



## Cacao extracts suppress tryptophan degradation of mitogen-stimulated peripheral blood mononuclear cells

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### ABSTRACT

**Ethnopharmacological relevance:** The fruits of *Theobroma cacao* L. (Sterculiaceae) have been used as food and a remedy for more than 4000 years. Today, about 100 therapeutic applications of cacao are described involving the gastrointestinal, nervous, cardiovascular and immune systems. Pro-inflammatory cytokine interferon- $\gamma$  and related biochemical pathways like tryptophan degradation by indoleamine 2,3-dioxygenase and neopterin formation are closely associated with the pathogenesis of such disorders. **Aim of the study:** To determine the anti-inflammatory effect of cacao extracts on interferon- $\gamma$  and biochemical consequences in immunocompetent cells.

**Materials and methods:** Effects of aqueous or ethanolic extracts of cacao were examined on mitogen-induced human peripheral blood mononuclear cells (PBMC) of healthy donors and on lipopolysaccharide-stimulated myelomonocytic THP-1 cells. Antioxidant activity of extracts was determined by oxygen radical absorption capacity (ORAC) assay.

**Results:** In mitogen-stimulated PBMC, enhanced degradation of tryptophan, formation of neopterin and interferon- $\gamma$  were almost completely suppressed by the cacao extracts at doses of  $\geq 5$   $\mu\text{g}/\text{mL}$ . Cacao extracts had no effect on tryptophan degradation in lipopolysaccharide-stimulated THP-1 cells.

**Conclusions:** There is a significant suppressive effect of cacao extracts on pro-inflammatory pathways in activated T-cells. Particularly the influence on indoleamine 2,3-dioxygenase could relate to some of the beneficial health effects ascribed to cacao.

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### 1. Introduction

Consumption of cacao or chocolate is very popular, from the ancient people of Olmec, Maya and Aztec cultures up to the present, and has been associated with regalement and a sense of delight. Especially the indigenous people of Central and South America still use the fruits of *Theobroma cacao* L. (Sterculiaceae) as a traditional medicine. Reviewing available literature concerning the historical use of cacao or chocolate for medicinal purposes revealed appetite

stimulating, relaxing and also mood-enhancing effects as the most consistent applications (Dillinger et al., 2000). Recently, dark chocolate was also demonstrated to induce coronary vasodilation, to improve coronary vascular function, and to decrease platelet adhesion within short time after consumption. These beneficial effects seem to go along with a significant reduction of serum oxidative stress and were positively correlated with changes in serum epicatechin concentration (Buijsse et al., 2006; Flammer et al., 2007). For all these effects, the extent of cacao present in chocolate is considered to be of ample importance.

Cacao refers to cocoa powder derived from the beans of *Theobroma cacao* L. (Sterculiaceae) by grinding and removing the cocoa butter from the dark, bitter cocoa solids. Several *in vitro* and *in vivo* studies suggest that the active compounds in cocoa exhibit protective effects against conditions such as cardiovascular disease and cancer, diseases which are also associated with inflammation and impaired immune function (Kris-Etherton and Keen, 2002; Steinberg et al., 2003; Yamagishi et al., 2003; Ramljak et al., 2005; Jourdain et al., 2006). Cocoa compounds were shown to improve or normalize, e.g., eicosanoid production (Schramm et al., 2001; Noreen et al., 1998), platelet activation (Rein et al., 2000; Holt et al.,

**Abbreviations:** 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; DMSO, dimethylsulfoxide; EGCG, Epigallocatechin-gallate; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; IDO, indoleamine 2,3-dioxygenase; IFN- $\gamma$ , interferon- $\gamma$ ; IL-2, interleukin-2; kyn/try, kynurenine to tryptophan ratio; LPS, lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; ORAC, oxygen radical absorption capacity; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; ROS, reactive oxygen species; TE, trolox equivalents; TLRs, toll like-receptors; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

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2002; Pearson et al., 2002), nitric oxide-dependent activities (Fisher et al., 2003; Heiss et al., 2003), and cytokine production (Heiss et al., 2003; Mao et al., 2000, 2002a, 2003). Thus, cocoa-derived products have the potential to positively modulate the inflammatory status that characterizes several chronic diseases.

