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Determination of Polycyclic Aromatic Hydrocarbon in Charcoal Beef Steak and Inhibitory Profile of Thyme Oil, Lactic Acid Bacteria and Marinating on their Existence

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EAM designed the project. EH performed the experiments and analyzed the data. GAKK developed the theoretical framework and wrote the manuscript.

Key words
Charcoal-grilled beef steaks, Polycyclic aromatic hydrocarbons, Lactic acid bacteria, Thyme oil, Marination

ABSTRACT

The main objective of this study was to investigate the extent of accumulation of PAHs in charcoal beef steak in El-Gharbia governorate (Egypt), as well as to reduce the level of contamination after using thyme oil, LABs and marination. A total of 40 random samples of charcoal-grilled beef steaks classified into 10 untreated and 30 treated samples were collected randomly from different markets in El-Gharbia governorate, Egypt. Each sample was wrapped in a plastic bag, in anicebox and transferred to the laboratory then the untreated samples were prepared for detection of 12 polycyclic aromatic hydrocarbons (PAHs) (benzo[a]pyrene; dibenzo [ae] pyrene; dibenzo [al] pyrene; dibenzo [ah] pyrene; dibenzo [ai] pyrene; indeno [1, 2, 3-cd] pyrene; benzo [ghi] perylene; cyclopenta (c, d) pyrene; benzo[a] anthracene; Dibenzo [a, h] anthracene; benzo[b] fluoranthene; chrysene). The treated samples were demonstrated as 3 untreated (control group); 9 samples treated with thyme oil at concentrations of 0.5, 1 and 1.5% (3 of each), 9 samples treated with bacterial cultures as *Streptococcus thermophiles*, *Lactobacillus bulgaricus* and *L. rhamnosus* (3 of each) and 9 samples treated with marinades (3 of each) represented by basic marinades that composed of sugar 330 g, water 65mL, onion 70g, turmeric 20g, lemongrass 15g, salt 15g, garlic 7g, coriander 2g and cinnamon 2g; commercial marinades were composed of Spices, onion 380g, garlic, salt 60g, sugar 210g, water 30mL and cooking oil 60 mL and then modified marinades that composed of basic marinade 520g and lemon Juice 25mL. Each group was examined for determination of their BaP, BaA, BbF and CHR polycyclic aromatic hydrocarbons (PAH4). The recorded results revealed that the mean values of PAH4 and total PAHs in untreated samples were 66.37±53.89 and 35.56±39.21µg/kg, respectively. Meanwhile, BaP and PAH4 residual concentrations in treated samples with thyme oil (0.5%, 1.0% and 1.5%); different cultures (*S. thermophilus, L. bulgaricus* and *L. rhamnosus*) and different marination technique (basic, commercial and modified) were 10.63±6.48; 19.83±14.19; 7.17±4.77; 13.8±9.42; 4.87±3.36; 8.58±6.32; 12.6±5.69; 24.13±16.02; 5.63±3.78; 10.87±7.34; 3.53±2.65; 6.43±4.66; 8.3±5.2; 15.73±10.55; 4.1±2.86; 7.77±5.53; 3.53±2.65 and 5.6±4.86µg/kg, respectively. Charcoal-grilled beef steaks treated with modified marination lead to a decrease in the levels of BaP and PAH4 in the examined samples.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), among other pollutants, have been released into the atmosphere as a result of rapid industrialization and urbanization (Mojiri *et al*., 2019). Because of their inherent characteristics, PAHs are persistent pollutants with a wide range of biological toxicity, and their removal from the atmosphere has long been of concern. Additionally, during residential and commercial food preparation, toasting, grilling, frying, baking, and roasting, PAHs are created (Rose *et al*., 2015). The accumulation of PAHs in charcoal beef steak is primarily caused by three factors: the smoke produced by incomplete combustion of the heat source that is connected to the surface of the charcoal beef steak; the pyrolysis of organic compounds (fat and protein) within the beef steak during the cooking process; and the heat supply itself (Zelinkova and Wenzl, 2015). Additionally, the fundamental mechanism of PAH creation is the production

