Università degli Studi di Padova

Padua Research Archive - Institutional Repository

Protective effects of ψ taraxasterol 3-O-myristate and arnidiol 3-O-myristate isolated from *Calendula officinalis* on epithelial intestinal barrier

Original Citation:

Availability:

This version is available at: 11577/3182471 since: 2017-01-28T08:18:49Z

Publisher:

Elsevier

Published version:

DOI: 10.1016/j.fitote.2016.01.007

Terms of use:

Open Access

This article is made available under terms and conditions applicable to Open Access Guidelines, as described at <http://www.unipd.it/download/file/fid/55401> (Italian only)

(Article begins on next page)

Accepted Manuscript

Protective effects of ψ taraxasterol 3-*O*-myristate and arnidiol 3-*O*-myristate isolated from *Calendula officinalis* on epithelial intestinal barrier

Stefano Dall'Acqua, Daniela Catanzaro, Veronica Cocetta, Nadine Igl, Eugenio Ragazzi, Maria Cecilia Giron, Laura Cecconelo, Monica Montopoli

PII: S0367-326X(16)30008-9
DOI: doi: [10.1016/j.fitote.2016.01.007](https://doi.org/10.1016/j.fitote.2016.01.007)
Reference: FITOTE 3337

To appear in: *Fitoterapia*

Received date: 23 November 2015
Revised date: 4 January 2016
Accepted date: 8 January 2016

Please cite this article as: Stefano Dall'Acqua, Daniela Catanzaro, Veronica Cocetta, Nadine Igl, Eugenio Ragazzi, Maria Cecilia Giron, Laura Cecconelo, Monica Montopoli, Protective effects of ψ taraxasterol 3-*O*-myristate and arnidiol 3-*O*-myristate isolated from *Calendula officinalis* on epithelial intestinal barrier, *Fitoterapia* (2016), doi: [10.1016/j.fitote.2016.01.007](https://doi.org/10.1016/j.fitote.2016.01.007)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Protective Effects of ψ Taraxasterol 3-*O*-myristate and Arnidiol 3-*O*-myristate Isolated from *Calendula officinalis* on Epithelial Intestinal Barrier

Stefano Dall'Acqua,^{*a} Daniela Catanzaro,^a Veronica Cocetta,^a Nadine Igl,^b Eugenio Ragazzi,^a
Maria Cecilia Giron,^a Laura Cecconelo,^a Monica Montopoli^a

^aDepartment of Pharmaceutical and Pharmacological Sciences, University of Padova, via Marzolo 5, Padova, Italy.

^bNATECO2 GmbH & Co. KG, Auenstrasse 18-20, 85283 Wolnzach, Germany.

Corresponding Author

Tel/Fax +39 049 827 5332/5336. E-mail stefano.dallacqua@unipd.it (S. Dall'Acqua).

Abstract

The triterpene esters Ψ taraxasterol-3-*O*-myristate (**1**) and arnidiol-3-*O*-myristate (**2**) were tested for their ability to protect epithelial intestinal barrier in an *in vitro* model. Their effects on ROS production and on trans-epithelial resistance were investigated on CaCo-2 cell monolayers both in basal and stress-induced conditions. Both compounds were able to modulate the stress damage induced by H₂O₂ and INF γ +TNF α , showing a potential use as model compounds for the study of new therapeutic agents for intestinal inflammations.

1. INTRODUCTION

Calendula officinalis L. (marigold) flower extracts are largely used for their anti-inflammatory effects especially for topical applications [1]. Furthermore the flower preparations were previously investigated for their anti-inflammatory, [1-4] antitumor-promoting [2], and cicatrizing effects [2]. Marigold extracts were also studied for their *in vitro* anti-HIV activity [5], hypoglycemic effects, gastric emptying inhibitory activity, and gastroprotective effects [3]. *C. officinalis* triterpenoids are considered as the most important anti-inflammatory principles of the extract [1,6] and studies reporting chemical modifications on triterpene in order to improve anti-inflammatory effect and to study structure-activity relationships were also reported [7]. Eleven triterpene alcohols isolated from Compositae Family flowers were studied in the mid-nineties for their inhibitory effects on TPA-induced inflammation in mice, showing inhibitory effects comparable to reference substances as indomethacin and hydrocortisone [8]. The interest in the triterpene derivatives as new therapeutic agents is growing and recently the synthetic oleanane triterpenoids were considered, due to their role of multifunctional drugs with possible applications for prevention and treatment of chronic diseases [9].

