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Advanced Formulation and Pharmacological Activity of Hydrogel of the Titrated Extract of *C. Asiatica*

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Titrated extract of *Centella asiatica* (TECA) contains three principal ingredients, asiaticoside (AS), asiatic acid (AA), and madecassic acid (MA). These components are known to be clinically effective on systemic scleroderma, abnormal scar formation, and keloids. However, one problem associated with administration of TECA is its low solubility in aqueous as well as oil medium. In this study, various nonionic surfactants and bile salts as anionic surfactant were tested and screened for solubilizing TECA with a view to developing topical hydrogel type of ointment which is stable physicochemically, and has better pharmacological effects. When TECA was incorporated into various nonionic surfactant systems, labrasol had the most potent capacity for solubilizing TECA. In cases of bile salt systems, Na-deoxycholate (Na-DOC) had foremost solubilizing capacity, even more than labrasol. In differential scanning calorimetric study, the peaks of AA, MA, AS and Na-DOC disappeared at the coprecipitate of 1% TECA and 1 % Na-DOC, suggesting the optimum condition of Na-DOC for solubilizing TECA. When the physicochemical stability of hydrogel containing this mixture was assessed, it was stable at room temperature for at least one month. Pharmacologically it significantly decreased the size of wound area at the 9th day when applied to the wound area of rat dorsal skin. Taken together, solubility of TECA was dramatically improved by using nonionic and anionic surfactant systems, and Na-DOC was found to be the most effective solubilizer of TECA in formulating a TECA-containing hydrogel typed ointment. Moreover this gel was considered to be applicable to clinical use for wound healing effect.

Key words: *Centella asiatica*, Asiatic acid, Asiaticoside, Madecassic acid, Bile salt, Hydrogel

INTRODUCTION

Centella asiatica (umbelliferae) is an ethnomedical plant used in different continents by diverse ancient cultures and tribal groups. *Centella asiatica* (a herb) has been used for hundreds of years as a traditional medicine of many Asian countries to improve wound healing. It has been reported that wound and ulcer healing are enhanced *via* promoting fibroblast proliferation and collagen synthesis by *C. asiatica* extract treatment in Europe (Maquart *et al.*, 1990). These properties are ascribed to the active ingredients, asiatic acid (AA), asiaticoside (AS), and madecassic acid (MA) as shown in Fig. 1 (Maquart *et al.*, 1999). And Madecassol, a formulation based on the titrated extract of *Centella asiatica* (TECA), prompted the proliferation of granulation and tensile strength through improving connective tissue formation, epithelization, and angiogenesis when applied locally on wounds in rat dorsal skins (Rosen *et al.*, 1967; Maquart *et al.*, 1999). These effects would provide with the metabolite effects which were required for fighting against local infection and essential to the restoration or building up of the connective tissue. Therefore, TECA is clinically effective on the systemic scleroderma, abnormal scar formation, and keloids (Kiesswetter, 1964). Also, TECA affects the enzymes which regulate the formation of mRNA-amino acid (e.g., proline, alanine) complex, a important intermediate in collagen biosynthesis (Beljanski and Vapaille, 1971). By these actions, TECA directly accelerate wound-healing process.

In spite of these excellent wound healing properties, it is difficult to formulate and deliver the naturally derived drug as an optimum system which can maximize its clinical effect with minimum side effects, because TECA is a sparingly soluble drug extracted from *Centella asiatica* in

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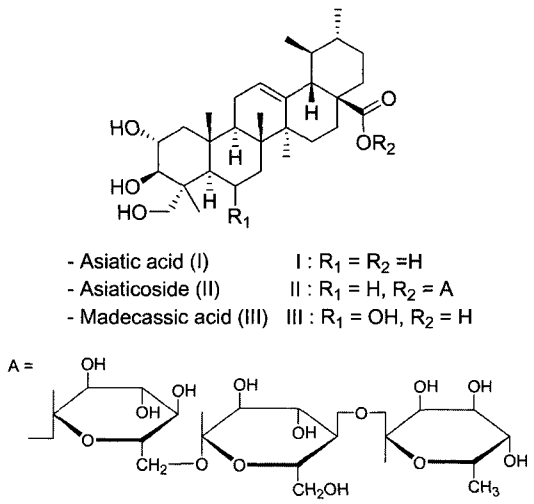

Fig. 1. Structure of three principal components in TECA (Asiatic acid, Asiaticoside, and Madecassic acid)

aqueous as well as oil medium.

