Isatis tinctoria L.-derived Petroleum Ether Extract Mediates Anti-inflammatory Effects via Inhibition of Interleukin-6, Interleukin-33 and Mast Cell Degranulation

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***Isatis tinctoria* L. (woad) has been used in medicine for centuries and has demonstrated anti-inflammatory effects. However, to date, no well-defined extracts with precise analysis of active substances have been developed. The aim of this study was to develop novel extracts of *Isatis tinctoria* L., and to characterize their active ingredients and anti-inflammatory properties. Various extracts of *Isatis tinctoria* L. were analysed for their active ingredients, and screened for anti-inflammatory effects using cyclooxygenase-2 activity assays. A petroleum ether extract was found to have the best effects, and was tested in a mouse model of acute allergic contact dermatitis. In the mouse model the petroleum ether extract resulted in significantly reduced ear swelling, oedema and inflammatory cell density. In mouse skin and human keratinocyte cultures, petroleum ether extract inhibited pro-inflammatory cytokine expression. Furthermore, human mast cell degranulation was significantly inhibited in LAD2 cell cultures. In conclusion, novel woad extracts were developed and shown to have anti-inflammatory properties in a contact hypersensitivity animal model and human keratinocytes. The production of such extracts and further characterization of their specific properties will enable determination of their potential dermatological effects in the treatment of inflamed and irritated skin.**

Key words: pruritus; atopic eczema; Isatis tinctoria L; anti-inflammatory agent; contact dermatitis.

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SIGNIFICANCE

The medicinal herb *Isatis tinctoria* (woad), which has been used in medicine for centuries, has demonstrated anti-inflammatory effects in recent studies. However, to date, no defined extracts with precise characterization of their active substance content have been developed. This study aimed to develop novel well-characterized extracts of woad, and to determine their anti-inflammatory properties in animal and human skin tissues. The production and precise characterization of such extracts will enable evaluation of their potential dermatological use in the treatment of irritated, pruritic skin.

The dye plant *Isatis tinctoria* L. (woad, family Brassicaceae) has been cultivated in Europe since ancient times. Woad is used for the manufacture of indigo dye (1) and has a long history of use as a medicinal herb in skin inflammation and infections (1–3). In recent studies woad extracts have demonstrated anti-inflammatory effects on allergen-induced airway inflammation (4) and in acute and subchronic hapten-induced models of oedema (5). Moreover, in a cell-based assay, a lipophilic extract of woad inhibited the activity of cyclooxygenase 2 (COX-2) and 5-lipoxygenase (5-LOX) (6, 7). The main and active substances in the woad extracts have been characterized recently (6, 8–10). Among these, alkaloid tryptanthrin seems to be the major compound that inhibits COX-2 (11) and 5-LOX (12) activity and demonstrates anti-inflammatory and anti-asthmatic properties. Tryptanthrin can also significantly reduce the level of thymic stromal lymphopoietin (TSLP) in lesional skin in an atopic eczema animal model, and thus can improve the clinical symptoms within this animal model (13). Indole acetonitrile derivatives, products from the enzymatic degradation of indole glucosinolates, are other major compounds in woad, which strongly inhibit production of nitric oxide and prostaglandin E2 (PGE2), release of tumour necrosis factor (TNF)-α, and interleukin (IL)-6 from macrophages, and show anti-inflammatory properties *in vivo* (14, 15). For the woad compound p-coumaric acid methyl ester, an anti-allergic effect, via inhibition of mast cell degranulation, could be detected, which is due to the modulation of hydrophobicity and alkyl chain bulkiness in RBL-2H3 cells (16). The benefit of topical p-coumaric acid methyl ester was recently shown in ultraviolet (UV)-induced inflammation in a mouse model (17).

Extracts of woad have been used in research and as ingredient of emollients for anti-irritative purposes. However, the mode of action of well-defined woad extracts and the composition of the active ingredients are little-known. There is a high unmet need for topical emollients with anti-inflammatory, anti-pruritic and anti-irritative effects for use in irritated and/or inflamed skin, especially in children and adults. The aim of the current study was therefore to develop standardized woad extracts

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with exact characterization and high reproducibility, and to determine their anti-inflammatory properties, for potential use in dermatology.

MATERIALS AND METHODS (see Appendix S1[1])

RESULTS

Profiling and analytical analysis of Isatis tinctoria *extracts*

Woad was planted under controlled conditions and cultivated by a specialized agricultural company between 2012 and 2014. Different materials (roots, stems, leaves) from the woad plant were harvested at different times during the 2-year growth phase. These were examined with regard to the parts of the plants, the harvest times and the genotypes, and the active substance concentrations measured by high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC). Due to optimization of the HPLC gradient and sample preparation, the limit of quantitation could be improved from 222 ng/ml to ~30 ng/ml for each standard substance. The limit of detection of active substances, calculated by SQS2000 software according to DIN 32 645, are listed in Table SI[1]. Three main substances were identified: p-coumaric acid methyl ester (pCM), 3-indole acetonitrile (IAN) and tryptanthrin (TRY). The baseline separation of the 3 compounds in standard solution is shown in **Fig. 1**A.

Confirmation of TRY, IAN and pCM identity in a sample was performed by matching the UV spectrum from 190 nm to 600 nm. Fig. 1B shows a petroleum ether extract in which TRY and IAN could be identified. Only pCM could not be clearly assigned in the extract. Here, the weak peak showed a slight deviation on the time axis. The screening of different ethanol extracts obtained from various pure genotypes of *Isatis tinctoria* L. leaves from different crops and harvest dates, regarding their extraction yield and TRY concentration, shows no distinct trend. However, the investigation revealed higher TRY and extraction yields in the first crop compared with the second crop in 9 out of 10 cases (Fig. 1C). Genotypes 3 and 9 were the most promising samples with regard to their yield of plant material and ratio of drug to extract. Mixed genotype samples at 6 harvest dates between July 2013 and September 2013 were also analysed, and showed overall more stable drug to extract ratios (Fig. 1C). Therefore, a mixed genotype (GT 20.08.2013) from the first crop was used for further studies.

