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In vitro Antimicrobial Activity Assessment of Zymox Otic Solution Against a Broad Range of Microbial Organisms

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ABSTRACT

The goal of this study was to assess the in vitro antimicrobial activity of Zymox Otic solution against a broad panel of microbial organisms. Serial twofold dilutions of Zymox Otic were used in microbroth dilution assays against 3 Gram-negative bacteria (Escherichia coli, Proteus mirabilis, and Pseudomonas aeruginosa), 4 Gram-positive bacteria (Staphylococcus aureus, Streptococcus intermedius, Streptococcus pyogenes, and Corynebacterium diphtheriae), and 2 fungi (Candida albicans and Malassezia pachydermatis). The results of these assays showed that Zymox Otic inhibited the growth of every organism tested at various test compound dilutions. Zymox Otic had the greatest amount of antimicrobial activity against S. pyogenes exhibiting a minimal inhibitory concentration (MIC) at a 1:32 dilution of the test compound. A 1:16 dilution of Zymox Otic effectively inhibited the growth of S. aureus, E. coli, S. intermedius, C. albicans, P. aeruginosa, C. diphtheriae, and M. pachydermatis; and a 1:8 dilution of Zymox Otic effectively inhibited the growth of P. mirabilis.

Materials and Methods

The materials and reagents were used in the study are shown in Table 1.

The minimal inhibitory concentration (MIC) of Zymox Otic against each microorganism was determined using microbroth dilution analysis. Bacterial and fungal strains were obtained from American Type Culture Collection (ATCC) and cultured according to the manufacturer's specifications. For each organism tested, 10 mL of sterile liquid broth media was inoculated with a freshly plated, single-colony isolate and incubated overnight to establish logarithmic growth. The specific media and growth condition used for the individual organisms is detailed in Table 2. After overnight incubation, the bacteria or fungus was pelleted by centrifugation (3100 x g, 5 min), the supernatant discarded, and the pellet suspended in 0.9% sterile saline solution (9 g/L sodium chloride) to an optical density at 625 nm equal to 0.1. This density was assumed to be equivalent to 1 x 108 colony-forming units (CFU)/mL for dosing purposes. The suspended inoculum was diluted 1:100 in sterile culture media to a concentration of 1 x 106 CFU/mL. One hundred microliters of the diluted inoculum was added to triplicate wells of a 96-well plate containing 100 µL of freshly prepared Zymox Otic solution serially diluted twofold in the respective sterile culture media. One hundred microliters of the diluted inoculum was also added to triplicate wells containing 100 µL of serial twofold dilutions of a positive control antibiotic and to triplicate wells containing 100 µL of media only. This inoculation scheme yielded final concentrations of each microbial organism estimated to be 5 x 105 CFU/mL and Zymox Otic solution ranging from a high-test dilution of 1:2 (inoculum mixed with 100 µL of undiluted Zymox Otic) to a low test of 1:2048. The plates were incubated 16 to 48 hours at 370C, and the inhibition of bacterial growth was determined by measuring the optical density at 625 nm for of each well using a Spectramax 384 plate reader. The MIC for each compound was defined as the lowest compound dilution that completely inhibited growth. The results are presented as the percentage of growth relative to the no-compound control and were calculated using the formula:

$$\frac{100\% \times OD_{625}X}{OD_{625}NC}$$

where $OD_{625}X$ is the average OD_{625} of the triplicate test samples and $OD_{625}NC$ is the average OD_{625} of the triplicate no compound control samples.