Recently many researches have been done for the purpose of solubilization of insoluble or only slightly soluble drug with the application of various nonionic surfactants such as labrasol, tween 80, PEG 400, and so on (Djordjevic *et al.*, 2004; Barreiro-Iglesias *et al.*, 2003 and 2004). Also the similar studies have been investigated using anionic surfactants such as Na-taurocholate, Na-taurodeoxycholate, Na-deoxycholate, and so on (Trotta *et al.*, 2003; Wiedmann and Kamel, 2002; Ringel *et al.*, 1998). Especially many delivery systems have been developed to improve the solubilization of drugs by these surfactants. One of the most promising agents is the system using the interaction of polymer and surfactant. Polymer/surfactant interactions may cause dramatic changes in the drug solubilizing capacity, rheological properties of polymer aqueous dispersions, and in drug diffusion and penetration through the skin and mucouses. In consequence, incorporation of polymer/surfactant opens a wide range of possibilities for developing drug delivery systems (Malmsten, 2002; Alvarez-Lorenzo and Concheiro, 2003).

Among the currently used polymers, the hydrogel has been widely used for preclinical and clinical studies (Patton and Palmer, 2005; Liu *et al.*, 2005). In the preparation of hydrogel typed ointment, Carbopol has been widely used both in pharmaceutical and cosmetic preparations (Contreras and Sanchez, 2002). Carbopol resins are cross-linked polyacrylic acids. They are hydrated in the presence of water, and the carboxylic acid groups in the molecules can dissociate in an aqueous system. The negative charges on the polymer backbone repel each other to cause dramatic polymer expansion. Its swelling and hydrophilic characteristics allow three main pharmaceutical applications; (i) as a thickening agent (Bonacucina *et al.*, 2004), (ii) as a suspending agent (Dolan *et al.*, 1960), and (iii) as an emulsifying agent (Islam *et al.*, 2004).

Therefore, in this study, it was pursued to improve water-solubility of TECA and optimize the efficiency of this drug by the hand of surfactant systems. And it was also pursued to develop topical hydrogel typed ointment which is stable physicochemically, and has better pharmacological effects.

MATERIALS AND METHODS

Materials

Titrated extract of *C. asiatica* (TECA composed of 40.4 % asiaticoside, 57.2% asiatic acid and madecassic acid) was provided from Dong-Kook Pharmaceutical Co.(Seoul, Korea) and Tween 80 (T), PEG 400 (P), propylene glycol (PG), labrasol (LS), labrafil (LF), Na-taurocholate (Na-TC), Na-taurodeoxycholate (Na-TDC), Na-cholate (Na-C), Na-glycocholate (Na-GC), and Na-deoxycholate (Na-DOC) were purchased from Sigma (St. Louis, MO).

HPLC Analysis

For the determination of TECA, a C18 ODS 4 μm column of 250 mm in length and 4.6 mm in internal diameter (YMC Co., Ltd. Japan), was used in conjunction with mobile phase consisting of 25% water and 75% methanol. The absorbance was measured at 214 nm. A constant volume (10 μL) of each sample was injected into the column *via* an autosampler. The signal was recorded and analyzed by a chromatopac integrator. The peak heights from each sample were measured to convert to the concentration.

Solubilization tests

Five nonionic surfactants (T, P, PG, LS, LF) were tested for water-soluble or miscible carrier system of TECA. TECA (50 mg) was added to nonionic surfactants of 50, 100, 200, and 300 mg, respectively. Water was added to the mixture to obtain final surfactant concentration of 5, 10, 20, and 30% (w/v). And five anionic surfactants (bile salts; Na-TC, Na-TDC, Na-C, Na-DOC, Na-GC) were also tested for water-soluble or miscible carrier system of TECA. 50 mg of TECA and 10, 50 or 100 mg of each bile salt were dissolved in 2 mL of methanol in a tube to coprecipitate. Then, the solvent was evaporated and samples were lyophilized for 24 h. And 1 mL of water was added to make final concentration of bile salt to be 1, 5, or 10% (w/v). The tubes were flushed with nitrogen, sealed and equilibrated for 24 h at room temperature under constant shaking. Subsequently, they were centrifuged at 5000 rpm for 5 min to separate unsolved TECA. Then the supernatant was filtered through a 0.45 mm membrane.

