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Effects of carvacrol on human fibroblast (WS-1) and gastric adenocarcinoma (AGS) cells in vitro and on Wistar rats in vivo

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Abstract

Carvacrol is a natural phenolic compound found in essential oils of *Lamiaceae* species. In the present study, an attempt has been made to elucidate the mechanism behind the anti-cancer potential of carvacrol on human gastric adenocarcinomas (AGS) by comparing its effects on cancer cells AGS to those on normal human fibroblast (WS-1) cells, in vitro. Cytotoxicity, reactive oxygen species (ROS) generation, glutathione (GSH) levels, genotoxicity, and apoptotic effects of carvacrol (0–600 μM) were studied in both cell lines. Additionally, the effect of high dose carvacrol (100 mg/kg BW) on the oxidative status was investigated in vivo. For this purpose, carvacrol was administered orally to male Wistar rats over a period of 60 days. Rats were weighed regularly. At the end of the experiment, rats were euthanized. Blood and stomach tissues were collected for biochemical and pathological examinations. The in vitro results showed significant differences in cell viability of AGS compared to WS-1 cells exposed to carvacrol. Also the extent of ROS generation, GSH reduction and DNA damage differed significantly between the cell lines studied ($P \leq 0.001$). The differences observed were statistically significant at all concentrations applied ($P \leq 0.001$). The results found in AGS cells were mirrored in the pathohistological findings obtained from animals of the in vivo experimental group. Changes in body weight, and oxidative stress index for plasma and stomach tissues of animals in this group were found to differ statistically significant from those found in the control group of Wistar rats ($P \leq 0.001$). The data obtained from our present study uncovered that carvacrol has the potential to cause toxic effects in both, AGS and WS-1 cells but more effectively in cancer cells than in normal cells. The carvacrol-mediated responses observed in the in vitro and in vivo experiments presented suggest a double-edged pro-oxidative effect. Via this mechanism carvacrol induced cytotoxicity, apoptosis, and DNA damage in a dose-dependent manner in both cancer and normal cells and these activities were higher in cancer cells than those of normal cells.

Keywords Apoptosis · Carvacrol · Human fibroblast cells · Human gastric adenocarcinoma cells · Pro-oxidant activity · Oxidative stress status

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Abbreviations

AO/EB	Acridine orange/ethidium bromide
Human AGS cells	Human gastric adenocarcinoma cells
ATP	Adenosinetriphosphate
DCFH-DA	2′, 7′-dichloro-dihydrofluorescein-diacetate
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
GC	Gastric cancer
GSH	Glutathione
IC_{50} level	The half maximal growth inhibitory concentration level
Na_3VO_4	Sodium orthovanadate
PBS	Phosphate buffered saline
ROS	Reactive oxygen species

TAS	Total antioxidant status
TBS-T	Tris-buffered saline with Tween
TOS	Total oxidative status

Introduction

The Mediterranean diet is generally considered to be a healthy diet which is primarily plant-based including fruit, vegetables, grains, legumes, nuts, spices, and olive oil [1]. A large number of herbs and aromatic plants are frequently used, especially from the family of *Lamiaceae* including Origanum, Satureja, Thymus, and Coridothymus species. A meta-analysis revealed that populations with Mediterranean diet habits show reduced incidence of cancer, cardiovascular, neurodegenerative, and chronic diseases in regard to morbidity and mortality [2]. Likewise, a cohort study showed a statistically significant lower incidence of gastric cancer in population with "Mediterranean-type diet" [3]. The important role of eating habits in the development of various cancer types has been described in several studies [4]. Gastrointestinal cancers are still the most important causes of morbidity and mortality among all cancer types. Gastric cancer (GC), a type of gastrointestinal cancer, is the forth most common cancer in the World [5] and thus an important public health burden. In 2016, 26,370 new cases and 10,730 deaths from gastric cancer are expected in the United States of America and even 133,999 new cases and 48,500 deaths in Japan [5, 6]. Mostly, gastric adenocarcinomas are diagnosed among gastric cancer patients.

Carvacrol (5-isopropyl-2-methylphenol) is a major compound of the essential oils in *Lamiaceae*, a plant family also called mint family with 236 genera and 6900–7200 species [7]. Carvacrol modulates metabolic enzymes and can protect or regenerate damaged organs [8]. In in vitro and in vivo studies, its anti-inflammatory, anti-obesity, antimicrobial, antioxidant, antidepressant, antinociceptive, and anti-cancer activities have been reported. Carvacrol is even able to prevent the proliferation of human ovarian adenocarcinoma cells as well as tumor cells resistant to chemotherapies including adriamycin, vincristine, and cisplatin [9]. On the other hand, it could be shown that combined loading of carvacrol and chemotherapy agents into human serum albumin nanoparticles may attack gastric cancer cells better than single drug-loaded nanoparticles in vitro [10]. Induction of cytotoxic effects was also found after exposure of human cervical cancer cell lines (HeLa and SiHa), human colon carcinoma cell lines (Caco-2, HCT116, and LoVo), human hepatoma cell line HepG2, human lung cancer cell line H1299, and human breast cancer cell line MCF-7, human acute promyelocytic leukemia cell line HL-60 to carvacrol [11–18]. It is important to note that carvacrol-induced cytotoxicity was also reported in studies conducted on normal cells such as V79 Chinese hamster lung fibroblast, healthy neuron, and Jurkat T lymphoma cells [18–20].

Reactive oxygen species (ROS) and free radicals in general are products of oxygen metabolism and essential for vital physiological functions [21]. ROS take part e.g., in cell signaling but excessive amounts can also cause alteration or even disruption of normal biological functions. Xenobiotics, including hydroxylamines, and phenolic compounds may lead to the formation of reactive intermediates by interacting with e.g., oxyhemoglobin or oxymyoglobin [22]. Oxidative stress occurs when there is an imbalance between ROS generation and/or activity and the antioxidant or scavenging defense. Glutathione (GSH) is known to act as an antioxidant that scavenges the intracellular ROS and therefore GSH level is an indicator for cell health [23]. Excessive ROS generation is understood to play a role in the onset and progression of many diseases including cancers. In such cases, macromolecules such as DNA, proteins, and lipids are destroyed. Polyphenols at higher doses may induce oxidative stress as well as due to excessive generation of ROS resulting in a pro-oxidant effect. For that reason, plants inducing ROS generation are able to activate apoptosis and cellular senescence [24, 25]. Thus, apoptosis causing agents are expected to be ideal anti-cancer drugs. Polyphenols at lower doses may protect the cells due to enhanced generation of GSH thereby exhibiting antioxidant effects. Apoptosis is a programmed and gene regulated cell death which is characterized by morphological and biochemical changes [26]. Several protein families such as the upstream *Bcl-2* family (e.g., the anti-apoptotic *Bcl-2* and pro-apoptotic *Bax*) and the downstream caspase family (e.g., *Caspase-9, Caspase-3*) take part in the regulation of apoptosis. Apoptosis differs from traumatic cell death that results from acute cellular injury, also called necrosis.