Screening for a potent extract

In the search for an effective woad extract, various extraction methods and solvents were tested. First,

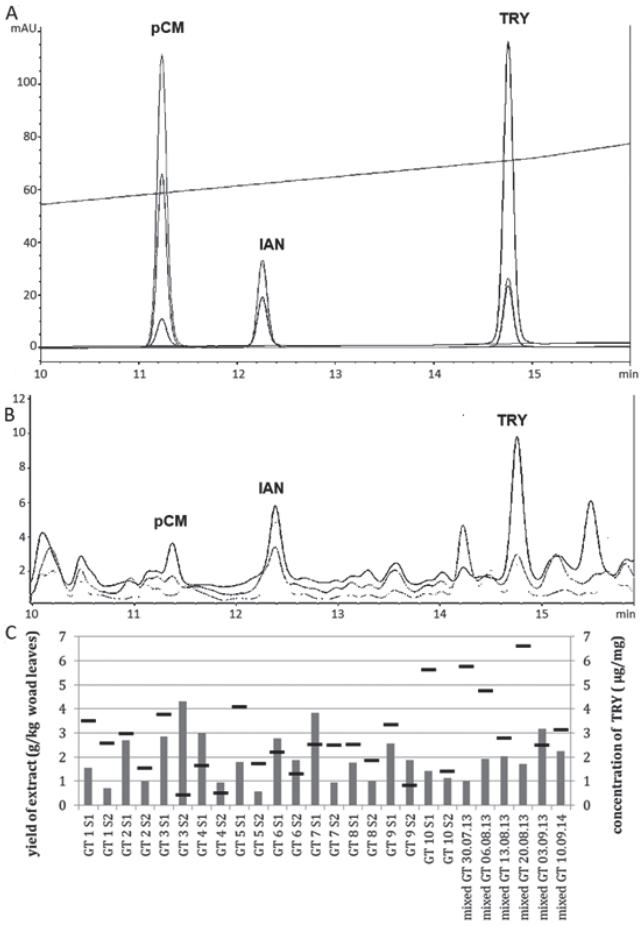

Fig. 1. High-performance liquid chromatography (HPLC) and screening of *Isatis tinctoria* L. genotypes. HPLC chromatogram of a (A) standard solution (3 µg/ml) with peaks for the 3 main components p-cumaric acid methyl ester (pCM), 3-indoleacetonitrile (IAN) and tryptanthrin (TRY) in the woad plant, and (B) the identified substances in the petroleum ether extract. In (B) the ingredients pCM and IAN were detected by retention time, since the 2 substances did not allow a clear assignment based solely on the ultraviolet (UV) spectra. (C) Yield of extraction (grey bars) and tryptanthrin (TRY) concentration (black slash) of various woad genotypes (GT) from first (S1) and second (S2) crop, in addition to the mixed genotypes on 6 different dates of harvest.

an ethanolic extract (extract 1) of leaves, harvested in 2012, was generated by accelerated solvent extraction (ASE). In this first step, isopropanol, dichloromethane and carbon dioxide were also tested as additional solvents, but did not have the desired results (data not shown). The other extracts (polar water fraction (extract 2), middle polar ethyl acetate fraction (extract 3) and lipophilic petroleum ether fraction (extract 4.1)) were obtained from leaves, harvested in 2013, through manual solid-liquid and liquid-liquid extraction. Attention was paid to high reproducibility and standardization during the extraction, as well as the colour and smell of the woad extracts for possible dermatological use. Generated extracts were tested towards their inhibitory performance against human COX-2 enzyme (**Fig. 2**A, B). The inhibitory potential of the extract 4.1 fraction (95% inhibition at 20 µg/ml) was higher than inhibi-

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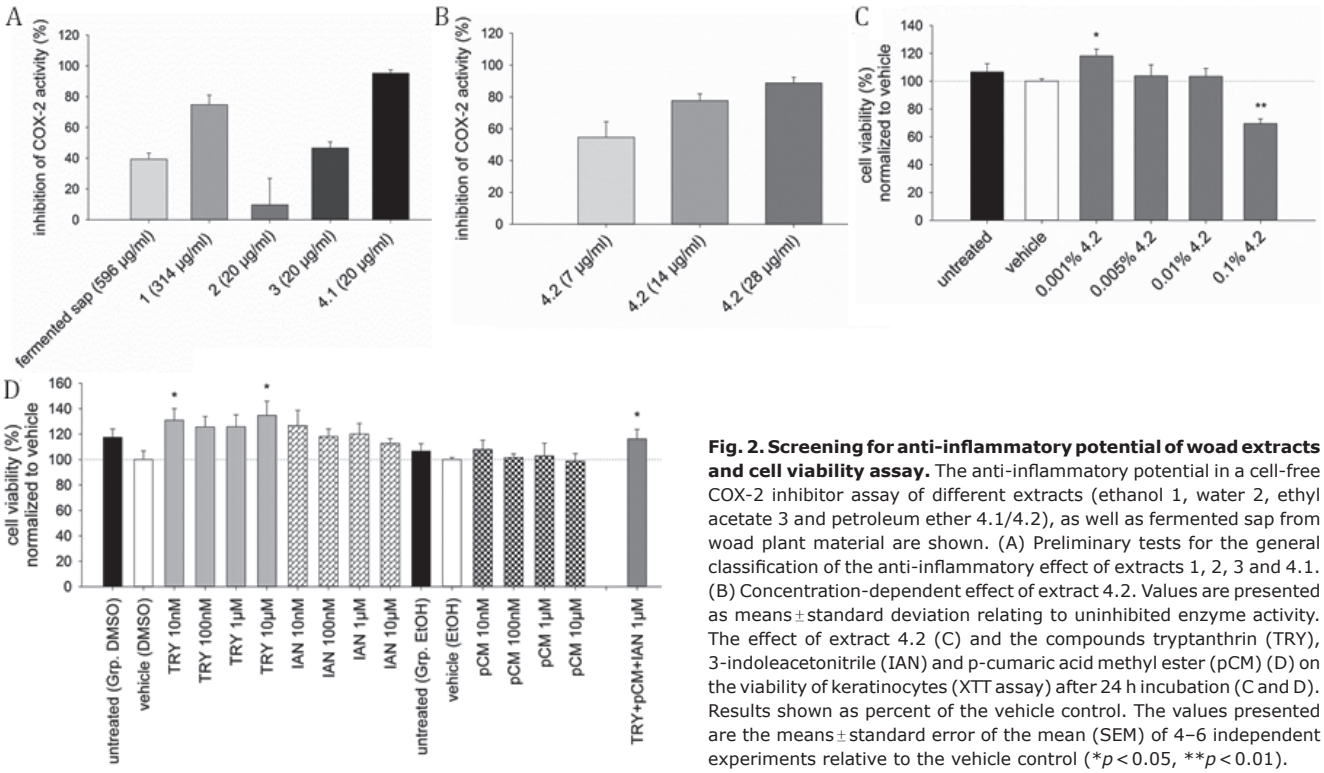

