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The Effectiveness of Processed Grapefruit-Seed Extract as An Antibacterial Agent: I. An *In Vitro* Agar Assay

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ABSTRACT

Objectives: Grapefruit-seed extract (GSE®) Citricidal® has, in recent reports, been reported to be successful in combating a variety of common infectious agents. In our study, drops of concentrated grapefruit-seed extract were tested for antibacterial properties against a number of gram-positive and gram-negative organisms.

Design: Sixty-seven (67) distinct biotypes were tested for their susceptibilities to the GSE as well as to 5 other topical antibacterials (Silvadene,® Sulfamylon,® Bactroban,® Nitrofurazone,® and Silvadene,® Nystatin). Wells were punched into Mueller-Hinton agar plates, which were then inoculated with the organism to be tested; each well was then inoculated with one of the antibacterial agents. After an overnight incubation period, the plates were checked for zones of bacterial susceptibility around the individual wells, with a measured susceptibility zone diameter of 10 mm or more considered a positive result.

Results: The GSE was consistently antibacterial against all of the biotypes tested, with susceptibility zone diameters equal to or greater than 15 mm in each case.

Conclusions: Our preliminary data thus suggest an antibacterial characteristic to GSE that is comparable to that of proven topical antibacterials. Although the GSE appeared to have a somewhat greater inhibitory effect on gram-positive organisms than on gram-negative organisms, its comparative effectiveness against a wide range of bacterial biotypes is significant.

INTRODUCTION

As bacterial species continue to develop resistance to a growing number of antibacterials, the interest in finding substances that can effectively support our beleaguered antibiotic arsenal has grown considerably. In patients with wounds susceptible to massive bacterial colonization, such as those encountered with severe thermal injury, the issue of bacterial resistance is of crucial importance (Lowbury, 1979). Because of the enormous bacterial load present in these patients, effective antibacterials must be potent and wide-ranging in the variety of organisms they will inhibit. In addition, because of the ease with which bacteria develop tolerant strains, an ability that can ultimately lead to drug resistance, one of the mea-

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sures of effectiveness for any new substance claiming to have antibacterial properties must be its effectiveness against a variety of different bacterial biotypes. This is particularly true for antibacterials applied topically to large open wound areas, such as severely burned skin.

Grapefruit-seed extract (GSE®) Citricidal® (BioChem Research, Lakeport, California) is one commercially available substance that has received some attention for possibly having antimicrobial properties. Testimonials from practicing physicians attest to this substance's effectiveness in treating a number of conditions resulting from infection, with its uses as a potent agent against *Candida albicans* receiving particular scrutiny (Gordon J, 1999). Other claims of effectiveness for GSE include successful treatment for dermatologic conditions such as dermatitis, warts, and poison ivy (Nutriteam; Nutribiotic, Lakeport, CA, 1999). GSE is made by first converting grapefruit seeds and pulp into an acidic liquid (Table 1). This liquid is loaded with polyphenolic compounds, including quercitin, helperidin, campherol glycoside, neohelperidin, naringin, apigenin, rutinoside, poncirin, etc. The polyphenols themselves are unstable but are chemically converted into more stable substances that belong to a diverse class of products called quaternary ammonium compounds. Some quaternary compounds, benzethonium chloride and benzalkonium chloride, for example, are used industrially as antimicrobials, but are toxic to animal life. The B vitamin choline is also a quaternary compounds, but is nontoxic and even essential for maintaining healthy neurologic function and fat metabolism (Table 2 and Figs. 1 and 2). Our study was undertaken to provide scientific data to ascertain whether or not GSE would (1) exhibit antibacterial properties at all, (2) effectively eliminate a wide range of organisms and biotypes, and (3) compare favorably or unfavorably with currently demonstrated topical antibacterials.