Apoptosis induced by carvacrol via direct activation of mitochondrial pathways was reported for various cancer cell lines [12, 15, 17, 18]. As a consequence, anti-cancer effects of carvacrol have raised interest in recent years. Generally, most of these studies are conducted in vitro and only a few have been demonstrating anti-cancer effects of carvacrol in vivo in lyomyosarcoma, hepatocellular and colon carcinoma models so far [27–29]. Hence, the clinical significance and direct impact on organs and organ function in patients still remain largely unknown. The potential value of carvacrol in clinical applications needs to be studied extensively in both, in vitro and in vivo systems. It is fundamental to investigate the anti-cancer effect of carvacrol including specificity and selectivity on cancer cell lines in comparison to the effects induced in normal cells. Anti-carcinogenic and anti-proliferative effects of carvacrol have been reported on different human cancer cell lines such as colon carcinoma, liver carcinoma, breast cancer, chronic myeloid leukemia, promyelocytic leukemia, lung cancer,

prostate cancer, larynx carcinoma cell lines [12, 15–18, 20, 30, 31]. A few genotoxicity studies were published using human hepatoma, human colon carcinoma, human gastric adenocarcinoma, human lymphocytes, V79 Chinese Hamster lung fibroblast, healthy neuron, and N2a cancer cells [11, 18–20, 31, 32]. To the authors' knowledge, no studies were performed on human fibroblast cells so far. In addition, no studies have been investigating carvacrol-induced cytotoxicity, genotoxicity, apoptosis, ROS generating, and GSH reducing effects on human gastric adenocarcinoma or fibroblast cells following longer exposure of these cells to carvacrol (e.g., 24 h). Likewise, no study yet was specifically reporting the effect of high-dose carvacrol on the oxidative status in vivo. Therefore, one aim of the present study was to investigate and compare the cytotoxic, genotoxic, apoptotic, and ROS generating effects of carvacrol on human gastric adenocarcinoma (AGS) and fibroblast (WS-1) cells in vitro. The second aim was to describe for the first time the in vivo effects of orally applied carvacrol in Wistar rats.

Materials and methods

Carvacrol

Carvacrol (W224502) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). For determination of the half maximal inhibitory concentration (IC_{50}) value, a range of concentrations was prepared [17, 20, 30]. Test solutions of carvacrol were prepared from the carvacrol stock solution (600 µM) for 10, 20, 30, 50, 100, 200, and 400 µM in dimethyl sulfoxide (DMSO), being the final DMSO concentration at below 0.1%.

Cell cultures

Human AGS and WS-1 cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Ham's F-12 (Kainghn's) Medium (Gibco Invitrogen Corporation, Carlsbad, CA, USA). The medium was supplemented with 10% FBS, and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) (Life Tech, CA, USA). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. When cells grown near confluence in 75 cm^2 plastic flasks, they were harvested. For performing the experiments, the AGS and WS-1 cells were plated in the 96-well with a density of 15×10^3 cells/ml, and in 6-well plates at a density of 18×10^4 cells/ml.

Cell viability assay

For determination of the number of viable cells in culture, the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, MI, USA) was used. The assay is based on quantification of the ATP presence, in the sense of identifying the cytotoxic effect of carvacrol on human AGS and WS-1 cells. The assay was performed as described earlier [31]. The amount of the ATP was measured using a luminometer (Varioskan Flash Multimode Reader, Thermo, Waltham, MA). The cell viability was expressed as the percentage compared with the negative control group designated as 100% [33]. The half maximal inhibitory concentration (IC_{50}) value was calculated from the concentration–response curves by non-linear regression analysis. All experiments were repeated three times, and standard deviation was within 5%.

DCFH-DA assay

Intracellular generation of ROS level was detected using an oxidant-sensitive fluorescent probe DCFH-DA (Sigma, St. Louis, MO, USA). Two-hundred microliters complete medium and 15×10^3/ml AGS and WS-1 cells were placed into 96-well plates. After 24 h of incubation, growth media were replaced with growth medium containing reduced fetal bovine serum (5% v/v). Plates were incubated with added test solutions (0–100 µM) of carvacrol for another 24 h. Cells were washed with cold PBS, and were incubated with 100 mM DCFH-DA for 30 min at 37 °C. DCF fluorescence intensity was measured using the fluorescence plate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, MA) at Ex./Em. = 488/525 nm. The measurements were carried out in triplicate, ensuring each time that the number of cells per treatment group had the same reproducibility. The results were reported as a percentage of relative fluorescence relative to control cells.

GSH assay

The GSH/GSSG-Glo assay (Promega, Madison, MI, USA) was used to determine and quantify GSH level in AGS and WS-1 cell lines. The assay is based on the luminescence measurements in a multiwell plate. One hundred microliters of complete medium and cells at a density of 15×10^3/ml were placed in each well of a 96-well microtiter plate. Cells were treated with test solutions of carvacrol (0–100 µM). The method was carried out according to Manufacturer's instructions. The light emitted in the presence of GSH was quantitated in relative light units. The intensity of emitted light quants was directly related to GSH content in the tested sample. All experiments were repeated three times and standard deviation was within 5%.

Acridine orange/ethidium bromide (AO/EB) staining

Apoptotic effect of carvacrol was studied using AO/EB fluorescent dyes at the cellular level. After placing 18×10^4/ml of AGS and WS-1 cells into a 6-well plate, 0–100 µM carvacrol were added, and incubated for 24 h. The cells were harvested, and washed twice with PBS. Finally, a solution of AO/EB fluorescent stain (Sigma, St. Louis, MO, USA) was added to the cell suspension, and the nuclear morphology was evaluated by fluorescence microscopy (Leica DM 1000, Solms, Germany) at Ex./Em. = 502/525 nm. Multiple photos were taken at randomly selected areas, and a minimum of 100 cells were counted. According to the method, the viable cells have normal green nuclei, apoptotic cells have green nuclei with fragmented chromatin, and necrotic cells have orange/red nuclei.

