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In vitro Bacteriostatic Effect of a Scaffold with a Mixture of *Hypericum perforatum* and *Azadirachta indica* Oil Extracts

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Author's contribution

Author MLI has designed all studies, collected and revised the data, written the manuscript and approved it.

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Short Research Article

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ABSTRACT

Background: Clinical evidences suggest antimicrobial activities of a new oil extract mixture of *Hypericum perforatum* and *Azadirachta indica*, included in a polymeric scaffold (or Hyperoil™ Polimeric Substrate - HPS).
Methods: Bacteriostatic activity was investigated on selected strains of *Staphylococcus aureus (ATCC® 12598™)*, *Pseudomonas aeruginosa* (ATCC® 10145™) and *Klebsiella ozaenae* AM through standardized *in vitro* tests.
Results and Conclusion: All bacterial strains were stabilized or reduced in size after 3 and 24 hours contact with the *HPS* if compared to the empty polymeric scaffolds.
Results showed the bacteriostatic effect of HPS that, added with its anti-inflammatory properties, could explain its tissue repair effects observed *in vivo*.

Keywords: Hypericum perforatum; Azadirachta indica; Staphylococcus aureus (ATCC® 12598™); Klebsiella ozaenae AM and Pseudomonas aeruginosa (ATCC® 10145™); bacteriostatic effect; scaffold; Hyperoil™ polymeric substrate.

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1. BACKGROUND

Nimh (*Azadirachta indica*) extracts have been used for centuries in the traditional Indian medicine; its oil, obtained with cold extraction from its berries, is also included in the Ayurvedic Pharmacopoeia of India [1]. Nimh oil contains diterpenoids and triterpenoids having cicatrizing [2], bacteriostatic [3], antifungal [4], immune-modulatory [5] and anti-inflammatory properties [6]. Nimh oil resulted relatively safe for external application in wounds [7].

The oil extract of *Hypericum perforatum* flowers was esteemed as one of the most popular remedy for excoriations, wounds and bruises [8]. This product showed anti-infective [9], antiphlogistic and cicatrizing activity [10] while anti-inflammatory effects of *H. perforatum* extracts were recently demonstrated [11] thus providing the rationale for using these preparations in legs wounds, together with nimh oil extract [12].

Hyperoil™ is a mixture of hypericum flowers extract (*H. perforatum*) and nimh oil (*A. indica*) produced by RIMOS S.r.L. Mirandola (MO) - Italy (Medical Device Class IIB CE0476), available as oil, gel, cream and gauze gel, that was recently used in complicated diabetic foot ulcers [13]. RIMOS developed a Hyperoil™ Polymeric Substrate (HPS) where Hyperoil™ oil was included in a polymeric scaffold, constituted by a fibrous membrane of poli (L-lactic) acid (PLLA), reproducing the biologic nano-structured fibrous matrix (extracellular matrix), whose fibers have a diameter from 10 to 300 nm. PLLA was chosen as it is a biocompatible, biodegradable and bio-absorbable product and, thus, used for the production of several biomaterials used for tissues regeneration [14].

Hyperoil™, together with improved metabolic control, was observed having recovered diabetic foot ulcers [13]. In patients with diabetes mellitus, foot infections in foot ulcers pose a significant risk, especially when patients are hospitalized for surgical treatments. These are complex infections commonly caused by *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *A. baumannii,* being the Gram-positive *Staphylococcus aureus* and the Gram-negative *Pseudomonas aeruginosa* the most common and difficult to be treated [15] especially when nosocomial infections occur [16]. *Klebsiella ozaenae* is an enterobacterial, frequent in nosocomial infections [17], continuously evolving to increase its resistance [18], thus representing a potentially difficult to be treated pathogen.

