

Placebo-controlled randomised clinical trial on the immunomodulating activities of low and high dose bromelain after oral administration – new evidence on the anti-inflammatory mode of action of bromelain

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8 (Running head: Immunomodulating effects of bromelain)

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Abstract

Bromelain is used for treatment of inflammatory diseases since decades. However, the exact mechanism of action remains poorly understood. While in vitro investigations have shown conflicting effects on the release of various cytokines, no in vivo data were available.

In this study, the effects on inflammation-related cytokines of two doses of bromelain were tested in a single dose placebo-controlled 3 x crossover randomised clinical trial. Cytokine circadian profiles were used to investigate the effects of bromelain on the human immune system by using stimulated whole-blood leukocytes.

The effects seen in these cultures demonstrated a significant shift in the circadian profiles of the Th1 cell mediator IFN γ ($p < 0.043$) after bromelain 3000 F.I.P., and trends in those of the Th2-type cytokine IL-5 as well as the immunosuppressive cytokine IL-10. This suggests a general effect on the antigen-specific (T cell) compartment of the human immune system.

Conclusion: This is the first time that bromelain was shown to modulate the cellular responses of lymphocyte after oral use. It is postulated that the immunomodulating effect of bromelain observed in this trial is part of its known anti-inflammatory activities. Further investigations will be necessary to verify the relevance of these findings to a diseased immune system.

Keywords

bromelain, cytokine, human, inflammation, immune system, clinical trial

1. Introduction

The use of pineapple bromelain in human medicine has been known for a long time (Taussig and Batkin, 1988). One of the major indications for this enzyme are inflammatory diseases (Brien *et al.*, 2006; Secor *et al.*, 2005), including those of autoimmune origin (Targoni *et al.*, 1999; Roep *et al.*, 2002). Currently, bromelain is used for the treatment of postoperative states, sports injuries and acute inflammation (Yuan *et al.*, 2006). It has been suggested that these effects may be the result of this protease acting either on soluble proteins (as α -2-macroglobulin, (Lauer *et al.*, 2001) or on proteins at the cell surface level (Engwerda *et al.*, 2001; Mynott *et al.*, 1999; Barth *et al.*, 2005). In vitro investigations using isolated LPS-stimulated peripheral blood mononuclear cells (PBMC) from healthy volunteers and patients revealed conflicting results after treatment of the cells with bromelain: In vitro, Barth *et al.* (Barth *et al.*, 2005) found an increase in IL-6, TNF α and INF γ , while IL-4 and IL-5 were not induced. Rose *et al.* (Rose *et al.*, 2005) also report a dose dependent induction of IL-6 production, while there was no effect on the production of IFN γ and IL-10. On the other hand the investigations by Huang *et al.* (Huang *et al.*, 2008) revealed a reduced TNF α , IL-1 beta and IL-6 production. However, only little reliable information is available on the effects bromelain triggers *in vivo* in humans, especially after oral ingestion.

Therefore, the aim of the study presented was to investigate the effects of bromelain on immune cell activities after oral ingestion by healthy volunteers.

The following cytokines and chemokines relevant to judge inflammatory processes were selected as relevant endpoints. IL-2 and IFN γ both pertain to the Th1-type of immune response (Schroder *et al.*, 2004), while IL-5 and IL-13 rather mark the Th2-driven reactions (O'Byrne, 2006). These two T helper cell types are seen mutual counter-regulators (Santoni *et al.*, 2008). Under the conditions chosen in these experiments, IL-6 and IL-10 are mainly produced by the cells of the native immune system (as a product of zymosan stimulation) and

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3 also can be seen as mediators that have opposite effects in the activation of immune cells (IL-
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5 6 being mostly of pro-inflammatory activity (Kishimoto, 2006), while one of the major
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7 functions of IL-10 is its inhibitory effect on cytokine synthesis (Mosser and Zhang, 2008). IL-
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9 8 and MCP-1 belong to the chemokines and can be seen the most prominent representatives
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11 of the both major sub-classes, the α - (or CXC) and the β -chemokines (CC), (Viola and Luster,
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13 2008).

