Inflammation Research

Dual inhibition of 5-lipoxygenase/cyclooxygenase by a reconstituted homeopathic remedy; possible explanation for clinical efficacy and favourable gastrointestinal tolerability

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Abstract. *Objective:* In order to elucidate potential anti-inflammatory activities of Zeel comp. N and its constituents, the inhibition of the synthesis of Leukotriene B_4 (LTB_4) and Prostaglandin (PGE_2) by 5-lipoxygenase (5-LOX) and cyclo-oxygenase 1 and 2 (COX 1 and 2) respectively were examined in vitro.
Materials: Human HL-60 cells, differentiated for 6–8 days with DMSO (1.2% v/v) were used for the 5-LOX assay. The COX activity assays were carried out with purified enzymes, COX 1 (ram seminal vesicles), COX 2 (sheep placenta) and with human THP-1 cells, differentiated for 24 h with PMA (50 nM).
Methods: LTB_4 and PGE_2 production in the 5-LOX and COX assays respectively were determined by enzyme linked immunoassays.
Results: A reconstituted Zeel comp. N combination as well as its constituent mother tinctures of *Arnica montana, Sanguinaria canadensis* and *Rhus toxicodendron* (*Toxicodendron quercifolium*) showed distinct inhibitory effects on the production of LTB_4 by 5-LOX (IC_{50} values of 10, 20, 2 and 5 µg/ml respectively) and on the synthesis of PGE_2 by COX 1 (IC_{50} values of 50, 80, 40 and 20 µg/ml respectively) and COX 2 enzymes (IC_{50} values of 60, 110, 50 and 20 µg/ml respectively). The mother tincture of *Solanum dulcamara* inhibited the production of PGE_2 by COX 1 (IC_{50} 40 µg/ml) and COX 2 (IC_{50} 150 µg/ml) but not production of leukotriene LTB_4 by 5-LOX.
Conclusions: The observed dual inhibition of both LOX- and COX-metabolic pathways may offer an explanation for the reported clinical efficacy and the favorable gastrointestinal tolerability of the original remedy Zeel comp. N.

Key words: Zeel comp. N – mother tinctures – in vitro assays – dual 5-LOX/COX inhibition

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Introduction

Gonarthrosis is a common, predominantly chronic, degenerative joint disease affecting about 30% of the population over 45 and more than 50% of people over 80 years of age [1]. The progressing erosion of cartilage in the joints is usually accompanied by inflammatory reactions and pain. Current symptomatic treatments of gonarthrosis include nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, hyaluronic acid and homeopathic medication as reviewed by Long and Ernst [2]. Because of the chronic nature of the disease, efficacy and side effects are important considerations when choosing a suitable long-term therapy. NSAIDs are the most widely used medication to effectively control inflammation and pain in arthritic disorders. However, long term use of most NSAIDs has a well documented history of adverse effects. These include abdominal pain, diarrhea, nausea and the more serious side effects of bronchospasm, gastrointestinal ulcers and bleeding [3–5].

NSAIDs exert their anti-inflammatory, antipyretic and analgesic actions by inhibiting the synthesis of prostaglandins by cyclo-oxygenase (COX). COX exists as two isoforms: COX 1, which is constitutively expressed in most cells under physiological conditions and COX 2, the form induced by pro-inflammatory agents such as cytokines, bacterial stimuli and tumor-promoting factors [6]. The preferential inhibition of COX 1 by traditional NSAIDs is thought to be responsible for their gastrotoxic effects by affecting the regulation of homeostatic functions in the gastric mucosa [6, 7]. A new generation of anti-inflammatory drugs is being developed, specifically targeting COX 2 induced prostaglandin production without influencing the homeostatic functions. However, while reducing the risk of gastropathy, recent research indicates that COX 2 specific inhibitors may not be free of adverse side effects. After reviewing the results of several randomized trials with COX 2 inhibitors including the VIGOR [8] and CLASS [9] studies, Mukherjee and colleagues concluded that a potential increase in cardiovascular event rates may be associated with the continuous use of these agents [10]. Furthermore, it has been reported that selective COX 2 inhibitors are also associ-

ated with adverse renal effects like sodium, potassium and water retention as well as decreases in renal function, similar to those of conventional nonselective NSAIDS [11, 12].

