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Antigenotoxic properties of *Eruca sativa* (rocket plant), erucin and erysolin in human hepatoma (HepG2) cells towards benzo(*a*)pyrene and their mode of action

Evelyn Lamy [a,*], Julia Schröder [a], Stefanie Paulus [a], Peter Brenk [a], Thorsten Stahl [b], Volker Mersch-Sundermann [a]

[a] University Medical Center Freiburg, Institute of Environmental Medicine and Hospital Hygiene, Breisacher Strasse 115b, 79106 Freiburg, Germany
[b] Landesbetrieb Hessisches Landeslabor, Abteilung V - Umwelt- und Spurenanalytik, Fachgebiet V.3 – Umweltanalytik, Glarusstr. 6, D-65203 Wiesbaden, Germany

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ABSTRACT

In recent years, rocket plant (*Eruca sativa*) has gained greater importance as a vegetable and spice, especially among Europeans. *E. sativa* is a member of the Brassicaceae, which is considered to be an important chemopreventive plant family. In the present study, we assessed the chemopreventive potency and underlying mechanisms of extracts of *E. sativa* in HepG2 cells. No genotoxic effect could be observed in HepG2 cells treated with up to 50 μl/ml plant juice for 24 h when using the comet assay. In antigenotoxicity experiments, *E. sativa* extract reduced the benzo(*a*)pyrene-induced genotoxicity in a U-shaped manner. This effect was accompanied by a significant induction of glutathione *S*-transferase. No significant suppression of B(*a*)P-induced CYP1A1 protein expression or enzyme activity could be observed. Chemical analysis of the plant material by gas chromatography identified the isothiocyanates erucin, sulforaphane, erysolin and phenylethyl isothiocyanate. Results derived with the single ITC compounds support the assumption that their synergistic interaction is responsible for the strong antigenotoxicity of the plant material. The present study provided an assessment of the bioactive effects of rocket plant extract in a human cell culture system. This could help to evaluate the balance between beneficial vs. possible adverse effects of rocket plant consumption.

1. Introduction

Rocket plant (*Eruca sativa*) is a member of the Brassica plant family, which comprises of more than 350 genera. *E. sativa* is widely used as vegetable and spice. Epidemiological and experimental data provide strong evidence that Brassicaceae species inhibit tumorigenesis (Lynn et al., 2006; van Poppel et al., 1999; Verhoeven et al., 1996; Verhoeven et al., 1997). The predominant non-nutrient compounds in Brassicaceae made accountable for this effect are enzymatic cleavage products of glucosinolates, namely isothiocyanates (ITCs) (Conaway et al., 2002; Hecht, 1999; Keum et al., 2005). ITCs are formed by the reaction of glycosinolates with the endogenous enzyme myrosinase, which is released by chopping or chewing the vegetables (Holst and Williamson, 2004). The protective effect of ITCs against genotoxicants is mainly attributed to their capabilities to modulate the activity of enzymes involved in biotransformation (Steinkellner et al., 2001a). Recent studies focusing on the identification of volatile and non-volative compounds of *E. sativa* found ten different ITCs in this plant (Bennett et al., 2006; Jirovetz et al., 2002). However, so far, no studies have been carried out that focus on the antigenotoxic effects of the plant material itself. Therefore, in the present study we investigated the genotoxicity and antigenotoxicity of crude *E. sativa* extract using the metabolically competent liver cancer cell line (HepG2) with the alkaline version of the comet assay. The comet assay has been used with that protocol in numerous studies, thereby delivering useful results for further and more detailed mechanistic studies. HepG2 cells are known for their phase I and II biotransformation enzyme activities that influence processes related to chemical carcinogenesis (Knasmuller et al., 2004; Mersch-Sundermann et al., 2004). HepG2 cells possess a functional Ah receptor (Roberts et al., 1990), inducible glutathione *S*-transferase (Dawson et al., 1985; Dierickx, 1989), sulfotransferase (Dierickx, 1989) and NAD(P)H: quinone oxidoreductase (Backman et al., 1991).

For antigenotoxicity experiments, we exposed HepG2 cells to benzo(*a*)pyrene [B(*a*)P], a polycyclic aromatic hydrocarbon (PAH),

Abbreviations: AITC, allyl isothiocyanate; ATP, adenosine triphosphate; B(*a*)P, benzo(*a*)pyrene; BITC, benzyl isothiocyanate; CYP1A1, cytochrome P450 1A1; d.w., dry weight; SCGE, single cell gelelectrophoresis assay, syn: comet assay; GST, glutathione *S*-transferase; ITC, isothiocyanate; LMP, low melting point agarose; MTBITC, 4-methylthiobutylisothiocyanate, erucin; PEITC, phenylethyl isothiocyanate; SFN, 4-methylsulfenylbutylisothiocyanate, sulforaphane.

* Corresponding author. Tel.: +49 761 270 8215; fax: +49 761 270 8203.
E-mail address: evelyn.lamy@uniklinik-freiburg.de (E. Lamy).

