Published in final edited form as:

Environ Toxicol Pharmacol. 2013 September ; 36(2): . doi:10.1016/j.etap.2013.04.017.

Benzo(a)pyrene modulates fluoranthene-induced cellular responses in HT-29 colon cells in a dual exposure system

Kelly L. Harris, Jeremy N. Myers, and Aramandla Ramesh*

Department of Biochemistry & Cancer Biology, Meharry Medical College, 1005 D.B. Todd Boulevard, Nashville, TN 37208, USA

Abstract

Our environment is contaminated with a diverse array of chemicals; one of which is polycyclic aromatic hydrocarbons (PAHs). While some PAHs are potent by nature, others undergo interactions such as additivity, synergism, antagonism or potentiation to manifest their toxicity. Therefore, the objective of this study was to investigate whether exposure to benzo(a)pyrene (BaP), a PAH compound influences the cytotoxicity and metabolism of fluoranthene (FLA; another PAH compound) using HT-29 cells. Cells cultured in Dulbecco's Modified Eagle Medium were treated with 1, 5, 10, 25 μ M BaP and FLA (0.01% dimethylsulfoxide as vehicle) individually and in combination over the course of 0–96 h. At the end of exposure, cells were stained with propidium iodide and the changes in cell cycle were analyzed using FACS analysis. Apoptosis was determined by caspase-3 assay. Post-incubation, samples were extracted and analyzed for FLA metabolites by reverse-phase HPLC with fluorescence detection. Cells exposed to BaP + FLA showed a marginal decrease in growth as compared to FLA alone and vehicle controls. Also, a decline in the percentage of cells in the S and G2 phases compared to G1 phase of cell cycle was noted when cells were treated with BaP and FLA together, compared to individual FLA treatment. The rate of FLA metabolism was more when cells were exposed to FLA in combination with BaP, compared to FLA alone. The enhanced biotransformation of FLA as a result of concomitant exposure to BaP may have implications for colon cancer risks arising from human dietary exposure to PAH mixtures through consumption of barbecued meat.

Keywords

Fluoranthene; Benzo(a)pyrene; Polycyclic aromatic hydrocarbons; Metabolism

1. Introduction

Recent years have witnessed a surge in the contamination of food by a family of compounds called polycyclic aromatic hydrocarbons. Being products of combustion, they contaminate the environment to a greater extent and contribute to the burden of environmentally-induced disease (Ramesh et al., 2011). Not only these compounds contaminate the dietary ingredients such as harvested agricultural produce, fish and products from livestock (Ramesh et al., 2012), they are also formed during food preparation processes and taint the food items.

The organ system that comes into contact first with ingested PAHs is the digestive system. Of late, the ability of PAHs to cause gastrointestinal (GI) cancers has attracted a lot of attention. Several studies with laboratory animal models and humans have implicated benzo(a)pyrene (a representative PAH compound) and other PAHs contributing to colon cancer (Diggs et al., 2011). However, the mechanisms have not yet been elucidated.

Ingested PAHs have to cross the intestinal barrier for absorption and subsequent breakdown through liver or localized metabolism in the GI tract itself. Disposition of some PAH metabolites in intestinal crypts (Herbst et al., 2006) and the ability of tumor cells to metabolize BaP (Diggs et al., 2012) lend credence to the assumption that localized metabolism of PAHs contribute to cytotoxicity and aid in the neoplastic transformation of polyps.

Seldom are humans exposed to a single PAH compound through dietary intake, but a multitude of PAHs. As it is a common phenomenon in mixture toxicity, some PAHs may have either synergistic or antagonistic effects, the manifestation of toxicity is dependent on the combination of PAHs present in the mixture and their relative proportions. Polycyclic aromatic hydrocarbon-induced carcinogenesis requires long term exposure to an individual PAH compound (Harris et al., 2009; Hakura et al., 2011; Wester et al., 2012) or mixtures (Culp et al., 1998). This issue brings to light the interactions between or among various PAHs and cytotoxicity. Therefore, the objective of this study was to assess how interactions in a binary exposure system affect the colon cell survival and proliferation. We have chosen BaP and FLA for this study because both were widespread toxicants as shown for cell culture systems (Le Hégarat et al., 2012; Wessel et al., 2012) and animal models (Knuckles et al., 2001, 2004). Additionally, the concentrations of BaP and FLA in meat products and agricultural produce (Maga, 1986; Ramesh et al., 2004) and their mean dietary intake (Martí-Cid et al., 2008) were reported to be higher than the other PAHs. While BaP is classified as a carcinogen (IPCS, 1998), FLA is considered as a non-classifiable carcinogen (EPA, 2001). Therefore, we have selected the HT-29 colon adenocarcinoma cells as a test system due to their extensive use to study cell toxicity (Sambruy et al., 2001), differentiation (Mitchell and Ball, 2004), and metabolism (Ebert et al., 2003).

