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Topical application of solubilized *Reseda luteola* extract reduces ultraviolet B-induced inflammation *in vivo*

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ABSTRACT

We investigated the skin tolerance and anti-inflammatory potential of a nanoparticular solubilisate of a luteolin-rich *Reseda* extract (s-RE) in two independent studies *in vivo*. *Reseda luteola* extract containing 40% flavonoids was solubilized with polysorbate, resulting in product micelles with a diameter of 10 (±1.5) nm. Standardized inflammation was induced by irradiating test areas on the back of healthy volunteers with defined doses of ultraviolet B (UVB). In the first study different concentrations of s-RE were tested in 10 volunteers to evaluate dose-dependency of anti-inflammatory effects of s-RE. In the second randomized, double-blind, placebo-controlled study a defined concentration of s-RE (2.5% w/w) was tested in 40 volunteers in comparison to the vehicle (glycerol) and hydrocortisone (1% w/w). s-RE dose-dependently reduced UVB-induced erythema when applied 30 min before irradiation. To a lesser extent, topical application of s-RE after irradiation also reduced UVB-induced erythema. s-RE was as effective as hydrocortisone, whereas the vehicle had no effect. Occlusive application of s-RE on non-irradiated test sites did not cause any skin irritation. Due to excellent skin tolerance combined with potent anti-inflammatory properties s-RE bears potential especially for the prevention but also for the treatment of inflammatory skin conditions such as UV-induced erythema.

1. Introduction

Flavonoids have a marked anti-oxidative potential and were intensively studied for their pharmacological effects, since they are part of many plants and of our daily nutrition. Luteolin is a flavone (Fig. 1) which occurs in many medicinal plants. While luteolin is only a minor flavonoid component in food, high amounts can be isolated from peanut hulls and from *Reseda luteola* L. that has been used as a dyeing plant due to its high luteolin content since ancient times [1]. Luteolin shows less pro-oxidant potential and apparently has a better safety profile than the flavonol quercetin, the best studied flavonoid [1–3]. Because oxidative processes are a major factor involved in photoageing and the development of skin cancer, the anti-oxidant properties of luteolin might be of interest for developing anti-cancer strategies [4–7]. Moreover, luteolin displays numerous anti-inflammatory effects at micromolar concentrations which cannot be completely explained by its anti-oxidant capacities. During the last years several anti-inflammatory mechanisms of luteolin have been identified. To name a few, luteolin inhibits prostaglandin D2 (PGD-2) and leukotriene release from human mast cells [8,9], suppresses COX-2 and pro-inflammatory cytokine expression [10–13] as well as the nuclear factor kappa B (NFκB) pathway [14]. Luteolin also inhibits inducible nitric oxygen synthase (iNOS) expression and NO production, and displays DNA-protective effects [15]. In a recent paper we have extensively reviewed the various anti-inflammatory effects and mechanisms of luteolin [1]. *In vivo* skin penetration studies with alcoholic solutions of luteolin, apigenin and apigenin-7-O-β-glucoside showed that luteolin is not only absorbed at the skin surface, but penetrates into deeper skin layers [16]. However, this indirect evidence of penetration has not been confirmed by standard penetration studies or clinical trials in man so far.

Because *R. luteola* is a natural source of luteolin and is easy to cultivate, we developed a luteolin-rich extract from *R. luteola* for the prevention or treatment of inflammatory skin diseases (e.g. sunburn). Due to poor solubility of the *Reseda* extract a nanoparticular solubilisate of the extract (s-RE) was used. The primary goal of

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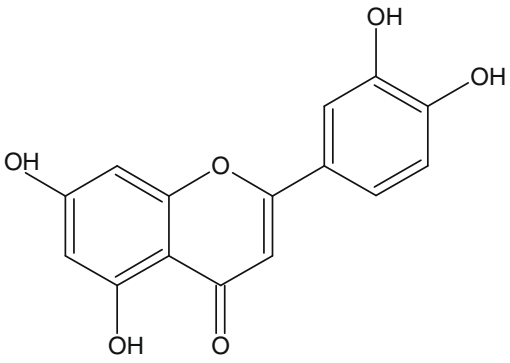

Fig. 1. Chemical structure of luteolin.

the clinical studies presented here was to investigate the reduction by s-RE of the ultraviolet B (UVB)-induced erythema *in vivo*. Additionally the skin tolerance of s-RE was assessed on non-irradiated skin.