14 15 16 **2. Methods**

17 18 **2.1. Study design and Volunteers**

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20 The trial was performed as a monocentric, partially placebo-controlled, double-blind,
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22 randomised, 3 x cross-over study at the Center for Pharmacology and Toxicology, Medical
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24 Faculty, University of Rostock. It was performed according to the German Drug Law
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26 (Arzneimittelgesetz), the GCP guideline CPMP/ICH/135/95, as well as the guideline
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28 2001/20/EG, and in compliance with the Declaration of Helsinki (EudraCT-Nr 2005-002302-
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30 28). After approval of the study by the ethics committee of the University of Rostock 15
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32 healthy male, no-smoking volunteers with an age of 18-50 years and a body mass index >17,5
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34 kg/m² to <30 mg/m² were included into the study. Volunteers were determined to be healthy
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36 on the basis of medical history, physical examination, routine clinical chemistry laboratory
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38 (including negative virus screen on hepatitis B, C and HIV and a negative drug screening) and
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40 electrocardiogram. Volunteers were excluded from participation if they had known allergy to
41
42 pineapple ingredients, used any drugs at present or up to 4 weeks prior to study start, had an
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44 acute infection at present or up to 2 weeks before the start of the study, underwent surgery or
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46 had larger injuries until 12 weeks before the study, had an vaccination within 4 weeks before
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48 the start of the study or had any chronic or inflammatory disease. Furthermore any self-
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50 medication with drugs or food supplements, excessive exercise, intake of pineapple or papaya
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52 (fruit and juice) and alcohol were prohibited throughout the study period.
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3 The study period consisted of three identical one week periods, in which the study medication
4 was provided in a randomized manner. The trial preparations (in the following scheme called
5 “X1, X2, ...”) were applied orally once a week with 1 week of wash-out in between. The
6 order of intake of the trial preparations bromelain 3000 F.I.P. units, bromelain 1000 F.I.P.
7 units and placebo were determined by a randomization scheme. TruCulture blood sampling
8 (TC) was done according to the scheme in table 1.
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16 On the day of trial drug intake the first blood sample was drawn before breakfast, for each
17 blood sample a new puncture was performed. No venous catheter was inserted and no blood
18 sample was drawn through a venous catheter. Blood donors who missed more than 1 time
19 point or developed an infection were excluded from this evaluation.
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23 24 25 **2.2. Trial medication**

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27 The trial drug medication was manufactured at Ursapharm Arzneimittel GmbH, Saarbrücken,
28 Germany. These were placebo tablets (lot No.: 004035), as well as two different
29 concentrations of bromelain tablets: 500 F.I.P. (Fédération Internationale Pharmaceutique)
30 units (lot No.: 018035) or 1000 F.I.P. units (lot No.: 005025). While those containing 500
31 F.I.P. units were the commercial product Bromelain-POS[®], as marketed by Ursapharm
32 Arzneimittel GmbH, the medication containing the higher dose, as well as the placebo tablets,
33 were produced separately as trial medication.
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36
37 The daily dose of 1000 F.I.P. units was achieved by applying 2 tablets of Bromelain-POS[®]
38 (open label), for the dose of 3000 F.I.P. units 3 tablets with 1000 F.I.P. units were given.
39 Thus, trial participants, physicians involved into the study and staff assessing the primary
40 outcome of the study were blinded with regard to the therapies bromelain 3000 F.I.P. units
41 and placebo.
42
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44 45 46 47 **2.3. TruCulture[®] blood collection and whole-blood culture system**

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49 The TruCulture[®] blood collection and whole-blood culture device was operated as described
50 in the manufacturer’s instruction manual (EDI GmbH). In brief, the TruCulture[®] syringe-
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3 tubes, containing a proprietary nutrient solution together with a combination of stimulants,
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5 were sent to the trial site at -40 °C on dry ice and stored at -20 °C at the trial site until use.
6
7 The blood leukocytes in this trial were stimulated by (a) zymosan, a β -glucan from yeast
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9 (Sigma-Aldrich, Deisenhofen, Germany), used at a final concentration of 300 μ g/mL; (b)
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11 *Staphylococcus enterotoxin B* (SEB, from the Bernhard Nocht Institute, Hamburg, Germany;
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13 final conc. 200 ng/mL); as well as (c) anti-CD28 antibodies (Beckmann-Coulter, Frankfurt,
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15 Germany, final conc. 1 μ g/mL). This combination of stimuli was used to activate
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17 simultaneously the phagocytes (granulocytes and monocytes) by zymosan, as well as the T-
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19 cells by SEB + anti-CD28, giving a very physiological pattern of activation. The TruCulture[®]
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21 syringe front end is a screw cap into which a male-type connector with a rubber septum is
22
23 inserted. Together with a special butterfly needle system (21G) attached to a female-type
24
25 connector that punctures this rubber septum during the blood draw (Sarstedt, Nümbrecht,
26
27 Germany), this ensures a closed state of these syringe-tubes, before as well as after the blood
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29 draw, thereby preventing the contamination of the cultures.
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34 After filling the TruCulture[®] tubes with blood they were immediately transferred into a
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36 desktop dry-block incubator (VLM Vogler, Bielefeld, Germany), where they were incubated
37
38 for 24h at 37 °C.
39

