[Title page] Original Article

Determination of polycyclic aromatic hydrocarbon content in heat-treated meat retailed in Egypt: Health risk assessment, benzo[a]pyrene induced mutagenicity and oxidative stress in human colon (CaCo-2) cells and protection using rosmarinic and ascorbic acids

Wageh Sobhy Darwish[1,2], Hitoshi Chiba[3], Waleed Rizk El-Ghareeb[2,4]

Abdelazim Elsayed Elhelaly[5,6], Shu-Ping Hui[1]

[1]Laboratory of Advanced Lipid Analysis, Faculty of Health Sciences, Hokkaido University, Kita 12, Nishi 5, Sapporo 060-0812, Japan

[2]Food Control Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt

[3]Department of Nutrition, Sapporo University of Health Sciences, Nakanuma Nishi 4-2-1-15, Higashi Ku, Sapporo 007-0894, Japan

[4]Department of Veterinary Public Health and Animal Husbandry, College of Veterinary Medicine, King Faisal University, Saudi Arabia

[5]Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Suez Canal University, Ismailia 41522, Egypt

[6]Center for Emerging Infectious Diseases, School of Medicine, Gifu University, Gifu 501-1193, Japan

Corresponding author: Shu-Ping Hui (Ph.D)

Laboratory of Advanced Lipid Analysis, Faculty of Health Sciences, Hokkaido University, Kita 12, Nishi 5, Sapporo 060-0812, Japan.

Email: keino@hs.hokudai.ac.jp

Running Head:

PAHs content in heat-processed meat with *in-vitro* adverse effects

Abstract

This study was undertaken to estimate the concentrations of the formed polycyclic aromatic hydrocarbons (PAHs) in heat-treated (boiled, pan-fried and grilled) meats collected from Egypt. Dietary intakes and cancer risks of PAHs among Egyptian adults were calculated. Benzo[a]pyrene (B[a]P)-induced mutagenicity and oxidative stress in human colon (CaCo-2) cell line and mechanisms behind such effects were also investigated. Finally, protection trials using rosmarinic (RMA) and ascorbic acids (ASA) were carried out. The results indicated formation of PAHs at high levels in the heat-treated meats. Calculated incremental life time cancer risk among Egyptian adults were 7.05179E-07, 7.00604E-06 and 1.86069E-05 due to ingestion of boiled, pan-fried and grilled meats, respectively. B[a]P-exposed CaCo-2 cells had high abilities for mutagenicity (490.05 ± 21.37 His+ revertants) and production of reactive oxygen species. RMA and ASA protected CaCo-2 cells via reduction of B[a]P-induced mutagenicity and oxidative stress and upregulation of phase II detoxification enzymes and xenobiotic transporters.

Keywords: Polycyclic aromatic hydrocarbons; benzo[a]pyrene; heat-treated meat; Egypt; Rosmarinic acid; Ascorbic acid

Chemical compounds studied in this article:

Benzo[a]pyrene (PubChem CID: 2336); Benz[a]anthracene (PubChem CID: 5954); Benzo[b]fluoranthene (PubChem CID: 9153); Benzo[k]fluoranthene (PubChem CID: 9158); Benzo[ghi]perylene (PubChem CID: 9117); Chrysene (PubChem CID: 9171); dibenz[a,h]anthracene (PubChem CID: 5889); Anthracene (PubChem CID: 8418); Ascorbic acid (PubChem CID: 54670067); Rosmarinic acid (PubChem CID: 5281792)

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are group of more than 100 fused ring aromatic compounds that are formed due to incomplete combustion of organic substances, pyrolysis of organic materials that are commonly used as energy sources, industrial incinerations, and released in tobacco smoke and car exhausts **(Kazerouni, Sinha, Hsu, Greenberg, & Rothman, 2001)**.

Heat-treatment of meat like grilling, barbequing, pan-frying and smoking may result in release of elevated concentrations of PAHs and contribute substantially to the human exposure to PAHs. Cooking method, temperature, time, fat content and oil influence the formation of PAHs. Grilling and charcoal-cooking release high concentrations of PAHs due to pyrolysis of organic matter at the high temperature **(Diggs, Harris, Rekhadevi, & Ramesh, 2012)**.

Humans are exposed to PAHs through different routes like ingestion, inhalation, dermal and occupational exposure. Food ingestion is considered the major route of exposure of humans to PAHs in non-smokers. Studies had shown that human exposure to benzo[a]pyrene (B[a]P), a major carcinogenic PAH, is significantly higher due to ingestion of contaminated foods, the concentration range in case of ingestion was (2-500 ng/day), while in case of inhalation, this range was (10-50 ng/day) **(Phillips, 1999)**.

Sixteen major PAHs were defined by the European Food Safety Authority **(EFSA, 2008)** based on their public health significance. For better understanding of the health risks associated with human exposure to PAHs via consumption of contaminated foods, EFSA had grouped PAHs into three groups based on their formation levels in foods, potential toxicity, mutagenic and carcinogenic activities. B[a]P and chrysene made a group called 2-PAHs group, while a group of B[a]P, chrysene, benz[a]anthracene, and benzo[b]fluoranthene referred as 4-PAHs group. The third group, 8-PAHs group, included 8 priority PAHs, namely, B[a]P, chrysene,

benz[a]anthracene, benzo[b]fluoranthene, anthracene, benzo[k]fluoranthene, benzo[ghi]perylene, and dibenz[a,h]anthracene **(EFSA, 2008)**. The International Agency of Research on Cancer (IARC) had classified PAHs according to their carcinogenicity to humans as carcinogen (Group 1) as B[a]P; probable carcinogen (Group 2A) as dibenz[a,h]anthracene; possible carcinogen (Group 2B) as benz[a]anthracene, benzo[b]fluoranthene and chrysene. This classification was based on sufficient evidences of carcinogenicity in experimental animals **(IARC, 2010)**.

There are epidemiologic evidences that high intake of meat, especially red heat-treated meat, increases the risk of colon and rectal cancers. It is estimated that diet contributes to more than 80 % of the known colorectal cancer cases **(Sinha et al., 2005)**. For instances, positive correlations were detected between the incidence of colorectal cancers and the lower fiber and higher meat intakes in a case-control study conducted at Sultan Qaboos University Hospital, Oman **(Mafiana, Al Lawati, Waly, Al Farsi, Al Kindi, &Al Moundhri, 2018)**. Additionally, **Buamden (2018)** reported that the highest incidences of colorectal cancer cases in the countries of Americas were in Uruguay, Barbados, Argentina and Cuba and were strongly associated with the availability of animal fat and red meat.

Grilling and barbequing of meat are very popular cooking practices in many Middle-Eastern countries including Egypt. Few reports investigated the formation of PAHs in heat-treated foods in Middle-Eastern countries. For instances, **Alomirah et al. (2011)** investigated the levels and the profiles of 16 PAHs in various grilled foods in Kuwait and estimated the dietary exposure of PAHs among Kuwaiti populations. However, the available information about the content of PAHs in the different heat-treated meats served in Egypt is scarce. In addition, the assessment of cancer risk among the Egyptian population due to consumption of such meat is less informed.

Ingestion of B[a]P-contaminated foods activates aryl hydrocarbon receptor (AhR) gene battery. AhR regulates a group of xenobiotic metabolizing enzymes (XMEs) that include phase I enzymes such as cytochrome P450 (CYP)1A subfamily, phase II detoxification enzymes such as UDP-glucuronosyl-transferase (UGT) 1A6, glutathione-S-transferase (GST) A1 and NAD(P)H: quinone oxidoreductase-1 (NQO1) **(Darwish, Ikenaka, Eldaly, & Ishizuka, 2010a)**. Phase III xenobiotic transporters such as multidrug resistance protein 1 (MDR1) and multidrug resistance associated protein 2 (MRP2) are also regulated via AhR and contribute to the excretion of B[a]P and its metabolites **(Kranz et al., 2014)**. In our previous report, it was declared that B[a]P exerts its mutagenic activities via modulation of XMEs in human liver (HepG2) cells **(Darwish, Ikenaka, Nakayama, Mizukawa, Thompson, & Ishizuka, 2018)**; however, B[a]P is positively associated with cancers in colon and rectum **(IARC, 2010)**. Therefore, in the current study, the effects of the food-relevant concentrations of B[a]P on human colon (CaCo-2) cell lines were examined.

Micronutrients such as rosmarinic (RMA) and ascorbic (ASA) acids are known for their antioxidant and anti-inflammatory properties **(Charalabopoulos et al., 2004; Venkatachalam, Gunasekaran, & Namasivayam, 2016)**; however, their antimutagenic activities and their modulatory effects on XMEs are less informed.

In sight of these factors, the objectives of the present study were firstly, to estimate the concentrations of the formed PAHs in the raw and heat-treated (boiled, pan-fried and grilled) meats collected from Egypt. Secondly, estimation of the daily intake and cancer risk of Egyptian adult population due to consumption of such meat was done. Thirdly, B[a]P-induced mutagenicity and oxidative stress in human colon (CaCo-2) cell lines and mechanisms behind that were also

investigated. Finally, protection trials against B[a]P-induced adverse effects using RMA and ASA were carried out.

2. Materials and Methods

2.1. Food samples collection

Random samples (n=100) were collected from different localities (restaurants or butcher shops) in Zagazig city, Egypt in the period of May-August 2017. Twenty-five samples from each of raw, boiled, pan-fried and charcoal grilled beef were purchased and stored at -20ºC until time of extraction and measurement of PAHs.

2.2. Chemicals and reagents

All used chemicals and reagents including methanol, ethanol, n-hexane, acetone, unhydrous sodium sulfate and potassium hydroxide were of analytical grade and purchased from Wako Pure Chemical Industries (Tokyo, Japan). Standards of 15 PAHs (acenaphthene, anthracene, benz[a]anthracene, benzo[a]pyrene, benzo[e]pyrene, benzo[b]fluoranthene, benzo[ghi]perylene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, naphthalene, phenanthrene and pyrene) were purchased from Accu Standard, Inc. (New Haven, USA).