During Th-1 type immune response, activated cells release large amounts of cytokines such as interleukin-(IL)-2 or interferon-(IFN)- $\gamma$ . Pro-inflammatory cytokine IFN- $\gamma$  is probably the most important multiplier of anti-microbial and anti-tumoral host defence producing a variety of physiological and cellular responses, e.g. induction of high amounts of anti-microbial and cytotoxic reactive oxygen species (ROS) by macrophages and other cells (Nathan, 1986). ROS are capable of interfering with various redox-sensitive intracellular signal-transduction cascades involving, e.g. activation of nuclear factor- $\kappa$ B (Schreck et al., 1991; Asehnoune et al., 2004), which leads to the production of further pro-inflammatory cytokines such as tumor necrosis factor-(TNF)- $\alpha$  (Min et al., 2003). Consequently, accumulation of ROS further amplifies Th1-type immune response, and thus appears as a positive regulator in addition to pro-inflammatory Th1-type cytokines.

In human macrophages, T-cell derived IFN- $\gamma$  induces also the enzyme indoleamine 2,3-dioxygenase (IDO), which converts tryptophan to kynurenines (Wirleitner et al., 2003) and formation of the immune activation marker neopterin, via induction of the enzyme guanosine-triphosphate-(GTP)-cyclohydrolase (Fuchs et al., 1988). Increased tryptophan degradation and neopterin production develop in patients during diseases which are associated with Th1-type immune activation such as infections, autoimmune diseases, malignant disorders, and during allograft rejection episodes (Murr et al., 2002). Higher neopterin concentrations are also associated with increased cardiovascular risk and they parallel the course of neurodegenerative disorders such as Parkinson's disease and Alzheimer's dementia (Blasko et al., 2007). IDO plays a central role in the suppression of intracellular bacteria and viruses during an antimicrobial immune response, as ongoing tryptophan degradation limits protein biosynthesis due to deprivation of this essential amino acid (Pfefferkorn, 1986; Ozaki et al., 1988). More recently, it has been demonstrated *in vitro* that also T cell proliferation is inhibited efficiently by IDO (Munn et al., 1999; Frumento et al., 2002). In patients, accelerated tryptophan degradation was found to parallel, and even to predict, the future course of several clinical conditions, including HIV infection, malignancy and autoimmune syndromes such as rheumatoid arthritis (Schroeksnadel et al., 2006a,b; Murray, 2003).

The essential amino acid tryptophan is not only required for protein synthesis, but also acts as a precursor for the biosynthesis of the neurotransmitter 5-hydroxytryptamine (5-HT; serotonin), which appears to be strongly involved in the pathogenesis of mood disorders and depression (Young and Leyton, 2002). Accordingly, activation of IDO seems to represent a link between the immunological network and the pathogenesis of depression, when the availability of tryptophan limits serotonin biosynthesis (Widner et al., 2002; Russo et al., 2003; Dantzer et al., 2008). If cacao extracts were able to interfere with IDO activation, it would correspond nicely to the effect of cocoa to improve mood.

In an approach to evaluate the effects of commercially available cacao on the T-cell/macrophage interplay, we studied the influence of cacao extracted in water or ethanol (30%) on tryptophan degradation in peripheral blood mononuclear cells (PBMC) stimulated with phytohaemagglutinin (PHA), which activates formation of pro-inflammatory cytokine IFN- $\gamma$  (Nathan et al., 1983) and subsequently tryptophan degradation and neopterin production (Weiss et al., 1999). In addition, effects of cacao extracts were also tested on lipopolysaccharide (LPS)-stimulated myelomonocytic

THP-1 cells, an appropriate model to study monocyte activation by another pro-inflammatory stimulus (Neurauter et al., 2003; Singh et al., 2005). To test for the antioxidant activity of cacao extracts, the Oxygen Radical Absorption Capacity (ORAC) assay was applied using fluorescein as a fluorescent probe (Ou et al., 2001).

## 2. Materials and methods

### 2.1. Chemicals

Ethanol (30%) and aqueous extracts of cacao were prepared from commercially available pure (100%) powdered cacao, produced from Western Africa *Theobroma cacao* (L.) beans (Bensdorp powdered cacao, Kraft foods, Vienna, Austria) and sterile filtered for cell culture experiments, which according to the manufacturer contains 185 mg/g protein, 140 mg/g carbohydrates, of which 18 mg/g is sugar, 210 mg/g fat of which 130 mg/g are saturated fatty acids, 290 mg/g fiber and 0.1 mg/g sodium, and according to J. Lied 17.2 mg/g total phenolics, 0.96 mg/g epicatechin, 0.4 mg/g protocatechuic acid and 0.32 mg/g procyanidin (Lied, 2002). Epigallocatechin-gallate (EGCG), ascorbic acid and Trolox were purchased from Sigma-Aldrich (Vienna, Austria) dissolved in dimethylsulfoxide (DMSO) and stored at  $-80^{\circ}\text{C}$ . Fluorescein, disodium salt (Anaspec, San Jose, CA) and 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH; Wako Chemicals, Germany) was dissolved in phosphate buffer (75 mmol/L; pH 7.4).