Results and Discussion

The results of our analysis indicated that Zymox Otic had antimicrobial activity against all organisms tested (Table 3 and Figs. 1 through 9). Zymox Otic had the greatest amount of antimicrobial activity against Streptococcus pyogenes, exhibiting an MIC at a 1:32 dilution of the original test compound. A 1:16 dilution of Zymox Otic effectively inhibited the growth of Staphylococcus aureus, Escherichia coli, Streptococcus intermedius, Candida albicans, Pseudomonas aeruginosa, Corynebacterium diphtheriae, and Malassezia pachydermatis; and a 1:8 dilution of Zymox Otic effectively inhibited the growth of Proteus mirabilis. All of the control antimicrobial compounds had effective MICs within published ranges for the organisms tested. The Zymox Otic compound formed a gelatinous precipitate at the highest concentrations (1:2 and 1:4 dilution, Figs. 1 through 9 panel C) leading to an increase in the measured optical density at the high-test concentrations relative to the intermediate concentrations. For Malassezia pachydermatis (Fig. 9, panels A and C), one well for each of the test concentration triplicates had a significantly higher OD_{625} than the other 2 replicates. Examination of these wells indicated that the fungus failed to evenly disperse during culturing and formed a mat on the top of the culture media. The presence of this mat inflated the optical density measured for these wells. Because this phenomenon was not observed in the no-compound control wells, the graph of the M. pachydermatis results that includes these data indicates an artificial enhancement of growth by the Zymox Otic compound at the higher concentrations (Fig. 9 panel C).

Table 1. Materials and Reagents Used in the Study

Staphylococcus aureus	ATCC	29213
Escherichia coli	ATCC	25922
Streptococcus pyogenes	ATCC	10389
Proteus mirabilis	ATCC	14153
Streptococcus intermedius	ATCC	27335
Candida albicans	ATCC	10231
Pseudomonas aeruginosa	ATCC	27853
Corynebacterium diphtheriae	ATCC	14779
Malassezia pachydermatis	ATCC	42756
96-well U-bottom plates	Falcon	353227
TSA II 5% sheep blood agar plates	Remel	01202
Disposable bacterial spreaders	VWR	WLS23286
Trypticase soy agar	BBL	211043
YM broth	Difco	271120
YM agar	Difco	271210
Sabouraud dextrose broth	Difco	238220
Nutrient broth	Difco	233000
Brain heart infusion media	Remel	452471
Sabouraud dextrose agar emmons	Remel	454471
Trypticase soy broth	BD	211768
Saline 0.9%	LC234601	Fisher
Mueller Hinton II broth (Cation adjusted)	BD	297310
Ampicillin	Fisher	BP1760-25
Gentamycin	Sigma	G1522
Amphotericin B	Fisher	BP264520
Bifinazole	Fisher	ICN15485601
Zymox Otic solution	Laclede	

Table 2. Growth Media and Culture Conditions Used to Culture and Assess the Antimicrobial Activity of Zymox Otic for Each Respective Organism

Staphylococcus aureus	Trypticase soy broth	37˚C/ambient air/16 hr
Escherichia coli	Trypticase soy broth	37˚C/ambient air/16 hr
Streptococcus pyogenes	Mueller Hinton II broth (Cation adjusted)	37˚C/ambient air/16 hr
Proteus mirabilis	Mueller Hinton II broth (Cation adjusted)	37˚C/ambient air/16 hr
Streptococcus intermedius	Brain heart infusion media	37˚C/5% CO_2/16 hr
Candida albicans	YM broth	37˚C/ambient air/24 hr
Pseudomonas aeruginosa	Mueller Hinton II broth (Cation adjusted)	37˚C/ambient air/16 hr
Corynebacterium diphtheriae	Trypticase soy broth	37˚C/ambient air/24 hr
Malassezia pachydermatis	Sabouraud dextrose broth	37˚C/ambient air/48 hr

Table 3. Minimal Inhibitory Concentration (MIC) of Zymox Otic and Control Compounds

Staphylococcus aureus	Ampicillin (µg/mL)	1
	Zymox (dilution)	1:16
Escherichia coli	Ampicillin (µg/mL)	16
	Zymox (dilution)	1:16
Streptococcus pyogenes	Ampicillin (µg/mL)	0.04
	Zymox (dilution)	1:32
Proteus mirabilis	Ampicillin (µg/mL)	2
	Zymox (dilution)	1:8
Streptococcus intermedius	Ampicillin (µg/mL)	25
	Zymox (dilution)	1:16
Candida albicans	Amphotericin B (µg/mL)	1.56
	Zymox (dilution)	1:16
Pseudomonas aeruginosa	Gentamicin (µg/mL)	0.78
	Zymox (dilution)	1:16
Corynebacterium diphtheriae	Ampicillin (µg/mL)	0.39
	Zymox (dilution)	1:16
Malassezia pachydermatis	Bifonazole (µg/mL)	0.1
	Zymox (dilution)	1:16

The listed MIC for each of the control compounds is given in µg/mL and the MIC for Zymox is listed as a dilution of the original starting material in the respective growth media

Figure 1. Determination of minimal inhibitory concentration of ampicillin (B) and Zymox Otic (C) against Staphylococcus aureus.