2.3. PAH extraction, analysis and quality assurance

PAHs were analyzed by the method described before **(Ikenaka, Ito, Heesoo, Watanabe, & Miyabara, 2008)** with slight modifications. Briefly, about 10 g of each meat sample was extracted with approximately 25 mL of 1 M KOH ethanol solution, and saponified for 10 h at 60ºC. The saponified solutions were then shake-extracted three times with n-hexane and the resulting hexane fractions containing PAHs were run through granular sodium sulfate, evaporated using a rotary evaporator and purified using silica gel column chromatography (2 g of 5% water containing silica gel). The obtained fractions were eluted by 100 mL of acetone/hexane = 1/99 (v/v), dried under a gentle nitrogen stream, and re-dissolved into 0.5 mL of methanol for HPLC analysis. HPLC analysis were performed using a Shimadzu LC20 series (Kyoto, Japan) equipped

with a fluorescence detector (RF-10AxL) and a ZORBAX Eclipse PAH (2.1 x 150 mm, 3.5lm, Agilent) as a separation column. Identification of PAHs was based on retention time, and quantification was performed by the use of external calibrations which were obtained with PAH solutions at seven concentration levels (0.01, 0.1, 1.0, 10.0, 100.0, 200.0 and 400.0 ng mL^{-1}) for each PAH. To evaluate the efficiency of the analytical procedures for the target compounds, spiking blank sample and heat-treated meat samples with the calibration standards (1.0, 10.0 and 100.0 ng/g) and all extraction and clean-up steps as for the samples were repeated. Recovery rates for each PAH congener tested were 85% (acenaphthene), 86% (anthracene), 88% (benz[a]anthracene), 91% (B[a]P), 85% (benzo[e]pyrene), 82% (benzo[b]fluoranthene), 88% (benzo[ghi]perylene), 85% (benzo[k]fluoranthene), 95% (chrysene), 88% (dibenz[a,h]anthracene), 103% (fluoranthene), 87% (fluorene), 84% (naphthalene), 91% (phenanthrene), and 84% (pyrene), respectively. The limits for detection (ng/g) of these PAHs were 0.02, 0.03, 0.04, 0.04, 0.02, 0.05, 0.04, 0.05, 0.02, 0.02, 0.04, 0.03, 0.05, 0.02, and 0.05, respectively. The relative standard deviations for replicate analyses (n = 3) were below 8%.

2.4. Health risk assessment of tested PAHs

2.4.1. Dietary Exposure Estimates

The carcinogenic risk of a PAH mixture is often expressed by its B[a]P equivalent concentration ($B[a]P_{eq}$), and the toxicity equivalency factors (TEFs) developed before **(Nisbet, & Lagoy, 1992)**. TEFs for the eight priority PAHs tested in this study were as following: anthracene (0.01), benz[a]anthracene (0.1), B[a]P (1.0), benzo[b]fluoranthene (0.1), benzo[ghi]perylene (0.01), benzo[k]fluoranthene (0.1), chrysene (0.01) and dibenz[a,h]anthracene (1.0).

The B[a]P Equivalent Concentration (BEC) of food was calculated from the following equation:

$$BEC = \sum C_i \, x \, TEF_i \quad (1)$$

where C_i is the concentration of each individual PAH congener ($_i$) in meat and TEF_i is the toxicity equivalency factor of each individual PAH congener ($_i$).

The Estimated Daily intake (EDI) of dietary PAHs (ng) was calculated as follows:

$$EDI = \sum BEC_i \; x \; IR \quad (2)$$

Where BEC_i is B[a]P equivalent concentration of each individual PAH congener ($_i$) in meat and IR is the ingestion rate of meat (g). To the best of our knowledge there is no clear surveillance data about consumption rate of different heat-treated meat in Egypt. Therefore, a theoretical ingestion rate for all kinds of heat-treated meat in Egypt was set at 76.71 g/day for adult population and used for calculation of the EDI of dietary PAHs. The selected ingestion rate is relatively within the same record of the Egyptian food balance sheet issued by economic affairs sector, ministry of Agriculture (EFBS, 2008) for the red meat consumption which was set as 28 Kg/capita/year.

2.4.2. Cancer risk estimates

The incremental lifetime cancer risk (ILCR) of Egyptian population caused by PAHs (in meat) was calculated according to **USEPA (2000)** from the following equation:

$$ILCR = EDI \; x \; EF \; x \; ED \; x \; SF \; x \; CF/(BW \; x \; AT) \quad (3)$$

ILCR is the incremental lifetime cancer risk. EF is the exposure frequency (365 days/year), ED is the exposure duration in life for adult set as 43. SF is the oral cancer slope factor of B[a]P which was set as 7.3. CF is the conversion factor, which was set as 10^{-6}. BW is the average adult body weight and was set as 70 Kg. AT is the average lifespan of carcinogens and set as 25550 days. All fixed values were according to the guidelines of **USEPA (2000)**.

2.5. Cell culture conditions and treatment

Human colon (CaCo-2) cells were grown in DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin mixture. Cells were maintained in a humidified atmosphere with 5% CO_2

187 at 37 °C. Cells were subcultured when reach 80-90% confluency (every 3-5 days). Once cell
188 confluency was reached, treatments were added to the medium. Cells were exposed to B[a]P under
189 three concentrations (1.0, 5.0 and 50.0 nM) for 24 h. The used concentrations of B[a]P were
190 relevant to that recorded in the foodstuffs tested in the present study or to the doses reported in
191 other *in-vitro* studies **(Chen, Li, Xu, & Zhou, 2014)**. Additionally, in protection studies, CaCo-2
192 cells were co-exposed to B[a]P (1.0 (low) and 50 (high) nM) and RMA (10 (low) and 100 (high)
193 μM) or ASA (10 (low) and 100 (high) μM) for 3, 6, 9 or 24 h. The concentrations of the tested
194 phytochemicals are used based on other reports of *in-vitro* studies **(Chen et al., 2014; Darwish,**
195 **Ikenaka, Nakayama, Mizukawa, & Ishizuka, 2016)**.

196 *2.6. Cell viability assay*

197 Cell viability was determined using CCK-8 assay (Dojindo Molecular Technologies,
198 Rockville, USA) in a clear a 96-well plate according to the manufacturer's instructions (n= 6 per
199 each treatment).

200 *2.7. Ames mutagenicity assay*

201 Ames assay was performed using *Salmonella typhimurium TA98* according to **Ames, &**
202 **Gold (1990)**, with slight modifications. The reaction mixture was done in a tube containing the
203 bacterium and co-factor S9 mixture. Human CaCo-2 cells exposed to B[a]P alone or combined
204 with either ASA or RMA were used to prepare S9 fractions according to the method published
205 before **(Darwish, Ikenaka, Ohno, Eldaly, & Ishizuka, 2010b)**. The reaction was continuous in
206 a water bath with shaker for 20 min at 37 °C, and then terminated by adding of top agar mixed
207 with 10% histidine-biotin mixture. Numbers of the grown revertant colonies were counted after
208 incubation for 48 h at 37 °C. Each experiment was run in duplicate and repeated at least five times
209 at different days.

2.8. Reactive Oxygen Species (ROS) measurement

The fluorogenic 2',7'-dichlorofluorescein diacetate (DCF-DA) (Sigma) was used as probe for measurement of ROS production in CaCo-2 cells **(Chen et al., 2014)**. The fluorescence intensity was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using a 96-well plate reader (Baxter, Deerfield, IL).

2.9. RNA isolation and quantitative RT-PCR

Total RNA was extracted according to the previously modified method **(Darwish et al., 2010b)**. In short, TRI reagent (Sigma-Aldrich) was used for lysis of CaCo-2 cells. Chloroform was then added for phase separation. The upper aqueous phase was mixed with equal volume of isopropanol. RNA pellets were precipitated by centrifugation at 10000*g* for 20 min at 4 °C. The pellets were then washed with 70% ethanol and dissolved using RNase-free H_2O. RNA concentrations and qualities were determined using a Nanodrop ND-1000 spectrophotometer (DYMO, Stamford, Conn., USA). For cDNA synthesis, ReverTraAce® qPCR RT Master Mix with gDNA remover (Toyobo Co. Ltd., Osaka, Japan) was used as described in the manufacturer's instructions. cDNA samples were stored at -20 °C for further analysis.

The mRNA expression levels of the phase I, II and III enzymes were determined using real-time reverse transcriptase-PCR (qRT-PCR), in Step One Plus Real-Time PCR system (Applied Biosystems, Foster, CA). The PCR mixture contained 2 μL of cDNA (600 ng), 5 μL Fast SYBR® Master mix, 5 μM of each primer, with RNase-free water added to a final volume of 10 μL. The reaction cycle comprised a holding stage for 20 s at 95 °C, followed by 40 denaturation cycles of 3 s at 95 °C and 30 s at 60 °C and 15 s extension at 95 °C. Single amplicon amplification was confirmed using melting curve analysis. The absence of primer dimers and genomic DNA amplification were confirmed by agarose gel electrophoresis. β-actin was used for normalization

by the comparative $^{\Delta\Delta}$Ct method. Each experiment was repeated at least three times, and n = 6 plates per each treatment. Primer sets for the selected targets were designed using Primer3Plus software (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and were displayed in **Table S1**. The efficiencies of the primers used in the present study ranged between 94-104%.

2.10. Statistical analysis

Statistical significances were evaluated for the content of the formed PAHs, B[a]P-induced mutagenicity and oxidative stress, and effects on mRNA expressions of detoxification enzymes using Tukey-Kramer honestly HSD (JMP program, SAS Institute, Cary, NC, USA) with $P < 0.05$ considered as significant.