Inflammatory bowel diseases (IBD) are intestinal chronic disorders with increasing incidence in Europe, Northern America and Asiatic countries. Ulcerative colitis (UC) and Crohn's disease (CD) are the most common types of chronic IBDs [10]. The diseases cause disruption of intestinal homeostasis, leading to deregulated inflammatory gut responses. As inflammation is intimately related to formation of reactive intermediates, including reactive oxygen and nitrogen species (ROS/RNS), oxidative stress has been proposed as a mechanism underlying the pathophysiology of IBD [11].

To control the inflammation and symptoms in IBD patients, aminosalicilates and corticosteroids are in general used, but in severe cases immunosuppressive drugs as well as anti-TNF α -monoclonal antibody can be prescribed [12]. Thus the anti-inflammatory and anti-ROS/RNS properties are considered important characteristics for potential therapeutic agents. In this area natural products may offer new opportunities, being still largely unexplored for their effect in inflammation, especially on intestinal epithelial tissues.

Triterpenoids are largely diffused in plant kingdom, and are present in numerous medicinal plants. Several classes of pentacyclic triterpenoids are found in nature differing for substituents and ring closures. For example oleanane, ursane and friedelanes (that are typical of conifers bark) possess antibacterial and antifungal actions that are strictly related to their role of secondary metabolites in plants [9]. Furthermore many edible plants contain pentacyclic triterpenes thus indicating that several of such compounds are relatively non toxic and can be ingested for long periods of time [9]. For these reasons, due to the documented anti-inflammatory effects, and for the well-known low toxicity, the marigold triterpenes may be ideal candidate as model compounds for the evaluation of novel anti-inflammatory agents for chronic intestinal inflammatory diseases treatment.

In this paper, we describe the protective effects of two different triterpene esters, namely Ψ taraxasterol-3-*O*-myristate (**1**) and arnidiol-3-*O*-myristate (**2**) on in vitro model of epithelial

intestinal barrier. The two considered triterpenes were both able to protect the intestinal cell lines from oxidative and inflammatory stress.

2. EXPERIMENTAL SECTION

2.1 General experimental procedures.

NMR spectra were recorded using a Bruker Avance III spectrometer operating at 400 MHz (^1H) and 100 MHz (^{13}C). Spectra were measured in deuterated chloroform at 303 K. The ^1H and ^{13}C chemical shifts were referenced to solvent signal [7.26 (^1H) and 77.00 (^{13}C)]. 2D -NMR measurements namely gs-COSY, gs-HSQC, gs HMBC were used to assign structures of isolated compounds. Mass spectra were obtained on a Varian 500 IT mass spectrometer equipped with an Atmospheric Pressure Chemical Ionization (APCI) source. Spectra were acquired in full scan in the range 50-2000 Da. For chromatographic purification an Agilent 1100 apparatus equipped with auto sampler, diode array detector and fraction collector was used. An Agilent 1260 apparatus equipped with auto sampler diode array and ELSD (Sedex LX 60) detector was used for purity determination.

2.2 Extraction and isolation

For the concentration of the triterpene ester fraction a preliminary extraction process with supercritical CO_2 (SCO_2) was operated in a R&D plant of NATECO₂. The use of SCO_2 for the extraction of *C. officinalis* triterpene was previously reported [13,14]. Before extraction the dried flowers were milled with a hammer mill to a particles size of < 2 mm and filled into the extraction basket. The extraction basket had a volume of 50 l and 10 kg of *C. officinalis* material per run were inserted. Extraction runs were performed with extraction pressures of 600 bars at 70 °C. Two separators were integrated in the extraction cycle to gain two fractions of extracted oleoresin. At the first separation step the pressure was reduced to 250 bar before decompression to the pressure of the

storage tank (60-70 bar) at the second separator. In the first fraction 1.5%, in the second separator 6.5% of oleoresin precipitated and the total yield of extracts was 8%.

