JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Fermentation Enhances the Biological Activity of *Allium cepa* Bulb Extracts

Agnès Millet,[†,∥] Evelyn Lamy,[‡,∥] Daniel Jonas,[‡] Florian Stintzing,[§] Volker Mersch-Sundermann,[‡] and Irmgard Merfort*[,†]

[†]Institute for Pharmaceutical Sciences, Department of Pharmaceutical Biology and Biotechnology, Albert-Ludwig-University Freiburg, Stefan-Meier-Strasse 19, 79104 Freiburg, Germany

[‡]Department of Environmental Health Sciences, University Medical Center Freiburg, Breisacher Strasse 115b, 79106 Freiburg, Germany

[§]WALA Heilmittel GmbH, Dorfstrasse 1, 73087 Bad Boll/Eckwälden, Germany

ABSTRACT: In this study, we compared the analytical fingerprint and bioactivity of three onion extracts, including an aqueous, a methanol, and a fermented aqueous extract. The extracts were characterized by HPLC-DAD, LC-MS, and GC-MS analyses. The antibacterial, antigenotoxic, and antiproliferative activity of these extracts was assessed by means of agar disk diffusion, bacterial growth kinetics, a comet assay, cell cycle distribution analysis, and cell viability testing. Both the aqueous and methanolic extracts showed a typical flavonol-fingerprint as assessed by HPLC measurements and showed little to no bioactivity. The fermented aqueous extract, which lacks the usual onion flavonoid profile, was found to be the most active in all of the assays. This finding indicates that metabolites of onion compounds, generated by lactic acid fermentation, may be more active than their precursor substances.

KEYWORDS: *Allium cepa, lactic acid fermentation, LC-MS, GC-MS, antibacterial, antigenotoxic, antiproliferative*

■ INTRODUCTION

Onions (*Allium cepa* L., Alliaceae) are among the oldest plants cultivated worldwide and are used both as a dietary component and for medical purposes.[1,2] They are used for the treatment and prevention of various diseases and disorders, such as asthma and heart disease, and disturbances of the gastrointestinal tract.[1,2] Epidemiological studies have provided evidence for a reduced cancer incidence as a result of onion intake. Onion consumption has been associated with a lower risk for developing stomach or breast cancer.[3,4] Other studies have shown the anticarcinogenic and chemopreventive potential of onions in multiple organs or cell lines.[5−8] Furthermore, their antioxidant and antibiotic properties have been reported.[1,2] These activities are mainly related to the thiosulfinates, volatile sulfur compounds that are responsible for the pungency of onions.[2,9,10] Additionally, onions are characterized by their high phenolic content, especially flavonoids; in fact, onions are regarded as one of the highest dietary sources of flavonoids.[2,11]

The antibacterial activity of *Allium cepa* is mostly due to organic sulfoxides;[2] however, quercetin and kaempferol have also been shown to display activity against several bacterial strains.[12] Individual quercetin oxidation products can inhibit the growth of MRSA strains and of *Helicobacter pylori*.[13] However, the antibacterial activity of onions is reported controversially. Whereas Indu et al.[14] detected no inhibition of growth in various bacterial strains, other studies report a low to moderate activity of onion crude extracts against both Gram-negative and Gram-positive bacteria.[15−17] While a slightly higher activity against Gram-positive strains compared with Gram-negative strains has been reported,[18] higher activity against Gram-negative bacteria has also been claimed.[12] Furthermore, the antibacterial activity of onions against *Streptococcus mutans* and *S. sobrinus*,[10] or even total inhibition of the pathogenic strains *Staphylococcus dysenteriae* and *S. aureus*, has been found following the application of a 4% aqueous onion extract.[19]

To clarify the contradictory results for the antibacterial activity of onion extracts and to enhance our knowledge on the chemopreventive activity of onions, we completed the following studies. Three different onion extracts were prepared and analyzed for their secondary metabolites as well as any other alterations during the different preparation processes. The antibacterial activity of the extracts, as well as the antigenotoxic and growth arresting potency on metabolically competent human cancer cells, was evaluated. Finally, the impact of the major constituents of the extracts on these aforementioned biological activities is discussed.

■ MATERIALS AND METHODS

Reagents. The following substances were used as a reference: catechol (99%; Sigma, Seelze, Germany), isoquercitrin (purum, TLC; Roth, Karlsruhe, Germany), quercetin dihydrate (>98%, HPLC; Sigma, Seelze, Germany), and L-tryptophan (>99%; Merck, Darmstadt, Germany). Methanol p.a. was purchased from Roth (Karlsruhe, Germany). DMSO (purity >99%, CAS 67-68-5) and benzo[*a*]pyrene (B(a)P) (purity >98%, CAS 50-32-8) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Gentamicin was purchased from

Received: October 13, 2011
Revised: January 31, 2012
Accepted: February 2, 2012
Published: February 2, 2012

© 2012 American Chemical Society

Serva (Heidelberg, Germany). Dulbecco's Minimal Essential Medium (DMEM) and fetal calf serum (FCS) were purchased from PAA (Darmstadt, Germany). Normal melting point agarose (NMA) was obtained from Merck (Darmstadt, Germany), and low melting point agarose (LMA) was obtained from Serva (Heidelberg, Germany).

Onion Extracts. *Fermented Aqueous Extracts (FAE).* A fermented aqueous *Allium cepa* extract was provided by WALA Heilmittel GmbH (Bad Boll/Eckwälden, Germany, batch AL06). This batch was chosen as representative of several phytochemically comparable batches (data not shown). The extract was prepared according to an official specification[20] using fresh yellow onion bulbs and whey in a ratio of 100:50 (w/w), respectively. Making use of the natural microbial flora of the plant and whey, spontaneous fermentation of this blend was performed over 3.5 days at 37 °C before separation of the liquid from the solid onion residue. The resulting turbid filtrate was again fermented for 3.5 days at room temperature and passed through a filter before storage for 12 months at 15 °C. The filtrate obtained was immediately frozen at 20 °C and thawed shortly before analyses. The final concentration was 0.8−0.9 g/mL fresh onion/mL extract.

Aqueous (AE) and Methanolic (ME) Extracts. A total of 50 g of fresh pieces (approximately 2 cm) of *A. cepa* bulbs, from the same onion batch as the fermented extract, were kept frozen at −20 °C. For preparation of the extracts, the onions were thawed, immediately cut into small pieces (1−3 mm) at room temperature, and thoroughly ground. Extracts were obtained by stirring 20 g of the pulp with 15 mL of either methanol (p.a.) or distilled water over a period of 1 h at room temperature. The mixtures were then filtered, and the residues were extracted two additional times. ME was evaporated to dryness in vacuo at a bath temperature of 40 °C, and AE was freeze-dried. Both were diluted in 20.0 mL of water. Ten-milliliter aliquots were kept frozen (−20 °C) until use. The final concentration of the extracts was 1.0 g/mL (fresh weight).

Instrumental Analyses. *HPLC−UV.* The extracts were diluted 1:2 in distilled water (v/v), centrifuged, and the supernatant was injected in the HPLC. The analysis was performed twice on an HP-1090 system series II (Hewlett-Packard, Palo Alto, California) equipped with an autosampler, a tertiary pump, and a photodiode array-detector. The analysis software was HP ChemStation (Agilent, version A.10.02, Santa Clara, Ca, USA). The Zorbax SB RP-column (C18; 3.5 μm; 250 × 4.6 mm; Agilent, Waldbronn, Germany) was connected to a Nova-Pak precolumn (C18; 4 μm; Waters, Eschborn, Germany). Detection was performed at 280 and 351 nm. A gradient was applied using aqueous 0.05% formic acid (v/v; solvent A) and methanol (HPLC grade; solvent B) with the following time course: 0−2 min, 0% B; 2−17 min, 0−45% B; 17−20 min, 45−80% B; 20−21 min, 80% B; 21−22 min, 80−100% B; 22−27 min, 100% B; 27−28 min, 100−0% B; 28−35 min, 0% B. The injection volume was 20 μL, and the flow rate was 1.0 mL/min.