Figure 1A

Ampicillin (µg/mL)	256	128	64	32	16	8	4	2	1	0.5	0.25	0
Sample 1	0.043	0.042	0.045	0.049	0.042	0.199	0.583	1.053	1.206	1.235	1.234	1.279

Sample 2	0.043	0.042	0.045	0.046	0.047	0.199	0.648	1.042	1.166	1.196	1.203	1.236
Sample 3	0.042	0.044	0.052	0.067	0.05	0.147	0.606	1.028	1.175	1.193	1.204	1.229
Average	0.043	0.043	0.047	0.054	0.046	0.182	0.612	1.041	1.182	1.208	1.214	1.248
% Control		3.4	3.4	3.8	4.3	3.7	14.6	49	83	95	97	100	100

Zymox (dilution)	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	0
Sample 1	0.271	0.054	0.066	0.057	1.119	1.09	0.985	0.95	0.926	0.939	1.024	1.223
Sample 2	0.108	0.256	0.117	0.062	1.054	1.092	0.995	0.95	0.952	0.942	1.042	1.217
Sample 3	0.177	0.066	0.062	0.061	1.074	1.096	0.996	0.979	0.967	0.983	1.058	1.272
Average	0.185	0.125	0.082	0.060	1.082	1.093	0.992	0.960	0.948	0.955	1.041	1.237
% Control		15.0	10.0	6.6	5.8	87.0	88.0	80.0	78.0	77.0	77.0	84	100

Figure 1C

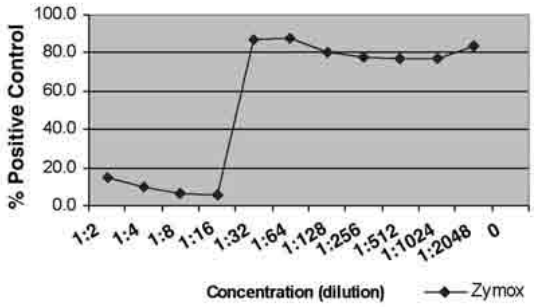


Figure 1B

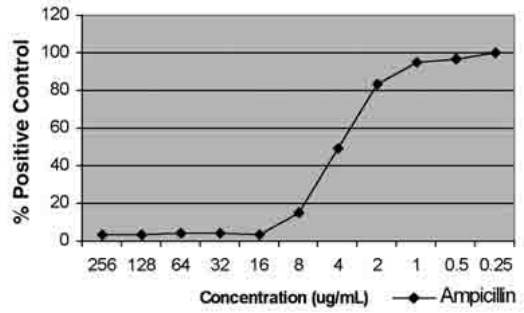


Figure 2A.

Ampicillin (µg/mL)	256	128	64	32	16	8	4	2	1	0.5	0.25	0
Sample 1	0.043	0.042	0.045	0.049	0.042	0.199	0.583	1.053	1.206	1.235	1.234	1.279
Sample 2	0.043	0.042	0.045	0.046	0.047	0.199	0.648	1.042	1.166	1.196	1.203	1.236
Sample 3	0.042	0.044	0.052	0.067	0.05	0.147	0.606	1.028	1.175	1.193	1.204	1.229
Average	0.043	0.043	0.047	0.054	0.046	0.182	0.612	1.041	1.182	1.208	1.214	1.248
% Control		3.4	3.4	3.8	4.3	3.7	14.6	49.1	83.4	94.7	96.8	97.2	100

Zymox (dilution)	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	0
Sample 1	0.271	0.054	0.066	0.057	1.119	1.09	0.985	0.95	0.926	0.939	1.024	1.223
Sample 2	0.108	0.256	0.117	0.062	1.054	1.092	0.995	0.95	0.952	0.942	1.042	1.217

Sample 3	0.177	0.066	0.062	0.061	1.074	1.096	0.996	0.979	0.967	0.983	1.058	1.272
Average	0.185	0.125	0.082	0.060	1.082	1.093	0.992	0.960	0.948	0.955	1.041	1.237
% Control		15.0	10.1	6.6	4.8	87.5	88.3	80.2	77.6	76.6	77.2	84.2	100

Figure 2. Determination of minimal inhibitory concentration of ampicillin (B) and Zymox Otic (C) against Escherichia coli.