2. Materials and methods

2.1. HT-29 colon cell culture

The HT-29 colon adenocarcinoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). It was cultivated in a T75 polystyrene cell culture flasks containing Dulbecco's Modified Eagle Medium (DMEM: Nutrient Mixture F-12 supplemented with 10% FBS and antibiotics-1% penicillin-streptomycin; Invitrogen) at 37°C in a 5% CO₂ incubator containing 50mL/LCO₂.

The HT-29 cells were seeded onto a 6 well plate or a T75 flask at a density of 20,000 cells/well or 1×10^6 cells respectively. The cells were then synchronized overnight by serum starvation (DMEM/F12 media, 1% FBS) method. Cells were then treated with either FLA or BaP and in combination as detailed below.

2.2. Cell growth

In order to analyze the effects of FLA on HT-29 cell growth, cells were treated with FLA or FLA + BaP concentrations as follows: untreated [no FLA but dimethylsulfoxide (DMSO: vehicle for FLA or FLA + BaP) added], DMSO (0.01%), 1, 5, 10, and 25 µM. The cells were exposed for 24, 48, 72, and 96 h time points. At each time point the cells were counted using a Coulter Counter (Beckman Coulter Inc., Palo Alto, CA).

2.3. Cytotoxicity

Cytotoxic effects of FLA and FLA + BaP on HT-29 cells were determined using a lactate dehydrogenase (LDH) kit (Abcam, Cambridge, MA). The HT-29 cells were seeded at a density of 2000 cells per well in a 96 well plate. After the initial plating, the cells were incubated at 37°C overnight (~12h) prior to being synchronized using starvation media. Subsequently, cells were treated with FLA & BaP at the concentrations and exposure time points mentioned above.

2.4. Apoptosis assay

In order to analyze apoptosis a Caspase-3 Colorimetric Protease Assay Kit (MBL International Corporation, Woburn, MA) was used. Cell seeding and test chemical exposure was done as mentioned above. After exposure to requisite time points, the apoptotic cells were assayed by measuring the chromophore p-nitroanilide (pNA) light emission using a xMark Microplate Spectrophotometer (BioRad, Hercules, CA) at 405 nm.

2.5. Cell cycle changes

In order to further explore the possibilities of cell growth inhibition, Fluorescence-Activated Cell Sorting (FACS) analysis was utilized. Cells were plated, synchronized, and treated as described previously. After exposure to FLA & BaP for the above-mentioned duration of time, cells were then fixed in 100% cold ethanol, incubated at 4°C for 1h and stained with propidium iodide (Sigma–Aldrich). After staining, 20 µl of 0.1% RNase in PBS was added to the cells and incubated at 4°C for 1 h prior to measuring the fluorescence. The cell preparations were analyzed using a FACS instrument (Becton Dickinson, Franklin Lakes, NJ) available in the Meharry BSL3/FACS core facility. The fluorescence was measured at an excitation wavelength of 488 nm with a blue laser and detected at channel FL2. All experiments were done in triplicate.

2.6. Extraction, identification and quantitation of benzo(a)pyrene metabolites

To enable extraction, cells pellets were lysed by the bead-beating technique as detailed in Myers et al. (2011). Lysed cell samples from control, FLA and BaP + FLA-exposed regimes were processed for analysis of BaP metabolites by liquid–liquid extraction and reverse phase high-performance liquid chromatography methods as described previously in Myers et al. (2011).

2.7. Statistical treatment of data

Data were analyzed by GraphPad Prism ver 5.0 Software (GraphPad, La Jolla, CA). A two-way analysis of variance (ANOVA) test was used to determine the effect of FLA and BaP + FLA dose on HT-29 cells. The differences among means were determined by using Bonferroni's *post hoc* test. The criterion for statistical significance was set at $p < 0.05$.