Western blotting

Apoptotic effect of carvacrol was detected by western blotting at the protein expression level. *Bax* (N-20), *Bcl-2* (C-21), *Caspase-3* (H-277), and *Caspase-9* (H-170) antibodies were provided by Santa Cruz Biotechnologies (Santa Cruz, CA, USA). After treatment with test solutions of carvacrol (0–50 µM), AGS and WS-1 cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1% aprotinin, 50 mM NaF, 0.1 mM Na_3VO_4), and proteinase inhibitor cocktail (Roche, Mannheim, Germany). The lysates were centrifuged at 13,000×g for 15 min at 4 °C. The pellet was discarded, and the supernatant containing the protein was transferred into a clean tube. Cell protein was measured by the Bradford Coomassie brilliant blue dye method [34]. All methods were performed as described earlier [31]. All samples were also blotted for β-actin (clone AC-15; Sigma-Aldrich, Dublin, Ireland) to normalize protein amounts. The expression level of proteins were detected using Amersham ECL Plus Western Blotting Detection Reagents (GE Health care, Piscataway, NJ, USA) and captured with an imaging system (Vilber Lourmat, France).

Alkaline single cell gel electrophoresis (SCGE, comet assay)

Genotoxic effect of carvacrol was investigated by the comet assay according to the method of Singh et al. [35], with slight modifications, as described earlier [31]. Before performing comet assay, the trypan blue exclusion test was used to determine the number of viable cells present in a cell suspension [36]. Test solutions of carvacrol (0–100 µM) were added in AGS and WS-1 cell cultures. Ten microliters of cell suspension was mixed with 90 µl of 0.6% low melting agarose, and added to the slides pre-coated with 1% normal melting agarose. According to Hartmann et al. [37], the percentage of DNA in the tail (% of tail DNA) was used as a parameter for measurement of DNA damage (DNA strand breaks). One hundred cells were scored per each sample after electrophoresis. All of the procedures was completed by the same biochemistry staff. DNA damage was analyzed by a single observer that was not aware of the subject's status.

In vivo experiment and samples

The experiment was performed at the Research Center of Experimental Animals from our university. All animals received human care in compliance with the guidelines for the protection of animals used for scientific purposes (Directive, 2010/63 EU, Decision, 2012/707/UE and RD 53/2013). All procedures have been approved by the Ethical Animal Experimentation Committee of the Bezmialem Vakif University under the licence number 2016/88.

Sixteen male Wistar rats 8 weeks of age were divided into 2 groups (8 rats/group): experimental and control group. Rats were fed with standard laboratory feed and water *ad libitum*. Rats in experimental group had oral gavage application of carvacrol (100 mg/kg body weight) once every 2 days until termination of the animal experiment. In our study, carvacrol administered to rats was 100 mg/kg body weight (BW) which corresponds to 0.1% of daily diets [38]. Body weight of rats was measured regularly once a 10 days until the end of 60 days. All animals were sacrificed by cervical dislocation after anesthesia with Ketamine/Xylazin 35–50/5–10 mg/kg BW. Blood samples were collected by intracardiac injection. All animals were macroscopically examined. Necropsy was carried out, and the stomach tissues were collected for biochemical and pathological investigations. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned and mounted on polylysine-coated glass slides. Specimens were stained with haematoxylin and eosin (HE). One 4-µm section of each submitted paraffin block was stained first with HE for diagnosis of cells. Stained sections were imaged using Nikon Eclipse Ci microscope and their photographs were taken on a NIS Elements Basic Research Microscope Imaging Software (Nikon Instruments Inc., Tokyo, Japan).

Immunohistochemistry

Sections obtained from stomach tissues of rats were analyzed using immunohistochemistry (IHC) staining. The antibodies against *Bax, Bcl-2* and *Caspase-9* (GeneTex Inc, Irvine, CA, USA) were used in this study. Anti ß-actin antibody was used as control. Serial sections (4 µm) were prepared from selected blocks on positive charged glass slides for IHC. Dewaxed and rehydrated sections were

washed with distilled water. Tissue antigen retrieval was performed by using microwave oven process. After inhibition of endogenous peroxidase activity by immersion in peroxide block solution (SensiTek HRP Anti-Polyvalent (DAB) Staining System, ScyTek Laboratories, Inc, Logan, UT, USA), sections were incubated with the primary antibody, washed thoroughly in PBS. All subsequent incubation steps were performed at room temperature, and slides were rinsed thoroughly with PBS between each step. Glasses were incubated with SensiTek HRP Anti-Polyvalent biotinylated secondary antibody (SensiTek HRP Anti-Polyvalent (DAB) Staining System, ScyTek Laboratories, Inc, Logan, UT, USA) followed by a streptavidin peroxidase complex (SensiTek HRP, ScyTek Laboratories, Inc, Logan, UT, USA) and washed with PBS. Finally, immune complexes were visualized by incubation with 0.01% H_2O_2 and 0.05% 3,3′-diaminobenzidine tetrachloride (DAB Chromogen substrate kit, ScyTek Laboratories, Inc, Logan, UT, USA).

Oxidative stress assay

In order to understand the effect of high-dose carvacrol on endogenous oxidative status, the oxidative stress assay was performed. Blood and stomach tissues obtained from rats were utilized to measure of total oxidative status (TOS) and total antioxidant status (TAS), and to calculate the oxidative stress index (OSI). Firstly, stomach tissue was homogenized, and blood was centrifuged at 1500×*g* for 30 min in order to obtain the plasma. The separated plasma was used for analysis of TAS and TOS levels using an automated colorimetric measurement method as described previously [39]. TAS was measured using a new-generation, stable and colored 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTSþ). ABTSþ is decolorized by antioxidants according to their concentrations and antioxidant capacity. This change in color was measured as a change in absorbance at 660 nm. This process was applied to an automated analyzer and the assay was calibrated with Trolox. The results were expressed as mmol Trolox equivalent/l. TOS was measured using an automated method which allowed the oxidants in the sample oxidize the ferrous ion-o-dianisidine complex into a ferric ion. The ferric ion forms a colored complex with xylenol orange in an acidic medium. The color intensity was measured spectrophotometrically which is related to the total quantity of oxidant molecules present. The assay was calibrated with hydrogen peroxide and the results were expressed as mmol hydrogen peroxide equivalent/l. The OSI values were calculated using the TOS/TAS formula (OSI = TOS/TAS).

Statistical analysis

In vitro and in vivo obtained data were analyzed using the Statistical Package for the Social Sciences version 16.0 (SPSS Inc, Chicago, IL, USA). All the data were expressed as mean ± SD of number of experiments. All experiments were performed in triplicate. Percentages were calculated in relation to control cells. Additionally, a non-linear regression analysis was performed for the estimation of the IC_{50} value of carvacrol in normal and cancer cell lines. All the data were tested using one-way ANOVA. Means of both cell lines were compared by Mann Whitney-U test. Pearson correlation coefficient test was performed to assess associations between ROS generation and cell viability parameters. A P value of ≤ 0.05 was considered to be statistically significant. Differences within and between animal groups were examined for statistical significance by one-way ANOVA and Student's T test or Mann Whitney-U test. Two-sided P values were calculated, and a value of $P \leq 0.05$ was considered to be statistically significant.