Inhibition of prostaglandin synthesis by NSAIDs is also associated with an increased production of leukotrienes by 5-lipoxygenase (5-LOX). Since the COX isozymes and 5-LOX share the same substrate arachidonic acid, inhibition of the COX pathways can lead to a shift towards the production of leukotrienes by 5-LOX due to increased substrate availability. Leukotrienes contribute to inflammatory processes and are implicated in the development of gastrointestinal ulcers [13]. Leukotrienes are also potent bronchoconstrictors and contribute to the NSAID-induced 'aspirin asthma' in susceptible patients [14]. Such complex side effects of traditional NSAIDs limit their applicability for long term treatment of arthritic disorders.

Zeel comp. N is a homeopathic medication based on a combination of plant products (*Arnica montana, Sanguinaria canadensis, Rhus toxicodendron* and *Solanum dulcamara*) and sulphur, used for the treatment of arthritic disorders. Efficacy and a favorable tolerability profile of Zeel comp. N have been demonstrated both in a multi-center, randomized, double blind verum-controlled parallel-group clinical study [1], and in a multi-center, randomized, single blind verum-controlled study [15]. Preliminary studies by Stancikova indicated that Zeel comp. inhibited the activity of human leukocyte elastase in vitro [16], but detailed evaluations of the activities of Zeel comp. N have not been carried out on a molecular level.

The aim of the present work was to investigate the mechanism of action of Zeel comp. N and its constituents by studying the inhibitory profiles for the synthesis of prostaglandins and leukotrienes in validated in vitro models.

Materials and methods

Materials

Chemicals and tissue culture supplies were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise stated. EIA Kits and purified cyclo-oxygenase 1 and 2 (sheep) were obtained from Cayman Chemical (MI, USA). HL-60 cells and THP-1 cells were obtained from I.A.Z. (Munich, Germany). Cytotoxicity kits were purchased from Biocat GmbH (Heidelberg, Germany). Zeel comp. N, mother tinctures (prepared after methods described in the German Homeopathic Pharmacopoea 2000 [17]) and homeopathic dilutions of mother tinctures (*Arnica montana, Sanguinaria canadensis, Rhus toxicodendron* and *Solanum dulcamara*) were obtained from Heel GmbH (Baden-Baden, Germany). As the original Zeel comp. N preparation (composition of injection solution (2.0 ml): Dil. D4 2.0 mg, Dil. D4 1.0 mg, Dil. D4 10 mg, Dil. D4 1.0 mg and Dil. D10 3.0 mg for *Arnica montana, Sanguinaria canadensis, Rhus toxicodendron, Solanum dulcamara* and sulphur respectively) can not be further concentrated for in vitro assays due to the presence of salts, Zeel comp. N was reconstituted from the mother tinctures under controlled conditions without the addition of NaCl. The concentrations of plant material in the mother tinctures were determined after drying the solutions under vacuum. Indicated assay concentrations refer to the weight of the plant material in solution.

5-LOX assay

Human HL-60 cells (myeloid leukemia, DSMZ No ACC 3) were kept at 37 °C in a humidified atmosphere with 5% CO_2 and cultured in com-

plete RPMI 1640 medium supplemented with 10% fetal calf serum and 1% (v/v) penicillin/streptomycin solution. Cells were differentiated for 6 to 8 days with DMSO (1.2% v/v). The 5-LOX activity assay was carried out as described by Bennet et al. [18]. Briefly, differentiated cells were harvested, suspended in PBS containing Ca^{2+} (1 mM) and glucose (1 mM) and distributed into a 96-well microtiter plate (1 × 10^6 cells/well). Stock solutions of test compounds in ethanol were diluted with PBS; the final ethanol concentration in the assay mix was 1.1% which had no effect on cell viability. After pre-incubation with sample or vehicle for 15 min at room temperature the reaction was started by adding calcium ionophore A 23187 (5 µM) and arachidonic acid (10 µM). All values are final concentrations. Negative controls were carried out without calcium ionophore stimulation. The assay mix (100 µl) was incubated for 15 min at 37 °C and terminated by adding 100 µl methanol containing HCl (1 M, 3% v/v) and placing the microtiter plate on ice. After centrifugation (340 × g) for 10 min the LTB_4 concentration in the supernatant was determined with an EIA kit (Cayman) according to the manufacturer's instructions. The effect of samples on the cell viability was determined with a WST-1 kit (Biocat) according to the manufacturer's instructions.

