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A standardized polyphenol mixture extracted from poplar-type propolis for remission of symptoms of uncomplicated upper respiratory tract infection (URTI): A monocentric, randomized, double-blind, placebo-controlled clinical trial

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ABSTRACT

Background: The most common symptoms of mild upper respiratory tract infections (URTIs) are sore throat, muffled dysphonia, and swelling and redness of the throat, which result from the inflammation process following acute bacterial or viral infection.
Hypothesis/purpose: As propolis is a natural resinous substance traditionally used to maintain oral cavity and upper respiratory tract health due to its antimicrobial and anti-inflammatory properties, the aim of this study is to evaluate the efficacy of an oral spray based on poplar-type propolis extract with a known and standardized polyphenol content, on the remission of the symptoms associated with mild uncomplicated URTIs.
Study design: A monocentric, randomized, double-blind, placebo-controlled clinical trial was performed.
Methods: This study was conducted in 122 healthy adults who had perceived mild upper respiratory tract infections. Participants, randomly assigned to receive either propolis oral spray ($N = 58$) or placebo ($N = 64$), underwent four visits (baseline = t0, after 3 days = t1 and after 5 days = t2 and after a follow-up of 15 days = t3) in an outpatient setting. Propolis oral spray total polyphenol content was 15 mg/ml. The dosage was 2–4 sprays, corresponding to 12–24 mg of polyphenols, three times for five days. The duration of the study was 8 weeks.
Results: After 3 days of treatment, 83% of subjects treated with propolis oral spray had remission of symptoms, while 72% of subjects in the placebo group had at least one remaining symptom. After five days, all subjects had recovered from all symptoms. This means that resolution from mild uncomplicated URTIs took place two days earlier, instead of taking place in five days as recorded in the control group. There was no relationship between the ingestion of propolis oral spray or placebo and adverse reactions.
Conclusion: Propolis oral spray can be used to improve both bacterial and viral uncomplicated URTI symptoms in a smaller number of days without the use of pharmacological treatment, leading to a prompt symptom resolution.

Abbreviations: CRF, Case report form; M.E.D., Multi dinamic extraction; URTIS, Upper respiratory tract infections.

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Introduction

The term Upper Respiratory Tract Infections (URTIs) is commonly used to describe acute infections of mucosa lining the upper respiratory tract caused by bacteria and viruses. Invasion of pathogens and their toxins induces an inflammatory response of the immune system, causing irritation and swelling, which may spread from the nasopharyngeal area to the sinuses, larynx, ear, epiglottis and lower airways. The associated diseases include the common cold, nasopharyngitis, acute medial otitis, pharyngitis, rhinosinusitis, laryngitis and laryngotracheitis (Yoon et al., 2017). URTIs are a very common condition being one of the most frequent causes of a physician's consultation worldwide. Upper respiratory tract infections are accountable for greater than 20 million missed days of school and greater than 20 million days of work lost, thus generating a large economic burden (Thomas et al., 2020).

The most prominent clinical symptoms of an URTI are cough, sore throat, runny nose, nasal congestion, headache, low-grade fever, facial pressure, sneezing, malaise and myalgias (Thomas et al., 2020). While patients recover spontaneously without therapy after about 7–10 days for most cases of uncomplicated URTIs, management of presumptive bacterial URTIs has focused on advising antibiotic drugs to avoid complications in the past. Nowadays, if symptoms persist, the main pharmacological treatments include nonsteroidal anti-inflammatory drugs showing antipyretic, analgesic, and anti-inflammatory properties, topical and systemic steroids used to reduce mucose swelling, dextromethorphan and codeine used as centrally acting cough suppressant in adults, and antibiotics in the case of detected bacterial infections (Smith et al., 2012). As these drugs are associated with notable adverse effects (AEs), the utilization of complementary and alternative remedies is widely used in both treatment and prevention of URTIs (Barrett et al., 2010; Brinckmann et al., 2003; Lucas et al., 2018).

Propolis has been commonly used worldwide as a traditional and ethnopharmacological medicine since ancient times (Yuksel et al., 2016) in particular in Europe (Italy, Germany, Switzerland, France, Greek, England, and Russia) (Jolly, 1978; Toreti et al., 2013; Wagh, 2013; Kuropatnicki et al., 2013; Monti et al., 1983; Murray and Pizzorno, 2005, Duke et al., 2017, Fedotova and Konovalov, 2019), China (Chan et al., 2013) Japan, North Africa, Sub Saharian Africa, Brazil, Australia (Bensoussan et al., 2005). In some countries, propolis is considered and regulated as a traditional medicine (e.g. Germany and Switzerland). In other parts of Europe, in the United States and Japan, propolis is regulated as a food supplement according to local laws. In Australia, propolis is regulated as a complementary medicine, having one or more active ingredients. Propolis is listed under Schedule 14 of the Commonwealth Therapeutic Goods Act 1989 (Bensoussan et al., 2005).

It consists of a natural resinous substance that bees collect from tree exudates and secretions, so as to build and protect their hives. Its composition is very complex and variable, depending on many factors such as geographical origin, types of vegetable sources, bee species, time and season of collection, and postharvest factors such as the extraction methods. Propolis consists of many active components (i.e. resins (50%), waxes (30%), essential oils (10%), pollen (5%), and other organic compounds (5%) including vitamins, minerals, and polyphenols, which can exert beneficial effects in URTIs, especially due to their anti-inflammatory effects (Zaccaria et al., 2017). Among minor components with healthy properties, flavonoids are the most important chemical class followed by phenolic acids such as caffeic, coumaric and ferulic acids, and caffeic acid phenethyl ester (CAPE).

Regarding the possible role of propolis in otorhinolaryngology, it has been found to be active against stomatitis and mouth ulcers. In addition, propolis showed beneficial effects in asthmatic patients (Kujumgiev et al., 1999; Tomazevic et al., 2013; Ali et al., 2011; Santos et al., 2005; Santos et al., 2008).

Despite propolis having been empirically used for centuries for the prevention and treatment of respiratory diseases, very few clinical studies have shown the beneficial effects of propolis against URTIs. As far as prevention of URTIs is concerned, a randomized, double blind, placebo controlled study on 430 children (age ranging from 1 to 5 years) showed a significant reduction of illness, (i.e. number of illness episodes, number of days with fever per child) in the group treated for 12 weeks with a herbal preparation containing propolis in combination with Echinacea and vitamin C, in comparison with the placebo group (Cohen et al., 2004). More recently, Marchisio et al. showed that propolis in combination with zinc reduced the recurrence of acute otitis media in 122 children (aged 1–5 years) with no problem of safety or tolerability (Marchisio et al., 2010). Regarding the possible role of propolis in the treatment of URTIs, the first study, which evaluated the effect of a preventative propolis treatment during the cold season against rhinopharyngitis in children, was published in 1995 (Crişan et al., 1995). Moreover, Di Pierro et al., showed that propolis complexed supplementation can reduce the severity of acute otitis media and reduce the rate of its evolution to more severe pathologies such as tracheitis, bronchitis, or rhinosinusitis in children (Di Pierro et al., 2016). More recently, Marti et al. showed that propolis nasal spray reduces the symptom intensity of acute rhinitis and common cold in children treated with propolis for 7 days without pharmacological treatments (Marti et al., 2017).

