Use of micronucleus and comet assay to evaluate the genotoxicity of oregano essential oil (*Origanum vulgare* l. *Virens*) in rats administered orally for 90 days.

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Running title: Genotoxicity study of oregano essential oil in rats

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Abstract

Essential oils from *Origanum spp.* exhibit antioxidant and antimicrobial activities making them suitable for use as food additives. The incorporation of oregano essential oil in active food packaging is under study; however, it has been not authorized for this purpose thus far. In order to fulfill the requirements of the European Food Safety Authority (EFSA), the aim of the present study was to determine the genotoxic potential of oregano essential oil using both micronucleus (MN) test and comet (standard and enzyme-modified) assays in Wistar rats treated with 50, 100 or 200 mg/kg body weight administered daily for 90 days. MN was performed in cells from the bone marrow and standard and enzyme-modified comet assays were conducted in stomach, liver and blood cells. The major compound detected in the analytical study of oregano essential oil from Origanum vulgare L. virens, was carvacrol (55.82%) followed by thymol (5.14%), as well as their precursors, γ-terpinene (16.39%) and ρ-cimne (4.71%). The results obtained in the genotoxicity assays indicated lack of effect in MN and standard comet assay under the conditions tested. Further, no apparent oxidative damage was observed in the enzyme-modified comet assay in any of the tissues examined of rats exposed to oregano essential oil for 90 days. Therefore, this oregano essential oil appears to be safe in Wistar rats and might be considered as a potential active material in food packaging industry.

Keywords: genotoxicity, micronucleus, comet assay, oregano essential oil, *in vivo*

Introduction

Herbs and spices containing essential oils (EO) are widely used as flavoring substances in food, soft drinks and beverages (Liju et al. 2013) and might be considered to be used as food additives as an attractive alternative to the synthetic ones in order to improve food shelf life (Hylgaard et al. 2012). Therefore, the safety of these EO needs to be assessed prior their utilization in the food industry either as additive in foods or as active substances in food packaging. In this regard, although antioxidant and antimicrobial studies regarding botanical preparation and/or its respective components are known (Bakkali et al. 2008; Burt 2004; Lang and Buchbauer 2012), toxicological data for EO are still scarce (Hwang and Kim 2012; Escobar et al. 2012, 2015), especially *in vitro* and *in vivo* data on genotoxic potential. Further, the disparity in these results needs to be investigated due to the lack of awareness if some of the compounds detected in the EO exhibit genotoxic, carcinogenic profile (Mellado-Garcia et al. 2016a). Among the predominant EO employed due to their extraordinary antimicrobial and antioxidants properties, the oregano essential oil (OEO) requires critical attention (Burt 2004; Olmedo et al. 2014; Viuda-Martos et al. 2009). This OEO is usually obtained from the aerial part of *Origanum* spp. by steam distillation, hydrodistillation or simultaneous distillation-extraction methods (Bayramoglu et al. 2008). OEO is included as well in the GRAS list by the FDA, although in Europe more studies are required to be approved for this purpose. Regarding OEO safety, varying findings were noted regarding *in vitro* toxicity potential of parent compound and main components as previously reported (Llana-Ruiz-Cabello et al. 2015). In general, the OEO tested did not display mutagenic activity with or without metabolic activation in the Ames test at the conditions tested (Ipek et al. 2005; De Martino et al. 2009; Gulluce et al. 2012); however, a reduction in mitotic index, chromosomal and nuclear irregularities were

found (Hamedo and Abdelmigid 2009). The nature of OEO makes it problematic for evaluation due to (1) its mixture in aqueous solutions, such as culture media of cell models, (2) results in turbid suspension due to hydrophobicity; and consequently, end-points of *in vitro* assays are difficult to determine (Hanlon et al. 2011). Hence, the assessment of their main compounds is an alternative when these are well defined (EFSA, 2009). Similarly to OEO, contradictory results were found regarding its main components, such as carvacrol and thymol (Llana-Ruíz-Cabello et al. 2015). Absence of mutagenic activity for carvacrol in the *Salmonella*/microsome reversion test (Stammatti et al. 1999) and in the sister chromatid exchange assay (Ipek et al. 2003) was reported. However, Ipek et al. (2005) and Llana-Ruíz-Cabello et al. (2014) demonstrated that carvacrol is a potent, direct-acting mutagen in the Ames test without and with external metabolic activation. Similar to mutagenic assays, *in vitro* studies of genotoxic activity of carvacrol provided contradictory observations because this substance induced both positive (Aydin et al. 2005a; 2005b; Llana-Ruíz-Cabello et al. 2014) and negative (Horváthová et al. 2006) results. In case of positive *in vitro* results EFSA urged to conduct *in vivo* tests. In addition, the Scientific Committee notes small number of substances that are negative *in vitro* might exert positive *in vivo* results as *in vitro* metabolic activation system does not encompass the full spectrum of potential genotoxic metabolites generated *in vivo* (EFSA, 2011). Consequently, *in vivo* data as well as an appropriate OEO profile characterization are necessary (EFSA 2009). In this regard, no apparent *in vivo* genotoxic effect for carvacrol was reported in rats using a combined micronucleus (MN) and comet assays (Llana-Ruiz-Cabello et al. 2016a).