3. Results and Discussion

3.1. Formation of PAHs in heat-treated meat

Cooking of meat primarily aims at removal of microbial food contaminants and safety of the final products served to consumers. In addition, cooking makes food more digestible and enhances the aroma and flavor as it undergoes chemical changes leading to formation of palatable cooked products. However, at the same time, heat treatment of meat, especially, at elevated temperatures is associated with the formation of several harmful compounds such as PAHs. Cooking of meat is considered as an art in Egypt and many Middle-Eastern countries. There is scarce information available about levels of PAHs in heat-treated meat and their related risk assessment in Egypt. The present study was carried out firstly to estimate the levels of PAHs in retailed heat-treated meat by the most common cooking methods in Egypt (boiling, pan-frying and grilling). In the current study, the estimated PAHs were grouped as follows: B[a]P group; total 2-PAHs group (B[a]P and chrysene), total 4-PAHs group (B[a]P, chrysene, benz[a]anthracene, and benzo[b]fluoranthene); total 8-PAHs group (B[a]P, chrysene, benz[a]anthracene, benzo[b]fluoranthene, anthracene, benzo[ghi]perylene, benzo[k]fluoranthene, and dibenz[a,h]anthracene); and total 15-PAHs group for the all tested PAHs. The achieved results revealed that, the total contents of the total 8-PAHs, 4-PAHs, 2-PAHs were on the following significant ($P < 0.05$) descending order: grilled > pan-fried > boiled > raw meat. The average residual concentrations (ng/g ww) of the total 2-PAHs were 2.81 ± 0.64, 1.08 ± 0.41 and 0.03 ± 0.01 in grilled, pan-fried and boiled meat, respectively. While the total 4-PAHs (ng/g ww) were 4.84 ± 0.94, 2.30 ± 0.71, 0.20 ± 0.10 and 0.06 ± 0.01 in grilled, pan-fried, boiled meat and raw meat, respectively; the total 8-PAHs (ng/g ww) were 9.27 ± 1.43, 4.49 ± 0.53, 0.85 ± 0.17 and 0.25 ± 0.07 in grilled, pan-fried, boiled meat and raw meat, respectively. B[a]P, a major pro-

mutagen and pro-carcinogen, is formed in the heat-treated meat with average residual concentrations (ng/g ww) of 2.66 ± 0.44, 0.98 ± 0.31 and 0.02 ± 0.01 in grilled, pan-fried and boiled meat, respectively **(Table 1)**. The frequency distribution of the tested PAHs showed that only two out of eight priority PAHs were detected in raw meat. However, all of the priority PAHs were detected in heat-treated meat **(Fig. S1)**. The residual concentrations of the detected B[a]P, total 2-PAHs and total 4-PAHs were in agreement with the maximum permissible limits (5 ng/g ww) of PAHs in grilled meat and meat products set by **EC (2006) and EFSA (2008)**. However, the total 8-PAH content, particularly for grilled meat exceeded that recommendation. The concentrations of B[a]P and 11 other PAHs in 322 commercial, meat products and 14 home-grilled meat samples from Estonia were comparable to that recorded in the present study and the highest PAH concentrations were detected in home-grilled pork samples **(Reinik, Tamme, Roasto, Juhkam, Tenno, & Kiis, 2007)**. Similarly, B[a]P concentrations were elevated in charcoal-barbecued chicken meat compared with that of roasted and raw chicken, with a concentration range of 0.09-6.94 ng/g ww in a charcoal cooked meat in the republic of Korea **(Chung, Yettella, Kim, Kwon, Kim, & Min, 2011)**. However, the recorded concentrations of B[a]P in the present study were much lower than that recorded in Turkey, where B[a]P concentrations (ng/g ww) were 43.80 ± 1.80, 31.33 ± 0.94, 62.60 ± 3.72 and 37.60 ± 3.84 in grilled and over-grilled lamb and beef meats **(Aygün, & Kabadayi, 2005)**. Additionally, **Olatunji, Fatoki, Opeolu, & Ximba, (2014)** recorded higher concentrations (0.07-46.67 ng/g) for benzo[k]fluoranthene, B[a]P, indeno[123-cd]pyrene and benzo[ghi]perylene in heat-processed meat in South Africa. The differences in the levels of the formed PAHs in the heat-treated meat are possibly due to several factors such as the cooking time, cooking temperature, the distance between meat and the fire, the type of the meat

287 cut and the fat content **(Kikugawa, 2004)**. Therefore, controlling of such factors might reduce the
288 formation of PAHs in the heat-treated meat and meat products.

289 *3.2. Human health risk assessment*

290

291 Parent compounds of PAHs are rapidly metabolized in the body; therefore, it is difficult to
292 extrapolate toxicity data from animal models to humans. Toxic equivalency factor is widely used
293 in the estimation of the potential risk of a PAH mixture **(Nisbet, & Lagoy, 1992)**. B[a]P is
294 commonly used as an indicator for PAHs contamination via calculation of B[a]P equivalent
295 concentration and subsequently ILCR **(Essumang, Dodoo, & Adjei, 2013)**. Consumption of well-
296 done meat by Egyptian consumers is in an increasing direction and subsequently, the risk for
297 exposure to carcinogenic PAHs is high. Therefore, the dietary intakes and the potential risk for
298 exposure to carcinogenic PAHs among the Egyptian population were calculated. B[a]P equivalent
299 concentrations ranged between 0.14-3.79 ng/g in the heat-treated meat. Additionally, EDI values
300 (ng/day) of PAHs based on B[a]P equivalent concentrations ranged between 11.01 in boiled meat
301 to 290.45 in grilled meat. However, EDI values drastically increased based on the average
302 concentrations of the total 8-PAHs as such values ranged between 65.20 to 711.18 ng/day **(Table**
303 **2)**. Such concentrations correspond well with that recorded in European Union countries as **EFSA**
304 **(2008)** reported that the median EDI values for B[a]P and total 8-PAHs were 235 and 1729 ng/day,
305 respectively. High intake of PAHs is linked to gastrointestinal tract cancer **(Essumang et al.,**
306 **2013)**; therefore, estimation of cancer risk was additionally calculated. The cancer risk estimates
307 among Egyptian adult population are considered for consumption of boiled meat (7.05179E-07)
308 and pan-fried meat (7.00604E-06), but it is alarming for consumers of grilled meat (1.86069E-05)
309 **(Table 2)**. Similarly, higher cancer risk (<1E-06) was reported in Ghana due to ingestion of several

kinds of demersal fishes **(Bandowe, Bigalke, Boamah, Nyarko, Saalia, & Wilcke, 2014)**. Additionally, **Duan et al. (2016)** reported that the median value of estimated ILCR attributable to PAH dietary intake was 6.65E-5 in the adult residents of Liaoning Province's Anshan City, China. However, lower ILCR values computed for male adults living in Catalonia (Spain) (**Martorell, Perelló, Martí-Cid, Castell, Llobet, & Domingo, 2010)** and Taiyuan (China) **(Xia et al., 2010)** and found to be 4.5E -06 and 4.04E-06, respectively. This variation in ILCR values can be explained, in part, by the fact that the exposure duration, the PAH contamination levels, and daily food consumption amounts were different in these studies. Therefore, it is highly recommended for Egyptian to reduce their daily intakes of heat-treated meat and it is advisable to control the cooking time and temperature **(Jiang et al., 2018; Li, Dong, Li, Han, Zhu, & Zhang, 2016)**.

3.3. Biological responses of human colon cells to food-relevant concentrations of BaP and protection trials using RMA and ASA

Food-relevant concentrations of the most toxic PAH, B[a]P, were added to CaCo-2 colon cell lines. The declared results in **Fig. 1** indicated that B[a]P did not affect the cell viability under the used dose range. However, S9 fractions prepared from the exposed cells showed clear dose-dependent mutagenic activities, in terms of production of histidine+ revertants in *Salmonella typhimurium* mutagenicity assay. Such B[a]P-induced mutagenesis in colon cells goes in agreement with our previous report in the human liver cells **(Darwish et al., 2018)**. In addition, **Diggs et al., (2012)** showed that colon tumors of ApcMin mice model can metabolize B[a]P to higher levels and increased the growth of the tumor tissue. In the current investigation, the tested concentrations of B[a]P produced ROS in a dose-dependent fashion in parallel to the induction of mutagenesis, suggesting that induction of oxidative stress is a possible mechanism for B[a]P-induced mutagenicity **(Fig. 1)**. Likely, production of ROS was linked to B[a]P-induced

carcinogenicity and cancer metastasis in the breast tissue of accumulative mouse model **(Guo, Xu, Ji, Song, Dai, & Zhan, 2015)**.

Several reports reported the protective effects of phytochemicals like curcumin, resveratrol, quercetin, β-carotene and retinol against B[a]P-induced genotoxicity and carcinogenicity in lung and liver cells **(Darwish et al., 2018; Malhotra, Nair, & Dhawan, 2012; Liu, Wu, & Zhang, 2015)**. However, there is little information available about the protective roles of RMA and ASA against B[a]P-adverse effects, particularly in the colon cell lines. In the present investigation, co-exposure of CaCo-2 cells to B[a]P and RMA or ASA at two different concentrations and for different times showed strong and significant antimutagenic effects for the tested phytochemicals **(Fig. 2A)**. In parallel, the used micronutrients showed clear antioxidant activities, in terms of reduction of the produced ROS in colon cells **(Fig. 2B)**, suggesting that the antioxidant abilities of both RMA and ASA might contribute to their antimutagenic activities. Similarly, ASA was considered as a strong inhibitor for B[a]P-induced carcinogenesis in Wistar rats **(Charalabopoulos et al., 2004)**. In addition, prenatal exposure to B[a]P reduced the birth weight among Norwegian children; while increasing the maternal dietary intake of vitamin C improved the fetal birth weight **(Duarte-Salles, Mendez, Meltzer, Alexander, & Haugen, 2013)**. Furthermore, RMA had effective protective roles against colon cancers in rats exposed to the colon-carcinogen, 1,2-dimethylhydrazine **(Venkatachalam et al., 2016)**.