Fig. 2. Screening for anti-inflammatory potential of woad extracts and cell viability assay. The anti-inflammatory potential in a cell-free COX-2 inhibitor assay of different extracts (ethanol 1, water 2, ethyl acetate 3 and petroleum ether 4.1/4.2), as well as fermented sap from woad plant material are shown. (A) Preliminary tests for the general classification of the anti-inflammatory effect of extracts 1, 2, 3 and 4.1. (B) Concentration-dependent effect of extract 4.2. Values are presented as means ± standard deviation relating to uninhibited enzyme activity. The effect of extract 4.2 (C) and the compounds tryptanthrin (TRY), 3-indoleacetonitrile (IAN) and p-cumaric acid methyl ester (pCM) (D) on the viability of keratinocytes (XTT assay) after 24 h incubation (C and D). Results shown as percent of the vehicle control. The values presented are the means ± standard error of the mean (SEM) of 4–6 independent experiments relative to the vehicle control (*$p < 0.05$, **$p < 0.01$).

tion by extract 1 fraction (75% inhibition at 314 µg/ml), extract 2 (10% inhibition at 20 µg/ml) and extract 3 (47% inhibition at 20 µg/ml). The tested extract 4.1 contained approximately 5.3 µg/mg TRY and a minor amount of IAN (< 1 µg/mg). The fermented sap, which was tested in parallel, contained the highest amount of IAN (22 µg/mg; TRY 0.1 µg/mg) and showed a mid-range inhibitory effect (~40% inhibition at 596 µg/ml) on COX-2 similar to extract 3 (Fig. 2A). The inhibitory effects of the single standard substances were lower than the inhibition of woad extracts with a similar concentration of these (data not shown).

Due to the small amount of extract 4.1, produced from approximately 132 mg/kg woad leaves, the extraction process was optimized. Extract 4.2 was obtained by the optimized extraction method, which had a 23-times higher yield of extract (3,066 mg/kg drug), but decreased content of TRY to 2.3 µg/mg. The content of IAN and pCM in extract 4.2 was below 100 ng/mg. In the assay, extract 4.2 (at a 1.5-fold concentration to that of extract 4.1) showed similar inhibitory effects as extract 4.1 (extract 4.1 95% inhibition at 20 µg/ml vs. extract 4.2 89% inhibition at 28 µg/ml). In addition, a concentration-dependent effectiveness of extract 4.2 was determined, whereby a concentration of 7 µg/ml could achieve a 45% inhibition of the COX-2 enzyme (Fig. 2B). A fat extract from fresh woad leaves also showed a high inhibition (80% inhibition at 25 µg/ml) in the COX-2 enzyme assay, with a very low active ingredient concentration at the same time (data not shown).

In order to precisely determine the effects of the woad extracts 1, 3 and 4.2, as well as of the 3 main compounds TRY, IAN and pCM on keratinocyte (Ha-CaT) viability, a cell proliferation assay (XTT) was performed. A gradient of the extracts and TRY, IAN or pCM was used to incubate HaCaTs for 24 h. A high cytotoxic effect on the keratinocytes was observed for the extracts 1 (< 40% viability vs control) and extract 3 (< 10% viability) at a concentration of 1 mg/ml (data not shown). For extract 4.2, no inhibitory effect was shown for lower concentrations (100 µg/ml to 10 µg/ml, Fig. 2C) and only the highest concentration of 1 mg/ml reduced cell viability slightly, to the level of 70% surviving cells ($p < 0.01$). As shown in Fig. 2D, TRY (10 nM and 10 µM) could significantly promote cell viability, just like a combination of TRY+pCM+IAN (each 1 µM). None of the other woad ingredients tested showed a significant proliferation-promoting or proliferation-reducing effect.

Analysis of the fatty acid profile showed an increased percentage in palmitic acid (C16:0) and gamma-linolenic acid (C18:3 n-6) in lipophilic extract 4.2. The proportion was approximately 25% (C16:0) or approximately 40% (C18:3 n-6) of the total fat. Similarities were found for the distribution in a leaf fat extract. Palmitic acid and gamma-linolenic acid were also increased here (Fig. S1[1]).