Differential scanning calorimetric study

Thermal analysis was performed using Perkin Elmer DSC 7 thermal analyzer system with a differential scanning calorimeter equipped with a computerized data station. All samples were weighed (5~6 mg) and heated at a scanning rate of 10°C/min between 50 and 300°C. Aluminum pans and lids were used for all the samples. Temperature calibration was performed periodically using indium and zinc as a standard.

Preparation of hydrogel

Surfactant-TECA solution (containing 1% (w/v) TECA in distilled water) was blended homogeneously with Carbopol 934 (2.0%, w/v), methyl paraben (0.2%, w/v), propyl paraben (0.2%, w/v) and sodium bicarbonate (1.68%, w/v) to neutralize the solution. The blending was continued until homogenous TECA-hydrogel was obtained.

Stability studies of TECA surfactant gel

The physical stability of the formulation of dissolved TECA-surfactant gel was evaluated by detecting crystallization of TECA or micelle aggregation under light microscope. 1% (w/v) TECA gels solubilized with 1% (w/v) Na-DOC or 10% (w/v) LS were prepared, and physical stability study was assessed under light microscope. The images of TECA/Na-DOC or TECA/LS gel formulation were taken immediately after preparation, 24 h, and one month of storage at room temperature. And the chemical stability of TECA in the gel formulations was assessed using reverse-phase HPLC to determine total TECA content of the formulation at different time points during the storage at room temperature. The gel formulation (1 g) was diluted adequately with methanol and centrifuged at 5000 rpm for 5 min and its supernatant was filtered through a 0.45 μm membrane, and quantified using HPLC method described above.

Pharmacological effect of TECA surfactant gel

This pharmacological effect was evaluated based on the dimension method modified from Habibipour *et al.* (2003). The dorsal skin of male Sprague-Dawley rats (170-200 g) was shaved with animal clippers. Under ether anesthesia, dorsal skin was wiped with alcohol and excised from each animal with sterile 7 mm-diameter punch. The excision area was located in each animal 3 cm below the neckline in the paravertebral location, separated by 1 cm from the spine. After the wounds were made, they were gently cleaned with alcohol cotton swabs to remove any exudate. All wounds were left open without dressing and the animals were returned to their individual cages. Rats were divided into four groups at random and the ointment (50 μL on each site) was applied using syringe and cotton swabs every day after the operation. Group 1 was not treated after operation (control group) and group 2 was treated with blank hydrogel (containing neither TECA nor surfactant). And group 3 was treated with hydrogel with TECA, and group 4 was treated with hydrogel containing TECA dissolved with Na-DOC. The surface area of the wound was measured with caliper at the 1st, 3rd, 5th, 9th day after the operation. The wound area was calculated by multiplying the major diameter of the wound by the minor diameter of the wound. And the effects of wound healing were quantified from the ratio of the wound area for the size before each treatment.

Statistical analysis

The data were expressed as the mean±S.D., and the statistical analysis was performed using one-way analysis of variance (ANOVA). To determine whether or not the differences between the profiles were significant, the Turkey's test was applied to the experimental values. A significance value of $p = 0.05$ was chosen.

RESULTS AND DISCUSSION

HPLC Analysis of TECA

The determination of the terpene acids (AA, MA) and the glycosides (AS) of *C. asiatica,* was done at the absorbance of 214 nm. Due to the large difference in polarity of the terpene acids and the glycoside, the two acids (AA, MA) and glycoside (AS) were well separated (Fig. 2). Without using a linear gradient of mobile phase, the three major compounds were separated in a single run. A flow rate of 0.8 mL/min resulted in 4.2, 12, and 19 minutes of retention time for AS, MA, and AA, respectively. The standard curves exhibited excellent linearity for the concentration range (10~2000 μg/mL) and regression analysis showed correlation coefficient greater than 0.97 (data not shown).