Figure 2C

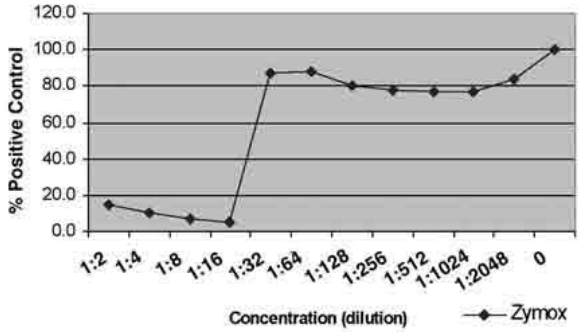


Figure 2B

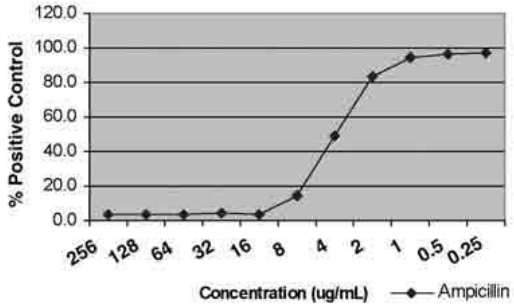


Figure 3. Determination of minimal inhibitory concentration of ampicillin (B) and Zymox Otic (C) against Streptococcus pyogenes.

Figure 3A.

Ampicillin (µg/mL)	5	2.5	1.25	0.625	0.31	0.156	0.078	0.039	0.019	0.009	0.005	0
Sample 1	0.309	0.307	0.306	0.308	0.304	0.307	0.303	0.306	0.699	0.942	1.008	1.054
Sample 2	0.311	0.304	0.306	0.306	0.31	0.307	0.303	0.304	0.819	0.996	1.059	1.097
Sample 3	0.309	0.308	0.306	0.306	0.307	0.305	0.305	0.306	0.81	0.998	1.071	1.119
Average	0.310	0.306	0.306	0.307	0.307	0.306	0.304	0.305	0.776	0.979	1.046	1.090
% Control		28.4	28.1	28.1	28.1	28.2	28.1	27.9	28.0	71.2	89.8	96.0	100.0

Zymox (dilution)	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	0
Sample 1	1.15	1.282	0.387	0.329	0.327	0.99	1.267	1.262	1.233	1.206	1.12	1.124
Sample 2	0.934	1.297	0.404	0.339	0.328	0.869	1.085	1.085	1.112	1.099	1.073	1.098
Sample 3	1.034	0.576	0.365	0.342	0.342	0.916	1.051	1.112	1.077	1.035	1.05	1.053
Average	1.039	1.052	0.385	0.337	0.332	0.925	1.134	1.153	1.141	1.113	1.081	1.092
%Control	95.2	96.3	35.3	30.8	30.4	84.7	103.9	105.6	104.5	102.0	99.0	100.0