2. Materials and methods

2.1. Isolation and characterization of R. luteola extract

R. luteola extract (Batch No. 0506.06) was provided from NIG (Magdeburg, Germany). In brief, the extract was obtained as follows: dried *R. luteola* from cultivation in Eastern Germany was subjected to a multistep extraction process (Fig. 2) in order to enrich the flavonoids. First, 1 kg dried plant material was pre-extracted exhaustively (7 times) with 7 l water to eliminate the bulk of unspecific water-soluble plant substances. In a second step the flavonoids were extracted 2 times from the residue with 7 l ethanol. Concentration and lyophilisation of the flavonoid-enriched solution resulted in 43.5 g dry extract with a flavonoid content of 32% (w/w). This residue was dissolved in 1.5 l ethanol and finally precipitated by addition of water, cooling and acidification. The resulting dry extract (31.5 g) was rich in flavones (40% w/w), especially luteolin (30% w/w), luteolin-7-O-glucoside (3% w/w) and apigenin (3% w/w) (see Fig. 3). The extract was analyzed on an analytical HPLC system consisting of a Hewlett Packard Series II 1090 Liquid Chromatograph equipped with a diode array detector E-3014 and a ChemStation for LC 3D. A Symmetry C-18, 3.5 μm, 150 mm × 4.6 mm (Waters Company, Germany) was used. Solvent A was acetonitril:H_2O:phosphoric acid (5:95:0.5, v:v:v), solvent B was acetonitril:H_2O:phosphoric acid (50:50:0.5, v:v:v). Elution was done with a linear gradient of 20–80% B in 35 min and 80% to 100% B in 1 min followed by an isocratic step of 100% B for 9 min. The flow rate was 0.7 mL/min, the injection volume 10 μL and detection was done at 350 nm. Luteolin, luteolin-7-O-glucoside, luteolin-3′-methylether and apigenin were purchased from Roth (Karlsruhe, Germany). Luteolin-3′,7-diglucoside was from HWI Analytik (Rheinzabern, Germany), luteolin-7-methylether was isolated from *Arnica* species as described [17] and luteolin-3′-O-glucoside was isolated from *R. luteola*. Identification was done by ^{1}H NMR and NMR data agreed with the literature [18]. A typical HPLC fingerprint of *R. luteola* extract is shown in Fig. 3. Stability testing using HPLC revealed that the flavonoids in s-RE are very stable in the solubilisate (<2% degradation after 1 year storage at rom temperature).

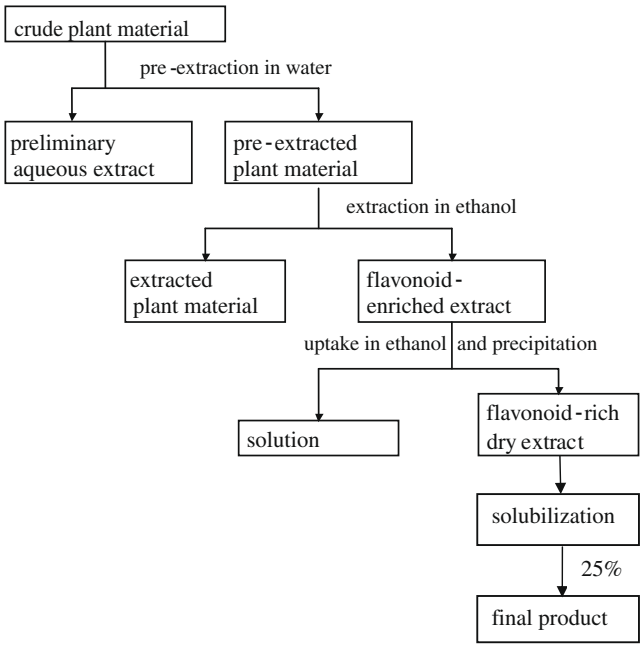

Fig. 2. Schematic flow chart of the *Reseda* extraction process.

