

RESEARCH PAPER

Topical anti-inflammatory of a gel containing combined natural extracts

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Abstract

A gel contained natural extracts, composed of an endonuclease from *Micrococcus luteus* and extracts from echinacea, licorice root, *Centella asiatica*, algae and cucumber with hyaluronic acid added, was assessed on its anti-inflammatory effect. *In vitro* permeation was tested through excised porcine skin using Franz diffusion cells, showed relatively high total phenolic compounds permeation at 12 and 14 h. The gel was none-to-low cytotoxic to human forehead fibroblasts. SDS-PAGE identified a band of collagen type 1 at 250 kDa in which the test gel exposed to the cultured cells also showed the same band. Croton oil-induced ear edema and tail flick tests in ICR mice showed similar anti-inflammatory and analgesic effects of the test gel to piroxicam gel and hydrocortisone cream during 2-4 h with a reduced effect of the test gel at 6 h. In conclusion, a gel containing combined natural extracts exhibited potentials for anti-inflammation.

Introduction

To date, numerous natural extracts for topical use have been claimed to possess anti-inflammation effect. For topical application of natural extracts, methods used to evaluate such efficacy including cell studies and skin permeation are needed. A gel

contained combined natural extracts was assessed on its topical anti-inflammatory effect. The natural extracts which were combined in the gel can potentially act as anti-inflammatory including *Micrococcus luteus* endonuclease, Echinacea

extract, licorice extract, *Centella asiatica* extract, algae and cucumber extracts. *M. luteus* endonucleus was shown to repair UV-irradiated DNA damage (Setlow et al., 1970). Echinacea extract affects phagocytic, but not specifically acquired, immune system (Percival, 2000). Licorice extracts contains anti-inflammatory glabridin (Yokota et al, 1998). Extracts of *C. asiatica* were shown to be effective anti-inflammatory with wound healing activity (Shetty et al, 2006, Zheng and Qin, 2007). Extracts from algae contain polyphenols and polysaccharides which were shown to be active as photo-chemoprevention on the skin (Hwang et al, 2006). Hyaluronic acid is a gel forming agent which can support wound healing (Brown and Jones, 2005).

In vitro methods are recommended for ethical reasons and feasibility using excised and reasonably standardized (Colipa, 1997). The stratum corneum is the rate limiting step to skin permeation and, therefore, required as the barrier membrane in an *in vitro* permeation protocol to mimic human skin conditions. Acute inflammation is an immediate response to injuries or damage of cells or tissues. Certain responses at the vascular epithelium as well as leukocytes can lead to major changes which express as cardinal signs of inflammation, i.e. erythema, edema, hyperalgesia and pain or eventually loss of function. Development from an acute inflammation to a chronic stage usually depends on the nature of the affected tissue and the responses of the body in eliminating the exudates or damage tissues. Croton oil which contains 12-o-tetradecanoylphorbol-13-acetate (TPA) and other phorbol esters as the main irritant agents is able to activate a cascade pathway of

arachidonic acid production which can stimulate release of inflammatory mediators such as histamine, serotonin, prostaglandins and leukotrienes via the pathways which involve cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes. COX and 5-LOX inhibitors and corticosteroids show topical anti-inflammatory action in croton oil or TPA induced skin inflammation in animals (Dulcetti et al, 2004). Typical tail-withdrawal reflex in rat induced by heat or pressure can also elicit inflammation as well as pain. For determine analgesic activity tail flick or tail immersion method has been selectively employed for testing analgesics which acts at the central nervous system. Latency for tail immersion reflects the analgesic activity, and is used to differentiate central from peripheral analgesics (Vogel, 2002). Collagen produced in cultured human fibroblasts was able to be separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and are used to monitor collagen production *in vitro* (Goldberg et al, 1972).

This study was intended to screen anti-inflammatory activities in animal models with collagen production using human fibroblasts and *in vitro* permeation of a gel, containing *M. luteus* endonuclease mixed with natural extracts of Echinacea, licorice, *C. asiatica*, algae and cucumber, in comparison with a gel containing non-steroidal anti-inflammatory and a steroid cream.

