

Neopein® and Improved Biopein® as Natural Preservatives

Frank S. D'Amelio, Sr., Youssef W. Mirhom, and Amy L. Dreyer
Bio-Botanica®, Inc., 75 Commerce Drive, Hauppauge, NY 11788

Abstract:

A Natural Preservative Ingredient "Biopein"⁽¹⁾ has been prepared for the purpose of using it as a preservative for various applications. The formula of "Biopein" has been improved by slightly changing the proportions of the different components when its efficacy was distinctly enhanced. The antimicrobial activity of improved "Biopein" and "Neopein" (Biopein from which the Cinnamon bark fraction was omitted) were tested against an array of microorganisms with different spectral 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/Propylparabens (MP) in ratio 5:4. The Minimum Inhibitory Concentration (MIC) was determined for each agent. It was found that improved "Biopein" has the lowest MIC (0.2%) followed in increasing order by "Neopein" (0.55%), PEA (0.60%), PE (1.00%), and MP (2.16%) according to their capability of inhibiting all the tested organisms. "Biopein" and "Neopein" can therefore, be used as effective natural alternatives to commonly used synthetic ingredients in appropriate formulations for product preservation. Their composition and use are patent pending.

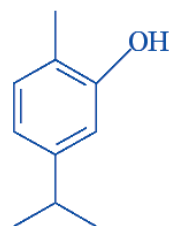
Introduction

Nowadays people are trying to stay away from everything synthetic, including preservatives, as much as possible. This is due to increasing complications arising from the use of synthetic ingredients as

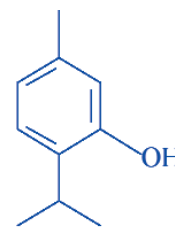
carcinogenicity, teratogenicity, liver, kidney, heart, respiratory or nervous system problems.

An effective natural antimicrobial formula "Biopein"⁽¹⁾ has been developed and proved to be very effective against tested microorganisms at a concentration as low as 0.3%. By slightly modifying the proportions of the ingredients we achieved an even lower concentration of 0.2%. The components of this improved blend are essentially the same as used in the original "Biopein"⁽¹⁾, but the proportions of the different ingredients were modified to optimize the synergistic effect. "Biopein" 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**).
- *Hydrastis canadensis* L. which contains Berberine and Hydrastine alkaloids (**Figure 5**).



Carvacrol



Thymol

Figure 1.

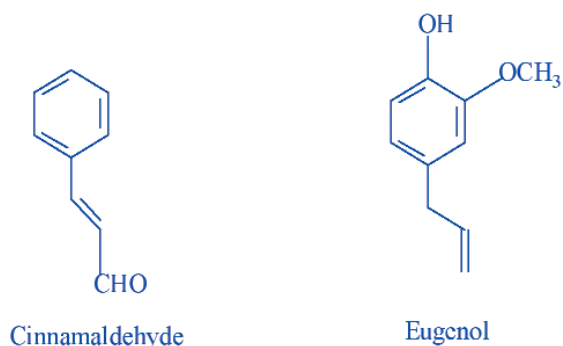


Figure 2.

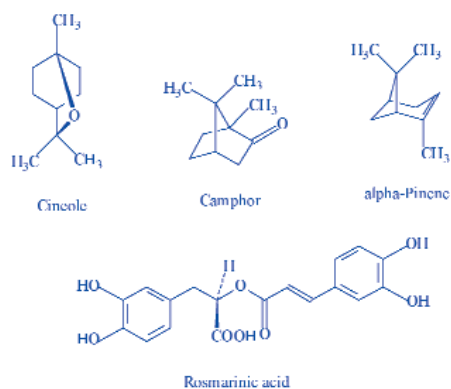


Figure 3.

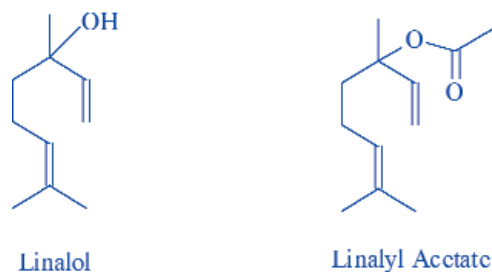


Figure 4.