### 2.2. Isolation and stimulation of human PBMC and THP-1 cells

PBMC were isolated from whole blood obtained from healthy donors, of whom informed consent was obtained that their donated blood unit was used for scientific purposes if not otherwise used. Separation of blood cells was performed using density centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). After isolation, PBMC were washed three times in phosphate buffered saline containing 0.2% EDTA [0.5 mmol/L]. Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), 1% of 200 mmol/L glutamine (Serva, Heidelberg, Germany) and 0.1% of gentamicin (50 mg/mL, Bio-Whittaker, Walkersville, MD) in a humidified atmosphere containing 5%  $\text{CO}_2$  for 48 h. This procedure was observed earlier to reveal best reproducible results when applied for testing of anti-inflammatory effects of compounds or drugs (Widner et al., 1997). Average tryptophan content in the supplemented RPMI 1640 medium was 31.5  $\mu\text{mol/L}$ . For each of the four experiments run in duplicates, PBMC were freshly prepared.

Isolated PBMC were plated at a density of  $1.5 \times 10^6$  cells/mL in supplemented RPMI 1640, preincubated for 30 min with or without cacao extracted in water or ethanol (30%) and stimulated or not with 10  $\mu\text{g/mL}$  PHA for 48 h.

The myelomonocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and was cultured in complete medium as described earlier (Neurauter et al., 2003). Cells were used from early passages and kept for <1.5 months. All THP-1 experiments were repeated at least twice and run in triplicates. The cells were regularly tested negative for mycoplasma.

### 2.3. Measurement of tryptophan, kynurenine, neopterin and interferon- $\gamma$ concentrations

After incubation of cells for 48 h, supernatants were harvested by centrifugation and tryptophan and kynurenine concentrations

were measured by high performance liquid chromatography (HPLC) using 3-nitro-L-tyrosine as internal standard (Widner et al., 1997). To estimate IDO activity, the kynurenine to tryptophan ratio (kyn/trp) was calculated and expressed as  $\mu\text{mol}$  kynurenine/ $\text{mmol}$  tryptophan (Widner et al., 1997). Neopterin concentrations were determined by ELISA (BRAHMS, Hennigsdorf/Berlin, Germany) according to the manufacturer's instructions with a detection limit of 2 nmol/L. In a subgroup of 3 PBMC experiments with 2 parallels, also concentrations of IFN- $\gamma$  were measured by ELISA (R&D International, Minneapolis, MN).

#### 2.4. Measurement of cell viability

After incubation of PBMC and THP-1 cells, cell viability was measured by MTT-test (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; Sigma, Vienna, Austria) and by trypan blue exclusion method in three experiments done in triplicates. No toxicity could be observed at the concentration range applied.

#### 2.5. Measurement of antioxidant activity (ORAC)

The ORAC-Assay (Ou et al., 2001) was carried out on a fluorometer (Fluoroscan Ascent; Labsystems). The reference compound Trolox was dissolved in 75 mmol/L phosphate buffer (pH 7.4). Aqueous and ethanol extracts of cacao powder were compared to EGCG and ascorbic acid, both dissolved in DMSO, as a control, and further dilutions of all tested samples were made in 75 mmol/L phosphate buffer (pH 7.4). In the final assay mixture (0.2 mL total volume), fluorescein ( $6.3 \times 10^{-8}$  M) was used as a target of free radical attack and 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) ( $1.9 \times 10^{-2}$  M) was used as a peroxy radical generator. 75 mmol/L phosphate buffer served as the blank, and Trolox (0.78, 1.56, 3.13, and 6.25  $\mu\text{mol/L}$ ) was used as the control standard. The fluorescence of fluorescein was recorded by a fluorometer every minute after the addition of AAPH for 35 min at 37 °C. All measurements were expressed relative to the initial reading. Final results were calculated using the differences of areas under the fluorescein decay curves (AUC) between the blank and a sample (Ou et al., 2001). The results were expressed as micromoles Trolox equivalents (TE) for pure chemicals and as Trolox equivalents/g (TE/g) for the cacao extracts.