Liver is the target organ for xenobiotics metabolism and detoxification in humans and animals. However, ingested contaminants such as PAHs are firstly absorbed through the intestinal tract. Therefore, gastrointestinal tract is considered as the first line of defense against xenobiotics in living organisms **(Darwish, Nakayama, Itotani, Ohno, Ikenaka, & Ishizuka, 2015)**. Nevertheless, few reports had investigated the biological responses of the intestinal cell lines to

food-borne mutagens. B[a]P is activated through epoxide hydrolase and aryl hydrocarbon receptor-induced phase I enzymes. This was confirmed, when mRNA expressions of intestinal phase I enzymes including CYP1A1 and CYP1B1 and epoxide hydrolase were elevated in a dose dependent manner **(Fig. 3)**. Interestingly, co-exposure of CaCo-2 cells to B[a]P and ASA or RMA significantly downregulated mRNA expressions of this group of enzymes **(Fig. 3A, B & C)**. In consistence with this result, ASA decreased the 2,3,7,8-tetrachloridibenxo-p-dioxin (TCDD)-induced CYP1A1 expression in human HepG2 cells **(Chang et al., 2009)**. Furthermore, downregulation of B[a]P-induced phase I CYP1A1 and CYP1A2 by phytochemicals such as β-carotene, β-apo-8-carotenal, retinol, and retinoic acid was reported in HepG2 cells **(Darwish et al., 2018)**. Phase II enzymes such as UGT1A6 and GSTA1 play major roles in B[a]P detoxification process through conjugation reactions for the formed reactive metabolites. The achieved results indicated that B[a]P significantly reduced mRNA expressions of UGT1A6 and GSTA1 in a concentration-dependent manner. Interestingly, RMA and ASA significantly upregulated mRNA expressions of UGT1A6 and GSTA1 to produce a state of balance between bio-activation and detoxification of B[a]P in colon cells **(Fig. 4A & B)**. NQO1 prevents the redox cycling of B[a]P quinone products, thus reducing ROS generation **(Yang, Yang, Ramesh, Goodwin, Okoro, & Guo, 2016)**. Both RMA and ASA upregulated NQO1 mRNA, this might explain the antioxidant abilities of these micronutrients against B[a]P-induced oxidative stress **(Fig. 4C)**. Upregulation of phase II and antioxidant enzymes might be considered as a protection mechanism against colon cancers. In this context, ASA was reported to induce NQO1 and GSTya in murine Hepa 1c1c7 exposed to heavy metals like arsenic, cadmium and chromium **(Elbekai, Duke, & El-Kadi, 2007)**. Likely, oral administration of RMA brought back the status of phase I and phase II detoxication agents and lipid peroxidation byproducts in 7,12-dimethylbenz(a)anthracene induced skin

carcinogenesis in Swiss albino mice **(Sharmila, & Manoharan, 2012)**. In addition, **Venkatachalam et al., (2016)** concluded that chronic supplementation of rats with RMA can protect against 1,2-dimethylhydrazine-induced colon cancers as they observed an increase in UGT and GST-dependent enzyme activities in RMA supplemented group. ATP-binding cassette (ABC) transporters such as MDR1 and MRP2 play important roles in cells in the process of biodetoxification and in the active efflux of Phase II metabolites of drugs and xenobiotics. In the current study, colon cells had elevated MDR1 and MRP2 mRNA expressions upon exposure to B[a]P as a method of bio-adaptation. Co-exposure of CaCo-2 cells to B[a]P and ASA or RMA significantly upregulated the gene expression of MDR1 and MRP2 **(Fig. 5A & B)**. In agreement with this result, MRP2-knockout mice had elevated concentrations of B[a]P and its metabolites and subsequently increased risk of B[a]P-induced carcinogenicity **(Kranz et al., 2014)**. In addition, ABC transporters played important roles for protection of cells against oxidative stress as indicated in marine ciliates and model plants such as *Brassica napus* **(Kim, Yim, Kim, Kim, & Lee, 2017; Zhang, Zhao, & Yang, 2018)**. Thus, induction of oxidative stress and modulation of XMEs are considered as possible mechanisms for B[a]P-related mutagenicity in the colon cells and the ameliorative effects of RMA and ASA might be through their antioxidant effects and upregulation of phase II and III enzymes in CaCo-2 cells.

It notes worthy to report that in the present work, the protective effects of RMA and ASA against B[a]P-induced mutagenicity and oxidative stress were confirmed at the *in-vitro* level. RMA and ASA were frequently added to the meat for their antioxidant, antimicrobial and preservative effects in order to increase the shelf life of meat with no alterations in the organoleptic characteristics of meat and meat products **(Choi et al., 2017; Hernández-Hernández, Ponce-Alquicira, Jaramillo-Flores, & Guerrero Legarreta, 2009)**. Therefore, future studies are needed

to confirm the antimutagenic activities of RMA and ASA and their effects on the formation of PAHs when used as marinades before cooking of meat.

4. Conclusions

This study indicated formation of different PAHs, particularly the carcinogenic species in the heat-treated meat retailed in Egypt. The heavy dietary intake of such contaminated meat may increase the cancer risk among the Egyptian population. Food-relevant concentrations of B[a]P induced mutagenesis and produced higher levels of ROS in CaCo-2 cells. B[a]P-induced mutagenicity might be attributed to the overexpression of the bio-activation of phase I XMEs and downregulation of biodetoxification phase II XMEs. To the best of our knowledge, this is the first study to report that micronutrients such as ASA and RMA can reduce B[a]P adverse effects in human CaCo-2 cells. The induction of phase II and III enzymes and downregulation of phase I enzymes are possible mechanisms for the protective roles of such phytochemicals. Therefore, it is highly recommended for people consuming high quantities of heat-treated meat to include RMA and ASA to their food menu.

Acknowledgments

This study was supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Conflicts of interest

The authors did not have any conflict of interest.

References

Alomirah, H., Al-Zenki, S., Al-Hooti, S., Zaghloul, S., Sawaya W., Ahmed, N., & Kannan, K. (2011). Concentrations and dietary exposure to polycyclic aromatic hydrocarbons (PAHs) from grilled and smoked foods. *Food Control, 22*, 2028–2035.

Ames, B. N., & Gold, L. S. (1990). Chemical carcinogenesis: Too many rodent carcinogens. *Proceedings of the Natural Academy of Sciences of the United States of America, 87 (19),* 7772–7776.

Aygün, S. F., & Kabadayi, F. (2005). Determination of benzo[a]pyrene in charcoal grilled meat samples by HPLC with fluorescence detection. *International Journal of Food Sciences and Nutrition, 56(8),* 581–585.

Bandowe, B. A., Bigalke, M., Boamah, L., Nyarko, E., Saalia, F. K., & Wilcke, W. (2014). Polycyclic aromatic compounds (PAHs and oxygenated PAHs) and trace metals in fish species from Ghana (West Africa): bioaccumulation and health risk assessment. *Environment International, 65,* 135–146.

Buamden, S. (2018). Association between food availability and mortality due to colorectal cancer in the Americas. *Salud Colectiva, 14(3),* 579–595.

Chang, H. J., Park, J. S., Lee, E. K., Kim, M. H., Baek, M. K., Kim, H. R., Jeong, H. G., Choi, S. Y., & Jung, Y. D. (2009). Ascorbic acid suppresses the 2,3,7,8-tetrachloridibenxo-p-dioxin (TCDD)-induced CYP1A1 expression in human HepG2 cells. *Toxicology In Vitro, 23(4),* 622–6.

Charalabopoulos, K., Karkabounas, S., Dimicco, P., Binolis, J., Charalabopoulos, A., Zelovitis, J., Avdikos, A., Metsios, A., Peschos, D., Agnantis, N. J., & Evangelou, A. (2004). The role of ascorbic acid, selenium, and glutathione on benzo[a]pyrene-

induced carcinogenesis in wistar rats. *Journal of B.U.ON.: Official Journal of the Balkan Union of Oncology, 9(2),* 187–92.

Chen, K. L., Li, H. X., Xu, X. L., & Zhou, G. H. (2014). The protective effect of rosmarinic acid on hyperthermia-induced C2C12 muscle cells damage. *Molecular Biology Reports, 41(8),* 5525–31.

Choi, Y. S., Kim, T. K., Jeon, K. H., Park, J. D., Kim, H. W., Hwang, K. E., & Kim, Y. B. (2017). Effects of Pre-converted nitrite from red beet and ascorbic acid on quality characteristics in meat emulsions. *Korean Journal for Food Science of Animal Resources, 37(2),* 288–296.

Chung, S. Y., Yettella, R. R., Kim, J. S., Kwon, K., Kim, M. C., & Min, D. B. (2011). Effects of grilling and roasting on the levels of polycyclic aromatic hydrocarbons in beef and pork. *Food Chemistry, 129,* 1420–1426.

Darwish, W. S., Ikenaka, Y., Eldaly, E., & Ishizuka, M. (2010a). Mutagenic activation and detoxification of benzo[a]pyrene in vitro by hepatic cytochrome P450 1A1 and phase II enzymes in three meat-producing animals. *Food and Chemical Toxicology, 48,* 2526–2531.

Darwish, W. S., Ikenaka, Y., Nakayama, S. M., Mizukawa, H., & Ishizuka, M. (2016). Constitutive Effects of lead on aryl hydrocarbon receptor gene battery and protection by β-carotene and ascorbic acid in human HepG2 cells. *Journal of Food Science, 81(1),* T275–81.

Darwish, W. S., Ikenaka, Y., Nakayama, S., Mizukawa, H., Thompson, L. A., & Ishizuka, M. (2018). β-carotene and retinol reduce benzo[a]pyrene-induced mutagenicity and oxidative stress via transcriptional modulation of xenobiotic metabolizing enzymes in

human HepG2 cell line. *Environmental Science and Pollution Research International, 25(7),* 6320–6328.

Darwish, W. S., Ikenaka, Y., Ohno, M., Eldaly, E., & Ishizuka, M. (2010b). Carotenoids as regulators for inter-species difference in Cytochrome P450 1A expression and activity in food producing animals and rats. *Food and Chemical Toxicology*, *48,* 3201–3208.

Darwish, W. S., Nakayama, S. M., Itotani, Y., Ohno, M., Ikenaka, Y., & Ishizuka, M. (2015). Metabolic activation of heterocyclic amines and expression of xenobiotic-metabolizing enzymes in the gastrointestinal tract of rats. *Journal of Food Science, 80(7),* T1627–32.

Diggs, D. L., Harris, K. L., Rekhadevi, P. V., & Ramesh, A. (2012). Tumor microsomal metabolism of the food toxicant, benzo(a)pyrene, in ApcMin mouse model of colon cancer. *Tumor Biology, 33(4),* 1255–60.

Duan, X., Shen, G., Yang, H., Tian, J., Wei, F., Gong, J., & Zhang, J. J. (2016). Dietary intake polycyclic aromatic hydrocarbons (PAHs) and associated cancer risk in a cohort of Chinese urban adults: Inter- and intra-individual variability. *Chemosphere*, *144,* 2469–2475.

Duarte-Salles, T., Mendez, M. A., Meltzer, H. M., Alexander, J., & Haugen, M. (2013). Dietary benzo(a)pyrene intake during pregnancy and birth weight: associations modified by vitamin C intakes in the Norwegian Mother and Child Cohort Study (MoBa). *Environment International*, *60,* 217–23.

Egyptian Food Balance Sheet (EFBS) issued by economic affairs sector, Central Administration for Agricultural Planning, Ministry of Agriculture (2008).

http://egypt.opendataforafrica.org/kddlloe/egypt-agriculture-sheet. Accessed on February 5th 2019.