COX assay

The COX activity assay with purified COX 1 (from ram seminal vesicles) and COX 2 (from sheep placenta) was carried out as described by Blasey et al. [19]. Briefly, COX 1 (2 U/ml) or COX 2 (1 U/ml), phenol (1 mM), hematin (1 µM), EDTH (1 mM) and Tris/HCl (100 mM, pH 8.0) were pre-incubated with sample or vehicle for 15 min at room temperature in 96-well microtiter plate. The reaction was started by adding arachidonic acid (10 µM) and incubating for 3 min at room temperature. All values are final concentrations in an assay volume of 50 µl. The reaction was terminated by adding 25 µl acetic acid (I M) and by placing the plates on ice immediately. After neutralization with 25 µl NaOH (I M) the PGE_2 concentration in the supernatant was determined with an EIA kit (Cayman) according to the manufacturer's instructions. The human monocytic line THP-1 (ATCC designation: TIB-202) was kept at 37 °C in a humidified atmosphere with 5% CO_2 and cultured in complete RPMI 1640 medium supplemented with 10% fetal calf serum and 1% (v/v) penicillin/streptomycin solution. Cells were differentiated for 24 h with PMA (50 nM). The cellular COX activity assay was carried out essentially as described by Barrios-Rodiles et al. [20]. Briefly, cells were harvested, distributed into a 96-well microtiter plate (1 × 10^5 cells/well) in the presence of PMA (10 nM) and incubated for 24 h to allow differentiation into adherent macrophages. Stock solutions of test compounds in ethanol were diluted with PBS; the final ethanol concentration in the assay mix was 1%, which had no effect on cell viability. After pre-incubation with sample or vehicle for 30 min at room temperature the cells were activated with LPS (100 ng/ml) for 24 h at 37 °C. The reaction was started by adding arachidonic acid (10 µM) and the assay mix (100 µl) was incubated for 15 min at 37 °C and terminated by adding 100 µl methanol containing HCl (1 M, 3% v/v) and placing the microtiter plate on ice. Negative controls were carried out without arachidonic acid. After centrifugation (340 × g) for 10 min the PGE_2 concentration in the supernatant was determined with an EIA kit (Cayman) according to the manufacturer's instructions. The effect of samples on the cell viability was determined with a WST-1 kit (Biocat) according to the manufacturer's instructions.

Data analysis

The degree of inhibition of LTB_4 or PGE_2 synthesis by 5-LOX or COX enzymes respectively was calculated in per cent relative to control experiments without samples. Measurements were carried out in duplicate and the experimental data are expressed as means with deviations from the mean. IC_{50} was defined as the concentration of inhibitor required to reduce the LTB_4 or PGE_2 synthesis by 50%; values were determined graphically from the dose-response curves.

Results

Inhibition of leukotriene synthesis

To evaluate the effects of the Zeel comp. N combination and its constituents on leukotriene formation the LTB_4 production in DMSO-differentiated and calcium-ionophore stimulated HL-60 cells was determined. Differentiated HL-60 cells express 5-LOX at a high level and react to stimulation with calcium ionophore with an increased production of leukotrienes

[18, 21]. A dose related reduction of LTB_4 production in response to co-incubation with test components therefore indicates an inhibition of 5-LOX activity in the cells.