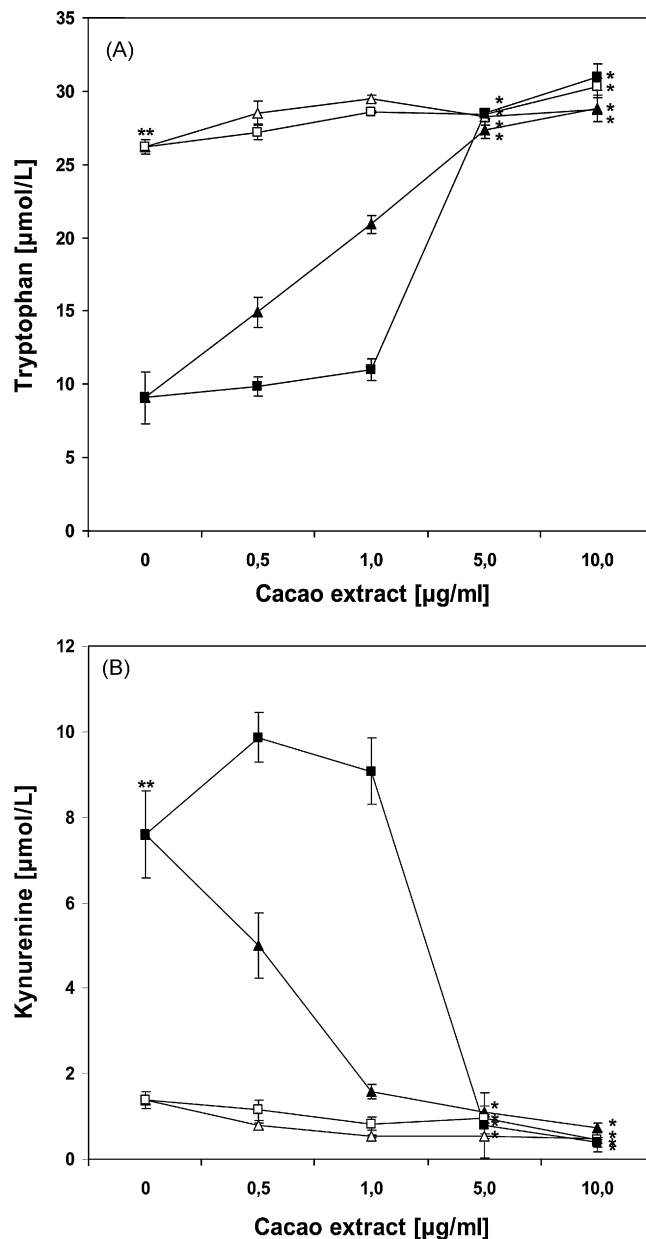
#### 2.6. Statistical analysis

For statistical analysis, the Statistical Package for the Social Sciences (version 14 SPSS, Chicago, IL, USA) was used. Because not all data sets showed normal distribution, for comparison of grouped data non-parametric Friedman test and Wilcoxon signed ranks test were applied. *p*-values below 0.05 were considered to indicate significant differences.

### 3. Results

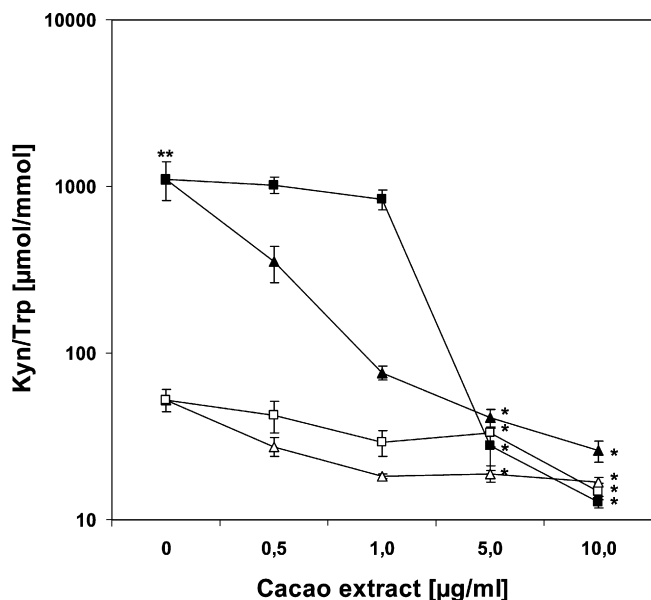
#### 3.1. Tryptophan metabolism in unstimulated- and PHA-stimulated PBMC

