A New Combined Antimicrobial Agent: Development and Application in Shoe Lining Leather

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Abstract: As the variety of microorganism in shoes, a single antimicrobial agent cannot inhibit all their growth. So, the development of a combined antimicrobial agent used in shoe lining leather is necessary. Considering the effective method to develop a combined antimicrobial agent using synergistic effect between different antimicrobial compounds, in this study, three antimicrobial compounds, viz. zinc(Π) complex of 2-amino benzothiazole and para-formyl phenoxyacetic acid (A), butyl-*p*-hydroxybenzonate (B) and triclosan (C), which have different antimicrobial spectrum and modes of action, were chosen as the active components to prepare a new combined antimicrobial agent. Firstly, the types of interaction between them were evaluated by the crossed filter paper strip method. Results show that there are synergistic effects between the three compounds. Then, the diameters of inhibition zones of their different combinations were determined to attain the optimal combination. Results show that, when the proportion of the three compounds is A:B:C＝0.1:0.63:0.27, the combination has the best overall inhibitory effect to moulds, bacteria and yeasts. Finally, as a new combined antimicrobial agent, the optimal combination was applied to the shoe lining leather by spraying, and the inhibitory effects of the treated leather against moulds, yeasts and bacteria were evaluated by the inhibition zone method and the inhibition ratio method. Results show that the inhibition zones with different diameters were formed for different microorganisms, and the diameters are in the range of 20mm to 35mm. When the leather contains 6.00 g kg^{-1} of the antimicrobial agent, the inhibition ratios for all the tested microorganisms are more than 80%, especially for *Penicillium* and *Staphylococcus aureus*, the inhibition ratios achieve 95.67% and 94.40%, respectively. When the content of the antimicrobial agent is increased to 9.30 g kg^{-1}, all the inhibition ratios are more than 90%.

Key words: combined antimicrobial agent; synergism; shoe lining leather; inhibition zone; inhibition ratio

1 Introduction

Microorganisms, such as moulds, yeasts and bacteria, are often observed in leather shoes and cause some skin diseases of feet and undesired odour. For example, in the moist climatic area of China, approximately 70% of adults were affected by athlete's foot in different degree, especially the young students and soldiers [1]. Also, statistical data indicated that 20-50% of population in the Western Europe suffered from foot mycelial (mycotic) diseases which are caused by fungal microorganisms [2]. So, it is necessary to develop an effective antimicrobial agent which can control the microbial growth in shoes. As the shoe lining leather, a kind of important shoe lining material, contacts tightly with the skin of feet, the addition of an antimicrobial agent to it may be helpful to build a clean environment in shoes, control the growth of microorganism and decrease the opportunity to suffer from skin disease of feet.

However, because of the different antimicrobial spectrum and toxicity problem, the normal leather fungicides, for example, 2-(thiocyanomethylthio) benzothiazole (TCMTB) is not suitable to be used in the shoe lining leather. So, it is necessary to develop a new antimicrobial agent used to inhibit the

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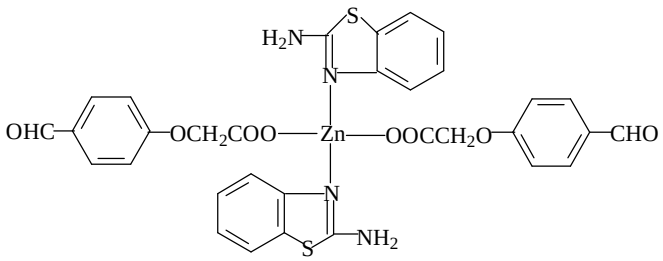
microorganism species in shoes. And because a single antimicrobial compound may not has enough inhibition effect for all the microorganism species in shoes, a hopeful direction is the development of the combined antimicrobial agent. Usually, a combined antimicrobial agent is composed of two or more antimicrobial compounds with varied modes of action in a proper proportion. Compared with a single antimicrobial compound, it has some advantages, for example, a wider antimicrobial spectrum, higher inhibitory effect and no resistance of antimicrobial agent [3].

