Coffee consumption induces GSTP in plasma and protects lymphocytes against (±)-anti-benzo[α]pyrene-7,8-dihydrodiol-9,10-epoxide induced DNA-damage: Results of controlled human intervention trials

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Abstract

A number of animal studies indicate that coffee protects against chemical induction of cancer; also human studies suggest that coffee consumption is inversely related with the incidence of different forms of cancer. The protective effects were attributed to induction of glutathione-S-transferases (GSTs) and protect against DNA-damage caused by (±)-anti-B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE), the DNA-reactive metabolite of benzo(a)pyrene. Ten participants consumed 1 L unfiltered coffee/d over 5 days. Before and after the intervention, saliva and blood were collected and the overall GST activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB). Additionally, GSTP and GSTA were determined in plasma with immunoassays. In blood, only weak (p = 0.042) induction of GST (CDNB) was found. Furthermore, pronounced (three-fold) induction of GSTP was observed in blood, whereas GSTA was not altered. No correlations were seen between induction of GST (CDNB) and GSTP activities and the GSTP1 genotypes of the participants. Also clinical parameters (creatinine, alanine, aminotransferase, aspartate aminotransferase, alkaline phosphatase), which are markers for organ damage, were monitored. None of them was altered by coffee, but serum cholesterol levels were slightly (not significantly) enhanced. In a second trial (n = 7), GSTP induction by unfiltered and paper filtered coffees, differing

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in cafestol and kahweol contents, were compared. The participants consumed 1 L coffee/d over 3 days. Again significant (three-fold) induction of GSTP was observed. The effects seen with the two coffees were identical, indicating that the diterpenoid concentrations are not responsible for the effects. In a further trial \((n = 7)\), the effect of coffee (unfiltered, 1 L/d; 5 days) on BPDE induced DNA-migration was studied in comet assays. A 45% reduction effect was observed. Our findings show that coffee induces GSTP in humans and indicate that consumption may lead to protection towards polycyclic aromatic hydrocarbons.

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1. Introduction

Coffee is among the most widely consumed beverages and its annual production is increasing [1]. As coffee contains a broad variety of potentially bioactive compounds, strong efforts have been made to study its effects on human health. A number of epidemiological studies indicate, that the consumption of coffee is inversely related to the incidence of colon cancer [2–5], additionally also protection towards other forms of cancer has been reported [6,7].

These observations are supported by data from animal studies which showed that coffee protects against dietary carcinogens such as nitrosamines and polycyclic aromatic hydrocarbons (PAHs) [8,9]. One of the most important mechanisms by which coffee and the coffee specific diterpenoids cafestol and kahweol \((C + K)\) protect against cancer appears to be induction of glutathione-S-transferases (GSTs) [10]. GSTs are a family of phase II enzymes which inactivate a broad variety of environmental and dietary toxins [11,12] including food carcinogens such as PAHs, aflatoxins and heterocyclic aromatic amines (HAAs). Recent studies indicate that the isozyme GSTP is highly protective towards \((\pm\)-anti-B[a]P)-7,8-dihydrodiol-9,10-epoxide (BPDE) which is the most important DNA reactive metabolite formed from benzo[\(a\)]pyrene (BaP) [13–15]. In a number of animal studies as well as in experiments with human derived cells [16], induction of GSTs by coffee and \(C + K\) was found and these effects were paralleled by protection towards chemical induction of tumors and DNA-damage [16–18]. Further evidence for the important role of GSTs in human cancer comes from a number of studies which show that functional polymorphisms of GST isozymes play a role in the aetiology of different forms of cancer [19,20].

Only a few studies are available in which the induction of GSTs by dietary factors was investigated in humans. It was shown that consumption of Brassica vegetables causes induction of overall GST and of specific isozymes in plasma of humans [21–23]. Only one comprehensive study on the effect of coffee is available: Grubben et al. [24] monitored the effect of unfiltered coffee on GST levels in colon mucosa cells. Neither the overall GST activity nor any of the isozymes GSTA, GSTP, GSTM were significantly induced, but a slight increase in the glutathione levels in the colorectal mucosa (8%) and plasma (15%) was found. Sreerama et al. [25] reported on induction of GST activity in saliva after coffee consumption but only one individual was monitored, therefore no firm conclusions can be drawn from this observation.