The results of the clinical trials reported and, more generally, literature data on propolis have the important limitation of not being easily comparable due to the high variability of the different types of propolis studied, which are obtained using different extraction methods showing different chemical compositions and therefore biological activities. To overcome the problem of propolis variability, we have used a new poplar-type propolis extract in our previous investigations, with a standardized polyphenol content obtained using a patented extraction method called Multi Dinamic Extraction (M.E.D.® propolis) (Volpi et al., 2017). M.E.D.® propolis is characterized by a reproducible chemical composition especially regarding the six main flavonoids, including galangin, chrysin, pinocembrin, apigenin, pinobanksin, and quercetin, with a relative concentration of about 40% (w/w) (Volpi et al., 2017). As far as phenolic acids are concerned, their relative concentrations ranges from 5% to 20% (w/w), with CAPE showing a relative concentration lower than 1% (w/w). Over the last decades, CAPE has been widely studied for its antimicrobic and anti-inflammatory properties, including antiviral activity, being able to inhibit the growth of Type A and B influenza virus by 95% and 92%, respectively, at the concentration of 8.8 μM, definitely higher than that found in propolis (Erdemli et al., 2015).

In our investigations, M.E.D.® propolis resulted to exert antioxidant and anti-inflammatory activities (Zaccaria et al., 2017) through an epigenetic mechanism of action, modifying the expression level of microRNAs and mRNA targets coding for antioxidant enzymes and pro-inflammatory cytokines. More recently, we showed that in experimental animals (adult male mice C57BL/6), the oral administration of M.E.D.® propolis is followed by the rapid absorption and metabolism of galangin and the induced adaptation of the antioxidant first line defense system. (Curti et al., 2019).

Considering that remission of URTI symptoms is the main reason for outpatient visits amongst adults within the first days of URTI and, to the best of our knowledge, no clinical trials have been performed to show the beneficial effects of propolis in reducing URTI symptoms in adults, and due to the fact that propolis exerts anti-inflammatory activity which is the initial cause of URTI symptoms, the aim of this study is to evaluate the effectiveness of a local treatment of M.E.D.® propolis oral spray in the remission of the associated symptomatology of URTIs by a monocentric, randomised double-blind placebo-controlled clinical trial.

Material and methods

Propolis oral spray and placebo

Propolis oral spray contains M.E.D.® propolis, vegetal glycerine

(10%) and natural flavours (< 1%). The M.E.D.® propolis used in this study is a hydro-alcoholic (6:4 v/v) solution obtained by the extraction of poplar-type raw propolis selected and worked in accordance with the Dynamic Multi Extraction patented method (N. 001,425,516)1 by B Natural srl (Corbetta, MI, Italy). According to the manufacturer's specifications, the propolis oral spray complies with European specifications for contaminants and microbiologic limits.

Placebo consists of a hydro-alcoholic (6:4 v/v) solution containing vegetal glycerin (10%), natural flavours (<1%) and commercial caramel color (E150) at a concentration so that the overall acceptable daily intake of the dietary colorant resulted to be less than 300 mg/kg of body weight /day (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 2011). Propolis oral spray and placebo were produced by B Natural (Corbetta (MI). ATS authorization 03/ATS (23/03/2016) and were packed in a 20 ml mouth spray bottles for oral application, indistinguishable in appearance, color, and flavor.

Polyphenol quantification in propolis oral spray using folin-ciocalteau's method

Total polyphenol content (TPC) of propolis oral spray was determined through Folin-Ciocalteau's method, using galangin as polyphenolic standard compound, as reported by Di Lorenzo et al. (Di Lorenzo et al., 2015). Propolis oral spray was analyzed in triplicate and the concentration of total polyphenols was calculated in terms of galangin equivalents, according to the following calibration curve: Absorbance (Abs) = 0.0014 concentration (g/ml) + 0.0756 (R^2 = 0.999), obtained from analyses of galangin solutions at different concentrations ranging from 10 to 700 mg/ml.

Propolis oral spray analysis by RP-HPLC–PDA–ESI–MSn

Chromatographic analyses were performed by means of the RP-HPLC-PDA-ESI-MSn method, set up by Zaccaria et al., 2019 (Zaccaria et al., 2019). These analyses were performed using an Agilent 1100 VL series mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA), which was further used on-line with HPLC equipment. The electrospray interface was set in negative ionization mode with the capillary voltage at 3500 V and a temperature source of 350 °C in full scan spectra (200–2200 Da, 10 full scans/s). Nitrogen was used as a drying (9 l/min) and nebulizing gas (11 p.s.i.). Software versions were 4.0 LC/MSD trap control 4.2 and Data Analysis 2.2 (Agilent Technologies, Inc., Santa Clara, CA, USA). Compound separation was obtained with an analytical Synergi Fusion RP-18 column (150 × 4.6 mm, 5 μm), equipped with a Hypersil Gold C18 precolumn (10 μ 2.1 mm, 5 μm), all produced by Phenomenex (Torrance, CA, USA). The mobile phase used was acidified water, with 0.1% formic acid (eluent A) and methanol (eluent B). The elution method was as follows: at 0 min 85% eluent A, from 85 to 60% in 30 min, from 60 to 45% in 35 min, from 45 to 38% in 5 min, from 38 to 0% in 15 min maintaining isocratic elution for 5 min. The run time was 110 min in total, including the reconditioning of the column. The flow rate was maintained at 1.00 ml/min, and the temperatures of the autosampler and column were kept at 4 and 33 °C, respectively. The volume of injection was set to 5 μl. Chromatograms were registered at 260 nm. The HPLC-ESI-MSn data were collected using Xcalibur software (Xcalibur 2.0, Thermo Fisher Scientific, Waltham, MA, USA).

Clinical trial design

A monocentric, randomised, double-blind, placebo-controlled clinical trial was performed by Samnium Medical Cooperative (Benevento, Italy) to evaluate the effects of propolis oral spray on an adult population suffering from uncomplicated forms of mild URTI diagnosed through a check-up by physicians and a throat swab.

The study was double-blind, both for the investigating physician and for the enrolled subjects. The participants received oral and written information concerning the study before they gave their written consent. Protocol, letter of intent of volunteers, and synoptic documents regarding the study were submitted to the Scientific Ethics Committee of ASL Benevento, Italy). The study was approved by the Committee (protocol number 152,869 of 18/12/2019) and carried out in accordance with the Helsinki declaration of 1964 (as revised in 2000). This study is listed on the ISRCTN registry (www.isrctn.com) with ID ISRCTN17594930 (doi.org/10.1186/ISRCTN17594930).