Solubilization of TECA in nonionic and anionic surfactant systems

To improve the solubility of a poorly water-soluble TECA, various anionic and nonionic surfactants were used to prepare micellar systems. The aqueous solubility of AS, MA, and AA in nonionic surfactant systems were shown in Fig. 3. In the absence of surfactant, the solubility of AS, MA, and AA in water is 149, 441, and 393 μg/mL, respectively, which is insufficient to provide the desired amount in hydrogel formulation. In the presence of surfactants, however, the solubility of AS, MA, and AA was increased in proportion to the concentration of surfactants. There were large differences in solubilizing capacity among nonionic surfactants. More TECA was solubilized in the presence of labrasol (LS) and Tween 80 (T), rather

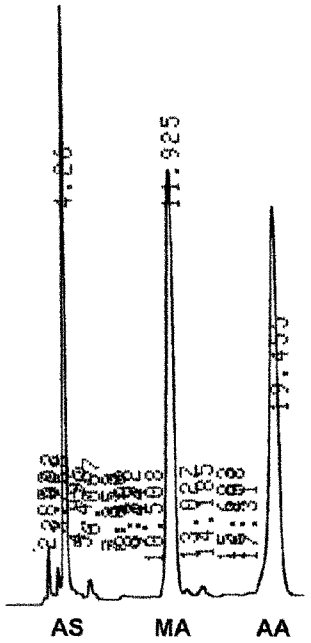

Fig. 2. HPLC chromatogram of TECA (AS, MA, and AA). The absorbance was measured at 214 nm and the three major compounds (AS, MA, and AA) were separated in a single run. A flow rate of 0.8 mL/min resulted in 4.26, 11.92, and 19.45 minutes of retention time for AS, MA, and AA, respectively.

than PEG 400, propylene glycol, and labrafil. In LS system, almost the MA and AA in the given condition (50 mg of TECA in the water) seemed to be solved out in the concentration of 5% (w/v), and the solubility of AS increased up to 3150 μg/mL with increase of LS concentration (Fig. 3).

The aqueous solubility of AS, MA, and AA in anionic surfactant systems were shown in Fig. 4. The solubility of AS, MA, and AA in anionic surfactant systems was dramatically increased by the surfactants. However, there were little differences in solubilizing capacity among the anionic surfactants systems except Na-DOC, which had the highest solubilizing capacity for TECA (Fig. 4). In Na-DOC system, almost the AS, MA and AA in the given condition (50 mg of TECA in the water) were dissolved in the concentration of 1% (w/v).

conventional dosage form for the intramuscular in- of TECA, PG-based TECA solution (Madecassol®), 10 mg/mL of TECA due to poor solubilty. In our vever, both the nonionic surfactant, LS and factant, Na-DOC could solubilize TECA at 0 mg/mL of concentration. Especially Na- ier solubilizing capacity for AS than LS us, 1% Na-DOC with 10 mg/mL of TECA an optimal dosage form for hydrogel