LC−MS. FAE was analyzed via LC-MS. The same extract dilution was used as mentioned above. The samples were injected twice and measured in the positive and in the negative mode. The HPLC system was an Agilent HPLC series 1200 (Agilent, Waldbronn, Germany) equipped with a degasser G1322A, a binary gradient pump G1312A, an auto sampler G1329A, a column oven G1316A, and a diode array detector G1315B. Using the same method as that described above, separation was achieved on a Zorbax SB RP-column, connected to a Security Guard Cartridge (C18; 4 × 2.0 mm, Phenomenex, Torrance, Ca, USA). The LC system was coupled to an HCT ultra ion trap (Bruker Daltonic GmbH, Bremen, Germany) with an ESI source operating in the negative mode. The full-scan mass spectra of the HPLC eluates were recorded during the chromatographic separation yielding $[M - H]^-$ ions. To gain further structural information, these ions were trapped and fragmented to yield the precursor product patterns of the analytes. MS^n data were acquired over the m/z-range from 50 to 2000 in the auto MS/MS mode with a compound stability and trap drive level of 100%. The instruments were controlled by Agilent Chemstation and EsquireControl Software (6.1).

GC−MS. One milliliter of extract was freeze-dried. Next, 1 mL of methanol was added to the residue. After centrifugation, the supernatant was used for analyses. GC−MS analyses were performed twice according to a previously described method.[21] The temperature program started at 60 °C, followed by a ramp of 10 °C/min to 220 °C, which was held for 10 min (complete run: 26 min). The injector and detector temperatures were set to 280 °C, the injection volume was 1.0 μL, and the split was 10:1. Mass spectra were recorded over the m/z-range from 40 to 400. The chromatograms were monitored in total ion mode. A NIST-library was available to compare MS-spectra and used to assign the majority of the peaks.

Pyruvate Measurement. The pyruvate analysis was performed as previously described.[22,23] The extracts were thawed at room temperature, centrifuged for 10 min (20,800*g*), filtered (0.45 μm syringe filter), and then diluted 1:10 (v/v) in distilled water. An aliquot (0.25 mL) of the filtrate was added to 0.5 mL of 2,4-dinitrophenyl hydrazine (DNPH; 0.0125% (w/v), in 2 mol/L HCl) and 0.75 mL distilled water in a reaction tube. The mixture was briefly vortexed and incubated at 37 °C for 10 min. A total of 2.5 mL of 0.6 mol/L NaOH was then added to the reaction tube, and the absorbance at 420 nm was recorded (Ultrospec 2100pro, Amersham Biosciences, Freiburg, Germany). To allow calculation of the pyruvate concentration from the onion samples, a standard curve was produced by sequential dilutions of a 1 mmol/L pyruvate solution, yielding a concentration range of standards from 0.03 to 0.8 mmol/L. The pyruvate concentration in onions (mmol/g fresh weight) was determined from the equation of the linear calibration standard.

Microbiological Assays. *Bacterial Cultures.* Five Gram-negative strains, including *Klebsiella pneumoniae* ATCC 700603, *Stenotrophomonas maltophilia* ATCC 13637, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Acinetobacter baumannii* ATCC 19606, and five Gram-positive strains including, *Staphylococcus aureus* ATCC 29213 and ATCC 43300, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, and *Enterococcus faecium* ATCC 6057 were used. Shortly before use, these bacterial strains were grown on blood agar (Heipha Dr. Müller GmbH, Eppelheim, Germany) and diluted to 10^8 cfu/mL in Tryptone Soy Broth (TSB, Oxoid, Basingstoke, UK) by means of turbidity measurements (MicroScan Turbidity Meter, Dade Behring, Chicago, USA).

Test Solutions. Ten milliliters of each onion extract was thawed, sterile filtered (0.2 μm), aliquoted into 1.5-mL reaction tubes (Roth, Karlsruhe, Germany), and immediately refrozen. The aliquots were allowed to reach room temperature before use. Distilled water served as a negative control, and octenisept wound desinfectant (Schülke-Mayr, Norderstedt, Germany) served as a positive control.

Agar Diffusion Assay. The 10^8 cfu/mL bacterial culture was diluted to 10^5 cfu/mL with TSB. This dilution was then poured and spread on an MH agar plate (Müller-Hinton agar, Merck, Darmstadt, Germany). The excess was pipetted off. Six cavities (6 mm i.d.) were punched in the agar after the plates had been dried for 15 min at room temperature. A total of 50 μL of the test solution was pipetted undiluted into each hole. After a 24-h incubation at 37 °C, the inhibition zone diameters were recorded with a ruler scaled in millimeters. This assay was performed twice with two replicates for each bacterial strain.

Bacterial Growth Kinetics. The 10^8 cfu/mL bacterial cultures were diluted to 10^4 cfu/mL with TSB. One part of the FAE, water, or octenisept as control was each mixed with nine parts of the prepared bacterial suspension. The solutions were then divided into three reaction tubes (Eppendorf, Wesseling-Berzdorf, Germany), which were incubated at 37 °C for 0, 4, and 24 h, respectively. At this point, a certain volume of the solution (mostly after dilution) was spread on an MH agar plate before incubation at 37 °C for 24 h. The cfu was counted, and the bacterial concentration (cfu/mL) was determined. This assay was performed twice with two replicates for all bacterial strains.

Genotoxicity, Antigenotoxicity, and Growth Inhibition Assays. *HepG2 Cells.* HepG2 cells were purchased from the