The clinical trial duration was 8 weeks. Participants underwent four visits (baseline = t0, after 3 days = t1, after 5 days = t2, and after the follow-up of 15 days = t3) in an outpatient setting. At baseline visit (t0) information on the sociodemographic, clinical and symptomatologic characteristics of the subjects was collected and reported in the case report form (CRF). In particular, the following URTI symptoms (presence/absence) were registered: sore throat, muffled dysphonia and swelling and redness of throat. Moreover, throat swabs were collected from physicians and transported to UNILAB SANNIO (San Giorgio del Sannio, Benevento, Italy) for microbiological analysis.

The physicians who carry out the enrolment were the family practitioners of the Samnium Medical Cooperative (Benevento, Italy), under the supervision of the principal investigator who performed the study. At the end of the baseline visit, the randomization sequence was generated by a statistician using STATA 16 software (Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC) and, the randomization list was kept hidden. Subjects were assigned to each treatment groups (propolis or placebo) by simple randomization (1:1 allocation ratio). It was not used stratification or blocking. The allocation sequence was kept hidden from the physician recruiting and evaluating participants using progressively numbered, opaque, sealed and stapled envelopes. The corresponding envelopes were opened only after the enlisted participants completed all baseline assessments. Both interventions were numbered according to the allocation sequence.

Propolis spray and placebo, made unrecognizable by identical packaging, color and taste, were given to the subjects. To the placebo group, 2–4 sprays of a hydro-alcoholic solution of dye E150 were administered during the baseline visit. Then, this treatment was repeated three times/day for 5 days in the subject's home. The propolis group was submitted to the same treatment with propolis oral spray. Clinical visits were carried out at t1 (after 3 days of treatment) and t2 (after 5 days of treatment) to evaluate the persistence of the symptoms (sore throat, muffled dysphonia and swelling and redness of throat) and health status. After the visits, the remission of symptoms was registered by the physicians on the CRF. After 5 days of treatment, subjects were followed up for 15 further days (t3). At the end of this follow-up period, a final throat swab was performed for the participants that were found to still be positive to bacterial infections. All data were compiled in the CRF by physicians.

Susceptibility test

Susceptibility tests were performed to determine the susceptibility of bacteria to antibiotics, at t0 (baseline visit) and t3 (after the follow-up of 15 days), both for the placebo and propolis group. To ascertain the incidence of the presence of pathogen strains resistant to antibiotics, the protocol provides for the determination of susceptibility to antibiotics of the bacteria occurring in the biological material taken from throat swab using the Kirby-Bauer method. The medium used was Mueller-Hinton (MH) agar, the commonly used microbiological grown medium for antibiotic susceptibility tests. To prepare the inoculum, 4–5 colonies grown on the primary isolation medium were suspended in 4–5 ml of Tryptic Soy Broth (enrichment broth), incubating for 2–6 h. Then, a bacterial suspension adjusted at the 0,5 MacFarland standard (see Clinical Laboratory Standards Institute document) was inoculated with a sterile swab on the MH agar surface. The antimicrobial-impregnated disks were placed on the agar surface, using sterile tweezers and the plates were incubated at 37 °C for 24 h. The results were evaluated by

diameter zone of inhibition around the disks

Study population

The number of participants recruited in this study (in December 2019) was 146, although the number of subjects actually involved was reduced to 122 due to not meeting the inclusion criteria. The subjects (58 in propolis group and 64 in placebo group) were recruited by the Samnium Medical Cooperative (Benevento, Italy). Subjects of both sexes, aged 18–77 years, were enrolled in December 2019 and were considered eligible for enrolment if they suffered from at least one of the following URTI symptoms: sore throat, muffled dysphonia and swelling and redness of throat. In addition, subjects were recruited only if these symptoms appeared the same day of the first baseline visit (t0). Pregnant women, women suspected of being pregnant, women who hoped to become pregnant, breastfeeding women, patients with allergies, cystic fibrosis, congenital or acquired immunodeficiency syndrome, history of asthma, serious renal disorders, cancer, cardiovascular diseases, systemic chronic disease and those considered unsuitable for the participation by the physician were excluded from the study. In addition, other exclusion criteria were the use of antibiotics and anti-inflammatory drugs within 72 h prior to enrolling in the study, and the use of immunological drugs within 4 weeks before the enrolment.

Evaluated variables

As sociodemographic characteristics, the age and gender of the participants were registered in the CRF. The primary endpoint was the remission of symptoms associated with URTIs, assessed at baseline (t0) and after visits at t1 (after three days) and t2 (after five days). A URTI diagnosis was made by the physician based on one or more URTI symptoms (sore throat, muffled dysphonia, swelling and redness of throat). As a secondary outcome, the persistence of positive throat swabs after the follow-up at 15 days was evaluated, to ascertain the incidence of the presence of pathogen strains resistant to antibiotics at the end of the follow-up (t3).

Statistical analysis

The sample size calculation was made using three 1-β power values equal to 0.95 and a significance level $\alpha = 0.05$. The sample size was determined to be 134 participants, allowing for a 15% drop out rate.

Descriptive statistics were used to characterize all survey items, using mean and standard deviation for the continuous variables, and numbers and frequency distributions for the categorical variables. Secondly, a univariate analysis was conducted by using χ^2 tests on 2×2 contingency tables to compare respiratory symptoms between treated and untreated subjects. We used Yates's correction when at least one cell of the table has an expected count smaller than 5. Thirdly, a multivariate logistic regression analysis was then conducted to determine the extent to which independent variables predicted the outcome of interest. We performed four logistic models, with the following outcome variables: remission or remission of all symptoms (Model 1), sore throat (Model 2), swelling and redness of throat (Model 3) and muffled dysphonia (Model 4) at three days. Independent variables included into the models were the following: age, gender, oral application of propolis (all Models), positive medical history for sore throat (Models 1, 3), positive medical history for muffled dysphonia (Models 1, 2, 3), positive medical history for swelling and redness of throat (1, 2), pharyngeal swab positivity (Models 1, 2, 3). The subject age was treated as a continuous variable, whereas all other predictors were treated as two-levels factors. The results of the logistic regression models were presented as Odds Ratios (ORs) with 95% confidence intervals (95% CIs). All reported values were based on two-tailed tests and were considered statistically significant at $p = 0.05$ or less. All data were coded and analysed using Stata software, version 15 (Stata Corp. Stata 2017. Stata Statistical Software. Release 15. College Station, TX: StataCorp LLC.).

