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REPORT ON ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF PREPARATION
"Probotanic Wild Oregano Oil"

''Baltik Junior''
Vučićev prolaz 20a
Belgrade

16 March, 2017, Belgrade

Subject: Response to the letter dated 5 February 2017

The company **''Baltik Junior''** addressed the Institute for Biological Research ''Siniša Stanković'' in Belgrade (hereinafter referred to as: IBISS) for their professional opinion on potential antibacterial and antifungal effect of "**Probotanic Wild Oregano Oil''** preparation.

Upon review of the documents submitted by the applicant, including literature and the processed laboratory data, we express our expert opinion as follows:

EXPERT OPINION

The sample of **"Probotanic Wild Oregano Oil**" was tested to the following Gram (+) and Gram (-) bacteria, *Streptococcus pyogenes* (IBRS S003), *Streptococcus salivarius*

(IBRS S006), *Streptococcus sanguinis* (IBRS S002), *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *Staphylococcus aureus* (MRSA) 11, *Proteus mirabilis* (clinical isolate), *Pseudomonas aeruginosa* (IBRS P001), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), *Listeria monocytogenes* (NCTC 7973), *Salmonella typhimurium* (ATCC 13311), *Salmonella enteritidis* (clinical isolate), *Salmonella infantis* (clinical isolate), *Escherichia coli* (ATCC 35210), *Enterobacter cloacae* (human isolate), *Enterococcus faecallis* (ATCC 19433), *Yersinia enterocolitica* (clinical isolate), *Serratia marcescens* (clinical isolate). For research of antifungal activity *in vitro* the following were used: *Candida albicans* (IBRS MH4), *Candida krusei* (IBRS lflacl), *Aspergillus niger* (ATCC 6275), *Aspergillus fumigatus* (human isolate), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC 12066) and *Trichoderma viride* (IAM 5061). All microorganisms were deposited in Mycotheca of Mycological laboratory, Department for plant physiology, Institute for Biological Research ''Siniša Stanković'', University of Belgrade. For positive control, the following antibiotics were used: Streptomycin, Ampicillin, Augmentin, Ciproxin and Klaricid and two mycotics: Bifonazole and Ketoconazole. *In vitro* microdilution method (Hanel and Raether, 1988; Soković et al, 2010) was used.

It was determined that **''Probotanic Wild Oregano Oil**'' had bacteriostatic and bactericidal effect, i.e. it inhibited but also prevented further growth of all tested Gram (-) and Gram (+) bacteria. The bacterial species most susceptible to the tested product was *Micrococcus flavus* with inhibiting concentration of 0.01 microg/ml and bactericide concentration of 0.015 microg/ml. The most resilient bacteria to the effect of the tested preparation were *Enterococcus faecallis* and *Serratia marcescens* with inhibiting/bactericide concentrations of 0,45/0,60 microg/ml. It was determined that this preparation had effect on all the tested bacteria in very low concentrations, even on the most resilient Gram (-) bacteria, among which is *Pseudomonas aeruginosa*, which is known to be one of the most resilient and resistant bacteria (Soković et al, 2010).

Streptomycin showed wide-range inhibiting activity, depending on the type of bacteria, varying from 1.5-100 microg/ml and bactericide activity varying from 3.0-200.0 microg/ml.

Ampicillin had inhibiting effect from 0.0007-400.00 microg/ml and bactericide effect from 0.0015-500.0 microg/ml, while Augmentin showed antimicrobial activity which was stronger than the two previously mentioned, with MIC ranging from 0.0015-20.0 microg/ml and MBC from 0.003-30.0 microg/ml. Ciproxin acted with MIC from 0.0007-45.00 and MBC from 0.0015-100.0 microg/ml, while Klaricid showed antimicrobial effect at MIC 0.007-50.0 microg/ml and MBC 0.003-100.0 microg/ml (Table 1, Graph 1).

The tested preparation ''Probotanic Wild Oregano Oil'' showed several times better antibacterial activity than the commercial drug streptomycin. The preparation also had antibacterial potential even stronger than Ampicillin (with the exception of *Streptococcus salivarius* and *E. faecallis*), stronger than Augmentin (except on *E. coli*), stronger than Ciproxin (except on *Streptococcus salivarius, Proteus mirabilis, Bacillus cereus, Salmonella entiritidis, E. coli*).

The tested preparation, in very low concentrations, had inhibiting and fungicidal effect on all the treated fungi. It showed its best activity on *Aspergillus fumigates* with MIC ranging from 0.10 microg/ml and MFC from 0.40 microg/ml. *Candida albicans* was the most resilient fungi to ''Probotanic Wild Oregano Oil'' with MIC 0.30 microg/ml and MFC 0.60 microg/ml.