40
41 At the end of this incubation the supernatants were separated by insertion of a valve separator
42
43 and the tubes were frozen at -20 °C until measuring the cytokine contents.
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45 **2.4. Mediator quantification**

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47 Cytokine and chemokine concentrations in the supernatants were determined by sandwich-
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49 type enzyme-linked immune-sorbent assays (ELISAs). These were performed strictly
50
51 according to the procedures suggested by the manufacturer using the matched antibody pairs
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53 for IL-2, IL-5, IL-6, IL-8, IL-10, IL-13, IFN γ , and MCP-1, but also the cytokine standards
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55 from R&D Systems.
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58 **2.5. Statistical methods**

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3 Only few data about circadian rhythms of the cytokines have been published (Petrovsky *et al.*,
4
5 1998) and no data on the effects of bromelain or its variability. The sample size of $n = 12$ was
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7 therefore a matter of agreement in this study that addressed complete new questions. The
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9 statistical analysis was performed at SCIRM, Fürth, and Ohm-University of Applied Sciences,
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11 Nuremberg.

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14 Statistical analysis: A thorough exploratory analysis of the data revealed that the considerable
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16 variability in the data of each cytokine was due to extreme differences between individual
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18 subjects (up to a factor of 70), while the within-subject variability was very small and mainly
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20 caused by the circadian rhythm (publication in preparation). Thus it was decided to *z*-
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22 transform the cytokine data individually for each subject for each cytokine. The resulting data
23
24 were then adjusted to zero at 8 a.m. of the medication day. For the analysis, a linear
25
26 mixed model with "group" and "time" as fixed effects and an interaction term "group*time"
27
28 was used to compare the effects of the medication and the changes during daytime and to
29
30 identify significant changes over the day. All *p*-values given below are Bonferroni-adjusted
31
32 for multiple comparisons.
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37 3. Results

38 12 volunteers with a mean age of 27.1 ± 4.3 years, a mean height of 183.9 ± 5.8 cm, a mean
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40 weight of 77.3 ± 10.3 kg and a mean body mass index of 22.8 ± 2.8 kg/m² finished the study
41
42 and were available for evaluation. Three volunteers were withdrawn from the study because
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44 of acute respiratory infection during the study period. For all cytokines investigated in the
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46 study, significant changes in the circadian profile have been established, with IFN- γ , IL-8, IL
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48 and IL-13 showing a profound diurnal increase ($p < 0,001$; $p = 0,008$, and $p < 0,001$,
49
50 respectively), and IL-10 as well as MCP-1 display a diurnal decrease (for both: $p < 0,001$).
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52 IL-6 has a morning and an afternoon peak ($p = 0,001$) and IL-5 a steady increase towards the
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54 evening hours ($p < 0,001$) (Schmolz *et al.*, paper submitted).
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3.1. IL-2, IL-6, IL-8, IL-13, MCP-1

The release of the cytokines IL-2, IL-6 and IL-13, as well as of the two chemokines, IL-8 and MCP-1, tested in this clinical trial did not show statistically significant drug effects in this trial (data not shown).

