Table 1 Composition of crude drugs of *Jatyadi Taila*[4-7]

Sanskrit Name	Nomenclature	Parts of use	Amount	Effects*
Jati	*Jasminum officinale* Linn. var. *grandiflorum* Bailey.	Leaf	10.66 g	(a), (c)
Nimba	*Azadirachta indica* A.Juss.	Leaf	10.67 g	(a), (c)
Patola	*Trichosanthes dioica* Roxb.	Whole	10.68 g	
Naktamala (karanja)	*Pongamia pinnata* (Linn.) Merr.	Leaf	10.69 g	(c)
Siktha (madhucchista)	beeswax	-	10.70 g	
Madhuka (yasti)	*Glycyrrhiza glabra* Linn.	Root	10.71 g	(a)
Kustha	*Saussurea lappa* C.B.Clarke	Root	10.72 g	(a)
Haridra	*Curcuma longa* Linn.	Root	10.73 g	(a), (b), (c)
Daruharidra	*Berberis aristata* DC	Stem	10.74 g	(b)
Katurohini (katuka)	*Picrorhiza kurroa* Royle ex Benth.	Rhizome	10.75 g	(a), (c)
Manjistha	*Rubia argyi* (H.Lév. et Vaniot) H.Hara ex Lauener	Stem	10.76 g	
Padmaka	*Cerasus cerasoides* (D.Don) S.Y.Sokolov	Heart Wood	10.77 g	(a)
Lodhra	*Symplocos racemosa* Roxb.	Stem Bark	10.78 g	(a), (b)
Abhaya (haritaki)	*Terminalia chebula* Retzius	Pericarp	10.79 g	(a), (b), (c)
Nilotpala (utpala)	*Nymphaea nouchali* Burm.f.	Flower	10.80 g	
Tutthaka (tuttha)	Copper sulfate	-	10.81 g	(c)
Sariva (sveta sariva)	*Hemidesmus indicus* R.Br.	Root	10.82 g	(a), (b)
Naktamala (karanja)	*Pongamia pinnata* (Linn.) Merr.	Seed	10.83 g	(b), (c)
Taila	*Sesamum indicum* Linn.	Oil	768 g	
Paniya	water		3.072 l	

*(a) Cooling or antipyretic effect; (b) anti-inflammatory effect; (c) disinfectant effect.

known that the IL-8 produced by keratinocytes enhances migration of neutrophil into injury sites as well as dermal inflammation.[10] Indeed, a typical steroidal anti-inflammatory agent such as dexamethasone strongly suppresses IL-8 production form keratinocytes.

Furthermore, the activities of *Jatyadi Taila* in NHEK were compared with those of original oils based on Kampo ointments (shiunko and chuoko) that have been used for anal fistula treatment in Japan. In Ayurveda, there is a principle of using plants for medical treatments that are grown in the environment where the target people live. Thus, we aimed to develop an original *Taila* made from Japanese plants that is suitable for Japanese people. Therefore, we focused on shiunko and chuoko and prepared a *Taila* using the crude drugs of these Kampo ointments.

Materials and Methods

Crude drugs: Small pieces of Lithospermi Radix (*Lithospermum erythrorhizon* Siebold et Zucc., lot: 91S0215), Angelicae Radix (*Angelica acutiloba* Kitagawa, lot: 8BJ1159), turmeric (*Curcuma longa* L., lot: 9183015), and Phellodendri Cortex (*Phellodendron amurense* Ruprecht, lot: 9291008) were purchased from Uchida Wakanyaku Co. (Tokyo, Japan). These crude drugs are compliant with JP 15.[11] Sesame oil (MD-24, MG-27) was purchased from Kosakai Seiyaku Co. (Tokyo, Japan). *Jatyadi Taila* was purchased from Nagarjum Pharmaceuticals Ltd. (Delhi, India).