In the present study we investigated the effect of unfiltered coffee on the GST levels in serum and saliva in a controlled study. The activity of the enzyme was measured with 1-chloro-2,4-dinitrobenzene (CDNB) which is a substrate for several isozymes [26], and provides information on the overall GST status. Furthermore, we also measured GSTA and GSTP with ELISAs, two important isozymes which can be induced by vegetables [23,27,28]. To elucidate if the inducibility of GST depends on the genetic background, the GSTM1 and GSTP1 genotypes of the participants were determined by PCR, furthermore several clinical parameters were measured in the serum to exclude that the induction effects are a consequence of toxicity and organ damage. In an additional trial we compared the effects of unfiltered and paper filtered coffee on GSTP induction to find out if the effects depend on the diterpenoid contents. With paper filtered coffee, the amounts of lipids are normally below 0.2% (<0.6 mg total diterpenes/cup), whereas levels up to 20% of oil (6–20 mg diterpenes/cup) have been reported for boiled (Scandinavian), French Press and metal filtered coffees [29–33].
As mentioned above, it is known that GSTs play a key role in the detoxification of PAHs which are an important class of DNA-reactive carcinogens. Therefore, we conducted a further trial in which the impact of coffee consumption on the sensitivity of peripheral lymphocytes towards BPDE induced DNA-damage was monitored in single cell gel electrophoresis assays (SCGE, comet assay).

2. Materials and methods

2.1. Design of the study

Ten healthy, non-smoking, non-vegetarians (three males, seven females) participated in the first trial. The design of the study is depicted in Fig. 1. Fourteen days before the start and during the study, the volunteers (mean age: 26 ± 4 years, b.w.: 75 ± 9 kg) had to refrain from consumption of cruciferous vegetables, coffee and tea. The participants were also asked to reduce the consumption of alcoholic beverages and fruit juices (not more than 200 mL/d), to consume limited amounts of fruits and vegetables (not more than 200 g/d) and not to perform physical exercises. 500 g of ground coffee (“Brasil sanft”, Eduscho-Kaffee GmbH, Vienna, Austria) were boiled in 10.0 L tap water for 5 min, subsequently, the coffee (designated as “unfiltered coffee”) was pressed through a metal mesh (Phillips, Vienna, Austria). All individuals consumed seven cups (in total 1.0 L) of coffee daily over 5 consecutive days. The design of the second intervention was identical as that of the first. Fourteen individuals (mean age: 25 ± 6 year, b.w.: 74 ± 10 kg) different from those involved in the first trial participated. Seven consumed unfiltered coffee (prepared as described above) and seven consumed coffee which had been filtered through a paper filter (“Melitta Aromapor”, Melitta Haushaltsprodukte GmbH&Co. KG, Minden, Germany) for three days. Before and at the end of the interventions, blood and saliva were collected. The study was approved by the Austrian Ethical Commission and informed consent was obtained from all participants.

The third trial was conducted under identical conditions as the first study but peripheral lymphocytes were used in this experiment for single cell gel electrophoresis assay and other participants were involved. The average age of the participants was 26 ± 6 years and their average body weight was 72 ± 8 kg.

2.2. Preparation of samples

A 4.0 mg DTT (Sigma–Aldrich, St. Louis, MO, USA) were added to 5.0 mL of sputum in plastic vials which were centrifuged at 9000 × g for 15 min. A 10.0 mL of blood were aspirated by venipuncture and centrifuged in heparinised glass vials (Becton-Dickinson, Plymouth, UK) at 760 × g for 10 min. The saliva supernatants and the plasma samples were stored in liquid nitrogen. For single cell gel electrophoresis assays, the lymphocytes were isolated by Ficoll centrifugation [35].

2.3. Enzyme measurements

Protein contents of saliva and plasma were determined spectrophotometrically according to Bradford [36] with the BIO-RAD® Protein Assay (BIO-RAD®, Munich, Germany). GST activity in saliva and plasma was determined according to Habig et al. [37] with CDNB as substrate. The chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). Spectropho-
metrical measurements were carried out in triplicate (Beckman DU 640 spectrophotometer, Fullerton, CA, USA). GSTA and GSTP contents were determined in plasma with quantitative immunoassays (HEPKIT\textsuperscript{TM}-Alpha and HEPKIT\textsuperscript{TM}-Pi, Biotrin International Ltd., Dublin, Ireland).