2.2. Solubilization of Reseda extract

The dry extract obtained from *R. luteola* L. is poorly soluble in water. It was therefore processed by AQUANOVA AG (Darmstadt, Germany) with polysorbate to obtain a water-soluble solubilisate. The resulting NovaSOL *Reseda* solubilisate (batch No. EW0099, herein after referred to as s-RE) contained 10% (w/w) of the *Reseda* extract and had a mean particle size of 10 nm (±1.5) as measured with Nano.Ultra (Particle Metrix GmbH, Meerbusch, Germany). The solution was miscible with water in every measure resulting in a crystal clear liquid with a very low turbidity (1.4 FNU on a scale from 0 to 2000 FNU), as measured with a Lovibond PC Checkit (Tintometer GmbH, Dortmund, Germany).

2.3. Human volunteers and ultraviolet irradiation

50 healthy volunteers participated in the two separate studies. The study protocol was approved by the ethics committee of the University of Freiburg and written informed consent was obtained from all subjects. Inclusion criteria were healthy, non smoking persons of both sexes, age > 18 years, skin types II and III according to the classification of Fitzpatrick [19]. Exclusion criteria were skin types I or IV, allergies, skin diseases, photosensitivity, sunbed tanning, metabolic diseases, use of any drugs (except contraceptives), alcohol consumption, infections, pregnancy, breast feeding, and participation in other studies during the last 2 months. UV radiation between 270 and 400 nm, peaking at 310 nm was delivered from 10 fluorescent UV-B lamps (Philips TL20 W/12) (Philips GmbH, Hamburg, Germany), housed in a UV 800 unit (Waldmann GmbH, VS-Schwenningen, Germany) [20]. After determination of the minimal erythema dose (MED) the irradiation dose was individually calculated for each volunteer (2.0 MED in the first trial and 1.5 MED in the second trial) (Table 1). Background erythema (T0) was measured in all test areas before treatment using a Mexameter MX 16 (Courage & Khazaka Electronics GmbH, Köln, Germany) as described [20].

2.4. Study protocols of the clinical trials

In the first study, test areas on the backs of 10 volunteers were occlusively treated with three different concentrations of s-RE in duplicate panels (Table 1). After 30 min one panel of the test areas was irradiated with 2.0 MED UVB, the other panel was left unirradiated. We have chosen the 2.0 MED as irradiation dose because

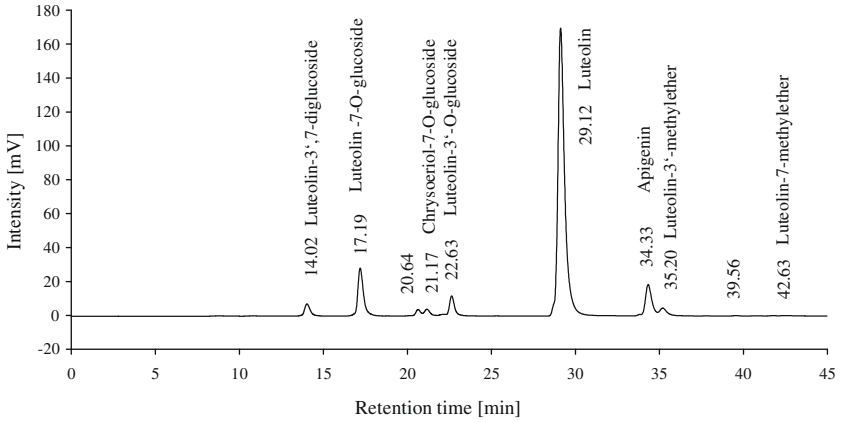

Fig. 3. HPLC fingerprint of the *R. luteola* extract.

Table 1
Basic information on the subjects and protocols of the two clinical studies.

