Natural Antimicrobial Agents: III. Suprapein™

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Abstract

A series of effective Natural Antimicrobial Agents have been developed with interesting characteristics. Biopein™[1,2] and Neopein™[2] have been described. Lately, Suprapein™ has been developed and tested against an array of bacteria and fungi with different susceptibilities. The organisms included gram positive *Staphylococcus aureus*, gram negative *Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, acid-fast bacterium *Mycobacterium smegmatis*, the Yeast *Candida albicans* and the filamentous mold *Aspergillus niger*. For comparison the following well-known synthetic preservatives were used viz. Phenoxyethanol (PE), Phenyl Ethyl Alcohol (PEA), and a combination of Methyl and Propyl Parabens (MP) in a ratio of 5:4. The Minimum Inhibitory Concentration (MIC) was determined for each agent. Suprapein™ had the lowest MIC (0.45%) followed in increasing order by PEA (0.60%), PE (1.00%) and MP (2.16%), according to their capability of inhibiting all the tested organisms. Suprapein™ can therefore, be used as an effective natural alternative to commonly used synthetic ingredients in appropriate formulations for product preservation. Its composition and use are patent pending.

Introduction

Consumers are staying away from anything synthetic, including preservatives. This is due to numerous unforeseen complications noticed or experienced as carcinogenicity, terratogenicity, liver, heart, respiratory or nervous system problems.

The Composition and MIC for Biopein™ and Neopein™ have been reported[1,2]. Suprapein™ has been introduced as a third member of the series of Natural Antimicrobial Agents, developed at Bio-Botanica, with different physical and chemical characteristics to give the formulator more choices to comply with his needs as to which preservative would be best suitable for the product.

Suprapein™ is an optimum synergistic combination of Botanical Fractions of the Following Herbs:

- *Origanum vulgare* L. and *Thymus vulgaris* L. which contain effective Phenolic ingredients, Carvacrol and Thymol (Figure 1).
- *Cinnamomum zeylanicum* Nees which contains mainly cinnamaldehyde and Eugenol (Figure 2).
- *Rosmarinus officinalis* L. which contains 1,8-Cineole, Camphor, alpha-Pinene and also small amounts of Rosmarinic Acid (Figure 3).
- *Lavandula officinalis* L. which contains Linalyl acetate and Linalol (Figure 4).
- *Mentha piperita* L. which contains Menthol, Menthyl Acetate and Menthone (Figure 5).
- *Citrus limon* L. which contains Limonene together with the Aldehyde geranial, neral and citronellal (Figure 6).
- *Hydrastis canadensis* L. which contains Berberine and Hydrastine alkaloids (Figure 7).
- *Olea europaea* L. which contains Oleuropein, the first secoiridoid compound to be isolated (Figure 8).

Martindale[3] reported comparatively high phenolic coefficients for certain Suprapein™ constituents viz. For Thyme, 15; for

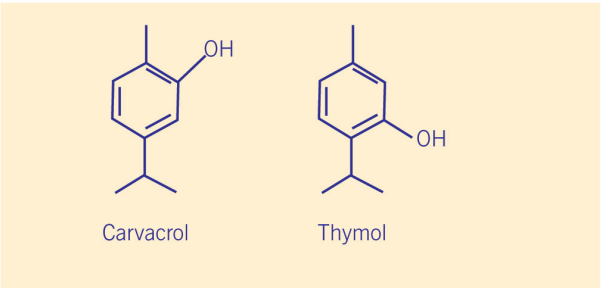
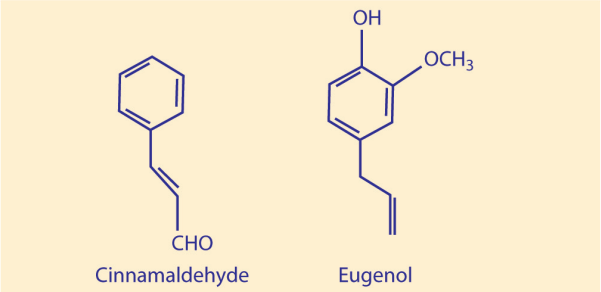


Figure 1.