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of free radicals by the abrasive combustion of food molecules, and these byproducts eventually accumulate in food products (Singh *et al*., 2016). Due to PAHs' potential to cause cancer, mutagenesis, and cytotoxicity. As a result, the European Commission has set limits for the amount of PAHs that can be found in certain food matrices. Limits for benzo [a] pyrene (BaP) and PAH4 in meat that has been heated through grilling or barbecuing are 5 ng g1 and 30 ng g1, respectively (EC, 2011). In addition, eight carcinogenic and genotoxic PAHs (PAH8), including chrysene, benzo (a) pyrene, benzo (b) fluoranthene, benzo (k) fluoranthene, dibenzo (a, h) anthracene, benzo (g, h, i) perylene, indeno (1, 2, 3-c, d) pyrene, and fluorene, had an average consumption across European (EFSA, 2008). The most extensively researched PAH is likely benzo [a] pyrene (BaP). BaP was classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen (IARC, 2015). As a result, the BaP determination has been frequently employed as an indication of the PAH content in environmental research (Janoszka, 2011). As a result, the most significant and widespread problem with regard to food safety is BaP contamination. Additionally, potential LAB detoxification mechanisms of carcinogens are supposedly done by physical binding between LAB strains and toxins, and the metabolism of LAB strains can reduce the danger of carcinogens. We tested whether LAB strains' dead pellets from heat-treated with B[a]p would have the same amount of binding power as their life pellets (Lili *et al*., 2017). Recent studies have shown that marinating meat can enhance meat quality while reducing the production of hazardous compounds (Garca-Lomillo *et al*., 2017). Ingredients having antioxidant characteristics that can help reduce the amount of PAHs in the system to a safe level have received particular attention (Viegas *et al*., 2014). Thyme (*Thymus vulgaris* L.) is a plant with significant commercial value that is primarily grown in the Mediterranean region. It is a fragrant plant belonging to the *Lamiaceae* family that contains an essential oil that is valuable and flavorful for cooking and has significant health benefits (Zengin and Baysal, 2015). Thyme extracts contain antioxidant properties, making them appropriate for use as sources of synthetic antioxidants in the food sector (Bergo *et al*., 2008). The inhibitory mechanisms against PAH produced during cooking have been attributed to the presence of phenolic components in thyme essential oil (Wang *et al*., 2019).

MATERIALS AND METHODS

Sampling

A total of 10 samples of charcoal-grilled beef steak (25gm each) were collected randomly from different markets in El-Gharbia Governorate, Egypt. The collected samples were wrapped in a plastic bag and put in a dry clean icebox, conveyed to the laboratory and analyzed for detection of polycyclic aromatic hydrocarbon.

Accurately, 30 samples of beef steak (25gm each) were classified into untreated control samples (3), 9 treated samples with thyme oil at concentrations of 0.5, 1 and 1.5% (3 of each), 9 treated samples with bacterial cultures as *S. thermophiles, L. bulgaricus* and *L. rhamnosus* at levels of 5×10^6 cfu/g (3 of each) and 9 treated samples with marinades represented by basic, commercial and modified type (3 of each). Basic marinades were composed of sugar 330 g, water 65mL, onion 70g, turmeric 20g, lemongrass 15g, salt 15g, garlic 7g, coriander 2g and cinnamon 2g. Commercial marinades were composed of spices (paprika, ginger, red chili and black pepper), onion 380g, garlic, salt 60g, sugar 210g, water 30ml and cooking oil 60 ml. Modified marinades were composed of basic marinade 520g and lemon juice 25mL.

Treatment of samples with thyme oil and bacterial cultures (Barbosa et al., 2009)

Each treated sample was dipped for 15 min in the prepared solutions of thyme oil (0.5%, 1% and 1.5%) and bacterial cultures (*S. thermophiles, L. bulgaricus* and *L. rhamnosus*). Then drained well for 5 min on a sterile stainless wire mesh screen. Control groups were dipped in sterile distilled water for the same period.

Treatment of samples with marinades

Before usage, the marinade's components were blended together in a blender and stored in a refrigerator (4°C) (at a maximum duration of 4 h). After thoroughly mixing them, they were applied to the beef steak samples and left for at least 4 hours at 4°C. The beef steak samples that had been marinated were roasted over charcoal until the color turned yellowish-brown (well done).

