The Application of Zanthoxylum Bungeanum Maxim's Antibacterial Component Extract in Cosmetics

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Abstract

The research object was to find a feasible and effective extraction method of antibacterial components in Yunnan pepper as well as to study and the bacteriostasis of such extract with respect to escherichia coli, Candida albicans, and Staphylococcus aureus, which are expected to be used as a theoretical basis of Yunnan pepper future application in cosmetics. Therefore, the antibacterial effects of Yunnan pepper extracts with various solvent used and technological parameter were compared and analyzed. The diameter of bacterial inhibition ring and Minimum Inhibitory concentration (MIC) was researched by using Oxford-cup tests and half dilution method. After collecting and analyzing the experimental data, the antibacterial effect of Yunnan pepper ethanol extract with respect to escherichia coli, Candida albicans, and Staphylococcus aureus was proved to the best, and the antibacterial effect of Yunnan pepper water vapor distillation extract is better than the antibacterial effect of Yunnan pepper petrol ether extract. The most effective technological parameter for ethanol extract was to extract 2h with 95% ethanol and 60℃extraction temperature. The inhibition zones of Yunnan pepper ethanol extract on escherichia coli, candida albicans and staphylococcus aureus were 26.09mm, 37.89mm and 21.58 mm, respectively, and MIC for escherichia coli, candida albicans and staphylococcus aureus were 6.25g/L, 3.125g/L and 12.5g/L. Yunnan pepper extract could be easily applied in various types of cosmetics as a partial replacement for chemical preservatives, such as phenoxyethanol, because of its efficient and multifunctional bacteriostasis.

In conclusion, Ethanol extract is the most effective method to extract Yunnan pepper, which can efficiently inhibit the growth of various microorganisms commonly found in cosmetics, and can thus be used as a natural cosmetic preservative system to partially, or even completely, replace chemical preservatives.

Keywords: Yunnan pepper extract, extraction method, bacteriostasis, cosmetic products.

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I. Introduction

1.1 Background information

Nowadays, people are seeking effective and safe cosmetics, and preservatives are one of the major sources of skin allergens in cosmetics. Formaldehyde and benzoic acid have already been banned in many countries. Nipkin ester preservative, which is widely used in cosmetics, has been doubted by scholars and consumers due to its potential safety problems. Even the use of chemical preservatives in cosmetics will be gradually limited. Therefore, developing a safe, mild and efficient preservative system from natural plants as a substitute of chemical preservatives is the main objective of this study.

Yunnan peppers' main constituents, including volatile oil, alkaloids, flavonoids, coumarins, and etc, have the functions of killing insects, relieving itchiness, inhibiting bacteria, and resisting oxidation. (付陈梅, 2003) Yunnan peppers contain bacteriostasis components that are commonly found in various plants, which make them a potential source of natural preservatives. (谢小梅[1], 2001)

1.2 Purpose

The application of Yunnan pepper as natural food preservatives has been well studied. For example, Song Liya (宋丽雅, 2016) found the antibacterial effect of Yunnan pepper ethanol extract with respect to six types of bacteria, including g. garcinia, pseudomonas aeruginosa, candida albicans and penicillium. Wang Yao (王瑶, 2017) showed that pepper ethanol extract has obvious inhibitory effect on acne-causing bacteria. Jiang Jiefang determined the MIC of pepper ethanol extract on 6 strains, including staphylococcus aureus, aspergillus flavus and escherichia coli, and the optimal technological parameters for ethanol extraction by using antibacterial spectrum. On the other hand, the bacteriostasis of Yunnan pepper with respect to 3 kinds of microorganisms commonly found in cosmetics—staphylococcus aureus, escherichia coli

and candida albicans—is relatively vague and rare, and most research results are not applicable in the field of cosmetics.

Yunnan pepper was selected as the ingredient for the research to study the extraction method of bacteriostatic active components in Yunnan pepper, and the bacteriostatic activity of Yunnan pepper extract with respect to staphylococcus aureus, escherichia coli and candida albicans in the field of cosmetics.