Mixtures or combinations of two or more antimicrobial compounds may bring synergistic effects, addition, antagonisms or indifference, depending on the characteristics of the substances and microorganisms under evaluation. During the process of development of a combined antimicrobial agent, the key step is the type evaluation of interaction between different antimicrobial compounds. In leather industry, G. M. NÚÑEZ et al in Argentina reported the type of interaction in the combination of TCMTB – OPP (orthophenylphenol) and TCMTB – CMC (parachlorometacresol) using the method of the minimum inhibitory concentration (MIC) [4]. An increase of inhibitory power was observed for both combinations and the tested moulds showed synergistic effects or potentiations. Researchers in Buckman Laboratories International, Inc. developed a unique potentiator (a dispersant with some surface active properties) to enhance leather fungicide performance [5]. When the potentiator was added before the fungicide, the extended preservation period of wet blue or retanned, dyed, and fatliquored stocks could be achieved. The potentiator is a material that has little antimicrobial activity itself. But, it can cause the destabilization of the cell membrane or cell wall of moulds, and the cell becomes more permeable to foreign fungicide molecules. So, more rapid death of mould cells will result and the performance of the fungicide is enhanced. In previous work, we studied the evaluation method of combination of leather fungicides by inhibition zone [3]. Compared with the MIC method, the inhibition zone method is a rapid and simple method, and it is suitable for the combination experiments with a lot of tested antimicrobial compounds and microorganism species. In this study, this method was applied to develop a new combined antimicrobial agent for shoe lining leather, and the tested microorganisms contained not only moulds but also yeasts and bacteria, which were isolated from leather shoes.

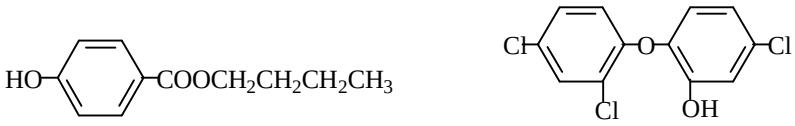
2 Experimental

2.1 Materials

The zinc(Π) complex of 2-amino benzothiazole and para-formyl phenoxyacetic acid (A) was synthesized by this laboratory and its composition percentage is more than 99%. Butyl-*p*-hydroxybenzonate (B) was purchased from Kelong Chemical Agent Plant (Chengdu, China), and triclosan (C), viz. 5-chloro-2-(2, 4-dichlorophenoxy) phenol, was provided by Sichuan Chemical Academy of Natural Gas. The Molecular structures of the three antimicrobial compounds are shown in Fig.1.



A



B **C**

Fig.1 Molecular structures of the three antimicrobial compounds

The shoe lining leather (black) used in this study was produced by a conventional process and no fungicide was added before. The tested microorganisms including bacteria, yeasts and moulds were isolated from leather shoes. *Escherichia coli* and *Staphylococcus aureus* were selected as the representative stains of bacteria. The tested yeast was *Rhodotorula mucilaginosa*, and the moulds were *Mucor*, *Penicillium* and *Aspergillus niger*. The filter paper used in these tests was qualitative from Fuyang Special Paper Co. (Hangzhou, China).

The culture mediums used in the experiments were Nutrient Agar medium for bacteria, Czapek-Dox medium for *Penicillium* and *Aspergillus niger*, and Potato medium (PDA) for *Rhodotorula mucilaginosa* and *Mucor*.

2.2 Preparation of tested microbial solutions and plates

Using sterile liquid Nutrient Agar, the tested solutions of bacteria were prepared by diluting the mature bacteria that had been incubated for 24h in an incubator (MJ-160Π, from Shanghai Yuejin medical apparatus and instruments factory) at 37℃. The concentrations of bacteria were adjusted to 10^6~10^7cfu/mL (colonies formed units per milliliter). For moulds and yeasts, a sterile transfer loop was used to scrape one or two loops of pure mould spores or yeasts off the fresh cultures, and the mould spores or yeasts were well dispersed in 100mL physiological saline solution (0.85%) after shaken for two hours in a water-bath oscillator (CHZ-8, produced by Jintan Fuhua Instrument Company Limited), thus, the tested solutions were prepared. The concentrations of moulds and yeasts were also 10^6~10^7cfu/mL.