Previously treated beef steak samples were labeled and every treated sample was separately packaged in sterile polyethylene bags. Each group was examined for determination of their BaP, BaA, BpF and CHR polycyclic aromatic hydrocarbons (PAH4).

Determination of polycyclic aromatic hydrocarbons (PAHS)

For the preparation, the following supplies were purchased at El-Gomhurya, Al-Amirya, Egypt: Potassium hydroxide, sodium chloride, sodium sulphate, cyclohexane (ECD tested), N, N-dimethylformamide, methanol (HPLC grade), and sodium chloride. Silica solid-phase extraction (SPE) tubes (500 mg) and ultra-pure water were created using a MilliQ filter system. AccuStandard provided the

15 PAH mixture (CT06513, USA).

The deuterated benzo [a]-pyrene-d_{12} solution in acetonitrile had a concentration of 50 mg/l, and the amount of cyclohexane in the analytical standard stock mix of PAHs was 1000 ng/l. At 4 °C, mixtures were kept.

Triplicates of 8 different mixed standard calibration solutions at concentrations of (0.1, 0.5, 1, 10, 25, 50, 75, and 100 ng/mL) were used for calibration. From 0.993-0.998, the correlation coefficient varied.

For extraction and preparation of samples the meat product under test had been fully homogenized. In order to accommodate the gas chromatography-mass spectrometry (GC-MS) detection method, the sample preparation approach was developed in accordance with Simko (2002) and Stumpe *et al*. (2008) with certain modifications. Each material under examination was ground into 20 g using anhydrous sodium sulphate in a mortar (2 g). Then, 60 mL of a (1-1) (v\v) hexane-acetone combination was squeezed into the mixture, filtered, and the tissue was extracted twice more. The filter paper was used to filter the combined organic solvent fractions using 1 g of anhydrous sodium sulphate. A silica SPE column that had previously been conditioned with cyclohexane received the extract (5 ml). Cyclohexane was used to rinse the flask (3 ml) and to elute the PAH (6 ml). The recovered fraction was transferred into a GC vial after being dissolved in 50 µl of cyclohexane and evaporated at 40 °C under a gentle nitrogen stream. Temperatures for the injector and detector were kept at 280°C and 300°C, respectively. The initial oven temperature was 100°C for two min, held at 280°C at a rate of 6°C/min, and held there for 15 min.

Statistical analysis

All experiments and analyses were performed in triplicate, and the results were reported as values of (mean, standard deviation and range). To analyze the data, Microsoft Office Excel 2016 and SPSS (17.0) system software was utilized. For statistical analysis, two-way ANOVA and independent-samples T-Test were utilized.

RESULTS AND DISCUSSION

In Egypt and other Arabian nations, charcoal grilling of beef steaks is popular in both restaurants and at home. that improve the digestibility, palatability, and organoleptic quality of meat; however, can also result in the formation of carcinogenic chemicals, including PAHs. Consequently, the determination of these chemicals provides information on potential health risks of dietary exposure to processed meat, which could be useful for the health management of the consumers (IARC, 2018).

Charcoal beef steak

The main focus of this study was on BaP and PAH4 (sum content of BaP, BaA, CHR and BbF). Also, the contents; of DaeP, DaIP, DahP, IcdP, DaiP, BghiP, CcdP, BaA and DahA were analyzed. Commission Regulation (EC, 2011) had set the maximum levels for PAH4 in meat products at 12 µg/kg and the maximum limit of BaA was 2 µg/kg.

The result reported in Table I demonstrated the concentrations of the sum of 12 PAHs in 10 charcoal beef steak samples that ranged from 4.8 to 105 µg/kg with a mean contamination level 35.56 ± 39.21µg/kg. All samples except one exceeded the maximum limits for BaP and the range was 1.5 to 20.8 µg/kg with a mean value 10.55±6.7 µg/kg and the range of sum PAH4 was 4.8 to 105 µg/kg with a mean value 66.37 ±53.89 µg/kg that also exceeded the maximum limits (12 µg/kg) set by European Union.

Table I. PAHs (µg/kg) in examined samples of charcoal meat beef steaks (*n=10).