Tolerance and safety assessment

In this investigation no specific toxicity studies have been performed. Nevertheless, for the evaluation of tolerance and safety of the intervention (propolis oral spray application), adverse events were monitored throughout the intervention period through spontaneous reporting of adverse events (AEs) by the participants to the relative physicians. At the end of the intervention period all subject data were evaluated by the principal investigator to determine the presence or absence of AEs.

Results

Propolis oral spray and placebo analysis

Propolis oral spray was submitted to Folin-Ciocalteau's assay to determine total polyphenol content, which resulted to be 15 mg/ml. Then propolis oral spray metabolic profile was determined by means of RP-HPLC-PDA-ESI-MSn (Fig. 1). The main polyphenols, non flavonoid compounds (caffeic, coumaric, ferulic, isoferulic acids, and caffeic acid phenethyl ester), and flavonoids, such as flavonols (galangin, quercetin, kaempferol, isorhamnetin,), flavones (chrysin, apigenin), flavonones (pinocembrin, pinobanksin), and their derivatives, were identified on the basis of their UV and mass spectra, matching the fragmentation patterns of standard molecules against the molecular ion and fragment ions (Table 1). After their identification, the main flavonoid species were determined by on-line HPLC–UV according to the method previously described Zaccaria et al., 2019. The concentration of the main flavonoids species resulted to be about 40% (w/w) of the total polyphenol content (Table 2).

Clinical trial

The study flowchart is reported in Fig. 2 according to the CONSORT PRO reporting guideline (Calvert et al., 2013). The number of participants involved in this study was 122. The treated group consisted of 58 (29 male and 29 female) subjects treated with propolis oral spray. The suggested daily dose was 2–4 sprays (corresponding to 0.8–1.6 ml of propolis oral spray and 12–24 mg/ml of polyphenols from M.E.D.® propolis) repeated three times/day for 5 days.

Upon receipt of the propolis spray and placebo at trial center, the shipment was inventoried ensuring that the information on the packing slip (inside and outside containers) matches accurately with what has been sent to the site, including the amount, batch numbers, manufacturing date, expiry date, name of manufacturer, quantity and storage conditions. Both interventions were stored in a locked cabinet in a locked room at environmental temperature accessible only to study staff. Access to the storage area were limited to essential research personnel. The following documentation was strictly maintained: entry and exit logbook and food supplement accountability logbook. The monitor reviewed drug accountability periodically and that final drug reconciliation to sponsor was performed.

The untreated group consisted of 64 subjects (25 male and 39 female) treated with placebo. The participants in the two groups had similar sociodemographic characteristics with no significant differences. At the baseline no difference between the two group was found. After the treatment we found a statistically significant difference between treated and untreated subjects. Subjects treated with propoli showed a higher remission of symptoms than subject in the placebo group.

The baseline characteristics of the subjects for each group are summarized in table 3. Mean ± standard deviation (SD) of subject age was 44 ± 14 years, and 68 of 122 (55.7%) were female. Moreover, in table 3, the number of subjects and the relative percentage reporting sore throat, muffled dysphonia, swelling and redness of throat and positive/negative throat swab are reported. After 5 days all the subjects in the study had

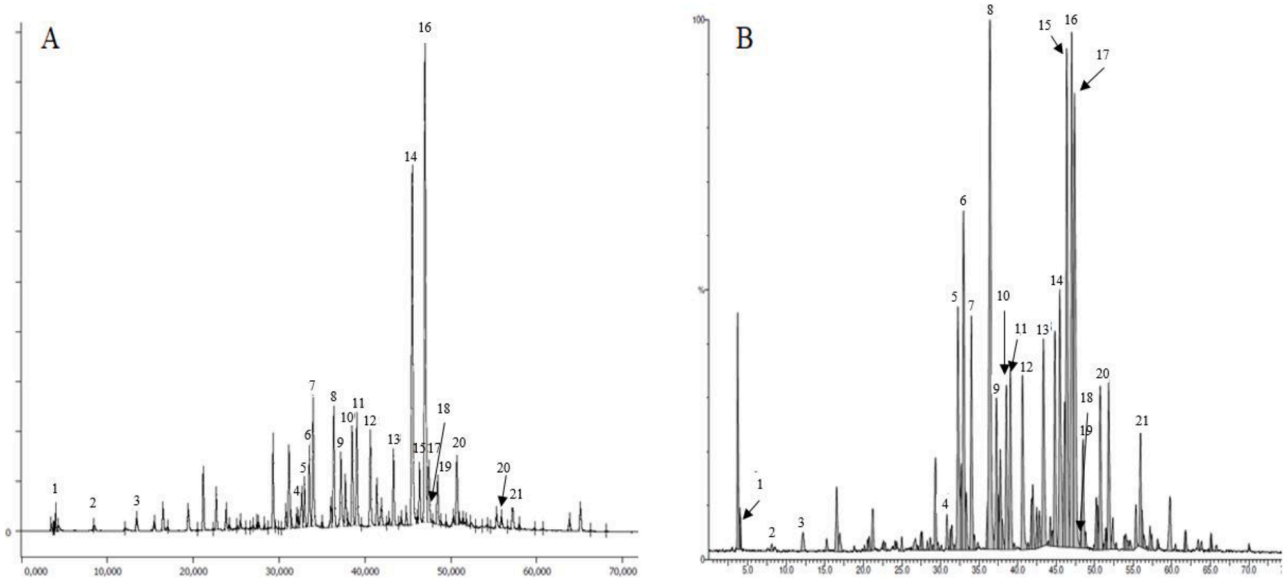

Fig. 1. Propolis oral spray: RP-HPLC chromatograms with UV-detection (A) and MS detection (B).

Table 1
Chromatographic and spectral properties of the compounds detected in propolis oral spray.

Peak number	RT (min)	UV absorption (λ_{max})	*m/z* [M-H]$^-$	Fragments (*m/z*)	Proposed structure
1	4.5	325	179	179, 46	Caffeic acid
2	7.6	310	163	119	Coumaric acid
3	12.1	322	193	193, 149, 134	Ferulic/Isoferulic acid
4	31.8	256	301	151, 179, 257, 273	Quercetin
5	32.5	288	315	300, 228	Quercetin 3-ME*
6	33.5	325	271	151, 165, 225, 253	Pinobaskin
7	34.3	267, 338	269	117, 149, 225	Apigenin
8	36.0	268, 294	285	257, 241	Kaempferol
9	37.1	254, 367	315	151	Isorhamnetin
10	38.0	290	299	284	Luteolin 5-ME*
11	39.0	290	329	314, 299, 285	Quercetin 5,7-DME**
12	41.1	260	283	239, 268	Galangine 5-ME*
13	43.5	290	315	300, 228	Quercetin 7-ME*
14	45.7	270	253	209	Chrysin
15	46.5	290	255	151, 187, 213	Pinocembrin
16	47.2	261, 351	269	227	Galangin
17	47.6	294, 318	313	253, 271, 299	Pinobanksin 3-O-Acetate
18	47.8	260	285	225,163, 105	Caffeic acid phenyl ester
19	48.5	264	283	269	Methoxychrysin
20	51.0	290	327	271, 253	Pinobanksin 3-O-Propionate
21	56.1	290	341	271, 253	Pinobanksin 3-O-Butyrate

* ME methyl ester.
** DME dimethyl ester.