The supernatants of unstimulated PBMC contained an average concentration of  $26.2 \pm 0.5$   $\mu\text{mol/L}$  tryptophan, which increased slightly to  $28.8 \pm 0.8$  or  $30.3 \pm 0.6$   $\mu\text{mol/L}$  after 48 h of treatment with 0.5–10  $\mu\text{g/mL}$  cacao extracted in water or ethanol, respectively (Fig. 1A). In parallel, the extracts also led to a modest decrease of kynurenine concentrations (Fig. 1B) and of kyn/trp (Fig. 2). Stimulation of PBMC with PHA [10  $\mu\text{g/mL}$ ] for 48 h led to a decrease of tryptophan concentrations in the supernatant to a level of  $9.1 \pm 1.8$   $\mu\text{mol/L}$  and a concurrent increase of kynurenine concentrations from  $1.4 \pm 0.2$  to  $7.6 \pm 1.0$   $\mu\text{mol/L}$  (both *p* < 0.005; Fig. 1A



**Fig. 1.** Concentrations of tryptophan (A) and kynurenine (B) in the supernatant of unstimulated (open symbols) PBMC and in cells stimulated with 10  $\mu\text{g/mL}$  phytohaemagglutinin (closed symbols) co-treated or not with increasing concentrations of cacao extracted in water (triangles) or 30% ethanol (squares). Results shown are the mean values  $\pm$  S.E.M. of four independent experiments run in duplicates (\*\**p* < 0.005, compared to unstimulated cells; \**p* < 0.05, compared to stimulated cells).

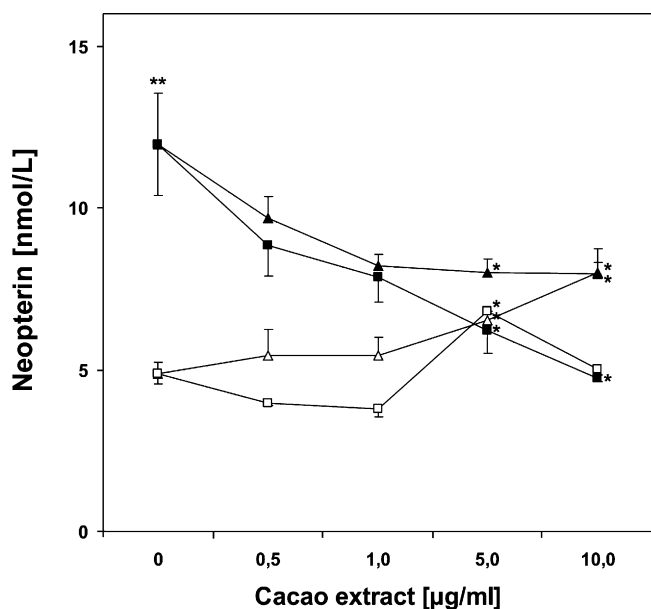
and B). Activation of IDO was indicated by an approximately 20-fold increase of kyn/trp in PHA treated cultures, as compared to unstimulated cells (*p* < 0.005; Fig. 2). Co-incubation with cacao extracted in water or ethanol dose-dependently, and at concentrations of  $\geq 5$   $\mu\text{g/mL}$  almost completely, suppressed mitogen-induced tryptophan degradation and reduced kyn/trp (Fig. 2). At these doses, tryptophan concentrations in the supernatants returned to concentrations comparable with unstimulated PBMC ( $27.4 \pm 0.6$  or  $28.5 \pm 0.4$   $\mu\text{mol/L}$ ) and kyn/trp even reached levels beyond unstimulated cells. Confirming earlier results (Neurauter et al., 2004), no influence of ethanol (up to 6% final concentration) was detected on tryptophan metabolism in stimulated PBMC and cell viability was not affected by the test substance at the concentrations used (data not shown).



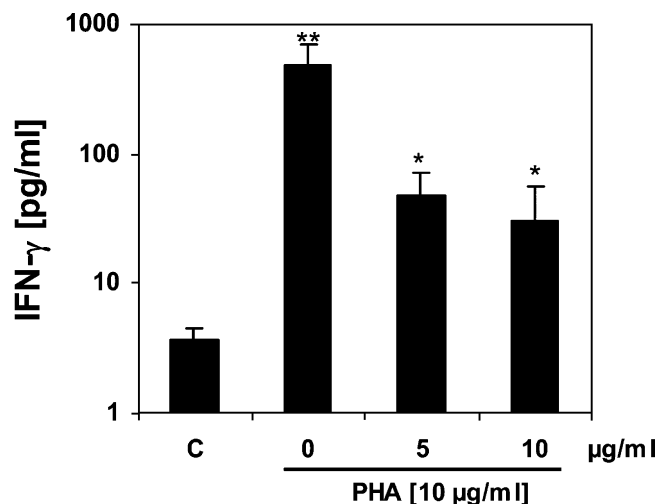
**Fig. 2.** Kynurenine to tryptophan ratio (kyn/trp) in unstimulated PBMC (open symbols) and in cells stimulated with 10 µg/mL phytohaemagglutinin (closed symbols) co-treated or not with increasing concentrations of cacao extracted in water (triangles) or 30% ethanol (squares). Results shown are the mean values  $\pm$  S.E.M. of four independent experiments run in duplicates (\*\* $p < 0.005$ , compared to unstimulated cells; \* $p < 0.05$ , compared to stimulated cells).