PAHs	Mean ± SD (Range)
BaP	10.55±6.7 (1.5-20.8)
Daep	UDL
DaIP	UDL
DahP	UDL
DaiP	UDL
IcdP	3.32±2.3 (1.2-6.1)
BghiP	5.17±3.23 (1.2-8.1)
CcdP	2.57±2.69 (0.19-5.1)
BaA	8.93±6.44 (1-19.4)
DahA	UDL
BbF	UDL
Chr	1.6±1.11 (0.4-2.6)
PAH4 sum	66.37±53.89 (4.8-105)
PAH sum	35.56±39.21 (4.8-105)

PAH4, BaA, Chr, BbF, and BaP; PAHs, polycyclic aromatic hydrocarbons; BaP, benzo [a] pyrene; DaeP, Dibenzo [ae] pyrene; DaIP, Dibenzo [al] pyrene; DahP, Dibenzo [ah] pyrene; DaiP, Dibenzo [ai] pyrene; IcdP, indeno [1, 2, 3-cd] pyrene; BghiP, benzo [ghi] perylene; CcdP, Cyclopenta (c, d) pyrene; BaA, benzo[a]anthracene; DahA, Dibenzo [a, h] anthracene; BbF, benzo [b] fluoranthene; Chr, chrysene; UDL, under detected limits; SD, standard deviation; n, Number of samples. Maximum level for PAH4 in meat products 12 µg kg and maximum level of BaP 2.0 µg/kg according to commission regulation (EC, 2011).

On the contrary, a lower concentration (0.11 to 3.93 µg /kg) in cooked meat products in Poland of BaP has been detected by Janoszka *et al*. (2004) and the total PAH content was within the range between 2.43 to 16.10 µg/kg. Furthermore Eldaly *et al*. (2016) did not detect benzo [ghi] perylene in the examined non-marinated charcoal-grilled meat samples, but detected benzo (a) anthracene and benzo (a) pyrene only with the maximum concentrations of 33.2 ± 4 and 26 ± 16 µg/kg, respectively. These variations in the concentration levels of PAHs were significantly attributed to the time of the grilling process, amount of fat content and high thermal contact led to the fat being dropped from the beef steak onto the flames resulting in generating of smoke, or due to pyrolysis of organic matter that form free radicals responsible for PAH formation.

Thyme oil treatment of charcoal beef steak

The data present in Table II showed that the mean concentration levels of BaP, and PAH4 in thyme oil added to the charcoal beef steak with three different concentration 0.5%, 1.0%, and 1.5% were 10.63±6.48; 19.83±14.19; 7.17±4.77; 13.8±9.42, and 4.87±3.36; 8.57±6.32 µg/kg^{-1}, respectively. The addition of 0.5%, 1.0%, and 1.5% of thyme oil in charcoal beef steak led the reduction of carcinogenic content of BaP, and PAH4 by 38%; 39%; 58%; 58%, and 71%; 74%, respectively as recorded in Table III. The more effective concentration for reduction of the carcinogenic PAHs in charcoal beef steak was 1.5%. These reduction percent may be attributed to the antioxidative effect of thyme oil on the charcoal beef steak. Furthermore, this study provides important information exploring the reduction effect of thyme oil on PAHs. So, the relationship between thyme oil concentrations and PAHs reduction rate needs further study.

Table II. Statistical analytical results of PAHs µg/kg (Mean±SD with ranges) in examined treated charcoal beef steak samples with thyme oil.

PHAS	Control	Thyme oil 0.5%	Thyme oil 1%	Thyme oil 1.5%
BaP	17.07±8.33 (9.7-26.1)	10.63±6.48 (5.2-17.8)	7.17±4.77 (3.3-12.5)	4.87±3.36 (2.1-8.6)
BaA	13.17±7.34 (6.8-21.2)	7.8±4.64 (4.1-13.0)	5.53±3.59 (2.8-9.6)	3.03±1.33 (1.9-4.5)
BbF	UDL	UDL	UDL	UDL
Chr	2.43±1.35 (1.1-3.8)	1.4±0.75 (0.6-2.1)	1.1±0.66 (0.4-1.7)	0.67±0.35 (0.3-1.0)
PAH4 sum	32.67±22.73 (7.3-51.2)	19.83±14.19 (4.2-31.9)	13.8±9.42 (3.3-21.5)	8.57±6.32 (2-14.6)

PAHs, polycyclic aromatic hydrocarbons; PAH4: BaP, BaA, BbF, and Chr; BaP, benzo[a]pyrene; BaA, benzo [a] anthracene; BbF, benzo[b] fluoranthene; Chr, chrysene; UDL, under detected limits; SD, standard deviation. Difference between treatment and control is significant ($p < 0.05$).

