Transdermal permeation research of ethanol extracts from *Lycopodium clavatum*

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Abstract. This paper aims to study the characteristics of transdermal absorption of alkaloid which is the main component in *Lycopodium clavatum*; and to investigate the effects of different concentrations of solvents and borneol of general penetration enhancer on the transdermal characteristics. The in vitro diffusion test was carried out with the drug transdermal diffusion tester. The abdominal skin of in vitro rat was selected as permeation barrier and the alkaloid content in the samples was determined by UV detection. In the results, 30% alcohol extracts from *Lycopodium clavatum* and 30% ethanol solution in 70% alcohol extracts could penetrate the abdominal skin of the sample rats. The borneol could promote such penetration and the cumulative infiltration capacity was 73.28µg and 82.09µg respectively. In conclusion, it is feasible and reliable to use total alkaloids as index to transdermal permeation research of alcohol extracts from *Lycopodium clavatum*. The alcohol extracts from *Lycopodium clavatum* have certain transdermal permeation research and the borneol can be used as an effective transdermal permeation promoter.

1. Introduction
A dried entire herb of lycopodium, lycopodiaceae[1], which consists of many alkaloids including, lycopodine, dehydro-lycopodine, *Lycopodium clavatum* alkaloid and other effective components[2]. Because of its obvious anti-inflammatory and analgesic effect [3] and effect of regulating immune function, it is widely used in traditional Chinese medicine compound preparation. At present, various domestic and foreign scholars have carried on many studies on chemical constituents and pharmacological aspects; although affirming the anti-inflammatory and analgesic effects of *Lycopodium clavatum*, but the specific components that make it work have not been clear. Furthermore, *Lycopodium clavatum* is mostly used in traditional Chinese medicine compound decoction and its efficacy has not been brought into full play. As a widely used anti-rheumatic drug, *Lycopodium clavatum* is worthy of further research and development due to its remarkable clinical efficacy and no toxic and side effects. In order to promote the study of *Lycopodium clavatum* in the preparations[4], we studied the transdermal absorption of the extracts of *Lycopodium clavatum*. Transdermal drug delivery system is a method of drug absorption through the skin, which is one of the hotspots in pharmaceutical research[5]. It can avoid gastrointestinal side-effects caused by oral
administration and the first-pass effect of the liver. It can administrate drugs at a constant rate for a long time and maintain an effective blood concentration. Also, it is easy to use, and the drug administration and sustained or controlled release can be interrupted at any time. The free lycopodine is fat-soluble and has not yet been reported in the study of transdermal absorption. Therefore, the traditional Chinese medicine *Lycopodium clavatum* was selected as the main subject of this experiment, and the in vitro skin penetration test of rats was completed. The experiment has laid a foundation for new preparations made by Chinese medicine through the preparation method of modern transdermal delivery system.

2. Materials and Methods

2.1. Instruments and reagent

TU-1810 UV-Vis spectrophotometer (Beijing Persee General Instruments Co., Ltd.), analytical balance (Mettler-Toledo Instruments (Shanghai) Co., Ltd., AL204), thermostatic water bath (HH), KQ3200DB type numerical control ultrasonic cleaner, electric thermostatic blast dryer (Shanghai Jing Hong Laboratory Instrument Co., Ltd., DHG-9123A), self-made horizontal diffusion cell, Magnetic Heating Agitator (Guohua Electric Appliance Co., Ltd, 78-1).

*Lycopodium clavatum* (a kind of dry herb with root which belongs to the lycopodium of lycopodiaceae), NaOH test solution (0.0500g NaOH was precisely weighed and put into a 100mL volumetric flask to complete the progress of constant volume), HCl test solution, pH6.8 buffer solution, bromothymol blue test solution (0.0500g bromothymol blue was precisely weighed and dissolved with 1.6ml NaOH solution , then put it into a 100mL volumetric flask to complete the progress of constant volume to obtain the bromothymol blue test solution), chloroform (analytically pure), physiological saline, 95% ethanol (analytically pure), 30% ethanol (analytically pure), 70% ethanol (analytically pure), ammonia water, matrine standard products (analytically pure), borneol.

2.2. Experimental animals

Healthy male and female Wister rats with weight of 150-200 g (Experimental Animal Center, Changchun University of Chinese Medicine).

2.3. Extraction of total alkaloids

The *Lycopodium clavatum* was pulverized into coarse powder at the first step. Two 50g of medicinal material then was added to a 1000mL round bottom flask with puring 500mL of 30% ethanol and 500 ml of 70% ethanol solution respectively into the flask and soaked for 10min. The liquid was heated and refluxed for 60min in a thermostat water bath with about 90°C temperature. Finally, the extracting solution was concentrated to the appropriate volume and then transferred to the evaporating dish to dry to constant weight at 80°C.