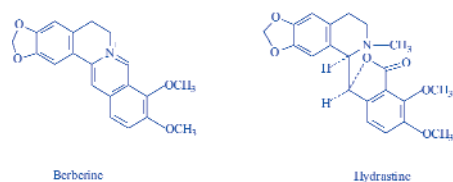


Figure 5.

Martindale⁽²⁾ reported comparatively high phenol coefficients for certain "Biopein" constituents viz. For Thyme, 15; for Cinnamon, 9; for Lavender, 5. The antimicrobial activity of Berberine and Hydrastine has been demonstrated⁽³⁾.

The different ingredients of "Biopein" were so chosen and mixed in adequate proportions not only to give a high level of antiseptic activity with minimum toxicity but also to offer minimal acceptable aroma when added in the low recommended concentrations.

Cinnamon bark fraction, which is present in "Biopein", was found to be specially effective against two resistant microorganisms namely *S. typhimurium* and *P. aeruginosa* with a MIC of 0.075 and 0.15% respectively. For regulatory reasons, some companies try to avoid using Cinnamic Aldehyde containing products. Therefore, we have developed another product "Neopein" not containing the Cinnamon bark fraction. Obviously, this was at the expense of the efficacy of the product. The MIC for *S. typhimurium* and *P. aeruginosa* was found to be 0.35 and 0.55% respectively, even after addition of Olive leaf fraction (*Olea europaea*) which contains Oleuropein which is a potent antimicrobial agent⁽⁴⁾. It is the first secoiridoid compound to be isolated with the following structure (Figure 6).

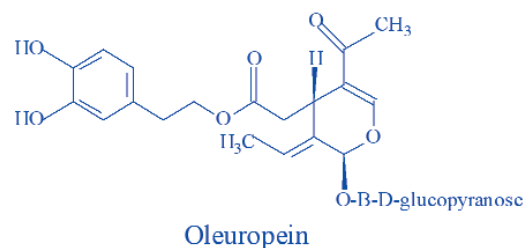


Figure 6.

In this report, the activity of improved "Biopein" and "Neopein" against selected bacteria, yeast and filamentous mold will be compared to certain commonly used synthetic preservatives and discussed.

Materials and Methods:

The Agar Dilution susceptibility method as described by Mitscher⁽⁵⁾ was used for the bacteria and yeast, while the Macrodilution Broth Susceptibility⁽⁶⁾ 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 slant. For each week, the organisms were cultured in 10ml of Tryptic Soy Broth (TSB). After incubation period (17hrs at 37°C for *S. aureus*, *E.*

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 |
| M. smegmatis ATCC 14468 | Undiluted |
| C. albicans ATCC 10231 | 1µl susp/10ml saline |
| P. aeruginosa ATCC 27853 | 1µl susp/10ml saline |
| A. niger ATCC 16404 | 1ml susp/10ml 0.1% Tween 80 in saline |

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 the "Biopein" and "Neopein" at an initial concentration of 0.15% v/v (1.5µl/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 labeled 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 $1 \times 10^4 - 1 \times 10^5$ 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, III). The MIC (Table IV) 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 growth (I) was recorded when there was total inhibition and broth in the tube appear clear. When a result of (I) was scored, the MIC was established by performing the appropriate dilutions (Table II, III). The MIC (Table IV) 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⁽⁷⁾ that the used microorganisms may only be affected at concentrations higher than 5%. Figure 7. shows the effect of MIC of "Biopein" and "Neopein" on the plate cultured microorganisms while Figure 8. Shows their effect on A. niger in the broth culture.

Table II: Antimicrobial Screening Results

| SAMPLE/ml Agar of Broth | BIOPEIN® | | | | | | | |
|-------------------------|-----------------|---|---|---|---|---|---|---|
| | MICROORGANISMS* | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 0.25 µl/ml | G | G | G | G | P | P | G | G |
| 0.5 µl/ml | G | G | G | P | I | I | G | P |
| 0.75 µl/ml | P | G | G | P | I | I | G | I |
| 1.0 µl/ml | I | I | I | I | I | I | P | I |
| 1.5 µl/ml | I | I | I | I | I | I | P | I |
| 2.0 µl/ml | I | I | I | I | I | I | I | I |

