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Does an Extract of Carob (*Ceratonia siliqua L.*) Have Chemopreventive Potential Related To Oxidative Stress and Drug Metabolism in Human Colon Cells?

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Phenolic ingredients of an aqueous carob extract are well characterized and consist of mainly gallic acid (GA). In order to assess possible chemopreventive mechanisms of carob, which can be used as a cacao substitute, effects on expression of genes related to stress response and drug metabolism were studied using human colon cell lines of different transformation state (LT97 and HT29). Stress-related genes, namely catalase (CAT) and superoxide dismutase (SOD2), were induced by carob extract and GA in LT97 adenoma, but not in HT29 carcinoma cells. Although corresponding protein products and enzyme activities were not elevated, pretreatment with carob extract and GA for 24 h reduced DNA damage in cells challenged with hydrogen peroxide (H_2O_2). In conclusion, carob extract and its major phenolic ingredient GA modulate gene expression and protect colon adenoma cells from genotoxic impact of H_2O_2. Upregulation of stress-response genes could not be related to functional consequences.

KEYWORDS: Carob; *Ceratonia siliqua L.*; gene expression; gallic acid; chemoprevention

1. INTRODUCTION

Colorectal cancer was the second most common cancer (>400 000 cases) after breast cancer and the second most common cause of death after lung cancer in Europe 2006 (*1*). For this reason, chemoprevention of this cancer is a major concern for improving public health. Ten years ago, epidemiological data pointed to positive effects of high consumption of vegetables, which became limited suggestive for vegetable and fruit consumption in the latest report of the World Cancer Research Fund (*2*). Basically, vegetables and fruits are high in concentrations of dietary fiber, polyphenols and other micronutrients. Investigation of both dietary fiber and polyphenols is challenging because both terms represent a group of rather heterogeneous substances. A recently published result of a large prospective case-control study showed a strong and linear inverse association of flavonoids with colorectal cancer risk, which makes it reasonable to study molecular mechanisms of this type of food ingredient (*3*).

Carob pods are the fruits of the carob tree that is cultivated in Mediterranean countries and used as a cocoa substitute in human foods (*4*). Carob is produced by water extraction of the pulp of carob pods to remove the majority of soluble carbohydrates (*5*). This process yields a product that contains mostly dietary fiber, as well as a phenolic fraction consisting of water-soluble and insoluble tannins, flavonol glycosides, and high contents of different forms of gallic acid (GA): free GA, gallotannins, and methyl gallate (*6*). It has already been shown that carob has antioxidative activity (*4*), modulates blood lipid profile in humans (*7*) and reduces growth of different cell lines (*8*). An aqueous extract of carob mainly consists of unbound GA (~73 wt %), but also catechin, myricetin rhamnoside, eriodictyol glycoside, quercetin glycoside and quercetin rhamnoside were detected (*8*). GA is ubiquitously distributed in vegetable food, mainly in its conjugated form. From this it is liberated by bacterial fermentation processes and is easily absorbed into blood circulation (*9*). After administration, GA reaches a maximum plasma concentration of about 2 μM after 1.3 ± 0.2 h and shows a half-life of about 1 h (*9, 10*). Comparable results after consumption of carob are not available. In addition, carob ingredients may reach the gut directly and some ingredients of carob such as GA, catechin and quercetin were already detected in fecal water of healthy individuals (*11, 12*). GA has been proven to be antioxidative using different methods (*13*). In cell based systems it was shown to be anti-apoptotic (*14*) as well as pro-apoptotic (*15*) and antiproliferative (*16*). Moreover, it inhibited H_2O_2 induced cytotoxicity (*17*), but shows cytotoxicity if applied at high (nonphysiological) concentrations due to formation of H_2O_2 in the tested cell culture medium (*18*).

In the present study, the impact of carob extract on the expression of genes related to drug metabolism and stress response in HT29 carcinoma and LT97 adenoma cells was studied using two pathway-related cDNA arrays. These genes are of interest to elucidate possible chemopreventive effects since the protein

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Table 1. Phenolic Composition of Carob Extract (in μM) Prepared in HT29 and LT97 Cell Culture Medium, Respectively, for Concentrations Used for the Experiments (Data Based on Ref *8*)