To test the inhibitory potential of the Zeel comp. N combination, it was reconstituted by combining plant material of the original mother tinctures of *Arnica montana*, *Sanguinaria canadensis*, *Rhus toxicodendron* and *Solanum dulcamara* with sulphur at a ratio of $2:1:10:1:3 \times 10^{-6}$ respectively, according to the ratio of the commercially available drug. As shown in Fig. 1 A, reconstituted Zeel comp. N combination
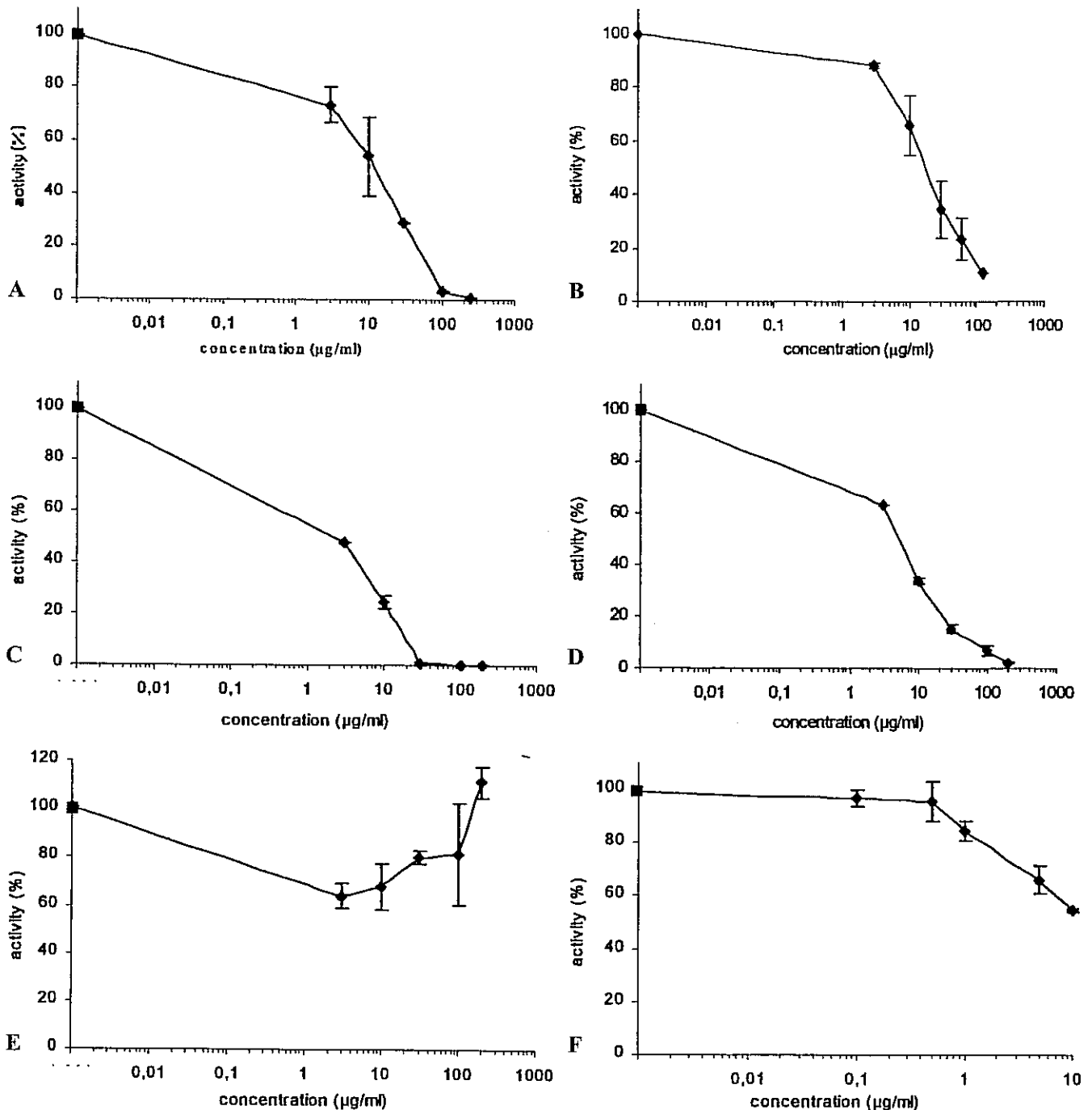

Fig. 1. Assay for the inhibition of LTB_4 production in calcium-ionophore stimulated cellular 5-LOX with **(A)** ZCN; **(B)** mother tincture of *Arnica montana*; **(C)** mother tincture of *Sanguinaria canadensis*; **(D)** mother tincture of *Rhus toxicodendron*; **(E)** mother tincture of *Solanum dulcamara*; **(F)** sulphur.

(ZCN) inhibited LTB_4 production with an IC_{50} value of 10 µg/ml.

As shown in Figs 1 B–1 E, with the exception of *Solanum dulcamara*, all plant derived components of ZCN inhibited the synthesis of LTB_4 by 5-LOX. The results show inhibition of the LTB_4 synthesis by extracts of *Arnica montana* (Fig. 1 B), *Sanguinaria canadensis* (Fig. 1 C) and *Rhus toxicodendron* (Fig. 1 D) with IC_{50} values of 20 µg/ml, 2 µg/ml and 5 µg/ml respectively. The extract of *Solanum dulcamara* did not inhibit LTB_4 synthesis (Fig. 1 E). Sulphur, the only mono-substance of ZCN, has a limited solubility and was only tested up to a concentration of 10 µg/ml in this assay. At this concentration the production of LTB_4 was inhibited by approximately 45% (Fig. 1 F).

Nordihydroguaiaretic acid (NDGA) a known inhibitor of the synthesis pathway of leukotrienes [22, 23] was used as a reference substance in the HL-60 assay system and revealed an IC_{50} value of 0.5 µM (results not shown). No cytotoxic effects of the compounds tested under the conditions of the 5-LOX assay were observed (results not shown).