In summary, extract 4.2 was found to be a promising candidate with a potential anti-inflammatory effect due to high COX-2 inhibition. All subsequent experiments were performed using extract 4.2 within the non-inhibitory range, up to a maximum concentration of 100 µg/ml.

Anti-inflammatory effect of petroleum ether extract in vivo

The *in vivo* potency and anti-inflammatory activity of the *Isatis tinctoria* extract was evaluated in an animal model of contact hypersensitivity (CHS), as a model of irritated and inflamed skin. Topical application of 100 µg/ml per ear extract 4.2, or 0.25 mg/ear TRY+pCM+IAN, 30 min before challenge and 24 h after challenge on mouse skin significantly inhibited acute oedema and inflammatory response in the mouse ear. During the late elicitation phase (within 24 h of the challenge) of the CHS, a significantly reduced ear swelling of 50% for TRY+pCM+IAN ($p < 0.05$) and 51% for extract 4.2 ($p < 0.05$) could be detected compared with the vehicle group. The inflammatory phase (up to 48 h) was diminished significantly, by 68% (TRY+pCM+IAN; $p < 0.001$) and 67% (extract 4.2; $p < 0.001$), respectively (**Fig. 3**B). Subsequent histological staining confirmed a reduction in swelling in the treated areas (Fig. 3A). Here, a significantly lower ear thickness compared with the vehicle group could be detected for both the combination of TRY+pCM+IAN ($p < 0.001$) and extract 4.2 ($p < 0.001$) (Fig. 3C). Furthermore, histological analysis revealed a lower number of mast cells after treatment with extract 4.2 ($p < 0.01$) or TRY+pCM+IAN ($p < 0.05$; Fig. 3A, Fig. S2[1]). Immunohistochemistry revealed a reduced number of CD4-positive T cells in the treated skin for extract 4.2 ($p < 0.05$) and for TRY+pCM+IAN ($p < 0.05$) (Fig. 3D, E).