	HT29 cell culture medium				LT97 cell culture medium			
	0.25 g/L	0.5 g/L	1 g/L	2 g/L	0.25 g/L	0.5 g/L	1 g/L	2 g/L
gallic acid	2.21	4.42	8.84	17.69	1.94	3.88	7.76	15.53
catechin	0.04	0.08	0.16	0.32	0.17	0.35	0.69	1.39
myricetin rhamnoside	0.08	0.16	0.33	0.66	0.08	0.15	0.31	0.62
eriodictyol glucoside	0.02	0.03	0.07	0.14	0.03	0.06	0.13	0.26
quercetin glucoside	0.02	0.04	0.07	0.14	0.03	0.05	0.11	0.22
quercetin rhamnoside	0.14	0.28	0.56	1.12	0.10	0.20	0.40	0.80
total	2.51	5.02	10.04	20.07	2.35	4.70	9.40	18.81

products may largely contribute to detoxification of carcinogenic factors as it has been discussed for the example of glutathione *S*-transferases (GST) and colorectal cancer (*19*). Concentrations of carob extract and GA used here were based on previously determined effective concentrations from cell proliferation assays and on present knowledge of physiological detectable range of GA (*8, 10*−*12*). Some genes were chosen for detailed real-time PCR analysis on account of their potential roles in chemoprevention and on account of their changed levels of expression in the cDNA arrays. Verification with real-time PCR (for *CAT*, *SOD2*, *peroxiredoxin* [*PRDX1*, *PRDX2*], *glutathione peroxidase* [*GPX1*], *X-ray repair, complementing defective, in Chinese hamster, 1* [*XRCC1*], *heat shock 70 kDa protein 8* [*HSPA8*] and *heat shock 90 kDa protein 1 alpha* [*HSPCA*]) was conducted at different time points, and effects were compared to results obtained with equimolar concentrations of GA. Additionally, expression of corresponding proteins or levels of activities of the resulting enzymes (for CAT and SOD2) and functional consequences (cellular protection against genotoxic H_2O_2) were studied using the different assays.

2. MATERIAL AND METHODS

2.1. Preparation of Carob Extract and GA. Preparation of GA (Fluka, Seelze, Germany) and carob extract are described in ref *8*. Concentrations of carob extract used in this study were based on previously determined effective concentrations from cell proliferation assays (*8*) and on present knowledge of physiological detectable range of GA (*9, 11, 12*). The phenolic composition of carob extract differed slightly in both cell culture media and is presented in **Table 1** for the applied concentrations. Because GA amounted to 74 wt % and 68 wt % in carob extract prepared with HT29 and LT97 cell culture medium, respectively, equimolar concentrations of GA were used additionally (**Table 1**). The aqueous extract of carob and the dilution of GA were prepared immediately before use.

2.2. Cell Lines and Culture Conditions. The human colon cell line HT29 was maintained according to ref *20*. The human colorectal adenoma cell line LT97 was described in ref *21* and was maintained in a culture medium (MCDB 302, Biochrom AG, Berlin, Germany) containing 20% of L15 Leibovitz medium, 1 μg/mL hydrocortisone, 10 μg/mL insulin, 5 nM sodium selenite (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), 2% FCS, 50 μg/mL gentamycin, 2 μg/mL transferrin, 0.4 mM L-glutamine (Invitrogen GmbH, Karlsruhe, Germany), 0.2 nM trijodo-L-thyronine (ICN, Ohio, USA), and 30 ng/mL epidermal growth factor (Merck Biosciences GmbH, Schwalbach, Germany). Both cell lines were maintained at 37 °C in a humidified incubator (5% CO_2, 95% humidity).

2.3. Treatment of Cells with Carob Extract and GA. Cells were grown in cell culture flasks until they reached ~70% confluence (24 h for HT29 cells and 2−4 days for LT97 cells). Then they were treated for 6−48 h with carob extract (0.5 or 2 g/L) or with GA concentrations corresponding to the amounts contained in the complete extract. After harvesting of the cells, total cell number and percentage of viability were determined with trypan blue (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in a hemacytometer.