Figure 3C

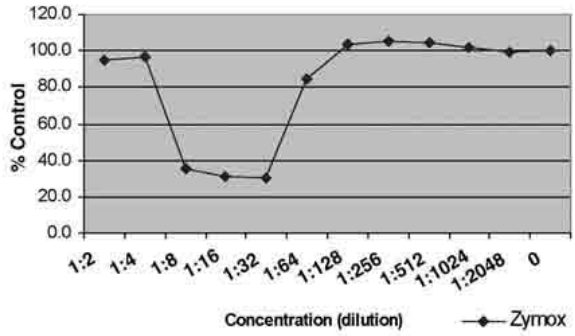


Figure 3B

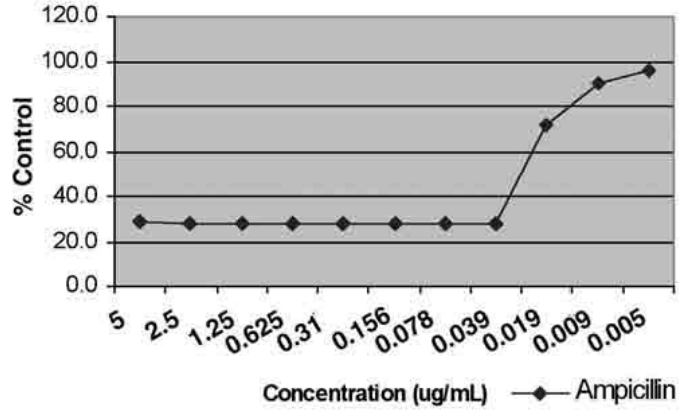


Figure 4. Determination of minimal inhibitory concentration of ampicillin (B) and Zymox Otic (C) against Proteus mirabilis.

Figure 4A.

Ampicillin (µg/mL)	100	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.09	0
Sample 1	0.057	0.046	0.045	0.046	0.049	0.054	0.1	0.958	1.104	1.063	1.041	0.966
Sample 2	0.045	0.043	0.048	0.046	0.048	0.05	0.091	0.935	1.154	1.115	1.075	0.881
Sample 3	0.046	0.043	0.048	0.045	0.045	0.05	0.095	0.931	1.136	1.067	1.052	0.837
Average	0.049	0.044	0.047	0.046	0.047	0.051	0.095	0.941	1.131	1.082	1.056	0.895
%Control	5.5	4.9	5.3	5.1	5.3	5.7	10.7	105.2	126.5	120.9	118.0	100

Zymox (dilution)	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	0
Sample 1	0.265	0.276	0.071	0.331	0.877	1.006	1.026	0.988	0.957	0.931	0.906	0.875
Sample 2	0.081	0.381	0.078	0.279	0.862	0.941	0.964	0.916	0.878	0.845	0.849	0.82
Sample 3	0.102	0.306	0.075	0.44	0.855	1.058	1.005	0.983	0.883	0.91	0.922	0.947
Average	0.149	0.321	0.075	0.350	0.865	1.002	0.998	0.962	0.906	0.895	0.892	0.881
%Control	17.0	36.4	8.5	39.7	98.2	113.7	113.4	109.3	102.9	101.7	101.3	100.0

Figure 4C

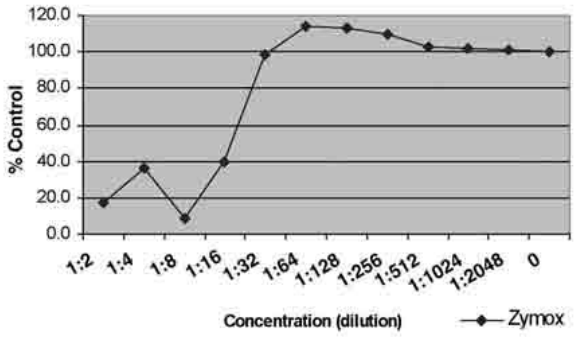


Figure 4B

Figure 5. Determination of minimal inhibitory concentration of ampicillin (B) and Zymox Otic (C) against Streptococcus intermedius.

Figure 5A.

Ampicillin (µg/mL)	100	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.098	0
Sample 1	0.059	0.068	0.13	0.208	1.497	1.62	1.654	1.611	1.554	1.629	1.612	1.633
Sample 2	0.065	0.068	0.071	0.152	1.347	1.595	1.525	1.512	1.508	1.519	1.525	1.531
Sample 3	0.063	0.065	0.069	0.166	1.271	1.508	1.556	1.53	1.521	1.524	1.527	1.615
Average	0.062	0.067	0.090	0.175	1.372	1.574	1.578	1.551	1.528	1.557	1.555	1.593
% Control		3.9	4.2	5.6	11.0	86.1	98.8	99.1	97.4	95.9	97.8	97.6	100