To prepare the plates containing bacteria, yeasts or moulds, 0.1mL tested solutions was evenly coated by a spreader on the sterile plates.

2.3 Development of the new combined antimicrobial agent

2.3.1 Evaluation of action type by crossed filter paper strip

The zinc(Π) complex of 2-amino benzothiazole and para-formyl phenoxyacetic acid (A), butyl-*p*-hydroxybenzonate (B) and triclosan (C) were dissolved respectively in the mixed solvent of dimethyl sulfoxide (DMSO) and acetone (1:9, v/v) at the concentration of 10.0 g L^{-1}. The filter paper used in these tests was cut into strips of 10mm×45mm. Thirty-five sterile filter paper strips were immersed in 50mL of each different solution. After 4 hours soaking, these strips were taken out by a sterile forceps and dried. Two strips that have been soaked in different solution were crossed at right-angles on the surface of the tested culture medium plate. The samples were incubated at 28℃(for yeast and moulds) or 37℃(for bacteria) for several days. When the clear inhibition area appeared, its shape around the two filter paper strips was observed and the action type was judged.

2.3.2 Measuration of diameters of inhibition zones for different combinations

A, B, C and their combinations were dissolved respectively in the mixed solvent of DMSO and acetone (1:9, v/v) with the concentration of 10.0 g L^{-1}. They were pairwise mixed as the following ratios

(v/v): 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9. The qualitative filter paper used in the experiment was cut into discs of 17.88mm in diameter. Seven discs were immersed in 10mL of each different mixed solution. After 4 hours, the discs were removed by a sterile forceps and air dried. The treated discs were placed in the center of the tested culture medium plate and incubated as above. At the end of the incubation, the diameters of inhibition zones around discs were measured by a caliper. According to the comparison with their diameters of inhibition zones for different microorganism species, the optimal proportion of the three compounds was attained and the combination was used as the antimicrobial agent for shoe lining leather in the following application experiment.

2.4 Application in shoe lining leather

2.4.1 Antimicrobial treatment of shoe lining leather

The combined antimicrobial agent was dissolved in the mixed solvent of dimethyl sulfoxide (DMSO) and acetone (1:9, v/v) to obtain solutions with the concentrations of 2.0, 3.0, 4.0, 5.0, 6.0 and 8.0 g L^{-1}, respectively. These solutions were evenly sprayed on the grain sides and flesh sides of the shoe lining leathers with a common airbrush. According to the weight of these solutions before and after spraying, the distribution of antimicrobial agent by weight and area in the shoe lining leather was calculated. After air drying, the treated leathers were cut into discs of 17.88mm in diameter under sterile conditions for the test of inhibition zones. For the inhibition ratio test, the samples were cut into chips (10mm×10mm).

2.4.2 Inhibition zone test

Using the sterile forceps, the tested discs of shoe lining leather were placed in the center of the contaminated plates and lightly pressed by sterile cotton poles to make them tightly appressed to the plates. These samples were incubated in the incubator until clear inhibition zones appeared. The temperature in the incubator was 37℃ for the bacteria and 28℃ for the moulds and yeasts. At the end of the incubation, the diameters of inhibition zones around discs were measured by a slide caliper.

2.4.3 Inhibition ratio test

In the inhibition ratio method, 2g chips of the treated leather and 0.2mL mixed seeded solution were added to a triangular flask containing 20mL sterilized physiological saline solution. Then, the triangular flask was shaken for 2 hours at 200r/min in the water-bath oscillator. The colony forming units (cfu) of the remained solution before and after oscillation were determined by plating technique. The inhibition ratio (*IR*) of the treated shoe lining leather was calculated by the formula (1).

$$IR = \frac{C_0 - C_t}{C_0} \times 100 \qquad (1)$$

where C_0 and C_t are the colony forming units of the solution before and after oscillation, respectively.

