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Fractionated lentisk oil as add-on therapy for preventing chronic rhinosinusitis recurrence

The dysbiosis hypothesis, the alteration of microbiota associated with perturbation of the local ecological landscape, has been suggested as a mechanism involved in most pathologies, including those occurring in the respiratory tract.[1, 2] Recent advancements in culture-independent molecular techniques have enhanced understanding of interactions between sinus microbiota and upper airway microenvironment demonstrating that during infection global bacterial load does not change in comparison with healthy controls, while biodiversity reduces.[3] Differences have been also demonstrated from a taxonomical perspective. Versus healthy controls, acute bacterial sinusitis is mostly characterized by the presence of microorganisms like *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pyogenes*; while *Staphylococcus aureus* and anaerobic organisms (*Prevotella* and *Porphyromonas*, *Fusobacterium*, and *Peptostreptococcus* spp.) are the commonest isolates in chronic rhinosinusitis (CRS).[4] The existence of a "pathological" microbiota in sinusitis does not explain the whole pathogenesis and/or the frequent antibiotic-resistant profile of these ailments. The existence of biofilms on the sinonasal mucosa of individuals with CRS has been proven by current biofilm identification methods and drugs potentially acting against biofilm formation have been proposed as a tool for counteracting CRS recurrence.[5] *Pistacia lentiscus* berry oil, commonly known as lentisk oil, represents a typical vegetal product of the Mediterranean area. In addition to its interesting alimentary properties, this product is widely used in folk medicine for oral and skin affections, in particular, acute gingivitis, pediatric skin infections (*i.e.* impetigo), and biofilm related infections often associated with *Streptococcus* spp.[6] Recent *in vitro* results have shown that lentisk oil, when fractionated by winterization processes to concentrate possible antibiofilm agents like anacardic acids, can indeed work as a "microbial modulating tool" possibly effective in the prevention of biofilm-associated diseases, also as add-on therapy to antibiotic treatment.[7] On this basis and after that the informed consent was given by all subjects involved, a placebo-controlled clinical study was conducted in patients affected by CRS (ethical board approval number, IAR20221123, was provided by the Italian Academy of Rhinology).[8] Inclusion and exclusion criteria, along with methods, are briefly described in Supplementary Digital Material 1 (Supplementary Text File 1). As shown in Figure 1, along with its high safety profile (no specific side effects were observed), the 30-day use of a fractionated lentisk oil-based medical device (Bactorinol®) has demonstrated to significantly improve most of the outcomes considered, including the quality of life (SNOT 22), nasal secretion occurrence and biofilm evidence. Also ciliary motility, supranuclear stria and bacterial cell number resulted to be improved. As regards to fractionated lentisk oil mechanism of action, previous *in-vitro* results, obtained by the Congo red method,[9] had demonstrated its possible role in reducing biofilm formation.[7] To confirm what clinically observed as regards to biofilm evidence, we have then used a different *in-vitro* method (crystal violet) able to detect also the demolition effect exerted by a specific substance.[10] According to the results obtained (Figure 2), fractionated lentisk oil significantly prevented biofilm forma-

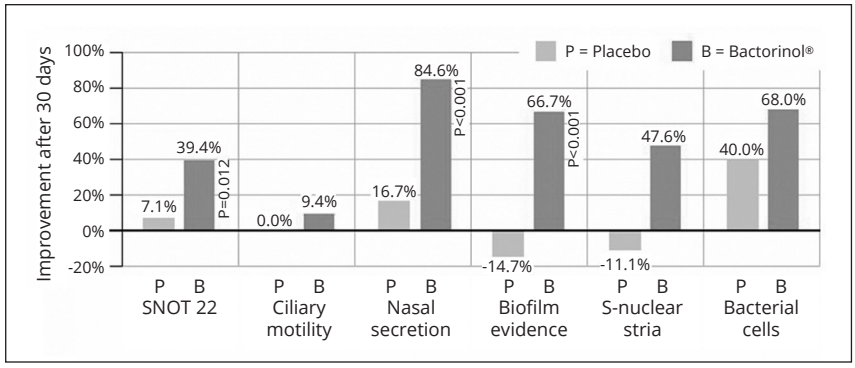

Figure 1.—Clinical outcomes variation (%) after 30 days of treatment in the two groups treated, besides daily nasal wash, with placebo or fractionated lentisk oil (Bactorinol®), respectively. Both treatments corresponded to 10 drops (5 each nostril) twice a day.
SNOT 22: Sino-Nasal Outcome 22; S-nuclear stria: supranuclear stria.