Table 2. Primer Sequences for Real-Time PCR

gene	forward	reverse
GAPDH	5′-ACCCACTCCTCCACCTTTGAC-3′	5′-TCCACCACCCTGTTGCTGTAG-3′
CAT	5′-TGGACAAGTACAATGCTGAG-3′	5′-TTACACGGATGAACGCTAAG-3′
SOD2	5′-GCCCTGGAACCTCACATCAAC-3′	5′-CAA CGC CTC CTG GTA CTT CTC-3′
GPX1	5′- GACTACACCCAGATGAACGA-3′	5′- ACGTACTTGAGGGAATTCAG-3′
PRDX1	5′- TCTAGCATGGGTCAATACAC-3′	5′- CCTTCATCAGCCTTTAAGAC-3′
PRDX2	5′-TAATGATTTGCCTGTGGGAC-3′	5′- TATTCCTTGCTGTCATCCAC-3′
XRCC1	5′-CAAAGGGAAGAGGAAGTTGG-3′	5′-GAGCTGGCAATTTAGGTCTC-3′
HSPA8	5′-TTTATGGTGGTGAATGATGCTG-3′	5′-CATTGGTAACAGTCTTCCCA-3′
HSPCA	5′-TGATAAGAACGACAAGTCTGTG-3′	5′-CATCTTCATCAATACCCAGACC-3′

2.4. Isolation of RNA. Total RNA was isolated from the cells using RNeasy Mini Kit (Qiagen, Hilden, Germany), dissolved in 50 μL of RNase free water and stored at −20 °C until further use. RNA quantification was done spectrophotometrically (Eppendorf BioPhotometer, Hamburg, Germany) and by using formaldehyde denaturing RNA gel electrophoresis (1.5%) to check integrity of rRNA and for DNA contamination before further processing.

2.5. Gene Expression Analysis Using cDNA Array. Gene expression profiles were studied with two pathway specific cDNA arrays from SuperArray Bioscience Corporation, each spotted with 96 target genes, negative controls and 4 different housekeeping genes (GEArray Q Series Human Drug Metabolism Gene Array HS-011 and GEArray Q Series Human Stress and Toxicity Gene Array HS-012, SuperArray Bioscience Corporation; Frederick, MD). One array contained genes encoding products involved in drug metabolism, namely phase I, phase II, and phase III enzymes. The other array was spotted with gene sequences related to stress and toxicity response, namely oxidative/metabolic stress, heat shock, proliferation/carcinogenesis, growth arrest, inflammation, DNA damage and repair and apoptosis. Workup of the gene arrays was done according to the manufacturer's protocol, and the techniques have been described in detail in ref *22*. Signal intensities were normalized by setting spot intensities of *GAPDH* to equal 100%. Only genes with signals above 1.5-fold of the blank in all three experiments were denoted as being expressed. Each set of experiments have been repeated three times independently.

2.6. Gene Expression Analysis Using Real-Time PCR. Expression of selected target genes was analyzed with 2.5 μg of total RNA converted into first-strand cDNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen GmbH, Karlsruhe, Germany). Real-time PCR was carried out using specific primer pairs and iQTM SYBR Green Supermix (Bio-Rad GmbH, Munich, Germany). All reactions were performed in duplicate. The PCR reaction profile included an initial denaturation of 2 min at 95 °C, 40 cycles of denaturing (30 s at 94 °C), annealing (30 s at 57−60 °C, depending on primer pairs), and extension (40 s at 72 °C), and finally an extension step of 10 min at 72 °C. Cumulative fluorescence was measured at the end of the extension phase of each cycle. Product-specific amplification was confirmed by melting curve analysis. Gene-specific primer sequences used for the quantification are shown in **Table 2**. Confirmation of the total gene specificity for primer nucleotides was performed using the NCBI-BLASTN search program. Gene expression of target genes was calculated on the basis of the housekeeping gene *GAPDH*. Changes of expression were determined as the fold change of the medium control.

2.7. Western Blot. Cytosols of cells treated with carob extract or GA were isolated by ultrasonic cell destruction and centrifugation (16000*g*) and subjected to SDS−PAGE. Separation was carried out using 12% polyacrylamide gel. The proteins were electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using a semidry blotter (CTI, Idstein/Taunus, Germany). Incubation with primary (antihuman CAT 60−65 kDa, dilution 1:1000, Calbiochem, antihuman SOD2 25 kDa, dilution 1:1000, LabFrontier, or antihuman Actin 42 kDa, dilution 1:20000, Sigma-Aldrich) and secondary antibodies (1:1000 dilutions, Dako, Hamburg, Germany) was carried out for 1 h at room temperature. For immunodetection, enhanced chemiluminescence was used. The level of actin served as an internal control. Films were scanned using "FluorS" imaging system (Biorad, Munich, Germany), and the relative protein expression levels were quantified by densitometric measurement.