Inhibition of prostaglandin synthesis

Effects of ZCN and its constituents on the production of prostaglandins were determined in in vitro assays with isolated COX enzymes. In order to distinguish between potential effects on the two isoforms of COX (COX 1 and COX 2), separate enzyme assays were carried out. An effect of ZCN on the production of PGE_2 was also tested in a more complex cellular model with PMA-differentiated human macrophages. A dose-related reduction of the synthesis of PGE_2 as a result of co-incubation with test compounds was used as a marker for the inhibition of COX.

As shown in Fig. 2 A and 3 A, ZCN inhibited both COX 1 and COX 2 with IC_{50} values of 50 µg/ml and 60 µg/ml respectively. ZCN also inhibited the production of PGE_2 in the macrophage model with an IC_{50} value of 10 µg/ml (Fig. 4).

All individual mother tinctures tested showed inhibitory effects on the prostaglandin synthesis. However, the inhibition of the two isoforms of COX varied for the different components of ZCN. Figures 2 A–2 E show inhibition of the COX 1 synthesis of PGE_2 by *Arnica montana* (Fig. 2 B), *Sanguinaria canadensis* (Fig. 2 C), *Rhus toxicodendron* Fig. 2 D) and *Solanum dulcamara* (Fig. 2 E) with IC_{50} values of 80 µg/ml, 40 µg/ml, 20 µg/ml and 40 µg/ml respectively. Generally the inhibitory effect of the mother tinctures was slightly weaker for the COX 2 enzyme. *Arnica montana*, *Sanguinaria canadensis* and *Solanum dulcamara* inhibited the PGE_2 production with IC_{50} values of 110 µg/ml, 50 µg/ml and 150 µg/ml respectively (Figs 3 B, 3 C and 3 E). In contrast, *Rhus toxicodendron* showed an equally strong inhibitory effect on both isozymes COX 1 and COX 2 with an IC_{50} value of 20 µg/ml for both enzymes (Fig. 2 D and 3 D). Sulphur, which could only be tested up to a concentration of 100 µg/ml in this system due to its poor solubility, did not inhibit either of the tested COX enzymes (Figs 2 F and 3 F).

Indomethacin, a common NSAID and a known inhibitor for both COX 1 and COX 2, was used as a reference compound in the COX assay systems [24, 25]. Assays with iso-lated enzymes with Indomethacin inhibited the synthesis of PGE_2 with IC_{50} values of 0.4 µM and 4.0 µM for COX 1 and COX 2 respectively. In the THP-1 system Indomethacin inhibited the production of PGE_2 with an IC_{50} value of 2 nM. In comparison, the production of LTB_4 by 5-LOX in the HL-60 system was inhibited by Indomethacin with an IC_{50} value of 140 µM (results not shown). No cytotoxic effects were observed for the compounds tested under the conditions of the COX assay (results not shown).

Discussion

The clinical efficacy and favorable tolerability of the original homeopathic medication Zeel comp. N for the treatment of gonathrosis have been shown in two multi-center, randomized, verum-controlled clinical studies [1, 15]. To elucidate the mechanisms of these effects, the possible specific influence of ZCN on the synthesis of leukotrienes and prostaglandins was evaluated on a molecular level in different in vitro models, using ZCN as well as its constituents. Inhibitory effects were shown in a cellular model for the production of leukotrienes by 5-LOX and in a test system for the prostaglandin synthesis with isolated COX 1 and COX 2. In both tests, ZCN as well as the majority of the individual components showed inhibitory activities of similar magnitudes (Figs 1–3). In a system with stimulated COX 2 expression in monocytic macrophages ZCN inhibited the biosynthesis of PGE_2 more efficiently than in assays with isolated COX enzymes (Fig. 4). The enhanced inhibition of COX observed in the cellular model indicates that ZCN may also exert some effects on the activity of COX 2 on a transcriptional or post-transcriptional level or may modulate the nuclear export of COX 2 mRNA [26, 20].

The individual components of the complex remedy, derived from the plants *Arnica montana*, *Sanguinaria canadensis* and *Rhus toxicodendron* showed distinct inhibitory effects on the production of leukotriene LTB_4 by 5-LOX as well as of prostaglandin PGE_2 by COX 1 and COX 2 enzymes. In contrast, *Solanum dulcamara* did not display inhibitory activity against 5-LOX and preferentially inhibited the COX 1 isoform (Figs 1 E, 2 E, 3 E). Sulphur, the only mono-substance of ZCN, showed some effect on the production of LTB_4 in the cellular test system, but did not inhibit the prostaglandin synthesis by isolated COX enzymes (Figs 1 F, 2 F, 3 F).