Table 1. NutriTeam's Method of Processing Grapefruit-Seed Extract, Citricidal®

1. Grapefruit pulp and seeds are dried and ground into a fine powder (by product of expeller-extracted grapefruit juice).
2. The grapefruit powder is dissolved in purified water and distilled to remove the fiber and pectin.
3. The distilled slurry is spray dried at low temperatures forming a concentrated grapefruit bioflavored powder.
4. It is then dissolved in vegetable glycerin and heated.
5. Food grade ammonium chloride (NH_4Cl) and ascorbic acid are added and this mixture is heated under pressure (NH_4Cl remaining in the final product 15% to 19%, Ascorbic acid ($C_6H_8O_6$) remaining 25 mg/q to 30 mg/q).
6. This compound undergoes catalytic conversion using natural catalysts (including hydrochloric acid [HCl] and natural enzymes).[a]
7. The slurry is cooled, filtered, and treated with ultraviolet light.

[a]Note: No HCl residue is present in the final product.

METHODS AND MATERIALS

All bacterial isolates were identified by Dade MicroScan® Inc. (Sacramento, CA) breakpoint panel groups (divided into gram-positive and gram-negative panels) which were incubated in the WalkAway 96 (Dade MicroScan® Inc.) for 24 to 48 hours (MicroScan Pos B.P. & Neg B.P, 1998). The colonies were then transferred from purity plates into turbidity standard (measures turbidities between 0.05 and 0.08) (MicroScan, 1991).

The bacteria were inoculated on 150-mm Mueller Hinton II agar plates, but before the plates were inoculated with the organisms, a sterile 6-mm biopsy punch was used to create seven wells in each plate. Each Mueller-Hinton plate was inoculated with a single identified bacterial isolate, and each well was filled with one of seven topical antimicrobials currently employed in the treatment of burns: (Heggers et al., 1990).

1. 1% Silvadene® cream (silver sulfadiazine, Marion Merrel, Dow, Inc., Kansas City, MO).
2. 1% Sulfamylon® cream (mafenide acetate, Bertex Pharmaceuticals, Sugarland, TX).
3. Sodium hypochlorite (NaOCl) 0.025% (Shriners Burns Hospital Pharmacy, Galveston, TX; Heggers et al., 1991).
4. Bactroban® ointment (2% mupirocin, SmithKline Beecham, Philadelphia, PA).

TABLE 2. ANALYSIS OF GSE® (CITRICIDAL®) AS DETERMINED BY BIO-CHEM RESEARCH LAKEPORT, CALIFORNIA

Test description	*Test results*	*Protocol*
Polyphenolic Compounds (quaternary compounds derived from grapefruit bioflavonoids)	59.3%	HPLC 017
Ascorbic acid	21.7%	AOAC
Glycerol	39.6%	
Heavy metals	11.0 mg/kg	Graphite furance Atomic absorption
Pesticides and PCBs	BDL	Gas chromatograph
Bacteria	Negative	Culture

HPLC, high-performance liquid chromatography; PCBs, polychlorinated biphenyls; BDL, below detectable limits.

5. 0.2% nitrofurazone ointment (Rugby Laboratories Inc., Rockville Center, NY).
6. Silvadene-Nystatin cream (a 50–50 preparation of 1% Silvadene and Nystatin ointment, Shriners Burns Hospital Pharmacy).
7. GSE (a Nutribiotic® product: 67% vegetable glycerine, 33% Citricidal, Lakepoint, CA; Table 2; Figs. 1 and 2).

All plates were incubated at 36°C for 20 to 22 hours, and the diameter of the zone of inhibition around each well was then measured to determine the antibacterial effectiveness of each of the individual agents. Using a metric ruler, measurements were taken of the diameter of the zones of inhibition from the point at which the bacteria began to grow on one side of the well, in a straight line across the center of the well, to the point at which the bacteria began to grow on the other side. Bearing in mind that the reported measurements of nongrowth include the 6 mm of space initially cleared in the agar by the biopsy punch to make the well, an inhibitory zone diameter of 10 mm or more was recognized as a positive result (Heggers et al., 1990).