3 Results and discussion

Due to the close contact between the shoe lining leather and human skin, besides the wide antimicrobial spectrum and good inhibitory effect, the antimicrobial agent should be of low or no toxicity. So, in the experiment, two antimicrobial compounds with low toxicity, viz. butyl-*p*-hydroxybenzonate (B) and triclosan (C), were chosen to test their combination. They all have good skin compatibility. The zinc(Π) complex of 2-amino benzothiazole and para-formyl phenoxyacetic acid (A) synthesized by this laboratory was also chosen because its LD_{50} for big rat skin is more than 5000mg/kg in the acute skin

toxicity tests and no other skin excitation irritation was observed. Furthermore, these compounds have different modes of action and antimicrobial spectrum. Butyl-*p*-hydroxybenzonate can inhibit the activities of respiratory enzymes and electron transfer enzymes in microorganism cell, and destroy cellular structure [6]. For triclosan, its action mode is, at first, the adsorption on the cell wall of microorganism, then, it penetrates the cell wall and reacts with lipid and protein in cell, which results in their denaturation and the discharge of cellular content with low molecular weight [7]. Butyl-*p*-hydroxybenzonate and zinc(Π) complex of 2-amino benzothiazole and para-formyl phenoxyacetic acid have better inhibitory effects to moulds, and Triclosan has better antibacterial activities.

3.1 Evaluation of action type of combinations

In the crossed filter paper strip method, the action type of combination between different antimicrobial compounds can be judged by the shape of inhibitory area. The corresponding relation between the inhibitory area and action type including synergism, additivity, indifference and antagonism was reported in our previous work [3]. After the three compounds were combined each other, their shapes of inhibitory area for six tested species are shown in Fig.2.

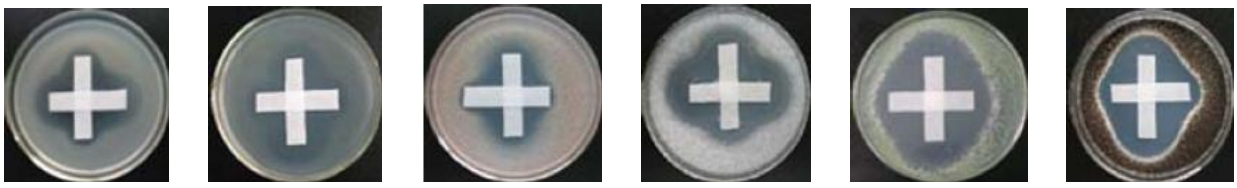
A and B:

Escherichia coli ***Staphylococcus aureus*** ***Rhodotorula mucilaginosa*** ***Mucor*** ***Penicillium*** ***Aspergillus niger***

A and C:

Escherichia coli ***Staphylococcus aureus*** ***Rhodotorula mucilaginosa*** ***Mucor*** ***Penicillium*** ***Aspergillus niger***

B and C:



Escherichia coli ***Staphylococcus aureus*** ***Rhodotorula mucilaginosa*** ***Mucor*** ***Penicillium*** ***Aspergillus niger***

Fig.2 Pictures of inhibition area of different combinations

As we know, the action type of combination can be influenced by the combination itself and the tested microorganisms. It can be seen from Fig.2 that there is no antagonism for all the combination and microorganisms. The combination of A and B shows synergism for all the six kinds of microorganism. For the combination of A and C, their action types have some difference for different species. The combination shows indifference for *Escherichia coli*, additivity for *Rhodotorula mucilaginosa* and

synergism for the other microorganisms. The action types of the combination of B and C are additivity for *Rhodotorula mucilaginosa* and synergism for the other tested stains. So, the combination of the three antimicrobial compounds is feasible and a combined antimicrobial agent may be obtained through the synergistic effect between them.

3.2 Diameters of inhibition zone for combinations with different proportions

At first, the diameters of inhibition zones for the two component combinations between zinc(Ⅱ) complex of 2-amino benzothiazole and para-formyl phenoxyacetic acid (A), butyl-*p*-hydroxybenzonate (B) and triclosan (C) were tested and the results are listed in Tab.1, Tab.2 and Tab.3.