The aim of this paper is to present the results of *in vitro* evaluations of the inhibitory bacteriostatic effect of HPS on *S. aureus* (ATCC® 12598™), *P. aeruginosa* (ATCC® 10145™) and *K. ozaenae* AM strains, to confirm bacteriostatic effects of its components, being maintained when included in PLLA, on bacteria possibly related with diabetic foot infections.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals used, including solvents, were of analytical grade. In details, PLLA (Lacea H.100-E) (average molecular weight by GPC = 8.4 x 10^4 g/mol, polydispersity index, PDI = 1.7) was supplied by Mitsui Fine Chemicals (Dusseldorf, Germany); dichloromethane (DCM) and dimethylformamide (DMF) were purchased from Sigma-Aldrich and used without further purification [19].

2.2 HPS

The Hyperoil™ oil mixture was incorporated in PLLA during the electrospinning polymeric scaffold production process to have HPS.

HPS was produced through an original electrospinning process, licensed by RIMOS S.r.L. Mirandola (MO) – Italy, to create a tissue-non-tissue, with fibers having a diameter of 600±200 nm (Fig. 1), simulating the extracellular matrix to build the environment where cells live. This biological fibrous matrix actively modulates the main processes that regulate cellular survival, proliferation and differentiation. The HPS had an average thinning of 50 micron to ensure its simplest usability.

The produced HPS was, then, meshed (by Mash-Graft II Tissue Expansion System, Zimmer Surgical, Dover OH, USA) to ensure surface expandability, tissue repair and the passage of the exudate through the HPS when used as matrix in patients with cutaneous ulcers.

The concentrations of Hyperoil™ oil included in the different polymeric scaffolds, to obtain HPS, were indicated as follows: 1.5% (HPS-1.5), 2.5% (HPS-2.5), 5% (HPS-5), 10% (HPS-10) or 25% (HPS-25) dilutions.

The weight percentage of Hyperoil™ oil included in the polymeric scaffolds was confirmed through a Differential Scanning Calorimetry (DSC) exam and resulted 0.0864 mg/cm^2 for HPS-1.5, 0.218 mg/cm^2 for HPS-2.5, 0.432 mg/cm^2 for HPS-5, 0.864 mg/cm^2 for HPS-10, and 2.160 mg/cm^2 for HPS-25.

HPS were sterilized with ethylene oxide and kept at + 4ºC before performing the experiments.

2.3 Bacterial Strains

2.3.1 *In vitro* bacterial assays on HPS

2.3.1.1 Bacterial strains and culture conditions

The microorganisms used in this study were *Staphylococcus aureus* (ATCC® 12598™) (*S. aureus*), *Pseudomonas aeruginosa* (ATCC® 10145™) (*P. aeruginosa*) and *Klebsiella ozaenae* AM (*K. ozaenae* AM). *K. ozaenae* AM was a laboratory isolate not-labelled strain provided by the "Zooprofilattico Institute of Pavia", Pavia, Italy.

P. aeruginosa (ATCC® 10145™) and *K. ozaenae* AM were routinely grown in Luria Bertani Broth (LB) (Difco, Detroit, MI, USA) whereas *S. aureus (ATCC® 12598™)* grew in Brain Heart Infusion (BHI) (Difco) overnight under aerobic conditions at 37ºC using a shaker incubator (New Brunswick Scientific Co., Edison, NJ, USA).

These cultures, used as source for the experiments, were reduced at a final density of 1x10^{10} cells/mL as determined by comparing the OD_{600} of the sample with a standard curve relating OD_{600} to cell number [20,21]. We used the cultures at the bacterial cell cycle indicated as the period required for replication (known as the C period) [22]. An overnight culture of each bacterial strain was diluted in fresh medium and cultured for 2-3 hrs in order to reach the OD600 around 0.6-0.7, indicative of a growth exponential phase.

2.3.1.2 Antibacterial activity

To examine the antimicrobial activity of each HPS sterilized, 200 µl (1x10^3 cells) of a growth exponential phase of an overnight diluted suspension of *S. aureus* (ATCC® 12598™), *P. aeruginosa* (ATCC® 10145™) and *K. ozaenae* AM cells was seeded on the surface of no Hyperoil™ oil (Control) or HPS-1.5, HPS-2.5, HPS-5, HPS-10 or HPS-25 and incubated for 1, 3 and 24 hrs, respectively [21].