Ⅱ. Literature Review

2.1 Yunnan Pepper Overview

Yunnan pepper (Zanthoxylum bungeanum Maxim) in the forms of Rutaceous shrub or small trees, is widely distributed throughout China as a special economic tree species with high medicinal value. The abundant chemical components in Yunnan peppers enable them to be used as medicine and condiments, which is recorded by *Compendium of Materia Medica* and *Chinese Pharmacopoeia*. Not only is the Yunnan pepper commonly used as food condiments due to its special stimulating taste, but it also has antipyretic, analgesic, antibacterial and antiseptic effects. (王振忠, 2006) (Kim, 2012)

The main functional components of Yunnan peppers include volatile oil, alkaloids, fatty acids, flavonoids, amino acids, lignans, and coumarins, as well as small amount of polyacids and triedodes (王振忠, 2006) (李惠勇，刘友平，张玲, 2008). Alkaloids (Gonzaga W, 2003), flavonoids (吴素蕊, 2005) and phenolic compounds (刘志芹, 2004) are the main constituents that serve an antibacterial effect, and organic solvent extraction and supercritical CO2 extraction are the two most common extraction methods to extract the functional components.

Guleria S (Sanjay G, 2013) extracted essential oil and methanol extract from the Yunnan pepper's leaves, which, according to research, can effectively inhibit Alternaria alternata, Alternaria brassicae, and Corn crescent fungus. Green pepper aroma components extracted by

Gao Fengjing (高逢敬[1], 2007) show bacteriostasis for various types of strains, except for escherichia coli and trichoderma viride.

2.2 Cosmetic Preservatives

Not only can chemical preservatives in cosmetics cause human skin irritation, allergy, inflammation and other potential safety risks (Schwensen J, 2016) , but they can also result in cumulative damage, such as unhealthy cuticles and accelerated aging skin. Natural preservative system, on the other hand, are milder, safer and it is more preferred by consumers all around the worldwide.

The evaluation methods of preservative effectiveness include the bacteriostatic circle test, MIC test and microbial challenge test

1.Bactericidal Test (diffusion method)
There are two methods—the filter paper method and the Oxford-cup method—with the identical fundamental method: Bacteria or fungi (or mixed) are evenly distributed on the appropriate culture medium. A preservative treated filter (or Oxford-cup) is placed on the medium. The anti-corrosion effect of the indicated preservative can be determined based on the diameter of the bacteriostatic circle formed around the filter (or Oxford-cup), and the longer the diameter is, the stronger the anti-corrosion effect. (刘文娟, 2016) (施昌松, 2006)

2. Minimum Inhibitory concentration (MIC)
The minimum bacteriostatic concentration is the minimum concentration that can inhibit the growth of microorganisms. MIC can quantify the anti-corrosion effect of the indicated preservative—the smaller the MIC value is, the stronger the anti-corrosion effect. (李素玉, 2015)

III. Method

3.1 Materials

Materials:

Zanthoxylum bungeanum Maxim (Yunnan pepper bought in the supermarket), ethanol, petroleum ether, beef extract, peptone, AGAR powder, normal saline, sucrose, glucose, and NaCl.

Strains Tested:

staphylococcus aureus, escherichia coli and candida albicans were all provided by Yunnan Xicao resources development co., LTD. Strains were placed in 4 ℃refrigerator after being cultured and activated for later use.

Major Equipments:

rotary evaporator, stirring extraction tank, steam distiller, draught drying cabinet, biochemical incubator, thermostat water bath, bechtop, vertical mode steam sterilizers, and super-centrifuge.