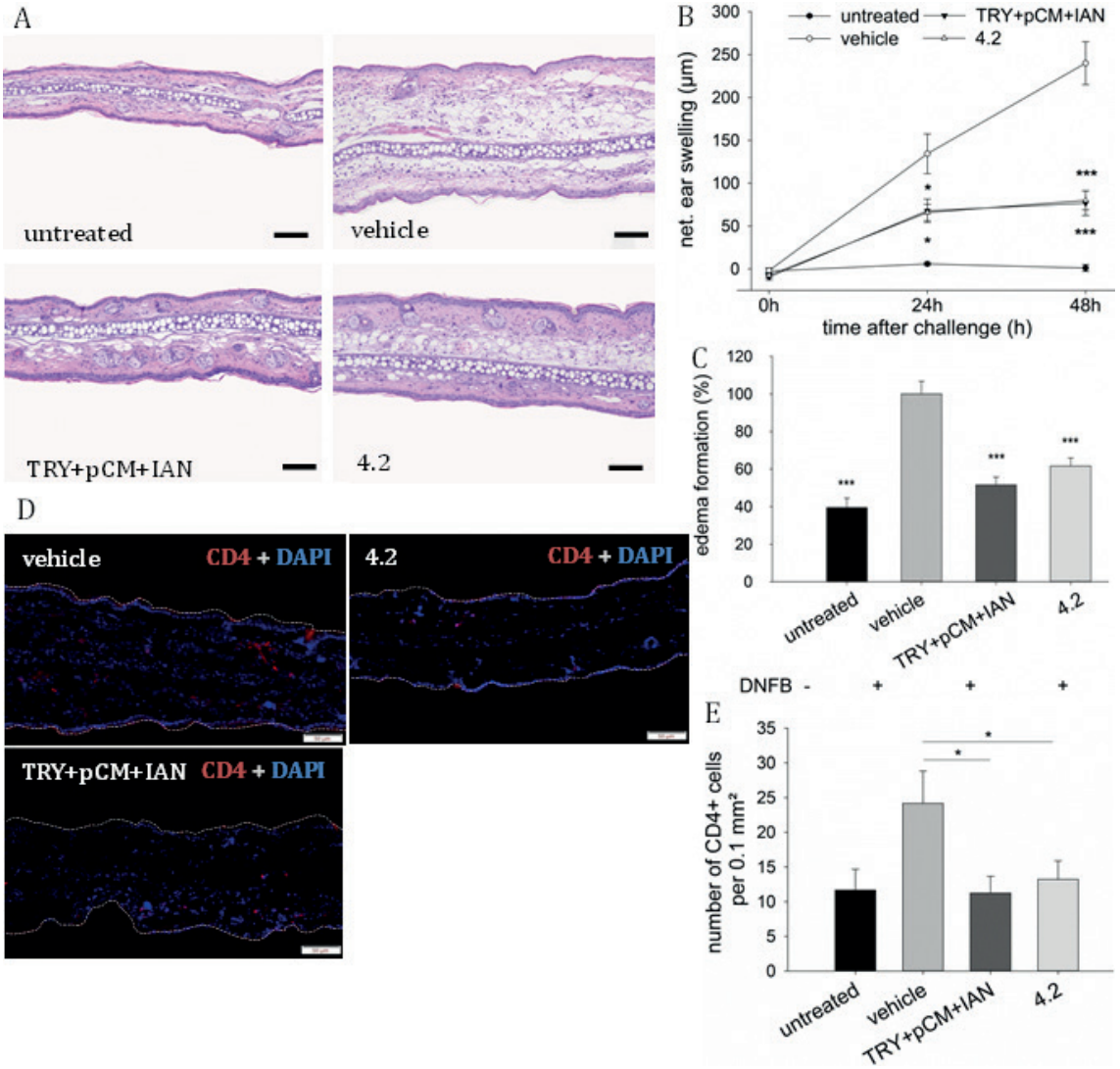

Fig. 3. Ear swelling, oedema formation and recruitment of inflammatory CD4+ cells in contact hypersensitivity (CHS) mouse model. (A) Representative haematoxylin and eosin staining in ears of wild-type mice ($n = 8–12$ mice per group) 48 h after challenge with 1-fluoro-2,4-dinitrobenzene (DNFB). *Scale bars*: 100 µm. Woad extract 4.2 and tryptanthrin (TRY), 3-indoleacetonitrile (IAN) and *p*-cumaric acid methyl ester (pCM) treated groups compared with vehicle (DNFB only) control are shown, as well as the statistical analysis of (B) ear swelling and (C) oedema formation in the ears. Results are presented as mean values ± standard error of the mean (SEM), $*p < 0.05$, $***p < 0.001$ vs. vehicle. (D) Representative immunofluorescence staining of mouse ears treated with TRY+pCM+IAN or 4.2 using an antibody against CD4 and nuclear staining (DAPI). *Scale bars*: 50 µm and (E) total number of CD4-positive cells per 0.1 mm^2 from 2–5 mice per group ($n = 3$ images per section). Plots are shown as means ± SEM; $*p < 0.05$ vs. vehicle (DNFB only).

Cytokine expression in vivo *and* in vitro *were reduced by petroleum ether extract and woad ingredients*

A significant reduction in the expression of the pro-inflammatory cytokines interleukin (IL)-1b and IL-6 was found in mouse skin after treatment with 4.2 (IL-1b $p<0.01$, IL-6 $p<0.001$) or TRY+pCM+IAN (IL-1b $p<0.05$, IL-6 $p<0.001$) compared with the vehicle control (**Fig. 4**B, D). TRY+pCM+IAN also significantly inhibited the expression of IL-4 ($p<0.01$) in the mice, but not extract 4.2 (Fig. 4C). mRNA expression of the alarmin cytokine IL-33 was only significantly affected by TRY+pCM+IAN ($p<0.05$, Fig. 4E), whereas expression of the IL-33 receptor interleukin 1 receptor-like 1 (IL1RL1=ST2) was down-regulated after treatment with extract 4.2 ($p<0.01$) or the main compounds ($p<0.05$) (Fig. 4F). Increased expression of interferon (IFN)-γ could be observed at the site of challenge in the treated mouse skin. Neither extract 4.2 nor TRY+pCM+IAN showed an effect on the increased IFN-γ expression measured 48 h after challenge (Fig. 4A).

In order to validate the effects observed in mouse skin, we also investigated expression of IL-6 and IL-33 in human keratinocytes derived from healthy donors after stimulation with the TLR3 agonist Poly I:C (for IL-6) or IFN-γ (for IL-33). Pretreatment with TRY ($p=0.07$) or TRY+pCM+IAN ($p<0.05$) reduced the Poly I:C-induced expression of IL-6 in the keratinocytes to approximately 50% compared with the vehicle (**Fig. 5**A). The effect of extract 4.2 was much more pronounced, leading to a decrease of 50% ($p<0.01$) for the highest extract concentration compared with the vehicle (Fig. 5B).

IFN-γ has an effect on expression of IL-33 in keratinocytes (18, 19). Dose kinetics determined a 20 ng/ml concentration of IFN-γ as suitable to stimulate the cells and induce IL-33 expression (Fig. S3[1]). TRY alone did not inhibit induction of IL-33 expression, but, in combination with pCM and IAN, or treatment with extract 4.2, led to a significant reduction in induced IL-33 mRNA expression ($p<0.01$ and $p<0.05$; Fig. 5C, D) compared with the vehicle group.

Petroleum ether extract inhibits degranulation of human LAD2 mast cells

Degranulation was measured by release of the enzyme β-hexosaminidase in LAD2 cells, which were stimulated either with the ion-carrier calcium ionophore (CI,