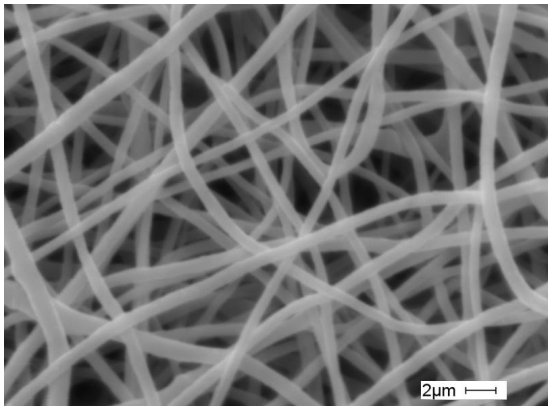

Fig. 1. Scanning electron microscopy picture of fibrous membrane of poli (L-lactic) acid (PLLA) constituting the polymeric scaffold structure

Tissue culture wells (TCPS) used as internal control of bacterial growth were incubated with the same cell suspension for 1, 3 and 24 hrs, respectively.

At the end of each incubation time, the bacterial suspension was serially diluted in its specific culture medium and plated on the Muller Hinton (Difco) agar plates, respectively. The plates were incubated for 24-48 hrs at 37ºC. No significant difference was observed in bacterial growth between cells grown on TCPS and the empty polymeric scaffolds. The data are expressed as number of CFU/mL.

2.4 Statistical Analysis

Results were expressed as mean number of bacterial colonies and standard error using Microsoft Excel worksheet. No formal statistical analyses were performed.

3. RESULTS AND DISCUSSION

The tested bacterial strains grew on the empty polymeric scaffolds without any significant difference if compared to the bacterial growth on the TCPS (data not shown). On the contrary, a variable reduction was observed after various times (1, 3 and 24 hours) of incubation on strains cultured with HPS containing different concentrations of Hyperoil™ (Figs. 2, 3 and 4) and compared to the polymeric substrate not containing the Hyperoil (Figs. 2, 3 and 4).

The experiments were performed using cells cycle synchronized bacterial cells: then the

results are related to the exponential phase of each bacterial culture. As previously reported, the bacterial cell cycle is traditionally divided into three stages: the period between division (birth) and the initiation of chromosome replication (the B period); the period required for chromosome replication (the C period); and the time between the completion of chromosome replication and the completion of cell division (the D period) [22]. To evaluate the efficacy of antibacterial activity of the HPS-Hyperoil it is important to perform the incubation with bacterial cells which are all in the growth exponential phase.

Antibacterial activity resulted dose-dependent through time for *S. aureus* (ATCC® 12598™) (Fig. 2) and *K. ozaenae* AM (Fig. 4) and, at a lesser extent, for *P. aeruginosa* (ATCC® 10145™) (Fig. 3). The maximal inhibition in the growth of bacterial strains was consistently observed after 3 hours, being for *K. ozaenae* AM the highest values at all tested doses if compared to *S. aureus* (ATCC® 12598™) and *P. aeruginosa* (ATCC® 10145™). The percent of reduction of cell viability on *P. aeruginosa* (ATCC® 10145™) (Table 2) and *S. aureus* 8325-4 (Table 1), resulted slightly lower in the 1st hour if compared to *K. ozaenae* AM (Table 3).

On the contrary, at higher incubation time (24h), the percent of reduction was kept higher on *P. aeruginosa* (ATCC® 10145™) (around 90-92%) and *S. aureus* (ATCC® 12598™) (around 90-94%) and resulted slightly decreased on *K. ozaenae* AM (between 80-88%). In particular, the effect on *P. aeruginosa* (ATCC® 10145™) was not dose-dependent after 24 hours being the HPS-25 the less effective on bacterial growth control (Fig. 3).