2.8. Enzyme Activities of CAT and SOD2. CAT activity was calculated according to Aebi et al. (*23*). Briefly, after treatment of LT97 cells for 24 and 48 h with carob extract or GA, cytosols were prepared as described above. Decomposition of 10 mM H_2O_2 was photometrically measured at 240 nm after addition of the cytosol for 2 min. Results were calculated on the basis of cytosolic protein content (*24*) which was not modified by the treatment.

SOD2 activity was calculated using Superoxide Dismutase Assay Kit II (Merck Biosciences, Darmstadt, Germany). This method utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The SOD assay measures all types of SOD. Inhibition of SOD1 using NaCN (3 mM) allowed detection of SOD2 activity. SOD2 is a mitochondrial enzyme, and destruction of mitochondria by ultrasonic treatment of the cells was verified by measurement of citrate synthase, which is commonly used as a quantitative marker enzyme of intact mitochondria.

2.9. Detection of DNA Damage. HT29 and LT97 cells were pretreated with 0.25, 0.5, 1 and 2 g/L carob extract or equimolar GA concentrations (**Table 1**) for 24 h. DNA damage was measured using single cell microgelelectrophoresis after challenging cells with 75 μM H_2O_2 for 15 min at 37 °C as it has been described elsewhere (*25*). The intensity of fluorescence in the comet tail (% tail intensity = TI) was used as the evaluation criteria. For each concentration, means of 150 cells were the basis for calculating effects in one experiment. Presented effects are the means of at least three independently reproduced experiments.

2.10. Statistics. The Prism software version 4.01 (Graph Pad, San Diego, CA) was used to calculate significances. Evaluation of modified gene expression was done by calculating the fold change, two-sided, paired *t* test for each gene and two-way ANOVA (matched) with Bonferroni posttests within the diverse functional groups. Real-time PCR, Western blot, enzyme activity and comet assay results were evaluated with one-way ANOVA with Bonferroni posttests for each test compound and time point separately against the medium control.

3. RESULTS

3.1. Effect of Carob Extract on Gene Expression Determined with Pathway Specific cDNA Arrays. HT29 carcinoma and LT97 adenoma cells were treated with carob extract (2 g/L for 24 h), and two different pathway specific cDNA arrays were applied to identify modulated gene expression. On the array membrane spotted with 96 genes encoding drug metabolizing enzymes, 50 and 59 genes were expressed in HT29 and LT97 cells, respectively. 48 genes were expressed in both cell lines (**Figure 1A**). Four genes were modified by treatment with carob extract in either HT29 or LT97 cells. These belonged to the 48 genes expressed in both cell lines and were *ATP-binding cassette, subfamily G, member 2* (*ABCG2*), *cytochrome P450 family 2 subfamily B polypeptide 6* (*CYP2B6*), *carbohydrate (N-acetylglucosamine 6-O)-sulfotransferase 7* (*CHST7*), and *microsomal glutathione S-transferase 3* (*MGST3*). *ABCG2* was downregulated after treatment with carob extract (fold change: 0.7 ± 0.4, in HT29). Its basic expression level was significantly higher in HT29 than in LT97 cells. *CYP2B6* and *CHST7* were both upregulated in HT29 cells (fold change 1.8 ± 1.0 and 14.1 ± 22.2, respectively). Their basic expression levels in both cell lines were not significantly different. In LT97 cells, *MGST3* was only marginally induced (1.2 ± 0.3). Due to high variation of the fold changes of these four genes, they were not selected for more detailed analysis.

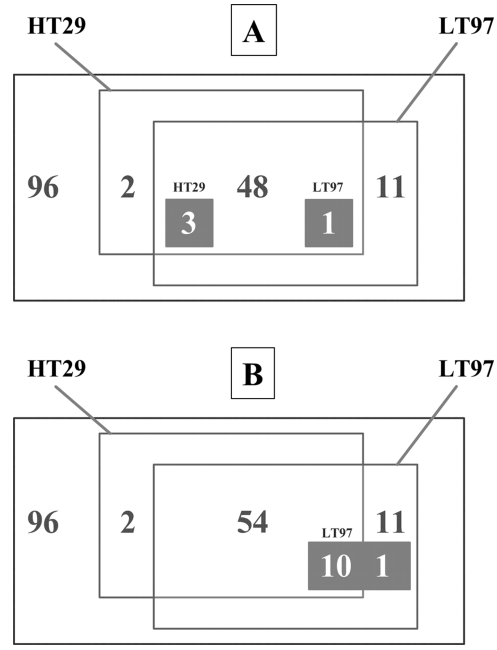

Figure 1. Venn diagram of available genes on cDNA array: (**A**) HS-011 (human drug metabolism gene array) and (**B**) HS-012 (human stress and toxicity pathfinder gene array). Black numbers indicate expressed genes in HT29 and LT97 cells and white numbers the amount of modulated genes in either cell line after treatment with carob extract for 24 h. Significant modulations were tested with two-way ANOVA and Bonferroni posttests within the functional groups, $n = 3$.