2.4. Method for determination of total alkaloids

2.4.1. Preparation of reference solution. 37.2 mg reference matrine was precisely weighed and put into a 10mL measuring bottle to be dissolved with methanol and diluted to the scale. The reference concentrated solution then can be got after shaking up. Next, 1ml reference solution was accurately absorbed and placed into a 10mL measuring bottle to be dissolved with methanol and diluted to the scale. The final reference solution can be extracted after shaking up (per 1ml solution contains 372 g matrine).

2.4.2. Preparation of solution for testing product. Appropriate amount of alcohol extract cream was selected and porphyrized, and screened by the second sieve. It was then precisely weighed and placed into a triangle flask with adding 10mL of 1% hydrochloric acid and plugged. Next, it was addressed through ultrasonic treatment (power 250W, frequency 40kHz) for 20 minutes and then filtered. The
following step was to add 5ml of 1% hydrochloric acid in residue and washed twice. Filter, combine filtrate, the solution was made to be pH10 with ammonia water after filtering. The solution was poured into a separate liquid funnel with adding chloroform to extract 3 times (adding 20mL, 10mL and 10mL respectively) and evaporated them to dry. In the final stage, the extract was dissolved with methanol and the progress of constant volume was finished to 25mL.

2.4.3. Linear relation study. The matrine solution of 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1.0mL were respectively absorbed and was placed in a separating funnel with adding 9ml buffer solution with pH6.8 and 1ml of 0.05% bromothymol blue in each funnel. Then, chloroform was added to extract 3 times (10mL, 8ml and 8ml for each time). Each time was oscillated for 5 minutes and placed for 10 minutes. All chloroform solutions were combined with and the volume was fixed to 25mL. The absorbance was measured at 415nm with methanol solution as blank. The linear relationship was investigated with taking the content of matrine (g/ml) as the abscissa and the absorbance value as the ordinate.

2.4.4. Determination of sample content. 1mL of each sample solution was precisely taken and placed in a separate liquid funnel with adding 9ml buffer solution with pH6.8 and 1ml of 0.05% bromothymol blue in each funnel. Then, chloroform was added to extract 3 times (10mL, 8ml and 8ml for each time). Each time was oscillated for 5 minutes, and placed for 10 minutes. All chloroform solutions were combined with and the volume was fixed to 25mL. The absorbance was measured at 415nm with methanol solution as blank.

2.5. Transdermal permeation research of ethanol extracts from Lycopodium clavatum

2.5.1. Preparation and preservation of in vitro rat skin. Healthy rats were drugged with ether and anesthetized with ultraflur. The hair on their back was removed by using a razor, and then the skin on their back was severed with tissue scissors. After being washed with normal saline, the skin of rats was preserved in a refrigerator at 4°C and used up within 1 week [6-7].

2.5.2. Transdermal permeation research. The skin surface liquid was dried with filter paper, the same rat skin was cut into 8 pieces of suitable size. The skin was respectively fixed them in the diffusion chamber and receiving chamber of transdermal absorption device and the cuticle faces the diffusion chamber. 30% ethanol saline solution was precisely injected to the receiving chamber to make the sample tube level above the skin (make sure the receiving fluid is in close contact with the skin).

The amount of added liquid was recorded and there are sampling pipes at the bottom of the receiving pool for the use of sampling, supplementing the receiving fluid and exhausting. Next, the saturated solution (close contact with the cuticle side of the skin without leaving cavity) was poured into the diffusion chamber with adding the alcohol extract. The electromagnetic thermostat blender was started with the temperature of (32±2)°C and the rotating speed of 100r/min after the step of that the sample was added to balance for 30min. 1ml of the receiving liquid was respectively extracted from the sampling tube of the receiving tank at the time of 2, 4, 6, 8, 10 and 12 hours, at the same time, 1 ml of 30% ethanol saline solution was added to the tube at each time and exhausted all bubbles. Using the blank skin as the reference sample (transdermal reference with solvent and additive only, no drugs added; other operations according to the test product). The sample solution was placed in a separating funnel with adding 1ml bromothymol blue solution and 9ml potassium hydrogen benzoate buffer (pH6.8). Then the chloroform was added to extract 3 times (10mL, 8ml, and 8ml for each time). Each time was oscillated for 5 minutes, and placed for 10 minutes. All chloroform solutions were combined with and the volume was fixed to 25ml. The absorbance was measured at 415nm with methanol solution as blank. The cumulative absorbance at each time point was calculated according to the formula: EI=Ei+I/ν∑Ei-1[8] and worked out the Cumulative concentration at each
time point. The Q of each time point is calculated based on the formula: \( Q = C \times V \) and the rate of penetration is obtained rely on Q-T equation.