3.2 Experimental method

3.2.1 Strain Culture

The experimental strains were inoculated in the corresponding liquid medium respectively, and cultivated to the log phase using shake incubator (37℃, 150 r/min). Strains were made into bacterium suspension using McFard Land. (Weerakkody N S, 2010)

3.2.2 Determining diameter of bacteriostatic zone with filter paper

The sterilized medium is poured into the culture dish under aseptic conditions. Strain suspensions were made separately with cultured strains, which were then evenly distributed into the corresponding petri dishes. Filter papers immersed in the extract were slightly dried and then put into the petri dish in an equilateral triangle (3 pieces each) and ensured to be in close contacting with the culture medium. Three copies were made for each strain sample. The bacteria were cultured in incubators at 37℃for 24h, the molds were cultured at 28℃for 48h, and the yeast was cultured at 30℃for 24h. The diameters (mm) of bacteriostatic circles were measured, and the average was calculated.

3.2.3 MIC test

Several sterilized test tubes were numbered in sequence. Yunnan pepper extract was twofold diluted with appropriate liquid culture medium, with concentrations of 100, 50, 25, 12.5, 6.25, 3.125,1.56 g/ L. The control group was established. 50L strain suspensions were added into each test tube and cultivated with incubator—bacteria at 37℃for 24h, mold at 28℃for 48h, and yeast at 30℃for 24h. Took the lowest concentration tube among the groups with no visible strain growth as MIC.

3.2.4 Antibacterial effects of different extraction methods.

Using escherichia coli, staphylococcus aureus and candida albicans as test strains, the bacteriostatic circles' diameter of Yunnan pepper ethanol extract, petroleum ether extract and water vapor distillation extract were studied with single factor experiment.

Yunnan peppers were dried and pulverized for 40 mesh. Ethanol, petroleum ether and distilled water was added respectively and stirred at 60℃for 2h. The solution was then pumped and filtered. After that filtrate was combined, decompressed and distilled. The solution was concentrated, and solvent was removed to get the corresponding pepper extract.

3.2.5 Extraction method analysis

3.2.5.1 The antibacterial effects analysis of different concentration of ethanol used in Yunnan pepper ethanol extract.

The diameters of bacteriostatic circles of 95% ethanol extract, 90% ethanol extract, and 85% ethanol extract with respect to Escherichia coli, Candida albicans, and Staphylococcus aureus were compared and studied by using single factor experiment (with extraction temperature: 60℃, extraction time: 3h, material proportion: 1:5, extracted 3 times each).

3.2.5.2 The antibacterial effects analysis of different extraction time used in Yunnan pepper ethanol extract.

The diameters of bacteriostatic circles 1h ethanol extract, 2h extract, and 3h extract with respect to Escherichia coli, Candida albicans, and Staphylococcus aureus were compared and studied

through using single factor experiment (with extraction temperature: 60℃, ethanol concentration: 95%, material proportion: 1:5, extracted 3 times each).

3.2.5.3 The antibacterial effects analysis of different extraction temperature used in Yunnan pepper ethanol extract.

The diameters of bacteriostatic circles of Yunnan pepper ethanol extract extracted at 40℃, 80℃, and 60℃with respect to Escherichia coli, Candida albicans, and Staphylococcus aureus were compared and studied by using single factor experiment (with extraction time 3h, ethanol concentration: 95%, material proportion: 1:5, extracted 3 times each).

3.2.5.4 The application of Yunnan pepper extract in cosmetics as natural preservative

Zanthoxylum bungeanum Maxi extract (extracted with extraction time 3h, ethanol concentration: 95%, material proportion: 1:5, extract temperature at 60℃) was added into lotion, gel and cream. The number of bacterial colonies in the samples were regularly recorded, which can be taken as the indicator of the anti-corrosion effect of natural preservatives used in the samples.

Lotion formula:

Chart 1.1 Lotion formula

Phase	Ingredients	%
A	Sterilize deionized water	To 100%
	Glycerinum	5
	Propylene glycol	12
	Polyethylene glycol-8	2
	Glyceryl polyacrylate	1
B	Low molecular weight hyaluronic acid	0.05
	Sterilize deionized water	5
C	Polyethylene glycol -90M	0.025
	Sterilize deionized water	3
D	hydrolyzed jojoba esters	1
E	Zanthoxylum bungeanum Maxi extract	Added according to chart 1.4

Beakers and agitators used in the experiment were cleaned with disinfecting alcohol. The water used in the experiment was sterilized deionized water and the operating environment was ensured to be clean.