Materials: Asian NHEK and serum-free medium for culturing NHEK were obtained from KOJ (Tokyo, Japan). A Human IL-8/NAP-1 ELISA Kit was obtained from eBioscience (San Diego, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Nacalai Tesque (Kyoto, Japan). Phorbol 12-myristate 13-acetate (TPA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was purchased from Takara-Bio (Shiga, Japan). Sodium dodecyl sulfate

(SDS), Tween 20, and the other regents were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Preparation of test samples: Besides Ayurvedic *Jatyadi Taila*, six kinds of oil based on chuoko and shiunko were prepared according to the descriptions in "The Ayurvedic formulary of India"[4] as described below (Table 2). Briefly, each powdered herb (12.5 g, filtered through a wire mesh of 850-μm diameter) was mixed with 400 ml of distilled water, and boiled for 30 min in a stainless-steel vessel using a gas heater with a low flame to reduce the volume by one-fourth. The decoction was then added to 100 ml of sesame oil that had previously been heated to 100℃ and cooled. The oil mixture was heated at 95-103℃ for 45 min until all the water had evaporated. After the heating, the oil mixture was filtered through gauzes, and the filtrate was allowed to cool at room temperature.

Table 2 Preparation of original oils based on chuoko and shiunko for *Kshara Sutra*

Sample code	ingredients
CO	*Curcuma longa* and sesame oil
PO	*Phellodendron amurense* and sesame oil
CPO	*Curcuma longa, Phellodendron amurense* and sesame oil
LO	*Lithospermum erythrorhizon* and sesame oil
AO	*Angelica acutiloba* and sesame oil
LAO	*Lithospermum erythrorhizon* , *Angelica acutiloba* and sesame oil
O	sesame oil (heated < 100℃)
J	*Jatyadi Taila* (purchased from India)

Fractionation of oil samples: To identify effective fractions, the oil samples and *Jatyadi Taila* were subjected to column chromatography on silica gel (10×70 mm) using an organic solvent system. Briefly, each oil sample (average, 1.57 g) was absorbed by silica gel and applied to column chromatography on silica gel using n-hexane/ethylacetate (EtOAc) (10:1 v/v; elution volume: 20 ml), 100% EtOAc (elution volume: 20 ml) and 100% methanol (20 ml) to give three corresponding fractions. Individual fractions were obtained after removal and evaporation of the respective solvent.

Measurement of IL-8 production in cultured NHEK: NHEK were maintained in serum-free medium for NHEK under 5% CO_2 at 37℃. NHEK were cultured in 96-well plates at 1×10^4 cells/well until subconfluence, and then preincubated with different concentrations of oil samples in keratinocyte basal medium without supplements for 48 h. After the incubation, TPA (10 ng/ml in ethanol) was added to each well to induce IL-8 production in the culture supernatants. The culture supernatants were collected after 48 h of stimulation with TPA, and measured for their levels of IL-8 by ELISA according to the manufacturer's instructions. In other cases, the cell viabilities were also measured by the MTT assay. Briefly, 20 μl of MTT solution (5% w/v in PBS) was added to the cells and incubated for 5 h at 37 ℃. After removal of the culture medium, the crystals of MTT-formazan were solubilized with 20% w/v SDS in 0.01 N HCl aqueous solution in a solution of dimethylsulfoxide (1:1 v/v). The absorbance was measured at 570 nm using a microplate reader, and the cell viability was calculated by comparing the absorbance with that of the non-treated control culture.

Statistical analysis: The mean ratios of cytokine production and cell viability were indicated as percentages of the controls, and determined from two or three independent experiments. Data are shown as the mean ± SD. Statistical significance was determined by Dunnett's multiple test after one-way analysis of variance (ANOVA) in comparison with the controls using statistical analysis software (KaleidaGraph™ ver. 4.00; Synergy Software, Reading, PA, USA). Differences were considered significant for values of $p<0.05$.

Results and Discussion

Although the Ayurvedic oil *Jatyadi Taila* is thought to be clinically effective for wound healing, there is no pharmacological evidence for its efficacy *in vivo* or *in vitro*. Therefore, we investigated how *Jatyadi Taila* affects biological functions, including cell viability and IL-8 production, in cultured NHEK *in vitro*.

First, we investigated the effects of *Jatyadi Taila* on the cell viability and IL-8 production in cultured NHEK co-stimulated with TPA. As shown in Fig. 1a,

the cell viability of NHEK treated with *Jatyadi Taila* at a concentration of 0.1% v/v was significantly reduced by 63.4%.