Results

Effects of carvacrol on cell viability of AGS and WS-1 cells

Carvacrol showed cytotoxic effects by significantly reducing cell viability of both cell line cultures in a dose-dependent manner ($P \leq 0.001$; Fig. 1 a). Moreover, differences were found to be significant between carvacrol exposed AGS and WS-1 cells (10–600 µM) ($P = 0.001$). IC50 values for carvacrol were 82.57 ± 5.5 µM on AGS and 138.1 ± 8.7 µM on WS-1 cells. Carvacrol at lower concentration (10 µM) caused statistically significant proliferation of WS-1 cells compared to untreated control cells ($P \leq 0.001$).

Effects of carvacrol on ROS generation in AGS and WS-1 cells

Dose-dependent ROS generating effect of carvacrol was found for both cells lines. Differences between AGS and WS-1 cells were statistically significant ($P \leq 0.001$). The measured relative fluorescences are shown in percentages (Fig. 1 b). A positive correlation was found between relative ROS level in AGS and WS-1 cells and increasing dose of carvacrol. However, when WS-1 cells treated with the lowest dose of carvacrol, it reduced the ROS level compared to the control group. This difference was also statistically significant ($P \leq 0.001$).

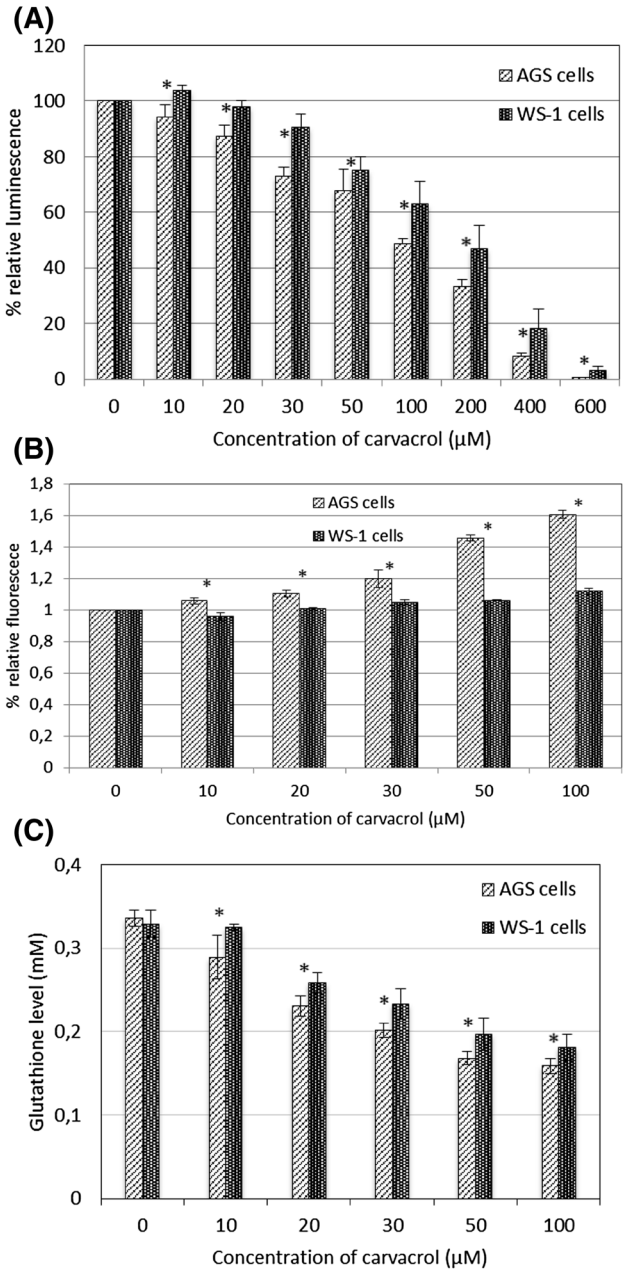

Fig. 1 **a** Cytotoxicity in human AGS and WS-1 cells exposed to carvacrol (0–600 µM) after 24 h. Carvacrol-induced cytotoxicity was in a dose-dependent manner. **b** ROS level in AGS and WS-1 cells exposed to carvacrol (0–100 µM) after 24 h was detected by DCFH-DA assay. **c** GSH level in AGS and WS-1 cells after 24 h of exposure to carvacrol (0–100 µM). All values are expressed as mean ± SD. *Differences were considered significant compared to the control group from $P \leq 0.001$

Effects of carvacrol on GSH level in AGS and WS-1 cells

Carvacrol induced dose-dependent reduction of GSH levels in both cell lines ($P \leq 0.001$). The measured relative luminescences are shown in percentages (Fig. 1c). A negative correlation was found between relative GSH levels and increased concentration of carvacrol. However, WS-1 cells exposed to carvacrol at lower concentration and WS-1 control cells showed the same GSH level.

Apoptotic effect of carvacrol on AGS and WS-1 cells detected by AO/EB staining assay

To evaluate whether the cytotoxic effect of carvacrol is caused by apoptosis, AO/EB staining was used to visualize nuclear changes and apoptotic characteristic body formation t. After staining the cells, were observed under a fluorescence microscope and counted to quantify apoptosis. After treatment with carvacrol (0–100 µM) for 24 h, cell morphology became abnormal in both cell lines with cell nuclear shrinkage, and chromatin condensation (Fig. 2a). The number of apoptotic and necrotic cells increased significantly in a dose-dependent manner, whereas the viable cells decreased significantly in both cell cultures ($P \leq 0.001$) (Fig. 2b). A significant difference was also found for necrotic cells at the highest dose ($P \leq 0.001$).

Apoptotic effect of carvacrol on AGS and WS-1 cells detected by western blotting

Expressional analysis of apoptotic (*Caspase-9, Caspase-3*), pro-apoptotic (*Bax*), and anti-apoptotic (*Bcl-2*) proteins have been studied in AGS and WS-1 cells by western blotting. Changes were investigated in cells treated with carvacrol (0–50 µM) after 24 h of incubation. Protein expressions for both cell cultures are demonstrated in Fig. 3a–c. The results revealed that in cells exposed to carvacrol, the ratio of *Caspase-3, Caspase-9*, and *Bax* proteins was significantly increased, whereas *Bcl-2* protein was decreased in a dose-dependent manner in both cell lines (Fig. 3c) ($P \leq 0.001$) suggesting that carvacrol induces apoptosis by decreasing *Bcl-2* protein levels, and increasing *Bax, Caspase-3*, and *Caspase-9* proteins level. However, *Bcl-2* level in WS-1 cells remained still higher than in AGS cells. *β-actin*, as a control, was positive in all samples.