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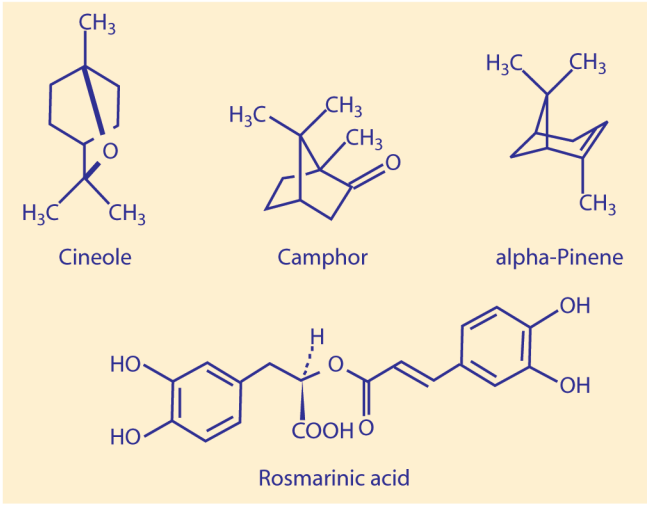
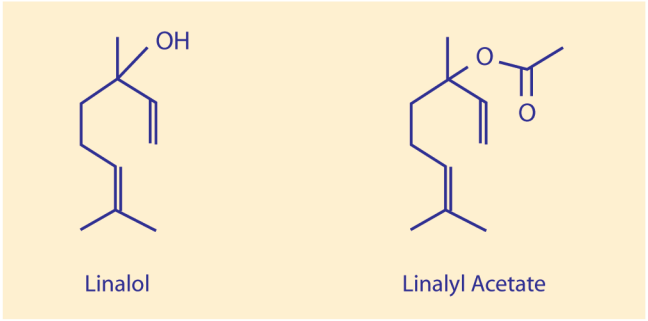


Figure 3.



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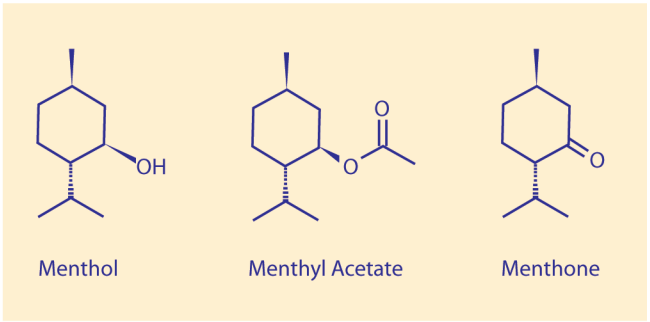

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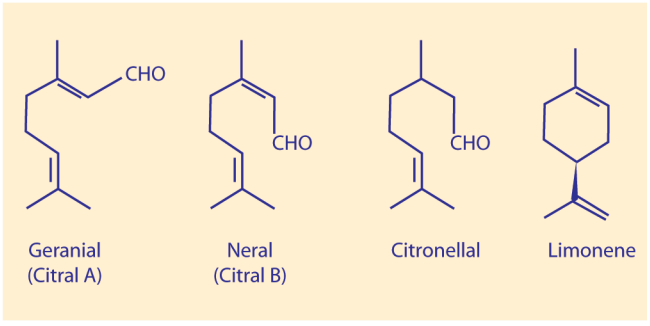

Figure 6.

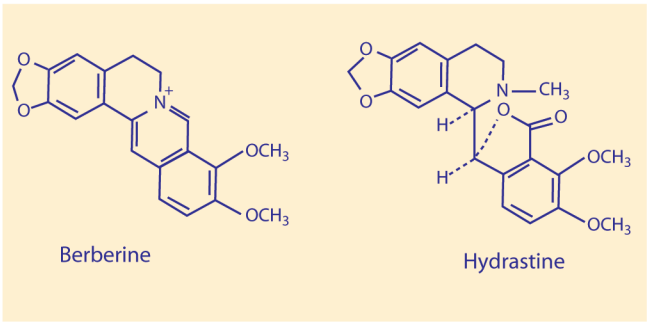

Figure 7.

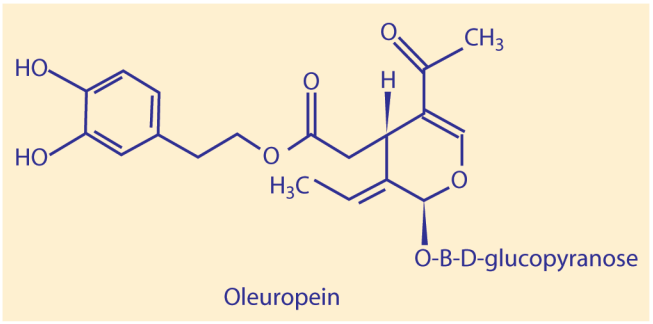

Figure 8.