Because of these different effects, the results of this study cannot sustain the bactericide effect, defined as the capacity of killing bacteria [23], of Hyperoil™ on *S. aureus* (ATCC® 12598™), *P. aeruginosa* (ATCC® 10145™) and *K. ozaenae* AM, but strongly sustain a bacteriostatic effect.

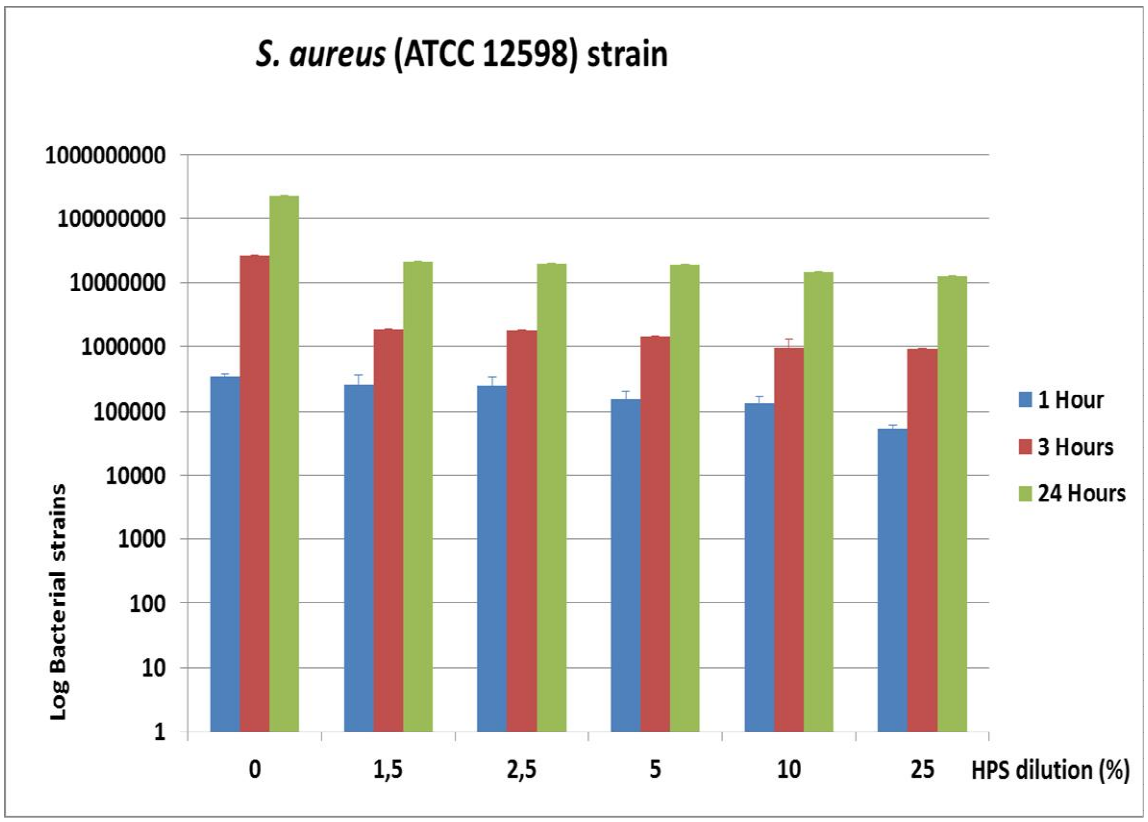

Fig. 2. *S. aureus* strain (Log) by time and HPS dilution

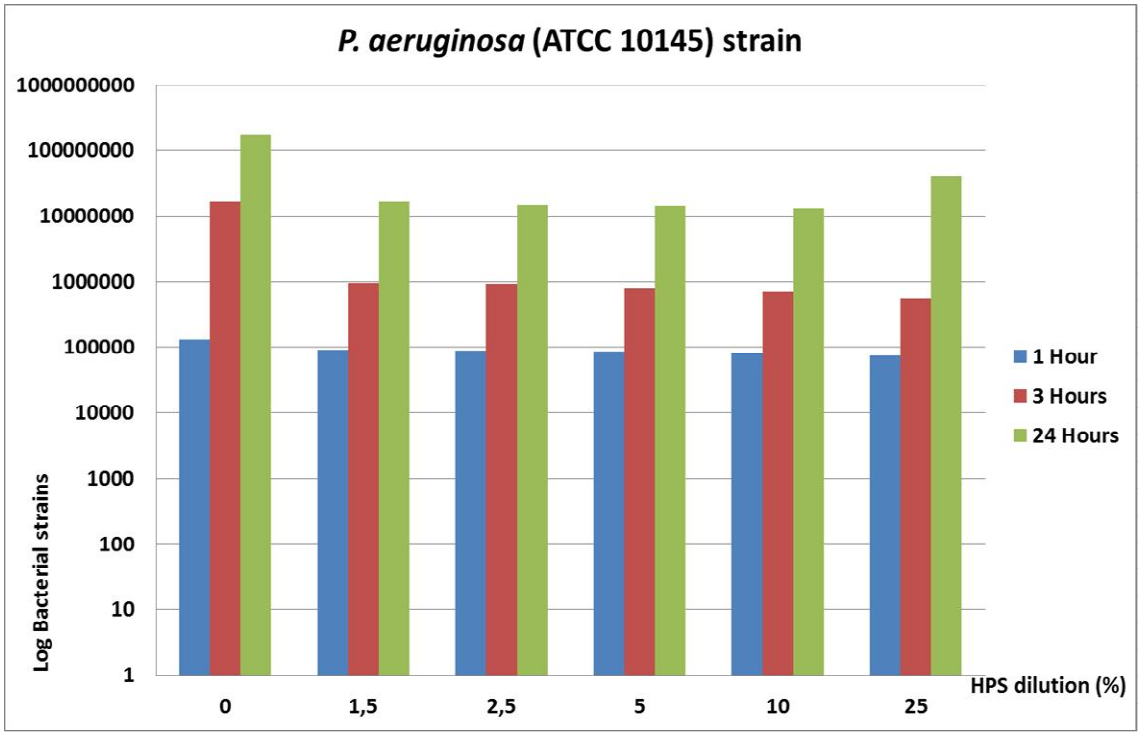

Fig. 3. *P. aeruginosa* strain (Log) by time and HPS dilution

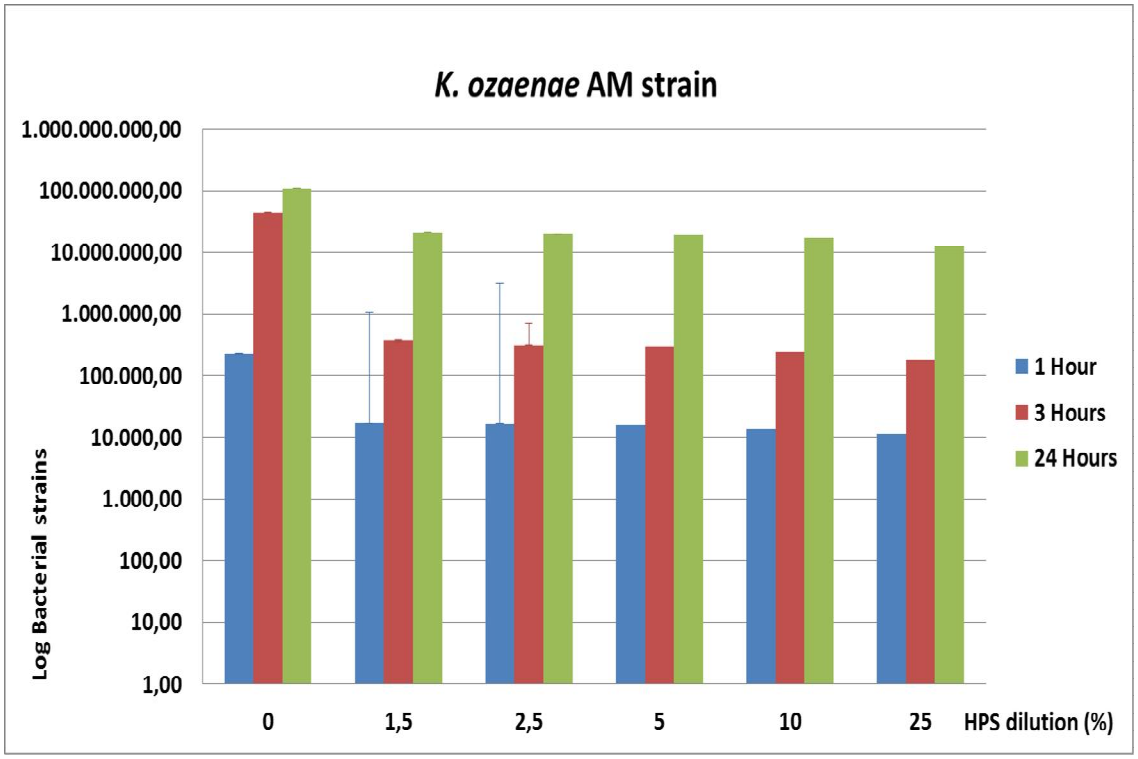

Fig. 4. *K. ozaenae* strain (Log) by time and HPS dilution

Table 1. Average (±SE) *S. aureus* strain by time and HPS dilution

HPS dilution (%)	*S. aureus* (ATCC® 12598™) strain 1 hour	SE	% reduction vs control	*S. aureus* (ATCC® 12598™) strain 3 hours	SE	% reduction vs control	*S. aureus* (ATCC® 12598™) strain 24 hours	SE	% reduction vs control
0	345333	37351	N/A	26800000	208167	N/A	226000000	3785939	N/A