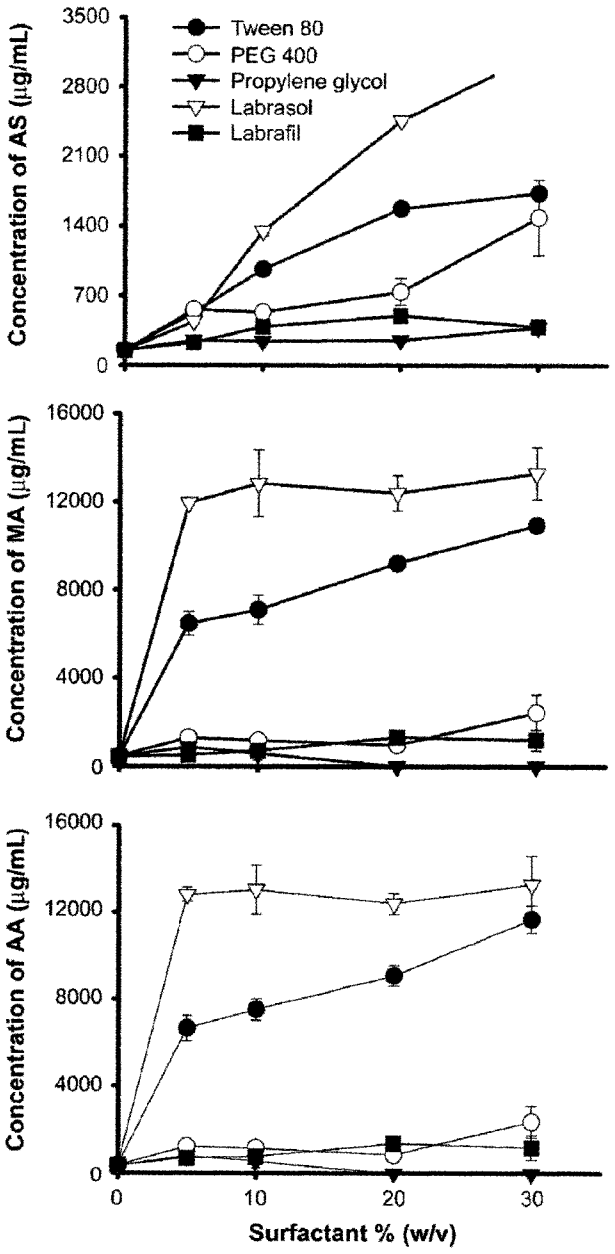

Fig. 3. Concentration of TECA (AS, MA, and AA) solubilized in various nonionic surfactants (mean ± SD, n = 3). Five nonionic surfactants (Tween 80, PEG 400, propylene glycol, labrasol, labrafil) were tested for water-soluble or miscible carrier system of TECA.

Differential scanning calorimetric studies on the coprecipitates of TECA and Na-deoxycholate

Typical DSC thermograms of the TECA/Na-DOC coprecipitate systems were depicted in Fig. 5. The main endo/exothermic peaks of AA, MA, and AS (line A, B, C, respectively) had an onset temperature of 233-243°C (cf. 230-233°C, Merck Index, 1996), which also appeared in the thermogram of the TECA/Na-DOC physical mixture systems (line F). Likewise, Na-DOC (line E) produced exothermic peak at about 200°C which al-

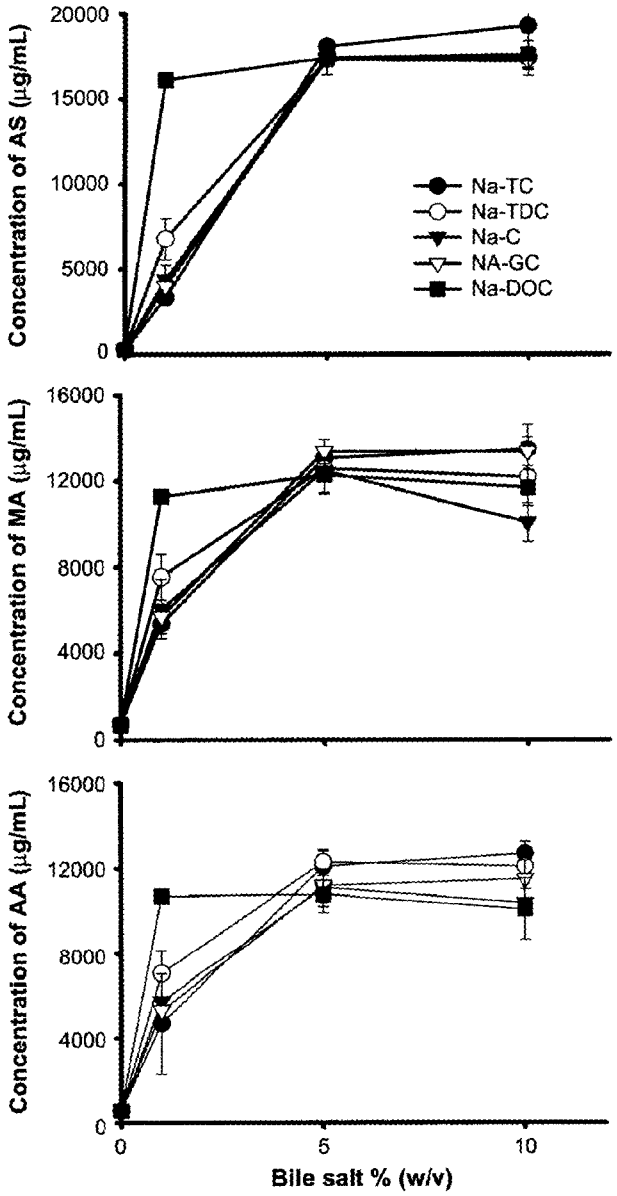

Fig. 4. Concentration of TECA (AS, MA, AA) solubilized in various anionic surfactants (mean ± SD, n = 3). Na-TC, Na-taurocholate; Na-TDC, Na-taurodeoxycholate; Na-C, Na-cholate; Na-GC, Na-glycocholate; Na-DOC, Na-deoxycholate