Zymox (dilution)	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	0
Sample 1	0.157	0.197	0.192	0.21	1.52	1.339	1.318	1.324	1.329	1.35	1.434	1.633
Sample 2	0.094	0.249	0.21	0.105	1.48	1.372	1.348	1.358	1.374	1.362	1.426	1.531
Sample 3	0.124	0.157	0.179	0.127	1.559	1.406	1.392	1.411	1.404	1.42	1.515	1.615
Average	0.125	0.201	0.194	0.147	1.520	1.372	1.353	1.364	1.369	1.377	1.458	1.593
% Control		7.8	12.6	12.2	9.2	95.4	86.1	84.9	85.6	85.9	86.5	91.5	100.0

Figure 5C

Figure 5B

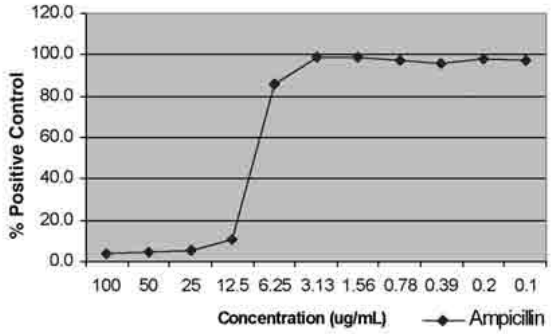


Figure 6. Determination of minimal inhibitory concentration of amphotericin B (B) and Zymox Otic (C) against Candida albicans.

Figure 6A.

Amphotericin B (µg/mL)	100	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.098	0
Sample 1 0.05	0.046	0.044	0.044	0.045	0.047	0.045	0.713	0.925	0.974	1.024	1.107	
Sample 2 0.056	0.061	0.047	0.059	0.052	0.051	0.046	0.855	0.947	0.92	1.013	1.153	
Sample 3 0.062	0.051	0.048	0.046	0.043	0.047	0.044	1.121	0.818	1.201	1.145	1.111	
Average 0.056	0.053	0.046	0.050	0.047	0.048	0.045	0.896	0.897	1.032	1.061	1.124	
%Control 5.0	4.7	4.1	4.4	4.2	4.3	4.0	79.8	79.8	91.8	94.4	100	

Zymox (dilution)	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	0
Sample 1 0.107	0.087	0.071	0.055	1.146	1.33	1.317	1.189	1.142	1.086	1.202	1.107	
Sample 2 0.103	0.087	0.065	0.057	0.942	1.177	1.139	1.141	1.125	1.049	1.003	1.153	
Sample 3 0.091	0.084	0.057	0.056	1.02	1.241	1.138	1.241	1.091	0.971	1.039	1.111	
Average 0.100	0.086	0.064	0.056	1.036	1.249	1.198	1.190	1.119	1.035	1.081	1.124	
%Control 8.9	7.7	5.7	5.0	92.2	111.2	106.6	105.9	99.6	92.1	96.2	100.0	

Figure 6C

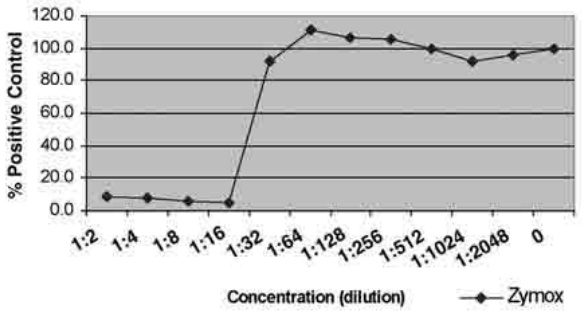


Figure 6B

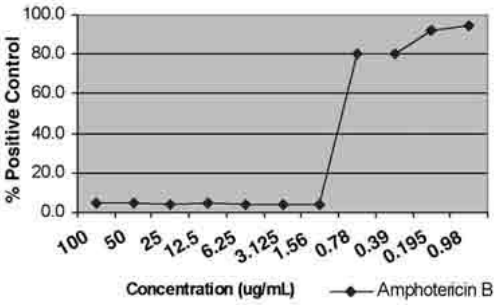


Figure 7. Determination of minimal inhibitory concentration of gentamicin (B) and Zymox Otic (C) against Pseudomonas aeruginosa.

Figure 7A.