The inhibitory effects observed in this study complement reported biological activities of the examined plant extracts in the literature. In preliminary evaluations, Stancikova et al. found that Zeel comp. inhibits the activity of human leukocyte elastase [16]. Preparations of *Arnica montana* have been shown to have anti-inflammatory properties by interfering with transcription factors NF-κB and NF-AT in in vitro and in vivo experiments [27, 28]. *Sanguinaria canadensis* was reported to display anti-inflammatory and antimicrobial properties [29]. No comparable activities have been reported for *Rhus toxicodendron* but preparations of other species of the same genus have shown antiviral as well as antimicrobial activities [30–32]. Tunon et al. showed that extracts of *Solanum dulcamara* completely inhibited the platelet factor induced exocytosis of elastase in vitro [33]. Such extracts
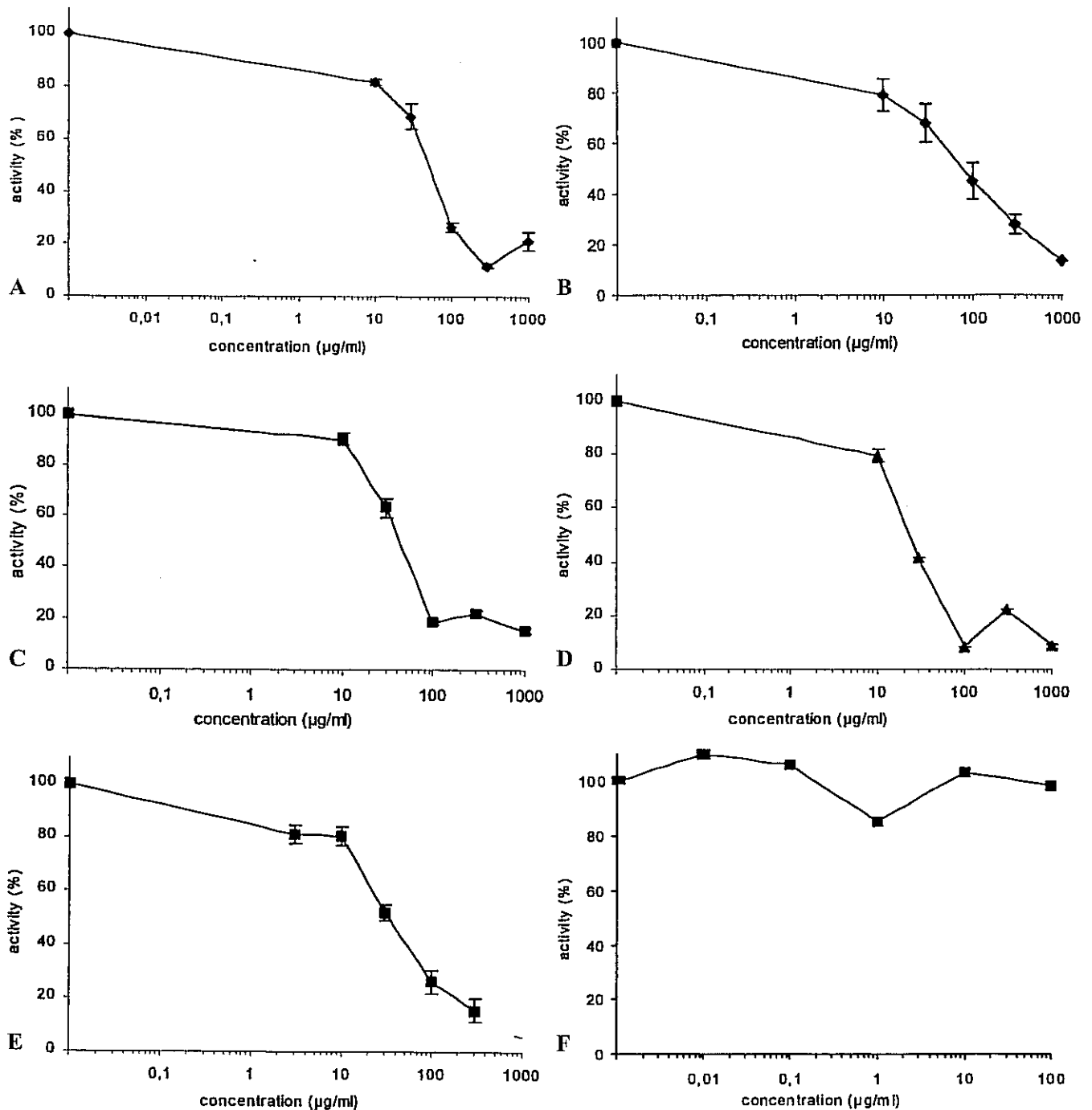
Fig. 2. Assay for the inhibition of PGE$_2$ production by purified COX 1 with (A) ZCN; (B) mother tincture of *Arnica montana*; (C) mother tincture of *Sanguinaria canadensis*; (D) mother tincture of *Rhus toxicodendron*; (E) mother tincture of *Solanum dulcamara*; (F) sulphur.

