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Synergistic interactions of chamomile flower, myrrh and coffee charcoal in inhibiting pro-inflammatory chemokine release from activated human macrophages

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ABSTRACT

Background: The herbal medicinal product Myrrhinil-Intest®, a combination of myrrh, chamomile flower extract and coffee charcoal is used for the maintenance therapy of inflammatory bowel disease. *In vitro* studies revealed that the herbal components influence chemokine signaling of human macrophages as part of an anti-inflammatory mechanism. However, the occurrence of synergistic effects remains unexplored.
Aim: The present study aims to investigate the synergistic effect of dual combinations of the plant extracts on the pro-inflammatory chemokine (CXCL13) release from activated human macrophages.
Methods: The single effect of myrrh, chamomile flower and coffee charcoal extract on CXCL13 release from lipopolysaccharide stimulated human macrophages was investigated using ELISA and IC_{50} values were determined. Budesonide served as positive control. To characterize the combined effect, IC_{50} values were used to prepare combinations of two plant extracts in different proportions to each other (3:5; 1:1; 5:3). Interpretation of the data was based on isobologram analysis and calculation of a combination index (CI).
Results: LPS-induced CXCL13 release from human macrophages was inhibited after treatment with myrrh (IC_{50} = 19 μg/ml), chamomile flower (IC_{50} = 82 μg/ml) and coffee charcoal (IC_{50} = 106 μg/ml) whereby the extent of inhibition was comparable to budesonide. All combinations of two plant extracts resulted in synergistic effects with varying magnitude (CI from 0.82 to 0.42). The combination of myrrh and coffee charcoal (ratio of 3:5) exhibited the strongest synergistic effect (CI = 0.42). Increasing amounts of coffee charcoal resulted in increased synergistic activity.
Conclusion: Synergistic effects between all plant components contribute to the anti-inflammatory activity of all dual combinations of the plant components and support the composition of the herbal combination.

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1. Introduction

Phytomedicine has gained much attraction in the last decades especially for the treatment of chronic, recurrent or serious diseases [1]. The chemically complex nature of herbal preparations provides a basis for targeting complex physiological mechanisms within a multi-target approach [2] and the occurrence of pharmacological interactions between the herbal components.

The intestinal immune system exhibits very complex physiological mechanisms as it has to provide a rapid, effective immune response against pathogens while maintaining tolerance towards food and commensal bacterial antigens. Disturbances in this delicate balance may result in aberrant inflammatory response leading to chronic intestinal inflammatory diseases such as inflammatory bowel disease (IBD) [3]. IBD comprises the chronic

Abbreviations: CAM, complementary and alternative medicine; CI, combination index; IBD, inflammatory bowel disease.

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relapsing inflammatory disorders Crohn's disease and ulcerative colitis and is thought to result from an aberrant and continuing immune response to microbes in the gut, catalyzed by the genetic susceptibility of the patient [4]. Because of their multifaceted pathophysiology, IBD are eligible to be treated by a multi-targeted approach, which is exhibited by most herbal therapies.

A recent clinical study suggests the use of the traditional herbal medicinal product Myrrhinil-Intest® containing myrrh (*Commiphora molmol* Engler, Burseraceae), chamomile flower (*Matricaria chamomilla* L., Asteraceae) and coffee charcoal (*Coffea arabica* L., Rubiaceae) for the maintenance therapy of remission in ulcerative colitis [5]. This double-blind study provides first evidence for a non-inferior application against the gold standard therapy mesalazine. However, the underlying pharmacological mechanisms and especially the occurrence of synergistic effects are widely unknown and remain subject of molecular biological investigations.

In vitro studies revealed, that chemokine signaling of human macrophages is influenced by myrrh, chamomile flower and coffee charcoal as part of an anti-inflammatory mechanism [6]. One specific target is the macrophage release of chemokine receptor ligand CXCL13 (also known as B cell attracting chemokine 1; BCA-1), which is believed to play a vital role in the development of chronic inflammation [7,8].

The aim of the present study was to investigate the pharmacological interaction of combinations of two out of the herbal components myrrh, chamomile flower and coffee charcoal on LPS-induced CXCL13 release from activated human macrophages. Thus, the effect of each individual component on the release of CXCL13 was determined first and mixtures of two extracts were then prepared based on the determined IC_{50} values and tested in ascending concentrations. Graphic analysis of the data based on the isobologram method according to Berenbaum [9] was performed to assess the type of interaction and a combination index introduced by Chou and Talalay [10] was determined to quantify the pharmacological interaction.

