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In-vitro evaluation of the antimicrobial activity of the product:

OZONIA® 10 CREAM

Dermatological cream made with ozonated sunflower oil (Ozonia 3000®).

Client:

Innovares s.r.l.

Via Risorgimento 5/B 42049 Sant'Ilario d'Enza (Re), Italy

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Introduction

Since 1973 the analysis and consulting activities of Laboratorio REI S.r.l. have focused on the agri-food, cosmetics and environmental protection sectors. With its 500m^2 laboratory, constantly updated technical staff and state-of-the-art analysis equipment, Laboratorio REI meets the needs of its clients in a professional manner, in both the effective execution of different types of analysis and in terms of technical evaluation and specific expertise on more complex issues that often accompany standard analytical determinations. In order to guarantee maximum reliability in sampling, performing analyses and validating the results, Laboratorio REI S.r.l. has obtained accreditation in accordance with standard UNI CEI EN ISO/IEC 17025*" General requirements for the competence of testing and calibration laboratories" from the Italian Accreditation Body, ACCREDIA,under the number 0508, dated October 2003.*

ACCREDIA accredits testing laboratories which operate in compliance with the prescriptions and requirements set out in standard UNI CEI EN ISO/IEC 17025 and in ACCREDIA, EA and ILAC documents, making sure they possess and maintain over time the necessary organizational, procedural, technical and professional requisites in such a way as to establish, in all the social and economic partners involved and especially in users and end consumers, a high level of trust in the work performed by said laboratories and in the value of the certificates of conformity issued by the same. This guarantee is provided through a four-year accreditation agreement signed between ACCREDIA and Laboratorio REI S.r.l. available for consultation at the the Laboratorio REI S.r.l. headquarters. The list of accredited tests is available on WWW.ACCREDIA.IT

OZONIA® 10 CREAM

Dermatological cream made with ozonated sunflower oil (Ozonia 3000).

RESEARCH PROJECT

In-vitro evaluation of the antimicrobial activity of the product

Innovares S.r.l. appointed Laboratorio REI S.r.l. to carry out an in-vitro study on the antimicrobial activity of the product OZONIA® 10 CREAM.

OZONIA® 10 CREAM performs a protective and eutrophic action for solutions of continuity and inflammation of the skin and mucosa, conditions which also require a preventive action against microbial colonization which hinders the healing process.

This study aims to evaluate the action of the compound on the specific micro-organisms, listed below (American Type Culture Collection, ATCC).

Escherichia coli (ATCC 8739) colonizes the human/animal gastrointestinal tract, faecal contamination indicator, rod-shaped, optional anaerobic/aerobic, certain strains are the etiologic agents of intestinal and extraintestinal diseases, as well as urinary tract infections, meningitis, peritonitis, septicaemia and pneumonia.

Pseudomonas aeruginosa (ATCC 9027) rod-shaped, purely aerobic, oxidase -positive, catalase -positive and with flagella polar making them motile. They are found in soil, water and on plants. They produce pigments such as pyocyanin which alter ciliary function, stimulates an inflammatory response and damages tissues. Pseudomonas aeruginosa is one of the most common to the extent that it is present in many samples of human faeces and more rarely also in the moister areas of the epidermis, such as the axilla and groin. It causes osteoarticular infections, external otitis and pneumonia. It is also responsible for folliculitis, eye infections such as conjunctivitis and endocarditis.

Staphilococcus. aureus (ATCC 6538) optional anaerobic/aerobic, usually found on the skin, mucosa of the front section of the nose and pharynx in most adults. Consequently an infection caused by the same can occur at any time. It is responsible for acute suppurative infections which may affect different parts of the organism: skin, skeleton, respiratory system, urinary system, central nervous system. Some strains can result in poisoning.