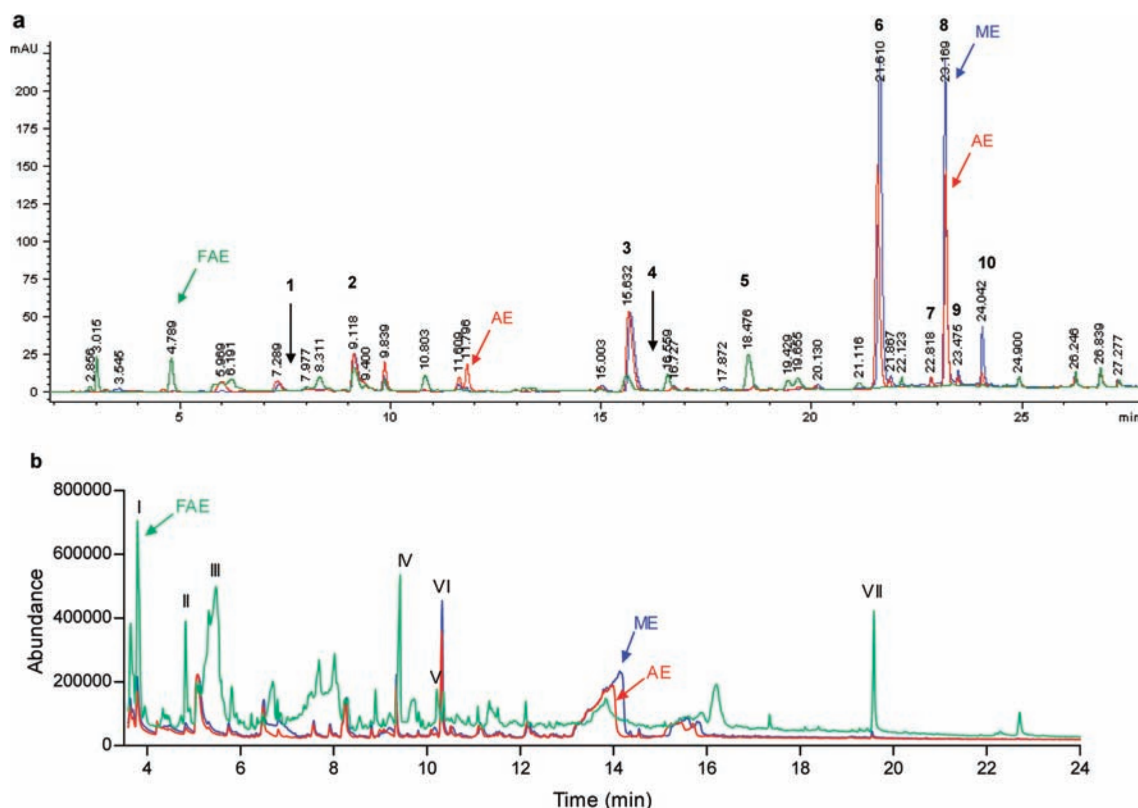

Figure 1. Overlay of HPLC–UV (280 nm; a) and GC–MS (b) chromatograms. The extracts, each diluted 1:2 (v/v) in water, are represented in blue, ME; red, AE; and green, FAE. For the assignment of the peaks in panel a using HPLC-UV and LC-MS, the following results were obtained: 1 = not identified; 2 = tyrosine; 3 = tryptophan; 4 and 5 = not identified; 6 = quercetin-3,4′-diglucoside; 7 = quercetin-3-glucoside; 8 = quercetin-4′-glucoside; 9 = isorhamnetin-4′-glucoside; and 10 = quercetin. For the assignment of the peaks in panel b by GC-MS the following compounds were identified: I = methylpyruvate; II = 3-hydroxymethylfuran; III = propylene glycol; IV = pyranone; V = catechol; VI = 5-hydroxymethylfurfural; and VII = palmitic acid.

Table 1. LC-MS and GC-MS Data of Compounds in Methanolic (ME), Aqueous (AE), and Fermented Aqueous Extracts (FAE) from Onion Bulbs[a]

				relative peak size in UV-chromatogram[b]			
	peak	Rt	compounds	ME	AE	FAE	*m/z* (rel. abundance)
LC-MS (ESI)	1	7.6	n.i.	+*	+*	+*	307 $[M+H]^+$, 217 ($+MS^2$); 305 $[M-H]^-$, 128 ($-MS^2$)
	2	9.1	tyrosine	–	–	–	182 $[M+H]^+$, 165 $[M-NH_2]^+$ ($+MS^2$); 180 $[M-H]^-$, 136 ($-MS^2$)
	3	15.6	tryptophan[c]	+	+	–	205 $[M+H]^+$, 188 $[M-NH_2]^+$ ($+MS^2$); 203 $[M-H]^-$
	4	16.3	n.i.	+*	+*	+*	295 $[M+H]^+$, 166 ($+MS^2$); 293 $[M-H]^-$, 128 ($-MS^2$)
	5	18.5	n.i.	–	n.d.	n.d.	231 $[M]^+$, 214 ($+MS^2$); 230 $[M-H]^-$
	6	21.6	quercetin-3,4′-di-*β*-glucoside	++	+	n.d.	325 $[diglucoside+H]^+$; 625$[M-H]^-$, 323 $[diglucoside-H]^-$
	7	22.8	quercetin-3-*β*-glucoside[c]	n.d.	–	n.d.	465 $[M+H]^+$; 463 $[M-H]^-$, 301 $[M-glucoside]^-$
	8	23.2	quercetin-4′-*β*-glucoside	++	+	n.d.	465 $[M+H]^+$; 463 $[M-H]^-$, 301 $[M-glucoside]^-$
	9	23.5	isorhamnetin-4′-*β*-glucoside	–	–	n.d.	163 $[glucose-H_2O+H]^+$; 477 [M-H][d]
	10	24.0	quercetin[c]	+	–	n.d.	303 $[M+H]^+$, 301 $[M-H]^-$; 179 ($-MS^2$), 151 ($-MS^3$)
GC-MS (EI)	I	3.8	methyl pyruvate	–	–	++	43 (100), 31 (16), 102 ([M], 12)
	II	4.8	3-hydroxymethylfuran	–	–	++	98 ([M], 100), 43 (82), 81 (49)
	III	5.5	propylene glycol	–	–	++	45 (100), 31 (38), 29 (20), 76 ([M], 0)
	IV	9.4	pyranone	–	–	++	144 ([M], 100), 43 (92), 101 (64)
	V	10.2	catechol[c]	n.d.	n.d.	+	110 ([M], 100), 64 (28)
	VI	10.3	5-hydroxymethylfurfural	++	++	–	97 (100), 126 ([M], 67), 41 (55)
	VII	19.6	palmitic acid	n.d.	n.d.	++	73 (100), 60 (71), 256 ([M], 59)

[a]++ = high; + = intermediate; – = traces; * = only detected in the MS. n.d. = not detected; n.i. = not identified. [b]Not quantified, relative amounts. [c]Identification by comparison with reference substances. [d]No aglycone detected.

German Collection of Microorganisms and Cell Cultures (DSMZ), Germany. The cells were cultured in low glucose DMEM supplemented with 15% fetal calf serum and 50 μg/mL gentamicin in a 5% CO_2 atmosphere at 37 °C. Cell line verification was conducted by microscopically checking the morphology of the cells and performing a growth curve analysis

dx.doi.org/10.1021/jf2041643 | J. Agric. Food Chem. 2012, 60, 2148–2156

on a regular basis. The cell culture tested negative for mycoplasma contamination.

Single-Cell Gel Electrophoresis (Comet) Assay. The SCGE assay, also known as the comet assay, was performed according to the guidelines developed by Tice et al.[24] with slight modifications as previously described.[25] Briefly, HepG2 cells were plated onto multiwell systems at a density of 3×10^5 cells/mL culture medium. The cells were exposed to the test substances for 24 h at logarithmic growth. For antigenotoxicity testing, the cell cultures were pretreated with the test substances for 24 h, the medium was removed by thorough washing with PBS, and the cells were subsequently exposed to 50 μM B(a)P for an additional 24 h. The samples ($n = 3$) were analyzed with a Leica fluorescence microscope (Leica DMLS, Solms, Germany; excitation filter, BP 546/10 nm; barrier filter, 590 nm) connected to a computerized image analysis system (Kinetic Imaging 5.5, Optilas, Munich, Germany). The Olive tail moment (OTM), defined as $(\text{Tail}_{\text{mean}} - \text{Head}_{\text{mean}}) \times \text{Tail}_{\%\text{DNA}}/100$, was calculated as an indicator of DNA damage. For each sample, 102 cells were analyzed.

Determination of Cell Viability. HepG2 cells were plated onto 12-well plates (Greiner bio-one, Germany) at a density of 1.5×10^5 cells/mL culture medium. After 48 h of growth, the cells were exposed to the test substances for 24 h at 37 °C and 5% CO_2. In combination experiments with B(a)P, the cells were pretreated with the extracts for 24 h before exposure to B(a)P (50 μM) for an additional 24 h. After treatment, the viability of the cells was determined by the vital dye exclusion test with erythrosin B.[26]

Measurement of the Sub-G1 DNA Content and Cell Cycle Distribution. Cellular DNA content was measured after permeabilization of HepG2 cells by fixation with 70% ethanol and staining of the DNA with PI master mix (40 μg/mL propidium iodide, 100 μg/mL RNase, DNase free, PBS), which allows for the identification of the generally called "sub-G1" or "hypoploid" peak of apoptotic cells on a DNA content frequency histogram. Analysis was performed using flow cytometry with a FACSCalibur (BD, Germany). The software Modfit was used to deconvolute the DNA frequency histograms, estimate the proportions of cells in particular phases of the cell cycle, and to quantify the percentage of apoptotic cells in the sub-G1 peak.

Statistical Analysis. For toxicity tests and combination experiments, the results were compared with the negative control and 50 μM B(a)P, respectively, using a Student's t test (*, $p \leq 0.05$; **, $p \leq 0.01$).