On the gene array which was spotted with genes encoding stress related enzymes, 56 and 65 genes of the 96 spotted genes were expressed in the medium controls of HT29 and LT97 cells, respectively (**Figure 1B**). 54 genes were detectable in both colon cell lines. Based on one-way ANOVA analysis within the functional groups, none of the stress related genes were affected by carob extract (2 g/L, 24 h) in HT29 cells. In contrast, 11 genes were significantly modulated in LT97 cells. Ten of these were also expressed, but not modulated by carob treatment in HT29 cells. Only *cytochrome P450 family 1 subfamily A polypeptide 1*, which was induced in LT97 cells, was not expressed in HT29 cells according to the cDNA array analysis. All genes that were significantly modulated in LT97 cells by carob treatment (using two-way ANOVA with Bonferronis posttest within the functional groups) are summarized in the Supporting Information. Some of them were also significantly modulated according to the *t* test. Both statistical methods bear some disadvantages, thus both tests were considered for identification of interesting target genes. Based on these statistical calculations and the extent and variation of the fold changes, 5 genes (*CAT*, *SOD2*, two heat shock proteins (*HSP*) and *XRCC1*) were selected for more in-depth analysis using real-time PCR.

3.2. Evaluation of cDNA Array Results of Five Selected Target Genes in LT97 Cells with Real-Time PCR. To cover not only the effective concentration of carob extract used for cDNA array analysis but also physiological concentration range noted for GA, one additional and four times lower concentration

Table 3. Confirmation of cDNA Array Results for *CAT* and *SOD2* in *LT97* Cells Treated with Carob Extract (0.5 g/L and 2 g/L) and GA (4 μM and 15.5 μM) for 6, 12, 24, and 48 h[a]

			CAT			SOD2		
			mean	SD		mean	SD	
6 h	carob extract	0.5 g/L	1.16	0.19		1.50	0.64	
		2 g/L	1.10	0.10		1.43	0.30	
	GA	4 μM	1.15	0.20		1.39	0.37	
		15.5 μM	1.11	0.14		1.01	0.40	
12 h	carob extract	0.5 g/L	0.60	0.15	*	0.73	0.14	
		2 g/L	0.82	0.18		1.15	0.18	
	GA	4 μM	0.85	0.17		0.77	0.01	
		15.5 μM	0.60	0.14	*	1.03	0.31	
24 h	carob extract	0.5 g/L	1.28	0.08	*	2.78	0.63	**
		2 g/L	1.09	0.11		2.25	0.26	*
	GA	4 μM	1.27	0.17	*	2.64	0.47	**
		15.5 μM	1.38	0.02	**	2.17	0.55	*
48 h	carob extract	0.5 g/L	0.71	0.17		0.76	0.19	
		2 g/L	0.81	0.17		0.98	0.47	
	GA	4 μM	0.66	0.11		0.64	0.17	
		15.5 μM	0.80	0.28		0.84	0.25	

[a] Results are normalized based on *GAPDH*, calculated to the medium control and expressed as fold change. Mean and SD of fold change of three independent experiments are presented. Statistical analysis was performed with one-way ANOVA with Bonferroni posttests for each test compound and time point separately against medium control, * $p < 0.05$, ** $p < 0.01$, $n = 3$.

of carob extract and equimolar concentrations of GA were applied and analyzed after additional time points, namely 6, 12, 24 and 48 h treatment of the cells.

Table 3 shows that induction of *CAT* after 24 h was confirmed for the lower concentration of carob extract (0.5 g/L) and for both GA concentrations. Modulation of *SOD2* gene expression detected with cDNA array was confirmed with real-time PCR for both concentrations of carob extract and GA, but induction was only detectable after treatment for 24 h (and not after 6, 12, and 48 h).