Procedure

1. Glycerinum, propylene glycol and glyceryl polyacrylate in phase A were measured and stirred until evenly dissolved. Deionized water was then added into the solution. After that, polyethylene glycol-8 was added and stirred until evenly dissolved.
2. Ingredients in phase B were mixed until evenly dissolved. Phase A solution was added and stirred until evenly dissolved.
3. Ingredients in phase C were mixed until evenly dissolved. Step 2 solution was added and stirred until evenly dissolved.
4. Hydrolyzed jojoba ester in phase D was added to Step 3 solution and stirred until evenly dissolved.
5. Preservative was added into the lotion according to chart 1.4, and stirred until evenly dissolved.

Gel formula

Chart 1.2 Gel formula

Phase	Ingredients	%
A	Sterilize deionized water	To 100%
	Glycerinum	5
	Xanthan gum	0.05
	EDTA-2NA	0.1
	Carbomer	0.48
	Glyceryl polyacrylate	0.05
B	Sodium hyaluronate	0.005
	Sterilize deionized water	1
C	Polyethylene glycol -90M	0.02

	Sterilize deionized water	2
D	Triethanolamine	0.48
E	Preservative	Added according to chart 1.4

Beakers and agitators used in the experiment were cleaned with disinfecting alcohol. The water used in the experiment was sterilized deionized water and the operating environment was ensured to be clean.

Procedure

1. Glycerinum, Xanthan gum, Carbomer and glyceryl polyacrylate in phase A were measured and stirred until evenly dissolved. Deionized water was then added into the solution. After that, EDTA-2NA was added and stirred until evenly dissolved.
2. Ingredients in phase B were mixed until evenly dissolved. Phase A solution was added and stirred until evenly dissolved.
3. Ingredients in phase C were mixed until evenly dissolved. Step 2 solution was added and stirred until evenly dissolved.
4. Triethanolamine in phase D was added to Step 3 solution and stirred until evenly dissolved.
5. Preservative was added into the gel according to chart 1.4, and stirred until evenly dissolved.

Cream formula

Chart 1.3 Cream formula

Phase	Ingredients	%
A	Sterilize deionized water	70-80
	Glycerinum	1-3
	Propylene glycol	1-5
	Sodium hyaluronate	0.01-0.1
B	Passion fruit seed essential oil	1-3
	Isononyl Isononanoate	1-2
	Decamethylcyclopentasiloxane	1-4

	PDMS	2-4
	Jojoba oil	1-3
C	Xianting G57	1-3
D	Preservative	Added according to chart 1.4

Beakers and agitators used in the experiment were cleaned with disinfecting alcohol. The water used in the experiment was sterilized deionized water, and the operating environment was ensured to be clean.

Procedure

1. Glycerinum, Propylene glycol, and Sodium hyaluronate in phase A were measured and stirred until evenly dissolved. Deionized water was then added into the solution and stirred until evenly dissolved. Put aside as solution 1
2. Ingredients in phase B were mixed until evenly dissolved.
3. Ingredients in phase C were added into phase B solution and stirred until evenly dissolved. Put aside as solution 2
4. Solution 2 was slowly added into Solution 1 and stirred until evenly dissolved.
5. Preservative was added into the cream according to chart 1.4, and stirred until evenly dissolved.

Chart 1.4 Preservatives

Groups	Phenoxyethanol	Yunnan Pepper extract	Hexanediol	Pentanediol
1	0	0	0	0
2	0	1	2	1
3	0	2	0	0
4	0.5	0	2	1

The lotion, gel and cream were made to sit for two days to allow the preservatives to evenly dissolve in the solution. The lotion was diluted with sterile water; and the number of bacteria, mold and yeast colonies were counted using dilution coating counting technique. This process was repeated every 7 days for 1 month.