Cinnamon, 9; For Rosemary, 6; for Lavender, 5 and for Lemon, 4. The antimicrobial activity of Berberine and Hydrastine has been demonstrated[4]. Olive leaf extract contains Oleuropein which is a potent antimicrobial agent[5].

The botanical extracts that make up Suprapein™ were so chosen and combined in adequate proportions not only to give a high level of antimicrobial activity with minimum toxicity but also to offer minimal acceptable aromatic notes when added to the products at the manufacturer's recommended low concentrations.

In this report, the activity of Suprapein™ against selected bacteria, Yeast and filamantous mold will be compared to certain commonly used synthetic preservatives and discussed.

Materials and Methods

The Agar Dilution susceptibility method as described by Mitscher[6] was used for the bacteria and yeast, while the Macrodilution Broth Susceptibility[7] method was used for the filamentous mold. The organisms used included the bacteria *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *S. typhimurium* ATCC 14028, *K. pneumoniae* ATCC 10031, *P. aeruginosa* ATCC 27853, *M. smegmatis* ATCC 14468, the yeast *C. albicans* ATCC 10231, and the filamentous mold *Aspergillus niger* ATCC 16404. The organisms were maintained on Tryptic Soy Agar (TSA) slants except for the mold, which was sustained on a Sabouraud Dextrose Agar (SDA) slant. For

Table I.

MICROORGANISM DILUTIONS	
S. aureus ATCC 29213	100µl susp/10ml saline
E. coli ATCC 25922	100µl susp/10ml saline
S. typhimurium ATCC 14028	100µl susp/10ml saline
K. pneumoniae ATCC 10031	100µl susp/10ml saline
P. aeruginosa ATCC 27853	1µl susp/10ml saline
M. smegmatis ATCC 14468	Undiluted
C. albicans ATCC 10231	1ml susp/10ml saline
A. niger ATCC 16404	1ml susp/10ml 0.1% Tween 80 in saline

each week, the organisms were cultured in 10ml of Tryptic Soy Broth (TSB). After an incubation period (17hrs at 37°C for *S. aureus, E. coli, S. typhimurium, K. pneumoniae, P. aeruginosa*; 48hrs at 37°C for *M. smegmatis*, and 7 days at 22°C for *Aspergillus niger*) the organism suspensions were diluted with 10ml of sterile saline (see Table I). The mold was diluted in a 0.1% Tween 80 solution in saline. The diluted organisms were then either inoculated onto the prepared sample plates with a 1µl loop (bacteria and yeast), or added to the prepared sample tubes with a 100µl pipette (mold).

Preparation of Samples

The PE, PEA and MP were initially screened at their commonly recommended effective concentrations {0.3% v/v (3µl/ml); 1% v/v (10µl/ml); and 0.18% w/v (1mg methyl and 0.8mg Propyl Paraben/ml) respectively} and Suprapein™ at an original concentration of 0.25% v/v (2.5 µl/ml)

For Bacteria and Yeast

1. Prepare 10ml tubes of TSA and allow to cool to 50°C
2. Add the calculated amount of sample to 100µl dimethylsulfoxide (DMSO) to achieve the specified concentration per ml TSA when 100µl of the DMSO solution are added to a test tube containing 10ml of TSA.
3. Vortex to homogenize the mixture in TSA
4. Pour "TSA+Sample" into a properly labeled Petri dish
5. Allow to cool overnight at room temperature.

For Filamentous Mold

1. Prepare 10ml tubes of Tryptic Soy Broth (TSB).
2. Add the calculated amount of sample to 100µl dimethylsulfoxide (DMSO) to achieve the specified concentration per ml TSB then 100µl of the DMSO solution is added to a test tube containing 10ml of TSB.
3. Vortex to homogenize the mixture in TSB.
4. Add 100µl of *A. niger* suspension to a tube of TSB not inoculated with any sample (positive control).

The prepared sample plates were divided into seven sections and labelled accordingly. The diluted organism suspensions were inoculated onto their appropriate section with a 1µl loop, streaking from the center to the outer edge. The plates were then incubated at 37°C for 48 hours, recording the results at 24 and 48 hours.