Candida albicans (ATCC 10231) is a saprobiontic fungus belonging to the saccharomycetes family. It is usually found in the mouth, the gastrointestinal tract and vagina,

Aspergillus brasiliensis (ATCC 16404) genus of fungus belonging to the trichocomaceae family, it can cause infections in humans and animals (aspergillosis): they affect therespiratory system (nose and lungs), and may spread to other organs (skin and ears).

Proteus mirabilis (ATCC 43071) belongs to the Enterobacteriaceae family, optional anaerobe, motile, causes inflammation, bacteremia and pneumonia.

Trichophyton mentagrophytes (ATCC 9533) zoophilous fungus (farm animals-pets), causes inflammatory lesions of the dermis and scalp.

Propionibacterium acnes (ATCC 11827) prefers anaerobic atmospheres, it is found on the human skin, causes acne and post-operative infections, inflammation of the spine.

The principle behind the antimicrobial study is to add a bacterial test suspension to a sample of the product being tested and keep it at 22°C for a set time.

Part 1) Experimental conditions

Culture media:
Nutrient broth: Brain Heart Infusion (BHI);
Nutrient broth: Saboraud dextrose Broth (SAB);
Letheen Broth Base Modified (MLB) diluent-neutralizer;
Solid culture medium for bacteria: Tryptone Soya Agar (TSA);
Solid medium for Yeasts and moulds, Saboraud Dextrose Agar (SDA).

Incubation in incubator at 22°C +/- 1°C.

Part 2) Preliminary testing of the diluent-neutralizer and validation

A bacterial test suspension is added to a sample of the OZONIA® 10 CREAM being tested to which a diluent is added with the capacity to immediately neutralize the bacterial activity of the preservative in the product.

Inactivation of the product's bacterial activity must be validated for each strain subjected to testing and for each of the experimental conditions selected.
The diluent, which returned a positive result, is MLB with a 1:10 dilution ratio.

Part 3) Preliminary testing of the bacterial contamination of the cosmetic product.

Mesophilic aerobic bacteria < 10 cfu/g according to the ISO 21149 accredited analysis method;
Yeasts and moulds: <10 cfu/g according to the ISO 16212 accredited analysis method;

Part 4) Inoculation of the strains in the sample of OZONIA® 10 CREAM

The microorganisms being tested were made to grow as follows:
Propionibacterium acnes in BHI medium for 48 -72 hours in anaerobic conditions at an incubation temperature of 35°C.

Aspergillus niger, Candida albicans in SAB medium for 24-48 hours at an incubation temperature of 32°C.

The remaining micro-organisms in BHI medium for 24 - 48 hours at an incubation temperature of 37°C.

The micro-organisms thus obtained are at a concentration of around 10^7 . Inoculation is then carried out separately with 0.2 ml in 20 g samples to achieve a final working concentration of approximately 10^3cells/ml. A positive control is also carried out alongside to check the correctness of the procedure.
To check the correctness of the preparation procedure, the inoculated sample is analysed after 1 hour by taking a 1-g sample to which 9 ml of MLB are added. This is followed by plate seeding on a specific medium with appropriate dilution.
Following this procedure, the inoculated sample is then left to incubate at 22°C in an incubator for subsequent sampling to be performed at set intervals.

Part 5) Results:

The table shows the results of the inoculum levels and the counts carried out at the set intervals. If the data is lower than the detection limit, the difference between the logarithms has been expressed as "greater than" (>).