In the present investigation, the chemical composition of OEO obtained from *Origanum vulgare* L. *virens* grown in Almería (Spain) was examined taking into account that botanical preparations need to be characterized due to variability. Further,

both MN and comet assays were conducted to evaluate *in vivo* potential genotoxic effects of OEO in bone marrow, as well as in stomach, liver, and blood cells of Wistar rats administered OEO for 90 days.

Materials and Methods

Supplies and chemicals

Chemicals, including Endo III and FPG, were purchased from Sigma-Aldrich (Madrid, Spain), VWR Eurolab (Madrid, Spain), C-viral S.L. (Seville, Spain) and BioWhittaker (Madrid, Spain).

Isolation of essential oil and gas chromatography analysis

The isolation of the OEO was performed in El Jarpil S.L. (Almería, Spain). Leaves and aerial parts of oregano (*Origanum vulgare* L. *virens*) were collected from Bédar (Almeria, Spain) in June 2012 and stored for 24-48 hr at 23ºC before distillation. A continuous water steam distillation extraction was performed for approximately 3.5 hr and the oil collected and stored at 4°C. The analysis was carried out according to Llana-Ruíz-Cabello et al. (2017), using a Hewlett Packard 5890 chromatograph interfaced to a Hewlett Packard 5970 mass selective detector (Hewlett Packard, Palo Alto, USA). Components found over 0.01% are presented in Llana-Ruíz-Cabello et al. (2017).

Animal housing and feeding conditions

The Ethical Animal Experimentation Committee of the University of Córdoba and the Junta de Andalucía approved the procedures developed in this study (project no. 05/10/2015-339). In addition, all animals received humane 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).

Forty-six Wistar rats (23 males and 23 females) strain Crl.WI (Han) were randomly distributed into 5 groups per gender (5 rats/group and 3 rats/positive control group), with similar mean body weight (297.5 ± 2 g for males and 188.7 ± 3.6 g for females) for each group, and individually housed in cages. Rats were fed with standard lab feed (Harlan 2014, Harlan Laboratories, Barcelona, Spain) and water *ad libitum*.

Experimental design and treatment

In order to reduce animal usage in genotoxicity testing, the European Food Safety Authority (EFSA) recommends to integrate both MN and comet assay into repeated-dose toxicity study (EFSA, 2011). Hence, in this investigation, the MN and comet assay, described in the Organisation for Economic Co-operation and Development (OECD) protocols (OECD 474 and 489, respectively) were developed associated into a repeated-dose toxicity study (OECD 408).

After acclimatization, animals were treated daily for 90 days with 50, 100 or 200 mg/kg/day of OEO incorporated to neutral gelatin according to Mellado-García et al. (2016a). These doses were selected according to an acute oral toxicity study "Up and Down Procedure" OECD 425 (OECD 2008) and after a palatability experiment which evidence that 200 mg/kg/day was the highest dose tolerated by animals. Finally, two additional spaced dose levels separated by a factor of 2 were employed. Neutral gelatin without OEO was used as negative control. Acute oral exposure was selected for the positive control group (three male and three female rats) which was administered 200 mg/kg/day ethylmethanesulfonate (EMS) using a stomach tube (Vygon, Ecouen, France). In order to obtain the positive controls for both assays three dosages were used as described by Bowen et al. (2011) and Mellado-García et al. (2016b).

Sample collection

Samples of bone marrow, stomach and liver were collected according to Mellado-García et al. (2016b). Blood samples were obtained by intracardiac injection (lightly anesthetized with isoflurane) and maintained in Vacutainer® sodium heparin tubes (Becton Dickinson, Rutherford, NJ).

Micronucleus (MN) assay

The recommendations of Corcuera et al. (2015) and the principles of OECD guideline 474 were followed to perform the mammalian erythrocyte MN test in bone marrow of Wistar rats exposed to OEO (OECD 2014a). Briefly, bone marrow extensions were performed on slides, fixed in methanol and stained with Giemsa (10 %). Then, the polychromatic erythrocytes (PCE) among total erythrocytes (normochromatic erythrocytes (NCE) + PCE) ratio and PCE among NCE ratio were determined by counting 500 erythrocytes per animal. The incidence of micronucleated immature erythrocytes (MNPCE) expressed as % MN was analyzed by counting a total of 5000 cells per animal.