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in a concentration of 50 μM. B(*a*)P is a strong mutagen and human carcinogen (IARC, 1983; 1987) and is ubiquitously distributed in the environment. The principle route of exposure for humans is through the diet (Phillips, 1999). To exert its genotoxic and/or carcinogenic potency, B(*a*)P has to be activated by CYP-450 monooxygenases to a variety of electrophiles that can covalently bind to DNA, RNA or proteins (Zytkovicz et al., 1981). Additionally, the generation of reactive B(*a*)P-intermediates, through the aryl hydrocarbon receptor (AhR)-dependent upregulation of CYP1A1, may lead directly to the formation of reactive oxygen species (ROS) (Elbekai et al., 2004). To elucidate the mechanisms underlying the antigenotoxic potency of *E. sativa* extract, we investigated the induction of total glutathione *S*-transferase, which plays a crucial role in the detoxification of numerous xenobiotics, e.g. B(*a*)P (Steinkellner et al., 2001b). Additionally, we determined the expression levels of cytochrome P4501A1 (CYP1A1) protein, one of the most important enzymes in the toxification of B(*a*)P and its modulation by the *E. sativa* extract or its ITC constituents. We also studied the effect of *E. sativa* extract on B(*a*)P-induced CYP1A activity since it is known that the ingredients of Brassicaceae, and ITCs in particular, can cause enzyme modulation on the posttranslational level by binding to the nucleophilic parts of CYP450 or by covalent protein modification (Goosen et al., 2000; Nakajima et al., 2001). GC–MS/MS analysis was carried out to determine the ITC levels in the *E. sativa* extract.

2. Materials and methods

2.1. Chemicals

DMSO (purity > 99%) (CAS 67-68-5), benzo[*a*]pyrene (B(*a*)P) (purity > 98%) (CAS 50-32-8) cyclohexane (p.a. quality), ethyl acetate (p.a. quality) salicylamide (purity > 99%) and resorufin (purity > 95%) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Normal melting point agarose (NMA) was from Merck (Darmstadt, Germany). Low melting point agarose (LMA) and gentamycin were obtained from Serva (Heidelberg, Germany). Dulbeccos Minimal Essential Medium (DMEM) and fetal calf serum (FCS) were from PAA (Pasching, Austria). B(*a*)P was dissolved in sterile DMSO. 4-Methylsulfinylbutyl isothiocyanate (purity > 98%) (CAS 4478-93-7), 4-methylsulfonlybutyl isothiocyanate (purity > 98%) (CAS 504-84-7) were purchased from LKT Labs (St. Paul, USA); allyl isothiocyanate (CAS 57-06-7) was purchased from Fluka, (Seelze, Germany); benzyl isothiozyanate (purity > 98%) (CAS 622-78-6) and phenlyethyl isothiocyanate (purity > 98%) (CAS 2257-09-2) were purchased from Sigma–Aldrich (Taufkirchen, Germany). 4-Methylthiobutyl isothiocyanate (purity > 99.9%) (CAS 4430-36-8) was synthesized by Dr. Mike Kotke, Dept. of Organic Chemistry, University of Giessen, Germany.

2.2. Plant material

E. sativa plant material was purchased in Giessen, Germany. For the cell culture experiments, the plant was crushed into very small pieces and the juice was subsequently squeezed out, sterile filtered (<0.22 μm) and stored at −80 °C until use. For GC–MS/MS analysis of the plant compounds, 10 g of the minced plant sample were extracted two times with cyclohexane/ethyl acetate (50:50, v/v) for 1 h and subsequently centrifuged (5000*g*, 10 min., 4 °C). The organic phase containing the ITCs was cleared through a 45 μm teflon filter. The dry weight was determined drying a 10 g sample of the material at 40 °C.

2.3. Chemical analysis by gas chromatography/mass spectrometry (GC–MS/MS)

Chemical analysis and quantification of the ITCs in the plant extract was done by a Varian gas chromatograph CP-3800 equipped with a CombiPAL auto sampler and a factorFour fused silica capillary column (VF-35 ms, 30 m × 0.25 mm (i.d.), 0.25 μm film thickness, Varian). The column temperature was set to 30 °C and held for 30 s. The temperature was increased to 90 °C at a rate of 5 °C/min and subsequently to 160 °C, both of which were held for 5 min, respectively. The final temperature of 280 °C was reached with an increase rate of 30 °C/min. This temperature was held for 10 min. The flow rate of the helium carrier gas was set to 1 ml/min. The injector temperature was set to 210 °C (splitless mode, injection volume = 1 μl). Detection was carried out by a Varian 1200 Quadrupole MS/MS, the transfer line was set to 210 °C. Mass spectra were obtained by electron ionisation at 70 eV, and mass scan was from 90 to 180. Compound quantification was calculated with an external standard calibration function for each compound.

2.4. HepG2 cells

HepG2 cells were kindly provided by Dr. Firouz Daroudi (“LUMC”, The Netherlands). The cells were cultured in low glucose DMEM supplemented with 15% fetal calf serum and 50 μg/ml gentamycin in a 5% CO_2 atmosphere at 37 °C.