Gentamicin (µg/mL) 100	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.098	0
Sample 1 0.042	0.041	0.041	0.041	0.041	0.042	0.042	0.045	0.446	1.268	1.289	1.412
Sample 2 0.044	0.042	0.043	0.041	0.042	0.042	0.042	0.046	0.478	1.182	1.241	1.343
Sample 3 0.048	0.042	0.053	0.042	0.04	0.039	0.039	0.055	0.399	1.136	1.225	1.394

Average	0.045	0.042	0.046	0.041	0.041	0.041	0.041	0.049	0.441	1.195	1.252	1.383
% Control		3.2	3.0	3.3	3.0	3.0	3.0	3.0	3.5	31.9	86.4	90.5	100

Zymox (dilution)		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	0
Sample 1	0.197	0.251	0.068	0.077	1.303	1.146	1.278	1.328	1.279	1.26	1.278	1.412	
Sample 2	0.093	0.278	0.081	0.074	1.194	1.121	1.233	1.259	1.25	1.284	1.264	1.343	
Sample 3	0.094	0.215	0.072	0.071	1.275	1.163	1.299	1.337	1.304	1.282	1.3	1.394	
Average	0.128	0.248	0.074	0.074	1.257	1.143	1.270	1.308	1.278	1.275	1.281	1.383	
% Control		9.3	17.9	5.3	5.4	90.9	82.7	91.8	94.6	92.4	92.2	92.6	100.0

Figure 7C

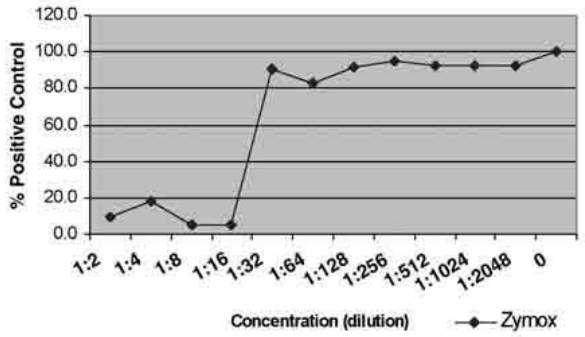


Figure 7B

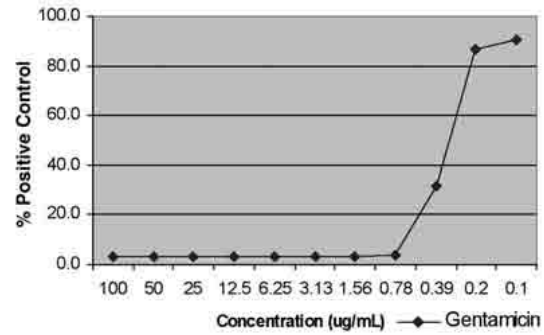


Figure 8. Determination of minimal inhibitory concentration of ampicillin (B) and Zymox Otic (c) against Corynebacterium diphtheriae.

Figure 8A.

Ampicillin (µg/mL)		100	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.098	0
Sample 1	0.05	0.049	0.049	0.048	0.048	0.049	0.049	0.049	0.05	0.157	0.215	0.22	
Sample 2	0.05	0.049	0.049	0.055	0.05	0.048	0.063	0.059	0.049	0.177	0.208	0.226	
Sample 3	0.048	0.047	0.049	0.049	0.049	0.048	0.048	0.048	0.048	0.171	0.208	0.236	
Average	0.049	0.048	0.049	0.051	0.049	0.048	0.053	0.052	0.049	0.168	0.210	0.227	
% Control		21.7	21.3	21.6	22.3	21.6	21.3	23.5	22.9	21.6	74.0	92.5	100

Zymox (dilution)		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	0
Sample 1	0.191	0.206	0.141	0.058	0.154	0.167	0.176	0.188	0.197	0.219	0.236	0.22	

Sample 2	0.101	0.268	0.196	0.061	0.172	0.204	0.216	0.225	0.227	0.232	0.246	0.226
Sample 3	0.12	0.126	0.18	0.064	0.175	0.231	0.225	0.233	0.239	0.25	0.256	0.236
Average	0.137	0.200	0.172	0.061	0.167	0.201	0.206	0.215	0.221	0.234	0.246	0.227
%Control	60.4	88.0	75.8	26.8	73.5	88.3	90.5	94.7	97.2	102.8	108.2	100.0