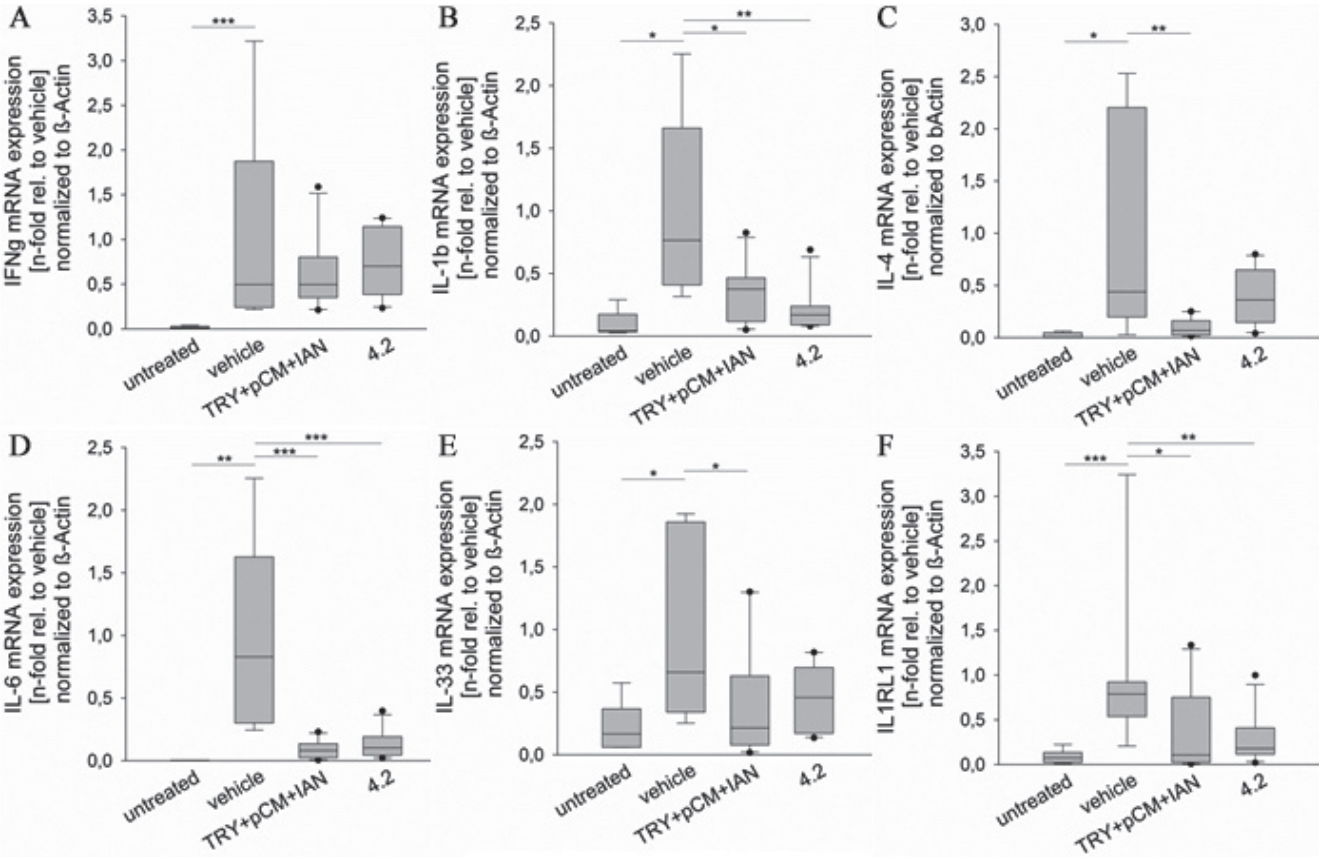

Fig. 4. Expression of pro-inflammatory cytokines after treatment with petroleum ether extract (4.2) or the compounds TRY, pCM and IAN in mouse ear. (A) Relative mRNA levels of the pro-inflammatory cytokines (A) interferon (IFN)-γ, (B) interleukin (IL)-1beta, (C) IL-4, (D) IL-6, (E) IL-33 and (F) ST2 in 4.2 or tryptanthrin (TRY), 3-indoleacetonitrile (IAN) and p-cumaric acid methyl ester (pCM)-treated mouse skin was measured 48 h after 1-fluoro-2,4-dinitrobenzene (DNFB) challenge ($n=8$–11 per each group) and normalized against beta-actin (β-Actin). *$p<0.05$, **$p<0.01$ ***$p<0.001$ vs. vehicle.

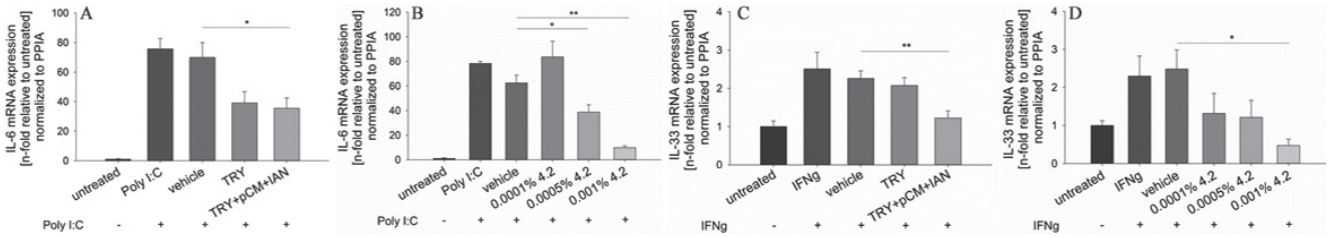

Fig. 5. Expression of pro-inflammatory cytokines after treatment with petroleum ether extract (4.2) or the compounds TRY, pCM and IAN in keratinocytes. Relative mRNA expression of interleukin (IL)-6 in primary human keratinocytes stimulated with poly I:C for 16 h and treated with (A) TRY (1 μM), TRY+pCM+IAN (each 1 μM) or (B) 4.2 (1, 5, 10 μg/ml). Results were normalized against peptidylprolyl isomerase A (PPIA). $n \geq 3$ experiments. Expression level of IL-33 in primary human keratinocytes stimulated with interferon (IFN)-γ for 16 h and treated with (C) TRY (1 μM), TRY+pCM+IAN (each 1 μM) or (D) 4.2 (1, 5, 10 μg/ml). $n \geq 3$ experiments; $*p < 0.05$, $**p < 0.01$ vs. vehicle.

2 μM; 20) or with neuropeptide substance P (SP, 30 μM; 20, 21). Extract 4.2 inhibited significantly and in a concentration-dependent way, the CI- (50 μg/ml and 75 μg/ml, $p < 0.001$) and SP- (50 μg/ml $p < 0.01$, 75 μg/ml $p < 0.001$) induced degranulation compared with the stimulation control alone (**Fig. 6**A). For the highest concentration of extract 4.2 (75 μg/ml) an inhibition of 40% (CI), respectively 30% (SP), compared with the untreated samples, was observed. Interestingly, the 3 woad compounds, TRY, pCM and IAN (10 nM to 10 μM), alone showed no effect on β-hexosaminidase release (see Fig. S4[1]). Treatment with a combination of the 3 ingredients (each 1 μM), however, led to a significant reduction in degranulation in our setting (Fig. 6B).