2.5. Determination of cell viability

HepG2 cells were plated onto 12-well plates (Greiner bio-one, Germany) at a density of 1.5×10^5 cells/ml culture medium. After 24 h of growth, the cells were exposed to *E. sativa* extract (3–50 μl/ml) or erysolin for 24 h at 37 °C and 5% CO_2. In combination experiments with B(*a*)P, the cells were pre-treated with *E. sativa* extract or erysolin for 24 h before exposure to B(*a*)P (50 μM) for another 24 h. After treatment, the viability of the cells was determined by erythrosin B (Bhuyan et al., 1976).

2.6. Single cell gel electrophoresis (comet) assay

For the genotoxicity testings of the plant extract and the *E. sativa*/B(*a*)P combination assays, the cells were treated as described in Section 2.5. These experiments were carried out (i) with a washing step before exposure of HepG2 cells to B(*a*)P treatment (ii) without washing of cells. Antigenotoxicity testing with erysolin/B(*a*)P was carried out with a washing step before B(*a*)P-exposure. The SCGE assay, also known as comet assay, was carried out as described by Singh and co-workers (1988) according to the guidelines developed by Tice and his group (2000) and Klaude et al. (1996) with slight modifications as described earlier (Lamy et al., 2004). The samples were analysed with a Leica fluorescence microscope (Leica DMLS; excitation filter: BP 546/10 nm; barrier filter: 590 nm) connected to a computerized image analysis system (Comet 5.5, Optilas, München, Germany).The Olive tail moment (OTM) was calculated as an indicator of DNA damage. One hundred and two cells per concentration were evaluated and the test was carried out three times. The reported OTM is mean ± standard deviation (SD) of three independent experiments. For the genotoxicity tests and the combination experiments, the significance was calculated in comparison to the negative control and 50 μM B(*a*)P, respectively, using the Student's *t*-test ($^{*}p \geqslant 0.05$, $^{**}p \geqslant 0.01$).

2.7. Measurement of glutathione S-transferase activity using CDNB

Total, i.e. cytosolic and microsomal GST activity in HepG2 cells was measured with a kit from Cayman Chemical (Grünberg, Germany) following the instruction manual. In short, 1×10^6 cells were harvested by scraping and centrifugation. The cell pellet was resuspended in cold PBS w/o Ca^{2+}/Mg^{2+} and lysed by sonication. After centrifugation (16,000*g*, 20 min., 4 °C), 20 μl of the supernatant were mixed with 20 μl reduced glutathione, 150 μl assay buffer and 10 μl 1-chloro-2,4-dinitrobenzene (CDNB).The absorbance was subsequently measured at 340 nm on a 96-well microtiter plate reader (Infinite M200, Tecan, Germany) in 1 min. intervals for 10 min. Samples and standards were assayed three times in duplicate and the concentration of total GST was calculated relative to the activity of control cells.

2.8. Determination of CYP1A1 protein levels by immunoblotting

HepG2 cells were seeded on culture flasks at a density of 1×10^7, grown overnight and treated with *E. sativa* extract (6, 25 and 50 μl/ml) or the ITCs for 24 h, washed three times with PBS and subsequently treated with 50 μM B(*a*)P for another 24 h. Next, the cells were harvested with a cell scraper, lysed by sonication on ice and centrifuged (14,000*g*, 15 min, 4 °C). The supernatant was again centrifuged (100,000*g*, 1 h, 4 °C) and the sediment containing the microsomes was kept at −80 °C until western blot analysis. Protein levels were assayed as described by Bradford (1976). Sample buffer (Biorad, Germany) containing DTT (0.12 M) was added to 20 μg of total microsomal protein, boiled at 95 °C for 5 min, separated by SDS PAGE (10%) and transferred to nitrocellulose membranes by electro-blotting. The samples were probed with an anti-human CYP1A1 monoclonal antibody of mouse, followed by treatment with an HRP-labeled anti-mouse secondary antibody. CYP1A1 containing microsomes from the human B lymphoblastoid cell line AHH-1 (Catno. 455111, BD Biosciences, Heidelberg, Germany) were used as positive control. The obtained blots were analysed by densitometric technique.

2.9. Determination of CYP1A activity in HepG2 cells using the ethoxyresorufin O-deethylase (EROD)-assay

The modulatory effect of *E. sativa* extract and its ITC constituents on B(*a*)P-induced CYP1A activity was assessed by the EROD assay according to Burke and coworkers (Burke and Orrenius, 1978). The assay is based on the O-deethylation of 7-ethoxyresorufin using fluorescence detection, whereby fluorescence intensity of the released resorufin is proportional to the CYP1A activity. HepG2 cells were seeded at a density of 5×10^4 cells/well in black 96-well plates (Greiner bio-one, Germany), treated with *E. sativa* extract (6, 25 and 50 μl/ml) or the ITCs for 24 h,