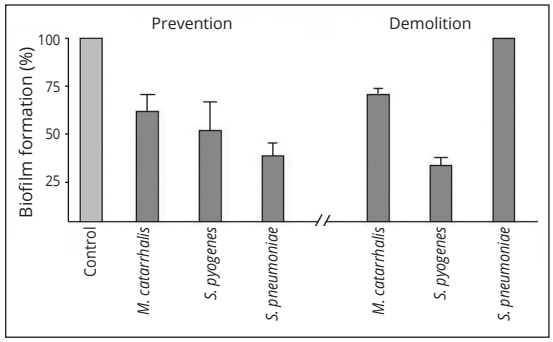

Figure 2.—*In-vitro* variation (%) of biofilm formation*, both in prevention and measured after its formation (demolition). *Crystal violet method: each well was stained with 0.1% crystal violet solution for 15 min at room temperature, washed twice with double distilled water, and dried thoroughly to quantify the biofilm. The biofilm was then solubilized with 33% (v/v) glacial acetic acid. Biofilm formation was quantified by spectrophotometer at a wavelength (OD) of 570 nm. The graph represents the average results obtained by experiments performed eight times.

tion in cultures of *M. catarrhalis*, *S. pyogenes* and *S. pneumoniae* (by 37%, 47%, and 56% respectively) also destroying the biofilm already formed in cultures of *M. catarrhalis* and *S. pyogenes* (by 30% and 60%) with no effect only on *S. pneumoniae* biofilm. The clinical outcomes observed, along with the *in-vitro* results got, clearly demonstrate that the use of the fractionated lentisk oil represents a possible means of supplementing medical therapies for the treatment of CRS especially with the aim of preventing recurrences. However, the rationale for using a medical device aimed at preventing recurrences implies its high safety profile. For this purpose, according to methods and ISO (International Organization for Standardization) documents previously described,[11-14] the fractionated lentisk oil has been subjected to an important series of verification tests which results have demonstrated that: 1) it is not absorbed by human mucosa (Supplementary Digital Material 2: Supplementary Table I); 2) it does not exhibit skin sensitization potential (Supplementary Digital Material 3: Supplementary Table II); 3) it is not irritant for nasal mucosa (Supplementary Digital Material 4: Supplementary Figure 1); and 4) it is not cytotoxic for human cells (Supplementary Digital Material 5: Supplementary Table III).

Both adult and pediatric chronic rhinosinusitis are indeed difficult to treat resulting in frequent recurrences and failures.[15] In western countries, sinusitis is one of the most common health complaints leading to a physician visit as well as one of the leading causes of antibiotic prescriptions. A recent survey in USA has demonstrated that there are up to 73 million restricted activity days in patients with sinusitis with a total direct medical costs of almost 2.4 billion dollars (not including surgery or radiographic imaging) per year, and about 20-25% of people demonstrated recurrency.[16] According to the results herewith presented, the use of a fractionated lentisk oil-based medical device (Bactorinol®) can be proposed as add-on therapy to prevent recurrency of CRS, both in adults and in children.

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Conflicts of interest

Francesco Di Pierro is the Scientific and Research Director in Pharmextracta SpA. Francesco Di Pierro is also the main author of the European patent describing the fractionated lentisk oil (N° 20205961.4 deposited on November 5th, 2020). The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' contributions

Conceptualization: Francesco Di Pierro, Giulia Monti, Alberto Macchi. Methodology: Giulia Monti, Alberto Macchi. Validation: Francesco Di Pierro, Nicola Zerbinati, Maria L. Tanda, Giulia Monti, Alberto Macchi. Formal analysis: Francesco Di Pierro, Alberto Macchi. Investigation: Giulia Monti, Alberto Macchi. Resources: Giulia Monti, Alberto Macchi. Data curation: Francesco Di Pierro, Alberto Macchi. Writing–original draft preparation: Francesco Di Pierro, Alberto Macchi. Writing–review and editing: Francesco Di Pierro, Nicola Zerbinati, Maria L. Tanda, Giulia Monti, Alberto Macchi. Visualization: Francesco Di Pierro, Nicola Zerbinati, Maria L. Tanda, Giulia Monti, Alberto Macchi. Supervision: Francesco Di Pierro, Nicola Zerbinati, Maria L. Tanda, Giulia Monti, Alberto Macchi. Project administration: Francesco Di Pierro, Nicola Zerbinati, Alberto Macchi. All authors read and approved the final version of the manuscript.