	Trial 1	Trial 2
Subjects included	10 subjects	40 subjects
Sex (m/f)	5/5	20/20
Mean age	24 years	27 years
Test preparations	Serial dilution of s-RE in water (0.625%, 1.25%, 2.5% s-RE, corresponding to 6.25%, 12.5% and 25% of the solubilisate)	2.5% s-RE (corresponding to 25% of the solubilisate) in glycerol compared to 1% hydrocortisone and placebo (glycerol alone)
Study protocol		
Visit 1 (day 1)	UVB test	
Visit 2 (day 2)	Evaluation of the UVB test, determination of the MED Calculation of the specific UVB irradiation dose Photometric measurement of the test areas (T0)	
	Application of s-RE (30 min)	UVB irradiation (1.5-fold MED)
	UVB irradiation (2-fold MED)	Application of s-RE (24 h)
Visit 3 (day 4)	Photometric measurement of the test areas (T1) (48 h after UVB)	
Results	1.25% and 2.5% s-RE significantly superior to placebo (water)	2.5% s-RE as effective as 1% hydrocortisone and significantly superior to placebo (glycerol)

dose–response testing revealed the strongest effect of s-RE when applied on test areas irradiated with 2.0 MED (data not shown). 48 h after irradiation the erythema of all test areas was measured photometrically a second time (Table 1). The second study was performed in a randomized, placebo-controlled, double-blind manner as described [20]. One panel of test areas on the back of 40 volunteers was irradiated with 1.5 MED prior to treatment. We have chosen 1.5 MED in this study because earlier studies of our group have shown that this irradiation dose is optimal when applying test substances with moderate anti-inflammatory activity after irradiation [20]. The irradiated and the non-irradiated test areas were treated occlusively for 24 h with 2.5% of s-RE in comparison to the vehicle (glycerol) and the positive control (1% hydrocortisone). After 48 h (T1) photometric measurements of the test areas were performed.

2.5. Statistical analysis

The data from each trial were analyzed separately. Skin erythema measurements were performed before treatment (T0) and 48 h after irradiation (T1). The raw data (means of triplicate scans) were processed electronically and were checked for correct data transfer and plausibility. The erythema index was calculated by subtracting the time zero values (T0) from the readings after 48 h (T1). The major readout parameter in both trials was the reduction of the UV-induced erythema by s-RE. The second parameter was the skin tolerability of the test substances on the non-irradiated test sites. Statistical analysis was performed using the Friedman test and the Wilcoxon test for pairwise comparisons. P-values $\leqslant 0.05$ were considered significant and were indicated in the figures by a single asterisk ($*$).

3. Results

3.1. Dose-dependent reduction of UV-erythema by s-RE

In the first study 3 different concentrations of s-RE were investigated (Table 1). s-RE dose-dependently reduced the skin erythema, the 1.25% and 2.5% concentrations of s-RE being significantly superior to the vehicle (Fig. 4). The test substances were also applied occlusively on non-irradiated test sites for 24 h in a separate panel. There was not any skin irritation, i.e. an increase in the skin reddening due to the application of s-RE.

3.2. Modification of UV-erythema by s-RE compared to hydrocortisone

After establishment of anti-inflammatory concentrations of s-RE the second study evaluated the effect of 2.5% s-RE in comparison to 1% hydrocortisone as a standard anti-inflammatory agent. In contrast to the first trial the test substances were applied after UVB irradiation. Again, after 48 h skin erythema was measured photometrically and the erythema index was calcu-