Bifonazole showed inhibiting activity from 100-200 microg/ml and fungicidal activity at 200-300 microg/ml. Ketoconazole had inhibiting effect at 50-1500 microg/ml and fungicidal at 100-2000 microg/ml. The tested preparation showed much stronger antifungal effect than commercial drugs (Table 2, Graph 2).

Taking into account that in the recent years resistance of microorganisms to the existing synthetic antibiotics has been more and more frequent, including the increasing toxicity of commercial preparations to human cells, there is a need for a new semi-synthetic or natural antimicrobial agents which would have no harmful effect on the health of people.

With regards to that, and on the basis of review of literature and performed *in vitro* analysis, the following **conclusion** can be drawn:

The tested preparation ''Probotanic Wild Oregano Oil'' showed good antibacterial and antifungal activity, several times greater than antibiotics (save for the mentioned exceptions and mycotics). It justified the use of the product ''Probotanic Wild Oregano Oil'' in prevention of various bacterial and fungal infections caused by the mentioned species. This conclusion is based on characteristics of this product, as well as the fact that the resistance of such microorganisms to natural preparations is far less.

References:

Hanel H. and Raether W. (1988): A more sophisticated method of determining the fungicidal effect of water-insoluble preparations with a cell harvester, using miconazole as an example. Mycoses 31, 148-154

Soković M, Glamočlija J, Marin D.P, Brkić D, van Griensven L.J.L.D (2010): Antibacterial Effects of the Essential Oils of Commonly Consumed Medicinal Herbs Using an *In Vitro* Model, Molecules, 15, 7532-7546

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The following bacteria were used in the research: *Streptococcus pyogenes* (IBRS S003), *Streptococcus salivarius* (IBRS S006), *Streptococcus sanguinis* (IBRS S002), *Staphylococcus aureus* (ATCC 25923), methicilin-resistant *Staphylococcus aureus* (MRSA) 11, *Proteus mirabilis* (clinical isolate), *Pseudomonas aeruginosa* (IBRS P001), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), *Listeria monocytogenes* (NCTC 7973), *Salmonella typhimurium* (ATCC 13311), *Salmonella enteritidis* (clinical isolate), *Salmonella infantis* (clinical isolate), *Escherichia coli* (ATCC 35210), *Enterobacter cloacae* (human isolate), *Enterococcus faecallis* (ATCC 19433), *Yersinia enterocolitica* (clinical isolate), *Serratia marcescens* (clinical isolate). For research of antifungal activity *in vitro* the following were used: *Candida albicans* (IBRS MH4), *Candida krusei* (IBRS 1flac1), *Aspergillus niger* (ATCC 6275), *Aspergillus fumigatus* (human isolate), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC 12066) and *Trichoderma viride* (IAM 5061). All microorganisms were deposited in Mycotheca of Mycological Laboratory, Department for Plant Physiology, Institute for Biological Research ''Siniša Stanković'', University of Belgrade. For positive control, the following antibiotics were used: Streptomycin, Ampicillin, Augmentin, Ciproxin and Klaricid and two mycotics: Bifonazole and Ketoconazole.

Table 1. Antibacterial activity of wild oregano essential oil (*Origanum minutiflorum*) - Probotanic and antibiotics (µg/ml)