For the purification of the target compounds 3.5 g of *Calendula* flowers SCO₂ extract was diluted with cyclohexane (5 mL) and was charged on a Varian Intelliflash Flash chromatograph. The stationary phase was a Silica column (Analogic Superflash SF25-120g). Sample was eluted using cyclohexane and 50 fractions of 15 mL were collected. Fractions were then collected on the basis of their TLC behaviour (Silica / eluents Cyclohexane 6: ethyl acetate 1) after spraying with vanillin and sulphuric acid and heating at 110 °C. Fraction 8-15 were collected together yielding 850 mg of material after solvent removal. Fractions were then analysed by HPLC and HPLC-MS in order to confirm the presence of triterpene esters. The residue was dissolved in methanol and used to perform the semipreparative HPLC. The stationary phase was an Agilent Eclipse XDB-C8 (9.4 x 250 mm, 5 μ). Elution was conducted starting with 75% methanol and going to 100% in 40 minutes. Flow rate was set at 3 ml/min. Chromatogram was recorded at 210 nm and portions of 100 μ L were repeatedly injected for peak collection. Peak eluted in the 12.5 to 35 minutes range. Ten peaks were collected representing the calenduladiol, arnidiol and faradiol esters of lauric, myristic and palmitic acid and the ψ taraxasterol-3-*O*-myristate. Identity of taraxasterol-3-*O*-myristate (21 mg) and arnidiol-3-*O*-myristate (25 mg) were confirmed by comparison of spectral data with those previously published [2,4,7,15,16]. Mass spectra of isolated compounds were also obtained using the APCI source. Purity of compounds used for biological activity was established to be >98% on the basis of HPLC-DAD analysis.

2.3 Intestinal cell monolayer preparation and treatment

Caco-2 cells were grown in high-glucose Dulbecco's modified Eagle's media (DMEM). Medium was supplemented with 10% FBS, 2% L-glutamine and 1% penicillin/streptomycin. Cells were

maintained under a humidified atmosphere of 5% CO₂ in air, at 37°C. Experimental inflammatory condition in Caco-2 cell monolayers was induced by the exposure for different times according to the assays, to 500 µM H₂O₂ or to 2.5 ng/ml recombinant human IFN-γ for 3 hours and then 10 ng/ml TNF-α were added.[17] Twenty-four hours pre-treatment with triterpenes(0.1-0.01 µM) was applied before the inflammatory stimuli. Reagents for cell cultures were from Lonza (Verviers, Belgium), whereas IFN-γ and TNF-α were from R&D Systems (Minneapolis, USA).

2.4 Cell viability assay

Cell viability was determined by the 3-(4,5-dimethyl- thiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St Louis, MO, USA) assay, as previously described [18]. Briefly, Caco-2 cells were seeded in 96-multiwells culture plates and treated with triterpenes (0.1-100 µM) for 24, 48 or 72 hours. The formazan absorbance was measured at 570 nm, using a Multilabel Plate Reader VICTOR™ X3(Wallac Instruments, Turku, Finland).

2.5 ROS fluorescence assay

ROS were quantified using 2',7'-dichlorofluorescein-diacetate (H₂-DCF-DA, Sigma-Aldrich, St Louis, MO, USA), as previously described [17]. Upon cleavage of the acetate groups by intracellular esterase and oxidation, the H₂.DCF-DA is converted to the fluorescent 2',7'-dichlorofluorescein (DCF). Briefly, the intestinal cells (5×10³) were seeded into 96-well plates and allowed to adhere overnight. ROS level was measured after the exposure to triterpenes or AKBA for 24 hours, in the absence or presence of 500 µM H₂O₂, and subsequent addition of 10 µM H₂-DCF-DA, further incubation for 30 min at 37°C and washing with phosphate-buffered saline (PBS). DCF fluorescence intensity was measured at excitation 485 nm - emission 535 nm, using a Multilabel

Plate Reader VICTOR™ X3 (Wallac Instruments, Turku, Finland). Fold increase in ROS production was calculated using the equation: $(F_{\text{treatment}} - F_{\text{blank}}) / (F_{\text{control}} - F_{\text{blank}})$, where F is the fluorescence reading.

