Penetration study of topical formulation containing

*Eulophia macrobulbon* extract

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**Introduction**

*Eulophia macrobulbon* (Parish & Rchb.f.) Hook.f. is a ground orchid found in several parts of Southeast Asia. The tubers are used as a herbal aphrodisiac¹, treatment for skin rash and insect bites². The pharmacological activities are vasorelaxant in the pulmonary of rat lungs³,⁴, pulmonary arteries and the corpus cavernosum isolated from human⁵, vasorelaxant on rat vessels, NOS activation, enhanced EDH production and endothelium-independent effects⁶. Acute oral toxicity on L⁰.⁵₀ level up to 2 g/kg of body weight is safe for oral use within 2 weeks. Chronic oral toxicity which 5, 50 and 500 mg/kg of body weight once daily are safety of consuming in 6 month³. The root extract is reported to reduce the production of TNF-α and IL-6, however, increase anti-inflammatory cytokine IL-10, and iNOS in LPS-stimulated macrophages⁷. The chemical constituent 1-(4'-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (1) extracted from the tuber showed the high PDE5 inhibitory activity (IC₅₀ = 0.12 μg/ml) as opposed to 0.014 μg/ml of IC₅₀ reported with sildenafil⁸. Thus, leading to a development of topical formulation containing the extract for treatment of erectile dysfunction.

Although oral therapies for ED are available, topical therapies are still under development. But topical therapies would be minimally invasive and would avoid the side effects associated with oral medications⁷. Topical formulation may provide benefit to patients with poor response to oral therapies or minimize the possible drug interactions with concurrent use of other medication.

A major obstacle to efficient drug delivery is the difficulty with which topical agents penetrate penile skin and fascial layers⁸. The horny cells at the stratum corneum contain a tight intercellular lipid matrix that impedes drug passage. Research carried out in 2012, focused on the use of permeation enhancers that would facilitate the penetration of resistant skin layers⁹. Permeation enhancers should increase drug penetration into the target tissue while simultaneously releasing the active agent at the site of action.

**Methods**

**E. macrobulbon extract**: The sample of *E. macrobulbon* tubers were collected from Prachinburi. The plant was identified by Asst.Prof.Dr. Anupan Kongbungkerd and the voucher specimen was kept at the Department of Biology in the Faculty of Science at Naresuan University. An extract was prepared using an alcohol and water-based process according to patent application No. 1503001282. The extracts contained not less than 0.6% of 1.

**Test formulations**: The basic formula contained 5% w/w of *E. macrobulbon* extract in water: PG: EtOH (10:40:50) with 5% chemical enhancers i.e. Transcutol® (diethylene glycol monoethyl ether), Neosolue™-aquilo (bis-ethoxydiglycol cyclohexane 1,4-dicarboxylate), Tween 80, IPM (isopropyl myristate), Oleic acid, and Limonene. Gel formulations were prepared by dispersing 1.5% carbopol 940 in a solvent containing water, propylene glycol, and ethanol at a ratio of 10:40:50, then neutralizing it with triethanolamine (TEA). *E. macrobulbon* was dissolved in the extracts as a gel with a concentration
of 5% w/w. The 5% penetration enhancers, Tween 80 and Neosolue™-aqulio, were added into the gel formulations.

**Skin permeation study**: Vertical Franz Diffusion cell was used for the skin permeation study. The synthetic membrane (Strat-M™) was mounted on the cell which filled with 5% tween 80 in pH 7.4 phosphate buffer. The medium was agitated at 440 rpm. The study was done at 32±1°C. 100 µl of formulation sample was applied on the membrane. Thus, 500 µl of medium was sampling at the predetermined time and replace with the fresh medium. The sample left on the membrane was removed by a cotton swab and determined for the amount of 1. The amount of 1 in the membrane was also determined by extracting with methanol.

**HPLC method for determination of 1**: A SHIMADZU LC solution equipped with solvent delivery pump (LC-20AT), UV/Vis detector (SPD-20A) and auto-sampler (SIL-20A) with 20 µL loop was used to determine the amount of 1. Data collection and analyses were performed using CLASS-VPTM System Software. The separation was performed with a reversed phase column Phenomenex Gemini-NX 5 µm C18 110Å 150x4.60 mm i.d., 5 µm particle size. The gradient mobile phase system of ACN: water was used. The gradient elution conditions applied were: 0.0-5.0 min, linear gradient 38% ACN; 12.5-13.5 min, linear gradient 100% ACN; and final washing up of the column with gradient 38% ACN for 6 min before reconditioning the column. In this system, the injection volume was 20 µl, the detection wavelength was set at 265 nm, and the flow rate was 1 ml/min. 1 was dissolved in methanol before injection.

**Results and Discussion**

The formulation was aimed to provide quick action upon application. Chemical enhancers were incorporated as to facilitate at least an increase of 5% in skin permeability of active compounds within 30 min of application. The basic formula contained water: PG:EtOH (10:40:50) with 5% chemical enhancers such as Transcutol® (diethylene glycol monoethyl ether), Neosolue™-aqulio (bis-ethoxydiglycol cyclohexane 1,4-dicarboxylate), Tween 80, IPM (isopropyl myristate), Oleic acid, and Limonene were tested for skin permeation. These improved skin permeation, especially formulations with Transcutol® and Neosolue™-aqulio which greatly increased skin permeability (Figure 1a).