Table III. Reduction percentage effect of different treatment on BaP and PAH4 for Charcoal Beef Steak. (*n=5).

Treatment	BaP %	PAH4 %
Thyme oil 0.5%	38%	39%
Thyme oil 1%	58%	58%
Thyme oil 1.5%	71%	74%
S. thermophilus	26%	26%
L. bulgaricus	67%	67%
L. rhamnosus	79%	80%
Basic marination	51%	52%
Commercial marination	76%	76%
Modified marination	79%	83%

Culture treatment of charcoal beef steak

The results recorded in the Table IV revealed that the mean concentration values of BaP and PAH4 after using different cultures treatment of charcoal beef steak with *S. thermophilus***,** *L. bulgaricus* and *L. rhamnosus* were 12.6±5.69; 24.13±16.02; 5.63± 3.78;10.87±7.34, and 3.53±2.65; 6.43±4.66 µg/kg, respectively. The reduction percent of BaPand PAH4 as recorded in Table III after the addition of different cultures such as *S. thermophilus, L. bulgaricus* and *L. rhamnosus* in charcoal beef steak were 26%; 26%; 67%; 67% and 79%; 80%, respectively. Moreover, *L. rhamnosus* was the more efficient culture for reducing the hazardous PAHs in charcoal beef steak. Furthermore, the type of wood, the smoking technique, the temperature at which it is burned, the nature of the smoke, and the length of time foodstuffs are exposed to the smoke all affect the amount of PAHs carried by the smoke particles (Duedahl-Olesen *et al*., 2006). In addition, elements including the distance from a heat source, the fuel used, the level of processing, as well as the length and kind of processing, affect how much PAHs are present. But processes like recycling, concentrating, crushing, and storage increase the amount of PAHs in different food products (Singh *et al*., 2016). Benzo [a] pyrene (BaP), which is regarded as a human cancer-causing agent, is the most researched PAH. In addition, according to the European Food Safety Authority, people in European Union member states consume the most meat and meat products on a daily basis (EFSA, 2008). In terms of quantifying these chemicals and determining what makes food sources more likely to include them, this underlines the importance of PAHs studies for smoked meat products. Additionally, *S. thermophilus and L. bulgaricus* were shown to lower PAHs concentration by 87.7 and 91.5 percent, respectively (Abou-Arab *et al*., 2015). Additionally, Hongfei *et al*.

(2017) looked at the possibility that using a combination of different LAB strains could help remove a variety of harmful substances. Additionally, some researchers hypothesized that certain cell-produced enzymes were responsible for the mechanism of decreasing hazardous chemicals (Fuchs *et al*., 2008). Therefore, after charcoal beef steak treatment with LAB suspension, a decrease in PAHs content could be accomplished. According to Abou-Baker *et al*. (2012), this process was brought on by the carcinogen's binding to the elements of cell walls. Exopolysaccharides were also noted by Tsuda *et al*. (2008) to be crucial in the removal of carcinogens.