RESULTS AND DISCUSSION

Phytochemical Analysis on Phenolic and Sulfur Constituents. Three different extracts, including a methanolic extract (ME), an aqueous extract (AE), and a fermented aqueous extract (FAE), were prepared from a single batch of fresh onions and studied. The flavonoid profiles of ME and AE were very similar (Figure 1 and Table 1). Quercetin glucosides, mainly quercetin-3,4′-diglucoside and quercetin-4′-glucoside, each amounted to approximately 45% of the total flavonols. To a lesser extent, the aglycone quercetin was detected in both extracts (ME and AE), thus corroborating the previous literature.[27,28] The flavonoid content in AE was slightly lower as in ME. In contrast, no flavonol glucosides and only traces of the aglycone quercetin could be detected in FAE, indicating the hydrolysis and further decomposition of these compounds. Furthermore, except for traces of catechol, no other decomposition products, such as the p-hydroxyacetophenone or p-hydroxyphenylpropionic acid previously detected in birch leaves extracted under the same fermentation conditions,[29] could be found in the present work. The absence of detectable metabolites in the fermented aqueous onion extract suggests rapid decomposition of the flavonols into smaller molecules, such as acetate, butyrate,[30] or even carbon dioxide.[31,32] These compounds were not detectable using the established HPLC-DAD, LC-MS, or GC-MS methods.

Allium extracts are also known to contain decomposition products of the genuine alk(en)yl cysteine sulphoxides (ACSOs).[9] These natural constituents are highly labile and volatile. No organic sulfur derivatives could be identified with the applied chromatographic methods. However, pyruvate has been proven to be formed when S-substituted L-cysteine sulfoxide derivatives interact with enzymes of the alliinase type.[23] Therefore, the content of pyruvate was determined photometrically as a substitute for the sulfoxides, using 2,4-dinitrophenylhydrazine (Table 2). This method allows the quantification of enzymatically liberated pyruvic acid, a compound that correlates with onion pungency.[22,23] Whereas moderate levels of pyruvic acid were detected in ME and AE (6.0 and 4.6 μmol/g fresh weight, respectively), which is in agreement with a recent report,[33] FAE did not contain any pyruvic acid. Additionally, we used GC-MS to analyze the composition of the extracts. Whereas pyruvic acid was not detectable via GC-MS, methyl pyruvate was assigned in each of the three extracts via the NIST database (m/z 102 [M], 43, 31). This result may be explained by the fact that pyruvic acid is methylated and that the methylated product does not react with 2,4-dinitrophenylhydrazine. Moreover, pyruvic acid can be further degraded during the fermentation process. Interestingly, the peak area of methyl pyruvate was significantly higher for FAE compared with ME and AE (Table 2). According to the GHP production protocol,[20] onions and whey serve as the starting point of fermentation. Pyruvate could be formed as an intermediate compound by lactic acid bacteria during lactate production in the fermentation process. However, despite the numerous possible lactate pathways, methyl pyruvate has not yet been reported as a possible intermediate of lactic acid fermentation.[34] Therefore, the subsequent generation of methyl pyruvate may be explained by the reaction of pyruvate with methanol, the latter being released from pectic substances during the fermentative process[35,36] as well as serving as the solvent for the GC-MS analyses.

Table 2. Photometric Determination of Enzymatically Produced Pyruvate in the Three Extracts and the Peak Areas of Methyl Pyruvate from the GC-MS Analyses

	pyruvate		methyl pyruvate
extract	(μmol/g f.w.[a])	RSD (%)	(peak area, m/z 102)
ME	6.02	9.90	396.363
AE	4.58	0.28	2581.50
FAE	0.56	5.74	1.1502.44

[a] f.w., fresh weight.

Biological Effects of Onion Extracts. In the agar diffusion assay, FAE induced a growth inhibition on all Gram-negative bacterial strains (Figure 2 a and b) and slightly reduced the growth of the Gram-positive strains. Individual isolated colonies could be observed within the zone of inhibition of the Gram-positive bacteria. The strongest bacterial activity was detected against the Gram-negative strain *A. baumannii*, with a zone of inhibition of 16.5 mm (50 μL extract). Interestingly, in this case, a double zone of inhibition was identified with a bacteria ring inside the inhibition zone. This result may be explained by the heterogeneous diffusion of the onion extract compounds in the agar, resulting in zones that were affected, whereas other zones exhibited no effects. However, it might also be possible that *A. baumannii* exhibits heterogeneous resistance to the

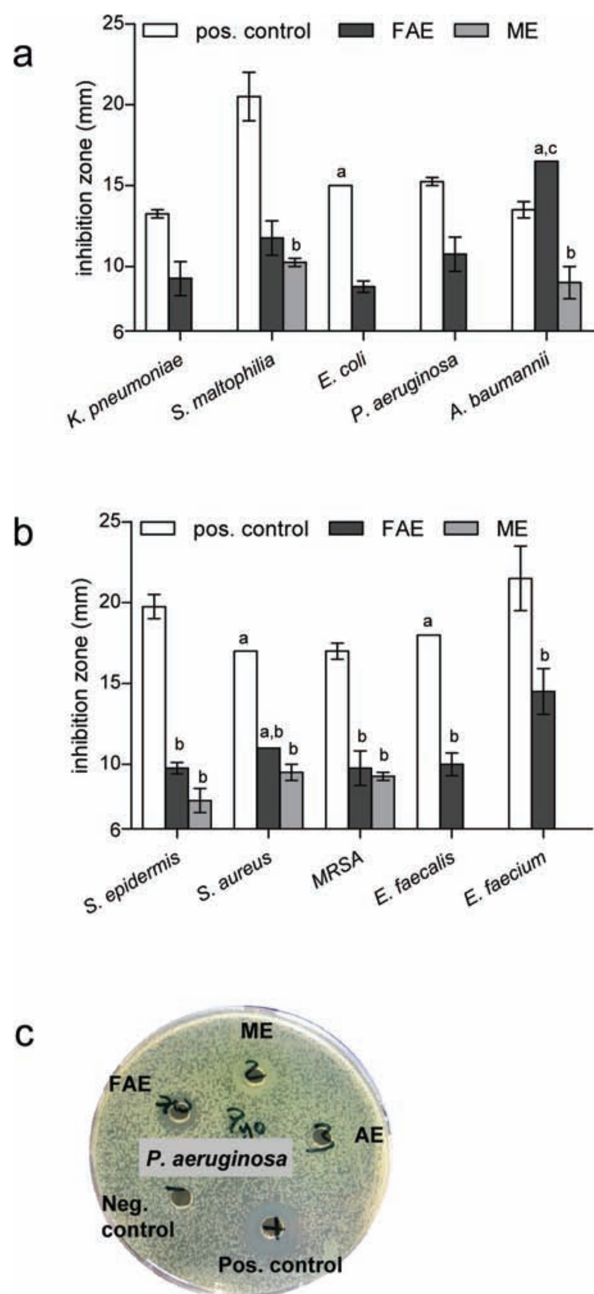

Figure 2. Agar diffusion assay. For five Gram-negative (a) and five Gram-positive (b) strains, the diameter of the zones of inhibition (±SD) for FAE (0.8−0.9 g/mL fresh weight), ME (1 g/mL fresh weight) and octenisept (0.1% octenidine diHCl, 2% 2-phenoxyethanol), as a positive control, are shown. The growth of *K. pneumoniae* was slightly induced by AE and ME. The growth of *P. aeruginosa* was strongly induced by ME; a = SD = zero, b = single colonies within the zone of inhibition, and c = double inhibition ring. An exemplary agar plate with *P. aeruginosa* (c) shows the growth inhibition by FAE and the positive control, no effect by AE and the negative control (sterilized water), and growth induction by ME.

extract. This type of an effect was observed for *Staphylococcus aureus* against vancomycin and methicillin.[37,38]

ME had a slight antibacterial effect on *S. maltophilia*, *A. baumannii*, *S. epidermis*, and *S. aureus*, as indicated by a modest reduction in bacterial growth (on a maximum diameter of 10 mm, Figure 2a and b); whereas, AE did not show any influence on these strains. Both extracts induced a slight growth of *K. pneumoniae*, and ME alone induced stronger growth of *P. aeruginosa* (on a diameter of 9−13 mm, Figure 2c).