Alternatively, the prepared sample tubes were inoculated with 100µl of the mold suspension, making the final concentration of the mold spores in each sample 1 x 10^4 – 1 x 10^5 spores/ml. The prepared tubes of TSB were incubated at 37°C for 5 days, recording the results at 3 and 5 days.

Recording the Results:

For Bacteria and Yeast: The results were scored in relation to the growth present on the positive control plate. Growth (G) was noted when there was full growth visible and the organism was not affected. Partial activity (P) was recorded when the organism was morphologically altered or growth was partially inhibited, and no growth (I) was recorded when there was total inhibition. When a result of (I) was scored, the MIC was established by performing the appropriate dilutions (Table II). The MIC (Table III) obtained was confirmed by 3 consecutive results.

For Filamentous Mold: The results were scored in relation to the growth present in the positive control tube. Growth (G) was noted when there was full growth visible (i.e., the tube appeared as cloudy as the positive control tube). Partial activity (P) was recorded when the sample tube of TSB was less turbid than the control tube, and no

Table II. **Antimicrobial screening results**

Suprapein™

Microorganisms	Sample (μl/ml)									
	0.25	0.50	0.75	1.0	2.0	3.0	3.5	4.0	4.5	5.0
S. aureus (gram positive)	G	G	G	P	I	I	I	I	I	I
E. coli (gram negative)	G	G	G	P	I	I	I	I	I	I
S. typhimurium (gram negative)	G	G	G	G	I	I	I	I	I	I
K. pneumoniae (gram negative)	G	G	G	I	I	I	I	I	I	I
P. aeruginosa (gram negative)	G	G	G	G	G	P	P	P	I	I
M. smegmatis (acid-fast)	P	I	I	I	I	I	I	I	I	I
C. albicans (yeast)	P	I	I	I	I	I	I	I	I	I
A. niger (mold)	P	I	I	I	I	I	I	I	I	I

Phenoxyethanol (PE)

Microorganisms	Sample (μl/ml)				
	1.0	2.5	3.0	5.0	10.0
S. aureus (gram positive)	G	G	G	G	I
E. coli (gram negative)	G	P	P	I	I
S. typhimurium (gram negative)	G	P	P	I	I
K. pneumoniae (gram negative)	G	P	P	I	I
P. aeruginosa (gram negative)	P	P	I	I	I
M. smegmatis (acid-fast)	P	I	I	I	I
C. albicans (yeast)	P	I	I	I	I
A. niger (mold)	P	I	I	I	I

Phenyl Ethyl Alcohol (PEA)

Microorganisms	Sample (μl/ml)				
	0.75	1.5	3.0	5.0	6.0
S. aureus (gram positive)	G	G	G	P	I
E. coli (gram negative)	G	P	I	I	I
S. typhimurium (gram negative)	G	P	I	I	I
K. pneumoniae (gram negative)	G	I	I	I	I
P. aeruginosa (gram negative)	P	P	P	I	I
M. smegmatis (acid-fast)	G	P	I	I	I
C. albicans (yeast)	P	P	I	I	I
A. niger (mold)	G	P	I	I	I

Table II. (continued)

Methylparaben and Propylparaben (MP)								
Microorganisms	**Sample (µl/ml)**							
	0.9	**1.8**	**3.6**	**5.4**	**7.2**	**10.8**	**16.2**	**21.6**
S. aureus (gram positive)	G	G	G	P	P	I	I	I
E. coli (gram negative)	G	G	G	G	P	P	I	I
S. typhimurium (gram negative)	G	G	G	G	P	P	P	I
K. pneumoniae (gram negative)	G	G	G	G	P	I	I	I
P. aeruginosa (gram negative)	G	G	G	P	P	P	P	I
M. smegmatis (acid-fast)	G	G	G	I	I	I	I	I
C. albicans (yeast)	G	G	G	I	I	I	I	I
A. niger (mold)	G	P	I	I	I	I	I	I

Abbreviations: G= growth, P= partial inhibition, I= inhibition (no growth). Results are scored in relation to the growth present on the positive control plate.

Table III.