DISCUSSION

Dry, pruritic or irritated skin is a frequent finding in many conditions, such as diseases associated with chronic pruritus (e.g. dry skin in elderly patients), paediatric genodermatoses, and in intervals between relapses of atopic dermatitis (AD) or psoriasis vulgaris. In these conditions, emollient use is advocated, while topical steroids and topical calcineurin inhibitors might not be appropriate or may induce side-effects (22). It has been shown that emollients can modify the inflammation and decrease the use of anti-inflammatory treatments in chronic pruritus entities, such as AD (23). Thus, emollients should be part of the therapy regimen in these conditions (24). The most promising emollients are these with mild anti-irritative and anti-inflammatory potential. In Chinese medicine, woad leaf and root preparation showed such a modification of inflammatory diseases and infections (3, 4). Therefore, we initiated this study in order to analyse whether woad extracts (*Isatis tinctoria L.*) might be beneficial for such preparations. This included systematic planting and harvesting of woad, isolation and analysis of the main ingredients, and analysis of their *in vivo* and in vitro anti-inflammatory properties.

This study examined a range of woad material from different harvest periods and different genotypes in order to find the optimal raw plant material for the extraction process. An important attribute of this study was the thorough analysis, which, in addition to a high yield of active ingredient and stability of the extracts, also took into account the colour and smell. These are all important factors for possible incorporation of the extract into an emollient. Small scales from dried leaves were examined with different extraction operations using Accelerated Solvent Extraction (temperature, content of solvent, number and duration of extraction cycles) and optimized with regard to the content of the ingredients.

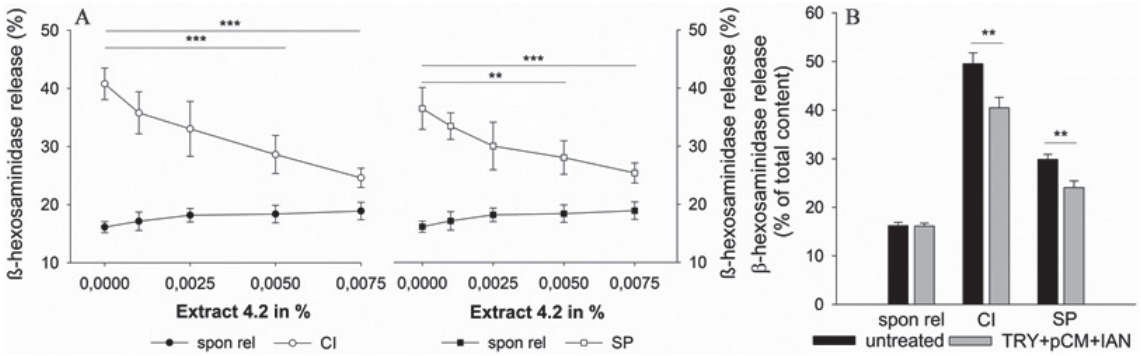

Fig. 6. Mast cell degranulation is reduced by woad extract treatment. Calcium ionophore- (CI) or substance P- (SP) stimulated LAD2 cells after pretreatment with: (A) petroleum ether extract 4.2 (10–75 μg/ml) from woad or (B) tryptanthrin (TRY), 3-indole acetonitrile (IAN) and *p*-cumaric acid methyl ester (pCM; each 1 μM). Shown is the spontaneous release (spon rel) vs. the release under stimulation (CI or SP) of β-hexosaminidase, measured by an enzymatic assay. Data are shown as mean ± standard error of the mean (SEM) from 4 independent experiments ($**p < 0.01$, $***p < 0.001$ vs. untreated).

Various ingredients from the woad plant, including 70 compounds in the leaves, have already been described in the literature (8, 9, 25, 26). In dried woad leaves we found the 3 main compounds tryptanthrin (TRY), 4-coumaric acid methyl ester (pCM) and 3-indole acetonitrile (IAN) and confirmed previous studies (8).

As a result of the extraction process and the subsequent optimization steps, 4 different extracts (ethanolic extract 1; water extract 2; ethyl acetate extract 3; petroleum ether, extracts 4.1 and 4.2), the fermented sap and a leaf fat extract were prepared and used in a COX-2 inhibitor assay to test their potential anti-inflammatory effect. In addition, the extracts were examined in a cell vitality assay. Lipophilic extract 4.2 was able to strongly inhibit the activity of COX-2 in a cell-free system and was thereby more effective than the 3 single substances, the other tested extracts (1, 2 and 3), the fermented sap or the leaf fat extract. This is consistent with the literature, in which a lipophilic woad extract is described as an inhibitor of the activity of COX-2 and 5-LOX (6, 7). In the final and most effective PE extract (4.2), however, only TRY could be detected at a significant amount, while the concentration of IAN and pCM was very low.

The results from the fatty acid profile indicate that the observed anti-inflammatory effect of the extracts may not be due to the active ingredients alone. The leaf fat extract can significantly inhibit COX-2 activity in our assay, with only a very low concentration of active ingredient. In both extracts, 4.2 and the leaf fat extract, the polyunsaturated fatty acid (n-6) gamma-linolenic acid (GLA) show a high percentage present. This new finding is particularly interesting, because GLA has anti-inflammatory properties and can inhibit COX-2 enzyme activity (SR1, SR2). GLA inactivates nuclear factor-kappaB and activator protein-1 by suppressing oxidative stress and the signal transduction pathway of phosphorylated extracellular signal-regulated kinase 1/2 and c-Jun N-terminal kinase-1 (SR2). Patients with AD have a low level of GLA, but adding GLA improves the clinical symptoms of the disease (SR3, SR4). The saturated fatty acid palmitic acid, also found in extract 4.2, increases expression of the COX-2 gene, but has no effect on the activity of the COX-2 enzyme (SR5, SR6).

Due to the strong potential anti-inflammatory effect, the low cytotoxic properties and the high yield of extract from the extraction process, extract 4.2 was chosen as the best candidate for subsequent studies.