3.2. IL-10 diurnal profile modulation after bromelain 3000

The daily profile of the release of IL-10 after treatment with bromelain 3000 F.I.P. showed a statistically significant difference to bromelain 1000 F.I.P. ($p = 0,028$) and a borderline significance vs. placebo ($p = 0,069$) (Fig. 1.). After placebo and Bromelain 1000, IL-10 decreased during morning hours and returned to baseline in the evening, while after Bromelain 3000, this decrease in the morning hours was only marginal. Thus Bromelain 3000 prolonged IL-10 production as compared to both other treatments. For the rest of the day, all groups showed similar courses.

3.3. IFN γ diurnal profile modulation after bromelain

For IFN γ , the placebo group demonstrated a peak in cell activity around noon, with a slow decrease towards the evening hours until evening (see Fig. 2). Both bromelain treatments did exhibit differences compared to placebo, but significance was found only with the higher dose ($p = 0,043$), which was apparently caused by a faster rise in cell activity in the morning and an increased secretion upon stimulation towards the evening. This latter activity seems to be prolonged even until the next morning.

3.4. Possible IL-5 diurnal profile modulation after bromelain 3000

Bromelain at 3000 F.I.P. units also seems to affect the production upon stimulation of IL-5, also a T-cell cytokine, although produced by Th2 lymphocytes. However, due to high variation, significance was not established ($p=0.146$). The reason why this increase in IL-5 secretion did not lead to a

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3 significant result seems to be the particularly high data scatter in the placebo group at time point 11 h
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5 (see Fig. 3).

7 **3.5. Tolerability of the study medication**

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9 The study medication was well tolerated. During the study no serious adverse events and no
10
11 study medication related adverse drug effects were observed.

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13 The three volunteers that were withdrawn from the study developed an acute respiratory
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15 infection, which was an exclusion criterion because it would have affected the cytokine
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17 release. The respiratory infection episodes were not regarded as being related to the
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19 medication.
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21

22 **4. Discussion**

23
24 Bromelain is a crude extract from the pineapple plant that contains, besides low molecular
25
26 weight compounds and other proteins, various closely related cysteine proteases. It is
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28 medicinally used for decades in the treatment of various diseases, such as injuries, like
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30 postoperative states, sinusitis and inflammatory diseases (Braun *et al.*, 2005; Inchingolo *et al.*,
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32 2010; Maurer, 2001; Yuan *et al.*, 2006). Especially the treatment of inflammatory diseases
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34 remains an important research area since the available therapeutic drugs are often not
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36 sufficiently efficient or side-effects are intolerable, all the more in cases of long-time therapy
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38 (Trelle *et al.*, 2011). Therefore this study aimed – for the first time – to characterize the
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40 effects of orally taken bromelain on immuno-modulation with regard to the cytokine secreting
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42 capacities of peripheral blood leukocytes.
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44

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46 One of the biggest obstacles in the characterisation of pharmacological effects on cells of the
47
48 human immune system is the well-known inter-individual variability in leukocyte activities
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50 (Yaqoob *et al.*, 1999; Wurfel *et al.*, 2005). By means of the highly standardised test system
51
52 we used (TruCulture[®]), the variability of the cell cultivation procedure could also be reduced
53
54 to a minimum (Schmolz *et al.*, paper submitted). An additional source of increasing the data
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56 scatter of especially investigations looking at short term kinetics (over 24 h), is the circadian
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3 changes in the reactivity of the cells of the human immune system. There are only a few
4 reports on such rhythmic changes in immune cell functions so far (Petrovsky *et al.*, 1998). We
5 controlled this factor by tight measurement intervals during the day and comparing the same
6 individuals after application of the two study drugs and placebo in randomised order. Finally,
7 we analysed the remaining variability, which was still considerable. It turned out that the
8 intra-individual levels were of remarkable stability over the whole study period of 4 weeks for
9 each volunteer, and that the inter-individual variability is a consequence of the fact that
10 obviously low, middle and high “producers” are existing, who differed up to a factor of 70,
11 depending on the cytokine analysed.
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13
14 Based on this finding of individual corridors of cytokine production levels within each
15 subject, z-standardisation of each individual’s cytokine data was the means to transform the
16 original data into a dataset with a mean of zero and a standard deviation of 1 to make them
17 comparable. Additionally, the data were adjusted to have their mean at 8 a.m. Thus, the
18 circadian profile is expressed in terms of deviation from the 8 a.m. value.
19