STRAIN	Unit of meas.	Inoculum	2 hours	4 hours	1 day	2 days	7 days
Escherichia coli	cfu/g	1.56×10^7	<10	<10	<10	<10	<10
	$\log_{10}$ cfu/g	7.19	<1.00	<1.00	<1.00	<1.00	<1.00
	log. reduction	/	6.19	6.19	6.19	6.19	6.19
Pseudomonas aeruginosa	cfu/g	9.6×10^6	<10	<10	<10	<10	<10
	$\log_{10}$ cfu/g	6.98	<1.00	<1.00	<1.00	<1.00	<1.00
	log. reduction	/	5.98	5.98	5.98	5.98	5.98
Staphilococcus. aureus	cfu/g	4.44×10^7	<10	<10	<10	<10	<10
	$\log_{10}$ cfu/g	7.64	<1.00	<1.00	<1.00	<1.00	<1.00
	log. reduction	/	6.64	6.64	6.64	6.64	6.64
Candida albicans	cfu/g	1.23×10^6	<10	<10	<10	<10	<10
	$\log_{10}$ cfu/g	6.08	<1.00	<1.00	<1.00	<1.00	<1.00
	log. reduction	/	5.08	5.08	5.08	5.08	5.08
Aspergillus brasiliensis	cfu/g	4.9×10^5	<10	<10	<10	<10	<10
	$\log_{10}$ cfu/g	5.69	<1.00	<1.00	<1.00	<1.00	<1.00
	log. reduction	/	4.69	4.69	4.69	4.69	4.69
Proteus mirabilis	cfu/g	2.31×10^7	<10	<10	<10	<10	<10
	$\log_{10}$ cfu/g	7.36	<1.00	<1.00	<1.00	<1.00	<1.00
	log. reduction	/	6.36	6.36	6.36	6.36	6.36
Trichophyton mentagrophytes	cfu/g	1.16×10^8	<10	<10	<10	<10	<10
	$\log_{10}$ cfu/g	8.06	<1.00	<1.00	<1.00	<1.00	<1.00
	log. reduction	/	7.06	7.06	7.06	7.06	7.06
Propionibacterium acnes	cfu/g	2.8×10^6	<10	<10	<10	<10	<10
	$\log_{10}$ cfu/g	6.45	<1.00	<1.00	<1.00	<1.00	<1.00
	log. reduction	/	5.45	5.45	5.45	5.45	5.45

For topical preparations, the Pharmacopea defines the criteria of acceptability for anitmicrobial activity in terms of the logarithmic reduction in live microorganisms against the initial value of the inoculum according to Table 5.1.3-2 and these are indicated in the Table.

		Logarithmic reduction			
	Criteria	2 days	7 days	14 days	28 days
Bacteria	A	2	3	-	No increase
	B			3	No increase
Yeasts and moulds	A	-	-	2	No increase
	B			1	No increase

The following table shows the reduction values as a % in relation to the inoculum.

STRAIN	**Unit of meas.**	**Inoculum**	**2 hours**	**4 hours**	**1 day**	**2 days**	**7 days**
Escherichia coli	cfu/g	1.56×10^7	100	100	100	100	100
Pseudomonas aeruginosa	cfu/g	9.6×10^6	100	100	100	100	100
Staphilococcus. aureus	cfu/g	4.44×10^7	100	100	100	100	100
Candida albicans	cfu/g	1.23×10^6	100	100	100	100	100
Aspergillus brasiliensis	cfu/g	4.9×10^5	100	100	100	100	100
Proteus mirabilis	cfu/g	2.31×10^7	100	100	100	100	100
Trichophyton mentagrophytes	cfu/g	1.16×10^8	100	100	100	100	100
Propionibacterium acnes	cfu/g	2.8×10^6	100	100	100	100	100

Part 6) Conclusions

The in-vitro experimental tests for the antimicrobial evaluation of OZONIA® 10 CREAM have provided a very positive outcome for the micro-organisms tested and without doubt it meets the recommended criteria of antimicrobial effectiveness against all the micro-organisms tested, as envisaged in Table 5.1.3-2 of the Pharmacopea for compounds intended for topical use.

Part 7) Bibliography

UNI EN ISO 11930 Evaluation Of The Antimicrobial Protection Of A Cosmetic Product.
EUROPEAN PHARMACOPOEIA Microbial contamination of non-sterile products 2.6.12 and Efficacy of antimicrobial preservation 5.1.3.

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