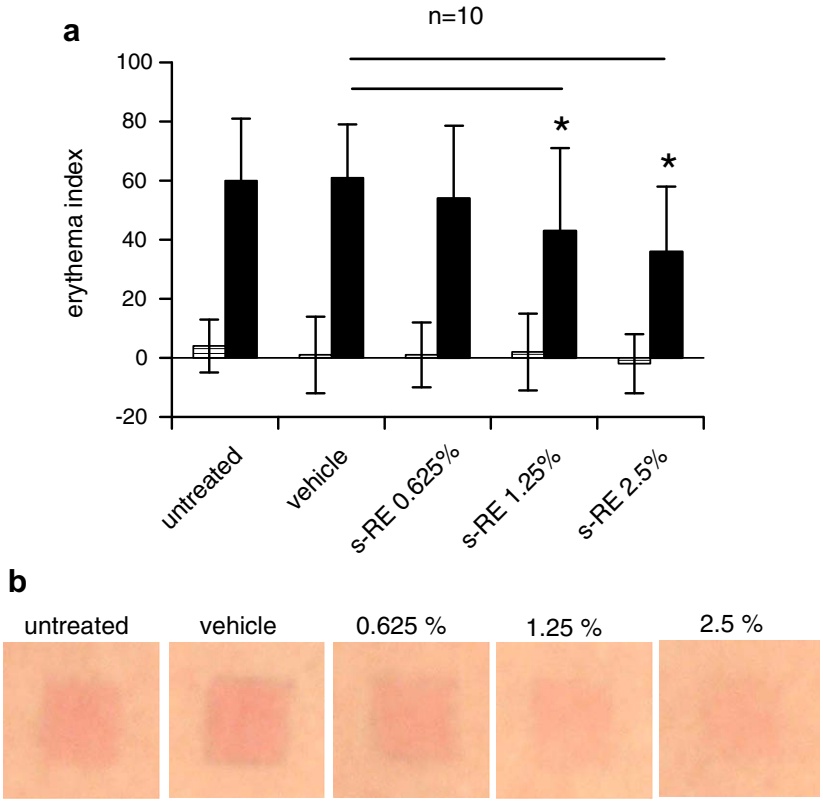

Fig. 4. Solubilized *R. luteola* extract (s-RE) dose-dependently reduces UVB-induced skin erythema. (a) Irradiated and non-irradiated test areas on the backs of 10 healthy volunteers were treated and mean ± SD are shown (∗, $p < 0.05$). Black bars, irradiated test panel; hatched bars, non-irradiated test panel. (b) Illustration of typical test reactions on the back of a volunteer.

lated after subtracting time zero readings. s-RE displayed a significant anti-inflammatory effect in comparison to the vehicle, to a similar extent as 1% hydrocortisone (Fig. 5). No skin irritating effect of the test preparations was observed on the non-irradiated test sites.

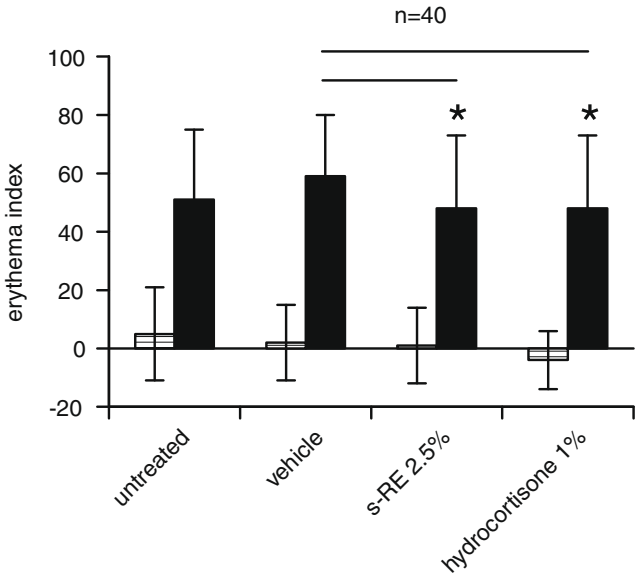

Fig. 5. Erythema reducing effect of 2.5% solubilized *R. luteola* extract (s-RE) compared to the vehicle (glycerol) and 1% hydrocortisone in the vehicle. Irradiated and non-irradiated test areas on the backs of 40 healthy volunteers were treated and mean ± SD are shown (∗, $p < 0.05$). Black bars, irradiated test panel; hatched bars, non-irradiated test panel.

4. Discussion

R. luteola L. is a plant whose main active ingredient, luteolin, has demonstrated anti-inflammatory effects in various *in vitro* and *in vivo* studies with animals [1]. Despite these experimental data, there are no clinical reports on the effectiveness of *R. luteola* L. or luteolin in humans. Therefore, the objective of the work presented here was to clinically assess anti-inflammatory properties of a luteolin-rich solubilized *Reseda* extract (s-RE) in a standardized test model in human volunteers *in vivo*. There are different methods to evaluate the anti-inflammatory potential of topical dermatologic preparations. The UV-erythema test is the most frequently used method in preclinical studies and screening tests for active compounds [20–22]. This test has proven especially useful in detecting compounds with corticosteroid-like properties [20].