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**Figure 1** Effect of penetration enhancer on the permeation of 1 from solution formulations (a) and gel formulations (b) which have *E. macrobulbon* extract 50 mg/ml and 5% penetration enhancer.
The penetration enhancers did not improve skin permeation during the first two hours but increased permeation of 1 after two hours which was the time required to effect the structure of skin and increase the diffusion of 1. Transcutol®, Neosolute®, and Tween 80 showed to enhance the permeation of 1. Transcutol® increases the drug solubility in the stratum corneum, improves vesicular bilayer fluidity and also acts as a penetration enhancer by reducing the barrier function of SC transiently. Neosolute® enhances the penetration of the active ingredients through the pores of the skin. Tween 80 is a surfactant that destroys chemical barrier in the skin, by dissolving fat in stratum corneum and can also effect to keratin within the stratum corneum.

On the other hand, Oleic acid, limonene, and IPM were decreased skin permeability of 1. Oleic acid is a fatty acid found abundantly in nature including in the human skin, lessens the barrier function of the skin and disrupt the intercellular lipid domains. It was found that oleic acid as a skin permeation enhancer did not increase the skin permeability of 1 more than the control group. This may be due to the amount of oleic acid that not be in the proper range. These four reports seem to be in agreement with our results.

IPM belongs to a fatty acid ester class of penetration enhancers, its mechanism of action varied with specific situations, and most frequently, IPM acted as a fluidizer to disrupt the highly organized intercellular lipids, thereby increasing the permeability of stratum corneum. However, using of IPM as a skin permeation enhancer in this study did not increase the skin permeability of 1 as compared with control group. This could be explained possibly due to this formulation like it did not stick to the skin or from the decreasing of solubility of 1 especially when the ethanol evaporates, and the effect of the appropriate concentration in the formulation.

Limonene modifies intercellular packing leading to the disruption of highly ordered lipid structures in the stratum corneum. In this study, Limonene even increased skin permeability by 45 min compared to the control group, however the permeability of 1 was unchanged thereafter. When observed at the skin found that the skins were dry and the accumulation of 1 in artificial skin was 51.5% at 4 hours. Limonene may change the structure of the skin and increase skin permeability in the first period. We hypothesized that the chemicals in E. macrobulbon extract may accumulate in the skin and become the barrier that prevent 1 to penetrate into the receiver solution.

TWEEN 80 and Neosolute® were selected for development of gel formulation due to enhanced permeation of 1 from E. macrobulbon extract and could be compatible with the gel formulation. From Figure 1b, the effect of permeation enhancers on the permeation of 1 from the gel formulations are similar to the effect of the penetration enhancers on the solution formulations by increasing the permeation of 1 after 2 hours. However, the formulation with Tween 80 do not differ from the control formulation. While 5% Neosolute® which has the best skin permeability in a long time (after 120 min).

But we do not choose because it is too long time, which is not suitable for actual use.

The topical drug formulations with E. macrobulbon extract in both solution and gel form delivered 1 through a synthetic membrane which was similar to human skin. It may be possible for 1 in E. macrobulbon extract to permeate through the skin to corpus cavernosum to treat ED, but the permeation of 1 still requires 4 hours, so further development to reduce the duration of permeation or development of a formulation that can deliver 1 via other routes, such as transurethral or oral medication, etc. is required.

However, the results showed that gel formulation with 5% Tween 80 provided optimum skin permeability of active compounds in a short time. The skin permeation for this formulation was 1.98% in 30 min and 2.24% in 60 min (% permeation at 30 and 60 min of 1 from gel with 5% Neosolute® was 1.54 and 2.08 respectively). The active dose of 1 was calculated with an IC50 of 0.12 μg/ml and the maximum dose before toxicity was 2.22 μg/ml. The amount of 1 in a 50 mg/ml formulation required to permeate to the target organ to treat erectile dysfunction within a safe range (therapeutics index) is 0.86% -15.85%. Thus, this formulation is likely to be active and safe, which could be tested further.

For future development of topical drug formulations targeting to increase the skin permeability of 1 by 5% within 30 min, the following parameter could be altered (I) increasing the amount of 1 in the formulation by using E. macrobulbon extract which had high 1 (II) adding 5% co-enhancers in the formulations as Transcutol® plus Neosolute™-aqueille, Transcutol® plus Tween 80, and Neosolute™-aqueille plus Tween 80 (III) incorporating only pure 1 for formulation to avoid interference of other compounds on skin permeability.

Conclusion
The results of the developed formula showed the gel formulation with 5% Tween 80 has the best skin permeability in the short time. The skin permeation for this medical formula was 1.98% at 30 min and 2.24% at 60 min, which was within the therapeutics index range. This may be a prototype or
preformulation to develop a drug formula for ED treatment in the future and further research can be done regarding in-vivo studies.

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