20
21 Results obtained within this study showed that all cytokines exhibited significant diurnal
22 changes with p-values < 0,02. (Schmolz et al., paper submitted). Although there are
23 investigations on circadian rhythms regarding leukocyte activities, it is difficult to compare
24 their data to ours, as they were using different cell culture systems and/or other activators
25 (Petrovsky and Harrison, 1998). However, when investigating effects of drugs on cytokine
26 production, circadian changes have to be taken into account; this is one of the important
27 general results of the study.
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30 The goal of the current trial was to describe any type of systemic effect that can be related to
31 the administration of bromelain on the cells of the human immune system, irrespective of the
32 underlying mechanism. The trial revealed a significant modulation in the release of IFN γ ,
33 seen as an increase in the afternoon hours (p<0.043 vs. placebo) after bromelain 3000 F.I.P.
34 For IL-10, the effect of group was significant (p = 0,019), but the Bonferroni-adjusted p-value
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3 vs. placebo was not significant ($p = 0.069$). With respect to IL-5 ($p=0.146$), the profiles
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5 differed obviously between bromelain 3000 F.I.P. and the other groups in the morning hours,
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7 but significance was not established ($p = 0.07$ for the group effect and $p = 0.146$ vs placebo).

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10 Larger group sizes are necessary to detect differences with a high level of a systematic difference.

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12 Only at the higher dose of bromelain (3000 F.I.P.) significant changes of the diurnal profiles
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14 were seen; they seem to affect both types of T helper cells, Th1 (IFN γ), as well as Th2 (IL-5).

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16 This suggests a general effect of this enzyme preparation on T lymphocytes, regardless of the
17
18 different sub-populations of these cells existing in the human body. The third mediator, IL-10,
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20 is rather known for its suppressive effects on cytokine synthesis (Mosser and Zhang, 2008)
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22 and – according to the type of leukocyte activation that was chosen in these cultures – can be
23
24 considered to be secreted by monocytes rather than by Th2 lymphocytes. As this effect was
25
26 most pronounced in the morning hours (while that on IFN γ had an additional late component
27
28 in the evening hours), one might assume that at least at its onset the bromelain-mediated
29
30 modulation of immune cell activities touches a broad spectrum of leukocyte sub-types, while
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32 the prolonged effect might affect mainly the T cells.

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34
35 The effects observed during bromelain treatment in this trial were detected in cultures that
36
37 involved a physiologic pro-inflammatory activation of immune cells, very similar to what
38
39 occurs during acute or chronic inflammation *in vivo*. It cannot be speculated, how these
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41 effects of bromelain would modulate the course of any type of inflammatory disease, at least
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43 because the treatment would usually involve repeated application of the medication. Despite
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45 that, two major findings can already be derived from these results:

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49 (a) Even after a single oral dose of bromelain significant changes in the response of
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51 physiologically stimulated immune cells were found.

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54 This demonstrates that the enzyme is able to either reach the blood circulation in an
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56 intact, biologically active form (as was suggested earlier by others (Castell, 1996;
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58 Desser *et al.*, 2001), or it acts indirectly, e.g. on the class of protease-activated

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3 receptors (PARs), which was shown for bromelain recently (Reddy and Lerner, 2010),
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5 thereby possibly stimulating intestinal epithelial cells which, in turn, could then
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7 change the activities of close-by cells of the mucosal immune system of the gut. In the
8
9 latter case, one further step in this reaction cascade would have to be postulated, which
10
11 could be a re-circulation of these cells, or a local secretion of systemically active
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13 mediators that consequently modulate the activities of leukocytes in the blood
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15 circulation (giving then rise to the changes seen in the whole-blood cultures of this
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17 clinical trial).
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21 (b) The fact that all of the effects reaching significant p-levels or showing clear trends
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23 were observed after applying 3000 F.I.P. units can be regarded an indicator of a
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25 concentration-dependency of these bromelain activities.
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30 31 **Conclusions**