Elbekai, R. H., Duke, J., & El-Kadi, A. O. (2007). Ascorbic acid differentially modulates the induction of heme oxygenase-1, NAD(P)H:quinone oxidoreductase 1 and glutathione S-transferase Ya by As(3+), Cd(2+) and Cr(6+). *Cancer Letters, 246(1-2),* 54–62.

Essumang, D. K., Dodoo, D. K., & Adjei, J. K. (2013). Effect of smoke generation sources and smoke curing duration on the levels of polycyclic aromatic hydrocarbon (PAH) in different suites of fish. *Food and Chemical Toxicology, 58,* 86e94.

European Commission (EC) (2006). Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union,* 364, 365–323.

European Food Safety Authority (EFSA) (2008). http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/724.pdf. Accessed on October 5th 2018.

Guo, J., Xu, Y., Ji, W., Song, L., Dai, C., & Zhan, L. (2015). Effects of exposure to benzo[a]pyrene on metastasis of breast cancer are mediated through ROS-ERK-MMP9 axis signaling. *Toxicology Letters, 234(3),* 201–10.

Hernández-Hernández, E., Ponce-Alquicira, E., Jaramillo-Flores, M. E., & Guerrero Legarreta, I. (2009). Antioxidant effect rosemary (*Rosmarinus officinalis L.*) and oregano (*Origanum vulgare L.*) extracts on TBARS and colour of model raw pork batters. *Meat Science, 81(2),* 410–7.

International Agency for Research on Cancer (IARC) (2010). Monographs on the evaluation of carcinogenic risk to humans, overall evaluation of carcinogenicity. *IARC Monogrophs on the Evaluation of Carcinogenic Risks to Humans, Suppl 92,* 33–814.

Ikenaka, Y., Ito, Y., Heesoo, E., Watanabe, E., & Miyabara, Y. (2008). Characteristics of accumulation pattern of polycyclic aromatic hydrocarbons in the organisms inhabited in Lake Suwa. *Journal of Environmental Chemistry, 18,* 341–352.

Jiang, D., Wang, G., Li, L., Wang, X., Li, W., Li, X., Shao, L., & Li, F. (2018). Occurrence, dietary exposure, and health risk estimation of polycyclic aromatic hydrocarbons in grilled and fried meats in Shandong of China. *Food Science and Nutrition, 6(8),* 2431-2439.

Kazerouni, N., Sinha, R., Hsu, C. H., Greenberg, A., & Rothman, N. (2001). Analysis of 200 food items for benzo[a]pyrene and estimation of its intake in an epidemiologic study. *Food and Chemical Toxicology, 39,* 423–36.

Kikugawa, K. (2004). Prevention of mutagen formation in heated meats and model systems. *Mutagenesis, 19,* 431–439.

Kim, H., Yim, B., Kim, J., Kim, H., & Lee, Y. M. (2017). Molecular characterization of ABC transporters in marine ciliate, *Euplotescrassus*: Identification and response to cadmium and benzo[a]pyrene. *Marine Pollution Bulletin, 124(2),* 725–735.

Kranz, J., Hessel, S., Aretz, J., Seidel, A., Petzinger, E., Geyer, J., & Lampen, A. (2014). The role of the efflux carriers Abcg2 and Abcc2 for the hepatobiliary elimination of benzo[a]pyrene and its metabolites in mice. *Chemico- Biological Interactions, 224,* 36–41.

Li, J., Dong, H., Li, X., Han, B., Zhu, C., & Zhang, D. (2016). Quantitatively assessing the health risk of exposure to PAHs from intake of smoked meats. *Ecotoxicology and Environmental Safety, 124,* 91–95.

Liu, Y., Wu, Y. M., & Zhang, P. Y. (2015). Protective effects of curcumin and quercetin during benzo(a)pyrene induced lung carcinogenesis in mice. European Review for *Medical and Pharmacological Sciences, 19(9),* 1736–1743.

Mafiana, R. N., Al Lawati, A. S., Waly, M. I., Al Farsi, Y., Al Kindi, M., & Al Moundhri, M. (2018). Association between Dietary and Lifestyle Indices and Colorectal Cancer in Oman: A Case-Control Study. *Asian Pacific Journal of Cancer Prevention: APJCP*. *19(11),* 3117–3122.

Malhotra, A., Nair, P., & Dhawan, D. K. (2012). Curcumin and resveratrol in combination modulates benzo(a)pyrene-induced genotoxicity during lung carcinogenesis. *Human & Experimental Toxicology, 31(12),* 1199–206.

Martorell, I., Perello, G., Marti-Cid, R., Castell, V., Llobet, J. M., & Domingo, J. L. (2010). Polycyclic aromatic hydrocarbons (PAH) in foods and estimated PAH intake by the population of Catalonia, Spain: Temporal trend. *Environment International, 36(5),* 424–432.

Nisbet, C., & Lagoy, P. (1992). Toxic equivalency factors (TEFs) for polycyclic aromatic hydrocarbons (PAHs). *Regulatory Toxicology and Pharmacology, 16,* 290–300.

Phillips, D. H. (1999). Polycyclic aromatic hydrocarbons in the diet. *Mutation Research, 443,* 139-47.

556 Olatunji, O. S., Fatoki, O. S., Opeolu, B. O., & Ximba, B. J. (2014). Determination of
557 polycyclic aromatic hydrocarbons [PAHs] in processed meat products using gas
558 chromatography - flame ionization detector. *Food Chemistry, 156,* 296–300.
559 Reinik, M., Tamme, T., Roasto, M., Juhkam, K., Tenno, T. & Kiis, A. (2007). Polycyclic
560 aromatic hydrocarbons (PAHs) in meat products and estimated PAH intake by children
561 and the general population in Estonia. *Food Additives and Contaminants, 24,* 429–437
562 Sharmila, R., & Manoharan, S. (2012). Anti-tumor activity of rosmarinic acid in 7,12-
563 dimethylbenz(a)anthracene (DMBA) induced skin carcinogenesis in Swiss albino
564 mice. *Indian Journal of Experimental Biology, 50(3),* 187–94.
565 Sinha, R., Cross, A., Curtin, J., Zimmerman, T., McNutt, S., Risch, A., & Holden, J. (2005).
566 Development of a food frequency questionnaire module and databases for compounds
567 in cooked and processed meats. *Molecular Nutrition & Food Research, 49,* 648–55.
568 United States Environmental Protection Agency (USEPA) (2000). Guidance for assessing
569 chemical contaminant data for use in fish advisories, EPA 823-B-00-008. Volume 2:
570 risk assessment and fish consumption limits. Third edition. Office of Water:
571 Washington,DC; http://water.epa.gov/scitech/swguidance/fishshellfish/techguidance/r
572 isk/volume2index.cfm
573 Venkatachalam, K., Gunasekaran, S., & Namasivayam, N. (2016). Biochemical and molecular
574 mechanisms underlying the chemopreventive efficacy of rosmarinic acid in a rat colon
575 cancer. *European Journal of Pharmacology, 1791,* 37–50.
576 Xia, Z., Duan, X., Qiu, W., Liu, D., Wang, B., Tao, S., Jiang, Q., Lu, B., Song, Y., & Hu, X.
577 (2010). Health risk assessment on dietary exposure to polycyclic aromatic

578 hydrocarbons (PAHs) in Taiyuan, China. *The Science of the Total Environment, 408(22),* 5331–5337.
579
580 Yang, F., Yang, H., Ramesh, A., Goodwin, J. S., Okoro, E. U., & Guo, Z. (2016).
581 Overexpression of catalase enhances benzo(a)pyrene detoxification in endothelial
582 microsomes. *PLoS ONE, 11(9),* e0162561.
583 Zhang, X. D., Zhao, K. X., & Yang, Z. M. (2018). Identification of genomic ATP
584 binding cassette (ABC) transporter genes and Cd-responsive ABCs in *Brassica napus*.
585 *Gene, 664,* 139–151.
586

587 **Table 1: Polycyclic aromatic hydrocarbon contents in heat-treated meat marketed in Egypt**

	Raw meat		Boiled meat		Pan-fried meat		Grilled meat	
	Range (Median)	**Mean ± SD**	**Range (Median)**	**Mean ± SD**	**Range (Median)**	**Mean ± SD**	**Range (Median)**	**Mean ± SD**
Acenaphthene	0.24-0.88 (0.49)	0.48 ± 0.20^{c}	1.11-2.18 (1.35)	1.44 ± 0.38^{b}	1.45-3.45 (2.56)	2.62 ± 0.58^{a}	2.05-5.21 (3.49)	3.65 ± 0.88^{a}
Anthracene	0.12-0.28 (0.17)	0.19 ± 0.05^{d}	0.08-0.55 (0.29)	0.31 ± 0.15^{c}	0.35-0.78 (0.53)	0.55 ± 0.14^{b}	0.25-2.25 (1.13)	1.24 ± 0.75^{a}
Benz[a]anthracene	0.04-0.09 (0.05)	0.06 ± 0.02^{d}	0.07-0.31 (0.11)	0.14 ± 0.07^{c}	0.42-1.08 (0.79)	0.72 ± 0.24^{b}	0.11-2.39 (1.31)	1.33 ± 0.61^{a}
Benzo[a]pyrene	BDT	BDT	0.01-0.05 (0.02)	0.02 ± 0.01^{c}	0.32-1.63 (0.96)	0.98 ± 0.31^{b}	1.67-3.53 (2.69)	2.66 ± 0.44^{a}
Benzo[e]pyrene	BDT	BDT	0.11-0.46 (0.18)	0.23 ± 0.12^{c}	0.22-0.78 (0.55)	0.54 ± 0.18^{b}	0.85-3.78 (2.17)	2.33 ± 0.84^{a}
Benzo[b]fluoranthene	BDT	BDT	0.02-0.12 (0.05)	0.06 ± 0.03^{b}	0.05-1.08 (0.51)	0.54 ± 0.31^{a}	0.06-1.52 (0.77)	0.72 ± 0.51^{a}
Benzo[ghi]perylene	BDT	BDT	0.05-0.22 (0.12)	0.12 ± 0.05^{c}	0.23-0.77 (0.46)	0.48 ± 0.18^{b}	0.64-1.06 (0.88)	0.86 ± 0.14^{a}
Benzo[k]fluoranthene	BDT	BDT	0.05-0.13 (0.08)	0.09 ± 0.03^{c}	0.33-1.55 (0.83)	0.84 ± 0.44^{b}	1.18-1.89 (1.62)	1.60 ± 0.24^{a}
Chrysene	BDT	BDT	0.01-0.02 (0.01)	0.01 ± 0.004^{c}	0.05-0.21 (0.09)	0.10 ± 0.04^{b}	0.04-0.26 (0.14)	0.15 ± 0.07^{a}
Dibenz[a,h]anthracene	BDT	BDT	0.03-0.16 (0.08)	0.09 ± 0.05^{c}	0.11-0.45 (0.24)	0.28 ± 0.11^{b}	0.55-0.95 (0.72)	0.74 ± 0.13^{a}
Fluoranthene	0.03-0.08 (0.05)	0.05 ± 0.01^{d}	0.11-0.35 (0.22)	0.22 ± 0.08^{c}	0.24-0.53 (0.44)	0.41 ± 0.11^{b}	0.25-0.86 (0.60)	0.61 ± 0.20^{a}
Fluorene	1.77-3.88 (2.25)	2.42 ± 0.61^{c}	1.14-3.25 (2.35)	2.31 ± 0.74^{c}	2.78-6.65 (5.01)	4.96 ± 1.17^{b}	6.28-14.23 (8.68)	9.41 ± 2.86^{a}
Naphthalene	0.64-3.52 (2.46)	2.28 ± 0.7^{6c}	6.19-13.27 (10.23)	10.11 ± 2.22^{b}	11.77-26.45 (17.97)	18.67 ± 5.88^{a}	7.29-42.11 (20.46)	23.26 ± 10.69^{a}
Phenanthrene	0.05-0.18 (0.13)	0.11 ± 0.04^{d}	0.12-0.65 (0.49)	0.47 ± 0.16^{c}	0.55-2.55 (1.86)	1.75 ± 0.64^{b}	1.88-4.85 (3.55)	3.64 ± 0.88^{a}