IL-8 is a chemoattractant cytokine produced by a variety of tissues as well as epidermal keratinocytes. It is well known that this cytokine attracts and activates inflammatory cells, such as neutrophils, in inflammatory regions.[9-10] Thus, studying the suppressive activity of IL-8 production by tested samples in cultured skin keratinocytes is a beneficial approach to elucidate their protective activities of skin damage. The level of IL-8 production in cultured NHEK co-stimulated with TPA was not affected by treatment with *Jatyadi Taila* (*J*) at the same concentration (Fig. 1b, J).

Next, we evaluated the effects of the original oils present in Kampo ointments such as chuoko and shiunko, which have been clinically applied for various types of skin damage as well as anal fistulas, for the same activities in cultured NHEK cells. Oils made from the crude drugs constituting chuoko or shiunko were made in accordance with Ayurvedic methods, and each oil at 0.1% and TPA were added to NHEK. As shown in Fig. 1a and 1b, the oil of turmeric with sesame oil (CO) significantly suppressed IL-8 production by 78.0%, and also decreased the cell viability of NHEK by 30.9% (Fig. 1, CO). The administration of Phellodendri Cortex with sesame oil (PO) showed a tendency to increase IL-8 production without increasing the cell proliferation (Fig. 1, PO). However, these activities were counteracted by the mixed oil of these crude drugs with sesame oil (Fig. 1, CPO).

On the other hand, the oil made from the crude drugs in shiunko did not show any significant effects on IL-8 production (Fig. 1b). The oils from Lithospermi Radix (LO) and Lithospermi Radix and Angelicae Radix (LAO) at a concentration of 0.1% slightly increased the ratios of cell viability in cultured NHEK (Fig. 1a, LO and LAO, respectively).

Fractions from the oils, including *Jatyadi Taila*, eluted by organic solvents, n-hexane/EtOAc (10:1), 100% EtOAc and 100% methanol, were tested for their influences on the cell viability and IL-8 production in cultured NHEK. As shown in Fig. 2, two different fractions (n-hexane/EtOAc=10:1 and 100% EtOAc) from sesame oil (O) did not affect the cell viability of NHEK, while the fraction eluted by methanol weakly, but sig-