3. Results

3.1. BaP does not modulate the effect of FLA on cell growth

Fig. 1 represents profiles from growth assays of HT-29 cells following FLA, and BaP + FLA exposure. No significant change was observed in each cell cycle phase among the various exposure concentrations from BaP exposure alone. No significant changes were observed among the time points and exposure concentrations in cells exposed to FLA alone, except the 25 µM concentration at 96 h. Cells exposed to 25 µM BaP + FLA demonstrated a significant reduction in growth at 96 h.

3.2. BaP enhances the cytotoxicity of FLA

Fig. 2 reveals the cytotoxicity of BaP and FLA to HT 29 cells. An exposure concentration-dependent increase in cytotoxicity was noticed for individual exposures of FLA at 48 and 72 h post exposure compared to 24 h. The 10 and 25 μM concentrations showed a statistically significant ($p < 0.05$) increase in cytotoxicity compared to the 1 and 5 μM concentrations. The combined treatment of BaP and FLA showed a trend similar to that of FLA individually but the cytotoxicity due to binary exposure was more pronounced than either one of them treated individually.

3.3. BaP marginally increases the apoptotic effects of FLA in HT-29 cells

Fig. 3 represents apoptosis profiles from Caspase 3 Analysis of HT-29 cells following FLA and FLA + BaP exposure respectively. No significant change was observed among the various exposure concentrations and time points. Fig. 3C represents the apoptosis profiles following exposure to BaP + FLA. There was no significant change in Caspase 3 activity among the exposure concentrations and time points except the 25 μM concentrations at 72 h, which showed significant apoptosis compared to 1 μM concentration of BaP + FLA.

3.4. BaP alters the effects of FLA on the cell cycle profiles of HT-29 cells in a concentration-and time-dependent manner

Fig. 4 represents cell cycle profiles from flow cytometry of HT-29 cells following FLA exposure. Except 24 h exposure to 1–25 μM FLA concentration at G1 phase, FLA did not exhibit any noteworthy effect on HT-29 cell cycle. A reduction in the G2 phase was shown at all the exposure periods, indicating that FLA blocks the cell cycle progression at G2/M transitions.

Fig. 5 represents cell cycle profiles following exposure to equimolar concentrations of FLA + BaP. The dual exposure demonstrates a cell cycle arrest similar to cells exposed to BaP alone. The percent of HT-29 cells in the S phase showed an increase over 24–96 h. Unlike cells exposed to BaP or FL alone, after 96 h there was a decrease in percent of cells treated with 10 μM and 25 μM concentrations in the G1 phase. From these results we conclude that BaP demonstrates an antagonistic effect on FL.

3.5. BaP exposure enhances the metabolism of FLA

Since most of the toxicity to cells induced by FLA or combination of BaP + FLA at 72 h post exposure, metabolite concentrations at this time point were shown. Fig. 6 shows metabolite concentrations generated by FLA alone and FLA + BaP in HT-29 cells. The 10 and 25 μM exposure concentrations produced greater quantities of metabolites compared to 1 and 5 μM concentrations and the differences were statistically significant ($p < 0.05$). In the presence of BaP, HT-29 cells generated significantly higher ($p < 0.05$) concentrations of FLA metabolites when compared to cells exposed to FLA alone (Figs. 6 and 7).

The FLA metabolites produced in the single (FLA) and binary (FLA + BaP) exposure systems were FLA 2,3-diol, trans-2,3-dihydroxy-1,10b-epoxy-1,2,3,10b tetrahydro FLA (2,3-DFLA), 3-hydroxy FLA, and 8-hydroxy FLA. The relative proportion of individual FLA metabolite types among total metabolites was also represented as percentages and depicted in Fig. 7. The HT-29 cells exposed simultaneously to both FLA & BaP generated higher proportion of FLA 2, 3-diol, and 2,3-DFLA when compared to cells that were exposed to FLA alone.

4. Discussion

Even though published reports indicate using a combination of PAHs to test the toxicity, those studies were limited to human mammary epithelial cells (Courter et al., 2007) and liver cancer cells (Tarantini et al., 2011), but not cells of the GI tract. Considering the substantial intake of PAHs through diet (reviewed in Ramesh et al., 2004) and their potential to cause colon cancer as revealed by laboratory and epidemiological studies (reviewed in Diggs et al., 2011), there exists a knowledge gap in the toxicity of PAH mixtures to cells of the GI tract. Therefore, we have studied the cellular responses (growth, cytotoxicity, apoptosis, and cell cycle changes) subsequent to FLA and FLA + BaP exposures in HT29 human colon cancer cells.