2.6 Trans-epithelial electrical resistance (TEER) assay

Caco-2 cell cultures were performed essentially as previously described[17]. Briefly, the cells were seeded on Transwell™ polyester membrane cell culture inserts (transparent PET membrane: 1.0 cm² growth surface area, 0.4 μm pore size; BD Falcon™) in 24-well plates and incubated with the appropriate culture media at 37 °C in a humidified atmosphere and 5% CO₂. Culture media were replaced every day until confluent monolayer was obtained. The TEER assay was performed in HBBS (Hanks' Balanced Salt solution, Lonza, Verviers, Belgium) after an equilibration period at room temperature. Treatments were added to the basal chamber without manipulating the apical media. Millicell® ERS meter, Millipore Corporation (Bedford, MA) connected to a pair of chopstick electrodes were inserted in the donor and receiver chambers and the 24 hours of TEER variation was recorded [17]. TEER was expressed as percentage of resistance, normalized to initial value.

3. RESULTS AND DISCUSSION

3.1 Calendula officinalis L. SCO₂ extract composition, and compound isolation

The SCO₂ extract of *C. officinalis* L. aerial part was obtained as a brownish resinous material with characteristic calendula smell. A preliminary direct infusion of the extract solution in APCI-MS in positive ion mode allowed the identification of different triterpene esters on the basis of their pseudomolecular ions as shown in Graph. 1A. In particular the presence of the pseudomolecular

ion at m/z $[M+H]^+$ 637 and at m/z $[M+H-H_2O]^+$ 663, 635 and 607 are ascribable to due to the presence of C-30 monohydroxyl triterpene miristate and to the typical C-30 dihydroxy triterpene mono esters of *C. officinalis* namely calenduladiol, adnidiol and faradiol as lauric, myristic and palmitic esters. Fragments ascribable to the fatty acid loss are also detected at m/z 407 and 409 respectively for dihydroxy and monodihydroxy triterpene derivatives. The HPLC-APCI-MS analysis allowed to detect several triterpene esters of *C. officinalis* as depicted in Graph. 1B. Repeated semipreparative HPLC-DAD separation (see material and methods) lead to the isolation of compound Ψ taraxasterol-3-*O*-myristate (**1**) and arnidiol-3-*O*-myristate (**2**) their APCI-MS spectra are reported in Graph 1C and 1D.

3.2 Effects of (**1**) and (**2**) on Caco-2 cells vitality.

We studied the effect, on Caco-2 intestinal epithelial cell monolayers, of the treatment with different concentrations of (**1**) and (**2**). The Caco-2 intestinal epithelial cell monolayers were treated for 24 and 48 hours with the triterpenes at four different concentrations (0.01-10 $\mu\text{g/ml}$). Fig.1 shows that the tested triterpenes did not significantly affect the viability of both the intestinal cell lines. The non-toxic behaviour of those compounds on Caco-2 cells (in the considered concentration 0.01-10 $\mu\text{g/ml}$) is comparable with the effects observed for 11-acetyl-keto-boswellic acid (AKBA) that we previously studied in the same model [17].

3.2 Effects of (**1**) and (**2**) on Caco-2 ROS production.

Abnormally high levels of ROS are produced in IBD and their destructive effects may contribute to the initiation and/or propagation of tissue damage [11]. Thus we studied the effects of (**1**) and (**2**) on ROS production in the same cell models by dichlorofluorescein acetate. The activity of triterpenes was evaluated (for 24 h) on ROS production in Caco-2 cell monolayers in basal condition and after exposure to H_2O_2 , as *in vitro* model of oxidative stress [19]. In the Caco-2 cells the treatment with the triterpenes (**1**) and (**2**) did not alter the basal intracellular ROS levels as shown in Fig 2.