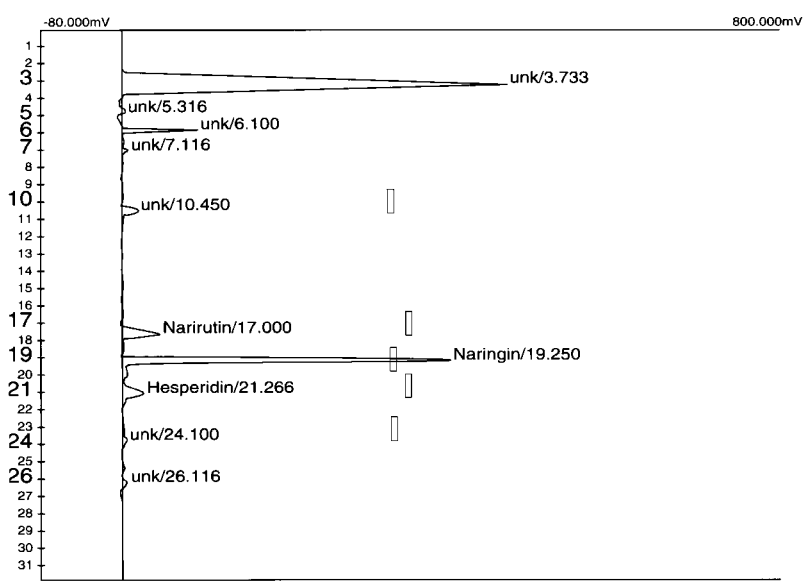

FIG. 1. High-performance liquid chromatography (HPLC) of polyphenols in grapefruit-seed extract (GSE) using column anth01. Retention number 1 at 15.350 is Eriocitrin, 96.96 ppm; 2 at 23.950 is Narirutin, 511.70 ppm; 3 at 27.250 is Naringin, 8830.27 ppm; 4 at 32.216 is Hesperidin, 286.14 ppm; 5 at 0.000 is Neohesperidin, 0.000 ppm (By permission from Bio/Chem Research, Data file pp 28706 chr, Lakeport, CA).

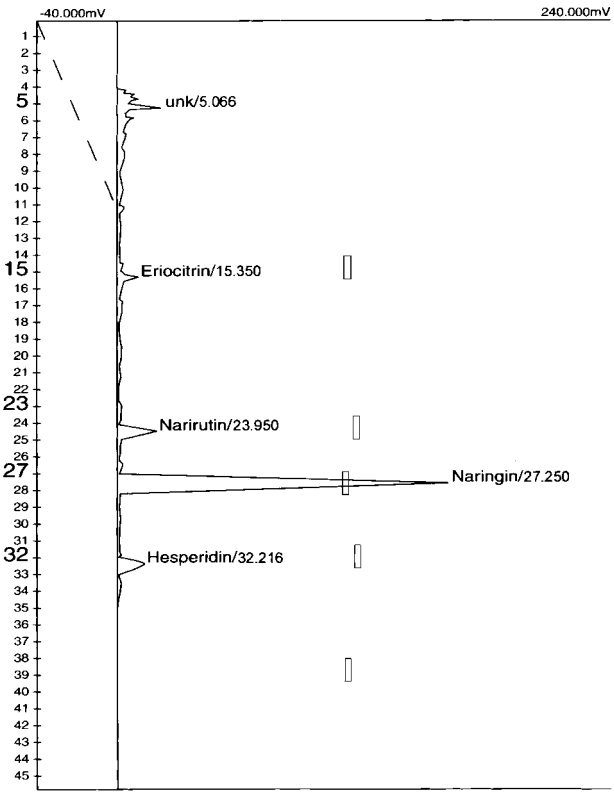

FIG. 2. High-performance liquid chromatography (HPLC) of flavonoids in grapefruit-seed extract (GSE) using column Flan 06. Retention number 1 at 0.000 is Eriocitrin, 0.00 ppm; 2 at 17.000 is Naritrutin 960.27 ppm; 3 at 19.250 is Naringin, 15016.58 ppm; 4 at 21.266 is Hesperidin, 614.48 ppm; 5 at 0.000 is Neohesperidin, 0.00 ppm (By permission from Bio/Chem Research, Data file Flav 28706a, Lakeport, CA).