Table 2
Relative percentage (% w/w) of the main flavonoid species in propolis oral spray determined by HPLC-UV.

Polyphenol	% (w/w)
Quercetin	1.5
Pinobanksin	2.2
Apigenin	1.8
Chrysin	14.0
Pinocembrin	2.0
Galangin	18.2
Sum of percentages	39.7

recovered from all symptoms, as expected considering that the first inclusion criterion was the diagnosis of uncomplicated forms of mild URTI. Fig. 3 shows the distribution of the three different symptoms in the treated and untreated groups after three days of watchful waiting. At t0, 8 people of the treated subjects and 7 of placebo group showed a positive throat swab. At t1, 17% of subjects who were treated with propolis had at least one symptom, while about 72% (RR: 2.93, CI: 1.95–4.42) of untreated subjects showed at least one symptom. Regarding the remission of single symptoms, similar results were obtained. In more detail, as far as a sore throat is concerned, this symptom was found in about 16% of subjects treated with propolis oral spray, while this value was 68% for the untreated group. (RR: 2.64, CI: 1.77–3.94). Moreover, 10% of subjects (*vs.* 71% in the untreated group) and 18% of subjects (*vs.* 83% in the untreated group) showed symptoms of muffled dysphonia (RR: 3.15, CI: 0.96–10.34) and swelling and redness of the throat (RR: 4.9, CI: 1.71–14.05), respectively.

After the follow-up (ended in January 2020) all the subjects with bacterial URTIs, in both treated and untreated groups, showed a negative throat swab. It was not possible to perform any statistical analysis after propolis treatment vs placebo since there were only 15 subjects with bacterial infections.

The results obtained from the univariate analysis show that only the treatment with propolis oral spray was related to resolution (resolution from all symptoms in treated group vs non treated group: $\chi^2 = 35.57$, df $= 1$, $p < 0.001$; resolution from sore throat in treated group vs non treated group: $\chi^2 = 28.38$, df $= 1$, $p < 0.001$; resolution from muffled dysphonia in treated group vs non treated group: $\chi^2 = 4.38$, df $= 1$, $p = 0.036$ after Yates's correction; resolution from swelling and redness of throat in treated group vs non treated group: $\chi^2 = 16.85$, df $= 1$, $p < 0.001$) (Table 4).

All the logistic models were significant (all symptoms: $\chi^2 = 46.51$, df $= 7$, $p < 0.001$; Sore throat: $\chi^2 = 34.21$, df $= 6$, $p < 0.001$; Swelling and redness of throat: $\chi^2 = 23.19$, df $= 6$, $p < 0.001$; Muffled dysphonia: $\chi^2 = 7.87$, df $= 3$, $p = 0.048$) and showed that the only variable related to resolution from all symptoms, as well as from single symptoms, was the

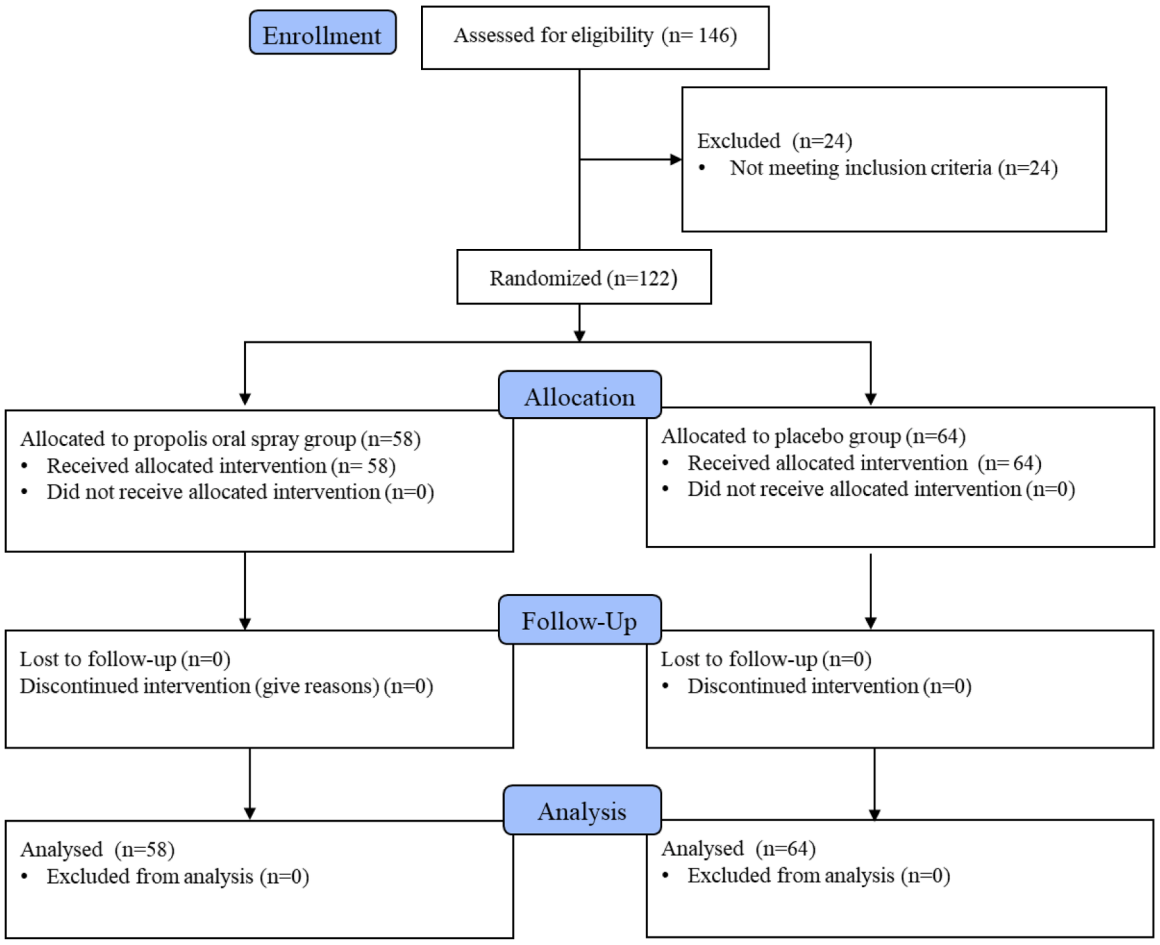

Fig. 2. CONSORT flow diagram.