Genotoxic effect of carvacrol on AGS and WS-1 cells detected by comet assay

The comet assay was used for the detection of carvacrol-induced DNA damage. Genotoxic effect was visualized as Comet formation (Fig. 4a). The percentages of tail DNA are shown in Fig. 4b for both cell lines. Significant differences in tail DNA formation were found between AGS and WS-1 cells exposed to carvacrol (0–100 µM) ($P \leq 0.001$).

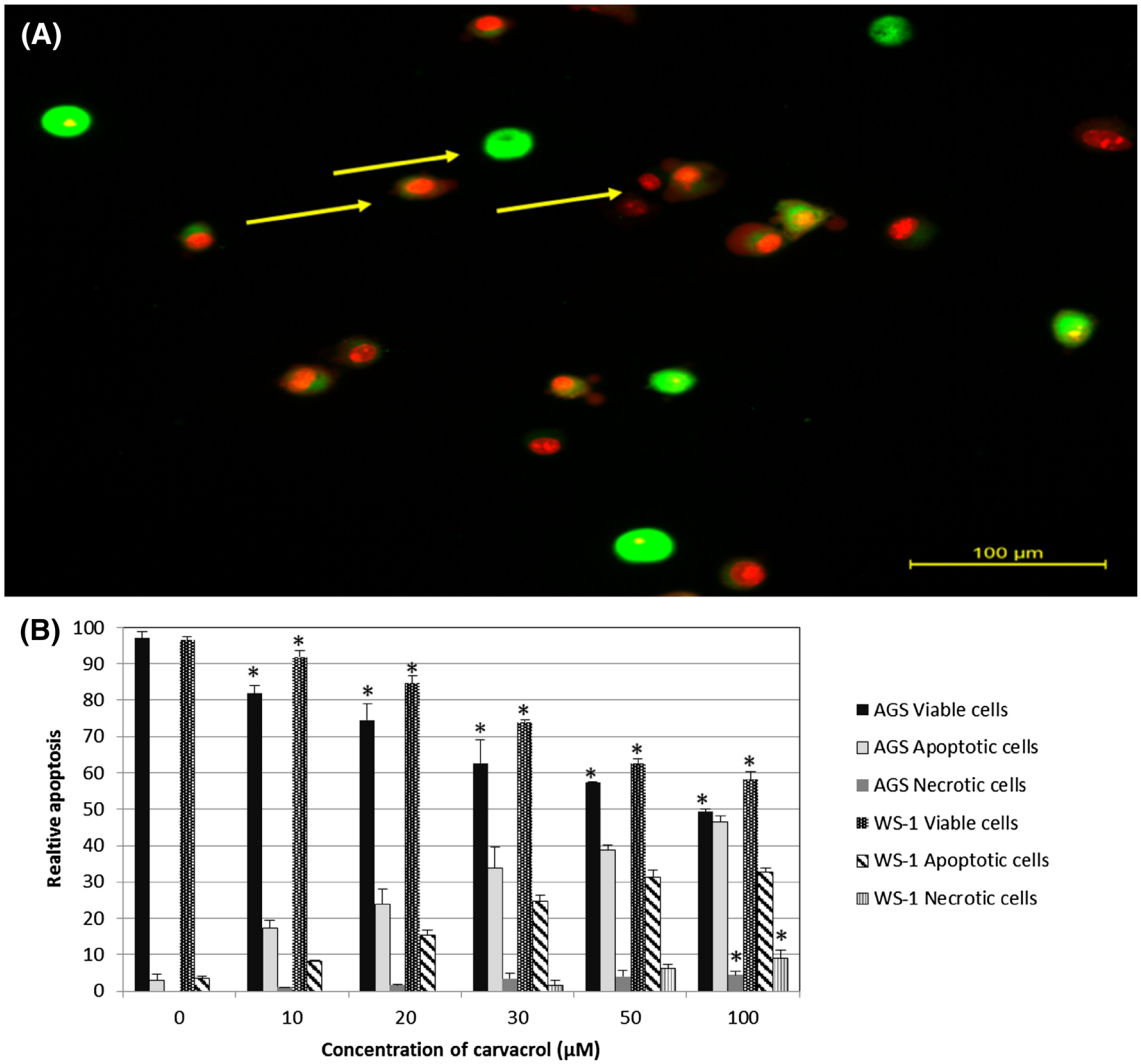

Fig. 2 Morphological changes in AGS and WS-1 cells exposed to carvacrol (0–100 µM) after 24 h. **a** A sample showing viable (green), apoptotic (orange), and necrotic (red) cells by acridine orange/ethidium bromide staining under florescence microscope. **b** Effect of carvacrol on cell viability in comparison with control cells are given in percentages. All values are expressed as mean ± SD. *Differences were considered significant compared to the control group from $P \leq 0.001$. (Color figure online)

In vivo activity of carvacrol

In order to investigate whether carvacrol induces oxidative stress in vivo, carvacrol was orally applied to Wistar rats over a period of 60 days. During the experiment, BW of rats ($n = 8$) increased significantly in the experimental group relative to animals of the control group ($n = 8$) ($P \leq 0.001$) (Fig. 5a). Also, significant differences were observed in BW of the experimental group for each measurement point ($P \leq 0.001$). At necropsy, rats from the control group revealed no pathological changes of organs or tissues, whereas rats from the experimental group showed tense abdomen, swollen stomach, and bowel with gas formation (Fig. 5b, c). Their stomach tissues were rubor and thinned. Stomach tissues from the experimental group stained with H&E showed infiltration of immune cells. Immunohistochemical staining of stomach tissues from the experimental group revealed that the expression of *Caspase-9* and *Bax* proteins in cells significantly increased, whereas the expression of *Bcl-2* protein was decreased. None of these effects were observed in animals of the control group. Results from the biochemical analyses showed that TOS and OSI levels in the plasma and stomach tissues of the experimental rats were significantly higher than those of control rats (Fig. 5d, e).