2.4. Determination of GSTM1- and GSTP1 genotypes

DNA was isolated from buccal cells with the QIA-GEN blood kit (QIAGEN, Hilden, Germany) and stored in elution buffer (Perkin Elmer Cetus, Norwalk, USA) at \(-20\) °C. Polymorphisms in the GSTM1 gene locus were determined with PCR as described by Bell et al.\cite{[38]} in which \(\beta\)-globin was co-amplified as an internal standard. Amplification products (\(\beta\)-globin 268 bp, GSTM1 215 bp) were resolved on 2% ethidium bromide-stained gel with minor modifications \cite{[39]}.

For the determination of the GSTP1 polymorphism PCR/FLP assays, with separation of PCR products on a 3% ethidium bromide-stained gel were carried out according to Harries et al.\cite{[40]} with minor modifications \cite{[39]}.

2.5. Blood parameters

Spectrophotometrical determinations of creatinine-, cholesterol-, alanine aminotransferase-, aspartate aminotransferase- and alkaline phosphatase contents were performed with an automated spectrophotometer (SYS 3 BM/Hitachi 747/737, Boehringer Mannheim, Germany) according to standard protocols\cite{[41–43]}.

2.6. Single cell gel electrophoresis assays

The lymphocytes were resuspended in phosphate buffered saline (pH=7.4) and exposed to 0.4\(\mu\)M (±)-anti-B[\(\beta\])P-7,8-dihydrodiol-9,10-epoxide (BPDE, obtained from Biochemisches Institut für Umweltkarzinogene, Germany, CAS: 58917-67-2) dissolved in DMSO (containing 0.1% trietylamine, Sigma, St. Louis) at 37° C for 30 min. Subsequently the cells were washed with PBS, centrifuged (8 min, 110 \(\times\) g) and transferred to agarose coated slides, which were prepared and further processed according to the protocol of Collins et al.\cite{[44]}.

Comet formation was monitored with a computer aided image analysis system (for details see Helma and Uhl\cite{[45]}).

Per experimental point, three cultures were prepared and from each 50 cells were analysed. Only cultures were evaluated in which the viability of the cells was \(\geq 80\%\) compared to untreated controls. The viability of the cells was determined with trypan blue\cite{[46]}.

2.7. Statistical analyses

To determine statistical differences of the enzyme activities before and after the interventions, analyses of variance (ANOVA) and Wilcoxon tests were carried out. Correlations between gender, genotype, blood parameters, body weight and GST induction were analysed with Spearman’s rank correlation test.

Comet tests in vitro with BPDE were tested by analysis of covariance, comparisons of doses against control were done by Dunett’s test. For statistical analyses of the SCGE intervention study, the median and 90th percentiles of the distribution of the tail lengths were calculated and subjected to an analyses of variance with control versus BPDE and time points (before coffee consumption and after consumption) as experimental factors and subjects of random factor. Although the data showed no significant deviation from normality they were log-transformed to reach homogeneity of variances. Post hoc tests of means of experimental conditions were done by Tukey’s HSD tests. For all comparisons a \(p\)-value below 0.05 was considered significant.

3. Results

3.1. GST (CDNB) measurements in blood and saliva

The results of GST measurements in saliva are shown in Fig. 2a. Before the intervention, the average activity was 29.47±14.00 mIU/mg. At the end of the study a 1.13-fold (not significant, \(p = 0.290\)) increase was detected.

The results obtained with the plasma samples are depicted in Fig. 2b. The enzyme activities were approximately three orders of magnitude lower than in saliva. The background activity before the intervention was 0.037±0.011 mIU/mg, after the intervention,
Fig. 2. (a) and (b) Effect of consumption of unfiltered coffee (1 L/P/d) on GST activities (mIU/min/mg) in saliva (a) and plasma (b). The enzyme activities were determined according to Habig et al. [37], protein contents were determined according to Bradford. [36] Numbers on the x-axis are codes of the different participants. Striped bars: GST activity before coffee consumption, black bars: activity after consumption. Bars indicate mean ± S.D. of three measurements in parallel, numbers indicate codes of the different participants.

it was significantly increased (0.040 ± 0.017 mIU/mg, \( p = 0.042 \)). It is notable that the inter-individual fluctuations were smaller than in saliva.

