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Intestinal formation of *trans*-crocetin from saffron extract (*Crocus sativus* L.) and *in vitro* permeation through intestinal and blood brain barrier

M. Lautenschläger[a], J. Sendker[a], S. Hüwel[b], H.J. Galla[b], S. Brandt[a], M. Düfer[c], K. Riehemann[d], A. Hensel[a,*]

[a] University of Münster, Institute for Pharmaceutical Biology and Phytochemistry, Corrensstraße 48, D-48149 Münster, Germany
[b] University of Münster, Institute for Biochemistry, Wilhelm-Klemm-Str. 2, D-48149 Münster, Germany
[c] University of Münster, Institute for Pharmaceutical and Medical Chemistry, Department of Pharmacology, Corrensstraße 48, D-48149 Münster, Germany
[d] Center for Nanotechnology/Institute of Physics, Heisenbergstraße 11, D-48149 Münster, Germany

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ABSTRACT

Aims: Extracts of saffron (*Crocus sativus* L.) have traditionally been used against depressions. Recent preclinical and clinical investigations have rationalized this traditional use. *Trans*-crocetin, a saffron metabolite originating from the crocin apocarotenoids, has been shown to exert strong NMDA receptor affinity and is thought to be responsible for the CNS activity of saffron. Pharmacokinetic properties of the main constituents from saffron have only been described to a limited extent. Therefore the present *in vitro* study aimed to determine if crocin-1 and *trans*-crocetin are able to pass the intestinal barrier and to penetrate the blood brain barrier (BBB). Additionally, the intestinal conversion of glycosylated crocins to the lipophilic crocetin had to be investigated. Experiments with Caco-2 cells and two different porcine BBB systems were conducted. Further on, potential intestinal metabolism of saffron extract was investigated by *ex vivo* experiments with murine intestine.

Methodology: *In vitro* Caco-2 monolayer cell culture was used for investigation of intestinal permeation of crocin-1 and *trans*-crocetin. *In vitro* models of porcine brain capillary endothelial cells (BCEC) and blood cerebrospinal fluid barrier (BCSFB) were used for monitoring permeation characteristics of *trans*-crocetin through the blood brain barrier (BBB). Intestine tissue and feces homogenates from mice served for metabolism experiments.

Results: Crocin-1, even at high concentrations (1000 μM) does not penetrate Caco-2 monolayers in relevant amounts. In contrast, *trans*-crocetin permeates in a concentration-independent manner (10–114 μM) the intestinal barrier by transcellular passage with about 32% of the substrate being transported within 2 h and a permeation coefficient of P_{app} $25.7 \times 10^{-6} \pm 6.23 \times 10^{-6}$ cm/s. *Trans*-crocetin serves as substrate for pGP efflux pump. *Trans*-crocetin permeates BBB with a slow but constant velocity over a 29 h period (BCEC system: P_{app} $1.48 \times 10^{-6} \pm 0.12 \times 10^{-6}$ cm/s; BCSFB system P_{app} $3.85 \times 10^{-6} \pm 0.21 \times 10^{-6}$ cm/s). Conversion of glycosylated crocins from saffron extract to *trans*-crocetin occurs mainly by intestinal cells, rather than by microbiological fermentation in the colon.

Conclusion: The here described *in vitro* studies have shown that crocins from saffron are probably not bioavailable in the systemic compartment after oral application. On the other side the investigations clearly have pointed out that crocins get hydrolyzed in the intestine to the deglycosylated *trans*-crocetin, which subsequently is absorbed by passive transcellular diffusion to a high extend and within a short time interval over the intestinal barrier. Crocetin will penetrate in a quite slow process the blood brain barrier to reach the CNS. The intestinal deglycosylation of different crocins in the intestine is mainly due to enzymatic processes in the epithelial cells and only to a very minor extent due to deglycosylation by the fecal microbiome. On the other side the fecal bacteria degrade the apocarotenoid backbone to smaller alkyl units, which do not show any more the typical UV absorbance of crocins. As previous studies have shown strong NMDA receptor affinity and channel opening activity of *trans*-crocetin the use of saffron for CNS disorders seems to be justified from the pharmacokinetic and pharmacodynamic background.

* Corresponding author. Tel.: +49 251 8333380; fax: +49 251 8338341.
E-mail address: ahensel@uni-muenster.de (A. Hensel).

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Abbreviations

A	apical
B	basolateral
BBB	blood-brain barrier
BCEC	brain capillary endothelial cells
BCSFB	blood cerebrospinal fluid barrier
CNS	central nervous system
DAPI	4′,6-diamidin-2-phenylindol
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
EVOM	epithelial voltohm meter
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NEAA	non-essential amino acids
NMDA	N-methyl- D -aspartate
P_{app}	apparent permeability coefficient [cm/s]
PBS	phosphate buffered saline
SE	hydroethanolic saffron extract
SGF	simulated gastric fluids
SIF	simulated intestinal fluids
TFA	trifluoroacetic acid
TEER	transepithelial electrical resistance