Upregulation of *XRCC1*, *HSPA8* and *HSPCA* after treatment with 2 g/L carob extract for 24 h, which was detected using cDNA array, was not confirmed with real-time PCR for either carob extract (0.5 and 2 g/L) or GA (4 and 15.5 μM, data not shown). In addition, expression of *PRDX1*, *PRDX2* and *GPX1* genes, which are not spotted on the cDNA arrays, but of putative interest regarding stress response as measured with the comet assay, was not modulated by carob extract and GA (**Table 4**).

3.3. Protein Expression of CAT and SOD2. Protein expression of both CAT and SOD2 was analyzed after treatment of LT97 cells for 24 and 48 h using Western blot. Neither carob extract (0.5 g/L and 2 g/L) nor GA (4 μM and 15.5 μM) modified the amount of either enzyme (data not shown).

3.4. Enzyme Activities. Neither CAT activity nor SOD2 activity was affected after 24 and 48 h treatment with carob extract (0.5 and 2 g/L) or GA (4 and 16 μM) in LT97 cells (data not shown).

3.5. Functional Consequences. To analyze whether treatment with either carob extract or GA caused an improved protection against oxidative stress, LT97 cells, and in comparison HT29 cells, were first treated for 24 h with either test compound and were then challenged with H_2O_2 (in the absence of the test compounds). Treatment of LT97 cells with 1 and 2 g/L carob extract (**Figure 2A**) or with 3.9 μM GA (equivalent to 0.5 g/L carob extract, **Figure 2B**) resulted in significantly reduced levels of DNA damage induced by H_2O_2. Neither carob extract nor GA induced DNA damage on its own. Treatment for 48 h also reduced H_2O_2 induced DNA damage, but effects

Table 4. Modulated Gene Expression of *PRDX1*, *PRXD2* and *GPX1* in LT97 Cells Treated with Carob Extract (0.5 g/L and 2 g/L) and GA (4 μM and 15.5 μM) for 6, 12, and 24 h Using Real-Time PCR[a]

			PRDX1		PRDX2		GPX1	
			mean	SD	mean	SD	mean	SD
6 h	carob extract	0.5 g/L	1.30	0.55	0.83	0.09	0.91	0.06
		2 g/L	1.23	0.12	1.14	0.38	1.12	0.16
	GA	4 μM	1.16	0.22	0.83	0.14	0.95	0.24
		15.5 μM	1.28	0.08	0.93	0.23	0.96	0.07
12 h	carob extract	0.5 g/L	0.81	0.09	1.27	0.31	1.64	0.99
		2 g/L	0.98	0.02	1.46	0.60	2.09	1.66
	GA	4 μM	0.71	0.04	1.59	0.69	1.93	1.58
		15.5 μM	1.01	0.09	1.23	0.24	1.58	1.00
24 h	carob extract	0.5 g/L	1.20	0.17	0.95	0.36	1.13	0.18
		2 g/L	1.08	0.26	1.24	0.36	1.09	0.36
	GA	4 μM	1.06	0.37	1.15	0.53	1.02	0.12
		15.5 μM	1.20	0.32	0.89	0.20	0.81	0.26

[a] Results are normalized based on *GAPDH*, calculated to the medium control and expressed as fold change. Mean and SD of fold change of three independent experiments are presented. Statistical analyses was performed with one-way ANOVA for each test compound and time point separately against the medium control, $n = 3$.