The rationale for BaP and FLA concentrations used in this study was based on environmental, lifestyle, occupational, and more importantly dietary relevance. People living in the vicinity of petrochemical facilities are exposed to 3.2–41 $\mu\text{g/kg}$ BaP and 9.0–78 $\mu\text{g/kg}$ FLA through soil (Abbas and Brack, 2006). People who either smoke or exposed to environmental tobacco smoke are exposed to 10 ng/cigarette of BaP/FLA (Grimmer et al., 1987) in addition to exposure through industrial (1.4–25 ng/m^3 ; Aries et al., 2008) and other occupational settings (cooking: 6.9 ng/m^3 ; Pan et al., 2008). From a dietary exposure standpoint, charbroiled meat, a rich source of PAHs has been reported to contain 26–42 $\mu\text{g/kg}$ BaP and 57–103 $\mu\text{g/kg}$ FLA (Maga, 1986). Accordingly, the mean dietary intake of FLA (13.66 $\mu\text{g/kg}$) was found to be far greater than BaP (1.28 $\mu\text{g/kg}$; Martí-Cid et al., 2008). Analysis of human samples revealed presence of PAHs in the μg range in tissues (WHO, 1998) and ng range in urine (Grainger et al., 2006; Aquilina et al., 2010). In the context of the above-mentioned exposure settings, it can be deduced that the concentrations of BaP and FLA used in the present study could easily reach bioavailable levels in the human body. Additionally the concentrations used in the present study (1–25 μM) also fall within the range (1–100 μM) used by other investigators for human colon carcinoma cells (Iwanari et al., 2002; Patten Hitt et al., 2002; Kim et al., 2005; de Waard et al., 2008).

One common feature that is shared by FLA and FLA + BaP is the exposure time-dependent cytotoxicity. Compared to 24 h, the 48 and 72 h exposure time points registered cytotoxic responses at high exposure concentrations. There is a general agreement in the cytotoxicity trend between FLA and BaP + FLA suggesting a common mechanism underlying the cytotoxic response. Changes in cell membrane integrity (Danion et al., 2011), and mitochondrial respiration (Rubin, 2001) may have a role to play in their cytotoxic ability. Since any cellular response requires metabolic activation of the PAH test chemical, it is reasonable to assume that high exposure concentrations and longer exposure times are necessary for PAH metabolism to elicit cytotoxicity (Schirmer et al., 1998). Our findings on metabolism of FLA support this assumption as the metabolite production increased with an increase in exposure duration and the effect seem more pronounced in the presence of BaP. Some of the metabolites generated may have contributed (discussed in detail elsewhere in this section) to the cytotoxicity of PAHs.

Even though, FLA has been reported to inhibit growth and induce apoptosis in human bone marrow-derived B cells (Hinoshita et al., 1992), T cell hybridomas (Yamaguchi et al., 1996), and HepG2 cells (Huang et al., 2012), in our cell system no marked inhibition was noticed. While FLA did not induce apoptosis on its own, addition of BaP to FLA failed to cause apoptosis of the cells except at 72 h. The lack of growth and apoptosis signifies arrests of these events are perhaps related to selective induction of genes specific for these pathways, favoring cellular quiescence. Our observations are in agreement with that of Raychoudhury and Kubinski (2003) and Ogba et al. (2005) who also reported the lack of apoptosis in FLA-exposed Sertoli and MCF-7 cells respectively.

Uncontrolled cell proliferation is one of the hallmarks of carcinogenesis. Upon FLA exposure, the percentages of cells remaining at the G1 phase were more than those of other treatment groups, which indicate that G1 phase serves as a restriction or checkpoint to govern the cell-cycle transition (Blagosklonny and Pardee, 2002). On the other hand, the percentage of cells remaining at the S phase showed a slight increase at 24, 48 h and maintained at the same level until 72 h and declined thereafter. Since progression beyond the G1 gate will cause the cell to undergo mitosis, after carrying out DNA synthesis and replication (Norbury, 2005), the considerable percent of cells remaining at the S phase increase the fixation of mutations and the likelihood of tumor development (Weiss et al., 2003). The smaller G2 phase is an indication of cell growth reaching a stationary phase at this stage. The cell cycle behavior of FLA, especially the prolonged arrest at S phase observed in our study is similar to that observed for human hepatoma (Wei et al., 2010), and mouse hepatoma Hepa 1c1c7 cells (Ko et al., 2004), MCF-7 breast cancer cells (Jeffy et al., 2000).