3.2. Measurement of GSTA and GSTP

To find out which GST isozymes were induced, immunoassays for GSTA and GSTP were conducted with the plasma samples. It can be seen in Fig. 3a that GSTA was not affected (\( p = 0.440 \)) whereas a pronounced, highly significant (\( p = 0.005 \)) increase in GSTP was observed (Fig. 3b). Before the intervention, the mean GSTP content was 16.34 ± 6.11 ng/mL, at the end of the study it was 50.06 ± 12.53 ng/mL.

3.3. Genotyping of the participants

From the ten participants of the first study, four had an intact \( \text{GSTM1} \) gene (participant numbers 4, 9, 12, 13), six were \( \text{GSTM*0} \) (6, 7, 15, 16, 17, 18), four (12, 13, 16, 17) had the wildtype \( \text{GSTP1} \) gene, the others (4, 6, 7, 9, 15, 18) were heterozygous.

The results of Spearman’s rank correlation tests showed no significant correlations between induction of GST activity in plasma and increased GSTP contents (\( p = 1.0 \)) and also no relation between induction of overall GST, GSTP and GSTA and the different genotypes.
3.4. Clinical blood parameters

Data of the measurements of the blood parameters (creatinine-, cholesterol-, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase) are not shown in detail. With exception of serum cholesterol, none of them was enhanced after coffee consumption. The cholesterol level was 195.2 ± 37.7 mg/100 mL before the begin of the study and was increased by 6.5% after the intervention (mean 207.3 ± 25.7 mg/100 mL). However, this effect was not statistically significant (*p* = 0.06).

3.5. Comparison of GSTP induction by filtered and unfiltered coffee

The results of the second trial in which the effects of unfiltered and paper filtered coffee on GSTP induction were compared are depicted in Fig. 4 a and b. It is known that the bioactive coffee compounds C + K are retained by paper filtration [33,34]. Both types of coffee were prepared from the same brand and boiled for 5 min. As mentioned above, the C + K values are substantially lower in paper filtered coffee. Nevertheless, identical GSTP induction effects were seen with both preparations.

3.6. Results of SCGE experiments

In order to define the optimal concentration of BPDE for the intervention trial, a dose response experiment was carried out with lymphocytes from one individual. The results are depicted in Fig. 5. It can be seen that the diol induced DNA-migration over the entire range tested; already at the lowest dose (0.25 μM) a significant effect was observed.

The results of the intervention trial are summarised in Fig. 6. In all participants the DNA-damage induced by BPDE was substantially reduced after coffee consumption. Overall the protection effect caused by coffee was 45% and highly significant (*p* = 0.0001). In control cultures (i.e. in cells which were not treated with the diol), no difference in DNA migration was seen before and after the invention (*p* = 0.84).

4. Discussion

More than 20 years ago Wattenberg [9] showed that feeding of coffee beans to rodents leads to an increase of the GST activity in different organs and protects against tumor induction by PAHs [9]. Also for green tea which is rich in phenolics, protective effects towards dietary carcinogens via this mechanism is well documented [47]. The results of the present study show that consumption of coffee leads also in humans to a pronounced increase in the activity of this detoxifying enzyme. Furthermore, we demonstrated that this effect is paralleled by substantial protection.
Fig. 5. Induction of DNA migration in human lymphocytes by BPDE. The cells were exposed for 30 min (at 37°C) to different concentrations of BPDE. Per experimental point three cultures were prepared and from each 50 cells were analysed. Stars indicate statistical significance (p ≤ 0.05).

Fig. 6. Impact of coffee consumption on induction of DNA migration by BPDE in human lymphocytes. The participants consumed 1 L of unfiltered coffee per day over a period of 5 days, before and after the intervention, the cells were isolated and treated with BPDE (0.4 μM) or with the solvent (DMSO) only for 30 min. Subsequently, the viability and DNA migration were monitored. The survival of the cells was in all cases ≥80% (data not shown). Bars indicate mean ± S.D. of three cultures in parallel. From each culture, 50 cells were analysed for DNA migration. Numbers on the x-axis indicate individual participants. White bars: DNA migration before coffee consumption in control cultures, shaded bars: DNA migration in control cultures after coffee consumption, grey bars: DNA migration induced by BPDE before coffee consumption, black bars: DNA migration induced by BPDE after coffee consumption.

towards DNA-damage caused by BPDE in peripheral lymphocytes.