Because FAE was the only one to show antibacterial activity in the agar diffusion test, it was further studied in a kinetic experiment using a bacterial suspension. A considerable reduction in cell colonies could be observed after 4 h when compared with the control (Figure 3). This effect was slightly more pronounced with the Gram-negative bacteria. Here, the number of colonies was between 10% and 20% of the negative control; whereas, in the case of the Gram-positive strains, the growth reduction was lower (30−55% of the negative control). After 24 h of incubation, FAE exhibited less or no antibacterial effect.

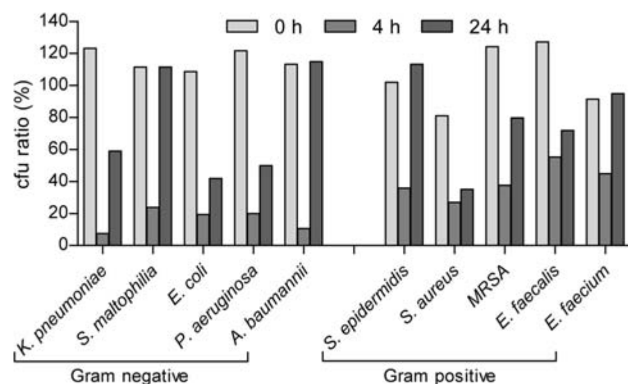

Figure 3. Growth kinetics of the Gram-negative and Gram-positive strains studied. The cfus (%) for the bacterial strains incubated for 0, 4, and 24 h with FAE (0.8−0.9 g/mL fresh weight), in relation to a water control, is reported.

All these findings clearly demonstrate that the mode of extraction may modify the specific composition, thus modulating the resulting antibacterial potency of the extract. This may also explain the various partly contradicting data from the literature. In two earlier reports, aqueous onion extracts were slightly more active against the Gram-positive strains tested (*Staphylococcus aureus*, *Bacillus cereus*, several *Streptococcus* species, and *Lactobacillus odontolyticus*) than against the Gram-negative species tested (*Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Klebsiella* species).[12,18] A slight antibacterial activity of the aqueous onion extracts against *E. coli* was only reported by Srinivasan et al.[17] and Elnima et al.[18] Another study used different concentrations of the aqueous onion extract and found no antimicrobial activity against all Gram-positive or Gram-negative strains tested.[14]

The antibacterial activity of onions is assumed to be primarily due to sulfoxides and other organic sulfur compounds. Unfortunately, the identification of these compounds was not possible in the present work because they are rather labile in solution and very volatile. Thus, it can only be speculated that sulfoxides and their degradation products are responsible for the moderate transient antibacterial activity that we observed. Santas et al. reported that quercetin and kaempferol and a flavonoid-rich onion extract display antimicrobial activity, mainly against Gram-negative bacteria.[12] Our findings are consistent with the assumption that flavonoids are generally not associated with a strong antimicrobial effect[39] because FAE contained only traces of quercetin. In addition, the antimicrobial activity of the extracts could be due to specific secondary metabolites formed by the respective bacterial strain. Finally,

short-chain organic acids resulting from lactic acid fermentation, such as acetate or butyrate, may also contribute to the stronger activity of FAE. These hypotheses should be addressed in further investigations.

Moreover, we evaluated whether the extracts possessed DNA damaging potential. As shown in Figure 4, 24 h-exposure of the

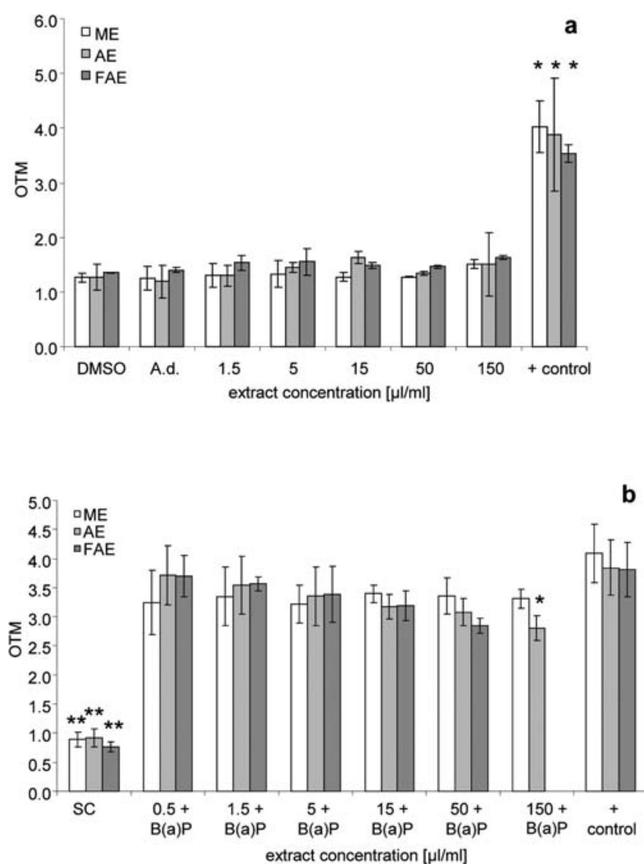

Figure 4. Effect of onion extract treatment on DNA migration, as determined by the SCGE assay: (a) HepG2 cells treated with the onion extract for 24 h and (b) HepG2 cells pretreated with the onion extract for 24 h before exposure to 50 μM B(a)P for an additional 24 h. The bars represent the mean OTM ± SD, (n = 3). *$p \leq 0.05$ and **$p \leq 0.01$ for a Student's t test comparing the extracts with the solvent control, 0.1% DMSO (a), or with 50 μM B(a)P (b). A.d., aqua dest; SC, solvent control = 0.1% DMSO; and + control, positive control = 50 μM B(a)P.

cells to any of the three extracts did not lead to an increase in DNA migration. The saturation limit of the extracts in the culture medium was reached at 150 μL/mL; therefore, higher concentrations were not tested in the assay.

In the antigenotoxicity testing, no protection against B(a)P-induced DNA damage was detectable with ME and FAE (Figure 4b). Because of massive cell loss, data evaluation of the highest concentration of FAE was impossible. Pretreatment of the cells with AE resulted in a weak concentration-dependent reduction of DNA damage, but the effect was significant at only 150 μL/mL.