Materials and methods

Materials: Standard collagen human type I (Sigma-Aldrich, U.S.A.), Dulbecco's modified eagle medium (D-MEM) was obtained from Invitrogen (U.S.A.), 75-cm² tissue culture flasks, 25-cm² tissue culture

flasks and 96-well plates from SLP Life Science (Korea), premium fetal bovine serum (FBS), penicillin/streptomycin/amphotericin B, trypsin/EDTA solution and MTT (3-(4,5-dimethylthiazol-2-yl-2-yl)-2,5-diphenyltetrazolium) from Invitrogen Cooperation (Gibco) (U.S.A.), phosphate buffer saline (PBS, Lonza, U.S.A.), electrophoretic equipment (Bio-Rad, Mini-Protean 3 Cell, U.S.A.), acetic acid (BHD Laboratory, England), acrylamide (Fluka, China), ammonium persulfate (Asia Pacific, Australia), 2-amino-2-(hydroxyl methyl)-1,3-propanediol (Tris, Sigma-Aldrich, Germany), bis-acrylamide (N,N'-methylenebisacrylamide, Fluka, U.S.A.), bromophenol blue (Ajax Finechem, Australia), Coomassie blue R250 (Bio-Rad, U.S.A.), 2-mercaptoethanol (Sigma-Aldrich, U.S.A.), protein molecular weight marker 10-250 kDa (Bio-Rad, U.S.A.), sodium dodecyl sulfate (SDS, VWR International, England) and tetramethyl ethylenediamine (TEMED) (Fluka, Belgium) were used as received.

Cultured cell study: Human fibroblast cells (subcultured from WL) were grown in 75-cm³ tissue culture flasks in complete media (10% FBS and 90% DMEM and 100 U/ml of penicillin, 100 µg/ml of streptomycin and 25 µg/ml of amphotericin B) in humidified CO₂ incubator (Water jacket CO₂ Incubator, U.S.A.) containing 5% CO₂ at 37°C. Cells were cultured to reach its plateau until about 10,000 cells/well in a 96-well plate.

Samples were freshly diluted from the test gel into phosphate buffer into a range of 10⁻³ - 10⁻⁶ dilution for treating the prepared cells which were then incubated at 37°C for 24 h. The incubated cells were subjected to MTT assay and the cultured media to collagen analysis.

MTT was added to the incubated cells and colorimetric detection of mitochondrial activity of the cells was measured at 550 nm (Bio-Rad, U.S.A.). Cell viability was estimated from the results obtained in comparison to the negative control (1% PBS in complete media) which was treated in the same well plates. Positive control (1% H₂O₂) confirmed cell responses of the same treatment.

Collagen type I standard was analyzed in comparison to the cultured media of the samples by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Acrylamide gel was prepared at 10% for use as separating and 4% as stacking gel and assembled to the gel apparatus. The samples were mixed with sample buffer containing SDS and heat the mixture at 100 °C for 5 min to denature the protein mix, followed by loading samples as well as the collagen type I standard and protein marker to the gel and running the electrophoresis at 25 mA for 45 min. The separated protein samples were subjected to 1 h staining with Coomassie Blue R-250, washing with destained solution and photographing of the gel.

Porcine ears were collected and pretreated within 2 h after sacrifice by soaking in 60°C water for 45 seconds. *In vitro* permeation was tested by using full-thickness skin from the ears of piglets (7-d old) as the barrier membrane and deionized water as the receptor medium controlled at 32 ± 1°C. The donor compartment was filled with 3 ml of the gel. Samples of the receptor medium were collected via sampling port and fresh medium of the same volume was replaced. The samples were subjected to determination of total phenolic content using the Folin-Ciocalteu

method (Singelton et al. 1999). The extracts were oxidized with Folin-Ciocalteu reagent, and neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 760 nm after 60 min. Using gallic acid as a standard total phenolic content (a range of the standard curves of 2.5-50 mg/L) was expressed as mg GA equivalent/ml, in comparison to calibration curves of the same day.

Animal study: Adult male ICR mice (weighing 30 ± 5 g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhonpathom, Thailand, and the animal handling were under supervision of the certified veterinarian of the Northeast Laboratory Animal Center, Khon Kaen University, Thailand. The study protocol has been reviewed and approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of the National Research Council of Thailand (AEKKU 83/2555). The animals were housed under natural conditions ($22 \pm 3^\circ\text{C}$, $50 \pm 5\% \text{RH}$, 12h/12h light-dark cycle) for 1 week prior to experimentation. Experiments were carried out on groups of 6 with free access to food and water in a Heating Ventilating and Air Conditioning (HVAC) system (Northeast Laboratory Animal Center, 2010).

Tail-flick test of the mice was conducted in randomly assigned groups ($n = 6$ each) of the test gel, 0.5% piroxicam gel and 1% hydrocortisone cream. The distal part of the tail (about 5 cm) of each mouse was topically applied with 0.1 g of the assigned topical preparation, left to dry for 60 min. The tail was subjected to expose to heat by

immersion in water controlled at $50 \pm 1^\circ\text{C}$. Tail-flick latency was to determine the time when animals flick the tails away from the hot water. Before administration of the treatments, each mouse was tested to measure a baseline time of response. The reaction times or tail-flick latencies were determined at 1, 2, 4 and 6 h after treatment of each drug. Anagesic activity was estimated as a percentage of change of tail flick latency from the base line level, as follows: (Yu et al, 2000).

$$\% \Delta \text{ latency} = \frac{L_{\text{after}} - L_{\text{before}}}{L_{\text{before}}} \times 100$$

Where L_{after} = tail flick latency after treatment and L_{before} = tail flick latency before treatment.