Pyrene	0.12-0.25 (0.15)	0.17 ± 0.05[d]	0.22-0.88 (0.58)	0.56 ± 0.21[c]	0.96-2.25 (1.86)	1.77 ± 0.41[b]	2.45-3.77 (2.70)	3.01 ± 0.58[a]
Total 2-PAHs	BDT	BDT	0.02-0.07 (0.03)	0.03 ± 0.01[c]	0.62-1.84 (1.05)	1.08 ± 0.41[b]	1.93-3.64 (2.77)	2.81 ± 0.64[a]
Total 4-PAHs	0.04-0.09 (0.05)	0.06 ± 0.01[d]	0.13-0.41 (0.20)	0.20 ± 0.10[c]	1.48-2.81 (2.28)	2.30 ± 0.71[b]	2.52-6.48 (5.21)	4.84 ± 0.94[a]
Total 8-PAHs	0.16-0.37 (0.23)	0.25 ± 0.07[d]	0.57-1.09 (0.84)	0.85 ± 0.17[c]	3.91-5.60 (4.48)	4.49 ± 0.53[b]	7.53-11.89 (9.08)	9.27 ± 1.43[a]
Total 15-PAHs	5.41-6.89 (5.63)	5.77 ± 0.44d	12.21-19.82 (15.89)	16.18 ± 2.24[c]	28.29-42.95 (34.74)	35.22 ± 5.14[b]	41.35-72.16 (54.71)	55.17 ± 9.23[a]

Residual concentrations of PAHs in heat-treated meat are expressed as ng/g ww. Values in the same row carrying different superscript letter are significantly different ($p < 0.05$) (n = 25 from each group of meat).

BDT: below detection limit

Table 2: Dietary intakes and cancer risk estimates due to consumption of heat-treated meat among Egyptian adult population

	Boiled-meat	Pan-fried meat	Grilled-meat
B[a]P equivalent concentration (ng/g)	0.14	1.43	3.79
EDI of BEC (ng/g/day)	11.01	109.36	290.45
EDI of total 8-PAHs (ng/g/day)	65.2	344.5	711.18
EDI of total 15-PAHs (ng/g/day)	1241.17	2701.73	4232.09
Cancer Risk Estimates	7.05179E-07	7.00604E-06	1.86069E-05

Figure legends

Fig. S1: Frequency distribution of the 8 priority polycyclic aromatic hydrocarbons in the examined heat-treated meat samples

Fig. 1: Effects of B[a]P on CaCo-2 cell viability, mutagenicity and oxidative stress

CaCo-2 cell viability was tested using CCK-8 assay. Mutagenicity of B[a]P reflects the mutagenic activity of B[a]P (0-50 nM) in *Salmonella typhimurium* TA98 mutagenicity assay. B[a]P induced oxidative stress: ROS production in CaCo-2 cells exposed to B[a]P (0-50 nM) was assayed using DCF-DA as a substrate. The data represent the average percentage ± SD relative to control (n=6). Columns with same color carrying different superscript letters either (A, B, C, D) or (a, b, c, d) are significantly different from each other ($P < 0.05$).

Fig. 2: Protective effects of ascorbic and rosmarinic acids against B[a]P induced mutagenicity and oxidative stress in human colon cell line

Ameliorative effects of ascorbic (ASA) and rosmarinic (RMA) acids (10 µM each) on B[a]P (1 (low) & 50 (high) nM) -induced A) Mutagenicity (%) B) Oxidative stress (%). Data are presented as means ± SD (n=6). All treatments at different incubation times (3, 6, 12 and 24 h) showed significant reduction with the control ($P < 0.05$).

Fig. 3: Changes in mRNA expressions of phase I enzymes in CaCo-2 cells exposed to B[a]P, ASA and RMA

The effects of co-exposure of CaCo-2 cells to B[a]P (1, 5 or 50 nM) and ascorbic (ASA) or rosmarinic (RMA) acids (10 & 100 µM each) on A) epoxide hydrolase-1, B) CYP1A1 and C) CYP1B1 mRNA expressions as determined by real-time RT-PCR. Data are presented as the mean ± SD (n=6). Columns with different superscript letters are significantly different from each other ($P < 0.05$).

Fig. 4: Changes in mRNA expressions of detoxification enzymes in CaCo-2 cells exposed to B[a]P, ASA and RMA

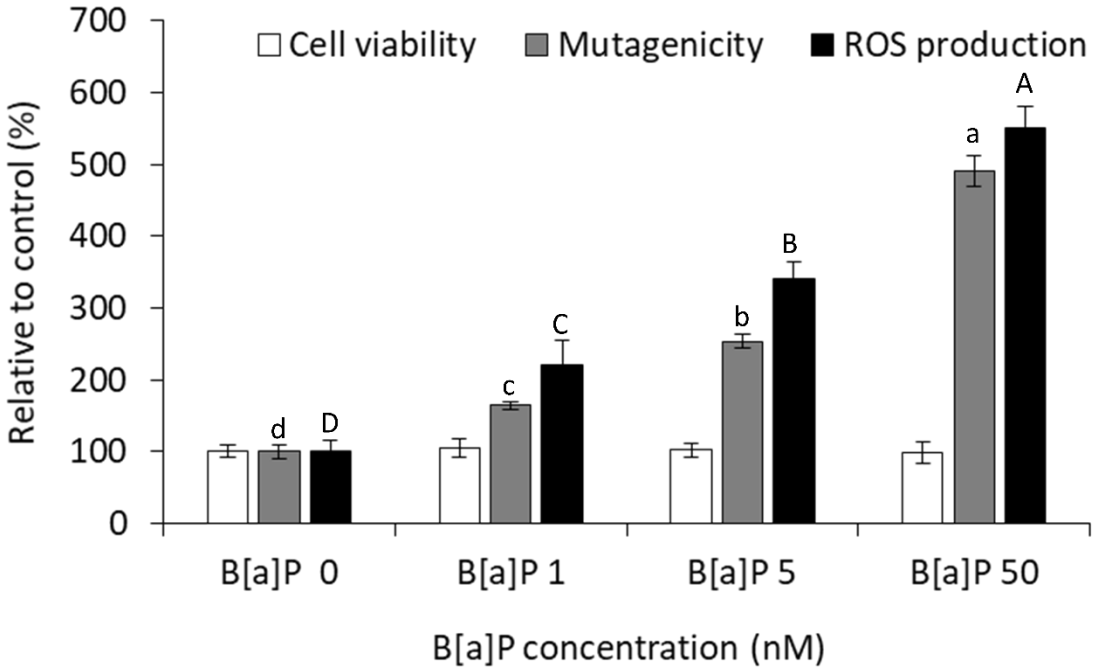
The effects of co-exposure of CaCo-2 cells to B[a]P (1, 5 or 50 nM) and ascorbic (ASA) or rosmarinic (RMA) acids (10 & 100 µM each) on A) UGT1A6, B) GSTA1 and C) NQO1 mRNA

expressions as determined by real-time RT-PCR. Data are presented as the mean ± SD (n=6). Columns with different superscript letters are significantly different from each other (*P <0.05*).