Discussion

GC is the third leading cause of cancer-related death in both sexes (8.8% of all deaths) worldwide [1]. Treatment includes surgery (removal of resectable gastric cancer),

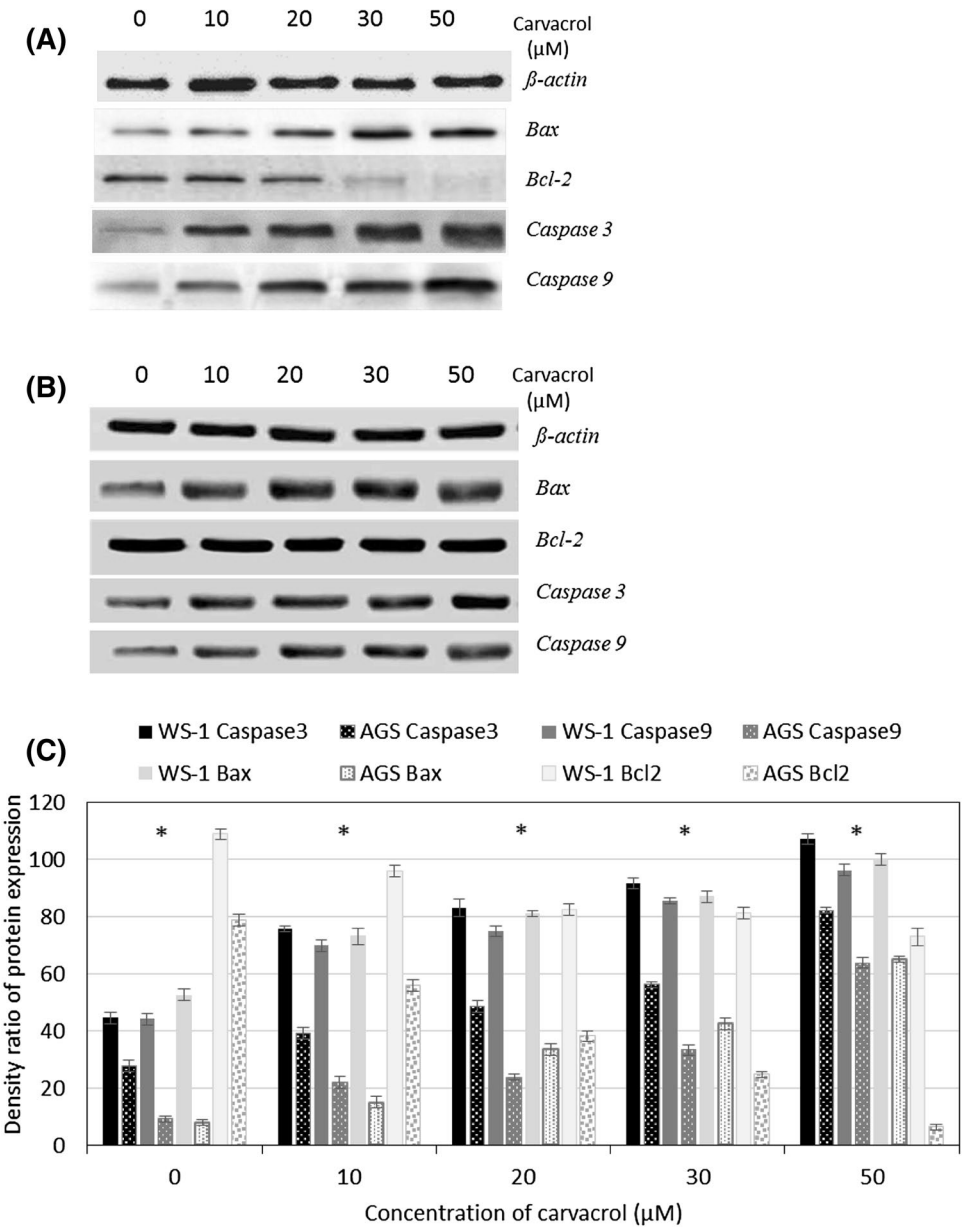

Fig. 3 Carvacrol-induced apoptosis was detected by western blotting. Human AGS and WS-1 cells were incubated with 0–50 μM of carvacrol. Protein expressions of *Bax, Bcl-2, Caspase-9*, and *Caspase-3* in comparison with *β-actin* are demonstrated in AGS cells (**a**) and WS-1 cells (**b**). Density ratios of expressed proteins are presented in percentages for both cells (**c**). All values are expressed as mean ± SD. *Differences were considered significant compared to the control group from $P \leq 0.001$

peri-/post-operative chemotherapy or chemoradiation. At later stages of cancer, even treatment may yield a poor prognosis with less than 25% overall 5-year survival [40]. Surgery remains the only curative therapy, whereas perioperative and adjuvant chemotherapy, as well as chemoradiation, can improve outcome of resectable gastric cancer with extended lymph node dissection [41]. In patients diagnosed with unresectable gastric cancer, extensive chemotherapy [42] or gene therapy [43] may help to shrink the tumor in order to allow curative resection since radical and complete resection represents the only potentially curative therapy for gastric cancer. For this purpose of improving effective cancer treatment, novel anti-cancer drugs must be developed [44]. Our strategy is to evaluate the biological effects of naturally occurring phenolic monoterpenes, especially carvacrol. In this regard, it is important to investigate whether or not carvacrol has the potential to increase ROS generation, cytotoxicity, genotoxicity, and apoptosis in human gastric adenocarcinoma cells. Even more important for the development of treatment strategies is to know whether these compounds could also impair healthy human cells and if so, to what extent.

To date, several natural phenolic compounds have been screened for their chemopreventive and anti-cancer treatment using different in vitro and in vivo experimental models [45]. It was suggested that polyphenols might have anti-cancer activity with an evidence of significantly inhibiting proliferation of cancer cells thereby inducing apoptosis.

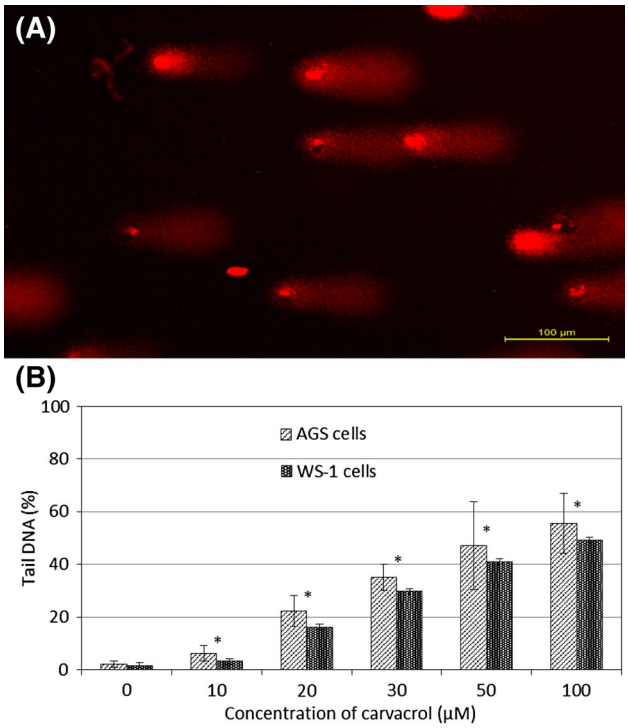

Fig. 4 Genotoxic effect of carvacrol on AGS and WS-1 cells was detected by comet assay. Cells exposed to (0–100 µM) carvacrol were analyzed after 24 h of incubation. **a** A sample showing increased comet formation (DNA damage). **b** Carvacrol-induced DNA damage was in a dose-dependent manner. All values are expressed as mean ± SD. *Significantly different from control ($P \leq 0.001$)

However, the clinical efficacy of these dietary agents needs careful evaluation in terms of targeting specific cancer cells and avoiding toxicological side effects on healthy cells [25].