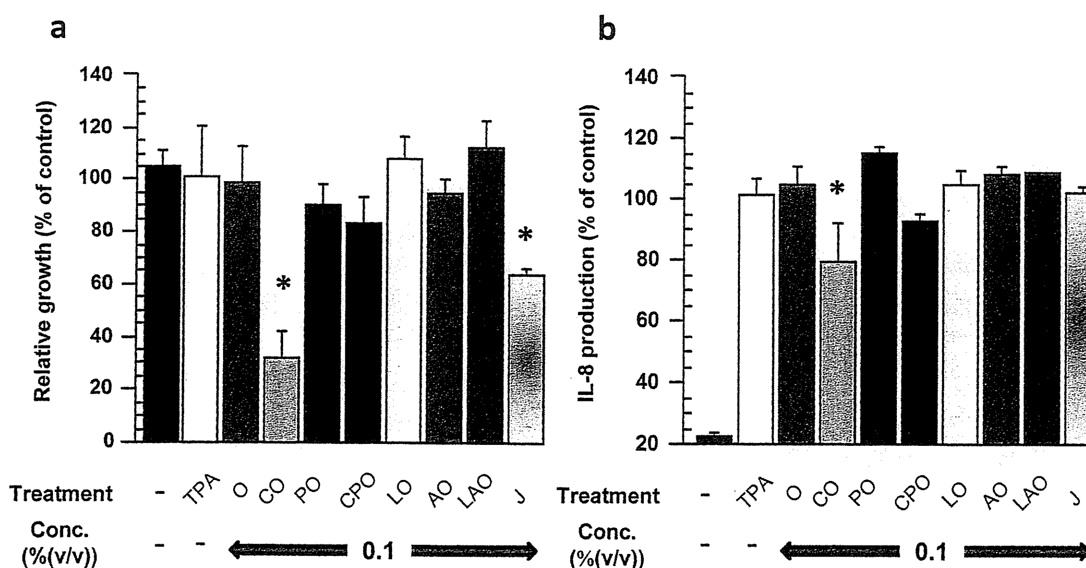

Fig. 1 Effects of oils made from the original crude drugs and *Jatyadi Taila* on cell proliferation and IL-8 production in cultured NHEK. Cells with or without treatment with the oils at a final concentration of 0.1% v/v were cultured for 48 h and co-stimulated with TPA (80 nM). After the incubation, the cell viability (a) and IL-8 secreted into the culture supernatants (b) were measured by the MTT assay or ELISA, respectively. *$p<0.05$ compared with the non-treated control culture. n=3. CO: *Curcuma longa* and sesame oil, PO: *Phellodendron amurense* and sesame oil, CPO: *Curcuma longa, Phellodendron amurense* and sesame oil, LO: *Lithospermum erythrorhizon* and sesame oil, AO: *Angelica acutiloba* and sesame oil, LAO: *Lithospermum erythrorhizon, Angelica acutiloba* and sesame oil, O: sesame oil (heated < 100 ℃), J: *Jatyadi Taila* (purchased from India).

nificantly, enhanced the proliferation of NHEK at a concentration of 0.1% in medium. The methanol fraction of CO strongly reduced the cell viability of NHEK (Fig. 2, CO-3). The fraction of PO eluted by methanol also significantly reduced the cell viability, although its activity was lower than that of CO (Fig. 2, PO-3). The methanol fraction of LO significantly increased the cell proliferation by 1.2-fold in contrast to the results obtained for the other samples. Despite the cytotoxicity of *Jatyadi Taila,* the three fractions from this oil did not show any significant toxicity against NHEK (Fig. 2, J-1-3).

We also evaluated the effects of the fractions on the IL-8 levels in cultured NHEK co-stimulated with TPA. Overall, the fractions from all oils containing the crude drugs as well as *Jatyadi Taila* suppressed the level of IL-8 production in NHEK. Although *Jatyadi Taila* did not inhibit the IL-8 production, the methanol fraction of *Jatyadi Taila* decreased IL-8 production by >70% (Fig. 3, J-3). The levels of IL-8 production in NHEK were significantly reduced by adding both the Methanol and EtOAc fractions of CO (Fig. 3, CO-3).

Jatyadi Taila is an oil remedy that contains 10 kinds of cooling or antipyretic herbs, six kinds of anti-inflammatory herbs, and eight kinds of disinfectant herbs (Table 1). We previously learned that the oil remedy *Jatyadi Taila* reduced inflammation and hastened recovery of the affected part of an anal fistula after *Kshara Sutra* ligation at Kshara Sutra Hospital in India (personal communication, Dr M.B. Kathia, Kshara Sutra Hospital, Mumbai). To investigate whether this Ayurvedic oil affects epithelial tissue NHEK, the cell growth and cytokine production in NHEK were measured under TPA-stimulatory conditions. However, direct administration of *Jatyadi Taila* showed cytotoxicity and did not affect the TPA-induced IL-8 production in cultured NHEK. The level of IL-8 production was strongly reduced by the methanol fraction of *Jatyadi Taila* without any significant cytotoxicity, suggesting that the anti-inflammatory active constituent was still present in this oil, and that the oil also contained not only suppressants of IL-8 production but also counteractive constituents.

To further apply Kampo ointments as Ayurvedic oils, we made six kinds of Ayurvedic oils based on the crude drugs of chuoko and shiunko, and investigated their effects on the cell viability and cytokine production in cultured NHEK. The typical Kampo ointment chuoko[12] is composed of turmeric (anti-inflammatory and antitumor activity[13]), Phellodendri Cortex (antioxidant activity[14]), beeswax, and sesame oil, and is used as a treatment for dermatitis owing to its anti-inflammatory, antipyretic, and anti-analgesic effects. Shiunko[15] is another Kampo ointment composed of Lithospermi Radix

