Supercritical Marigold Flower CO₂-Extract - Evergreen in Evidence Based Cosmetic Application

Authors: Dr. P. May, Dr. K.-W. Quirin, Flavex Naturextrakte GmbH, Rehlingen, Germany

Abstract
Marigold (Calendula) has gained increasing attention in recent years due to new scientific evidence of the old well-known healing forces of the plant in dermatological and cosmetic applications. Supercritical CO₂-extraction proves state-of-the-art technology for producing highly active Calendula extracts. The pentacyclic triterpene alcohols such as faradiol-3-monoesters and monols such as taraxasterols are important marker ingredients and have been identified as the anti-inflammatory principles which are especially enriched by CO₂-extraction. The Calendula CO₂-extract thus delivers the base for active and well standardised cosmetic preparations especially for ointments which have a long tradition in the self-medication of skin diseases and in cosmetics. In contrast to other asteraceae, marigold does not contain sesquiterpene lactones which might have a sensitising potential.

Introduction
Marigold (Calendula officinalis L.) – not to be confused with tagetes – is an annual unpretentious plant of 30-60 cm height, growing wildly or cultivated in Mediterranean countries, North Africa, Asia, North and Middle America. The active parts are the yellow to dark orange flowers which can be harvested during the whole summer season.

The marigold flower, along with the arnica and the chamomile flower, is one of the best known medical plants. All three belong to the same asteraceae plant family and have similar physiological properties but Calendula, in contrast to the other Asteraceae flowers does not contain any sesquiterpene lactones which may cause allergies. Among the various species of the genus Calendula, only Calendula officinalis and C. arvensis are used worldwide, mainly the officinalis variety.

The therapeutic use of Calendula flowers and ointments goes back at least to Hildegard von Bingen in the 12th Century and it has been mentioned in many popular medical books for almost a hundred years[1,2].

The plant is listed in many Pharmacopoeias such as European Pharmacopoeia, British Herbal Pharmacopoeia, German Pharmacopoeia, U.S. Pharmacopoeia and in the German Commission E and European Scientific Co-operative on Phytotherapy (ESCOP) monographs. Indications are inflammations of the skin and oral mucosa, wound healing and antimicrobial activity, especially in ointments for medical cosmetics and skin care products. Health Canada lists Calendula in the Health Products Category.

Figure 1a. Calendula officinalis
Figure 1b. Calendula officinalis
The main ingredients of Calendula officinalis flowers are 2-10% triterpene saponins, free and esterified triterpene alcohols with approximately 4% diols, free triterpene monols and approximately 0.8% triterpene triols\(^3\). The most relevant triterpene alcohols are listed in Table 1 and their molecular structures are given in Figure 2.

<table>
<thead>
<tr>
<th>Monols</th>
<th>Diols</th>
<th>Triols</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Amyrin</td>
<td>Arnidiol</td>
<td>Heliantriols</td>
</tr>
<tr>
<td>β-Amyrin</td>
<td>Calenduladiol</td>
<td>Ursatriol</td>
</tr>
<tr>
<td>Lupeol</td>
<td>Brein</td>
<td>Longispinogenin</td>
</tr>
<tr>
<td>Taraxasterol</td>
<td>Faradiol</td>
<td></td>
</tr>
<tr>
<td>ψ-Taraxasterol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Relevant Triterpene Alcohols of Calendula officinalis

Further ingredients of Calendula officinalis flowers are 0.2-0.9% flavonoids\(^4\), 1.5% and more carotenoids\(^5,6\), polysaccharides\(^7\), 0.6% sterols\(^8\), 0.2-0.3% essential oil\(^9\), 0.1% phenolic acids\(^10\) and traces of coumarins\(^11\).

**Extraction Methods - Advantage of CO\(_2\)-Extraction**

Calendula preparations are traditionally obtained by direct extraction of the flowers with the lipid base of the ointment i.e. peanut oil, wool fat or lard. These maceration procedures have the disadvantages of providing only very diluted extracts, of extracting only a part of the ingredients and sometimes of applying high temperatures but they already give a clear reference to the lipophilic nature of the active principles. Other procedures are the more hydrophilic extractions with ethanol/water or with propylene glycol.