The present study was conducted under controlled dietary conditions. This has the advantage that individual fluctuations can be minimized. We found in earlier investigations that GST levels as well as DNA migration vary strongly when the measurements are conducted without dietary control. Due to the reduction of these fluctuations and the design of the study as an intervention trial it is possible to get meaningful results with a lower number of participants as those required in studies which are based on comparisons of different groups. Also in earlier human studies in which the impact of dietary factors on GST levels and on DNA-damage in lymphocytes were measured, similar or even smaller group sizes as in the present study were used [27,28,44,48–51]. The induction of GSTP as well as the protective effect towards DNA-damage caused by BPDE was seen in ≥90% of the participants and are statistically highly significant. For the negative results (lack of GST (CDNB) induction in saliva and lack of GSTA induction in plasma) we calculated the statistical power of our study to detect a 25% alteration (increase or decrease) of these parameters; for GST (CDNB) it was 84% and for GSTA it was 88%, respectively. This shows that the number of participants in our experiments was sufficient to detect relevant alterations. Only a few earlier studies on the induction of GSTs in humans by dietary factors are available. Apart from...
the coffee study by Grubben et al. [19] who did not find an effect on GSTs in colon mucosa after coffee consumption, all other investigations concerned the effects of vegetables. For example Nijhoff et al. [27,28] reported induction of GSTA with Brussels sprouts in males but not in females, more recently Lampe et al. [52] found induction effects by Brassica and Allium diets. Also with these vegetables, cancer protective effects were seen in animal experiments which were attributed to induction of GSTs [53]. The human glutathione biotransformation system consists of four major classes of GSTs namely GSTA, GSTM, GSTP and GSTT which differ in their tissue distribution and also in their substrate specificity [54]. In the aforementioned vegetable studies, the increase in overall GST was found in spectrophotometric assays with CDNB which is a substrate for different isozymes except GSTT [54]. Immunoassays showed induction of GSTA in the plasma [28,52] whereas GSTP levels were not altered [28], only in an experiment with intestinal biopsies, a significant induction of both isozymes was observed in rectal cells after consumption of Brussels sprouts [27]. In the present study, a different induction pattern was observed, i.e. the GSTA levels were not affected whereas a drastic increase in GSTP was observed.