Tab.1 Diameters of inhibition zones of different combinations of A and B (mm)

Proportion	*Escherichia coli*	*Staphylococcus aureus*	*Rhodotorula mucilaginosa*	*Mucor*	*Penicillium*	*Aspergillus niger*
A	19.65	20.50	17.88	18.27	26.30	17.88
9:1	19.86	21.10	20.47	20.21	30.46	29.06
8:2	20.40	20.61	22.42	26.04	29.28	21.52
7:3	21.09	24.81	29.55	29.30	35.35	30.05
6:4	21.54	21.08	23.78	34.84	35.37	30.11
5:5	19.61	22.45	26.42	32.71	41.38	29.32
4:6	20.32	24.81	33.50	32.76	48.42	32.27
3:7	19.18	24.81	28.97	36.34	45.72	34.35
2:8	19.07	23.01	30.26	32.79	42.88	34.30
1:9	18.85	23.75	31.62	32.03	47.66	34.96
B	18.52	22.05	28.60	32.93	46.69	35.58

Tab.2 Diameters of inhibition zones of different combinations of A and C (mm)

Proportion	*Escherichia coli*	*Staphylococcus aureus*	*Rhodotorula mucilaginosa*	*Mucor*	*Penicillium*	*Aspergillus niger*
A	19.65	20.50	17.88	18.27	26.30	17.88
9:1	24.93	29.42	22.45	21.07	32.36	22.84
8:2	25.15	33.29	22.29	21.66	32.92	23.93
7:3	25.40	31.97	22.57	22.32	34.36	24.27
6:4	26.48	32.58	22.93	22.26	34.46	25.95
5:5	26.92	31.44	22.63	23.62	34.94	25.04
4:6	28.69	35.39	23.41	23.52	35.72	26.33
3:7	29.36	35.11	24.63	23.43	32.61	24.87
2:8	30.71	31.26	25.49	20.84	33.10	24.91
1:9	30.86	32.87	25.66	20.51	35.01	25.86
C	30.71	34.96	27.32	19.61	38.80	24.53

Tab.3 Diameters of inhibition zones of different combinations of B and C (mm)

Proportion	*Escherichia coli*	*Staphylococcus aureus*	*Rhodotorula mucilaginosa*	*Mucor*	*Penicillium*	*Aspergillus niger*

B	18.52	22.05	28.60	32.93	46.69	35.58
9:1	23.69	29.43	30.27	31.81	43.09	36.36
8:2	25.83	32.71	28.43	31.81	44.68	35.91
7:3	28.31	35.45	29.06	29.90	42.78	33.63
6:4	28.76	34.32	25.68	28.57	39.50	32.70
5:5	28.48	35.62	26.34	25.35	41.97	30.70
4:6	29.17	37.49	24.73	26.61	42.26	30.64
3:7	29.09	36.57	25.99	22.83	42.34	28.72
2:8	28.64	34.36	21.44	23.12	39.49	27.68
1:9	30.17	35.20	21.51	23.13	39.08	26.50
C	30.71	34.96	27.32	19.61	38.80	24.53

Comparing the diameters of inhibition zones in the above three tables, it can be seen that butyl-*p*-hydroxybenzonate (B) has the best inhibitory effects to fungi, triclosan (C) has the best antibacterial activities, and the integrated antimicrobial effects of zinc(Π) complex of 2-amino benzothiazole and para-formyl phenoxyacetic acid (A) is the worst. The proportion of combination has prominent influence to its inhibitory effects. For the different kinds of tested microorganism, the same combination has different diameters of inhibition zones, and there is no combination which has the best antimicrobial effects for all the tested species. For example, the diameters of inhibition zones of the combination of A: B = 4: 6 for *Escherichia coli*, *Staphylococcus aureus*, *Rhodotorula mucilaginosa* and *Penicillium* are bigger than that of A and B, its diameter of inhibition zone for *Mucor* is similar to that of B, and for *Aspergillus niger*, its diameter of inhibition zone is a littler shorter than that of B. After overall comparison of the inhibitory effects of all the combinations to the tested microorganism, three optimal combinations of A: B = 4: 6, A: C = 3: 7 and B: C = 7: 3 were selected to carry on the following tests of three components combination. The diameters of inhibition zones of combinations of A: B = 4: 6 and C, A: C = 3: 7 and B, B: C = 7: 3 and A were listed in Tab.4, Tab.5 and Tab.6, respectively.