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History

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SUPPLEMENTARY DIGITAL MATERIAL 1

Inclusion Criteria

100 patients were selected (57 males and 43 females), over 18 years old with a diagnosis of Chronic Rhinosinusitis, with a nasal polypoid score ≤1. All patients came to our attention complaining with more than 5 recurrences per year, determining low quality of life.

Exclusion Criteria

We excluded patients with a Nasal Polypoid score > 2, with less than 5 episodes of recurrence per year and a SNOT 22 score lower than 10. Patients affected by immunity, neoplastic, neurological and/or psychiatric diseases and/or with inability to undergo therapy were not included into the study.

Methods

100 Patients with chronic rhinosinusitis were enrolled and divided into two groups by randomization methods using the random number table. All patients underwent nasal endoscopy, assessment of their SNOT 22 questionnaire, cytological specimen examination, and ciliary motility evaluation at T0 (enrollment visit) and T1 (30 days after).

During both visits all these criteria were evaluated. The control group was treated for 30 days with nasal wash with a specific probe (Rinowash®) and 10 drops of placebo twice daily. The treated group was treated for a 30-day period with nasal wash with a specific probe and 10 drops of lentisk oil (formulated as Bactorinol® drops; Pharmextracta, Pontenure, PC, Italy) twice a day.

At the end of treatment, all patients underwent nasal endoscopy, SNOT 22, cytological examination and ciliary motility evaluation.

The Rinowash® probe

This tool is a specific terminal for aerosol therapy of the upper airways. Connected to the traditional device for aerosol therapy with a compressor, it allows a complete and accurate treatment of the upper airways in 1-3 minutes, for the treatment of allergic and non-allergic rhinitis, rhinosinusitis, nasal polyposis. Producing particles with a diameter greater than 10 microns, it acts exclusively at the level of the upper airways, nebulizing 5 ml of solution in one minute. The Technical specifications is Operating pressure from 1 to 3.5 bar, Operating flow from 5 to 20 L/min with a MMAD 18 μm.

SNOT 22

To visualize the SNOT 22 questionnaire: https://www.farragutent.com/wp-content/uploads/snott-22.pdf

SUPPLEMENTARY DIGITAL MATERIAL 2

Supplementary Table I.

Nasal absorption of free fatty acids and triglycerides contained in Bactorinol® nasal drops

% Absorption (vs initial amount)	**T = 1h**	**T= 3h**	**T=24 h**
Caffeine (positive control)	15.42 ±0.81	34.06 ± 2.21	-
FFA and TG from Bactorinol® nasal drops	-	-	0

In vitro analysis was performed using a reconstructed human nasal epithelium model (EpiAirway™). After 24 hours of prolonged exposure, the impact on barrier integrity and cell viability was assessed by measuring transepithelial electrical resistance (TEER). Next, absorption was evaluated using the highest non-toxic formulation applied to the apical compartment of EpiAirway™. After 24 hours, absorption was determined by measuring free fatty acids (FFAs) and triglycerides (TGs) in the basolateral compartment. Caffeine was used as a positive control, and HBSS was used as a negative control. FFA and TG concentrations were determined using gas chromatography-mass spectrometry (GC-MS), while caffeine absorption was assessed by measuring its absorbance using a spectrophotometer. All experiments were performed in triplicate. The absorption of caffeine at 1 hour and 3 hours confirmed epithelial functionality. No FFAs or TGs from Bactorinol® nasal drops were absorbed.

Supplementary Table II.