Croton oil-induced ear edema of each mouse was obtained by applying 0.5 mg/cm² of croton oil, i.e. 0.01 ml of 5% croton oil in acetone, to the posterior region of right ear, while the same amount of acetone onto the left ear used as within (negative) control. About 1 min after the pretreatment, each treated area was topically applied with each assigned topical preparation, i.e. the test gel, 0.5% piroxicam gel or 1% hydrocortisone cream. Thicknesses of both left and right ears at the pretreated areas were measured at predetermined time intervals using a digital vernier caliper (Mitutoya, Japan) at 1, 2, 4 and 6 h after treatment of each drug. Anti-inflammatory activity was expressed as a percentage of edema inhibition (%inhibition) of each mouse using an equation (Manconia et al., 2009), as follows:

$$\% \text{ inhibition} = \frac{T_{\text{control}} - T_{\text{test}}}{T_{\text{control}}} \times 100$$

In vitro testing of a topical formulation

where T_{control} = ear thickness at negative control treatment (left ear, mm) and T_{test} = ear thickness at treated site after treatment (right ear, mm).

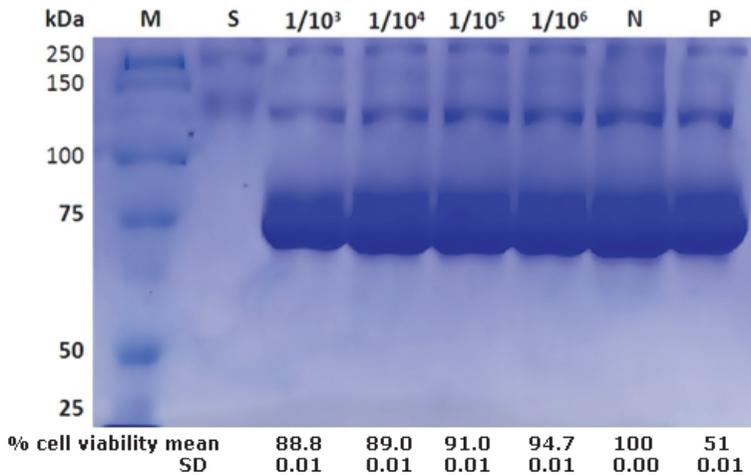


Figure 1 SDS-PAGE of samples of cultured media of human fibroblasts after 24 h-treated with dilutions of the test gel of $1/10^3 - 10^6$ in lanes 3- 6, compared with the protein maker (M, lane 1), standard collagen type I (S, lane 2), negative control (N, lane 7) and positive control (P, lane 8). Mean and SD of cell viability (%) of the human fibroblasts was displayed at the relevant lane.

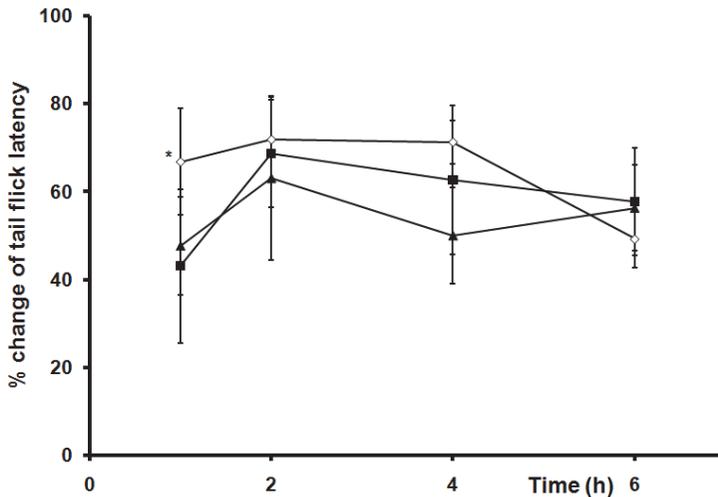


Figure 2 Changes in tail-flick latency after topical applications of the test gel (◇), hydrocortisone cream (▲) and piroxicam gel (■) using tail flick test in mice (* $p < 0.05$ compared to hydrocortisone cream).