In summary, no relevant antigenotoxic activity (with or without flavonoids) against B(a)P-induced DNA damage, even at very high concentrations, was evident. Our results contrast the previously reported chemopreventive effects of *Allium* vegetables related to the regulation of drug metabolizing enzymes and antioxidant activity.[40−42] Thus, a methanolic extract of *Allium cepa* was proven to induce quinone reductase, an important detoxification enzyme in murine hepatoma Hepa1c1c7 cells.[43] In another study, glutathione peroxidase and glutathione reductase activities increased in the erythrocytes of rats fed onion extracts.[44] Flavonoids, which are the main ingredient in onions, are suggested to possess chemopreventive properties, such as the DNA-protective effect of quercetin against B(a)P found in HepG2 cells.[45]

In addition to their chemopreventive action, the onion extracts were studied for their potential chemotherapeutic properties, i.e., cytotoxic and/or cytostatic efficacy against tumor cells. Cell viability, as assessed by erythrosin B, was not remarkably affected by either of the extracts (Figure 5a). In

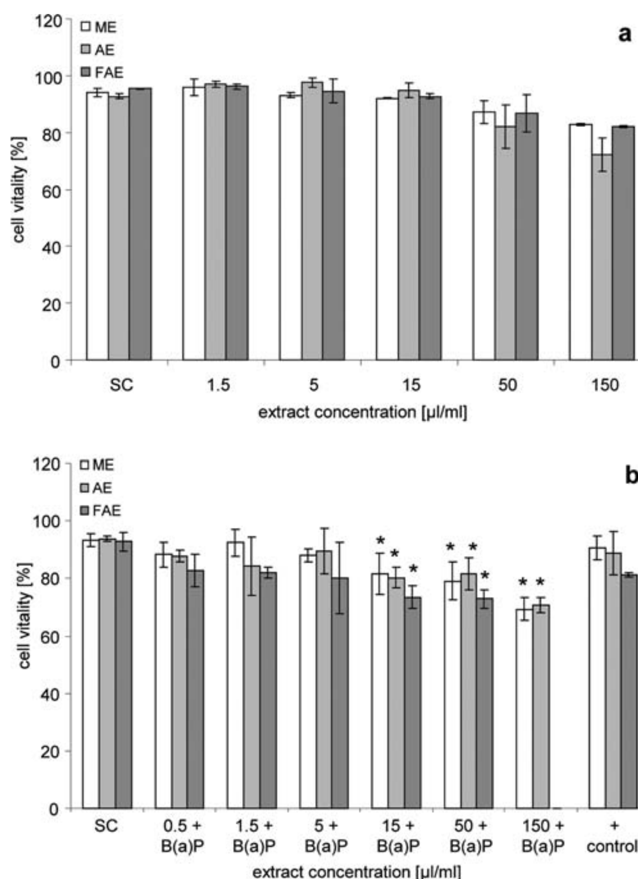

Figure 5. Vitality assessment of HepG2 cells after extract treatment for 24 h (a) and extract/B(a)P combination treatment (b). The bars represent the mean values ± SD (n = 2). SC, solvent control = aqua dest (a) or 0.1% DMSO (b). * $p \leq 0.05$ for Student's test comparing the treatments with the solvent control.

combination experiments with B(a)P, a continuous reduction in vitality was detectable; however, the loss was only remarkable in cells pretreated with 150 μL/mL of FAE, concurring with the results from the comet assay (Figure 5b).

Furthermore, the cell cycle of HepG2 cells was not influenced by ME and AE (Figure 6a and b). Interestingly, although no considerable toxicity was indicated by erythrosin B, cell treatment with FAE led to a significant cell arrest at the G0/G1 phase. At a concentration of 50 μL/mL FAE, the cell population at G0/1 phase increased by 15%, while the number of S phase cells dropped by 11% when compared with control cells (Figure 6c). In accordance with this observation, a strong apoptotic response of 47% above control was evident at

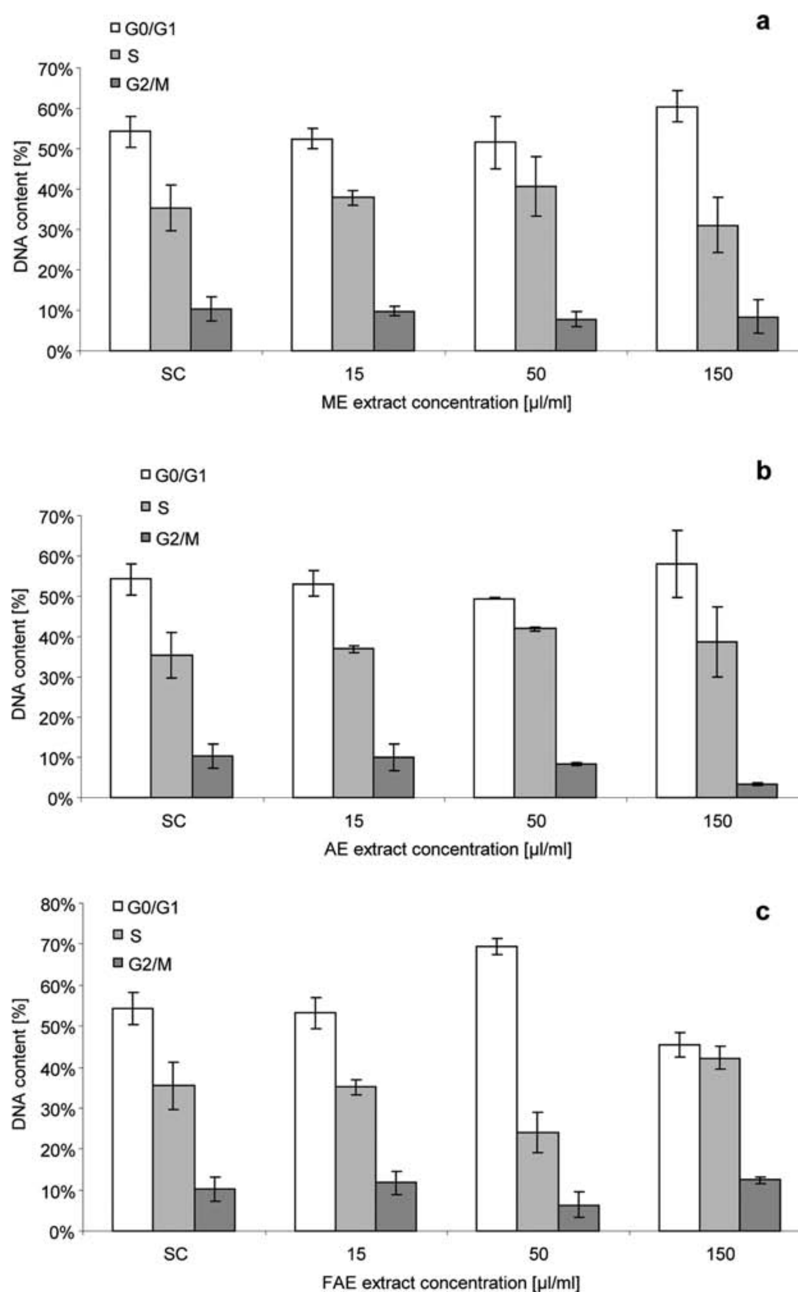

Figure 6. Effect of onion extracts on the cell cycle. HepG2 cells were treated with the extract for 24 h and analyzed using flow cytometry. The bars represent the mean values ± SD (n = 3). SC, solvent control = aqua dest.

concentrations greater than 50 μL/mL FAE (Figure 7). Weak apoptosis (approximately 2-fold of control) was detected after treatment of cells with ME for 24 h but not with AE (Figure 7).

These results are in line with the previous data found in the literature. Yang et al. provided evidence that the antiproliferative capacity of onion extracts against cancer cells differs widely between varieties, some of which are completely inactive.[46] This was demonstrated in HepG2 as well as in Caco-2 cells in a concentration range comparable to those used in our current study. However, the bioactivity detected by Yang et al. could not be clearly attributed to the phenolic or flavonoid content.[46] Additionally, Votto et al. also showed that a 24-h cell treatment with a crude onion extract induced the weak death of human Lucena MDR erythroleukemic and K562 cancer cells but only at high concentrations.[47] Further experiments conducted by this group revealed that neither the aqueous extract nor the methanolic extract, or quercetin alone could account for the cytotoxic effect. The authors speculated that cell death was triggered by the DNA damage signaling pathway. However, due to the lack of effect in the comet assay, this hypothesis can be excluded in our study. In another investigation, the aqueous extracts of onions were shown to induce apoptosis in Jurkat T leukemia and PC-3 prostate cancer cells. This finding correlated with a specific inhibition of proteasomal chymotrypsin-like activity.[48]

Previous studies performed with onion oil demonstrated a growth inhibitory potency in the lung cancer cell line A549, presumably via the production of reactive oxygen species.[49] Furthermore, the proliferation of human promyelocytic leukemia HL-60 cells was also inhibited by onion oil.[50]

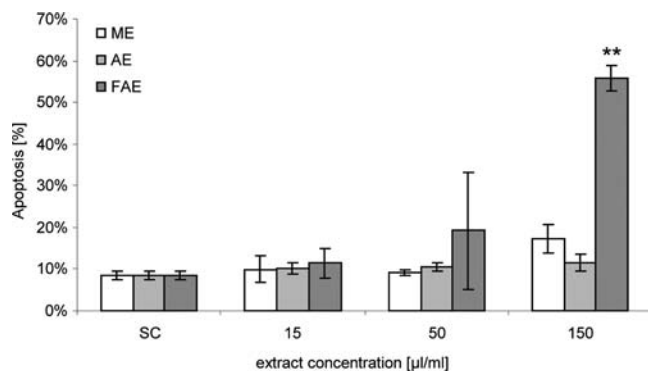

Figure 7. Effect of onion extracts on apoptosis induction. HepG2 cells were treated with the onion extract for 24 h and analyzed for their subG1 DNA content using flow cytometry. The bars represent the mean values ± SD (n = 3). * $p \leq 0.05$ for the Student's t test comparing the extracts with the solvent control (SC), aqua dest.