peared in the thermogram of the physical mixture of TECA/Na-DOC (line F). But in the TECA/Na-DOC coprecipitate system (TECA : Na-DOC = 1:0.5 w/w, line G), the peak of TECA appeared weakly and shifted to about 220°C which could not be observed in the TECA/Na-DOC coprecipitate system (TECA : Na-DOC = 1:1 and 1:2, line H and I). These results suggest that TECA in the physical mixture and 1:0.5 coprecipitate was partly in crystalline state, whereas either TECA in the 1:1 or 1:2 coprecipitate was in an amorphous state.

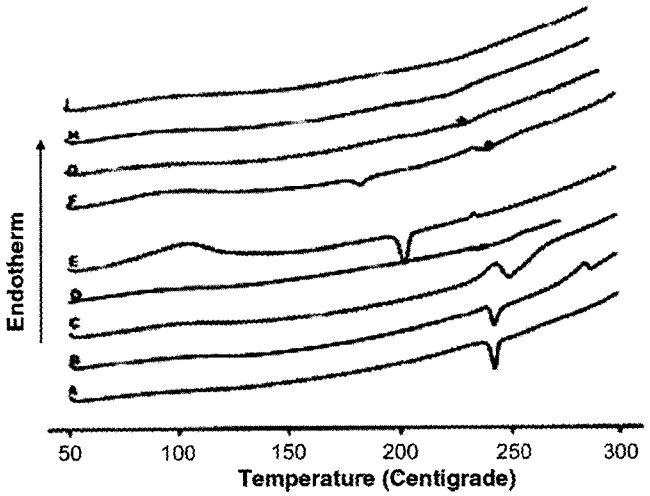

Fig. 5. DSC thermograms of TECA (AS, MA, and AA), Na-DOC, physical mixture and coprecipitates. A, AA; B, MA; C, AS; D, standard; E, Na-DOC; F, physical mixture (TECA : Na-DOC = 1:1 w/w); G, coprecipitate (TECA : Na-DOC = 1:0.5 w/w); H, coprecipitate (TECA : Na-DOC = 1:1 w/w); I, coprecipitate (TECA : Na-DOC = 1:2 w/w).

Physicochemical stability study of the gel formulation

1% (w/v) TECA gel solubilized with 1% (w/v) Na-DOC or 10% (w/v) LS were prepared and physical stability was assessed under light microscope. And the images of TECA/Na-DOC or TECA/LS gel formulation were taken immediately after preparation, 24 h, and one month of storage at room temperature. In TECA/LS gel formulation, a large number of TECA needles were observed after storage for 24 h (Fig. 6B), whereas no crystals or aggregate was observed after storage for one month in TECA/Na-DOC (Fig. 6C) similar to freshly prepared gel (Fig. 6A). For chemical stability study of the gel formulation, HPLC assay methods were used to estimate the TECA concentration in gel formulation (dissolved with 1% Na-DOC). The TECA remained chemically stable in the gel formulation for at least one month at room temperature. Neither additional peaks nor reduction of TECA content was made as shown in the chromatograms (Fig. 7).

Pharmacological study

The rats were healthy throughout the experimental period after the treatment of each formulation. The size of open wound was enlarged shortly after operation. However, the size decreased in all groups from 3rd to 9th day (data not shown). The size of wound area on 9th day showed significant difference by the treatment of the gel formulation of TECA/Na-DOC, i.e., 21% decrease compared to control, blank gel, or unsolved TECA gel formulation (Table I). So this gel formulation of TECA/Na-DOC was considered to be applicable to clinical use for wound healing effect.