Tab.4 Diameters of inhibition zones of different combinations of A/B and C (mm)

Proportion	*Escherichia coli*	*Staphylococcus aureus*	*Rhodotorula mucilaginosa*	*Mucor*	*Penicillium*	*Aspergillus niger*
A/B	18.88	23.28	33.68	32.68	48.17	32.55
9:1	25.54	28.85	30.64	29.91	46.01	35.18
8:2	32.74	32.44	31.79	29.45	46.36	32.87
7:3	27.88	33.29	33.21	27.41	46.27	30.47
6:4	30.16	39.57	28.64	27.73	43.62	30.22
5:5	36.98	41.95	30.79	30.05	44.48	28.09
4:6	34.42	39.37	28.48	28.58	45.92	27.07
3:7	32.96	36.56	28.48	29.02	43.07	27.05
2:8	37.17	40.72	27.79	25.37	43.49	25.28
1:9	33.06	36.71	27.36	26.65	44.30	25.74
C	31.02	35.13	27.31	20.87	39.21	24.53

Note: A/B indicate the combination of A:B = 4:6.

Tab. 5 Diameters of inhibition zones of different combinations of A/C and B (mm)

Proportion	*Escherichia coli*	*Staphylococcus aureus*	*Rhodotorula mucilaginosa*	*Mucor*	*Penicillium*	*Aspergillus niger*
A/C	29.68	35.15	24.50	23.01	32.55	25.01
9:1	29.97	35.43	22.55	22.92	44.85	26.14
8:2	31.99	35.71	22.91	24.28	45.04	26.47
7:3	36.12	43.93	26.64	24.45	44.80	30.86
6:4	29.85	33.95	24.36	25.36	45.69	31.80
5:5	31.87	35.22	25.49	27.08	46.56	31.44
4:6	28.96	33.60	23.15	27.16	47.56	31.41
3:7	33.16	39.09	24.90	28.29	48.13	30.66
2:8	27.60	32.91	26.19	32.38	44.97	36.01
1:9	23.08	30.55	28.34	32.25	45.52	35.73
B	18.22	22.62	28.50	32.92	46.59	35.32

Note: A/C indicate the combination of A:C = 3:7.

Tab.6 Diameters of inhibition zones of different combinations of B/C and A (mm)

Proportion	*Escherichia coli*	*Staphylococcus aureus*	*Rhodotorula mucilaginosa*	*Mucor*	*Penicillium*	*Aspergillus niger*
B/C	28.74	35.03	29.18	29.62	42.65	33.54
9:1	38.16	42.15	27.30	30.31	44.21	33.20
8:2	33.56	33.17	30.80	30.95	43.72	31.70
7:3	35.33	38.58	28.12	28.62	43.73	31.90
6:4	37.84	52.68	26.46	28.34	43.56	31.23
5:5	31.71	35.32	28.73	28.62	41.91	32.55
4:6	39.25	39.13	24.26	29.31	40.79	30.52
3:7	32.52	37.02	19.21	27.64	40.24	27.22
2:8	29.29	30.51	22.30	24.15	39.65	24.03
1:9	31.42	39.58	18.09	21.86	37.00	23.27
A	19.83	20.39	17.88	18.18	26.81	17.95

Note: B/C indicate the combination of B:C = 7:3。

For the shoe lining leather, the combined antimicrobial agent should have overall inhibitory effects for moulds, yeasts and bacteria, which is the main aspect of our consideration. So, the combination of A: B: C = 0.1: 0.63: 0.27 is chosen as the active component of the combined antimicrobial agent. Compared with the single antimicrobial compound, its inhibitory effects to fungi are similar to that of butyl-*p*-hydroxybenzonate (B), and much better than that of zinc(Π) complex of 2-amino benzothiazole and para-formyl phenoxyacetic acid (A) and triclosan (C); its diameters of inhibition zones for bacteria are bigger than that of each single antimicrobial compound. Compared with the three two-component combinations, its diameters of inhibition zones for fungi are a little shorter than that of the combination of A: B = 4: 6, but much bigger than that of the other two combinations; and for bacteria, its inhibitory effect is the best. Therefore, the three component combination has good overall inhibitory effect to moulds, yeasts and bacteria.