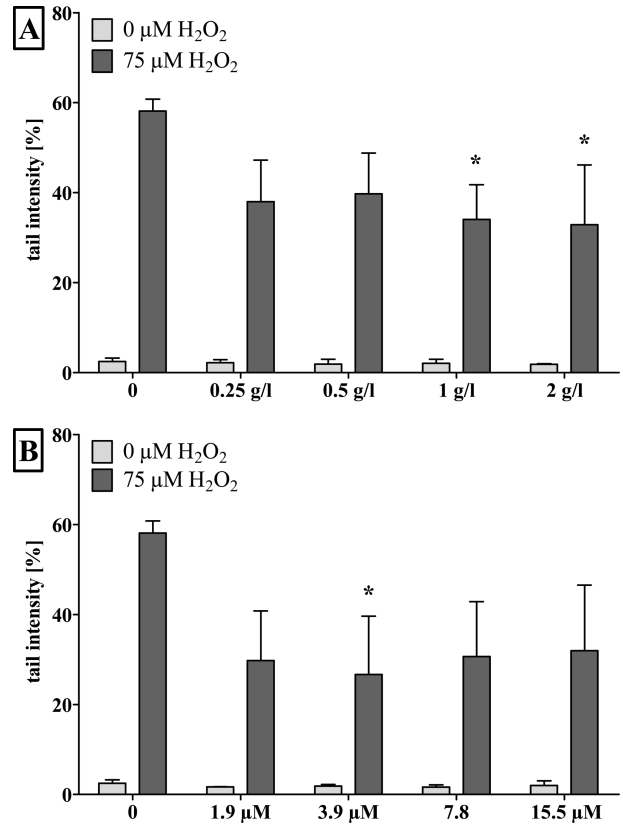

Figure 2. Modulation of H_2O_2 induced DNA damage by pretreatment with (**A**) carob extract (0.25−2 g/L) and (**B**) equimolar concentration of GA (1.9−15.5 μM) for 24 h in LT97 cells. Statistical analysis was performed with one-way ANOVA Bonferroni posttests, * $p < 0.05$, $n = 3$.

for both carob extract and GA were not significant anymore (data not shown). Equivalent treatment of HT29 cells did not result in any observable protective impact (**Figure 3**). No cytotoxic impact of either substance was detected at the applied concentrations.

Polyphenols are also known for their direct antioxidative capacity. For this reason carob extract and GA were also investigated for their ability to protect LT97 cells against H_2O_2 induced DNA damage following simultaneous treatment. Even

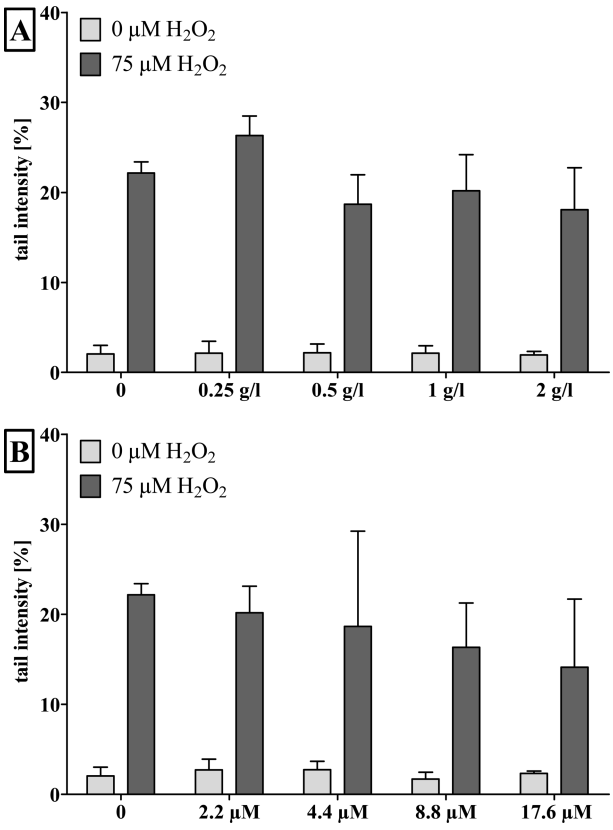

Figure 3. Modulation of H_2O_2 induced DNA damage by pretreatment with (**A**) carob extract (0.25−2 g/L) and (**B**) equimolar concentration of GA (2.2−17.6 μM) for 24 h in HT29 cells. Statistical analysis was performed with one-way ANOVA, $n = 4$.

though GA reduced induced DNA damage this result failed to reach statistical significance (data not shown).

4. DISCUSSION

Carob contains a spectrum of polyphenols (*6*). A water soluble fraction of carob reduced the number of HT29 colon carcinoma as well as LT97 colon adenoma cells in a time and dose dependent manner (*8*). These effects were based on the inhibition of cell proliferation in both cell lines and thereby represent a basic mechanism of cancer chemoprevention.