The *in vivo* potency and anti-inflammatory activity of the *I. tinctoria* extract was evaluated in a CHS animal model (27, 28). Both the *I. tinctoria* extract (4.2) and TRY+pCM+IAN- mixture (1 μM each) showed a similar reduction in ear swelling in the classical 24–48 h CHS effector response. This was confirmed by a dramatic reduction in oedema formation using histological investigation. In addition, a reduced number of mast cells could be detected in the treated mouse ears. This was shown both after treatment with the 3 active ingredients and with extract 4.2. Furthermore, 48 h after the challenge, the number of CD4+ T cells at the site of treatment (extract 4.2 and TRY+pCM+IAN) was reduced almost to the level of the untreated control As far as we know, this could be shown for the first time for woad extracts. Our results are in line with other lipophilic woad extracts in acute and subchronic mice models of skin inflammation (5), which demonstrated a similar reduction in inflammation.

The gene expression of IFN-γ in lesional mouse skin was not influenced by extract 4.2 or TRY+pCM+IAN, while the 3 main components inhibited IL-4 expression. Brattström et al. (4) demonstrated that a carbon dioxide *I. tinctoria* extract inhibits production of the Th2 cytokines IL-4 and IL-5 in allergen-induced airway inflammation. TRY alone also reduces the production of IL-4 by Th2 cells, whereas IFN-γ production by Th1 cells is not affected (29). Furthermore, TRY inhibits the differentiation of purified CD4+ T cells to a Th2 phenotype (29). According to the findings to date, the woad ingredients with TRY mainly affect the Th2 cell population and the corresponding cytokines.

Furthermore, we were able to show that extract 4.2 and TRY+pCM+IAN significantly reduce IL-1beta and IL-6 gene expression in the DNFB-treated mouse. Also, in normal human epidermal keratinocytes a significantly reduced IL-6 expression by extract 4.2 and TRY+pCM+IAN treatment was found after Poly I:C-induction. IL-1beta and IL-6 are important pro-inflammatory mediators; IL-1beta is involved in cell processes such as proliferation, differentiation, apoptosis and induction of COX-2 and IL-6 release (30). Both cytokines play a key role in the formation of acute contact dermatitis (ACD), and the levels of their transcripts correlate with the intensity of an experimentally induced contact dermatitis (31, 32). CD4+ T cells are prevalent in inflammatory lesions and can produce IL-6, which is relevant to the acute phase of atopic eczema (AE), for example (33, 34). Monoclonal antibodies against IL-6 reduce the signs of AD (reduction of EASI) and diminish pruritus within 6 weeks (34). The relationship between IL-6 and pruritus is also described in some pruritic diseases in which the severity of pruritus correlates with the serum levels of IL-6 (35, 36).

As new findings for woad extracts we were able to show that treatment with extract 4.2 and TRY+pCM+IAN significantly reduced the IL-33 (TRY+pCM+IAN) and ST2 (both) mRNA in the lesional skin during CHS. IL-33 is a member of the IL-1 cytokine family and may play a crucial role in the acute and chronic phases of inflammation in AD and contribute to ACD (18, 19). It is known that IL-33 and its receptor ST2 are increased in lesional skin of AD (37). In human keratinocytes, we detected a significant inhibition in IFN-γ-induced IL-33 gene expression by the petroleum ether extract and the 3 woad components. IL-33 can be induced not only in epidermal keratinocytes from AD patients by IFN-γ (18,

19), but may increase IFN-γ release itself by interaction with activated T cells. This potential regulatory loop may contribute to chronicity of disease (18). Woad ingredients do not seem to directly affect IFN-γ, but decreased expression and release of IL-33 may lead to interruption of the regulatory loop.

To date, only a few ingredients from *I. tinctoria* are known to affect mast cell degranulation. For indolin-2-one, inhibition of C48/80-induced histamine release was demonstrated in rat peritoneal mast cells (38), whereas TRY does not cause this induced degranulation (38). Kiefer et al. (39) confirmed the results of indolin-2-one in murine bone marrow-derived mast cells. In contrast, TRY causes a decrease in IgE-mediated degranulation in rat basophilic leukaemia cells (29). This may indicate different mechanisms of the inhibitory effect, which are still unclear for woad extracts and require further investigation. In our study, TRY alone also had no effect on degranulation of the human mast cell line LAD2 cells. Combination with the other 2 ingredients, IAN and pCM, however, led to a significant reduction in β-hexosaminidase secretion. Extract 4.2 demonstrated to be a very effective inhibitor of mast cell degranulation, as it decreases CI- and SP-induced release of β-hexosaminidase in LAD2 cells in a concentration-dependent manner. To our knowledge, such an effect of woad extracts on human mast cells has not been shown previously. As effector cells for allergic reactions, mast cells are involved in the development of inflammatory processes and skin lesions in AD and ACD (40). Thus, our extract can inhibit the mast cells and reduce the actual inflammatory reactions.

In summary, we have developed a novel woad extract that displays anti-inflammatory properties by down-regulation of inflammatory cytokines, such as IL-6 and IL-33, inhibiting mast cell responses, COX-2 activity, and has further proven its efficacy in an ACD mouse model. *In vivo*, petroleum ether extract 4.2 showed comparable efficacy to the 3 woad ingredients TRY, pCM and IAN, and was found to be more effective in the *in vitro* experiments. Compared with a composite of TRY+pCM+IAN, the natural and defined extract also displays high reproducibility. Fractionation of the crude extract to a petroleum ether extract could compensate for the variability in raw material to produce an extract with a consistently anti-inflammatory effect. We cannot rule out that parts of the observed effects of the extract are due to additional low-concentrations of substances in the extract. A precise analysis of extract 4.2 with regard to other ingredients would provide the basis for further studies. This could be a useful extension of our understanding of the mode of action of woad extracts. Further preclinical and clinical studies in patients with inflamed, irritated and pruritic skin are required; however, the results of the current study suggest that standardized woad extracts with a high content of TRY may be promising substances for use in dermato-cosmetics in inflamed, irritated and dry skin.

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