Fig. 5: Changes in mRNA expressions of xenobiotic transporters in CaCo-2 cells exposed to B[a]P, ASA and RMA

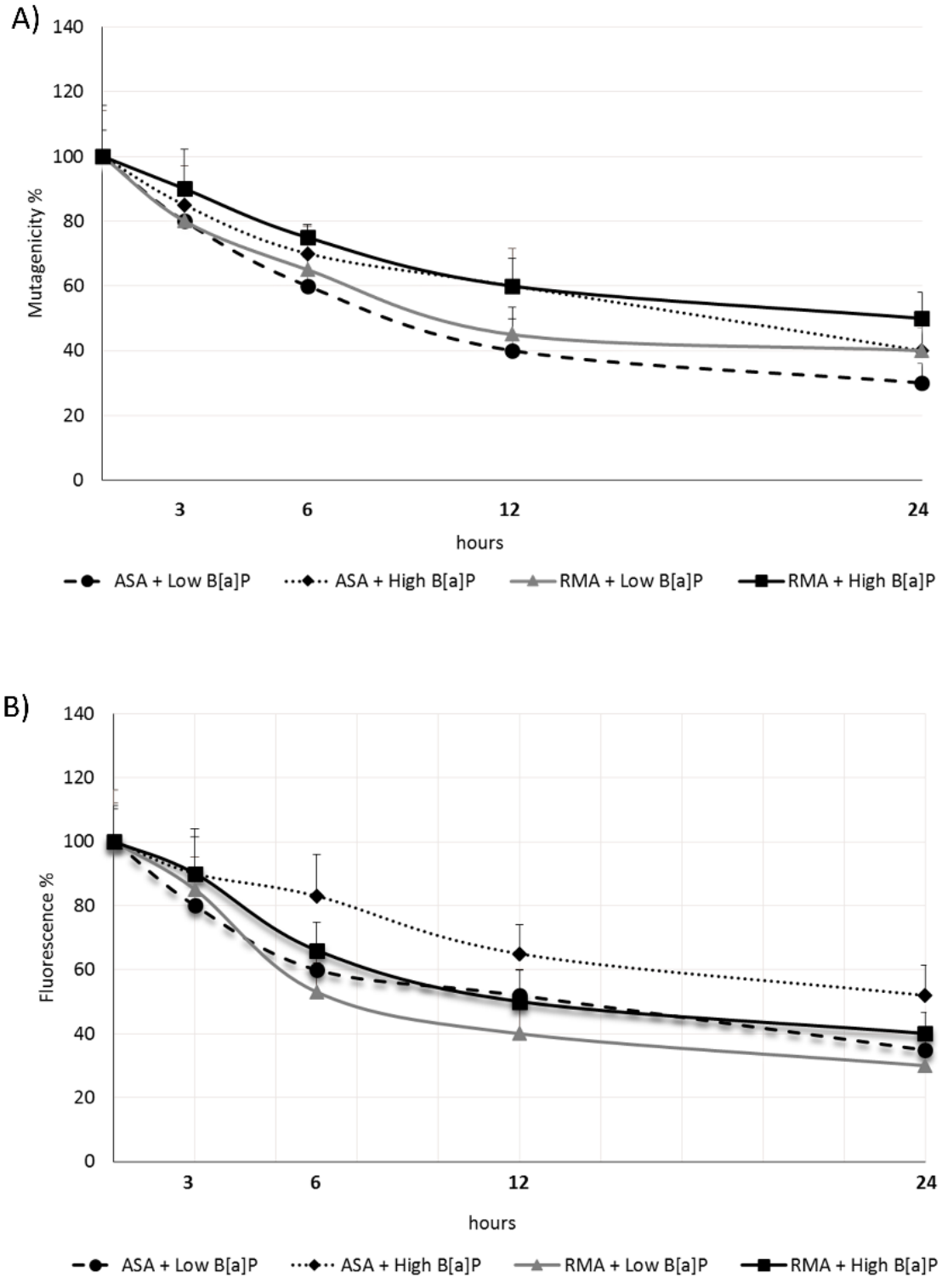
The effects of co-exposure of CaCo-2 cells to B[a]P (1, 5 or 50 nM) and ascorbic (ASA) or rosmarinic (RMA) acids (10 & 100 µM each) on A) MDR1 and B) MRP2 mRNA expressions as determined by real-time RT-PCR. Data are presented as the mean ± SD (n=6). Columns with different superscript letters are significantly different from each other (*P <0.05*).

638 **Fig. 1.**


Cell viability
Mutagenicity
ROS production
Relative to control (%)
700
600
500
400
300
200
100
0
d
D
c
C
b
B
a
A
B[a]P 0
B[a]P 1
B[a]P 5
B[a]P 50
B[a]P concentration (nM)

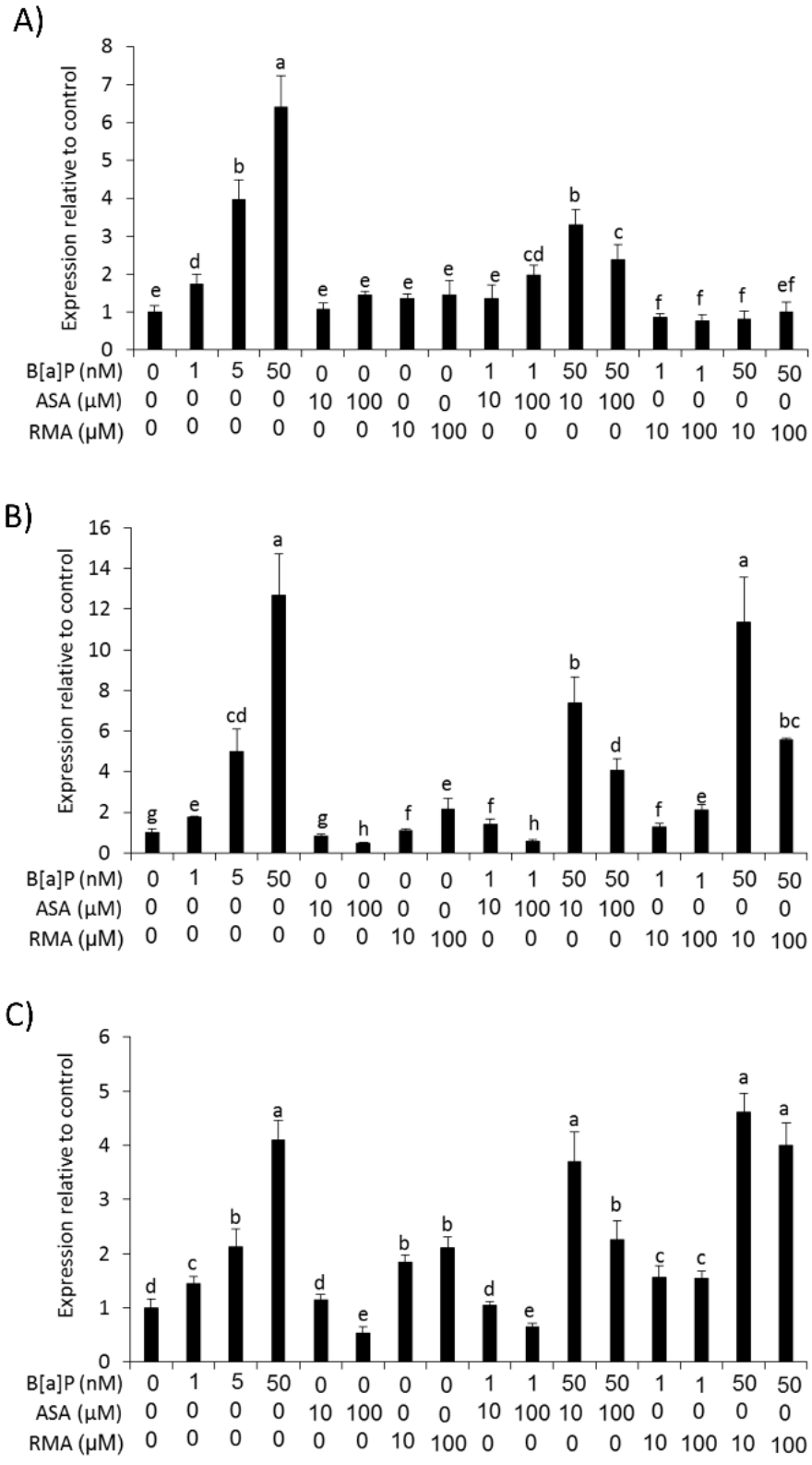
639

640 **Fig. 2.**


A)
Mutagenicity %
140
120
100
80
60
40
20
0
3
6
12
24
hours
ASA + Low B[a]P
ASA + High B[a]P
RMA + Low B[a]P
RMA + High B[a]P
B)
Fluorescence %
140
120
100
80
60
40
20
0
3
6
12
24
hours
ASA + Low B[a]P
ASA + High B[a]P
RMA + Low B[a]P
RMA + High B[a]P

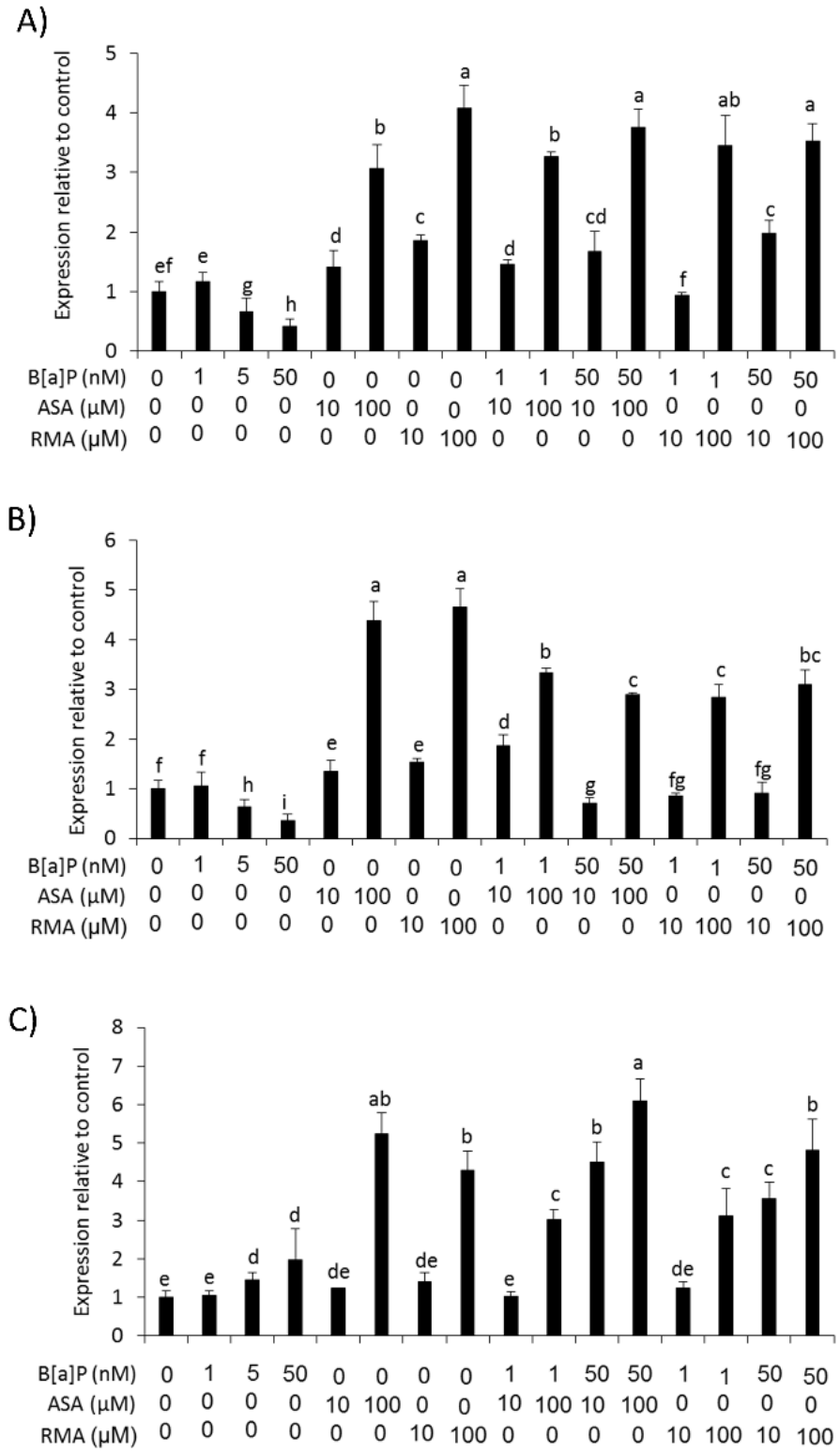
641

642 **Fig. 3.**


A)
Expression relative to control
B[a]P (nM)
ASA (μM)
RMA (μM)
B)
Expression relative to control
B[a]P (nM)
ASA (μM)
RMA (μM)
C)
Expression relative to control
B[a]P (nM)
ASA (μM)
RMA (μM)