1,5	264067	93544	23,5%	1903333	17638	92,9%	21233333	384419	90,6%
2,5	247033	87469	28,5%	1820000	10000	93,2%	20066667	88192	91,1%
5	151550	56706	56,1%	1463333	33333	94,5%	19400000	208167	91,4%
10	133000	34078	61,5%	944000	406041	96,5%	14600000	360555	93,5%
25	53250	6083	84,6%	928000	15308	96,5%	12666667	384419	94,4%

HPS: Hyperoil Polimeric Substrate; Control = PS polymeric substrate without Hyperoil; N/A Not Applicable; % percentage; SE Standard Error

Table 2. Average (±SE) *P. aeruginosa* strain by time and HPS dilution

HPS dilution (%)	*P. aeruginosa* (ATCC® 10145™) strain 1 hour	SE	% reduction vs control	*P. aeruginosa* (ATCC® 10145™) strain 3 hours	SE	% reduction vs control	*P. aeruginosa* (ATCC® 10145™) strain 24 hours	SE	% reduction vs control
0	130333	10333	N/A	16466667	3286504	N/A	175333333	2905933	N/A
1,5	91400	305	29,9%	970666	6333	94,1%	16633333	409606	90,5%
2,5	88766	2533	31,9%	923666,7	31991	94,4%	15000000	152752	91,4%