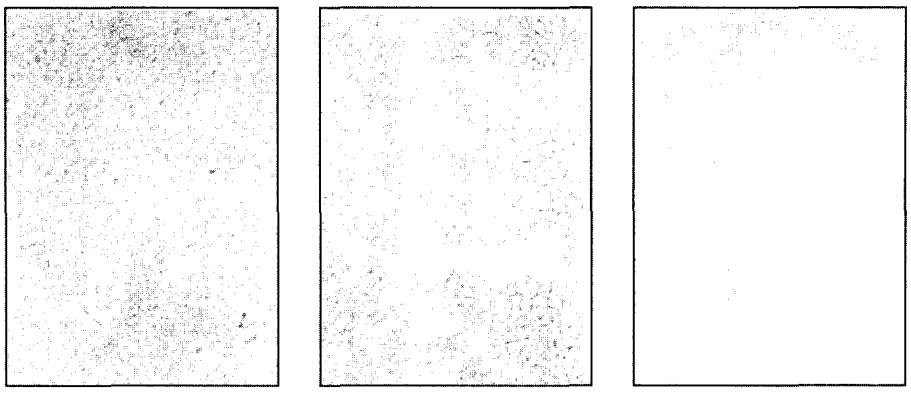

Fig. 6. Morphology of TECA gel formulation. TECA was incorporated into hydrogel of solubilized form, and observed under light-microscope, where TECA concentration is 1% (w/v). A) freshly prepared gel (all the TECA was dissolved in both LS and Na-DOC formulations), B) the gel containing 10% (w/v) LS after storage for 24 h at room temperature (crystalline is observed), and C) the gel containing 1% (w/v) Na-DOC after storage for one month at room temperature.

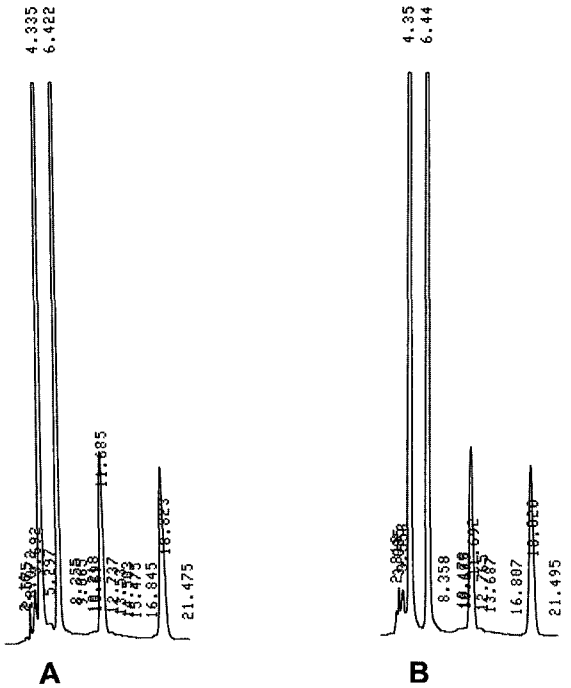

Fig. 7. HPLC chromatograms of TECA components in the gel formulation after storage for one month at room temperature. A) the fresh gel, B) the gel after the storage for one month

Table I. The effect of each formulation upon the wound area on the 9th postoperative day (mean ± SD, n=10 for each group)

Group	The Ratio of wound area
Control	0.354 ± 0.005
Blank gel	0.339 ± 0.007
Unsolved TECA gel	0.392 ± 0.008
TECA/Na-DOC gel	0.245 ± 0.008*

*; significant difference ($p < 0.05$)

CONCLUSION

In this study, various nonionic surfactants and bile salts as anionic surfactant were tested and screened for solubilizing TECA with a view to developing topical hydrogel type of ointment which is stable physicochemically, and has better pharmacological effects. The labrasol (LS) and Na-deoxycholate (Na-DOC) improved the solubility of the TECA the most among the nonionic surfactant and anionic surfactant systems, respectively. And in Na-DOC surfactant system, the ratio of 1:1 in weight was optimum for solubilization of the TECA through coprecipitate method. The gel formulation of the dissolved TECA/Na-DOC was physicochemically stable for at least one month. Pharmacologically the TECA formulation showed better wound healing effect. Therefore this gel formulation of TECA/Na-DOC was considered to be applicable to clinical use for wound healing effect.

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