Figure 8C

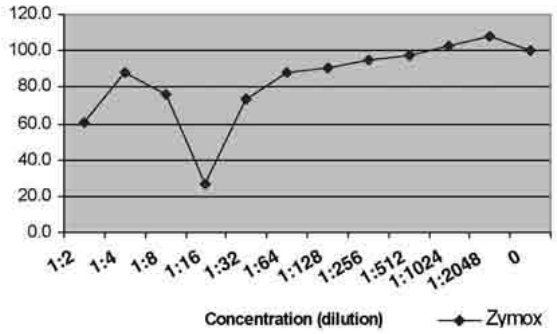


Figure 8B

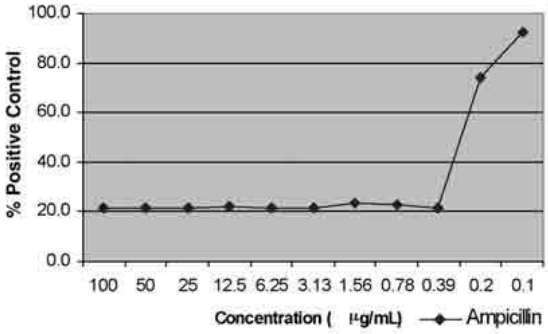


Figure 9. Determination of minimal inhibitory concentration of Bifonazole (B) and Zymox Otic (C) against Malassezia pachydermatis.

Figure 9A.

Bifonazole (mg/mL)	25	12.5	6.25	3.125	1.56	0.781	0.39	0.195	0.0975	0.049	0.024	0
Sample 1	0.056	0.062	0.06	0.074	0.064	0.058	0.059	0.07	0.065	0.061	0.504	0.573
Sample 2	0.058	0.06	0.061	0.083	0.075	0.077	0.076	0.075	0.064	0.233	0.545	0.631
Sample 3	0.051	0.053	0.057	0.061	0.072	0.058	0.078	0.0650.078		0.075	0.064	0.162	0.544
Average	0.055	0.058	0.059	0.073	0.070	0.064	0.071	0.073	0.068	0.119	0.404	0.583
% Control		9.4	10.0	10.2	12.5	12.1	11.0	12.2	12.4	11.7	20.5	69.3	100

Zymox (dilution)	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	0
Sample 1	0.282	0.2	0.159	0.07	0.18	1.743	1.747	0.428	1.954	1.894	1.954	0.573
Sample 2	0.509	0.231	0.149	0.068	0.061	0.584	0.5	0.377	0.435	0.583	0.462	0.631
Sample 3	0.497	0.193	0.177	0.065	0.16	0.416	0.496	1.894	0.5	0.527	0.536	0.544
Average	0.429	0.208	0.162	0.068	0.134	0.914	0.914	0.900	0.963	1.001	0.984	0.583
%Control	73.7	35.7	27.7	11.6	22.9	156.9	156.9	154.4	165.3	171.9	168.9	100.0

Figure 9C

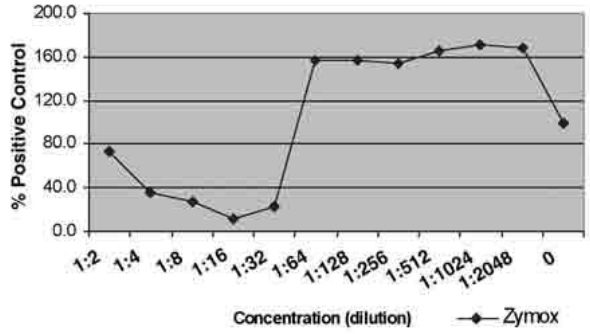
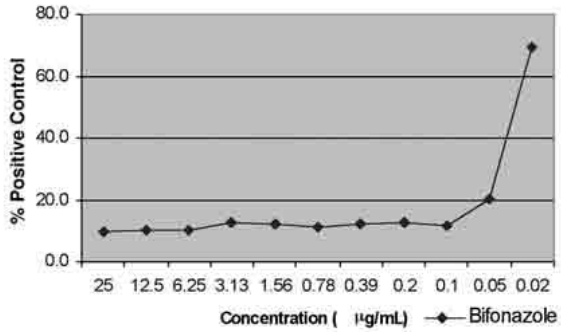


Figure 9B



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