Table 3
Characteristics of the study population: demographic and clinical data at baseline.

Characteristics	Number of observations	%	Treated(n = 58)	Untreated(n = 64)
Gender				
Male	54	44.3	29	25
Female	68	55.7	29	39
Age 44 ± 14 (18–77) *			44 ± 14 (18–77) *	44 ± 5 (18–77) *
Positive medical history for sore throat				
Yes	102	83.6	46	56
No	20	16.4	12	8
Positive medical history for muffled dysphonia				
Yes	17	13.9	10	7
No	105	86.1	48	57
Positive medical history swelling and redness of throat				
Yes	40	32.8	22	18
No	82	67.2	36	46
Throat swab				
Positive	15	12.3	7	8
Negative	107	87.7	51	56

* Data are presented as the mean ± standard deviation (range).

oral application of propolis. No associations were found between the recovery of symptoms after 3 days and the origin of the infection (bacterial or viral), or indeed gender or age. As regards model 1, 2 and 3, the remission of all symptoms (OR: 17.58, CI: 6.42–48.18, $p < 0.001$), sore throat (OR: 14.45, CI: 4.93–42.3, $p < 0.001$), and muffled dysphonia (OR: 50.33, CI: 4.88–519,22, $p < 0.001$) at three days (t1) was

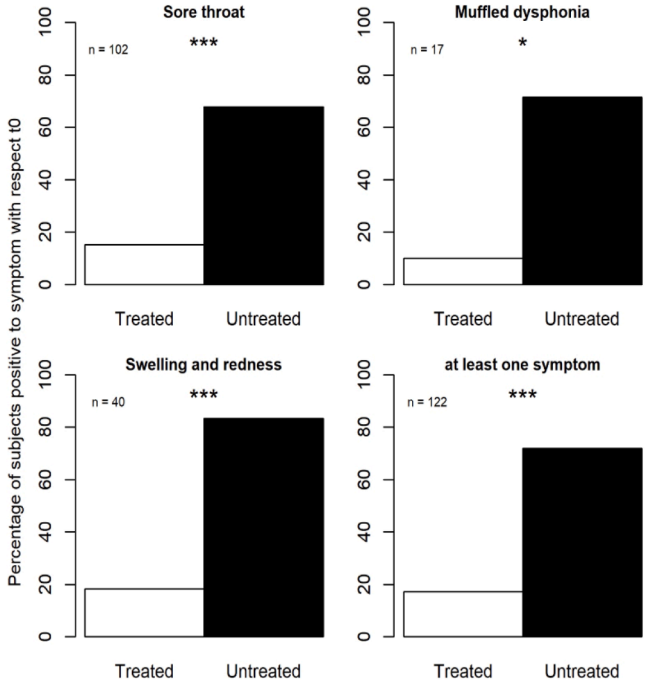

Fig 3. Number of patients with URTI symptoms at t0 and t1; n is for the whole sample including treated and untreated subjects; *: $p < 0.05$, ***: $p < 0.001$.

Table 4
Profile of patients who had a remission of all symptoms (122 Obs), sore throat, swelling and redness of throat, and muffled dysphonia after three days of treatment.

Models	OR	SE	95% CI	*p*
Model 1: All synthoms				
Gender	1.90	0.92	0.75–4.90	0.173
Age	1.02	0.02	0.99–1.05	0.227
Positive medical history for sore throat	3.45	2.70	0.75–15.95	0.113
Positive medical history for muffled dysphonia	1.63	1.11	0.43–6.22	0.471
Positive medical history for swelling and redness of throat	0.98	0.57	0.31–3.06	0.966
Propolis oral spray application	17.58	9.04	6.42–48.18	<0.001
Positive throat swab	0.68	0.47	0.17–2.62	0.571
Model 2: Sore throat				
Age	1	0.02	0.97–1.04	0.711
Gender	1.96	1.03	0.70–5.49	0.2
Positive medical history for muffled dysphonia	2.90	2.40	0.57–14.71	0.2
Positive medical history for swelling and redness of throat	0.75	0.46	0.22–2.48	0.632
Propolis oral spray application	14.45	7.92	4.93–42.3	<0.001
Positive throat swab	1.67	1.32	0.35–7.88	0.516
Model 3: Swelling and redness of throat				
Age	1.05	0.04	0.97–1.12	0.217
Gender	1.62	1.50	0.26–9.94	0.603
Positive medical history for sore throat	3.39	3.41	0.47–24.34	0.224
Positive medical history for muffled dysphonia	3.98	4.87	0.36–43.83	0.258
Propolis oral spray application	50.33	5.93	4.88–519.22	0.001
Positive throat swab	1.42	2.12	0.08–26.47	0.812
Model 4: Muffled dysphonia				
Gender	1.41	2.09	0.08–25.78	0.816
Age	0.96	0.061	0.85–1.09	0.510
Propolis oral spray application	25.63	36.36	1.59–413.28	0.022

significantly associated with the oral application of propolis vs placebo. In model 4 the remission of swelling and redness of throat at three days was significantly associated with the oral application of propolis vs placebo (OR: 25.63, CI: 1.59–413.28, $p = 0.022$), too, but in this model with 17 subjects, the only adjustable variables in the model are age, sex and the treatment with propolis.

During the five days of treatment no subjects reported adverse effects (AEs) related to application of propolis, including absence of oral mucosal allergies, and the principal investigator judged that the application of propolis oral spray was considered well tolerated.

Discussion

Mild uncomplicated URTIs are acute bacterial or viral infections inducing inflammation of the upper airways responsible for characteristic symptoms, which are commonly resolved without pharmacological treatment after about one week. Nevertheless, statistics show that URTIs are a cause of absences from work and daily activities. Propolis is traditionally considered a useful remedy for the treatment of URTI but scientific evidence is very limited. In addition, the high variability and low reproducibility of the chemical composition of propolis make it impossible to correlate the content of bioactive compounds with its efficacy. Therefore, in the present study, a monocentric, double-blind, placebo controlled clinical trial was conducted to demonstrate the effects of a poplar-type propolis extract, with a standardized polyphenol content, in the remission of URTI symptoms. The results of this clinical study clearly show that the application of propolis oral spray assists in three days remission of the most common symptoms of URTIs, in comparison to a placebo group showing a statistically lower incidence of symptom remission after three days of watchful waiting. This means that symptoms resolution, which in the studied adult population commonly occurs after 5 days, was two days early.

Our results are in agreement with those obtained by Di Pierro et al. that demonstrated the beneficial effects of propolis used as an add-on therapy in cases of acute otitis media and viral pharyngitis in 56 children (Di Pierro et al., 2016). This clinical study highlighted the efficacy of propolis in the reduction of the length and severity of symptoms in cases of non-streptococcal pharyngitis and in tracheitis, bronchitis, and rhinosinusitis after 72 h of treatment with propolis, decreasing the need for administration of antipyretics and anti-inflammatory drugs in children, thus reducing the risks of possible side effects to the liver and gastric mucosa. Similar results were also obtained by Marti et al. in a pilot non controlled study on a population of children (Marti et al., 2017). In this case, nasal application of propolis spray induced the resolution of symptoms after 4 days of treatment.