| SAMPLE/ml Agar of Broth | NEOPEIN® | | | | | | | |
|-------------------------|-----------------|---|---|---|---|---|---|---|
| | MICROORGANISMS* | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 0.5 µl/ml | G | G | G | G | G | G | G | G |
| 0.75 µl/ml | G | G | G | G | P | G | G | P |
| 1.0 µl/ml | G | G | G | I | I | I | G | I |
| 1.5 µl/ml | P | G | G | I | I | I | G | I |
| 2.0 µl/ml | I | I | P | I | I | I | G | I |
| 3.0 µl/ml | I | I | P | I | I | I | P | I |
| 3.5 µl/ml | I | I | I | I | I | I | P | I |
| 4.0 µl/ml | I | I | I | I | I | I | P | I |
| 4.5 µl/ml | I | I | I | I | I | I | P | I |
| 5.0 µl/ml | I | I | I | I | I | I | P | I |
| 5.5 µl/ml | I | I | 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 negative control plate.

*Microorganisms: (1) *Staphylococcus aureus*
 (2) *Escherichia coli* (3) *Salmonella typhimurium*
 (4) *Klebsiella pneumoniae* (5) *Mycobacterium smegmatis*
 (6) *Candida albicans*
 (7) *Pseudomonas aeruginosa* (8) *Aspergillus niger*

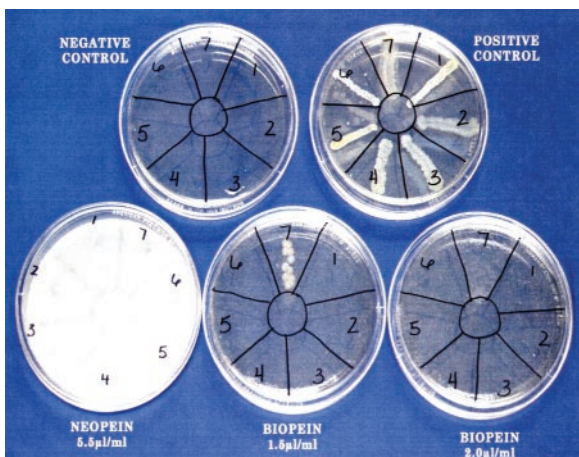


Figure 7: All tested microorganisms are inhibited by 0.2% Biopein or 0.55% Neopein (note that the plate has been reversed to show complete inhibition)

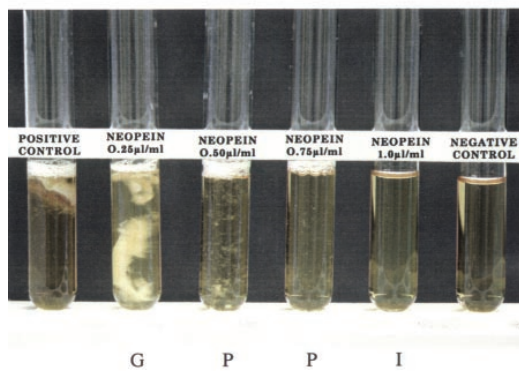
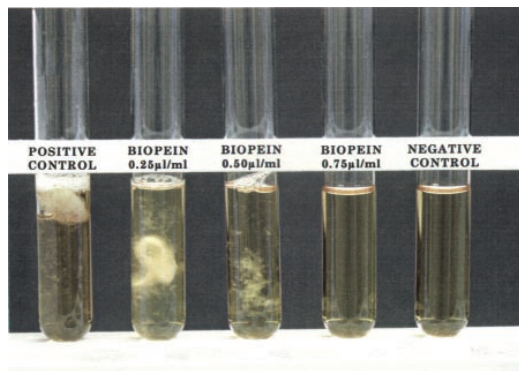


Figure 8: Anti-mold Activity of Different Concentrations of Biopein or Neopein.

Table III: Antimicrobial Screening Results

| PHENOXYETHANOL (PE) [®] | | | | | | | | |
|----------------------------------|-----------------|---|---|---|---|---|---|---|
| SAMPLE/ml Agar of Broth | MICROORGANISMS* | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1.0 µl/ml | G | G | G | G | P | P | P | P |
| 2.5 µl/ml | G | P | P | P | I | I | P | I |
| 3.0 µl/ml | G | P | P | P | I | I | I | I |
| 5.0 µl/ml | G | I | I | I | I | I | I | I |
| 10.0 µl/ml | I | I | I | I | I | I | I | I |