32 The immunomodulating effect of bromelain 3000 F.I.P. demonstrated in this study is an initial
33
34 step towards the understanding of this natural compound, as it could be demonstrated that
35
36 effects on the immune system are present after a single application. Further studies should
37
38 analyse repeated applications and effects in inflammatory conditions to close the gap between
39
40 *in vitro* results and empirical use.
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43 In a remarkable set of animal experiments, Baez and coworkers (Baez *et al.*, 2007) have
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45 demonstrated, *in vivo*, convincing antitumor effects of the substance. It is a serious and
46
47 valuable task to elaborate the mechanisms behind these results in order to lay the grounds of
48
49 their transfer to human cancer treatment and other immune system related diseases, where
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51 bromelain has a more or less empirical role as adjunct therapy.
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53 Taken together, the benefits of the medicinal product bromelain are - and might become even
54
55 more - a valuable constituent of a modern (supportive) treatment of inflammatory diseases.
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58 The results of these experiments support the idea of bromelain having a “multiple-target
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activity profile”, similar to what is claimed for several other herbal preparations. This is postulated to have advantages over the inhibition of single targets, especially in the treatment of complex diseases, such as chronic inflammation.

Further investigations will be necessary to evaluate the pharmacodynamics of repeated bromelain application and the spectrum of effects under disease situations.

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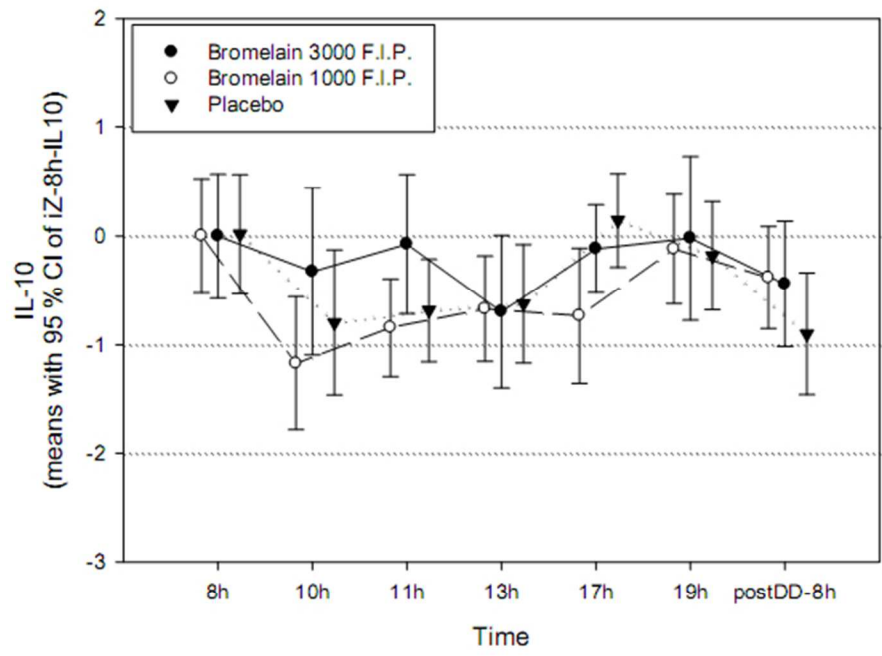
For Peer Review

Table. 1: Drug application and sampling scheme

Time	Mon	Tue	Wed	Thu	Fri	Sat	Sun	Mon	Tue	Wed	etc.
Drug	--	X1	--	--	--	--	--	--	X2	--	
8:00	TC	TC	TC	--	--	--	--	TC	TC	TC	
10:00		TC							TC		
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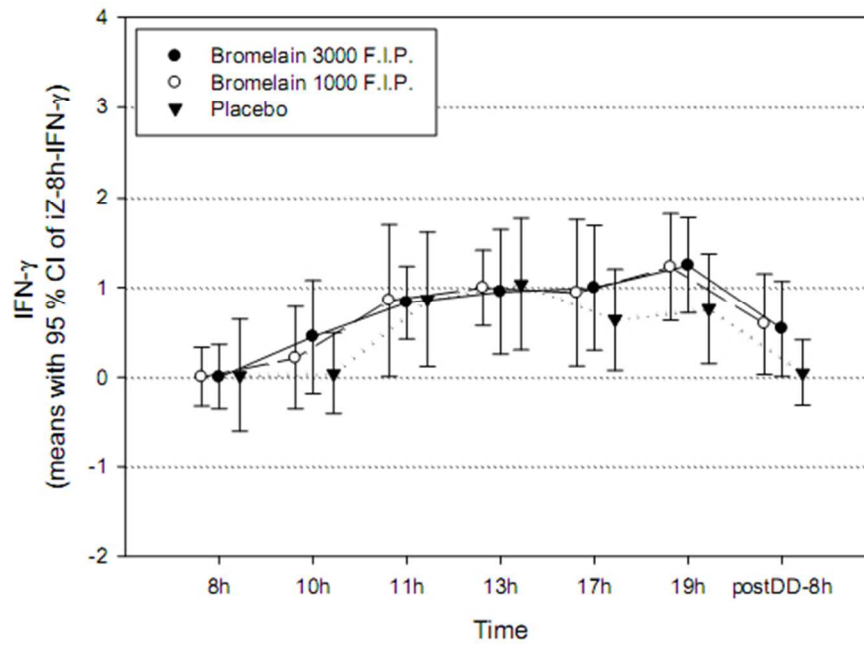
X1: drug 1; X2: drug 2; TC: TrueCulture blood sampling