### 3.2. Neopterin formation in unstimulated- and PHA-stimulated PBMC

After an incubation period of 48 h, the supernatants of unstimulated PBMC contained an average neopterin concentration of  $4.9 \pm 0.3$  nmol/L. Upon treatment of the cells with 10 µg/mL cacao extracted in water or 5 µg/mL of cacao extracted in ethanol, neopterin concentrations increased to a level of  $7.9 \pm 0.8$  and  $6.8 \pm 0.4$  nmol/L, respectively (both  $p < 0.05$ ; Fig. 3). Stimulation of



**Fig. 3.** Neopterin formation in unstimulated (open symbols) PBMC and in cells stimulated with 10 µg/mL phytohaemagglutinin (closed symbols) co-treated or not with increasing concentrations of cacao extracted in water (triangles) or 30% ethanol (squares). Results shown are the mean values  $\pm$  S.E.M. of four independent experiments run in duplicates (\*\* $p < 0.005$ , compared to unstimulated cells; \* $p < 0.05$ , compared to stimulated cells).



**Fig. 4.** Concentrations of interferon (IFN)- $\gamma$  secreted into the supernatant of unstimulated PBMC (C) and cells stimulated with 10 µg/mL phytohaemagglutinin for 48 h, co-treated or not with increasing concentrations of cacao extracted in water. Results shown are the mean values  $\pm$  S.E.M. of three independent experiments run in duplicates (\*\* $p < 0.005$ , compared to unstimulated cells; \* $p < 0.05$ , compared to stimulated cells;  $n = 3$ ).

PBMC with PHA [10 µg/mL] strongly induced neopterin production to a level of  $12.0 \pm 1.6$  nmol/L ( $p < 0.005$ ; Fig. 3), and co-incubation with the cacao extracts decreased mitogen induced neopterin production significantly, in a dose-dependent manner ( $p < 0.05$ ; Fig. 3). Co-incubation with 10 µg/mL cacao extracted in ethanol completely suppressed mitogen-induced neopterin production. No influence of ethanol (up to 6% final concentration) was detected on neopterin production in stimulated PBMC (data not shown).

### 3.3. Release of Th1-type cytokine IFN- $\gamma$

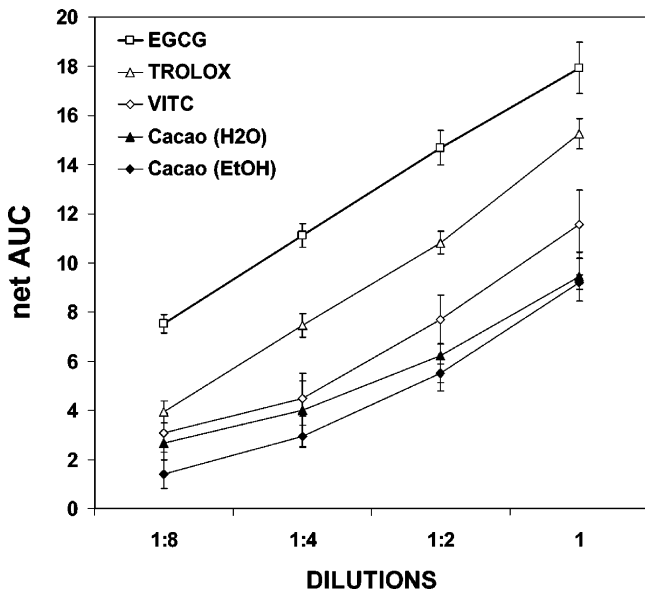
Concentrations of IFN- $\gamma$  released into the supernatants of PBMC was significantly higher in PHA [10 µg/mL]-stimulated cells ( $483 \pm 216$  ng/L) compared with unstimulated controls ( $3.7 \pm 0.88$  ng/L;  $n = 6$ ,  $p < 0.005$ ) resulting in an about 130-fold increase of IFN- $\gamma$  production upon mitogen stimulation for 48 h. Subsequently, co-incubation with cacao extracted in water efficiently, and almost completely suppressed PHA induced IFN- $\gamma$  secretion to a level of  $47.5 \pm 23.4$  ng/L at doses of 5 or  $30.8 \pm 24.8$  ng/L at doses of 10 µg/mL (both  $p < 0.05$ ; Fig. 4).