Cytotoxic and apoptotic effects of carvacrol observed in this study were in a dose-dependent manner but this effect was more effective on human AGS cells than human WS-1 cells. Other studies reported also cytotoxic and apoptotic effects of carvacrol on different cancer and healthy cell lines [11–20, 30, 31]. In these studies, carvacrol induced cell death by apoptosis and necrosis. Similarly, in our study, apoptosis as well as necrosis were observed in human AGS and WS-1 cells exposed to carvacrol over a period of 24 h. Phenolic compounds can affect the cellular redox status because of their pro-oxidant capacity. This may result in cytotoxicity, apoptosis, and/or necrosis following ROS generation [18, 46].

Increased ROS generation has been seen in the onset and progression of cancer [21, 24]. Phenolic compounds are chemical substances which may have either antioxidant or pro-oxidant effects depending on conditions such as cell resistance, concentration, and time. Pro-oxidants can induce oxidative stress either by generating ROS or by inhibiting or decreasing the antioxidant status of cells [14, 18, 46, 47]. However, the toxic mechanism of carvacrol seems to be linked to mitochondrial membrane damaging by permeabilization, resulting in a pro-oxidant status and induction of apoptosis thereafter [12, 14–18, 46, 47]. A few studies reported that carvacrol at lower concentrations protects the human glioblastoma, human colon carcinoma, and human acute promyelocytic leukemia cells against the ROS [18, 46, 47], whereas its higher concentrations induce an oxidative stress following increased ROS generation. Although there is important research data about the anti-proliferative and apoptotic effects of carvacrol as well as its antioxidant effect, limited information exists on its pro-oxidant activity in cancer and healthy cells. The GSH level is an indicator for cell health [23]. The GSH is an antioxidant which scavenges the intracellular ROS. Therefore, the effects of carvacrol on intracellular ROS and GSH level were examined in the present study. A positive relationship was found between cytotoxicity and increased ROS generation after 24 h of exposure to carvacrol at higher concentrations, whereas there is a negative relationship between cytotoxicity and decreased GSH level. This result was in agreement with earlier reports [18, 46, 47]. It has to be pointed out that in contrast to higher doses of carvacrol, low concentrations of this phenolic compound induced increased proliferation of fibroblast cells compared to untreated control cells. This effect was accompanied by reduced ROS and unaltered GSH levels relative to untreated control cells. None of these effects were seen in gastric adenocarcinoma cells. In contrary, AGS cells were negatively affected by carvacrol even at its lowest concentration. This observation seems to be important for designing in vivo studies using appropriate dose rates for optimizing the treatment outcome while minimizing drug-induced side effects.

The main effect of carvacrol on cancer cells seems to be the initiation of apoptosis which in general is a physiological cell death that is programmed by different proteins. Previously, it has been reported that carvacrol induces apoptosis by direct activation of the mitochondrial pathway in human glioblastoma DBTRG-05MG, human hepatoma HepG2 and human metastatic breast cancer MDA-MB 231 cells [12, 15–18, 47]. In human glioblastoma DBTRG-05MG and in human acute promyelocytic leukemia cells carvacrol induced apoptosis through the activation of *Caspase-3* [18, 47]. In MDA-MB 231 cells, it was shown to act by decreasing the mitochondrial membrane potential, release of *Cytochrome-c* from mitochondria, increase in multicaspase activity, inactivation of *PARP*, and the decrease of *Bcl-2*/*Bax* ratio [12]. The apoptotic effect of carvacrol on HepG2 cells was confirmed by the activation of *Caspase-3*, cleavage of *PARP*, and decreased *Bcl-2* gene expression [15]. Additionally, it was reported that carvacrol-treated human breast cancer cell line MCF-7 showed an induction of apoptosis through increased expression levels of *p53, Bax, Caspase-9,*