Furthermore (**2**) significantly reduced ROS generation induced by the oxidative stimulus ($p = 0.01$) at all the tested concentrations, while for (**1**) this effect resulted significant only at the lowest concentrations (0.01 $\mu\text{g/ml}$). As a comparison a well known antioxidant, the N-acetyl-cystein (NAC), was used and the two compounds presented lower activity compared to NAC. In order to compare our triterpene activity a further reference compound was used namely the acetyl-11-keto-boswellic acid (AKBA) that we recently studied and was able to reduce H_2O_2 oxidative stimulus ROS generation in Caco-2 cells. As shown in Fig 3 at the two tested concentrations (0.01 and 0.1 $\mu\text{g/mL}$) AKBA significantly decreased, about 50% compared to controls, ROS generation. These data suggest a different mode of action of the two tested triterpene esters compared to AKBA due to the limited effect of (**1**) and (**2**) on ROS generation induced by the H_2O_2 oxidative stimulus.

It is widely accepted that the epithelial barrier function is impaired in patients with inflammatory bowel disease (IBD). The intestinal permeability is defined as the ability of compounds to cross the intestinal mucosa through the paracellular junction areas restricted. Studies with different inert non-metabolized probes indicate that paracellular permeability is increased in Crohn's disease (CD) [20]. This increase in permeability has been shown in patients with active inflammatory bowel disease (IBD) but there is no evidence of a return to normal function when the disease is in remission. Furthermore, the increase of the permeability may precede the onset of inflammation [21]. To assess a potential role of such triterpenes as possible new agents in the treatment of intestinal inflammatory diseases, their effect towards the intestinal permeability was studied using transepithelial resistance measurements (TEER) in an *in vitro* model, as we previously described [17].

3.3 TEER measurements.

We performed the TEER measurements on cells studying the effects of (**1**) and (**2**) at two concentrations (0.01 and 0.1 $\mu\text{g/ml}$) due to the non toxic effect on cells. Both the compounds did not

alter the permeability as shown by the TEER measurements compared with control (Fig 4). Furthermore stimulus with H_2O_2 or $INF\gamma+TNF\alpha$ were used in the *in vitro* model in order to study the ability of (1) and (2) to modulate oxidative stress and inflammatory conditions, respectively using AKBA as reference compound. As showed in Fig.5, the three triterpenes significantly increased the trans-epithelial resistance in inflammatory and in oxidative damage-induced conditions. A decreased TEER value was observed after treatment with H_2O_2 (-50%). On the other hand, the treatment with the two triterpenes (0.1 and 0.01 $\mu g/ml$) significantly increased the TEER values compared to H_2O_2 treatment, indicating a lower permeability in the presence of the triterpenes. At 24 hours, the treatment at both concentrations 0.01 and 0.1 $\mu g/ml$ produced a significant change in TEER compared to H_2O_2 ($p = 0.01$ towards H_2O_2 as a reference) resulting in >90% of initial TEER also at lower dose. The effect of (1) and (2) compared to the AKBA, was very similar showing a new activity for those triterpene esters (Fig 6.).

The natural triterpene can be considered promising compounds in this therapeutic area, in fact previous reports indicated that α and β -amyrin showed a systemic anti-inflammatory action of in TNBS-induced colitis Swiss albino mice [22], and in the dextran sulfate sodium (DSS)-induced colitis in mice [23]. Their mechanism of action was at least in part related to interaction with ability to interact with cannabinoid system as also reported in other study [24]. Also the *Boswellia*-derived triterpene AKBA was previously investigated for its positive effects on intestinal inflammation [25], and our group recently demonstrated a further role in the protective effect on epithelial permeation [17]. The various natural derivatives may exert different effects due to multiple mechanisms of action due to their variation in the chemical structures, thus further studies are needed in order to study possible structure-activity relationships and the role of the esterification with fatty acid on bioactivity.

This observation opens an additional investigation view on the molecular mechanism of triterpene activity. In fact (1) and (2) were able to modulate the *in vitro* indices of dysfunction in the used *in vitro* models, which have a practical evidence in the clinical manifestation of IBD.