### 3.4. Neopterin formation and tryptophan metabolism in unstimulated- and LPS-stimulated THP-1 cells

Experiments with LPS [1 µg/mL]-stimulated THP-1 cells revealed significantly elevated tryptophan degradation and neopterin production compared to unstimulated cells (all  $p < 0.05$ ), which however did not change upon addition of cacao extracted in water or ethanol (details not shown).

### 3.5. Antioxidant capacity of tested cacao extracts

Antioxidant capacities of the pure chemicals EGCG ( $5.48 \pm 0.5$  µmol TE), Trolox ( $0.98 \pm 0.1$  µmol TE) and vitamin C ( $0.93 \pm 0.1$  µmol TE) as reference standards and the aqueous and ethanolic extracts of cacao are shown in Fig. 5. Both preparations, cacao extracted in water or ethanol, showed potent antioxidant capacity with relative ORAC values of  $737 \pm 64.9$  and  $694 \pm 55.2$  TE/g, respectively. No significant differences were observed between the aqueous and ethanolic extracts.



**Fig. 5.** Area under the curve (net AUC) of cacao extracted in water or 30% ethanol compared to reference standards epigallocatechingallate (EGCG), Trolox and vitamin C (VITC) at different dilutions (stock solution of EGCG (1.36  $\mu\text{M}$ ), Trolox (6.25  $\mu\text{M}$ ), VITC (3.54  $\mu\text{M}$ ) and cacao extracts (31.3  $\mu\text{g}/\text{mL}$ ). The net AUC = AUC<sub>sample</sub> – AUC<sub>blank</sub>; the AUC was calculated by the equation previously described by Ou et al. (2001). Results shown are the mean values  $\pm$  S.E.M. of four concentrations and six independent experiments.

#### 4. Discussion and conclusions

The present study shows that commercially available cacao powder, extracted in either water or 30% ethanol, dose-dependently, and at concentrations of  $\geq 5 \mu\text{g}/\text{mL}$  almost completely, suppressed mitogen-induced degradation of tryptophan in PBMC. The production of IFN- $\gamma$  and neopterin by PHA-stimulated PBMC was also strongly suppressed by the cacao extracts, which directs to a down-regulatory effect of cacao compounds on T-cells. Treatment of LPS-stimulated myelomonocytic THP-1 cells did not reveal any effect of the added cacao extracts on tryptophan degradation and neopterin production. Consequently, the suppressive effect of cacao extracts seems to be directed on T-cells rather than on monocytic cells induced by a distinct proinflammatory pathway via toll like-receptors (TLRs).

The results of our study with respect to the suppression of mitogen-stimulated IDO activity and IFN- $\gamma$  production in PBMC by the cacao extracts, agree well with the available literature on their immunosuppressive and anti-inflammatory effects. Other groups showed that cocoa flavonols mediate various anti-inflammatory effects in PHA-stimulated PBMC such as inhibition of IL-2 (Sanbongi et al., 1997; Heiss et al., 2003), and IL-4 (Mao et al., 2002b) or stimulation of IL-1 $\beta$  (Mao et al., 2000) and IL-5 (Mao et al., 2002b). Most of the described mechanisms of action of cocoa have been ascribed to the polyphenolic compounds present in high amounts in cocoa beans, among others particularly the flavan-3-ol monomers epicatechin, catechin, gallocatechin or epigallocatechin and their oligomeric derivatives known as procyanidins can be found (Porter et al., 1991; Natsume et al., 2000). Many studies have suggested that flavonoids have the capacity to act as antioxidants *in vitro* due to their ability to reduce free radical formation and to scavenge free radicals (Middleton et al., 2000). Miller et al. (2006) used the ORAC assay to determine the antioxidant capacity of cocoa and chocolate products from major brands in the United States and found that the natural cocoa powders contained the highest levels of antioxidant capacity with an ORAC value between 720 and 875  $\mu\text{mol}$  Trolox equivalents/g. The ORAC values of our cacao extracts were similar to

those found in the study of Miller et al. Although flavanol-rich cocoa has the potential to augment an individual's antioxidant defence system, there are, as likely as not, other cellular mechanisms by which cocoa-based products may affect human health. Suppression of the release of IFN- $\gamma$  and its down-stream biochemical pathways agrees well with earlier findings by us and others showing several antioxidants such as vitamin C and E, the stilbene resveratrol but also of green and black tea or wine to exert suppressive properties on stimulated PBMC similar to cacao extracts (Zvetkova et al., 2001; Tan et al., 2005; Wirleitner et al., 2005; Schroecksnadel et al., 2007; Winkler et al., 2007). Preliminary data also showed an inhibitory capacity of procyanidin B2 (4,8''-Bi-[(+)-epicatechin, cis,cis''-4,8''-Bi(3,3',4',5,7-pentahydroxyflavane) on PHA-stimulated degradation of tryptophan in PBMC (to be published).