The best technology for preparing lipophilic extracts is supercritical CO\(_2\)-extraction\(^12\). This procedure manages to obtain the complete lipophilic spectrum of plant constituents without any dilution, without the stress of high temperatures, without decomposition of ingredients and without leaving any solvent residues. The CO\(_2\)-extract is composed of 100% of Calendula ingredients and can be added in a precise dosage to dermatological or cosmetic preparations.

**Composition of the CO\(_2\)-Extract**

The composite flowers with petals and calyx are used for CO\(_2\)-extraction. The yield is about 5% corresponding to a drug/extract ratio of 20:1. The active substances in the CO\(_2\)-extract are esters of pentacyclic triterpene monols and diols derived from the 4-taraxene structure. Among them faradiol is present in the CO\(_2\)-extract at 98% as monoester and at 2% as diester of mainly myristic (51%), palmitic (28%) and lauric (8%) acids.

Faradiol esters appear to be the most relevant compounds due to their high activity and due to their quantitative prevalence of about 20% in the CO\(_2\)-extract. Further components in the CO\(_2\)-extract are taraxasterols, sterols, essential oil and carotenoids.

The Calendula CO\(_2\)-extract has a dark orange colour and is rich in carotenoids containing e.g. β-carotene, esterified xanthophylls...
Natural Ingredients

The carotenes have antioxidant properties reacting with free radicals and are believed to stimulate the granulation and formation of the skin’s cell tissue in the wound healing process. The CO₂-extraction of *Calendula* carotenoids is described in detail elsewhere\(^{(13)}\). There is also some essential oil present in the *Calendula* extract responsible for the characteristic hay-like flavour. Some tenths of substances have been identified with sesquiterpene alcohols like α-cadinol as major components\(^{(14)}\).

The supercritical CO₂-extract is unique in terms of concentration and composition of bioactive ingredients. It is also available in organic quality and it meets the strict criteria of natural cosmetic standards e.g. BDIH, COSMOS, ICADA, NATRUE, NPA, and NSF ANSI 305.

**Pharmacology**

A number of studies have evaluated the anti-inflammatory efficacy of *Calendula officinalis* and related this activity to the presence of triterpene alcohols, especially to faradiol esters and taraxasterol\(^{15-20}\).

Investigations conducted by Della Loggia et. al. have clearly demonstrated that the CO₂-procedure is effective in yielding an active extract by verifying its topical anti-inflammatory activity in comparison to a hydroalcoholic (70%) extract of the same raw material\(^{18}\). The test was based on the inhibition of the croton oil-induced dermatitis of the mouse ear and the results are summarised in Table 3.

The hydroalcoholic extract has a mild dose dependent effect reaching 20% of oedema inhibition at a dose of 1200 μg/ear. The same concentration of the CO₂-extract is 3.5 times more effective with 70.7% inhibition of the inflammatory response. Also on Drug Equivalent base there is a 70% better activity of the CO₂-extract compared to the hydroalcoholic product.

Further investigations published by Della Loggia et. al.\(^{19}\) revealed the composition of the active molecules by means of a bioassay-oriented fractionation of the *Calendula* CO₂-extract using the mouse ear test described above. It is evident from the investigations that CO₂ extraction concentrates the relevant anti-inflammatory compounds of the *Calendula* flower. The content of faradiol monoesters should be taken as a rational parameter for quality control and the standardisation of *Calendula* preparations\(^{18,21}\).

Plotting the activity of the CO₂-extract and a triterpene enriched fraction (TEF) measured as oedema inhibition (%) against the log of the applied doses expressed as crude drug equivalents in mg gave a correlation of excellent linearity (Figure 3). The TEF was obtained by column chromatography in order to remove the carotenoids as completely as possible.