5	84700	3572	35,0%	808000	44635	95,1%	14400000	709459	91,8%
10	81200	2886	37,7%	728333	14813	95,6%	13333333	328295	92,4%
25	72933	5944	44,0%	574666	63878	96,5%	40236667	29387860	77,1%

HPS: Hyperoil Polimeric Substrate; Control = PS polymeric substrate without Hyperoil; N/A Not Applicable; % percentage; SE Standard Error

Table 3. Average (±SE) *K. ozaenae* strain by time and HPS dilution

HPS dilution (%)	*K. ozaenae* AM strain 1 hour	SE	% reduction vs control	*K. ozaenae* AM strain 3 hours	SE	% reduction vs control	*K. ozaenae* AM strain 24 hours	SE	% reduction vs control
0	228667	4702	N/A	43033333	1049338	N/A	110333333	3179797	N/A
1,5	17067	698	92,5%	375667	14193	99,1%	21233333	384419	80,8%
2,5	16633	669	92,7%	314667	40043	99,3%	20066667	88192	81,8%
5	16100	1000	93,0%	302667	41914	99,3%	19400000	208167	82,4%
10	14067	1510	93,8%	246667	32028	99,4%	17733333	371184	83,9%
25	11800	551	94,8%	186000	9165	99,6%	12666667	384419	88,5%