4. Conclusion

This study shows that the triterpene esters Ψ taraxasterol-3-*O*-myristate (1) and arnidiol-3-*O*-myristate (2) (0.01 $\mu\text{g/mL}$) were able to maintain TEER levels during inflammatory and oxidative damage-induced conditions, showing a protective effect on epithelial permeation. Compared to AKBA, negligible effect is observed on ROS production thus suggesting that at least in part a different mode of action can be postulated for these compounds. Considering the well-known anti-inflammatory properties of these compounds and their relatively poor toxicity, the present findings indicate that these triterpenes have some potential as protective agents on intestinal permeability.

Further studies are needed to explore possible structure-activity relationships as well as to assess safety and efficacy of such compounds on more complex models of IBD or other intestinal inflammatory diseases.

ACKNOWLEDGMENT

We would like to gratefully acknowledge Unifarco s.p.a. for their donation of dried *Calendula* flowers used for the extraction.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This work was supported by grants from the University of Padova (CPDA124517/12 to MM; UNIPDex60% n. 60A04-0443 to ER, and MM, UNIPDex60% n. 60A04-9128/14 to MCG and MM, UNIPDex60% 60A04-8917/12 and UNIPDex60%A04-8445/13 to SDA). DC fellowship was supported by the grant n.CPDR134012 from the University of Padova. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare no competing financial interest.

Chart 1. Structures of the isolated compounds, taraxasterol-3-O-myristate (1) and arnidiol-3-O-myristate (2).

Graph 1. **A)** APCI-MS direct infusion of SCO₂ extract of *C. officinalis*; main peaks are tentatively assigned on the basis of the *m/z* values. **B)** HPLC-APCI-MS of SCO₂ extract of *C. officinalis*; main peaks are assigned on the basis of the *m/z* values and retention times. **C)** APCI-MS spectra of compound (1). **D** APCI-MS spectra of compound (2).

Figure legends

Figure 1. Effect of triterpenes on cell viability measured by MTT assay in Caco-2 intestinal epithelial cell lines. Results are expressed as absorbance Units, mean $\pm$ SEM ($n = 3$), normalized to values in untreated cells.

Figure 2. Effect of triterpenes on ROS level in Caco-2 cells. ROS were measured after 24 hours of incubation in untreated cells and after 500 μ M H₂O₂ exposure. Data are expressed as mean $\pm$ SEM percentage of basal (100%) DCF fluorescence intensity (FI) of $n = 4$ experiments. * $p < 0.05$ treatment vs H₂O₂ ** $p < 0.01$ treatment vs H₂O₂.

Figure 3. Effect of AKBA(A) and NAC (B) on ROS level in Caco-2 cells. ROS were measured after 24 hours of incubation in untreated cells and after 500 μ M H₂O₂ exposure. Data are expressed as mean $\pm$ SEM percentage of basal (100%) DCF fluorescence intensity (FI) of $n = 4$ experiments. ** $p < 0.01$ AKBA vs control; *** $p < 0.0001$ AKBA vs control; §§§ $p < 0.0001$ H₂O₂ vs control.

Figure 4. Effect of triterpenes on transepithelial electrical resistance (TEER) in Caco-2 cells. Data are expressed as mean $\pm$ SEM percentage of baseline TEER ($n = 3$ experiments).

Figure 5. Effect of triterpenes on transepithelial electrical resistance (TEER) in Caco-2 cells after 24h treatment with $\text{INF}\gamma$ + $\text{TNF}\alpha$ and H_2O_2 . Data are expressed as mean $\pm$ SEM percentage of baseline TEER ($n = 3$ experiments); $*p<0.05$, $p<0.01$, $***p<0.0001$ vs stimuli; $§§§p<0.0001$ vs control.**

Figure 6. Effect of AKBA on trans-epithelial electrical resistance (TEER) in Caco-2 cells after 24h treatment with $\text{INF}\gamma$ + $\text{TNF}\alpha$ and H_2O_2 . Data are expressed as mean $\pm$ SEM percentage of baseline TEER ($n = 3$ experiments); $*p<0.05$, $p<0.01$, $§§§p<0.0001$.**