<table>
<thead>
<tr>
<th>Components</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faradiol esters</td>
<td>17.25</td>
</tr>
<tr>
<td>Sum of taraxasterol, Amyrin and Lupeol</td>
<td>10.13</td>
</tr>
<tr>
<td>- with alpha-amyrin</td>
<td>1.2-1.8</td>
</tr>
<tr>
<td>- with beta-amyrin</td>
<td>1.1-1.7</td>
</tr>
<tr>
<td>- with Lupeol</td>
<td>0.5-0.9</td>
</tr>
<tr>
<td>Sum of sterols</td>
<td>2.1-2.5</td>
</tr>
<tr>
<td>Essential Oil</td>
<td>1.5-1.7</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>0.85-1.2</td>
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Table 2. Composition of Marigold Flower CO₂-total Extract

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<table>
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<tr>
<th>Substance</th>
<th>Dose μg/ear</th>
<th>D. E.</th>
<th>Oedema Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroalcoholic Extract</td>
<td>300</td>
<td>1.05</td>
<td>9.3</td>
</tr>
<tr>
<td>“</td>
<td>600</td>
<td>2.1</td>
<td>12.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>1200</td>
<td>4.2</td>
<td>20.0</td>
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<tr>
<td>CO₂-extract</td>
<td>75</td>
<td>1.8</td>
<td>14.7</td>
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<td>“</td>
<td>150</td>
<td>3.6</td>
<td>30.7</td>
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<tr>
<td>“</td>
<td>300</td>
<td>7.2</td>
<td>44.0</td>
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<td>“</td>
<td>600</td>
<td>14.4</td>
<td>58.7</td>
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<tr>
<td>&quot;</td>
<td>1200</td>
<td>28.8</td>
<td>70.7</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>120</td>
<td>/</td>
<td>73.3</td>
</tr>
</tbody>
</table>

D.E. = Drug Equivalent (mg of crude drug corresponding to the applied dose)

Table 3. Anti-inflammatory Activity of *Calendula officinalis* Extract
Free monols, especially ψ-taraxasterol, also showed synergistic anti-inflammatory activity but their contribution to the effect of the extract is of lesser importance due to their lower concentration\(^{(19,20)}\). Inhibitions of oedema produced by topical application of faradiol 3-myristate, faradiol 3-palmitate and ψ-taraxasterol were 46%, 45% and 49% respectively at 240μg/cm\(^2\) and 65%, 66% and 86% at 480μg/cm\(^2\) \(^{(20)}\). In another study, using 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to induce ear inflammation in mice, topically applied taraxasterol and ψ-taraxasterol dose-dependently inhibited inflammation with ID\(^{50}\) values of 0.3 and 0.4 mg/ear respectively, compared to 0.3 mg/ear for indomethacin and 0.03 mg/ear for hydrocortisone\(^{(22)}\).

The topical application of the pentacyclic triterpene alcohol α-amyrin caused a dose-related inhibition of both the ear oedema (ID\(^{50}\) value: 0.31 mg/ear) and the influx of polymorphonuclear cells (ID\(^{50}\) value: 0.45 mg/ear). It also prevented the increase of the proinflammatory cytokine interleukin-1β levels in response to topical application of TPA in mice ear. The maximal obtained efficacy was in all cases very similar to the steroidal anti-inflammatory drug dexamethasone\(^{(23)}\).

The Calendula flower CO\(_2\)-extract also has a relatively high concentration of phytosterols. Sterols are not only essential for skin structure formation but they are also precursors of hormones with a number of positive effects. They stabilise cell membranes and prevent dehydration. The skin revitalises naturally and becomes soft and smooth. Phytosterols stimulate the collagen synthesis and thus help to protect the skin against harmful environmental stress. In a recent study the photoprotective effect of phytosterols was described\(^{(24)}\). In-vitro tests with human keratinocytes have demonstrated that phytosterols inhibit UV-induced MMP-1 expression and down-regulation of COL1A1 and COL1A2 genes responsible for collagen synthesis. The findings were recently confirmed in a human study\(^{(25)}\). Generally reduced sterol content in keratinocytes increases the susceptibility to UV-stress and the incidence of detrimental skin disorders. Phytosterols had similar protective efficacy on cell membranes as cholesterol.