IV. Result and Justification

4.1 The antibacterial effects to of Yunnan pepper extract with different solvent used.

The antibacterial effects on Escherichia coli, Candida albicans, and Staphylococcus aureus of Yunnan pepper ethanol extract, petroleum ether extract and water vapor distillation extract were compared, as shown in figure 1. Three types of extract all show bacteriostasis to Escherichia coli, Candida albicans, and Staphylococcus aureus. And the antibacterial effects of these three extracts were ranked: ethanol extract > petroleum ether extract > water vapor distillation extract. Therefore, Yunnan pepper ethanol extract was selected for further study.

	Ethanol extract	Petroleum ether extract	Water vapor distillation extract
Escherichia coli	22	16.7	13.8
Candida albicans	24.6	18.3	16.4
Staphylococcus aureus	18.5	12.6	13.5
Water control	0	0	0

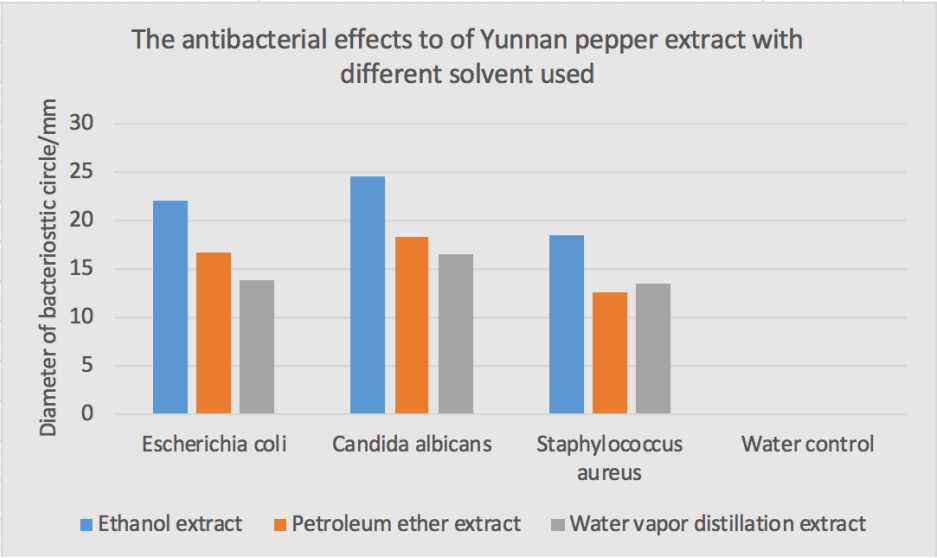

Figure 1

4.2 The antibacterial effects of Yunnan pepper ethanol extracts with different ethanol concentration used

The antibacterial effects on Escherichia coli, Candida albicans, and Staphylococcus aureus of Yunnan pepper ethanol extracts with 95%, 90%, and 85% ethanol concentration used were compared, as shown in figure 2. Pepper ethanol extract with 95% ethanol concentration used shows the best antibacterial effect. Therefore, 95% concentration ethanol was used for the further study.

	95% Ethanol	90% Ethanol	85% Ethanol
Escherichia coli	24	20.2	21
Candida albicans	28.9	24.5	22
Staphylococcus aureus	18.9	17.2	18.1
Water control	0	0	0