References

- [1] B.P. Muley, S.S. Khadabadi, N.B. Banarase, Phytochemical constituents and pharmacological activities of *Calendula officinalis* Linn (Asteraceae): A review, *Tropical Journal of Pharmaceutical Research*. 8 (2009) 455-465.
- [2] M. Ukiya, T. Akihisa, K. Yasukawa, H. Tokuda, T. Suzuki, Y. Kimura, Anti-inflammatory, anti-tumor-promoting, and cytotoxic activities of constituents of marigold (*Calendula officinalis*) flowers, *J. Nat. Prod.* 69 (2006) 1692-1696.
- [3] M. Yoshikawa, T. Murakami, A. Kishi, T. Kageura, H. Matsuda, Medicinal flowers. III. Marigold. (1): Hypoglycemic, gastric emptying inhibitory, and gastroprotective principles and new oleanane-type triterpene oligoglycosides, calendasaponins A, B, C, and D, from egyptian *Calendula officinalis*, *Chemical and Pharmaceutical Bulletin*. 49 (2001) 863-870.
- [4] K. Zitterl-Eglseer, S. Sosa, J. Jurenitsch, M. Schubert-Zsilavec, R. Della Loggia, A. Tubaro, M. Bertoldi, C. Franz, Anti-oedematous activities of the main triterpendiol esters of marigold (*Calendula officinalis* L.), *J. Ethnopharmacol.* 57 (1997) 139-144.
- [5] Z. Kalvatchev, R. Walder, D. Garzaro, Anti-HIV activity of extracts from *Calendula officinalis* flowers, *Biomedicine and Pharmacotherapy*. 51 (1997) 176-180.
- [6] R. Della Loggia, A. Tubaro, S. Sosa, H. Becker, S. Saar, O. Isaac, The role of triterpenoids in the topical anti-inflammatory activity of *Calendula officinalis* flowers, *Planta Med.* 60 (1994) 516-520.
- [7] H. Neukirch, M. D'Ambrosio, S. Sosa, G. Altinier, R. Della Loggia, A. Guerriero, Improved anti-inflammatory activity of three new terpenoids derived, by systematic chemical modifications, from the abundant triterpenes of the flowery plant *Calendula officinalis*, *Chemistry and Biodiversity*. 2 (2005) 657-671.
- [8] T. Akihisa, K. Yasukawa, H. Oinuma, Y. Kasahara, S. Yamanouchi, M. Takido, K. Kumaki, T. Tamura, Triterpene alcohols from the flowers of compositae and their anti- inflammatory effects, *Phytochemistry*. 43 (1996) 1255-1260.
- [9] K.T. Liby, M.B. Sporn, Synthetic oleanane triterpenoids: Multifunctional drugs with a broad range of applications for prevention and treatment of chronic disease, *Pharmacol. Rev.* 64 (2012) 972-1003.
- [10] E.G. Quetglas, A. Armuzzi, S. Wigge, G. Fiorino, L. Barnscheid, M. Froelich, S. Danese, Review article: The pharmacokinetics and pharmacodynamics of drugs used in inflammatory bowel disease treatment, *Eur. J. Clin. Pharmacol.* 71 (2015) 773-799.
- [11] H. Zhu, Y.R. Li, Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: Updated experimental and clinical evidence, *Exp. Biol. Med.* 237 (2012) 474-480.
- [12] K.M. Taylor, P.M. Irving, Optimization of conventional therapy in patients with IBD, *Nat. Rev. Gastroenterol. Hepatol.* 8 (2011) 646-656.
- [13] M. Hamburger, S. Adler, D. Baumann, A. Förg, B. Weinreich, Preparative purification of the major anti-inflammatory triterpenoid esters from Marigold (*Calendula officinalis*), *Fitoterapia*. 74 (2003) 328-338.