The protective effect of a base cream prepared with Calendula flower supercritical CO\(_2\)-extract according to DAC against sodium lauryl sulfate induced contact dermatitis was tested on 20 healthy volunteers in a 4-day repetitive irritation test. A statistically significant protective effect was observed\(^{(26)}\).

In a randomised controlled trial the positive effects of ointment with lipophilic marigold extract on venous ulcer epithelialisation in the lower leg could be demonstrated. The total surface of ulcers decreased by 41.7% compared to 14.5% in the placebo group. The results suggest that C. officinalis extract accelerates wound healing significantly\(^{(27)}\).

**Mechanisms of Action of the Anti-inflammatory and Anticancer Activities of Triterpene Alcohols**

An increasing number of scientific studies document the interrelation between proinflammatory states and tumour genesis and promotion\(^{(28,29)}\). Phytochemicals with strong anti-inflammatory effects are interesting for the investigation of cancer-preventive activity. In recent years triterpenoid alcohols have attracted interest because of their good efficacy and low toxicity\(^{(30)}\).

The triterpene esters in Calendula extracts have similar benefits as the well-known triterpene acids, e.g. oleanolic and ursolic acids with the same pentacyclic structure. They are recommended in the case of inflammatory conditions, have immunomodulatory efficacy and modulate pathways involved in cancer formation and proliferation.
The anti-inflammatory and cancer-preventing activity of faradiol esters, taraxasterol, amyrin and lupeol, which all are constituents of the Calendula CO2-extract, has been demonstrated in different scientific studies\(^\text{31,32}\).

A triterpene enriched Calendula fraction given orally to mice inoculated with Ehrlich mouse carcinoma prevented the development of ascites and increased survival time compared to the control\(^\text{33}\).

Taraxasterol and faradiol esters showed strong inhibitory activity against TPA-induced inflammation in mice. These compounds inhibited markedly the tumour-promoting effect of TPA damaged skin after initiation with 7,12-DiMethylBenz(\(\alpha\))Anthracene (DMBA)\(^\text{31}\).

Anti-inflammatory mechanisms which play a role in anticarcinogenic activity include inhibition of NF-\(\kappa\)B, COX-2\(^\text{34,35}\), arachidonic acid metabolism\(^\text{36}\), decreased expression of inflammatory cytokines IL-1\(\beta\), IL-6, and TNF-\(\alpha\), resulting in growth inhibition of cancer cell lines\(^\text{37}\) and down-regulation of enzymes, such as protein kinase C which mediates inflammation and tumour-cell proliferation\(^\text{37}\). The following table gives an overview of amyrin and lupeol molecular targets.

Topical application of \(\alpha\)-amyrin dose-dependently inhibited TPA-induced increase of prostaglandin E2 (PGE2) levels and COX-2 expression in the mouse skin. The evaluation of NF-\(\kappa\)B pathway revealed that topical treatment with \(\alpha\)-amyrin is able to prevent IkB\(\alpha\) degradation, p65/RelA phosphorylation and NF-\(\kappa\)B activation. Moreover, \(\alpha\)-amyrin applied topically inhibited in dose-dependent manner the activation of upstream protein kinases, namely extracellular signal-regulated protein kinase (ERK), p38 mitogen-activated protein kinase (MAPK) and protein kinase C (PKC)\(\alpha\) after a topical TPA treatment. Collectively, the present results suggest that topical skin application of \(\alpha\)-amyrin exerts a strong and rapid inhibition of TPA-induced inflammation\(^\text{38}\).