To ascertain the cause of the zone of precipitation exhibited by the GSE compound, a variety of quaternary compounds (benzethonium and benzalkonium chloride) were introduced into the 6-mm agar wells and incubated overnight at 35°C.

RESULTS/DISCUSSION

The GSE showed a remarkable consistency in its action against a wide range of organisms. Of the 67 biotypes tested, all 67 (100%) were susceptible to the GSE, with no zone of inhibition of less than 15 mm reported. Both gram-positive and gram-negative organisms were effectively managed by the GSE, with the various strains of staphylococci and enterococci (n = 46) averaging the greatest susceptibilities (*Staphylococcus aureus*, 24 mm; *S. epidermidis*, 26 mm; *S. haemolyticus*, 22 mm) (Table 3). Among the gram-negatives (n = 60) notoriously resistant *Pseudomonas aeruginosa* also gave evidence of being susceptible to the GSE, although the measured zones of inhibition from the *Pseudomonas* biotype isolates were among the lowest of any of the tested organisms, averaging 16 mm (Table 4).

Figures 3 and 4 are representative of a gram-positive and a gram-negative organisms response (*S. aureus* and *P. aeruginosa*, respectively).

One curious finding in our study was the presence of a discolored zone around the GSE well in every plate. Given the fact that some of the plates had an additional inhibitory area outside the pale zone while others did not, it might be reasonable to suggest that this ubiquitous discolorization is the result of some kind of precipitating reaction with the combination of constituents in GSE with the agar components, particularly the phenobic compounds. In some cases (*Pseudomonas*, for example), the discolored zone was the only area around the GSE well that showed no bacterial growth (Figs. 3 and 4). In those instances, the determination as to whether it is something in the GSE itself, or rather some byproduct of the GSEs reacting with the chemical substances in the agar; that is affecting the bacteria is less clear; results of 15- or 16-mm zones of inhibition should be interpreted with this reservation in mind.

A possible explanation for the GSEs lethality to bacteria may lie in its relatively low pH (Table 3). We measured the pH of the GSE to be 5.5, and this acidic property could be crucial in establishing an environment prohibitory to bacterial life (and may also play a role in creating the discolored zones mentioned above). The quaternary compounds caused what appeared to be precipitation of magnesium and sodium salts present in the media, a characteristic of phenolic compounds. It is a well-established fact that the quaternary compounds are profound antimicrobial agents often used as compounds of sterilization of sharp instruments (Kolmer, et al, 1951; United States. Navy Dept. Bureau of Medicine and Surgery, 1953).

Under these experimental conditions, the GSE compared favorably to the topical an-

Table 3. Mean Sensitivity Zones of Topical Antibacterials Against 46 Gram-Positive Isolate Zone Sizes (in mm)