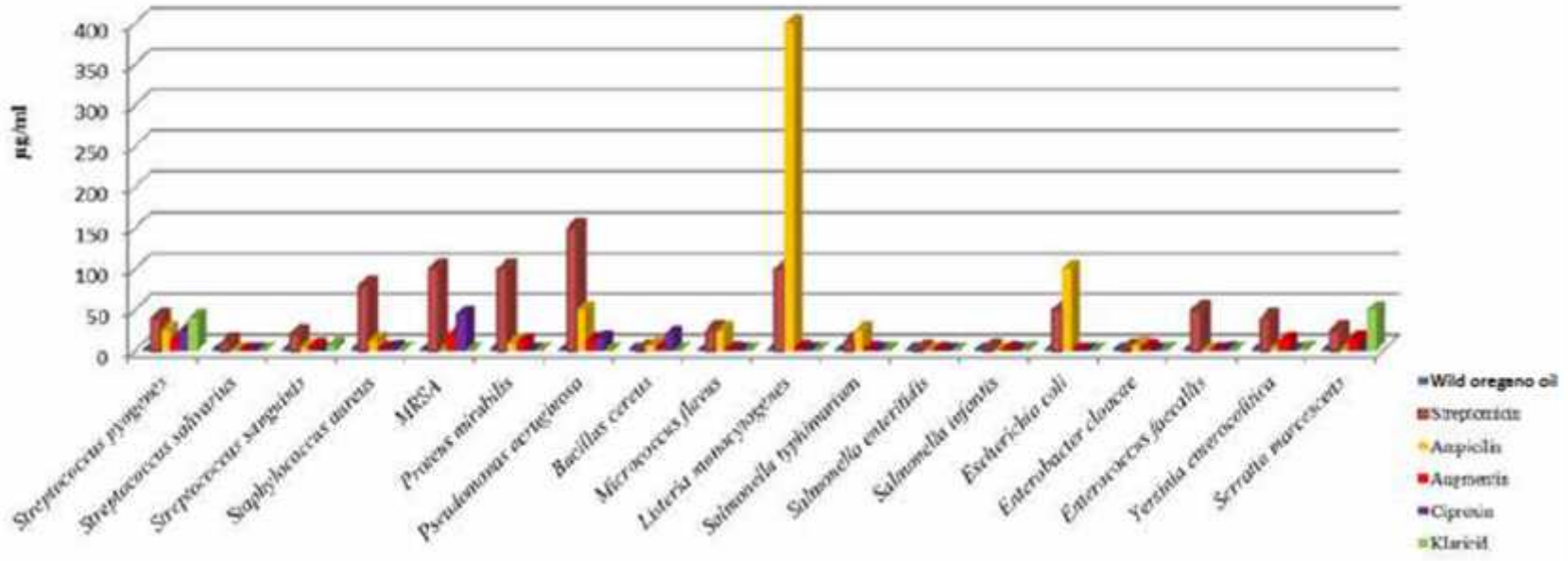
	Wild oregano oil MIC MBC	Streptomycin MIC MBC	Ampicilin MIC MBC	Augmentin MIC MBC	Ciproxin MIC MBC	Klaricid MIC MBC
Streptococcus pyogenes	0.20 0.30	40.0 80.0	25.0 100.0	12.5 25.0	25.0 100.0	40.0 50.0
Streptococcus salivarius	0.04 0.075	10.0 20.0	0.0007 0.0015	0.2 0.3	0.3 0.6	0.007 0.015
Streptococcus sanguinis	0.10 0.15	20.0 40.0	4.0 6.0	5.0 10.0	0.15 0.3	6.0 12.0
Staphylococcus aureus	0.60 1.20	80.0 160.0	12.5 25.0	2.0 3.0	3.0 6.0	0.5 0.6
meticilin-rezistant *Staphylococcus aureus*	0.10 0.15	100.0 -	- -	20.0 30.0	45.0 60.0	0.02 0.03
Proteus mirabilis	0.04 0.075	100.0 200.0	10.0 12.5	10.0 12.5	0.007 0.015	0.003 0.007
Pseudomonas aeruginosa	0.30 0.6	150.0 200.0	50.0 100.0	12.5 25.0	15.0 30.0	0.15 0.3
Bacillus cereus	0.075 0.15	1.50 3.00	6.0 25.0	3.0 6.0	20.0 30.0	0.0015 0.003
Micrococcus flavus	0.01 0.015	25.0 50.0	25.0 40.0	1.25 2.5	0.02 0.03	0.015 0.03
Listeria monocytogenes	0.30 0.60	100.0 200.0	400.0 500.0	3.0 6.0	0.6 1.2	0.3 1.2
Salmonella typhimurium	0.30 0.60	12.5 25.0	25.0 50.0	2.0 2.5	0.6 2.5	0.6 1.2
Salmonella enteritidis	0.30 0.60	1.50 3.0	0.03 0.06	1.0 1.5	0.15 0.3	0.02 0.03
Salmonella infantis	0.10 0.15	3.0 6.0	0.03 0.06	1.5 3.0	0.02 0.03	1.5 2.5
Escherichia coli	0.075 0.15	50.0 100.0	100.0 200.0	0.0015 0.003	0.007 0.015	0.007 0.015
Enterobacter cloacae	0.30 0.60	3.0 6.0	6.0 12.0	4.0 6.0	0.02 0.03	0.5 0.6
Enterococcus faecalis	0.45 0.60	50.0 60.0	0.015 0.030	0.2 0.3	1.2 2.5	0.5 0.6
Yersinia enterocolitica	0.20 0.60	40.0 50.0	3.0 6.0	12.5 25.0	0.0007 0.0015	2.0 3.0
Serratia marcescens	0.45 0.60	25.0 50.0	1.0 1.5	15.0 25.0	1.5 3.0	50.0 100.0