Isolation of single-cell suspensions for the comet assay

Single cell suspensions from stomach and liver were isolated based upon the method of Corcuera et al. (2015) and Mellado-García et al. (2016b). Briefly, tissues were washed with Merchant´s buffer (MB) (0.14 M NaCl, 1.47 nM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 10 mM Na_2EDTA, pH 7.4) and medium discarded. After that a portion of each tissue were homogenized in cold at 4^0C. Finally, homogenates were centrifuged, filtered and mixed with 5ml buffer until slide preparation. Heparinized blood samples were mixed v/v (1/1) with phosphate buffered saline (PBS) solution and lymphocytes isolated using histopaque® (Sigma-Aldrich, Madrid, Spain) and centrifuged at 1200g for 30 min. Finally, cells were washed twice with PBS and re-suspended in PBS at a concentration of 2 x 10^5 cells/ml.

Standard and enzyme-modified comet assay

For blood samples, 30 µl cells suspension was mixed with 140 µl 0.5% low-melting point agarose and 12 drops of 5 µl were placed on a microscope slide. For stomach and liver cell suspensions were mixed with 1% low-melting point agarose and mixtures placed on a microscope slide in the same manner as blood samples. Then, the standard comet assay was performed as previously described by Mellado-García et al. (2016b) and according to the recommendations of OECD guideline 489 (OECD, 2014b). Combining the comet assay with restriction enzymes such as Endo III, which responds to oxidized pyrimidines, and formamidopyrimidine (DNA) glycosylase (FPG) , which recognizes altered purine bases including 8-oxo-guanine, enabling detection of lesions that are not severe breaks (Azqueta et al., 2009). Briefly, 30 µl cell suspension were mixed with 140 µl pre-warmed low-melting-point agarose in PBS, and immediately 12 aliquots of each cell suspension were placed on agarose precoated glass slides. After lysis, slides were washed three times for 5 min with enzyme buffer (40 mM HEPES, 0.1M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8). Subsequently, two gels in each slide were exposed to 30 µl lysis solution, enzyme buffer alone (buffer F), buffer F containing FPG (10 U/ml) or buffer F containing Endo III (14 U/ml) for up to 30 min in a metal box at 37 ºC. Then, nuclei were denatured and electrophoresis performed. Finally, DNA was neutralized in PBS, washed with water and fixed with 70% and absolute ethanol before staining. Images of at least 100 randomly selected nuclei per animal were analyzed with the image analysis software Comet Assay IV (Perceptive Instruments, UK). The % DNA in tail represents DNA strand breaks and oxidized damage in DNA bases. Results obtained for control groups exhibited 10 % DNA in tail indicating that cells are in satisfactory conditions for comet assay (Collins 2004).

Statistical analysis

Results from the MN assay are presented as mean ± SD for each group of animals and statistical analysis was performed using the analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. For the standard and enzyme-modified comet assay, means ± SD were calculated and total scores of different groups compared using the non-parametric Kruskal–Wallis test followed by the Mann–Whitney U-test when the first test showed differences. Analysis was performed using Graph-Pad InStat software (GraphPad Software Inc., La Jolla, USA). The criterion for significance was set at $p<0.05$.

Results

No mortality occurred during the study. Body weight gain or food/water consumption were not significantly altered through the experiment. Additional data is available in Llana-Ruiz-Cabello et al. (2017).

Chemical composition of OEO

Gas chromatography-mass spectrometry (GC-MS) analysis of *Origanum vulgare* L. *virens* essential oil resulted in identification of 108 compounds, representing 99.9% of the essential oil. Based upon GC-MS analysis data for *O. vulgare* L. *virens* essential oil: carvacrol (55.82%), p-cimene (16.39%), thymol (5.14%), γ-terpine (4.71%) and β-cariophyllene (2.40%) were found to be the main components, comprising 84.46% of the sample.

Micronucleus (MN) assay

Results obtained in the MN assay of rats exposed to OEO are presented in Table 1. In comparison with their respective controls, the PCE/total erythrocytes ratio exhibited significant differences from 100 mg/kg in male rats and from 200 mg/kg in female rats.

Regarding PCE/NCE ratio in males, a significant decrease was observed at 100 mg/kg, while in females this significant reduction was noted at the highest dose assayed. Finally, the % of MN in immature erythrocytes was not markedly affected in all groups of both genders. In contrast, treatment with EMS (positive control) induced a significant increase in the frequency of MN.

Standard and enzyme-modified comet assay

No marked DNA strand breaks were observed in stomach (Figure 1A), liver (Figure 1B) and blood samples (Figure1C) in the standard comet assay. Similarly, no significant alterations were detected in the frequency of Endo III or FPG-sensitive sites in any tissue. However, a significant elevation was found in 200 mg/kg EMS (positive control) treated groups.