Organism / *GSE® Conc.*	*Zone Sizes (in mm)*						
	Number Tested	*Silvadene®*	*Sulfamylon®*	*Bactroban®*	*Nitrofurazone*	*Silvadene/Nystatin*	
Staphylococcus aureus	11	20 ± 0.59*	33 ± 1.1	37 ± 6.3	33 ± 1.0	16 ± 0.62	24 ± 0.35
Staphylococcus epidermidis	7	22 ± 0.46	33 ± 0.69	30 ± 4.5	34 ± 5.0	19 ± 0.61	26 ± 1.1
Staphylococcus haemolyticus	16	20 ± 0.24	31 ± 1.7	30 ± 4.4	37 ± 0.51	16 ± 0.44	22 ± 0.21
Staphylococcus capitis	2	24 ± 0.50	30 ± 2.0	52 ± 3.0	41 ± 4.0	27 ± 1.5	27 ± 4.0
Staphylococcus sciuri	1	20	33	14	38	15	22
Staphylococcus salivarius	1	21	43	45	30	15	29
Enterococcus casseliflavus	1	14	28	40	25	11	26
Enterococcus faecalis	6	15 ± 0.43	29 ± 1.2	27 ± 0.99	15 ± 2.4	10 ± 0.8	24 ± 0.42
Enterococcus faecium	1	17	31	43	15	11	31
Total	46						

Mean value ± standard error of the mean.

Table 4. Mean Sensitivity Zones of Topical Antibacterials Against 60 Gram-Negative Isolates, Zone Sizes (in mm)

Organism / *Conc.*	*Zone Sizes (in mm)*						
	Number Tested	*Silvadene®*	*Sulfamylon®*	*Bactroban®*	*Nitrofurazone*	*Silvadene/Nystatin*	*GSE®*
Pseudomonas aeruginosa	15	18 ± 0.69*	34 ± 1.0	12 ± 0.62	9 ± 0.55	17 ± 0.75	16 ± 0.24
Pseudomonas fluor putida	1	17	32	14	7	17	16
Providencia rettgeri	4	15 ± 0.29	27 ± 0.65	22 ± 0.48	17 ± 0.82	12 ± 0.29	16 ± 0.29
Providencia stuartii	1	15	23	25	18	12	15
Morganella morganii	2	14 ± 0.50	23 ± 2.5	16 ± 1.5	25 ± 0.50	10 ± 0.0	18 ± 1.0
Escherichia coli	3	16 ± 0.0	18 ± 0.67	24 ± 0.33	29 ± 1.2	15 ± 0.88	16 ± 0.0
Klebsiella pneumoniae	1	19	24	23	24	15	16
Ac. baum/haem	14	16 ± 0.40	26 ± 0.72	15 ± 0.36	18 ± 0.78	13 ± 0.32	17 ± 0.0
Enterobacter taylorae	2	13 ± 4.0	23 ± 4.0	23 ± 7.5	23 ± 1.5	11 ± 3.5	18 ± 1.5
Enterobacter cloacae	17	12 ± 0.56	23 ± 0.91	25 ± 1.2	22 ± 1.4	10 ± 0.48	16 ± 0.11
Total	60						

Mean value ± standard error of the mean.

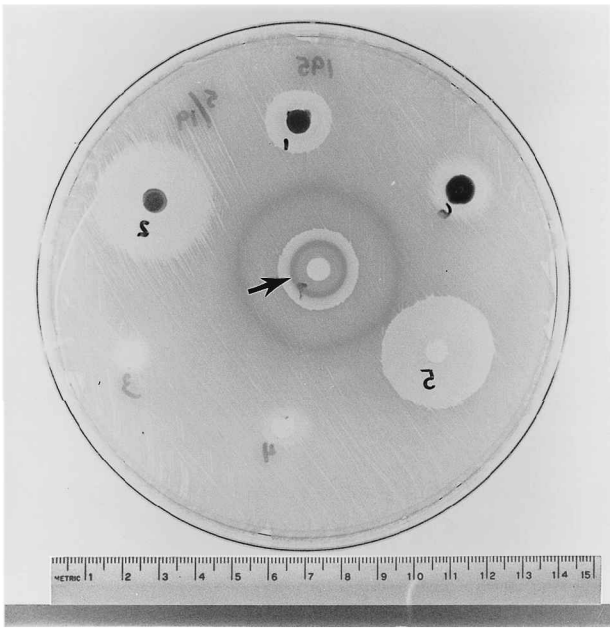

FIG. 3. Representative of *Staphyloccus aureus* susceptibility to the known topical antimicrobials compared to grapefruit-seed extract (GSE) (center well). Zone of precipitation devoid of bacterial growth represented by the arrow. Antimicrobials beginning with top well and going from left to right are (1) Silvadene; (2) Sulfamylon®; (3) NaOCl; (4) Bactroban; (5) Nitrofurazone; (6) Silvadene/Nystatin.