- no activity

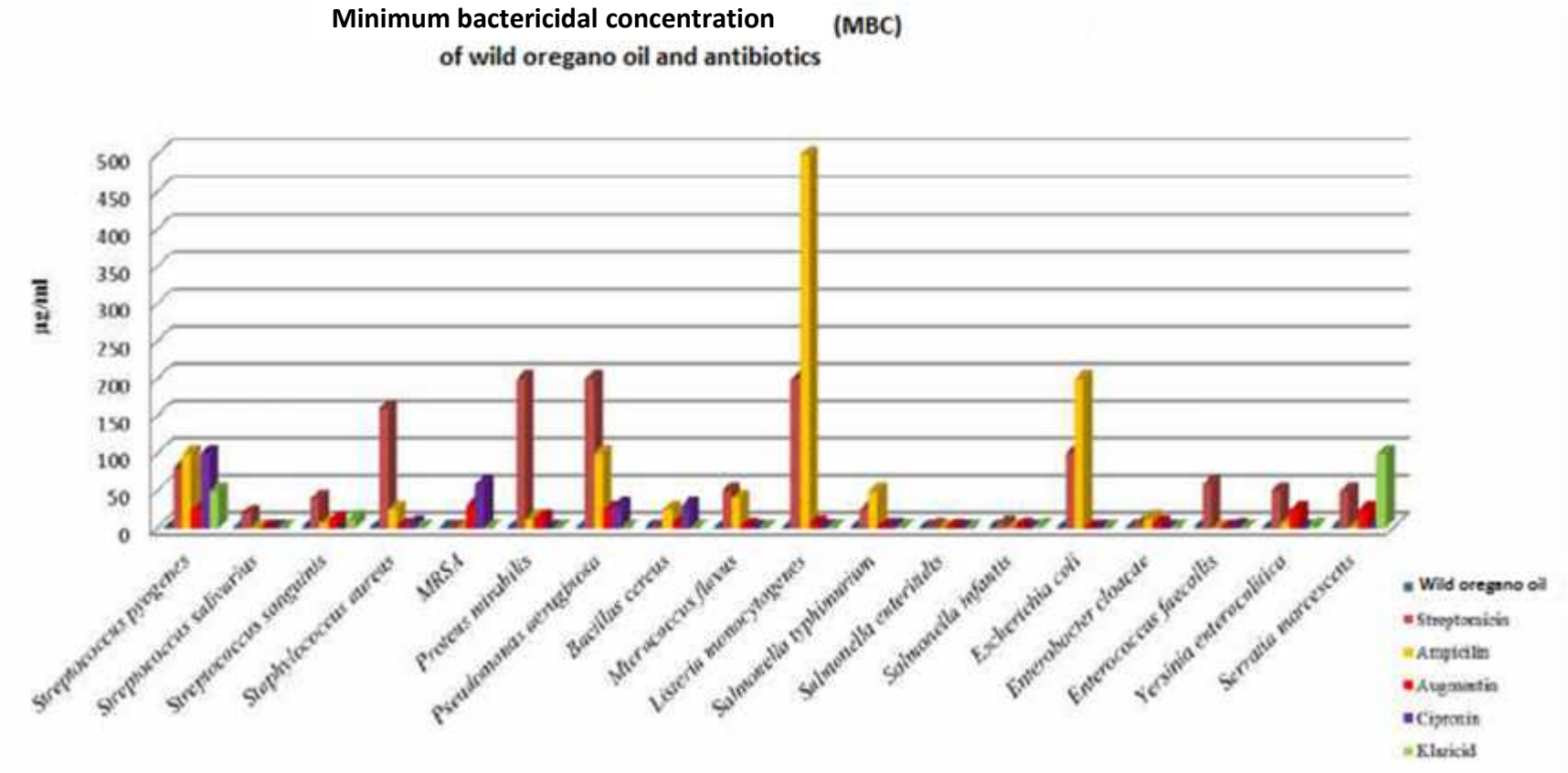
Table 2. Antifungal activity of wild oregano essential oil (*Origanum minutiflorum*) - Probotanic and mycotics (µg/ml)

	Wild oregano oil MIC MFC	Bifonazol MIC MFC	Ketokonazol MIC MFC
Aspergillus niger	0.02 0.04	150 200	200 500
Aspergillus fumigatus	0.01 0.04	150 200	200 500
Aspergillus versicolor	0.02 0.04	100 200	200 500
Aspergillus ochraceus	0.04 0.075	150 200	1500 2000
Trichoderma viride	0.02 0.04	150 200	1000 1000
Candida albicans	0.30 0.60	200 300	1500 2000
Candida kruzei	0.075 0.15	100 200	50 100

Minimum inhibitory concentration (MIC) of wild oregano oil and antibiotics



Minimum bactericidal concentration (MBC)
of wild oregano oil and antibiotics



Minimum inhibitory (MIC) and fungicidal (MFC) concentrations of wild oregano oil and mycotics