HPS: Hyperoil Polimeric Substrate; Control = PS polymeric substrate without Hyperoil; N/A Not Applicable; % percentage; SE Standard Error

The activity against the Gram positive *S. aureus* (ATCC® 12598™), the Gram negative *P. aeruginosa* (ATCC® 10145™) being common and often resistant strains in skin and hospital infections [24], where Hyperoil™ scaffolds are expected to be used, could explain the successful results observed while suing Hyperoil™ on diabetic foot ulcers [13]. On the other hand, the activity on *K. ozaenae* AM, has a particular importance considering the wide number of identified antibiotic-resistant strains of this enterobacterium, frequently linked with opportunistic infections of opened wounds [25]. The particular structure of the polymeric scaffold might not ensure a-priori the efficacy of Hyperoil™ when meshed with the PLLA structure. The results of this study support *in-vitro* the observed clinical results.

4. CONCLUSION

HPS, being Hyperoil™ included in a highly biocompatible scaffold of poly-lactic acid, showed bacteriostatic effects on the tested *S. aureus* (ATCC® 12598™), *P. aeruginosa* (ATCC® 10145™) and *K. ozaenae* AM strains.

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COMPETING INTERESTS

Maria Letizia Iabichella acts as research consultant for RIMOS S.r.L. Mirandola (MO) - Italy.

REFERENCES

1. Government of India Ministry of Health and Family Welfare Department of Ayush. The Ayurvedic Pharmacopoeia of India. The Ayurvedic Pharmacopoeia of India. 2012;5:119. 1. part- I, Access date October 28, 2012. Available:http://www.ayurveda.hu/api/API-Vol-5.pdf
2. Dos Santos ACG, Rodrigues OG, De Araujo LVC, Dos Santos SB, De C. Guerra RDMSN, Feitosa MLT, Teixeira WC, Santos-Ribeiro A. Use of neem extract in the control of acariasis by *Myobia musculi* Schranck (Acari: Miobidae) and *Myocoptes musculinus* Koch (Acari: Listrophoridae) in mice (*Mus musculus* var. albina L.). Neotropical Entomology. 2006;35:269-272.
3. Narayanan AS, Raja SSS, Ponmurugan K, Kandekar SC, Natarajaseenivasan K, Maripandi A, Mandeel QA. Antibacterial activity of selected medicinal plants against multiple antibiotic resistant uropathogens: a study from Kolli Hills, Tamil Nadu, India. Beneficial Microbes. 2011;2:235-243.
4. Murthy SP, Sirsi M. Pharmacological studies on Melia *Azadirachta indica*. Indian Journal of Physiology and Pharmacology. 1958;2:387-396.
5. Upadhyay SN, Dhawan S, Garg S, Talwar GP. Immunomodulatory effects of neem (*Azadirachta indica*) oil. Int J Immunopharmacol. 1992;14:1187-93.
6. Athar A, Saikat H, Hirekodathakallu T, et al. Novel Anti-inflammatory Activity of Epoxyazadiradione against macrophage migration inhibitory factor. Journal of Biological Chemistry. 2012;287:24844-24861.
7. Tandan SK, Gupta S, Chandra S, Lal J, Singh R. Safety evaluation of *Azadirachta indica* seed oil, a herbal wound dressing agent. Fitoter. 1995;66:69-72.
8. Fernie WT. Herbal simples. Bristol: John Wright & Co.; 1897.
9. Aizenman BE. Antibiotic preparations from *Hypericum perforatum*. Kiev: Mikrobiol. Zh. 1969;31:128-33.
10. Bombardelli E, Morazzoni P. *Hypericum perforatum*. Fitoterapia. 1995;62:43-68.
11. Koeberle A, Rossi A, Bauer J, Dehm F, Verotta L, Northoff H, Sautebin L, Werz O. Hyperforin, an anti-inflammatory constituent from St. John's Wort, inhibits microsomal prostaglandin E(2) synthase-1 and suppresses prostaglandin E(2) formation *in vivo*. Front Pharmacol. 2011;2.
12. Läuchli S, Hafner J, Wehrmann C, Frenc LE. Post-surgical scalp wounds with exposedbone treated with a plant-derived wound therapeutic. J Wound Care. 2012;21:228-33.
13. Iabichella ML. The use of an extract of *Hypericum perforatum* and *Azadirachta indica* in advanced diabetic foot: An