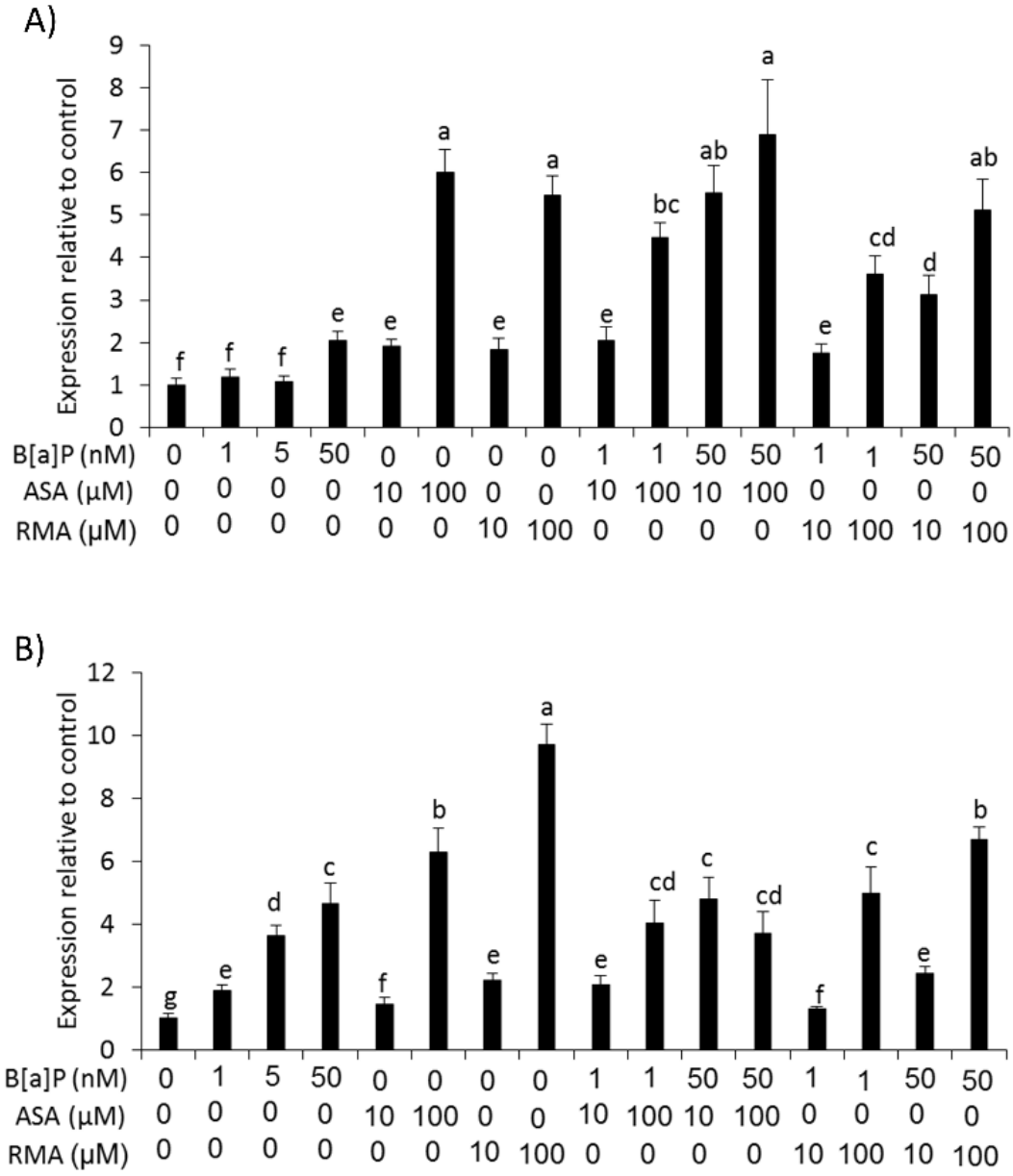
643

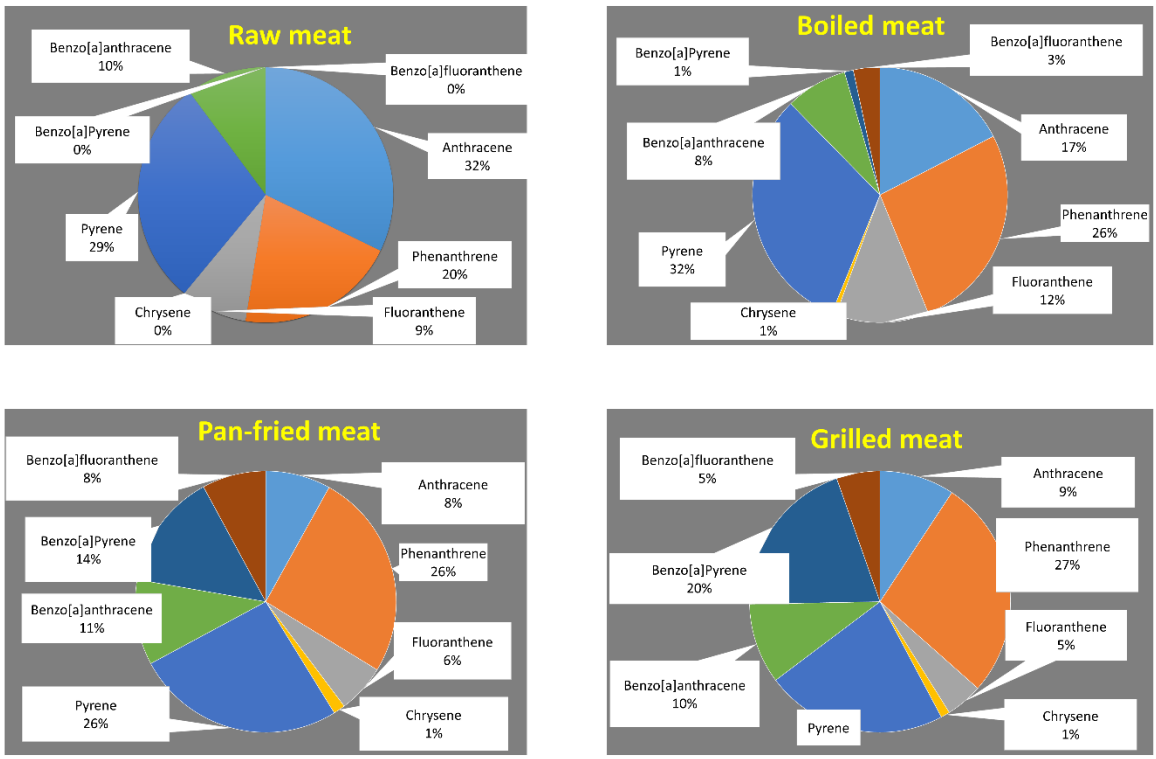
644 **Fig. 4.**


A)
Expression relative to control
B[a]P (nM)
ASA (µM)
RMA (µM)
B)
Expression relative to control
B[a]P (nM)
ASA (µM)
RMA (µM)
C)
Expression relative to control
B[a]P (nM)
ASA (µM)
RMA (µM)

645

Fig. 5.


A)
Expression relative to control
B[a]P (nM)
ASA (µM)
RMA (µM)
B)
Expression relative to control
B[a]P (nM)
ASA (µM)
RMA (µM)



Supplementary Figure 1

651 **Supplementary Table 1: Primers used in the present study**

Target	Sequence
Human Epoxide hydrolase-1	F- 5′- GAGCCTGCGAGCCGAGAC -3′
	R- 5′- CGTGGATCTCCTCATCTGACGTTT-3′
Human CYP1A1	F- 5′- CTATCTGGGCTGTGGGCAA -3′
	R-5′- CTGGCTCAAGCACAACTTGG -3′
Human CYP1B1	F- 5′-CTTTCGGCCACTACTCGGAG-3′
	R- 5′-CTCGAGGACTTGGCGGCT-3′
Human UGT1A6	F 5’-CATGATTGTTATTGGCCTGTAC-3’
	R 5’-TCTGTGAAAAGAGCATCAAACT-3’
Human GSTA1	F- 5′- CAGCAAGTGCCAATGGTTGA-3′
	R- 5′- TATTTGCTGGCAATGTAGTTGAGAA-3′
Human NQO1	F- 5′- GGATTGGACCGAGCTGGAA-3′
	R-5′- AATTGCAGTGAAGATGAAGGCAAC-3′
Human MDR1	F- 5′- CTGCTTGATGGCAAAGAAATAAAG-3′
	R- 5′- GGCTGTTGTCTCCATAGGCAAT-3′
Human MRP2	F- 5′- ATGCTTCCTGGGGATAAT-3′
	R- 5′- TCAAAGGCACGGATAACT-3′
Human β-actin	F- 5′- CTGGCACCCAGGACAATG-3′
	R-5′-GCCGATCCACACGGAGTA-3′.

652

653