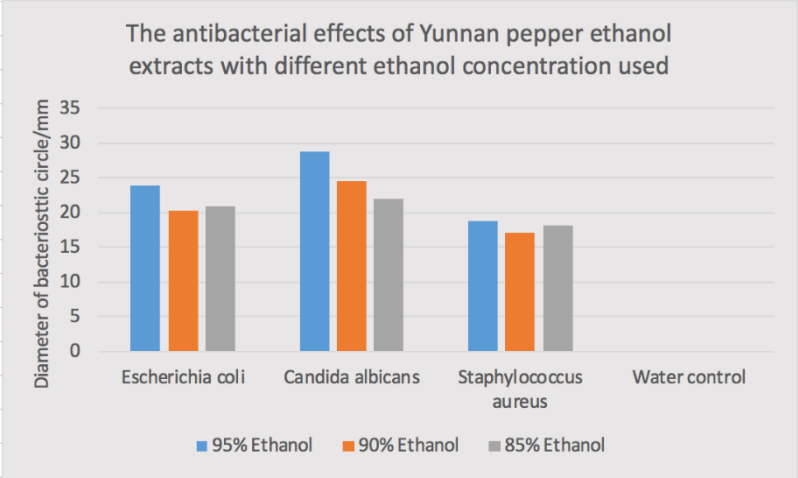

Figure 2

4.3 The antibacterial effects of Yunnan pepper ethanol extracts with different extraction time

The antibacterial effects on Escherichia coli, Candida albicans, and Staphylococcus aureus of Yunnan pepper ethanol extracts with extraction time of 1h, 2h, and 3h were compared, as shown in figure 3. Pepper ethanol extracts with 3h and 2h extraction time show almost the same antibacterial effect. Therefore, 2h was used as the extraction time in the following study to minimize the time consumption.

	Extraction time 1h	Extraction time 2h	Extraction 3h
Escherichia coli	18.3	24.6	24.9
Candida albicans	24.5	32.6	32.8
Staphylococcus aureus	14.4	19.4	19.5
Water control	0	0	0

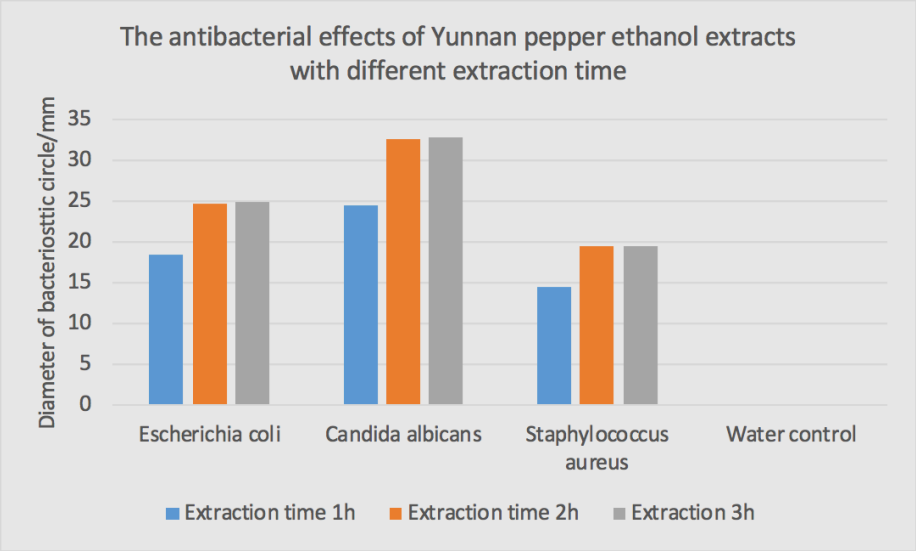

Figure 3

4.4 The antibacterial effects of Yunnan pepper ethanol extracts with different extraction temperature

The antibacterial effects to Escherichia coli, Candida albicans, and Staphylococcus aureus of Yunnan pepper ethanol extract with extraction temperature at 40℃, 60℃and 80℃were compared, as shown in figure 4. Three types of extracts all show bacteriostasis to Escherichia coli, Candida albicans, and Staphylococcus aureus, among which pepper ethanol extract with extraction temperature at 60℃shows the best antibacterial effect. Therefore, Yunnan pepper ethanol extract extracted at 60℃was selected for further study.

	40℃	60℃	80℃
Escherichia coli	26.09	25.1	24.3
Candida albicans	37.89	35	32.5
Staphylococcus aureus	21.58	18	19.1
Water control	0	0	0