unexpected outcome. BMJ Case Reports; 2013. DOI: 10.1136/bcr-2012-007299.
14. Jagur-Grodzinski J. Polymers for tissue engineering, medical devices, and regenerative medicine. Concise general review of recent studies. Polymers for Advanced Technologies. 2006;17(6):395-418.
15. Mendes JJ, Leandro C, Mottola C, Barbosa R, Silva FA, Oliveira M, Vilela CL, Melo-Cristino J, Górski A, Pimentel M, São-José C, Cavaco-Silva P, Garcia M. In vitro design of a novel lytic bacteriophage cocktail with therapeutic potential against organisms causing diabetic foot infections. J Med Microbiol; 2014. PII: jmm.0.071753-0. DOI: 10.1099/jmm.0.071753-0.
16. Nguyen TK, Milch H. *Klebsiella* and *Enterobacter* strains derived from hospital infections. I. Correlation between species, phage type and antibiotic sensitivity. Acta Microbiol Acad Sci Hung. 1981;28(1):67-81.
17. Ji X1, Jin P, Chu Y, Feng S, Wang P. Clinical characteristics and risk factors of diabetic foot ulcer with multidrug-resistant organism infection. Int J Low Extrem Wounds. 2014;13(1):64-71. DOI: 10.1177/1534734614521236. Epub 2014 Feb 10.
18. Brisse S1, Fevre C, Passet V, Issenhuth-Jeanjean S, Tournebize R, Diancourt L, Grimont P. Virulent clones of *Klebsiella pneumoniae*: identification and evolutionary scenario based on genomic and phenotypic characterization. PLoS One. 2009;4(3):e4982. DOI: 10.1371/journal.pone.0004982. Epub 2009 Mar 25.
19. Saino E, Focarete ML, Gualandi C, Emanuele E, Cornaglia AI, Imbriani M, Visai L. Effect of electrospun fiber diameter and alignment on macrophage activation and secretion of proinflammatory cytokines and chemokines. *Biomacromolecules*. 2011;12(5):1900-11.
20. Del Curto B, Brunella MF, Giordano C, Pedeferri MP, Valtulina V, Visai L, Cigada A. Decreased bacterial adhesion to surface-treated titanium. Int J Artif Organs. 2005;28:718-30.
21. Petrini P, Arciola CR, Pezzali I, Bozzini S, Montanaro L, Tanzi MC, Speziale P, Visai L. Antibacterial activity of zinc modified titanium oxide surface. Int J Artif Organs. 2006;29:434-42.
22. Wang JD, Levin PA. Metabolism, cell growth and the bacterial cell cycle. Nat Rev Microbiol. 2009;7(11):822-7.
23. Pankey GA, Sabath LD. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. Clin. Infect. Dis. 2004;38(6):864–70.
24. Mohammadzadeh R1, Ahmadiyan N. Skin infection management using novel antibacterial agents. Adv Pharm Bull. 2013;3(1):247-8.
25. Livermore DM, Yuan M. Antibiotic resistance and production of extended-spectrum beta-lactamases amongst *Klebsiella* spp. from intensive care units in Europe. J Antimicrob Chemother. 1999;38(3):409-24.

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