Marinade treatment of charcoal beef steak

Moreover, the results of the current study outlined in Table V demonstrated that the total means concentration levels of BaP and PAH4 by the different marination in the treatment of charcoal beef steak with basic marination, commercial margination and modified marination were 8.3±5.2, 15.73±10.55, 4.1±2.86, 7.77±5.53, 3.53±2.65, and 5.6±4.86 µg/kg, respectively. The reduction percent in the treated charcoal beef steak marinated with basic marination, commercial marination and modified marination for BaP and PAH4 was 51%, 52%, 76%, 76% and 79%, 83%, respectively Table III. The modified marinade treatment was the more effective marinade method in the reduction of the carcinogenic PAHs in charcoal beef steak. These lower results of PAHs in the modified marinade treatment of beef steak were attributed to the effect of the modified marination ingredients led to tenderizing the beef steak, making the protein easier to rapid digest and cook in addition to preventing the moisture to be lost and the beef meat did not dry out and toughen up. So, in reducing the time needed to complete cooking these results were agree with those reported by Farhadian *et al*. (2012). In addition to decreasing the fat droplets come in contact with charcoal and therefore prevent the smoking formation and PAHs accumulation on the surface of beef steak. Additionally, the basic marinade treatment with lemon juice added had a beneficial impact on the concentration of PAHs. These phenomena might result from variations in the samples' acidic content. Additionally, the kinetics and mechanism of nonenzymatic browning may be affected by the acidity (Maillard reactions). As pH rises, the reaction rate typically rises as well (Li *et al*., 2021). According to research on the kinetics and mechanism of PAH synthesis, PAHs play a role in the fragrance products of Maillard reactions (Britt *et al*., 2004). However, according to Nursten (2005), lemon juice includes organic substances, such as sulphur dioxide, that are frequently employed to prevent the Maillard processes and may help to lower the content of PAHs in the beef steak samples.

Table IV. PAHs µg/kg in examined treated samples of charcoal beef steak with different cultures.

PHAS	Control		*S. thermophilus*		*L. bulgaricus*		*L. rhamnosus*	
	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD
BaP	9.7-26.1	17.07±8.33	8.5-19.1	12.6±5.69	2.7-9.9	5.63±3.78	1.4-6.5	3.53±2.65
BaA	6.8-21.2	13.17±7.34	4.8-15.4	9.37±5.45	2.1-7.4	4.33±2.75	1.2-3.9	2.43±1.37
BbF	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL
Chr	1.1-3.8	2.43±1.35	1.2-3.0	2.17±0.91	1.2-3.9	0.9±0.2	0.2-0.8	0.47±0.31
PAH4 sum	7.3-51.2	32.67±22.73	6.5-37.8	24.13±16.02	2.7-16.9	10.87±7.34	1.4-10.6	6.43±4.66

For abbreviation see Table II. *S. thermophiles, Streptococcus thermophiles; L. bulgaricus, Lactobacillus bulgaricus; L. rhamnosus, Lactobacillusrhamnosus.* Difference between treatment and control is significant ($p < 0.05$).

Table V. PAHs µg/kg in examined charcoal beef steak samples treated with different marination technique.

PHAS	Control		Basic		Commercial		Modified	
	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD
BaP	9.7-26.1	17.07±8.33	3.8-14.0	8.3±5.2	1.8-7.3	4.1±2.86	1.4-6.5	3.53±2.65
BaA	6.8-21.2	13.17±7.34	3.2-10.3	6.03±3.76	1.6-5.1	3.13±1.79	0.9-3.0	1.77±1.1
BbF	UDL	UDL	UDL	UDL	UDL	UDL	UDL	UDL
Chr	1.1-3.8	2.43±1.35	0.6-2.1	1.4±0.75	0.4-0.7	0.53±0.15	0.1-0.6	0.3±0.26
PAH4 sum	7.3-51.2	32.67±22.73	4.2-24.9	15.73±10.55	1.6-12.3	7.77±5.53	0.9-10.6	5.6±4.86

For abbreviation see Table II. Basic marination: sugar 333 g, water 60g, onion 65mL, turmeric 15g, lemon grass 10g, salt 10g, garlic 5g, coriander 1g and cinnamon 1g. Commercial marination: Spices, onion 375g, garlic, salt 50g, sugar 200g water 25mL and cooking oil 50 mL. Modified marination: Basic marinade 500g and lemon juice 20mL. Difference between treatment and control is significant ($p < 0.05$).

CONCLUSION

This research showed that addition of thyme oil in charcoal beef steak prior to grilling led to reduction of carcinogenic content of BaP, and PAH4 and the more effective concentration for reduction of the carcinogenic PAHs in charcoal beef steak was to 1.5% concentration. Concerning to treated beef steak with lactic acid bacteria (LABs), the more effective culture in the reduction of the carcinogenic PAHs in was to *L. rhamnosus*. Furthermore, marinated with lemon juice had a significantly higher effect on reduction of PAHs formation. It is recommended that charcoal beef steaks treated should be marinated prior to grilling to minimize the hazards of PAHs on human health.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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