have a history of traditional use against fever, rheumatism and pain.

Together, these reported effects reveal an interesting activity profile with dual inhibition of COX 1 and 2 as well as 5-LOX, both major pathways of the arachidonic acid metabolism. Such dual inhibitors are reported to have several advantages over the widely used NSAIDs which preferentially inhibit COX. Among the advantages are a broad range of anti-inflammatory properties as well as a reduction of the NSAID-associated commonly observed gastro-

intestinal (GI) ulcerogenic activity and bronchospasms [34, 35].

In particular, GI injuries such as peptic ulceration, perforation and bleeding [36, 37] are major disadvantages of NSAID therapies. The formation of leukotrienes by 5-LOX during treatment with NSAIDs has been implicated in the development of GI injuries [38, 39]. Asako and colleagues reported that the LTB$_4$ triggered chemotactic mechanism of leukocyte adherence to gastric microvessels may be an important component in the pathogenetic mechanism of
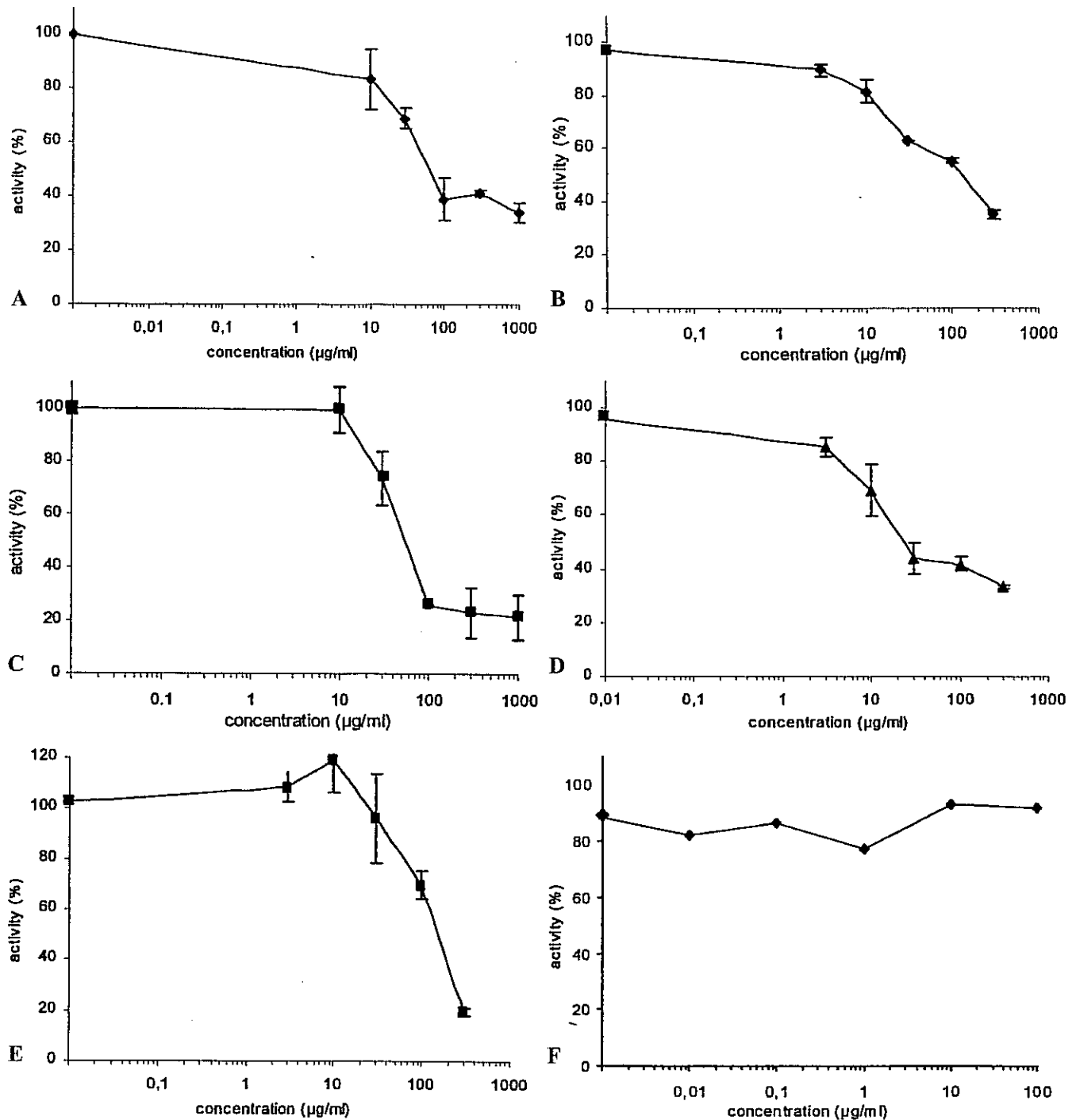
Fig. 3. Assay for the inhibition of PGE_2 production by purified COX 2 with (A) ZCN; (B) mother tincture of *Arnica montana*; (C) mother tincture of *Sanguinaria canadensis*; (D) mother tincture of *Rhus toxicodendron*; (E) mother tincture of *Solanum dulcamara*; (F) sulphur.