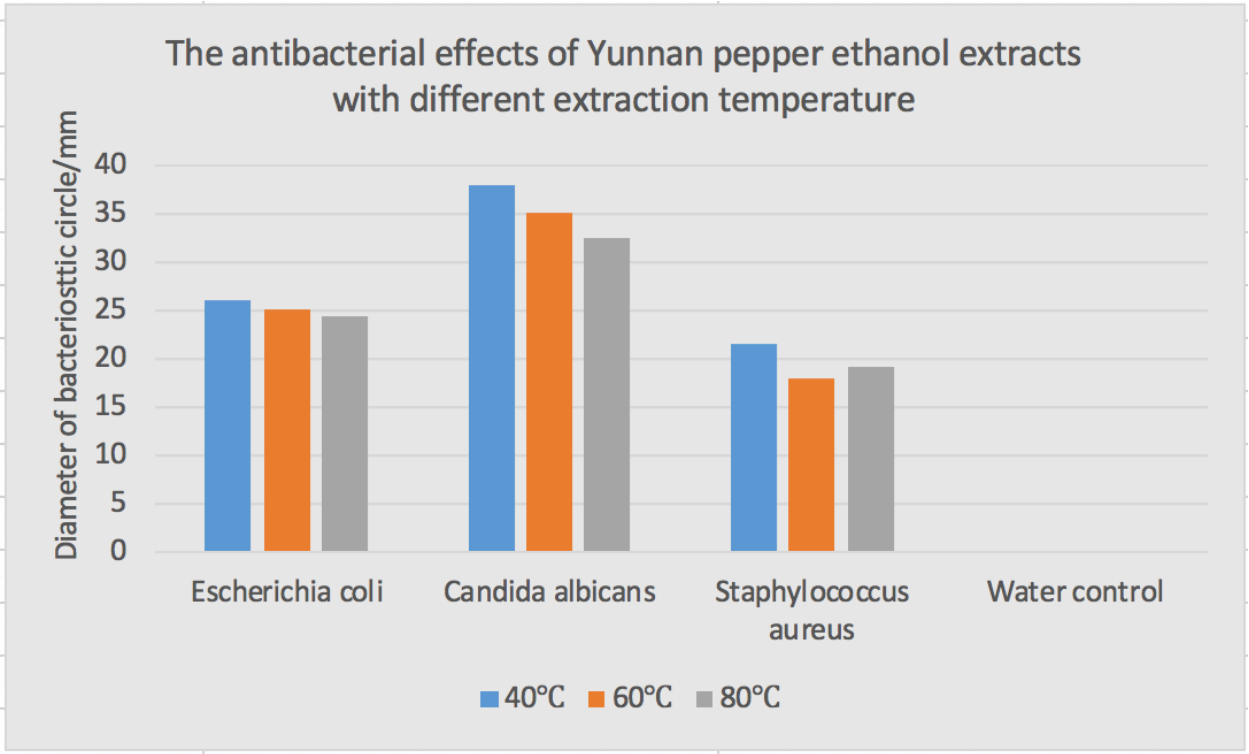

Figure 4

4.5 MIC test for Yunnan pepper ethanol extract with optimal extraction conditions

Chart 5 MIC test for 3 strains

	Escherichia coli	Candida albicans	Staphylococcus aureu
95%Yunnan pepper ethanol extract	6.25g/L	3.125g/L	12.5g/L

According to Chart 5, Yunnan pepper ethanol extract shows bacteriostasis to Escherichia coli, Candida albicans, and Staphylococcus aureus, and Candida albicans was the most successfully inhibited.

4.6 The anti-corrosion effects of Yunnan pepper extract in lotion, gel and cream

Group 1 with no preservative added; group 2 with 1% Yunnan pepper extract, 2% ethylene glycol, 1% pentanediol added; group 3 with 4% Yunnan pepper extract added; group 4 with 1% phenoxyethanol, 2% ethylene glycol, 1% pentanediol added.

	phenoxyethanol	Yunnan pepper extract	ethylene glycol	pentanediol
Group 1	0%	0%	0%	0%
Group 2	0%	1%	2%	1%
Group 3	0%	4%	0%	0%
Group 4	1%	0%	2%	1%