However, in contrast to the extracts in the present study, onion oil is a highly concentrated lipophilic distillate containing no flavonoids but rather high amounts of organic sulfur compounds.

In summary, of the extracts we examined, FAE possessed the best growth inhibitory potency. FAE also showed the best antibacterial activity, which leads to the suggestion that the same components are responsible for the positive response in the different bioactivity end points. Although the common flavonols, quercetin-4′-glucoside and quercetin-3,4′-diglucoside, are present in onions and are both biologically important, AE and ME were not active in the utilized assays. In contrast, FAE was biologically active. This may be due to organic acids or metabolites formed from flavonols and organosulfur compounds during lactic acid fermentation. It remains unclear if the activity of the extracts are from a single compound or the concerted action of several compounds; however, this warrants further investigation in the future.

AUTHOR INFORMATION

Corresponding Author

*Phone: +49-761-203-8373. Fax: +49-761-203-8383. E-mail: irmgard.merfort@pharmazie.uni-freiburg.de.

Author Contributions

[‖]These authors contributed equally to this work.

Funding

We are grateful to WALA Heilmittel GmbH (Bad Boll/Eckwaelden, Germany) for financial support. E.L. is funded by an academic grant from the European Social Fund and the Ministry of Science, Research and Arts Baden-Württemberg, Germany.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to WALA Heilmittel GmbH (Bad Boll/Eckwaelden, Germany) for providing the fermented aqueous onion extract and onion bulbs. We thank B. Schuler for performing the pyruvate tests, I. Engels for her support with the microbiological assays, and J. Bertrams for processing the LC-MS data. Furthermore, we are grateful to D. Lawrie-Blum for proofreading the manuscript.

REFERENCES

(1) Corzo-Martinez, M.; Corzo, N.; Villamiel, M. Biological properties of onions and garlic. *Trends Food. Sci. Technol.* **2007**, *18*, 609−625.

(2) Lanzotti, V. The analysis of onion and garlic. *J. Chromatogr., A* **2006**, *1112*, 3−22.

(3) Challier, B.; Perarnau, J. M.; Viel, J. F. Garlic, onion and cereal fibre as protective factors for breast cancer: a French case-control study. *Eur. J. Epidemiol.* **1998**, *14*, 737−747.

(4) Dorant, E.; van den Brandt, P. A.; Goldbohm, R. A.; Sturmans, F. Consumption of onions and a reduced risk of stomach carcinoma. *Gastroenterology* **1996**, *110*, 12−20.

(5) Fukushima, S.; Takada, N.; Hori, T.; Wanibuchi, H. Cancer prevention by organosulfur compounds from garlic and onion. *J. Cell. Biochem. Suppl.* **1997**, *27*, 100−105.

(6) Niukian, K.; Schwartz, J.; Shklar, G. In vitro inhibitory effect of onion extract on hamster buccal pouch carcinogenesis. *Nutr. Cancer* **1987**, *10*, 137−144.

(7) Niukian, K.; Schwartz, J.; Shklar, G. Effects of onion extract on the development of hamster buccal pouch carcinomas as expressed in tumor burden. *Nutr. Cancer* **1987**, *9*, 171−176.

(8) Perchellet, J. P.; Perchellet, E. M.; Belman, S. Inhibition of DMBA-induced mouse skin tumorigenesis by garlic oil and inhibition of two tumor-promotion stages by garlic and onion oils. *Nutr. Cancer* **1990**, *14*, 183−193.

(9) Block, E. The organosulfur chemistry of the genus *Allium* - Implications for the organic-chemistry of sulfur. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 1135−1178.

(10) Rose, P.; Whiteman, M.; Moore, P. K.; Zhu, Y. Z. Bioactive S-alk(en)yl cysteine sulfoxide metabolites in the genus *Allium*: the chemistry of potential therapeutic agents. *Nat. Prod. Rep.* **2005**, *22*, 351−368.

(11) Nemeth, K.; Piskula, M. K. Food content, processing, absorption and metabolism of onion flavonoids. *Crit. Rev. Food. Sci. Nutr.* **2007**, *47*, 397−409.

(12) Santas, J.; Almajano, M. P.; Carbo, R. Antimicrobial and antioxidant activity of crude onion (*Allium cepa*, L.) extracts. *Int. J. Food Sci. Technol.* **2010**, *45*, 403−409.

(13) Ramos, F. A.; Takaishi, Y.; Shirotori, M.; Kawaguchi, Y.; Tsuchiya, K.; Shibata, H.; Higuti, T.; Tadokoro, T.; Takeuchi, M. Antibacterial and antioxidant activities of quercetin oxidation products from yellow onion (*Allium cepa*) skin. *J. Agric. Food Chem.* **2006**, *54*, 3551−3557.

(14) Indu, M. N.; Hatha, A. A. M.; Abirosh, C.; Harsha, U.; Vivekanandan, G. Antimicrobial activity of some of the South-Indian spices against serotypes of *Escherichia coli*, *Salmonella*, *Listeria monocytogenes* and *Aeromonas hydrophila*. *Braz. J. Microbiol.* **2006**, *37*, 153−158.

(15) Abdou, I. A.; Abouzeid, A. A.; Elsherbe, M. R.; Elgheat, Z. H. A. Antimicrobial activities of *Allium sativum*, *Allium cepa*, *Raphanus sativus*, *Capsicum frutescens*, *Eruca sativa*, *Allium kurrat* on bacteria. *Qual. Plant Mat. Veg.* **1972**, *22* (1), 29−35.

(16) Hughes, B. G.; Lawson, L. D. Antimicrobial effects of *Allium sativum* L (garlic), *Allium ampeloprasum* L (elephant garlic), and *Allium cepa* L (onion), garlic compounds and commercial garlic supplement products. *Phytother. Res.* **1991**, *5*, 154−158.

(17) Srinivasan, D.; Nathan, S.; Suresh, T.; Lakshmana Perumalsamy, P. Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. *J. Ethnopharmacol.* **2001**, *74*, 217−220.

(18) Elnima, E. I.; Ahmed, S. A.; Mekkawi, A. G.; Mossa, J. S. The anti-microbial activity of garlic and onion extracts. *Pharmazie* **1983**, *38*, 747−748.

(19) Al Delaimy, K. S.; Ali, S. H. Antibacterial action of vegetable extracts on growth of pathogenic bacteria. *J. Sci. Food Agric.* **1970**, *21*, 110−112.

(20) *German Homeopathic Pharmacopoeia*; Special Production Protocols, Protocol 34a; Deutscher Apotheker Verlag: Stuttgart, Germany, 2009.