| PHENYL ETHYL ALCOHOL (PEA) [®] | | | | | | | | |
|---|-----------------|---|---|---|---|---|---|---|
| SAMPLE/ml Agar of Broth | MICROORGANISMS* | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 0.75 µl/ml | G | G | G | G | G | P | P | G |
| 1.5 µl/ml | G | P | P | I | P | P | P | P |
| 3.0 µl/ml | G | I | I | I | I | I | P | I |
| 5.0 µl/ml | P | I | I | I | I | I | I | I |
| 6.0 µl/ml | I | I | I | I | I | I | I | I |

| METHYLPARABEN AND PROPYLPARABEN [®] | | | | | | | | |
|--|-----------------|---|---|---|---|---|---|---|
| SAMPLE/ml Agar of Broth | MICROORGANISMS* | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 0.9 mg/ml | G | G | G | G | G | G | G | G |
| 1.8 mg/ml | G | G | G | G | G | G | G | P |
| 3.6 mg/ml | G | G | G | G | G | G | G | I |
| 5.4 mg/ml | P | G | G | G | I | I | P | I |
| 7.2 mg/ml | P | P | P | P | I | I | P | I |
| 10.8 mg/ml | I | P | P | I | I | I | P | I |
| 16.2 mg/ml | I | I | P | I | I | I | P | I |
| 21.6 mg/ml | I | I | 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 negative control plate.

*Microorganisms: (1) *Staphylococcus aureus* (2) *Escherichia coli* (3) *Salmonella typhimurium* (4) *Klebsiella pneumoniae* (5) *Mycobacterium smegmatis* (6) *Candida albicans* (7) *Pseudomonas aeruginosa* (8) *Aspergillus niger*

Results and Discussion:

The MIC for "Biopein", improved by simply changing the proportions of the different ingredients, is compared to the commonly used preservatives tested on each individual microorganism and presented in **Table IV**. "Biopein" showed the lowest MIC of 0.2%, (was 0.3% before improvement) capable of inhibiting all the tested organisms (i.e., gram positive, gram negative, acid-fast bacteria, yeast and *A. niger*). Only 0.075% was required to inhibit the mold and 0.1% to inhibit all microorganisms except *P. aeruginosa* while 0.2% was a sufficient concentration to inhibit all the tested microorganisms. "Neopein" on the other hand, was not as potent as Biopein because omitting Cinnamon bark fraction made it less effective against *S. typhimurium* (MIC, 0.35%) and *P. aeruginosa*

(MIC, 0.55%). The activity against *A. niger* (8) remained comparatively low with MIC of 0.10%. It is to be noted that no preservative comes close to Neopein and Biopein with a MIC of 0.10 and 0.075% respectively against *A. niger* as compared to 0.25, 0.30 and 0.36% for PE, PEA and MP respectively.

Since *S. aureus* (1) 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). Biopein was able to inhibit *S. aureus* at a MIC of 0.10% and Neopein at 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* (2), *S. typhimurium* (3), and *K. pneumoniae* (4) are gram

Table IV : Minimum Inhibitory Concentration (MIC)

| | (Sample/ml agar or Broth) | | | | |
|------------------------------|---------------------------|---------------------|---------------------------|------------------------------------|---|
| | Biopein® (µl/ml) | Neopein® (µl/ml) | Phenoxyethanol (µl/ml) | Phenyl Ethyl Alcohol (µl/ml) | Methylparaben & Propylparaben (mg/ml) |
| S. aureus ATCC 25213 | 1.0 | 2.0 | 10.0 | 6.0 | 10.8 |
| E. coli ATCC 25922 | 1.0 | 2.0 | 5.0 | 3.0 | 16.2 |
| S. typhimurium ATCC 14028 | 1.0 | 3.5 | 5.0 | 3.0 | 21.6 |
| K. pneumoniae ATCC 10031 | 1.0 | 1.0 | 5.0 | 1.5 | 10.8 |
| M. smegmatis ATCC 14468 | 0.5 | 1.0 | 2.5 | 3.0 | 5.4 |
| C. albicans ATCC 10231 | 0.5 | 1.0 | 2.5 | 3.0 | 5.4 |
| P. aeruginosa ATCC 27853 | 2.0 | 5.5 | 5.0 | 5.0 | 21.6 |
| A. niger ATCC16404 | 0.75 | 1.0 | 2.5 | 3.0 | 3.6 |

negative rods that cause gastroenteritis and a variety of infections throughout the body. "Biopein" and "Neopein" were able to inhibit all three at relatively low concentrations viz. 0.10% for *K. pneumoniae*, 0.10 and 0.20% respectively for *E. coli*, 0.10 and 0.35% respectively for *S. typhimurium*.