Chart 6 Strain colonies in lotion

Group	Strain colony	Day 2	Day 9	Day 16	Day 23	Day 30
1（cfu/ml）	Bacteria	0	3-4	＞10	＞10	＞10
	Mold and yeast	0	3	9-10	＞10	＞10
2（cfu/ml）	Bacteria	0	0	0	1	3-4
	Mold and yeast	0	0	0	0	1-2
3（cfu/ml）	Bacteria	0	0	0	0	0
	Mold and yeast	0	0	0	0	0
4（cfu/ml）	Bacteria	0	0	0	0	0
	Mold and yeast	0	0	0	0	0

Chart 7 Strain colonies in gel

Group	Strain colony	Day 2	Day 9	Day 16	Day 23	Day 30
1 (cfu/ml)	Bacteria	0	1-2	7-9	＞10	＞10
	Mold and yeast	0	1	5-8	＞10	＞10
2 (cfu/ml)	Bacteria	0	0	0	1	3
	Mold and yeast	0	0	0	0	1-2
3 (cfu/ml)	Bacteria	0	0	0	0	0
	Mold and yeast	0	0	0	0	0
4 (cfu/ml)	Bacteria	0	0	0	0	0
	Mold and yeast	0	0	0	0	0

Chart 8 Strain colonies in cream

Group	Strain colony	Day 2	Day 9	Day 16	Day 23	Day 30
1 (cfu/ml)	Bacteria	0	3-5	＞10	＞10	＞10
	Mold and yeast	0	2-3	＞10	＞10	＞10
2 (cfu/ml)	Bacteria	0	0	0	1	2-4
	Mold and yeast	0	0	0	1-2	4
3 (cfu/ml)	Bacteria	0	0	0	0	0
	Mold and yeast	0	0	0	0	1
4 (cfu/ml)	Bacteria	0	0	0	0	0
	Mold and yeast	0	0	0	0	0

As shown in chart 6, 7, and 8, group 1 with no preservative added shows higher possibility of strain infection; group 4 with chemical preservatives added and group 3 with Yunnan pepper ethanol extract added shows high bacteriostasis, and group 2 with both natural and chemical preservatives also shows higher anti-corrosion effect than group 1. Therefore, the extract of antibacterial components in plants does efficiently inhibit the growth of strains and can be used exclusively or combined with chemical preservatives in cosmetics.

V. Conclusion and evaluation

Solvent extraction technology was used in the experiment to extract the antibacterial components in Yunnan pepper. The optimal extraction method is using 95% ethanol, with an extraction temperature of 60℃, extraction time 2h, and extract for 3 times. The extract acquired under this condition shows the best bacteriostasis. Besides, 95% concentration ethanol has good solubility and fluidity, which is suitable for cosmetic products.

5.1 Conclusion

5.2 Conclusion

5.2.1 Best technological parameters and solvent

The best solvent for Yunnan pepper extraction is 95% ethanol; optimal extraction time is 2h; optimal extraction temperature at 60℃.

5.2.2 Bacteriostasis and MIC tests

Yunnan pepper ethanol extract shows bacteriostasis to Escherichia coli, Candida albicans, and Staphylococcus aureus, and Candida albicans was most successfully inhibited. The MIC value for Escherichia coli, Candida albicans, and Staphylococcus aureus was 6.25g/L, 3.125g/L and 12.5g/L respectively.

5.2.3 Preservative system in cosmetics

Zanthoxylum bungeanum Maxim ethanol extract can be used as preservative system in cosmetics.

5.3 Further research

5.3.1 Further research on Yunnan pepper and other plants' bactriostasis

There might be more plants with bacteriostasis that need to be studied. Besides, extraction methods now are limited, which can result in some negligence of antibacterial contents in plants.

5.3.2 The antibacterial effect of specific components

The plant extract used in the experiment was not able to be further purified, thus the relationship between antibacterial effect and plant components could not be clearly tested. The target site in plants and enzymes and amino acids in the plant cells that are responsible for bacteriostasis are not well studied.

5.3.3 Decolorization and deordorization

Yunnan pepper extract can be decolorized and deodorized to be better used as preservative in a broader range of cosmetic products. The safety and skin affinity of Yunnan pepper extract should be further analyzed.

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