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Plant extracts in cell-based anti-inflammatory assays—Pitfalls and considerations related to removal of activity masking bulk components☆

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ABSTRACT

Plants used in traditional medicine represent an important source of new lead compounds. However, cell-based *in vitro* screening assays with plant material are hampered by the complex nature of plant extracts as mixtures of active and inactive components. Bulk constituents, such as chlorophyll and polyphenols were previously shown to interfere with several biological *in vitro* assays. Their influence on anti-inflammatory cell-based testing systems has not been thoroughly investigated. Hence, the present study was aimed at comparing different procedures for the removal of bulk constituents from plant extracts and examining the influence of their elimination on selected cell-based anti-inflammatory assays.

Malva sp. and *Glechoma hederacea* L., two plants used in traditional European medicine for the treatment of inflammatory disorders, were subjected to three different methods for the removal of chlorophyll and polyphenols, respectively. Removal of bulk constituents was confirmed by HPLC and mass spectrometry. Extracts were tested before and after the purification procedure, to determine their potential to inhibit the activation of the transcription factor NF-κB in reporter gene assay and to interfere with the secretion of the chemokine IL-8 after stimulation of endothelial cells with tumor necrosis factor (TNF-α) or lipopolysaccharide (LPS). Removal of chlorophyll from tested extracts led to a strong decrease in the anti-inflammatory activities, due to loss of bioactive constituents. In contrast, the effect of the polyphenol-free extracts was either not changed or significantly increased, depending on the purification method used. The study concluded that clearance of bulk compounds represents a valuable strategy for cell-based *in vitro* anti-inflammatory evaluation of plant extracts. Liquid–liquid partitioning was identified as the optimal method for the elimination of both chlorophyll and polyphenols. It is recommended that removal of chlorophyll from extracts always be accompanied by HPLC profiling to detect a possible loss of active constituents.

1. Introduction

Plant-derived natural products represent an important source of new compounds effective against various diseases including inflammatory disorders (Cragg and Newman, 2006; Newman and Cragg, 2007). As underlined by Heinrich, one of the characteristics of natural product extracts is that they represent complex mixtures

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of active, partially active and inactive components (Heinrich, 2010). When testing plant extracts in biological *in vitro* assays, a potential interference of ubiquitous substances, such as chlorophyll and bulk polyphenols (*e.g.* tannins) should be considered since they may generate false positive or false negative results. For example, chlorophyll is known to interact with fatty acids and also to exhibit anti-oxidative effects (Cho et al., 2000; Park et al., 2007; Potterat and Hamburger, 2006). Tannins on the other hand, form tight complexes with metal ions, proteins and polysaccharides. They also act as anti-oxidants and were shown to interfere with cell-based *in vitro* assays addressing anti-bacterial, anti-fungal, anti-viral and anti-parasitic activity (Cos et al., 2006; Tan et al., 1991; Wei et al., 2011; Zhang et al., 2010). Therefore, we hypothesized that bulk components influence the readout of inflammation-related cell-based *in vitro* assays. Plant extracts as well as plant-derived polyphenols have been repeatedly shown to modulate prominent inflammatory mediators (Dvorak et al., 2006; Hamalainen et al., 2007; Nam, 2006). However, thorough investigations regarding the effect of chlorophyll and bulk polyphenols on cell-based anti-inflammatory assays are lacking. Hence, the evaluation of a possible interference of bulk plant constituents with these assays is highly relevant.

The present work was aimed at a systematic investigation of the effects of extract purification, using various methods, on anti-inflammatory assays. The study was carried out with two selected species, *Malva* sp. (*Malvae folium* according to the European Pharmacopeia) and *Glechoma hederacea* L., used in traditional European medicine against inflammatory disorders and with proven anti-inflammatory activity (Vogl et al., 2013). Six methods for extract purification were evaluated, and the influence of the elimination of bulk constituents such as chlorophyll and polyphenols on the bioactivities of plant extracts was determined. Finally, the necessity for removal of bulk constituents from plant extracts prior to *in vitro* evaluation of anti-inflammatory activity was discussed.

2. Results and discussion

In vitro test systems for inflammation have their limitations and advantages. While they are not able to reproduce intrinsic inflammation and also fail to mimic the ever-changing *in vivo* inflammatory environment, they are commonly preferred to *in vivo* experiments due to ethical reasons, lower cost and effort as well as higher speed and throughput (Swinney and Anthony, 2011; Winyard, 2003).

In vitro models include both cell- and protein-based assays. While the latter are relatively cheap, fast, and easy to perform, they seldom deliver results that are transferable to more complex systems, such as cells. Compounds identified as hits in protein-based models may be unable to enter cells and reach the relevant target protein. Thus, cellular systems are overall more time- and cost-effective for identification of lead compounds.