Another mechanism that refers to improvement of cellular protection was investigated here. Among genes found to be modulated in LT97 cells, modulation of *CAT* and *SOD2* was in part confirmed using real-time PCR. Further analysis on protein and activity levels was performed since these proteins may contribute to detoxification of ROS and colorectal carcinogenesis is associated with serious oxidative stress (*26*). For both enzymes modulation by dietary factors is known and linked to carcinogenesis (*27, 28*). Elevated mRNA levels could lead to higher protein amounts. However, protein levels of CAT and SOD2 did not change following treatment with carob extract and GA after 24 or 48 h and activity of neither enzyme was increased. While SOD2 activity was measured specifically by inactivation of SOD1, detection of CAT activity rather measures the overall ability of the cells to decompose H_2O_2 (*23*). Besides CAT, there are two other classes of peroxidases in the cells, namely glutathione peroxidases (GPX) and peroxiredoxins (PRDX) (*29*). PRDX is irreversibly inactivated during in vitro enzyme reactions (CAT activity assay) due to overoxidation and cannot be detected using CAT activity assay, but GPX might interfere and influences CAT activity results (*29*). Because of the protective activity of a preincubation with carob extract and GA observed with the comet assay, gene expression of *GPX1*, *PRDX1* and *PRDX2*, that were not available on both arrays, was therefore also analyzed using real-time PCR (after treatment for 6, 12 and 24 h), but they were not modulated in LT97 cells. For this reason, there are probably other antioxidative mechanisms that lead to reduced DNA damage by pretreatment with carob extract and GA. In contrast to carob extract, GA tended also to be directly antioxidative. This observation is in line with a study by Glei et al. in which the single compound chlorogenic acid protected HT29 cells from H_2O_2 induced DNA damage after short time incubation while the complex green coffee extract did not (*20*). Neither carob extract nor GA showed possible pro-oxidant properties based on genotoxicity at applied concentrations (*30*).

Table 5. Content of GA, catechin and quercetin [in μM] in fecal samples of several individuals (*11, 12*) and concentrations detected in carob extract prepared in HT29 and LT97 cell culture medium (*8*)[a]

	fecal content		carob extract	
	Jenner (2005)	Halliwell (2005)	Klenow (2008)	
	$n = 5$	$n = 15$	2 g/L	0.5 g/L
GA	0.17−1.40	0.17−2.31	17.69/15.53	4.42/3.88
catechin	0.02−0.31	0.00−0.31	0.32/1.39	0.08/0.35
quercetin	0.16−1.30	0.00−2.31		

[a] Quercetin was not detected as an aglycon in the extract but as glycosides.

Predictability of in vivo effects from in vitro results is often difficult. In part it is based on nonphysiological concentrations applied in vitro (*18*). In this study concentrations of GA were used that are in the range of achievable reported plasma concentrations (*9*). Catechin, applied in a range that refers to the normal daily intake (35 mg), results in plasma concentrations below 0.1 μM, which is also comparable to applied concentrations via carob extract (*10*). Anyway, it is even more likely that carob acts directly in the gastrointestinal tract. GA, catechin and other compounds are detectable in fecal water (without supplementation) in concentrations that comply with contents in carob extract applied in this study (**Table 5**) (*8, 11, 12*). In addition, polyphenols are metabolized in the gut. Further studies are needed to identify the metabolites and their impact on gut health.

In conclusion, the effects of carob extract in LT97 adenoma cells point to mechanisms of cellular protection against factors of oxidative stress, in particular H_2O_2. In contrast to the inhibition of cell proliferation by carob extract (*8*), modulation of gene expression and protection against induced DNA damage could be linked to GA as the main ingredient of carob extract. Furthermore, these protective effects were not detected in HT29 carcinoma cells, in which this could be linked to unwanted chemoresistance. Protection against oxidative stress in adenoma cells might defend against or slow down the carcinogenic process. Since the upregulation of genes encoding for proteins that are involved in deactivating ROS was not accompanied by modulation of protein and activity levels, other mechanisms for protection against H_2O_2 need to be identified.

ABBREVIATIONS USED

ABCG2, ATP-binding cassette, subfamily G, member 2; CAT, catalase; CHST7, carbohydrate (*N*-acetylglucosamine 6-*O*)-sulfotransferase 7; CYP2B6, cytochrome P450 family 2 subfamily B polypeptide 6; GA, gallic acid; GPX, glutathione peroxidase; HSP, heat shock protein; HSPA8, heat shock 70 kDa protein 8; HSPCA, heat shock 90 kDa protein 1 alpha;

H_2O_2, hydrogen peroxide; MGST3, microsomal glutathione *S*-transferase 3; PRDX, peroxiredoxin; SOD2, manganese superoxide dismutase; TI, tail intensity; XRCC1, heat shock 70 kDa protein 8 and heat shock 90 kDa protein 1 alpha.

Supporting Information Available: Table of significant results in LT97 cells treated with carob extract (2 g/L) for 24 h using cDNA arrays. This material is available free of charge via the Internet at http://pubs.acs.org.

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