2.5.3. **Observation of penetration enhancers.** Same as the transdermal permeation experiment, 1%, 2% and 5% borneol were added in the diffusion chamber respectively to observe the osmotic effect.

3. **Results**

3.1. **Linear relationship study**

The matrine solution of 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1.0mL were respectively absorbed. After dealing with such solutions, the absorbance was measured at 415nm with methanol solution as blank. The linear relationship was investigated with taking the content of matrine (\( \mu g/ml \)) as the abscissa and the absorbance value as the ordinate. The results showed that matrine has a good linear relationship within the range of 2.976-14.88\( \mu g/ml \) by applying the regression equation of \( y=0.0507x+0.0674 \), and \( R^2=0.9975 \).

3.2. **Determination of sample content**

The 1ml solution of the sample was placed in a separating funnel. After dealing with such solutions, the absorbance was measured at 415nm with methanol solution as blank. The results showed that in 30% ethanol extract, the concentration of alkaloids was 7.455\( \mu g/ml \) and the total content of alkaloids \( M=1.86375mg \) with the percentage of 3.7089mg/g in the sample solutions; in 70% ethanol extract, the concentration of alkaloids was 2.2604\( \mu g/ml \) and the total content of alkaloids \( M=0.5651mg \) with the percentage of 1.1284mg/g in the sample solutions.

3.3. **Transdermal permeation research of ethanol extracts from Lycopodium clavatum**

Transdermal permeation research of 30% alcohol extract from lycopodium clavatum was carried out by adding 1%, 2% and 5% borneols in the diffusion chamber to investigate its osmotic effect. The results are shown in Table 1 and Fig. 1.

| Time (h) | 30% ethanol | 30% ethanol+2% borneol | 30% ethanol+5% borneol |
|---------|--------------|------------------------|------------------------|
| 2       | 0.06789      | 0.06909                | 0.06833                |
| 4       | 0.06902      | 0.07146                | 0.06884                |
| 6       | 0.07032      | 0.07396                | 0.06985                |
| 8       | 0.07147      | 0.07654                | 0.07088                |
| 10      | 0.07269      | 0.07913                | 0.07205                |
| 12      | 0.07414      | 0.08182                | 0.07328                |
Transdermal permeation research of 70% ethanol alcohol extract of from lycopodium clavatum was carried out by adding 1%, 2% and 5% borneols in the diffusion chamber to investigate its osmotic effect. The results are shown in Table 2 and Fig. 2.

### Table 2. Cumulative permeance of accelerants with different concentrations (70% alcohol extract)

| Time (h) | 30% ethanol | 30% ethanol+2% borneol | 30% ethanol+5% borneol |
|---------|-------------|------------------------|------------------------|
| 2       | 0.06840     | 0.06759                | 0.06901                |
| 4       | 0.06921     | 0.06853                | 0.07129                |
| 6       | 0.07056     | 0.06970                | 0.07384                |
| 8       | 0.07156     | 0.07183                | 0.07646                |
| 10      | 0.07271     | 0.07408                | 0.07923                |
| 12      | 0.07396     | 0.07637                | 0.08209                |

4. Discussion
The transdermal absorption process of drugs is divided into three stages: the drug is released from the matrix; the drug penetrates the cuticle barrier; and the drug is absorbed by blood vessels through the epidermis and the dermis[9]. There are many factors affecting the transdermal absorption of drugs, such as the oil-water partition coefficient, the relative molecular weight of drugs, the melting point of drugs and other physical and chemical properties[10].

The total alkaloids content and solubility of 30% and 70% ethanol extracts were significantly different, but there was no significant difference in terms of the transdermal permeation. It therefore can draw a conclusion that the transdermal permeation of such alkaloids is the skin controlled release type, and the transdermal permeation is not able to improve by merely increase the alkaloids content and solubility.

The borneol had an obvious osmotic effect on the alcohol extract of lycopodium clavatum, and the osmotic effect improved with the increase of concentration, but the osmotic effect decreased when the concentration reached and exceeded 2%. The reason may be that borneol can increase cuticle permeability, which can promote the transdermal penetration of drugs. When the concentration of borneol is too high, it can increase the solubility of alkaloids in skin and weaken the transdermal penetration of drugs.

5. Conclusion
The drug transdermal diffusion test instrument was applied to the in vitro diffusion test for the transdermal absorption of lycopodium clavatum extract. The abdomen skin of rats was used as an osmotic barrier to determine the alkaloid content in the samples. The results showed that the borneol had the effect of promoting osmotic effect on the extract of lycopodium clavatum, and the cumulative permeance was 73.28µg and 82.09µg, respectively. Which indicated that the extract of lycopodium clavatum had certain transdermal permeation, and the borneol could be regarded as a more effective transdermal permeation promoter of lycopodium clavatum.

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