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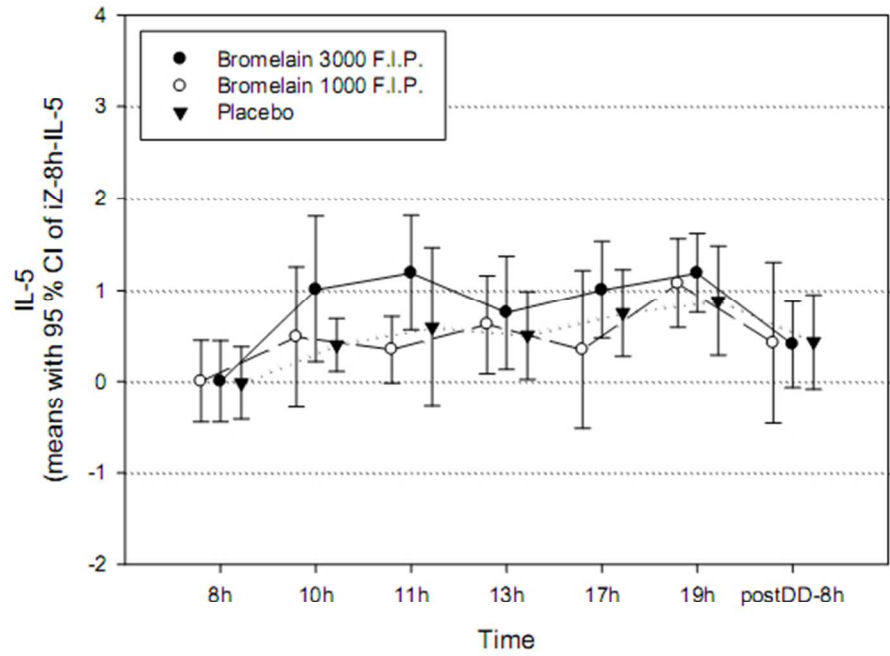
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Review

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7 Figure 1: Means with 95 % confidence intervals in the circadian profile of leukocyte activity of n = 12
8 healthy volunteers regarding the experimentally stimulated release of IL-10 for placebo, bromelain
9 1000 and 3000 F.I.P. units. After bromelain 3000 F.I.P, the levels of IL-10 are statistically significantly
10 elevated compared to bromelain 1000 F.I.P. ($p = 0,028$), but not to placebo ($p = 0,069$) due to the
11 inhibition of the decrease between 10 a.m. and 11 a.m. (all p-values are Bonferroni-adjusted).
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14 Figure 2: Means with 95 % confidence intervals in the circadian profile of leukocyte activity of n = 12 healthy
15 volunteers; test of the experimentally stimulated release of IFN- γ for placebo, bromelain 1000 and 3000 F.I.P.
16 units. After bromelain 3000 F.I.P. units, IFN- γ levels were increased especially in the afternoon hours ($p =$
17 $0,043$; Bonferroni-adjusted).
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20 Figure 3: Means with 95 % confidence intervals in the circadian profile of leukocyte activity of n = 12 healthy
21 volunteers regarding the experimentally stimulated release of IL-5 for placebo, 1000 and 3000 F.I.P. units
22 bromelain, respectively. The differences between bromelain 3000 F.I.P and placebo were statistically not
23 significant ($p = 0.146$).
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