GSTP is the most widely distributed enzyme of all GSTs and the most abundant form in many tissues except in the liver [55]. It is dominating for example in the lungs [56] and in the rectum [27], also in lymphocytes its activity is higher as that of other GSTs [54]. Approximately 50% of the Caucasians carry a mutation in the GSTP gene [40] and it is known that polymorphisms exist in humans in exons 5 and 6 which have functional effects on the gene product resulting in decreased enzyme activity [37–40]. A number of studies indicate that these polymorphisms are associated with increased risks for different forms of cancer in humans for example in the lung [61–64], breast [65,66], oesophagus [67], prostate [40,68], bladder [40], tests [40] and colon [69]. It is notable that some other studies failed to find correlations between GSTP polymorphisms and specific forms of cancer, for example no associations were seen in two other studies on colon cancer [61,70] and two Swedish investigations did not detect associations with prostate cancer incidence. Nevertheless, the overall evidence strongly suggests that the GSTP status has an impact on cancer risks in humans. The observation that coffee consumption reduces BPDE induced DNA-damage was not unexpected. The protective effect of GSTP towards chemical carcinogens was shown earlier in a number of genotoxicity studies which indicated that this enzyme detoxifies representatives of different classes of DNA-reactive carcinogens. Ryberg et al. [62] found increased hydrophobic DNA-adducts in lung tissues of cancer patients (smokers) who had the GG phenotype. The GG phenotype (GSTP1-valin variant) causes formation of an altered enzyme protein which has lower affinity to CDNB, but a greater affinity towards other substrates (bromosulphthalein and ethacrylic acid) [71]. In another study with smokers, no effect of GSTP1 mutations on adduct formation in peripheral white blood cells was found, but in combination with GSTM1 null genotypes a significant increase was detected [72]. No such combination effects were seen in a similar study in lung cells, but again in blood cells the adduct levels were increased in smokers with the GSTM1 null genotype and GSTP1 (Ile105/Val105 or Val105/Val105). These findings were explained by the fact that GSTP catalyses the detoxification of BPDE (the ultimate DNA-reactive metabolite of 3,4-benz[alpyrene (B(a)P) and other diols [73–75]). Consistent with these biochemical data it was shown that GSTP transfected cells are more resistant towards BPDE induced DNA-damage than normal cells [13–15] and enhanced skin tumorigenesis by 7,12-dimethylbenz[a]anthracene (DMBA) was found in knockout mice lacking class GSTP [76]. GSTP plays also an important role in protection towards other DNA-reactive carcinogens. In workers exposed to styrene oxide, a higher number of HPRT mutations was found in individuals with heterozygosity of the GSTP1 gene [77]. These effects towards styrene oxide were further confirmed in in vitro micronucleus experiments with human lymphocytes [78]. Protective effects of GSTP were also reported against the heterocyclic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in experiments with human liver and prostate cells [79,80], towards the UV mimetic compound 4-nitroquinoline-1-oxide (4-NQO), alkylating agents [81–84] and against different DNA-reactive anticancer drugs such as cis-platin, carbo-platin and thiopeta [85].
The results described in the last paragraphs indicate that GSTP plays an important role in the protection towards DNA-reactive carcinogens (in particular towards PAHs) and that altered activity (due to polymorphisms) is associated with increased cancer risks. The strong increase in the activity of this isozyme (two- to six-fold) seen in the present study supports the assumption that coffee consumption has beneficial effects in humans in terms of reducing their cancer risks and is supported by the results of the SCGE experiments which clearly show a protective effect of coffee consumption towards BPDE induced DNA-damage in peripheral human lymphocytes. It is notable, that we observed also pronounced enzyme induction effects in individuals who were heterozygous in the \textit{GSTP1} gene. This indicates that the upregulation is not affected by base mutations in the \textit{GSTP1} allele.

In the present study we were interested initially in overall GSTP induction effects in the body and not in the alterations of this enzyme in the lymphocytes. However, it is not possible to rule out on the basis of the present findings in which cells/organisms induction effects take place. This question will be clarified in further animal studies which are in progress. It is known that extracellular GSTP induction may be due to toxic effects but since no alterations in the clinical parameters were seen and no changes of the viability of the lymphocytes before and after coffee consumption were observed, this possibility can be excluded. As mentioned above, strong protective effects towards BPDE were seen in the lymphocytes. Since it is known that GSTP protects against DNA damage and that lymphocytes possess this enzyme, this can be taken as indication that GSTP induction takes also place intracellularly in the blood cells.

One of the mechanisms which may account for the GST induction is the increase in the glutathione concentrations by coffee consumption in plasma which was seen in two human intervention studies [19,86]. It is conceivable that higher co-substrate levels lead to an enhanced activity of the enzyme.

The results of the second intervention trial in which we compared the effects of unfiltered and paper filtered coffee on GSTP induction were unexpected. As shown in Fig. 3 no significant difference in GSTA induction was seen although the two types of coffee differed substantially in their diterpenoid contents due to different preparation procedures [29–33]. In many earlier studies, it was claimed that the DNA- and cancer protective effects of coffee as well as the induction of GST and other enzymes might be due to the coffee specific diterpenoids C + K [17]. However, it was also shown by Wattenberg and Lam [18] that C + K account only for 40% of GST (CDNB) inducing activity of coffee in the mouse liver and Esposito et al. [86] found induction of glutathione after consumption of coffees with low C + K contents. Since C + K leads to hypercholesteremic effects [29,30] it is important that the increase in the cancer protective enzyme system was also seen after consumption of a coffee preparation which had low diterpenoid levels. In this context, it is notable that it has been postulated that, apart from C + K other coffee components, in particular polyphenols such as chlorogenic acids and melanoidins produced during the roasting process, may also account for GSH and GST induction [86–88].

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