As far as tolerance and safety assessment are concerned, it is well known that propolis is a safe bee product in use since 300 years B.C. in traditional medicine worldwide (Miguel et al., 2011). Moreover, propolis is well tolerated, as it has been also used in toothpastes, mouthwash products, cough syrups and oral pills (Koo et al., 1999). This study confirms the safety of propolis, as no subjects reported AEs related to propolis treatment and all participants considered propolis oral spray application to be well tolerated.

This work has limitations and strengths. The main limitation is represented by the low number of patients with URTIs of bacterial origin, shown by the low number of patients with positive throat swabs at t0, which prevents the assessment of significant differences between the treated and untreated groups after the follow-up period, as all patients showed a negative throat swab.

As reported in the study by Thomas et al., bacteria may cause roughly 15% of sudden onset pharyngitis presentations as in our study, where we found about 12.3% positive throat swabs.

Actually, no statistical difference was found between the participants of this study and the participants reported in Thomas et al. ($\chi^2 = 0.495$, $df = 1$, $p = 0.48$).

The muffled dysphonia and swelling and redness of throat were also significantly low at baselines, but these results are in line with the common symptoms of URTIs reported in the recent literature, as described above. Regarding muffled dysphonia, in the model 4 calculated with 17 subjects, the remission was significantly associated with the oral application of propolis vs placebo and resulted to be more simle in comparison with the other models.

On the other hand, the major strength of this study are that, 1) to the best of our knowledge, it was the first double blind, controlled interventional study of the effects of a propolis oral spray on the symptoms of uncomplicated form of mild URTIs, as the other published studies were retrospective or non-controlled studies; and 2) it was the first clinical trial in which a propolis with a well-known content of polyphenols (M.E.D.® propolis) was studied. Moreover, it is the first one to involve an adult population for which a more rapid course of URTIs means a shorter sickness leave and a faster return to work and normal daily activities. After 5 days, all participants recovered from the symptoms, while most of the subjects who received propolis application recovered from the symptoms after 3 days. Therefore, propolis oral application reduces the time for disappearance of symptoms.

In conclusion, propolis oral spray can be used to improve both bacterial and viral mild uncomplicated URTI symptoms in a lower number of days without the use of symptomatic treatment leading to a more immediate resolution. Future studies including only subjects with URTIs of bacterial origin must be conducted to unravel the possible effect of propolis oral spray on the incidence of antibiotic resistant bacteria.

Author contributions

M.D.A., M.D., and G.C.T. designed the clinical study, analyzed and interpreted the data; M.D. C.E. and E.U.G. drafted the manuscript; B.B., G.C., and A.R.B. conducted the clinical study; C.E. and E.U.G. wrote the

documents for Ethic Committee, monitored the clinical study, and collected and analyzed the data; F.G. set up the chromatographic analysis protocol, was in charge of the analysis of propolis, and write the part of the manuscript regarding the chromatographic analysis; R.S. performed the statistical analysis; V.Z. set up the composition and prepared propolis oral spray and placebo and write part of the "Introduction" and "Materials and Methods" sections. All authors approved the final version of manuscript.

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Cristina Esposito: Investigation, Methodology, Software, Writing - original draft, Writing - review & editing. **Emanuele Ugo Garzarella:** Investigation, Methodology, Software, Writing - original draft, Writing - review & editing. **Bruno Bocchino:** Investigation, Methodology. **Maria D'Avino:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing. **Giuseppe Caruso:** Investigation, Methodology. **Antonio Riccardo Buonomo:** Investigation, Methodology. **Roberto Sacchi:** Software, Writing - original draft. **Fabio Galeotti:** Investigation, Methodology. **Gian Carlo Tenore:** Conceptualization, Supervision, Writing - original draft. **Vincenzo Zaccaria:** Investigation, Methodology. **Maria Daglia:** Conceptualization, Supervision, Writing - original draft.

Declaration of Competing Interest

V.Z. is a B Natural srl employee. The other authors declare no conflict of interest.