**Cosmetic Application**

*Calendula* ointments are recommended for the treatment of damaged skin. They stimulate granulation, enhance the formation of cell tissue, accelerate wound healing, have an anti-inflammatory and pain killing effect. *Calendula* is used to cure decubitus, burns, eczema, bee stings, swelling and inflammation. *Calendula* preparations are also traditionally used to treat inflammation of the veins, varicose veins and hemorrhoids, the positive therapeutic effects being proven by several medical studies.

*Calendula* cream is used to treat sensitive, dry and chapped skin. The cream improves the resistance against environmental chemicals or mechanical irritations, restores the hydration equilibrium, has an anti-ageing effect, helps to repair cellular damages and creates a smooth and beautiful surface. Furthermore *Calendula* is applied for the preparation of tonic face lotions, sun-creams, aftershave products and lipsticks. The positive properties are also used in soaps, hair shampoos and bath additives for dry skin. Last but not least, *Calendula* extracts can be used in mouth care products and tooth pastes. The *Calendula* CO2-extract is thus an excellent tool to formulate a wide range of active high grade cosmetics.

**Toxicology**

It should be pointed out that *Calendula* extracts, against common opinion, do not contain any sesquiterpene lactones which may cause allergies\(^\text{48}\). Thus *Calendula* extracts do not irritate sensitive skin. *Calendula* extracts are non-toxic and no secondary effects have been observed. The topical administration of preparations of *Calendula* flowers can be regarded as safe, especially at therapeutic doses. The Cosmetic Ingredient Review (CIR) panel confirms that *Calendula officinalis* flower extracts are safe as used in cosmetic formulations in concentrations up to 6%. They also were not irritating, sensitising, or photosensitising in animal or clinical tests but may be mild ocular irritants.

<table>
<thead>
<tr>
<th>Triterpene</th>
<th>Targets</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amyrin</td>
<td>NF-(\kappa)B, IL-(\beta), COX-2, CREB, ERK, PKC, P38 MAPK</td>
<td>(^\text{38,39})</td>
</tr>
<tr>
<td>Lupeol</td>
<td>NF-(\kappa)B, cFLIP, survivin, Bax, caspase-3, caspase-9</td>
<td>(^\text{40-49})</td>
</tr>
</tbody>
</table>

Table 4. Amyrin and Lupeol Molecular Targets
Natural Ingredients

The CIR Expert Panel concluded that these ingredients are safe for use in cosmetics in the usage practices and concentration given in this amended safety assessment. More details about Calendula concerning the botany, chemistry, pharmacology, toxicology and therapeutic applications are given in a comprehensive monograph.

Conclusion

Calendula flower preparations have remarkable healing power. This confirms the traditional use for treatment of wounds and all kinds of inflammatory skin problems and diseases. Supercritical CO2-extraction is the state-of-the art technology for concentrating the bioactive ingredients of Calendula flowers. The CO2-extract contains the lipophilic triterpene enriched fraction with faradiol esters as the main components. The efficacy of faradiol esters is supported by further triterpene monols and triols, especially taraxasterols and amyrins. The triterpene alcohols are highly active anti-inflammatory ingredients and additionally they have a positive effect on key biochemical pathways influencing ageing, tumour genesis and promotion. The perfect blend of bioactive components makes the supercritical Calendula extract an ideal ingredient for natural cosmetics with protective and restorative efficacy.

References

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Authors’ Biographies

Dr. Peter May studied chemistry at the University of Saarland and received his Ph.D. in 1991 in metal organic chemistry. Dr. May is the author of several publications about plant science and phytotherapy. Peter has worked at Flavex Naturextrakte GmbH for over 10 years within the Sales and Marketing Department.

Dr. K.-W. Quirin is a chemist and received his Ph.D. in 1984 from the University of Saarland, Institute of Pharmacognosy and Analytical Phytochemistry. He holds two scientific prizes and is also the author of a number of book chapters and many articles.

Dr. Quirin has been CEO of Flavex Naturextrakte GmbH for 28 years, a company producing specialty botanical extracts for cosmetics and food supplements on the basis of supercritical CO2-extraction.