M. smegmatis (5) is an acid-fast bacterium similar to *M. tuberculosis*, which is an intracellular parasitic bacterium that is always associated with infection and is highly communicable. "Biopein" and "Neopein" were able to inhibit this acid-fast bacterium at concentrations of 0.05 and 0.10% respectively while other preservatives needed concentrations reaching 10 times higher compared to "Biopein" and 5 times higher compared to "Neopein".

C. albicans (6) 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. "Biopein" and "Neopein" were able to inhibit the yeast at concentrations of 0.05 and 0.10% respectively while PE, PEA and MP could inhibit it at concentrations

reaching 10 or 5 times higher respectively.

P. aeruginosa (7) is a gram-negative rod that may cause infection whenever moisture can accumulate including wounds, burns and catheters. It is also resistant to many antibiotics; the MIC for "Biopein" is quite low only 0.20% while for "Neopein" it is 0.55%. This also demonstrates how much the Cinnamon bark fraction is important against this resistant organism and its omission decreases the effectiveness of the product about 3 folds. 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 "Biopein" and "Neopein" was 0.075 and 0.10% respectively while it was much higher for PE, PEA and MP being 0.25, 0.30 and 0.36% respectively.

In conclusion, "Biopein" and "Neopein" are proprietary blends, which showed the ability to inhibit a variety of organisms including possible pathogenic organisms that may be introduced into the products, at a lower concentration than certain commonly used synthetic preservatives. "Biopein" and "Neopein" have demonstrated themselves to be effective broad-spectrum antimicrobial agents. The choice of one or the other depends on the regulations applied. Their composition and use as natural alternatives for products preservation are in a patenting process.

Literature Cited:

1. F.S. D'Amelio, Sr., Y.W. Mirhom and A.L. Dreyer, *Cosmetics and Toiletries Manufacture Worldwide*, pp 17-21, Aston Publishing group, UK (2003).
2. W.H. Martindale, *Perfum. Essent. Oils Rec.* 1, 266 (1910).
3. A.Y. Leung and S. Foster, *Encyclopedia of Common Natural Engredients*, 2nd ed. p. 282, Publisher John Willey & Sons, Inc. NY (1996).
4. M. Walker, *Health Foods Business*, 43(8), 37 (1997).
5. L.A. Mitscher, *J. Nat Prod.*, 35, 157(1972).
6. S. Allen, W. Janda and E. Koneman, *Diagnostic Microbiology*, 2nd ed. pp 628-630, Lippincot Co. Philadelphia (1992).
7. J.R. Zgoda and J.R. Porter, *Pharmaceutical Biology*, 39(3), 221 (2001).

Acknowledgement:

The authors are indebted to Josephine Perricone, Frank D'Amelio, Jr., and Dean D'Amelio for their keen

interest in this work and their generous support, to William Wilson for putting the facilities of his laboratory at their disposal, to Wen W. Zhang for Technical Assistance and to Dr. Muhammad M. Qureshi for editing the manuscript.

About The Authors:

Frank D'Amelio, Sr. has over 35 years of experience in the botanical industry. He is the founder and CEO of Bio-Botanica®, and is an associate referee on botanical drugs for the association of Analytical Chemists. He is the author of a number of published articles and a book: "Botanicals: A Phytocosmetic Desk Reference". Member of IFT, AOAC and ACS.

Dr. Youssef Wissa Mirhom is the Vice President of Research and Development at Bio-Botanica, Inc. and C. Sc. O. He is also Emeritus Professor of Pharmacognosy and Medicinal Plants. He has supervised a considerable number of scientific projects including 9 M. Sc. and Ph. D. degrees. He has 68 original scientific publications and 2 books on medicinal plants. He has lectured at more than 48 national and international conferences and has served on international committees including the Expert Committee of the World Health Organization on Traditional Medicine and Primary Health Care (East Mediterranean Region).

Amy L. Dreyer is the Microbiology laboratory director at Bio-Botanica, Inc. with nine years previous experience in medical microbiology. She is certified by the American Society of Clinical Pathologists (ASCP).