients, as primary endpoints for treating influenza patients while one of the secondary endpoints included the time required to alleviate both fever and the severity of clinical symptoms (Wang et al., 2010).

4.2. The main findings of the Cretan study

In our randomized clinical trial, we have compared the essential-oil extract of three Cretan aromatic plants with placebo; our main finding is that the plant extract did not show a statistically significant benefit or harm in patients with upper respiratory illness of any cause, although a slight albeit non-significant amelioration of symptoms was detected in the treated patients. This is further more evident in virus-positive patients. In details, the following primary or secondary endpoints of the trial are:

- The proportion of patients who presented symptom-free on day seven is higher in the intervention (90%) than in the control group (70%), a result close to statistical significance. Interestingly, and in spite of the small number of patients, a favorable effect was found in the subgroup of H1N1 influenza-infected patients.
- The proportion of patients whose CRP levels were normalized by the end of the follow-up period is significant in the intervention ($p=0.016$), but not within the placebo group ($p=0.125$).

In addition, we found a Number Needed to Treat (NNT) of 20 in the total population decreasing to 5 in the virus-positive population, meaning that at least one out of six virus-positive patients received the beneficial effect of our preparation.

4.3. Limitations and strengths of the study

Two key issues should be taken into consideration, prior to any safe conclusions on the effectiveness of the Cretan extracts in our population: (1) the definition of common cold that in our study was based on the Jackson score is based on a subjective assessment and could have potentially introduced selection bias. We are not certain to what extent non-infectious diseases have been included in the study sample. This may explain the high number of patients without a virus-infection (resulting in a sample size far lower than that required to detect a medium-large effect size—see *Methods*). The possible inclusion of non-infectious diseases could seriously have affected the design of the study that assumed a high proportion of patients with virus infection. (2) The primary endpoint of this trial was also based on self-reported symptoms and it is known that subjective self-assessment might also be another potential source of bias. Furthermore, the self-healing nature of the disease and the small duration of the symptoms presents additional difficulties in the exploitation of the data.

In this report, we provide only clinical data, without any attempt to decipher underlying mechanisms of action, explaining the anti-viral properties of our preparation. However, preliminary data suggest a direct in vitro anti-viral action of our preparation. In a recent review it was highlighted that the use of certain herbal extracts rich in polyphenols could play an important role in controlling virus outbreaks and alleviating their symptoms (Hudson, 2009). Actually, we are in the process of further delineating the direct anti-viral action of our preparation, as well as of some of its specific constituents, detected after an NMR analysis, although we strongly believe that the beneficial effect might be attributed to a combined effect of more than one constituent, as we have previously reported in the case of wine extracts (Damianaki et al., 2000; Kampa et al., 2000).

The present study is the first RCT on the subject. Randomization was successful in creating comparable baseline groups in terms of other disease prevalence, demographic, anthropometric and laboratory (biochemical, hematological and immunological) data. In addition, the proportion of patients completing the study was very high. However, there are certain limitations that need to be considered in the interpretation of results. Our sample is limited in size (~100 participants) and becomes even smaller when focussing on patients with a documented viral infection. Even in the sub-sample of virus positive patients, our sample was very heterogeneous as several types of viruses

were identified. However, the effect of our extract might be underestimated, since a sample of 48 subjects with a virus-documented infection does not provide enough statistical power, to respond to the question of the potency of our preparation in a large spectrum of common cold and influenza viruses. This can only be addressed through laboratory estimation of the potency of our preparation on virally-infected cells (which is under investigation) and through a larger analysis, permitting the collection of a significantly higher number of virus-infected patients.

4.4. Impact of the study

The use of herbs is extensively diffused among local cultures and it represents a time-line between ancient and modern traditions. Tradition and culture can further create ideal synergies in engaging the pharmaceutical industry towards innovative herbal-oriented research and possibly new products. However, our main intention was to introduce and promote research based on indigenous knowledge of the European regional flora, especially from the island of Crete, considered as a herbarium of the Mediterranean flora. We do not, of course, suggest to the health practitioners that they replace existing guidelines about the use of anti-viral treatment in influenza virus, although the use of specific anti-viral agents has been recently criticized, nor we suggest the combined use of anti-viral drugs and herbal extracts, which may lead to unwanted drug-herb interactions (Yang et al., 2012). The study findings arrive on a time where much criticism has been published on the effectiveness of the neuraminidase inhibitors (Jefferson et al., 2014). Very few adverse effects were reported in our study group. Indeed, our current laboratory data suggest that our preparation is effective in a broad spectrum of viruses, at doses similar or higher to those reported here.

5. Conclusions

Compared with placebo the essential-oil extract of three Cretan aromatic plants provided no detectable statistically significant benefit or harm in the patients with upper respiratory illness, although descriptive differences were identified in favorable direction mainly in the virus-positive population. In general, our RCT study provides promising new data in the field of the use of aromatic plants and their therapeutic potential as anti-viral therapeutics, and calls for additional basic and interventional research either for prevention or treatment of common cold and influenza in larger group of patients.

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Authors' contributions

CL, SP and EC conceived and shaped the idea. AB, JM, CL, and EKS prepared the first draft of the manuscript. JM was responsible for designing the randomization procedure. AB participated in the study, and was responsible for data entry and data analysis under the supervision of JM. GD participated in the study as coordinator and reviewed the manuscript. NM, SPD, and GS were responsible for the biochemical and immunological analysis. GKT and HEK were responsible for GC–MS analyses of specimens. CL provided clinical details and technical input, revised the manuscript and

performed editing and format changes throughout the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supporting information

Supplementary data (Supplemental Tables) associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jep.2015.01.030>.

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