Introduction

Saffron, the dried stigmata of *Crocus sativus* L., has been used in traditional medicine (Ferrence and Bendersky 2004) against spasms, bronchospasm, menstruation disorders, liver disease, pain, insomnia, digestive ailments and as stimulant, aphrodisiac, antidepressant and for supportive treatment of cancer (Schmidt et al. 2007). Several clinical pilot studies proved significant antidepressant effects of saffron extracts; a recent meta-analysis, including 5 randomized, controlled studies, indicated significant improvement of symptoms in patients with major depressions (Hausenblas et al. 2013). Recent studies have shown neuroprotective effects of saffron extract with *trans*-crocetin – the deglycosylated metabolite of crocins – being the active ingredient. This cytoprotective activity of *trans*-crocetin is due to the high affinity of this apocarotenoid to the phencyclidine binding site of the NMDA receptor (Lechtenberg et al. 2008). This antagonistic effect at central NMDA receptors leads to an inhibition of postsynaptic potentials and inhibition of glutamatergic transmission, as recently shown in rat cortical brain (Berger et al. 2011). Besides the antidepressive effects saffron extracts and especially its main secondary metabolite crocin has been used for the treatment of coronary diseases (for review see Kamalipour et al. 2011; Joukar et al. 2010) due to its potential antihyperlipidemic effects (Lee et al., 2005), inhibition of cardiac Ca^{2+} channels (Boskabady et al. 2008) and pronounced antioxidant reactivity (Mashmoul, 2013), Main secondary metabolites of saffron extracts are about 0.5% of volatile compounds with safranal as main constituent (Schmidt et al. 2007; Lechtenberg et al. 2008). The typical spicy taste of saffron originates from picrocrocin (5–15%), the biosynthetical precursor of safranal. The main constituents of saffron constitute a series of apocarotenoids, typically carotenoid-glycosyl esters of a C_{20}-dicarboxylic acid, the so-called crocins (Fig. 1). Crocins account for up to 30% of dry weight and are responsible for the intense yellow color of saffron (Schmidt et al. 2007; Lechtenberg et al. 2008). Non-glycosylated C_{20}-dicarboxylic acids, with *trans*-crocetin as lead compound can be found only in traces in saffron of good quality (Lechtenberg et al. 2008).

From the pharmacokinetic point of view it has been assumed that crocin is excreted largely through the intestinal tract following oral administration; on the other side the intestinal tract serves as an important site for crocin hydrolysis (Xi et al. 2007) and bioavailability of isolated crocetin after oral administration has been shown in healthy volunteers (Umigai et al. 2011). Xi et al. (2007) showed that orally administed crocin is not absorbed in rats after a single dose or repeated doses and crocin is excreted largely through the intestinal tract following oral administration. Until now it is still unclear to which extent crocins are metabolized in the intestinum to the deglycosylated crocetin, in which gastrointestinal compartments this occurs and whether this is due to fecal metabolism. The following study aimed to investigate the underlying permeation mechanisms across the intestinal epithelial barrier by using Caco-2 cells (Hubatsch et al. 2007) and to clarify if *trans*-crocetin permeates the blood brain barrier under *in vitro* conditions by using two different blood brain barrier models (porcine blood capillary endothelial cells, BCEC (Franke et al. 2000) and blood cerebrospinal fluid barrier, BCSFB (Angelow et al. 2004)).

Materials and methods

Materials

Saffron, grown in Iran, purchased from the International Mumbai Saffron Trading Exchange, batch No. 7002, was identified by macroscopic and microscopic investigation against a saffron reference sample. Full analytical characterization was performed by UPLC® according to Lautenschläger et al. (2014). A voucher specimen (IPBP291) has been deposited in the documentation center of the Institute of Pharmaceutical Biology and Phytochemistry, Münster, Germany.

Standardized hydroethanolic saffron extract (SE), *trans*-crocin-1 and *trans*-crocetin were prepared as described recently (Lautenschläger et al. 2014).

If not stated otherwise, solvents, reagents and consumables were obtained from VWR (Darmstadt, Germany). Propranolol HCl was obtained from Acros Organics (Geel, Belgium), Lucifer Yellow CH dilithium salt, DAPI and EDTA from Sigma–Aldrich Chemie (Buchs, Switzerland), Verapamil HCl from Fluka Chemie (Buchs, Switzerland), TexasRed®-X Phalloidin from Molecular Biotechnology (MoBiTec, Göttingen, Germany). FBS Hyclone® was purchased from Thermo Fischer Scientific Inc. (Waltham, MA, USA), NEAAs were from GE Healthcare (Chalfont St Giles, Buckinghamshire, UK), Ezetimibe from Molekula GmbH (München, Germany) and all other components for *in vitro* cell culture were from PAA Laboratories (Pasching, Austria). Tissue culture plastics were supplied by Sarstedt (Nümbrecht, Germany). Transwell® plates (12 well plate, 12 mm diameter, 0.4 μm pore size, polycarbonate membrane) were obtained from Corning Costar® (New York, USA). Test solutions were sterilized prior use by 0.2 μm syringe filter polypropylene (VWR, Darmstadt, Germany).

Caco-2 in vitro *cell culture*

Cultivation of Caco-2 cell line (ECACC, European Collection of Cell Culture, Salisbury, UK, LOT 07/G/006; 6/08/07 P46), absorption studies as well as respective evaluations were performed according to Hubatsch et al. (2007) (with minor modifications by Zumdick et al. (2012)) and quantitative calculations were carried out according to Tavelin et al. (2002).

Cells from passage number 70–82 were maintained in 75 cm^2 plastic culture flasks in 10 ml of Dulbecco's Modified Eagle's Medium, supplemented with 10% FBS Hyclone®, 1% NEAA and 1% penicillin/streptomycin (100×), cultivated in a humidified incubator at 37 °C, 5% CO_2 until 70–90% confluency was achieved.

For subcultivation the cell monolayer was treated for 5 min with 0.25% trypsin/0.2% EDTA. Cell counting was performed by electric current exclusion (CASY® cell-counter, Schärfe Systems, Germany).

For transport experiments cells were seeded at a density of 2×10^5 cells/ml on Transwell® plates and grown for 21–23 days to