In both studies presented here s-RE effectively reduced the UVB-induced erythema regardless if it was applied to the skin before or after irradiation. However, there is a discrepancy between the efficacy of s-RE in both trials. Compared to the vehicle 2.5% s-RE is more effective when applied before irradiation (first trial), even though a higher UVB dose has been used. This discrepancy may be explained by the different readout systems of both trials. In the first study s-RE was applied before irradiation. Therefore, the well known radical scavenging properties of luteolin certainly contribute to the reduction of the UV-erythema, because UV-erythema is largely caused by irradiation-induced reactive oxygen species. In the second study, when s-RE was applied after irradiation, anti-oxidative effects only play a minor role in the reduction of the erythema. In this system rather anti-inflammatory effects of

luteolin play a role, such as inhibition of NFκB and COX-2 [1,12–14]. From both studies, we can learn that because of its radical scavening activity s-RE bears great potential for preventing UV-induced erythema when applied before irradiation. On the other hand, to a lesser extent s-RE also may reduce UV-induced erythema after irradiation due to its anti-inflammatory activity. Although this effect is not as pronounced compared to application before irradiation it was significant and comparable to 1% hydrocortisone. The findings from both studies confirm the topical bioavailability of luteolin *in vivo* that has already been suggested in a previous penetration study in human volunteers [16]. The *Reseda* extract tested here is only poorly soluble in aqueous solvents. Therefore we have used an AQUANOVA-solubilisate in the present studies. The solubilisates are carrier solutions, which transport the active substance in so-called product micelles. They have several important advantages. First, even non-polar substances are rendered water soluble; second, the particle size of the resulting product micelles is very small (10 nm for s-RE) which enhances their ability to penetrate into deeper skin layers. Finally, they are stable in aqueous solutions with respect to pH and temperature. In the first study a dose-dependent inhibition of the UV-induced inflammation by s-RE could be demonstrated, and in the second study s-RE proved as effective as the anti-inflammatory 'gold standard' hydrocortisone. This illustrates that the active compound, i.e. luteolin, is not only released from the micelles, but also penetrates into the upper layers of the skin. The product micelles are produced using polysorbate as an emulsifier. One might speculate that the topical application of an emulsifier on the skin might cause irritation and inflammation. However, the skin tolerability of the *Reseda* solubilisate on the non-irradiated test sites was excellent. Even the sensitive photometric measurement of skin reddening did not reveal any irritation with any of the concentrations of the solubilisate. The skin tolerance of the solubilisate was comparable to that of the solvents, i.e. distilled water and glycerol. AQUANOVA-solubilisates thus exhibit excellent properties as carriers of active substances. Penetration studies with AQUANOVA vitamin E-solubilisates, retinol-solubilisates and coenzyme Q10-solubilisates revealed excellent penetration down to the basal layers of the stratum corneum for all topical formulations tested [23]. This is in accordance with other AQUANOVA-solubilisate bioavailability studies that have demonstrated effective uptake of micellized tocopherol by humans [24], of coenzyme Q by nematodes [25], and of coenzyme Q10 by mice [26].

It would be interesting to know if s-RE not only reduces UVB-induced inflammation, but if it also can reduce irradiation-induced DNA damage. Although we have not addressed this question in the present work, there are several lines of evidence that suggest that luteolin, the main compound in s-RE, displays DNA-protective effects and also might protect cells from irradiation-induced damage. First, luteolin displays DNA-protective effects against reactive oxygen species and melphalan-induced DNA damage in HMB-2 cells as shown by Horvathova et al. [15]. Second, luteolin prevents chemical-induced carcinogenesis in rats [7]. Finally, recent reviews have highlighted that luteolin generally bears great potential for cancer prevention and therapy [4,5].

In summary, we have shown here for the first time that topical application of a solubilized luteolin-rich extract from *R. luteola* (s-RE) effectively reduces UVB-induced skin inflammation. Due to its excellent skin tolerance, s-RE bears promising potential for the treatment and prevention of inflammatory skin conditions such as sunburn. Additionally, s-RE might protect DNA, collagen and other structural skin components from solar radiation-induced damage, which needs to be clarified in further studies.

Conflict of interest

C.M. Schempp and A. Wähling hold a patent on the production and dermatological use of luteolin-rich *Reseda* extract. The other authors declare no conflict of interest.

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