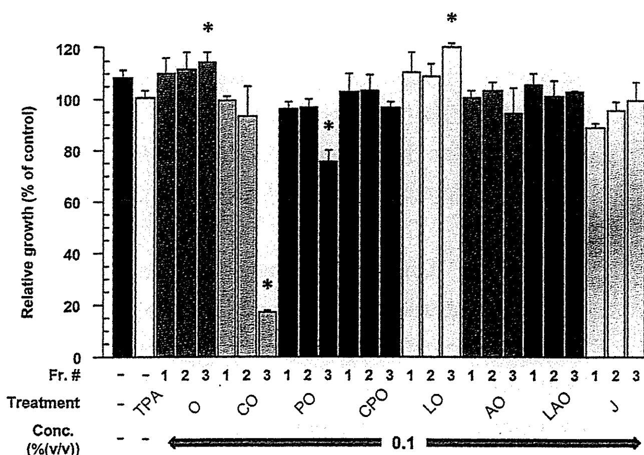

Fig. 2 Effects of three fractions of the original oils or *Jatyadi Taila* on cell proliferation in cultured NHEK. Fractions separated from the oils (final concentration, 0.1% v/v) were added to the cell culture system with or without TPA for 48 h. After the incubation, the cell viability was measured by the MTT assay. **1**: Fraction eluted by EtOAc/n-hexane (10:1); **2**: fraction eluted by EtOAc; **3**: fraction eluted by methanol. *$p<0.05$ compared with the non-treated control culture. $n=3$.

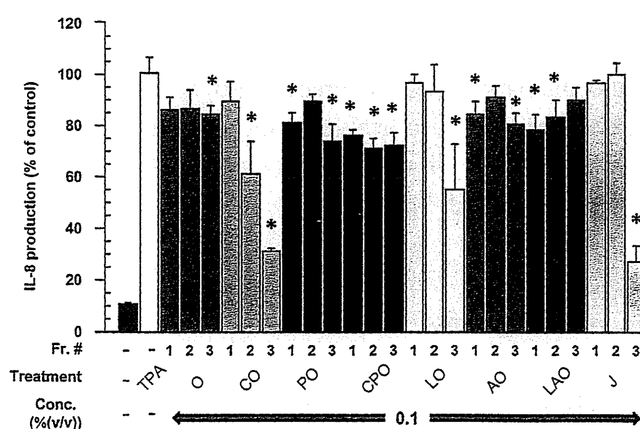

Fig. 3 Effects of three fractions of the original oils or *Jatyadi Taila* on IL-8 production in cultured NHEK. Fractions separated from the oils (final concentration, 0.1% v/v) were added to the cell culture system with or without TPA for 48 h. After the incubation, the IL-8 level in the culture supernatant was measured by ELISA. **1**: Fraction eluted by EtOAc/n-hexane (10:1); **2**: fraction eluted by EtOAc; **3**: fraction eluted by methanol. *$p<0.05$ compared with the non-treated control culture. $n=3$.

(anti-bacterial activity,[16] healing acceleration,[17] and anti-inflammatory activity[18]), Angelicae Radix (antipyretic and anti-inflammation activity[19]), beeswax, lard, and sesame oil, and is used to treat skin troubles, such as ulcer, eczema, burns, and piles. Among the tested oils, CO as the basis of chuoko suppressed IL-8 production in NHEK accompanied by significant cytotoxicity. Interestingly, the oil made from turmeric and Phellodendri Cortex (CPO) counteracted the cytotoxicity and suppression of IL-8 production by turmeric. Furthermore, although the original oils (PO, CPO, LO, AO, and LAO) had no activities, suppressive effects on IL-8 production were observed when NHEK were treated with their organic solvent-soluble fractions. It can therefore be proposed that Ayurvedic oils based on Kampo ointments, CPO and LAO, would be applicable for anal fistula surgery after *Kshara Sutra* ligation. In addition, LO moderately enhanced the proliferation of NHEK, while CPO did not, indicating that LO or LAO might have any kind of constituents for hastening the recovery of the skin damage.

Since the oil *Jatyadi Taila* has been optimized for Indian people, we developed an original *Taila* for Japanese people. And we examined the pharmacological effects of *Taila* itself for developing the substitute of it, because the effect showed up by the synergistic effect of various ingredients in Ayurvedic treatments. To obtain more beneficial activity of the oils, we are currently investigating the effects of the oils on the dose-response and combination ratios of crude drugs in the same culture system, and also analyzing the comprehensive active ingredients of the oils as well as the original Ayurvedic *Jatyadi Taila*.

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