In Vitro skin sensitization assay with reconstructed tissues EpiDerm

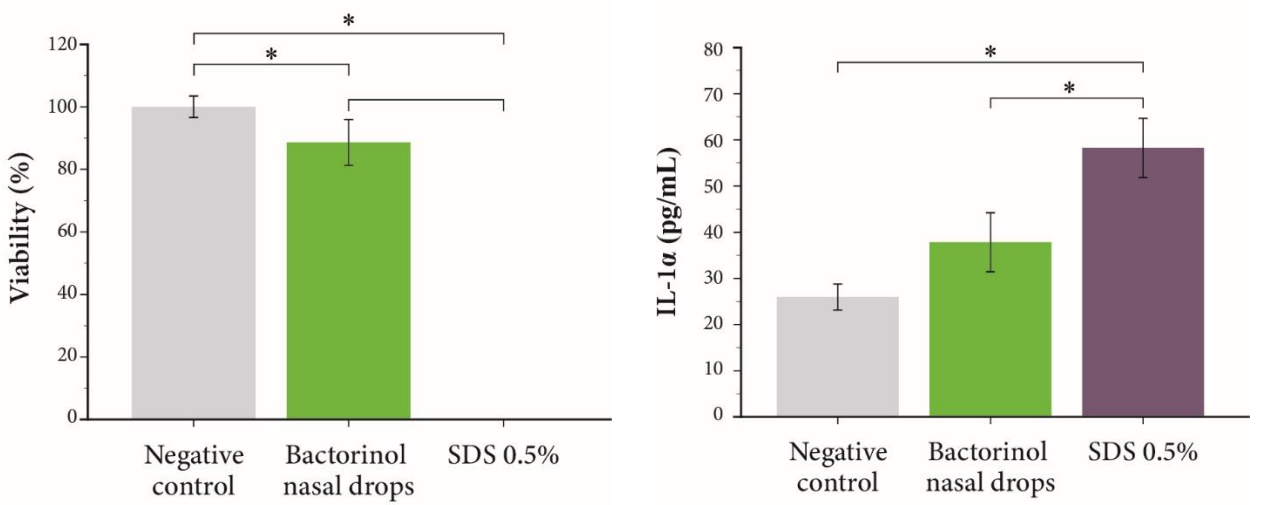
Test Substance	**Viability (%)**	**IL-18 S.I.**
Untreated control	100	1.0
Vehicle control	128.7	0.7
Bactorinol® nasal drops	118.4	0.8
DNCB 2 mg/ml (positive control)	9.2	2.7

Bactorinol® nasal drops was applied on the EpiDerm in duplicate, for 24 hours at 37 ± 1°C and 5 ± 1 % CO2. After treatment, release of IL-18 into medium was measured by ELISA test, whereas tissues were treated with MTT in order to evaluate cell viability. According to the applied laboratory protocol, a test substance is considered as potentially sensitizer if it induces ≥2.5- fold increase in IL-18 when compared to a negative control (S.I.) at cell viability ≥5% and ≤40%. Considering the results in the table below, Bactorinol® nasal drops did not exhibit skin sensitization potential, as shown in the table below.

SUPPLEMENTARY DIGITAL MATERIAL 4

Supplementary Figure 1.

Evaluation of the in vitro irritant effect of Bactorinol® nasal drops



The irritation test was performed using an in vitro reconstructed human model mimicking nasal epithelium (EpiAirway™). The potential irritant effect was determined by assessment of the viability and of the pro-inflammatory cytokine IL-1α, following exposure to the formulation. All tests were repeated in triplicate. HBSS and SDS were used as negative and positive control, respectively. The nasal mimicking epithelia was incubated with Bactorinol® nasal drops at 37°C from 24 h to mimic prolonged exposure. After the treatment, cell viability was assessed by MTT assay and IL-1α released was quantified by ELISA assay.

SUPPLEMENTARY DIGITAL MATERIAL 5

Supplementary Table III.

Test for in vitro cytotoxicity

Time point: 2 hours ± 10 min	**Grades of cytotoxicity**				**Reactivity**
	Filter 1	**Filter 2**	**Filter 3**	**Filter 4**	
Negative control (NC)	0	0	0	0	None
Positive control (PC)	3	3	3	3	Moderate
Bactorinol® nasal drops	0	0	0	0	None

Bactorinol® nasal drops were applied in 4 replicates onto borosilicate microglass discs. These discs were then placed in contact with cells using a diffusion method through cellulose acetate filter. Incubation was carried out at 37°C for 2h. Cytotoxicity was quantitatively evaluated by measuring succinate dehydrogenase activity which is able to reduce a yellow tetrazolium solution to a purple formazan compound. The amount of formazan produced is directly proportional to cell viability, allowing the correlation of the number of live cells to the intensity of the resulting color. Positive control with SDS and negative control with medium were used.