tibacterials. Against *S. aureus*, for example, the GSE created a wider zone of inhibition (24 mm) than did Silvadene (20 mm), and Silvadene-Nystatin (16 mm), but was not as effective as Sulfamylon (33 mm), Bactroban (37 mm), or Nitrofurazone (33 mm) (Table 3). Against *P. aeruginosa*, the GSE zone of inhibition (16 mm) was wider than that of Bactroban (12 mm), and Nitrofurazone (9 mm), and smaller than that of the Silvadene (18 mm), the Sulfamylon (34 mm), and Silvadene-Nystatin (17 mm) (Table 4).

Uniquely the antimicrobial effects of NaOCl (Test well 3) was negligible. This was because of the fact that its potency lasts only 24 hours and we failed to introduce a new solution during each test sequence (data not shown).

What these preliminary results indicate is that GSE has bactericidal effects against a wide range of both gram-positive and gram-negative organisms, and, judging from its comparison against proven topical antibacterials, may hold some promise as a topical antibacterial agent. Whether or not these experimental results have any meaningful medical application awaits further *in vivo* studies, but the scientific data gathered in this study suggest that it is conceivable. Currently studies are underway to determine the *in vitro* effectiveness and potential toxicity of GSE and at what dilution it remains effective and at what concentration does it remain antibacterial and non-toxic (Heggers et al., 1991).

CONCLUSIONS

Our results indicate that GSE has bactericidal effects against a wide range of gram-positive and gram-negative organisms. Under these experimental conditions, the effectiveness of GSE compares favorably to that of proven topical antibacterials. Whether or not these experimental results have any meaningful medical application awaits further *in vivo* studies, but the scientific data gathered in this study suggest that it is conceivable.

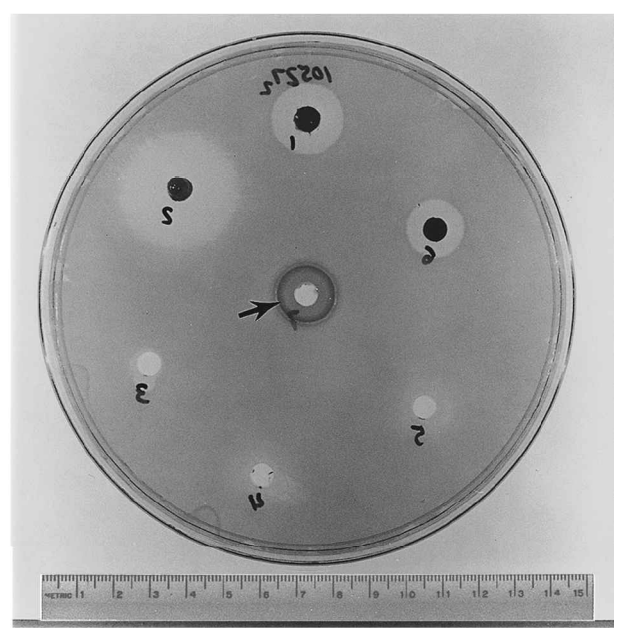

FIG. 4. Representative of *Pseudomonas aeruginosa* susceptibility to the known antimicrobials compared to grapefruit-seed extract (GSE) (center well). Zone of precipitation devoid of bacterial growth represented by the arrow. Antimicrobials beginning with top well and going from left to right are (1) Silvadene; (2) Sulfamylon; (3) NaOCl; (4) Bactroban; (5) Nitrofurazone; (6) Silvadene/Nystatin.

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