NSAID-induced mucosal injury [38]. Furthermore, the NSAID-induced shunting mechanism of arachidonic acid to the 5-LOX pathway has been linked to an increased production of leukotrienes [14]. These 5-LOX metabolites act as potent bronchoconstrictors and are reported to contribute to 'aspirin asthma' and related respiratory diseases [40, 41].

In agreement with this hypothesis dual inhibitors capable of inhibiting both COX and 5-LOX pathways are reported to display advantageous pharmacological profiles [42]. Tries

and colleagues reported broad anti-inflammatory properties, superior gastric tolerability and anti-asthmatic activity of the COX/LOX dual inhibitor ML3000 [23, 43]. Therefore, the balanced dual inhibition of both COX- and LOX-metabolic pathways by ZCN and several of its components demonstrated in this study points to an advantageous pharmacological profile of the preparation. To assess the contribution of the individual constituents of ZCN to the overall effect of the reconstituted remedy and to identify possible additive or syn-
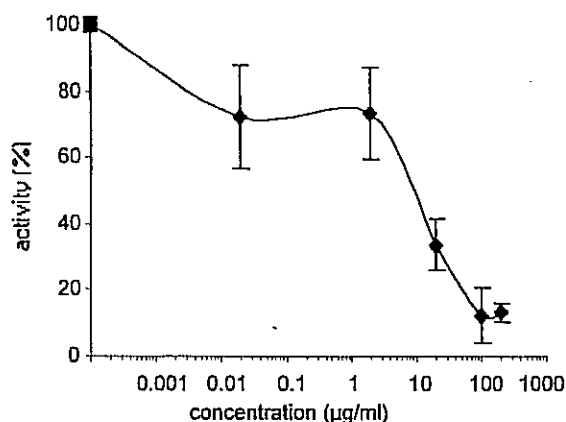
Fig. 4. Assay for the inhibition of PGE$_2$ production by COX in LPS stimulated monocytes with ZCN.

ergistic mechanisms further investigations will have to be carried out with various ratios of constituents. The present study does not fully explain the use of *Solanum dulcamara* in the traditional preparation of Zeel comp N, since it did not display inhibitory activity against the production of LTB$_4$ by 5-LOX and showed relatively weak inhibition of COX 2. However, the reported therapeutic efficacy of the original remedy may also be based on synergistic effects caused by nonbioactive constituents, which may improve solubility, absorbtion rate and bioavailabilty of the active components. Eventhough, the IC$_{50}$-values observed in this in vitro study are significantly higher than the concentrations of the ingredients in the commercial drug, the results may offer an explanation on a molecular level for the reported clinical efficacy and the favorable tolerability of the original homeopathic remedy. However, the dose-effect relationship between the active ingredients of the homeopathic remedy and the physiologically relevant concentrations as well as the effects of the individual constituents on solubility, absorbtion rate and bioavailabilty will have to be elucidated in further in vivo studies.

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