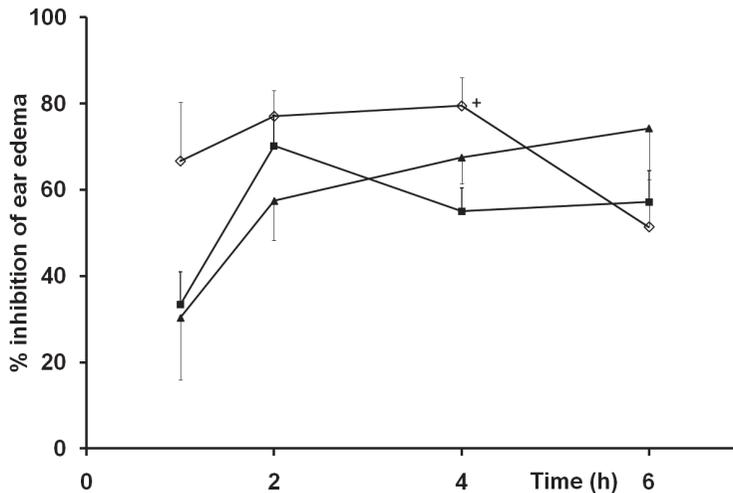


Figure 3 Croton oil-induced ear edema tested in mice after topical applications of the test gel (◇), hydrocortisone cream (▲) and piroxicam gel (■) (n = 6 each). (* $p < 0.05$ compared with piroxicam gel).

Results

Cytotoxicity test by MTT indicates non-corrosive effect from the test gel with combined natural extracts in various concentrations from 10^{-3} – 10^{-6} dilutions to human forehead fibroblasts. Also, SDS-PAGE showed significant increases of proteins at 75, 125, and 250 kDa as shown in Figure 1. The protein bands at 125 kDa from the samples were similar to the standard collagen type I. The intensities of the 125 kDa were reduced when cell viability was lower than 90%, suggesting that collagen type I could be related to the function of the cultured cells. However, more studies are needed to quantitatively monitor for confirmation.

Analgesic activity tested by hydrocortisone cream showed a significant increase in changes of tail flick latency from negative control at about 60% during 1-4 h which was decline at 6 h. Hydrocortisone cream

and piroxicam gel showed tail flick latency increase at 2-6 h. In comparison to the positive controls, tail flick latency of the test gel was significant different from that of hydrocortisone cream at 1 h ($p < 0.05$) but not at other duration. However, a drop trend of the test gel at 6 h should be noted.

Croton oil contains TPA which mediates inflammatory chemicals as well as COX enzymes in which corticosteroids like hydrocortisone and NSAID like piroxicam show topical anti-inflammatory. Hydrocortisone cream could significantly inhibit ear edema at 2, 4, and 6 h. This was also the case with piroxicam gel. The test gel showed anti-inflammation with ear edema inhibition of 67, 77, 80 and 51%, at 1, 2, 4 and 6 h, respectively. Results from the test gel showed only one significant difference from that of piroxicam gel at 4 h ($p < 0.05$).

In vitro permeation of the test gel could detect 15.9 ± 8.7 and 25.1 ± 13.5 mgGA equivalent/ml of the test gel at 12 and 24 h. This information suggests potential permeation of the polyphenols from the combined herbal components through the skin. Since the determination of total phenolic content by UV spectrophotometry is not highly sensitive, so the receptor media collected at less than 12 h of the study did not give reliable value, thus further studies by using highly sensitive instruments might give more conclusive results.

Conclusion

The test gel contained natural extracts, composed of an endonuclease from *Micrococcus luteus* and extracts from echinacea, licorice root, *Centella Asiatica*, algae and cucumber with hyaluronic acid showed anti-inflammatory potentials *in vitro* and in animal tests. *In vitro* permeation through excised porcine skin showed relatively high total phenolic compounds permeated. About 90% of the human forehead fibroblast cells remained viable upon exposure to up to the concentration of the gel as high as 1%. It is unlikely that the level of permeation to reach the viable cells would be as high as 1% of the test gel, thus, the test gel should be concluded as a none-to-low cytotoxic effect of this topical product. SDS-PAGE using collagen type I as the standard identified at 250 kDa showed similar result when a 10^{-6} dilution of the test gel was exposed to the cultured cells, suggesting the existence of collagen. Croton oil-induced ear edema and tail flick tests in ICR mice showed similar anti-inflammatory and analgesic effects of the test gel to piroxicam gel and hydrocortisone cream during 2-4 h. However, a tendency of the reduction of the anti-inflammatory

and analgesic effects of the test gel at 6 h was observed, suggesting that the test gel is not a prolonged-release product or the effect of natural extracts is transient. It is concluded combined natural extracts in a gel formulation exhibited potentials for anti-inflammation.

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