One explanation that could be advanced for the FLA + BaP-induced cell cycle block at the G1 and S phases is as follows. Even though the cells were initially gated at G1, the blockage may be leaky, allowing cells to reenter the cell cycle and resume progression, which was blocked again at S phase. The possibility of apoptosis induction by FLA occurring independent of G1 phase arrest is ruled out because of the non-responsiveness of cells to FLA-induced apoptosis. The differential effects of BaP + FLA on colon cell cycle could be attributed to changes in the expression of cell cycle regulatory proteins (Hsieh et al., 2002) which we have not explored in this study. Nonetheless, our data on apoptosis and cell growth together with cell cycle support our contention that FLA in combination with BaP is a cell cycle blocker.

The differential metabolic behavior of FLA and FLA in the presence of BaP could be explained by the relative ability of these toxicants to induce drug metabolizing enzyme (cytochrome P450) activity, with BaP apparently showing more potency (Phillips and Grover, 1994; Sjogren et al., 1996). Induction of CYP enzymes by BaP may have modulated the enzymatic metabolic activation of FLA thereby enhancing its carcinogenic potential. Our findings are in agreement with that of Vaca et al. (1992) and Huderson et al. (2010) who have shown that co-treatment with BaP induces the microsomal metabolism of FLA to precursors of reactive metabolites. Bio-transformation of FLA by tumor cells may lead to production of intracellular metabolites (Buesen et al., 2002; Myers et al., 2011) whose production rate may have enhanced due to the action of BaP. Co-exposure to both BaP and FLA has been shown to enhance the metabolism of FLA in adipose (Huderson et al., 2010) and colon tumor (Diggs et al., 2012) tissues of a colon cancer mouse model. This situation may aid in the progression of disease as well. Evidence is available in support of the involvement of metabolism in toxicity-associated inflammatory diseases such as cancer. Fluoranthene and BaP through metabolic activation induce reactive oxygen species (ROS) production, which also induces inflammatory responses in colon (Uno and Makishima, 2009; Khalil et al., 2010).

The FLA metabolite types identified in our study are in agreement with published literature, which used microsomes from intestinal, hepatic and extra-hepatic tissues (Walker et al., 2006; Huderson et al., 2010; Diggs et al., 2012). While colon cells could metabolize FLA, the generation of FLA 2,3-diol in greater proportions when cells were treated with FLA together with BaP is noteworthy due to the ability of this metabolite to bind to cellular macromolecules such as DNA and proteins (Babson et al., 1986; Hutchins et al., 1988).

Our findings were in line with the synergistic responses observed when various PAH compounds were co-exposed with BaP. Coexposure to BaP increased tumor latency period

when PAH mixtures of anthracene, chrysene, pyrene, fluoranthene and phenanthrene were used in mouse skin bioassays (Warshawsky et al., 1993). Binary mixtures of dibenzo(a,e)pyrene, dibenzo[a,l]pyrene, dibenzo[a,h]-anthracene, benzo[b]fluoranthene, fluoranthene or 1-methylphenanthrene with BaP showed synergistic interaction in DNA binding potencies compared to individual compounds (Hughes and Phillips, 1990). Similarly, binary mixtures of benzo(b)fluoranthene, dibenz(a,h)anthracene or indeno(1,2,3-ed)pyrene were found to modulate the genotoxicity of BaP as revealed by the enhanced formation of BaP-DNA adducts in HepG2 cells (Tarantini et al., 2011). These findings lend credence to our supposition that the presence of another PAH compound in a mixture will have a bearing on the metabolism of the chemical in question. Additionally, our earlier studies have shown an enhanced metabolism of FLA when microsomes from adipose tissue (Hudson et al., 2010) and colon tumors (Diggs et al., 2012) were exposed to BaP and FLA together.