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References

Ali, H.S., Abdul Rasool, B.K., 2011. Propolis buccal paste in treatment of aphtous ulceration: formulation and clinical evaluation. Asian J. Pharm. Clin. Res. 4, 29–33.
Barrett, B., Brown, R., Rakel, D., Mundt, M., Bone, K., Barlow, S., 2010. Echinacea for treating the common cold: a randomized trial. Ann. Intern. Med. 153, 769–777.
Bensoussan, B., Myers, S., Cooke, M., and Chera, P.A., 2005. A Review of Complementary Medicines in NSW.
Brinckmann, J., Sigwart, H., van Houten, Taylor, L., 2003. Safety and efficacy of a traditional herbal medicine (Throat Coat) in symptomatic temporary remission of pain in patients with acute pharyngitis: a multicenter, prospective, randomized, double-blinded, placebo-controlled study. J. Altern. Complement. Med. 9, 285–298.
Calvert, M., Blazeby, J., Altman, D.G., Revicki, D.A., Moher, D., Brundage, M.D., CONSORT PRO group., 2013. Reporting of patient-reported outcomes in randomized trials: the CONSORT PRO extension. JAMA 309, 814–822.
Chan, G.C., Cheung, K.W., Sze, D.M., 2013. The immunomodulatory and anticancer properties of propolis. Clin Rev Allergy Immunol 44 (3), 262–273.
Cohen, H.A, Varsano, I., Kahan, E., Sarrell, E.M., Uziel, Y., 2004. Effectiveness of an Herbal Preparation Containing Echinacea, Propolis, and Vitamin C in Preventing Respiratory Tract Infections in Children. Arch. Pediatr. Adolesc. Med. 158, 217–221.
Crişan, I., Zaharia, C.N., Popovici, F., Jucu, V., Belu, O., Dascălu, C., Mutiu, A., Petrescu, A., 1995. Natural propolis extract NIVCRISOL in the treatment of acute and chronic rhinopharyngitis in children. Rom. J. Virol. 46, 115–133.
Curti, V., Zaccaria, V., Tsetegho Sokeng, A.J., Dacrema, M., Masiello, I., Mascaro, A., D'Antona, G., Daglia, M., 2019. Bioavailability and In Vivo Antioxidant Activity of a Standardized Polyphenol Mixture Extracted from Brown Propolis. Int. J. Mol. Sci. 20, 1250.
Di Lorenzo, A., Nabavi, S.F., Sureda, A., Moghaddam, A.H., Khanjani, S., Arcidiaco, P., Nabavi, S.M., Daglia, M., 2015. Antidepressive-like effects and antioxidant activity of green tea and GABA green tea in a mouse model of post-stroke depression. Mol. Nutr. Food Res. 60, 566–579.
Di Pierro, F., Zanvit, A., Colombo, M., 2016. Role of a proprietary propolis-based product on the wait-and-see approach in acute otitis media and in preventing evolution to tracheitis, bronchitis, or rhinosinusitis from nonstreptococcal pharyngitis. Int. J. Gen. Med. 9, 409–414.
Duke, C.C., Tran, V.H., Duke, R.K., Abu-Mellal, A., Plunkett, G.T., King, D.I., Hamid, K., Wilson, K.L., Barrett, R.L., Bruhl, J.J., 2017. A sedge plant as the source of Kangaroo Island propolis rich in prenylated p-coumarate ester and stilbenes. Phytochemistry 134, 87–97.
EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS); EFSA Journal 2011. Scientific Opinion on the re-evaluation of caramel colours (E 150 a,b,c,d) as food additives., 9.
Erdemli, H.K., Akyol, S., Armutcu, F., Akyol, O., 2015. Antiviral properties of caffeic acid phenethyl ester and its potential application. J. Intercult. Ethnopharmacol. 4 (4), 344–347.
Fedotova, V.V., Konovalov, A.D., 2019. Indian Journal of Pharmaceutical Education and Research 53, 4.
Jolly, V.G., 1978. Propolis varnish for violins. Bee World 59 (4), 158–161.
Koo, H., Rosalen, P.L., Cury, J.A., Park, Y.K., Ikegaki, M., Sattler, A., 1999. Effect of Apis mellifera Propolis from Two Brazilian Regions on Caries Development in Desalivated Rats. Caries Res 33, 393–400.
Kujumgiev, A., Tsvetkova, I., Serkedjieva, Y., Bankova, V., Christov, R., Popov, S., 1999. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J. Ethnopharmacol. 64, 235–240.
Kuropatnicki, A.K., Szliszka, E., Krol, W., 2013. Historical aspects of propolis research in modern times. Evidence-based complementary and alternative medicine, 964149.
Lucas, S., Leach, M., Kumar, S., 2018. Complementary and alternative medicine utilisation for the management of acute respiratory tract infection in children: a systematic review. Complement. Ther. Med. 37, 158–166.
Marchisio, P., Esposito, S., Bianchini, S., Desantis, C., Galeone, C., Nazzari, E., Pignataro, L., Principi, N., 2010. Effectiveness of a propolis and zinc solution in preventing acute otitis media in children with a history of recurrent acute otitis media. Int. J. Immunopathol. Pharmacol. 23, 567–575.
Marti, J., López, F., Gascón, I., Julve, J., 2017. Propolis nasal spray effectively improves recovery from infectious acute rhinitis and common cold symptoms in children: a pilot study. J. Biol. Regul. Homeost. Agents. 31, 943–950.
Miguel, M.G., Antunes, M.D., 2011. Is propolis safe as an alternative medicine? J. Pharm. Bioallied Sci. 3, 479–495.
Monti, M., Bertt, E., Carminati, G., Cusini, M., 1983. Occupational and cosmetic dermatitis from propolis. Contact Dermatitis 9, 163.
Murray, M., Pizzorno, J., 2005. Text Book of Natural Medicine. Churchill Livingstone – Elsevier, London.
Santos, V.R., Gomes, R.T., de Mesquita, R.A., de Moura, M.D., França, E.C., de Aguiar, E. G., Naves, M.D., Abreu, J.A., Abreu, S.R., 2008. Efficacy of Brazilian propolis gel for the management of denture stomatitis: a pilot study. Phytother. Res. 22, 1544–1547.
Santos, V.R., Pimenta, F.J., Aguiar, M.C., Carmo, Do, M.A., Naves M.D., Mesquita, R.A., 2005. Oral candidiasis treatment with Brazilian ethanol propolis extract. Phytother. Res. 19, 652–654.
Smith, S.M., Schroeder, K., Fahey, T., 2012. Over-the-counter medications for acute cough in children and adults in ambulatory settings. Cochrane Database Syst Rev 8. CD001831.
Thomas, M., Koutsothanasis, G.A., Bomar, P.A., 2020. Upper Respiratory Tract Infection. StatPearls Publishing, StatPearls. Treasure Island (FL).
Tomazevic, T., Jazbec, J., 2013. A double-blind randomised placebo-controlled study of propolis (bee glue) effectiveness in the treatment of severe oral mucositis in chemotherapy treated children. Complement. Ther. Med. 21, 306–312.
Toreti, V.C., Sato, H.H., Pastore, G.M., Park, Y.K., 2013. Recent progress of propolis for its biological and chemical compositions and its botanical origin Evidence-Based Complement. Alternat. Med., 697390
32 Volpi, N., Fachini, A., 2017 Procedimento Per L'ottenimento di Estratti Integrali di Propoli Ricchi in Polifenoli e Dotati di Attività Antibatterica e Sua Applicazione Nella Prevenzione e Trattamento di Processi Infettivi di Origine Batterica. Ufficio Italiano Brevetti e Marchi No. 0001425516, 02/02/2017.
Wagh, V.D., 2013. Propolis: a wonder bees product and its pharmacological potentials Adv. Pharmacol. Sci., 308249
Yoon, Y.K., Park, C.S., Kim, J.W., Hwang, K., Lee, S.Y., Kim, T.H., Park, D.Y., Kim, H.J., Kim, D.Y., Lee, H.J., Shin, H.Y., You, Y.K., Park, D.A., Kim, S.W., 2017. Guidelines for the Antibiotic Use in Adults with Acute Upper Respiratory Tract Infections. Infect. Chemother. 49, 326–352.
Yuksel, S., Akyol, S., 2016. The consumption of propolis and royal jelly in preventing upper respiratory tract infections and as dietary supplementation in children. J. Intercult. Ethnopharmacol. 5, 308–311.
Zaccaria, V., Curti, V., Di Lorenzo, A., Baldi, A., Maccario, C., Sommatis, S., Mocchi, R., Daglia, M., 2017. Effect of Green and Brown Propolis Extracts on the Expression Levels of microRNAs, mRNAs and Proteins, Related to Oxidative Stress and Inflammation. Nutrients 9, 1090.
Zaccaria, V., Garzarella, E.U., Di Giovanni, C., Galeotti, F., Gisone, L., Campoccia, D., Volpi, N., Arciola, C.R., Daglia, M., 2019. Multi Dynamic Extraction: an Innovative Method to Obtain a Standardized Chemically and Biologically Reproducible

Polyphenol Extract from Poplar-Type Propolis to Be Used for Its Anti-Infective Properties. Materials (Basel) 12, 3746.