In vitro assays used for the study of anti-inflammatory activity of drug candidates target key events of the inflammatory response, such as cytokine-induced IKK activity and NF-κB activation, induction of adhesion molecules, such as ICAM-1 and E-selectin, and TNF-α- and IL-1-induced secretion of matrix metalloproteinases (Winyard, 2003). NF-κB signaling in general, plays a well-established role in regulating the inflammatory response (Perkins, 2007; Pober and Sessa, 2007). Hence, the expression of an NF-κB-dependent reporter gene in HEK cells, and secretion of one key endogenous NF-κB target gene, the chemokine IL-8 from endothelial cells, have been chosen as main inflammatory readouts for the present study. Both models are well suited for screening purposes, easy to perform, sensitive, reproducible, and compatible with a high throughput (Fan and Wood, 2007).

In literature, bioactivity of plant extracts is sometimes described upon application of very high test concentrations (up to the mmol/L range). A long standing question is whether effects seen at such high concentrations are physiologically relevant. In order to increase the chance for identifying compounds with relevant and selective activity, the maximum concentration applied in our study was 10 μg/mL for extracts and fractions. Screening results were considered positive when the inhibition of NF-κB and IL-8 expression was higher than 50% compared to the response after stimulation with LPS or TNF-α. As positive control we used two different known NF-κB inhibitors, BAY 11-7082 and parthenolide, which abolished the expression of the NF-κB driven luciferase reporter and of IL-8 in a concentration range consistent with the published literature (Bork et al., 1997; Pierce et al., 1997).

To assess whether chlorophyll and polyphenols alone could influence the anti-inflammatory assays, representative compounds from both chemical classes were tested.

Neither chlorophyll A or B, nor the polyphenols tannic acid, epicatechin gallate and rosmarinic acid influenced the TNF-α-induced NF-κB activation and IL-8 expression at the concentration of 10 μg/mL (Fig. 1a–d). Only epicatechin gallate weakly inhibited the TNF-α-induced IL-8 expression at 10 μg/mL (Fig. 1d). Moreover, these compounds did not activate NF-κB or induced IL-8 expression when applied to unstimulated cells (data not shown).

In a next step, different methods for elimination of bulk constituents from plant extracts were tested and compared. Three chlorophyll removal (CR) methods were used on a dichloromethane (DCM) extract of *Malva* sp.: solvent partitioning between DCM and MeOH/H_2O (CR1), preparative TLC (CR2), and column chromatography (CR3). The highest extract yield was provided by the method CR2, followed by CR3 and CR1 (Table 1). As shown by HPLC profiling with ELSD and UV detection, all three methods successfully eliminated the typical chlorophyll degradation product pheophorbide A, identified as peak 4 (Figs. 2 and 3). Additionally, this purification process resulted in the enrichment of more polar constituents by using methods CR1 and CR3 (Fig. 3). However, compounds other than chlorophyll were also removed. The ELSD detection showed that peak 1, identified as linolenic acid and found to be the main component of the extract, was also removed, as well as peak 2, identified as linoleic acid (Fig. 2). Palmitic acid, peak 3, was depleted by the methods CR1 and CR2 but enriched by the method CR3. This last compound could not be visualized in the HPLC-UV chromatogram due to the lack of chromophores (Fig. 3). Overall, our data demonstrate that HPLC-ELSD profiling linked to the purification step could detect the loss of potentially active compounds. Of the three methods, solvent partitioning CR1 was the most effective, rapid and cost effective.

For polyphenol removal (PR), precipitation with gelatin/NaCl, polyvinylpyrrolidone (PVP) or caffeine was found to be inadequate, since these procedures nonspecifically eliminate compounds with phenolic groups including flavonoids, which is not desired. A methanolic extract (MeOH) of *G. hederacea* was purified by liquid–liquid partitioning (PR1) as well as by chromatography on polyamide using two elution methods (PR2, PR3). Method PR2 led to the highest extract yield, followed by PR3 and PR1 (Table 1). HPLC analyses and comparison with pure reference compounds revealed that all three methods yielded extracts with reduced content of polyphenols as indicated by the elimination of rosmarinic acid, identified as peak 6 (Fig. 4). In addition, compounds different to polyphenols, such as the cyclic hydroxamic acid 2-benzoxazolinone (peak 5) identified by LC-MS, were enriched by the purification procedures. The liquid–liquid partitioning method PR1 revealed to be the most rapid, robust and cost effective.

With regard to the anti-inflammatory activity, the differences between the depletion methods were rather pronounced for the elimination of polyphenols. Liquid–liquid partitioning method PR1

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