- [14] D. Baumann, S. Adler, S. Grüner, F. Otto, B. Weinreich, M. Hamburger, Supercritical carbon dioxide extraction of marigold at high pressures: Comparison of analytical and pilot-scale extraction, *Phytochem. Anal.* 15 (2004) 226-230.
- [15] M. Ukiya, T. Akihisa, H. Tokuda, H. Suzuki, T. Mukainaka, E. Ichiishi, K. Yasukawa, Y. Kasahara, H. Nishino, Constituents of Compositae plants: III. Anti-tumor promoting effects and cytotoxic activity against human cancer cell lines of triterpene diols and triols from edible chrysanthemum flowers, *Cancer Lett.* 177 (2002) 7-12.
- [16] H. Neukirch, M. D'Ambrosio, J. Dalla Via, A. Guerriero, Simultaneous quantitative determination of eight triterpenoid monoesters from flowers of 10 varieties of *Calendula officinalis* L. and characterisation of a new triterpenoid monoester, *Phytochem. Anal.* 15 (2004) 30-35.
- [17] D. Catanzaro, S. Rancan, G. Orso, S. Dall'Acqua, P. Brun, M.C. Giron, M. Carrara, I. Castagliuolo, E. Ragazzi, L. Caparrotta, M. Montopoli, *Boswellia serrata* Preserves Intestinal Epithelial Barrier from Oxidative and Inflammatory Damage, *PLoS One.* 10 (2015) e0125375.
- [18] E.M. Nagy, S. Sitran, M. Montopoli, M. Favaro, L. Marchiò, L. Caparrotta, D. Fregona, Zinc(II) complexes with dithiocarbamate derivatives: Structural characterisation and biological assays on cancerous cell lines, *J. Inorg. Biochem.* 117 (2012) 131-139.
- [19] N. Wang, G. Wang, J. Hao, J. Ma, Y. Wang, X. Jiang, H. Jiang, Curcumin ameliorates hydrogen peroxide-induced epithelial barrier disruption by upregulating heme oxygenase-1 expression in human intestinal epithelial cells, *Dig. Dis. Sci.* 57 (2012) 1792-1801.
- [20] L. Shen, L. Su, J.R. Turner, Mechanisms and functional implications of intestinal barrier defects, *Dig. Dis.* 27 (2009) 443-449.
- [21] M.C. Arrieta, L. Bistritz, J.B. Meddings, Alterations in intestinal permeability, *Gut.* 55 (2006) 1512-1520.
- [22] C.E. Vitor, C.P. Figueiredo, D.B. Hara, A.F. Bento, T.L. Mazzuco, J.B. Calixto, Therapeutic action and underlying mechanisms of a combination of two pentacyclic triterpenes, and β -amyirin, in a mouse model of colitis, *Br. J. Pharmacol.* 157 (2009) 1034-1044.
- [23] I. Matos, A.F. Bento, R. Marcon, R.F. Claudino, J.B. Calixto, Preventive and therapeutic oral administration of the pentacyclic triterpene α,β -amyirin ameliorates dextran sulfate sodium-induced colitis in mice: The relevance of cannabinoid system, *Mol. Immunol.* 54 (2013) 482-492.
- [24] K.A.B. Simão Da Silva, A.F. Paszcuk, G.F. Passos, E.S. Silva, A.F. Bento, F.C. Meotti, J.B. Calixto, Activation of cannabinoid receptors by the pentacyclic triterpene α,β -amyirin inhibits inflammatory and neuropathic persistent pain in mice, *Pain.* 152 (2011) 1872-1887.
- [25] C.F. Krieglstein, C. Anthoni, E.J.M. Rijcken, M. Laukötter, H.-. Spiegel, S.E. Boden, S. Schweizer, H. Safayhi, N. Senninger, G. Schürmann, Acetyl-11-keto- β -boswellic acid, a constituent of a herbal medicine from *Boswellia serrata* resin, attenuates experimental ileitis, *Int. J. Colorectal Dis.* 16 (2001) 88-95.

ψ-taraxasterol 3-O-myristate (1)

arnidiol 3-O-myristate (2)

Chart 1

Graph 1

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Protective Effects of ψ -Taraxasterol 3-O-myristate and Arnidiol 3-O-myristate

Isolated from *Calendula officinalis* on Epithelial Intestinal Barrier.

Graphical abstract