3.3 Application in shoe lining leather

In the leather industry, fungicides are often added in the process liquor of chrome tanning or post-tanning processes such as fat-liquoring. This addition method has some disadvantages. For example, the fungicides are incompletely absorbed by leather, which results in some environmental concerns. In addition, the distribution of fungicides may be not even in the different parts of leather due to the small amount of addition. For the new combined antimicrobial agent that was prepared by mixing zinc(Π) complex of 2-amino benzothiazole and para-formyl phenoxyacetic acid (A), butyl-*p*-hydroxybenzonate (B) and triclosan (C) according the proportion of combination of A: B: C = 0.1: 0.63: 0.27, If the above addition method were adapted, the absorption of each antimicrobial component may has some difference, which can cause the change of combination proportion in the shoe lining leather, and the performance of the combined antimicrobial agent may decrease.

In this experiment, the combined antimicrobial agent was applied to the shoe lining leather by spraying. Compared with the conventional addition method, the spraying method can assure the changeless of combination proportion and avoid the problem of low absorptivity and uneven distribution. The inhibitory effects of the treated leather against moulds, yeasts and bacteria were evaluated by the inhibition zone method and the inhibition ratio method.

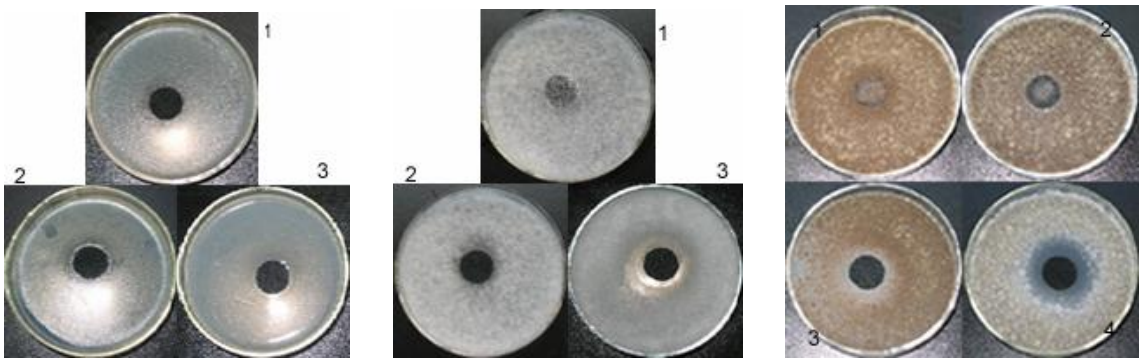
Tab. 7 Diameters of inhibition zones of shoe lining leather

Conc. (g L^{-1})	Distribution		Species					
	g m^{-2}	g Kg^{-1}	*Escherichia coli*	*Staphylococcus aureus*	*Rhodotorula mucilaginosa*	*Mucor*	*Penicillium*	*Aspergillus niger*
2.0	1.26	6.00	O	O	O	×	19.54	×
3.0	1.96	9.30	20.11	26.65	O	14.00	23.11	O
4.0	2.73	13.32	21.35	28.77	O	O	25.25	18.36
5.0	3.32	17.04	22.47	30.16	O	O	28.11	22.36
6.0	4.10	21.23	23.09	31.56	20.59	20.92	30.20	25.39
8.0	5.75	28.92	24.91	34.26	22.80	23.58	32.17	29.37

Note: O indicates that the area around the disc is covered by yeasts, but there is no growth on the leather disc. × indicates that there is mould growth on the leather disc.