The greater levels of FLA in the environment, and its increased dietary intake relative to BaP notwithstanding, the presence of a significant difference in metabolite concentrations between FLA and FLA + BaP treatment groups indicate that FLA is a weak mutagen and the observed effects (responses) endorse that viewpoint. Fluoranthene on its own may not be able to exert cytotoxicity to a significant extent, but presence of potent PAH compounds like BaP appears to enhance the potency of FLA as it relates to tumor promotion. Therefore, from the standpoint of carcinogenesis, BaP co-exposure may not be beneficial in a real world situation when both these toxicants are ingested or inhaled in significant amounts. If the results obtained *in vitro* could be extrapolated to *in vivo* situation, BaP co-treatment appears to enhance the generation of reactive metabolites of FLA which in a dual exposure scenario may influence tumor progression albeit at a slower pace.

Acknowledgments

This research was supported by grants 5R01CA142845-04 (AR) from the National Cancer Institute (NCI), and 5R25GM059994-11 from the National Institute of General Medical Sciences (NIGMS), and G12RR003032 (for funding FACS/BSL3 Core Facility at Mehary) from the National Center for Advancing Translational Sciences (NCATS), all of which are components of the National Institutes of Health (NIH). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIH. Our thanks are also due to the Southern Regional Education Board, Atlanta, Georgia for the award of Doctoral Research Fellowships to Ms. Kelly Harris and Dr. Jeremy Myers. Technical assistance from and Ms. Qiuja Shao is also acknowledged.

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Fig. 1.

Growth of HT-29 colon cells exposed to or fluoranthene (FLA) or a combination of benzo(a)pyrene (BaP) and fluoranthene. HT-29 cells were treated with the above-mentioned toxicants at 1, 5, 10 and 25 μM for 24, 48, 72 and 96 h. Post exposure to toxicants, the cells were counted using a Beckman Coulter Counter. Data from triplicate determinations were used. Asterisks denote statistical significance ($*p < 0.05$) compared to control.

Fig. 2.

Cytotoxicity of HT-29 colon cells exposed to FLA, and BaP + FLA. HT-29 cells were treated with the above-mentioned toxicants at 1, 5, 10 and 25 μ M for 24, 48, 72 and 96 h. Post exposure to toxicants, the cytotoxicity was assayed by using the lactate dehydrogenase assay kit. Data from triplicate determinations were used. Asterisks denote statistical significance (* $p < 0.05$) compared to control.

Fig. 3.

Apoptosis of HT-29 colon cells exposed to 1, 5, 10 and 25 μ M FLA, and BaP + FLA for 24, 48, 72 and 96 h. Apoptosis of cells were determined using the Caspase-3 assay kit. Data from triplicate determinations were used.

Fig. 4.

Cell cycle changes in HT-29 colon cells exposed to 1, 5, 10 and 25 μ M FLA for 24, 48, 72 and 96 h. Cell cycle changes were analyzed using the Fluorescence-Activated Cell Sorting (FACS). Data from triplicate determinations were used.

Fig. 5.

Cell cycle changes in HT-29 colon cells exposed to 1, 5, 10 and 25 μ M FLA + BaP for 24, 48, 72 and 96 h. Cell cycle changes were analyzed using the Fluorescence-Activated Cell Sorting (FACS). Data from triplicate determinations were used.

Fig. 6.

Metabolism of FLA and FLA + BaP in HT-29 colon cells exposed either individually or to a mixture of these toxicants. Post exposure to FLA or FLA + BaP, the cells were harvested, subjected to liquid-liquid extraction and samples were analyzed by reverse phase HPLC with fluorescence detection for metabolite analysis. Data from triplicate determination of metabolite concentrations from individual exposure concentration of parent compound or mixture were compared. Asterisks denote statistical significance ($*p < 0.05$) among various treatment groups indicated by bars.

Fig. 7.

Metabolite composition of FLA. And FLA + BaP in HT-29 colon cells exposed either individually or to a mixture of these toxicants. Post exposures to FLA or FLA + BaP, the cells were harvested, subjected to liquid-liquid extraction and samples were analyzed by reverse phase HPLC with fluorescence detection for metabolite analysis. Values are expressed as the percentage of individual metabolite types among the total metabolites (sum of individual concentrations of FLA 2,3-diol; trans-2,3-dihydroxy-1,10b-epoxy-1,2,3,10 tetrahydro FLA [2,3-DFLA]; 3-hydroxy FLA; 8-hydroxy FLA) formed.