At high concentrations (25  $\mu\text{g}/\text{mL}$ ) cocoa flavonols were also reported to slightly stimulate the secretion of TNF- $\alpha$  in unstimulated and stimulated PBMC (Mao et al., 2002a), and interestingly also in our experiments using unstimulated PBMC the cacao extracts induced a faint but significant stimulatory effect on neopterin production at concentrations of  $\geq 5 \mu\text{g}/\text{mL}$ . A similar enhancing effect on neopterin production in resting PBMC was observed earlier with green and black tea extracts prepared from *Camellia sinensis* (L.) (Zvetkova et al., 2001). This effect may possibly arise from an artefact resulting from oxidation of cocoa polyphenols in the cell culture media by air oxygen followed by the generation of superoxide anion and H<sub>2</sub>O<sub>2</sub>, which has been shown for high doses of epicatechin and other flavan-3-ols (Long et al., 2000). In contrast, both cacao extracts diminished tryptophan degradation also in unstimulated cells, most probably by the inhibition of spontaneous IDO activity. Lower kynurenine concentrations were observed together with higher tryptophan levels resulting in a significant decrease of kyn/trp at doses of  $\geq 5 \mu\text{g}/\text{mL}$ .

There are a number of reports implicating a role of cytokine-induced IDO in psychiatric diseases (Sandyk, 1992; Young, 1993; Wirleitner et al., 2003; Dantzer et al., 2008), and several studies showed, that mood is negatively influenced by the depletion of tryptophan (Young et al., 1985; Delgado et al., 1994; Reilly et al., 1997). If our *in vitro* findings would also hold true for the *in vivo* situation, data imply that cacao extracts are able to slow-down inflammation-associated tryptophan degradation and thus improve tryptophan availability for serotonin production. Such a scenario is well in line with mood enhancing properties of cocoa products, e.g., a capacity to improve mood, lift spirits and make people feel-good. In atypical depression and in seasonal affective disorder, chocolate craving was reported to be a form of self-medication (Wurtman and Wurtman, 1989) and in having an impact on brain neurotransmitters, chocolate has been characterized to have antidepressant benefits (Parker et al., 2006). Accordingly, several psychoactive constituents including anandamines, caffeine or phenylethylamine have been identified in cocoa (Hurst et al., 1982; DiTomaso et al., 1996). On the one hand, we cannot exclude that these biogenic amines may also affect the stimulation capacity of PBMC, on the other hand, the achievable plasma level of these compounds after ingestion of a typical serving of a cocoa product is assumed to be too low. Nevertheless, inhibition of IDO activity by cacao may mimic a kind of oral tryptophan supplementation which has been shown to result in enhanced concentrations of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the cerebrospinal fluid (Bender, 1983) and conversely diets devoid of tryptophan resulted in impaired cerebral serotonin formation (Delgado et al., 1990).

It should be mentioned that such a potential beneficial effect of cacao has been gathered from *in vitro* experiments only, which cannot simply be extrapolated to the *in vivo* situation. However, at least in the gastrointestinal tract one can assume the existence of all the compounds present in cacao at effectual concentrations where they

could also increase the availability of tryptophan and production of serotonin. A great proportion of serotonin (about 95%) in the human body is synthesized and stored in the gastrointestinal tract acting as a paracrine messenger to modulate sensation, secretion and motility (Gershon and Tack, 2007). According to this, ingestion of cacao products or administration of polyphenols present in cacao could play an important role in the modulation of tryptophan availability and consequently the disposability of serotonin. Furthermore, the antioxidant capacity of cacao products may locally shift the redox equilibrium in the gastrointestinal tract, which could then be of benefit for the intestine and the whole organism.

On the basis of our findings regarding the suppression of mitogen-induced degradation of tryptophan due to an inhibition of activated IDO by the tested cacao extracts, we propose another mechanism for the mood elevating effect of cacao-based products: Their capacity to enhance the availability of tryptophan for serotonin synthesis may improve quality of life, especially in patients suffering from inflammatory conditions.

### Conflict of interest

The authors declare that they have no conflicts of interest.

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