It can be seen from Tab.7 that, as the concentration of the antimicrobial agent is improved from 2.0 to 8.0 g L^{-1}, the distribution in weight of the antimicrobial agent in the leather increases from 6.00 to 28.92 g Kg^{-1}, and the distribution in area increases from 1.26 to 5.75 g m^{-2}. The diameters of inhibition zones of the leather sample also increase gradually, but for different tested microorganism, there are some differences. For *Penicillium*, when the concentration of the antimicrobial agent is 2.0 g L^{-1}, namely, its content is 6.00 g Kg^{-1} in the leather, a clear inhibition zone with the diameter of 19.54mm was observed; when the concentration is increased to 8.0 g L^{-1}, the diameter are 32.17mm. For *Aspergillus niger*, as shown in Fig.3, there is mould growth on the surface of the leather at 2.0 g L^{-1}, but the extent of growth is much smaller than that of the blank control, which shows the treated leather has moderate inhibitory effect; when the concentration is increased to 3.0 g L^{-1}, no mould growth is observed; and there is a clear inhibition zone at 4.0 g L^{-1}. For *Mucor*, the inhibitory effect of the shoe lining leather is not so good. There is no growth on the surface of leather when the concentration is 4.0 g L^{-1}, and to form inhibition zone, the concentration should be increased to 6.0 g L^{-1}. For *Rhodotorula mucilaginosa*, when the

concentration of the antimicrobial agent is 6.0 g L^{-1}, there is an inhibition zone with the diameter of 20.59mm. For bacteria, when the concentration is 3.0 g L^{-1}, the inhibition zone for *Staphylococcus aureus* is bigger than that of *Escherichia coli*, and their diameter are 26.65mm and 20.11mm, respectively.



Escherichia coli ***Mucor*** ***Aspergillus niger***

Fig.3 Pictures of inhibition zones

(***Escherichia coli*****:** 1– blank control; 2– 3.0 g L^{-1}; 3– 4.0 g L^{-1}. ***Mucor*****:** 1– blank control; 2– 4.0 g L^{-1}; 3– 8.0 g L^{-1}. ***Aspergillus niger*****:** 1– blank control; 2– 2.0 g L^{-1}; 3– 4.0 g L^{-1}; 4– 6.0 g L^{-1}.)

The main reason for the difference of diameters of inhibition zones to the different tested microorganisms is that the antimicrobial agent itself has different antimicrobial spectrum. In addition, the difference is caused by the different characteristics of microorganism species themselves. When the concentration is increased, the extents of increase for each kind of tested species are different. The extents of increase are small for *Escherichia coli*, *Rhodotorula mucilaginosa* and *Mucor*, but for *Staphylococcus aureus*, *Penicillium* and *Aspergillus niger*, the extents are much large. So, the size of inhibition zone for different microorganisms can not be as the only proof to judge the inhibitory effect of the treated leather to different microorganisms. So, it is necessary to evaluate quantitatively the inhibitory effect of the treated leather.

Tab.8 Inhibition ratios of the shoe lining leather (%)

Conc. (g L^{-1})	*Escherichia coli*	*Staphylococcus aureus*	*Rhodotorula mucilaginosa*	*Mucor*	*Penicillium*	*Aspergillus niger*
2.0	88.94	94.40	82.28	87.41	95.67	86.77
3.0	92.24	99.42	91.90	94.07	98.92	95.13
4.0	93.29	99.57	95.74	98.78	99.57	98.06

As shown in Tab.8, when the concentration of the antimicrobial agent is 2.0 g L^{-1}, the shoe lining leather already shows good inhibitory effect, especially for *Penicillium* and *Staphylococcus aureus*, the inhibition ratios reach 95.67% and 94.40%, respectively. As the concentration is increased to 3.0 g L^{-1}, all the inhibition ratios are beyond 90%, and there is little difference for the different microorganisms. According to the results of inhibition ratio test, the inhibitory sequence of the shoe lining leather against the tested microorganisms can be obtained, namely, *Staphylococcus aureus*, *Penicillium*> *Aspergillus niger*, *Mucor*> *Rhodotorula mucilaginosa*, *Escherichia coli*.

4 Conclusions

The three antimicrobial compounds, viz. zinc(Π) complex of 2-amino benzothiazole and para-formyl phenoxyacetic acid (A), butyl-*p*-hydroxybenzonate (B) and triclosan (C) show synergistic effect to inhibition the growth of different microorganisms, and the new antimicrobial agent prepared by combined them at a proportion of A:B:C＝0.1:0.63:0.27 has good overall inhibitory effect to bacteria, moulds and yeasts, which was proved by its application results on shoe lining leather. Considering the great diversity of microorganism in shoes, the new combined antimicrobial agent should be a good choice for shoe lining leather.

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