2. Materials and methods

2.1. Plant extracts

Powdered myrrh obtained from the dried resin of mainly *Commiphora molmol* ENGLER, compliant with specifications in the European Pharmacopoeia monograph, and powdered coffee charcoal obtained from special roasted beans of Coffea arabica L. was provided by Lomapharm® Rudolf Lohmann GmbH KG, Emmerthal, Germany (Myrrhe Gum EB/BP pulv., batch-no. JA0167; Carbo Coffea EB 6, batch-no. JB0142, Lomapharm, Emmerthal, Germany). An ethanolic myrrh extract was prepared by extraction of the powdered resin under reflux with ethanol (96% v/v) at 90 °C for 60 min and subsequent steam distillation after suspension in water. Coffee charcoal extract was prepared by aqueous extraction of the powdered substance with hot water for 15 min. The chamomile flower dry extract (EtOH 60 % m/m; DER: 4–6:1; batch-no. HC0070) used in the finished dosage form was provided by Lomapharm® Rudolf Lohmann GmbH KG, Emmerthal, Germany.

2.1.1. Plant extract characterization with HPLC-DAD and HPLC–MS

HPLC chromatograms were recorded for all extracts and reference standards (apigenin, caffeine, ferulic acid, caffeic acid, nicotinic acid, trigonelline hydrochlorid, chlorogenic acid, neochlorogenic acid, kyrptochlorogenic acid, *p*-coumaric acid, theobromine, theophyillin, cafestol; purity ≥99%, HPLC; Sigma) on a Dionex Ultimate 3000 HPLC System (Thermo Scientific) with 717plus Autosampler (Waters). Chromatographic data were collected and integrated using a Waters 996 photodiode array detector (PDA). For HPLC–MS analysis an Agilent 1100 system consisting of binary pump, autosampler and variable wavelength detector was coupled to an Bruker Esquire 3000plus (ESI–MS) system. Spectra were recorded in negative and positive ion mode. A Nucleodur 100-5-C_{18} ec column (250 mm × 4 mm) purchased from Macherey-Nagel (Germany) operated at 25 °C was used for separation.

Gradient elution with methanol 0.1% (eluent A) and aqueous formic acid 0.1% (eluent B) was performed as follows: 0–10 min 5 % eluent A (isocratic); 10–90 min 5% eluent A → 100% eluent A; 90–150 min 100% eluent A (isocratic); 150–170 min 100% eluent A → 5% eluent A; 170–180 min 5% eluent A (isocratic); flow rate: 0.5 mL/min. HPLC-DAD chromatograms, UV spectra and molecular weight of selected peaks are presented in Supportive Information (Figs. 1–3S).

2.2. Cell culture and LPS stimulation

The human leukemic cell line THP-1 (ATCC, TIB-202) [11], which is commonly used to model macrophage function [12], was cultured in RPMI 1640 (Gibco, Life Technology) supplemented with fetal calf serum (FCS, 10%; Sigma Aldrich) and penicillin/streptomycin (P/S, 1%; Sigma Aldrich) at standard cell culture conditions. Differentiation to macrophage-like cells was induced with phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich) in a concentration of 100 ng/ml for 48 h. THP-1 cells were then stimulated with LPS (100 ng/ml, lipopolysaccharide from *E. coli* 0111:B4; Sigma-Aldrich) to induce a pro-inflammatory response as it has been demonstrated before [13].

2.3. Quantification of protein release

To determine the influence of the plant extracts on CXCL13 release, the plant extracts and combinations thereof were incubated simultaneously with LPS (100 ng/ml) in their respective concentration for 24 h. The anti-inflammatory glucocorticoid budesonide (1 nM; Sigma-Aldrich) served as positive control because it is a therapeutic option in the treatment of IBD. After incubation, protein concentration in the cell free supernatants was determined using CXCL13 ELISA Duo Set (R&D Systems). The assay was performed according to manufacturer's instructions.

2.4. Cell viability assay (MTT assay)

Simultaneous cytotoxicity controls were performed with the treated cells to ensure a stable cell viability throughout the assays. Thus, an MTT assay was used, to determine the relative amount of viable cells which are able to convert yellow soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) to purple formazan. Briefly, after macrophages were stimulated with LPS, the supernatant has been removed and remaining cells were treated with 100 μL MTT (0.3 mg/ml in PBS) per well for two hours. Untreated cells and cells treated with Triton X (0.1%) served as control. After the incubation period, cells were lysed by addition of 100 μL SDS lysis buffer. After complete cell lysis, the amount of resulting purple formazan was detected spectrophotometrically at 570 nm.

2.5. Determination of interaction between plant extracts

To assess the interaction of the plant extracts with regard to CXCL13 release inhibition from activated macrophages, concentration-response curves were first determined for all individual extracts and IC_{50} values were determined.

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