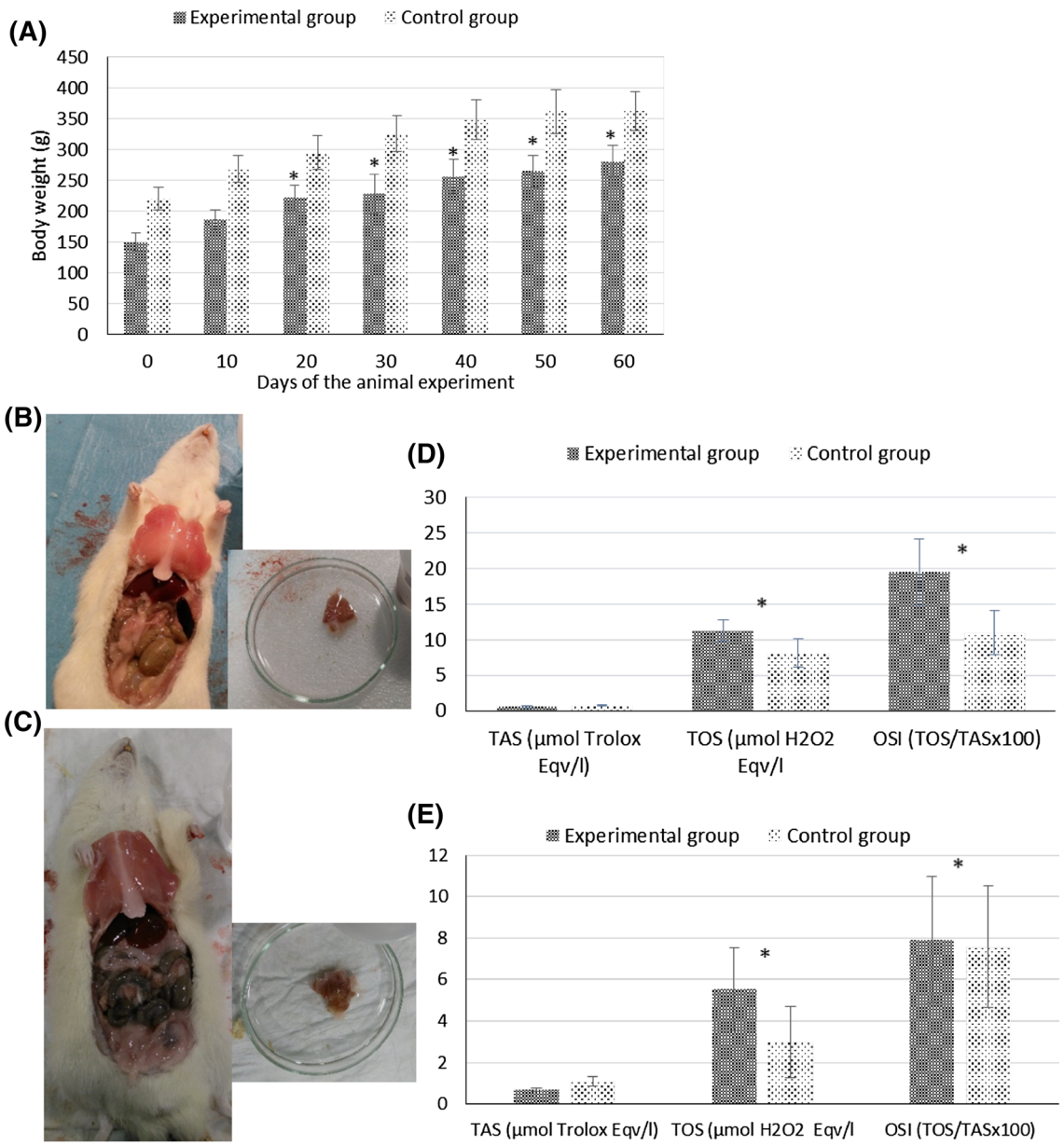

Fig. 5 Results from the in vivo effects of high dose carvacrol are presented. **a** Body weight changes in both rat groups. **b** Macroscopic appearance of a rat and its stomach from the experimental group at the necropsy. **c** Macroscopic appearance of a rat and its stomach from the control group at the necropsy. **d** Measured TAS, TOS, and OSI values in plasma of both rat groups. **e** Measured TAS, TOS, and OSI values in stomach tissues of both rat groups. All values are expressed as mean ± SD. *Significantly different from control ($P \leq 0.001$)

Caspase-6, Caspase-3 proteins, and decreased *Bcl-2* level [16]. As an initiator protein *Caspase-9* and as an effector protein *Caspase-3* are in the cascade of apoptosis, *Bax* promotes the apoptosis process, and *Bcl-2* is thought to protect cells from apoptosis [26]. To elucidate the mechanism in gastric adenocarcinoma, we aimed to investigate and compare the expression levels of such proteins by western blotting in carvacrol-treated cancer cells (human AGS cells) and in healthy cells (human WS-1 cells). Protein levels of *Bcl-2* decreased in both treated cell lines but *Bcl-2* level was significantly higher in WS-1 cells than in AGS cells. The obtained results were in agreement with earlier reports. To our knowledge, our study is the first report describing *Caspase-9* and *Caspase-3* activity of carvacrol in human AGS and WS-1 cells. Down-regulation of *Bcl-2* and up-regulation of *Bax, Caspase-9*, and *Caspase-3* protein expression in human AGS cells may draw attention to further potential assessment of carvacrol as an ideal therapeutic agent for gastric adenocarcinomas. The observed effect that anti-apoptotic *Bcl-2* remained upregulated in WS-1 cells makes

it even more attractive to speculate that carvacrol may serve as a future anti-cancer treatment option.

For more detailed understanding of the underlaying mechanisms of carvacrol-induced cell impairment, the comet assay was performed which is widely used as biochemical marker for DNA damage in the sense of genotoxicity [48]. Polyphenols at lower concentrations may have antioxidant effect, whereas their higher concentrations may induce DNA damage [49]. Carvacrol showed after 24 h of incubation genotoxic effect on human AGS and WS-1 cells in a dose-dependent manner. This result is in agreement with the previous reports described for mouse myoblast and neuron cancer cells exposed to carvacrol [20, 50]. Opposite to these results, a study reported carvacrol at lower concentrations not to be associated with a DNA-damaging effect on HepG2 and Caco-2 cell lines [11]. This concentration-dependent effect of polyphenols may be a target for mitochondrial membrane permeabilization. Polyphenols at higher concentrations could damage, and permeabilize the mitochondrial membranes, oxidize the antioxidants, and react as a pro-oxidant, followed by DNA damage. It seems that the conversion from antioxidants into pro-oxidants occurs at higher concentrations [19, 49]. Therefore, we investigated this pro-oxidant effect also in vivo by oral application of high-dose carvacrol (100 mg/kg BW) to Wistar rats. The results showed that rats from the experimental group had higher oxidative stress in stomach tissues as well as in their blood than rats from the control group. The observed increase in BW in the experimental group is thought to be caused by the pathological alterations presented as swollen abdomen, and gas-filled stomach and bowels at necropsy. These results were also confirmed by the pathohistological examination of tissues showing significant differences in alteration between samples of the experimental and control group. Further in vivo studies need to explore whether low concentrations of carvacrol can be better tolerated by healthy cells, as observed in our in vitro experiments, while being still effective against malignant cells such as AGS cells.

Conclusions

To our knowledge, this is the first study that presents and compares cytotoxic, genotoxic, apoptotic, ROS generating, and GSH decreasing activities of carvacrol on human AGS and WS-1 cells. In addition, the pro-oxidant activity of carvacrol was investigated in vivo on Wistar rats after application of high doses. Carvacrol inhibited cell proliferation, and induced cytotoxicity, DNA damage, apoptosis, ROS generation and decreasing GSH level in a dose-dependent manner. Moreover, it could also be demonstrated that the antiproliferative and apoptosis inducing effects of carvacrol are regulated by *Bax, Bcl-2, Caspase-3*, and *Caspase-9* proteins controlling the apoptosis of cells. There was a close negative relationship between cell viability and ROS level. Results of in vitro and in vivo investigations revealed that a dose-dependent effect of carvacrol on cancer and healthy cells can probably be related to the increasing ROS generation, *via* its pro-oxidant activity. Taken together, the carvacrol-mediated responses observed in the in vitro and in vivo experiments presented suggest a double-edged effect. *Via* a pro-oxidative mechanism carvacrol is able to harm cancer cells and thus may qualify as a candidate for the development of future gastric cancer treatment strategies. Nevertheless, it has to be taken into consideration that carvacrol used in higher concentrations may also induce harmful side effects in normal healthy cells. Further research has to address this concentration-dependent double-edged mechanism.

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Author contributions AGB and AD wrote the paper; AGB and AK conceived and designed the study; AGB, EMG, ISE, and MGB did the experiments; AGB and EMG collected and analyzed the data. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no actual or potential conflicts of interest.

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