(21) Millet, A.; Stintzing, F.; Merfort, I. Validation of a GC-FID method for rapid quantification of nicotine in fermented extracts prepared from *Nicotiana tabacum* fresh leaves and studies of nicotine metabolites. *J. Pharm. Biomed. Anal.* **2009**, *49*, 1166−1171.

(22) Abayomi, L. A.; Terry, L. A. Implications of spatial and temporal changes in concentration of pyruvate and glucose in onion (*Allium cepa* L.) bulbs during controlled atmosphere storage. *J. Sci. Food Agric.* **2009**, *89*, 683−687.

(23) Schwimmer, S.; Weston, W. J. Onion flavor and odor - enzymatic development of pyruvic acid in onion as a measure of pungency. *J. Agric. Food Chem.* **1961**, *9*, 301−304.

(24) Tice, R. R.; Andrews, P. W.; Singh, N. P. The single cell gel assay: a sensitive technique for evaluating intercellular differences in DNA damage and repair. *Basic Life Sci.* **1990**, *53*, 291−301.

(25) Lamy, E.; Kassie, F.; Gminski, R.; Schmeiser, H. H.; Mersch-Sundermann, V. 3-Nitrobenzanthrone (3-NBA) induced micronucleus formation and DNA damage in human hepatoma (HepG2) cells. *Toxicol. Lett.* **2004**, *146*, 103−109.

(26) Krause, A. W.; Carley, W. W.; Webb, W. W. Fluorescent erythrosin-B is preferable to trypan blue as a vital exclusion dye for mammalian-cells in monolayer-culture. *J. Histochem. Cytochem.* **1984**, *32*, 1084−1090.

(27) Bonaccorsi, P.; Caristi, C.; Gargiulli, C.; Leuzzi, U. Flavonol glucosides in *Allium* species: A comparative study by means of HPLC-DAD-ESI-MS-MS. *Food Chem.* **2008**, *107*, 1668−1673.

(28) Vagen, I. M.; Slimestad, R. Amount of characteristic compounds in 15 cultivars of onion *Allium cepa* L. in controlled field trials. *J. Sci. Food Agric.* **2008**, *88*, 404−411.

(29) Millet, A.; Stintzing, F.; Merfort, I. Flavonol quantification and stability of phenolics in fermented extracts from fresh *Betula pendula* leaves. *J. Pharm. Biomed. Anal.* **2010**, *53*, 137−144.

(30) Schneider, H.; Schwiertz, A.; Collins, M. D.; Blaut, M. Anaerobic transformation of quercetin-3-glucoside by bacteria from the human intestinal tract. *Arch. Microbiol.* **1999**, *171* (2), 81−91.

(31) Kallianos, A. G.; Petrakis, P. L.; Shetlar, M. R.; Wender, S. H. Preliminary studies on degradation products of quercetin in the rat's gastrointestinal tract. *Arch. Biochem. Biophys.* **1959**, *81*, 430−433.

(32) Walle, T.; Walle, U. K.; Halushka, P. V. Carbon dioxide is the major metabolite of quercetin in humans. *J. Nutr.* **2001**, *131*, 2648−2652.

(33) Lee, E. J.; Yoo, K. S.; Jifon, J.; Patil, B. S. Characterization of shortday onion cultivars of 3 pungency levels with flavor precursor, free amino acid, sulfur, and sugar contents. *J. Food Sci.* **2009**, *74*, 475−480.

(34) Ganzle, M. G.; Zhang, C. G.; Monang, B. S.; Lee, V.; Schwab, C. Novel metabolites from cereal-associated lactobacilli - Novel functionalities for cereal products? *Food Microbiol.* **2009**, *26*, 712−719.

(35) Matsuura, Y.; Matsubara, K.; Fuchigami, M. Molecular composition of onion pectic acid. *J. Food Sci.* **2000**, *65*, 1160−1163.

(36) Wucherpfennig, K.; Bitsch, I. Bewertung von Methylalkohol in klaren und naturtrüben Apfelsäften unter Berücksichtigung moderner Entsaftungstechnologie. *Flüssiges Obst* **2004**, *71*, 456−460.

(37) Liu, C.; Chambers, H. F. *Staphylococcus aureus* with heterogeneous resistance to vancomycin: Epidemiology, clinical significance, and critical assessment of diagnostic methods. *Antimicrob. Agents Chemother.* **2003**, *47*, 3040−3045.

(38) Ryffel, C.; Strassle, A.; Kayser, F. H.; Bergerbachi, B. Mechanisms of heteroresistance in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **1994**, *38*, 724−728.

(39) Cushnie, T. P. T; Lamb, A. J. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents* **2005**, *26*, 343−356.

(40) Munday, R.; Munday, J. S.; Munday, C. M. Comparative effects of mono-, di-, tri-, and tetrasulfides derived from plants of the *Allium* family: redox cycling in vitro and hemolytic activity and Phase 2 enzyme induction in vivo. *Free Radical Biol. Med.* **2003**, *34*, 1200−1211.

(41) Munday, R.; Munday, C. M. Induction of phase II enzymes by aliphatic sulfides derived from garlic and onions: an overview. *Methods Enzymol.* **2004**, *382*, 449−456.

(42) Perchellet, J. P.; Perchellet, E. M.; Abney, N. L.; Zirnstein, J. A.; Belman, S. Effects of garlic and onion oils on glutathione peroxidase activity, the ratio of reduced/oxidized glutathione and ornithine decarboxylase induction in isolated mouse epidermal cells treated with tumor promoters. *Cancer Biochem. Biophys.* **1986**, *8*, 299−312.

(43) Xiao, H.; Parkin, K. L. Isolation and identification of potential cancer chemopreventive agents from methanolic extracts of green onion (*Allium cepa*). *Phytochemistry* **2007**, *68*, 1059−1067.

(44) Roldan-Marin, E.; Krath, B. N.; Poulsen, M.; Binderup, M. L.; Nielsen, T. H.; Hansen, M.; Barri, T.; Langkilde, S.; Cano, M. P.; Sanchez-Moreno, C.; Dragsted, L. O. Effects of an onion by-product on bioactivity and safety markers in healthy rats. *Br. J. Nutr.* **2009**, *102*, 1574−1582.

(45) Kang, Z. C.; Tsai, S. J.; Lee, H. Quercetin inhibits benzo[a]pyrene-induced DNA adducts in human Hep G2 cells by altering cytochrome P-450 1A1 gene expression. *Nutr. Cancer* **1999**, *35*, 175−179.

(46) Yang, J.; Meyers, K. J.; Van der Heide, J.; Liu, R. H. Varietal differences in phenolic content and antioxidant and anti proliferative activities of onions. *J. Agric. Food Chem.* **2004**, *52*, 6787−6793.

(47) Votto, A. P. S; Domingues, B. S.; de Souza, M. M.; da Silva, F. M. R.; Caldas, S. S.; Filgueira, D. M. V. B.; Clementin, R. M.; Primel, E. G.; Vallochi, A. L.; Furlong, E. B.; Trindade, G. S. Toxicity mechanisms of onion (*Allium cepa*) extracts and compounds in multidrug resistant erythroleukemic cell line. *Biol. Res.* **2010**, *43*, 429−438.

(48) Chen, M. S.; Chen, D.; Dou, Q. P. Inhibition of proteasome activity by various fruits and vegetables is associated with cancer cell death. *In Vivo* **2004**, *18*, 73−80.

(49) Wu, X. J.; Stahl, T.; Hu, Y.; Kassie, F.; Mersch-Sundermann, V. The production of reactive oxygen species and the mitochondrial membrane potential are modulated during onion oil-induced cell cycle arrest and apoptosis in A549 cells. *J. Nutr.* **2006**, *136*, 608−613.

(50) Seki, T.; Tsuji, K.; Hayato, Y.; Moritomo, T.; Ariga, T. Garlic and onion oils inhibit proliferation and induce differentiation of HL-60 cells. *Cancer Lett.* **2000**, *160*, 29−35.