Minimum Inhibitory Concentration (MIC) (sample/ml agar or Broth)				
	Suprapein™ (µl/ml)	**Phenoxyethanol** (µl/ml)	**Phenyl Ethyl Alcohol** (µl/ml)	**Methylparaben & Propylparaben** (mg/ml)
S. aureus ATCC 25213	2.0	10.0	6.0	10.8
E. coli ATCC 25922	2.0	5.0	3.0	16.2
S. typhimurium ATCC 14028	2.0	5.0	3.0	21.6
K. pneumoniae ATCC 10031	1.0	5.0	1.5	10.8
P. aeruginosa ATCC 27853	4.5	5.0	5.0	21.6
M. smegmatis ATCC 14468	0.5	2.5	3.0	5.4
C. albicans ATCC 10231	0.5	2.5	3.0	5.4
A. niger ATCC 16404	0.5	2.5	3.0	3.6
MIC to Inhibit All Organisms	**0.45%**	**1.0%**	**0.6%**	**2.16%**

growth (I) was recorded when there was total inhibition and broth in the tube appear clear. When a result of (I) was scored (Table II), the MIC was established by performing the appropriate dilutions. The MIC (Table III) obtained was confirmed by 3 consecutive results.

DMSO has been used to solubilize the test samples and help to diffuse the Lipophilic ingredients into the media. DMSO was used at a concentration not exceeding 1%. It was reported[8] that the used microorganisms may only be affected at concentrations higher than 5%.

Results and Discussion

Suprapein™ proved to be a well balanced synergistic combination of Botanical fractions fully complying with the restrictions of the 7th Amendment, Annex III and possessing potent antimicrobial activity. The MIC of *A. niger, C. Albicans, M. smegmatis*, and *K. pneumoniae* did not exceed 0.05% and was as low as 0.2% for *S. aureus, E. coli* and *S. typhimurium* and only 0.45% for the comparatively resistant *P. aeruginosa*.

Since *S. aureus* is frequently part of the normal human flora, it can become an opportunistic pathogen causing infections ranging from food poisoning to skin infections to toxic shock syndrome (TSS). Suprapein™ was able to inhibit *S. aureus* at a MIC of 0.20% whereas, all other preservatives tested required much higher concentrations, 1.0% for PE, 0.6% for PEA and 1.08% for MP.

The three coliform bacteria, *E. coli, S. typhimurium*, and *K. pneumoniae* are gram negative rods that cause gastroenteritis and a variety of infections throughout

the body. Suprapein™ was able to inhibit all three at a relatively low concentrations viz. Only 0.05% for *K. pneumoniae*, 0.20% for *E. coli* and *S. typhimurium*, while other preservatives needed concentrations ranging from 1.5 to 10 times higher.

M. smegmatis is an acid-fast bacterium similar to *M. tuberculosis*, an intracellular parasitic bacterium which is always associated with infection and is highly communicable. Suprapein™ was able to inhibit this acid-fast bacterium at a concentration of 0.05% while other preservatives needed concentrations reaching 5 to 10 times higher.

C. albicans is the species of yeast most often isolated from clinical specimens and can cause infection of the skin, nails and mucous membranes. It is also a causative source of diaper rash and certain vaginal and gastrointestinal infections. Suprapein™ was able to inhibit the yeast at a concentration of 0.05% while PE, PEA and MP could inhibit it at concentrations reaching 5 to 10 times higher.

P. aeruginosa is a gram-negative rod that may cause infection whenever moisture is present and can accumulate in wounds, burns and catheters. It is also resistant to many antibiotics; the MIC for Suprapein™ is 0.45%, while the MIC for PE, PEA and MP was found to be 0.50, 0.50 and 2.16% respectively.

Aspergillus species produce a variety of mycotoxins as aflatoxins and sterigmatocystin that pose a potential threat to human and animal health causing hepatocellular carcinoma. As representative of this genus, *A. niger* which grows on different food crops and is less toxic has been selected for testing the antimold activity, and cautiously extrapolating the results obtained to other dangerous filamentous molds. For instance, it was found that the MIC for Suprapein™ was 0.05% while it was much higher for PE, PEA and MP being 0.25, 0.30 and 0.36% respectively.

In conclusion, Suprapein™ is a proprietary synergistic combination of botanical extracts, which show the ability to inhibit a variety of organisms including possible pathogenic organisms that may be introduced into products. Suprapein™ can be used at lower concentrations than other commonly used synthetic preservatives. Suprapein™ has demonstrated itself to be an effective broad-spectrum antimicrobial agent. Its composition and use as natural alternative to synthetic preservatives is patent pending.

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