Discussion

Origanum vulgare L. EO, as well as other EO, is intended to be used as additive in food and food packaging due to its antimicrobial and antioxidant properties (Ipek et al. 2005). In fact, the effectiveness of this OEO included in polylactic acid (PLA) was identified in lettuce packaged with this active film (Llana-Ruiz-Cabello et al. 2016b). However, the lack of data on genotoxicity, reproductive and developmental toxicity is delaying the approval of these substances as food additives by the European Commission. In this regard, EFSA (2016) reported that genotoxic potential of the botanical preparation and its respective components needs to be clarified prior to authorization in Europe (EFSA, 2016).

Chemical variations in composition of EO are widely known (Burt 2004) but the sources of this variability are hard to determine. Németh-Zamboriné (2016) indicated the factors that influence the composition of these complex mixtures may be classified

as abiotic such as soil, light intensity, temperature or water supplies; or biotic, which are related to the genetic differences observed in species from the same botanical genus. The extraction method used to obtain the EO plays a prominent role in the final qualitative and quantitative composition of these substances (Azmir et al. 2013). Consequently, the EO obtained from different sources or by varying extraction techniques might account for differing properties, activities or toxicological behavior. In this regard, the guidance on safety assessment of botanicals suggests that the chemical composition of EO need to be provided with emphasis on the concentrations of constituents of relevance for safety assessment (EFSA 2009). The compositions of different OEO were previously determined by (Mezzoug et al. 2007; Bostancioglu et al. 2012; Lukas et al. 2015). Although the quantity of the major compounds seems to be variable, most investigators agreed that carvacrol, thymol and their precursors, c-terpinene and ρ-cymene, are the major constituents present in oils obtained from *Origanum vulgare* species (Mezzoug et al. 2007; Bostancioglu et al. 2012; Lukas et al. 2015). Our data are in agreement with these findings showing that carvacrol (55.82%), followed by its precursor ρ-cimene (16.39%) are the main components in the EO obtained from *Origanum vulgare* L. *virens*. A ratio of carvacrol/thymol 10:1 was found, although this may vary between approximately 1:1 (Mezzoug et al. 2007; Özkan & Erdogan 2011), and 50:1 (Bostancioglu et al. 2012).

In relation to the potential use in the food industry of OEO, the toxicological profile needs to be determined. There are apparently no *in vivo* studies focused on the genotoxicity of the complete OEO mixture, but individual components have been assessed. In this regard, EFSA (2011) encouraged integration of genotoxicity test, such as comet assay and MN test; into repeated-dose toxicity studies (RTD) because the combined measurement of genotoxicity endpoints offers the possibility for an improved

interpretation of genotoxic findings since such data may be evaluated in conjunction with routine toxicological information obtained in the RTD study. In the present investigation, an absence of significant differences with respect control group in % of MN and in the DNA strand breaks in the standard comet assay were observed in rats exposed daily for 90 days to OEO. Further, no oxidative damage was found in the enzyme-modified comet assay in any of the tissues investigated of the animals. This finding is particularly interesting because polyphenolic plant extracts belonging to the *Lamiaceae* family, such as oregano, exhibit both antioxidant and prooxidant behavior depending on the concentration used (Samec et al. 2015). Therefore, a positive result in this assay may have been expected. In the case of the MN assay, evidence of exposure of bone marrow is required to show when negative results are obtained (OECD 474, 2014). In this context, one of the findings included a reduction of the PCE/NCE ratio in bone marrow, which was noted in the present study, confirming that exposure of animals occurred.

The *in vivo* results obtained in the present experiment are useful to ensure whether the genotoxic potential *in vitro* is expressed *in vivo*. *In vitro* studies related to the mutagenicity/genotoxicity of different extracts obtained from *Oreganum vulgare* are scarce and the existing studies did not give rise to safety concerns with respect to genotoxicity (Hamedo and Abdelmigid 2009). Ipek et al. (2005) and De Martino et al. (2009) reported that OEO did not induce mutagenic effects on TA98 and TA100 *Salmonella thyphimurium* strains. Similar results were noted for *S. thyphimurium* TA1535 and TA1537 and *Escherichia coli* wP2uvrA (Gulluce et al. 2012). In contrast, Hamedo and Abdelmigid (2009) reported reduction in the mitotic index and chromosomal and nuclear irregularities in *Vicia faba* seeds exposed to 0.1% OEO using the chromosomal aberration (CA) test.

In relation to the components of OEOs most studies regarding genotoxicity were conducted using carvacrol, one of the active and major compounds, which seems to be responsible for antioxidant and antimicrobial properties. The *in vitro* genotoxicity of carvacrol was predominantly assessed and contradictory results reported (Stammatti et al. 1999; Ipek et al. 2003; Aydin et al. 2005a; 2005b; Horváthová et al. 2006; Llana-Ruíz-Cabello et al. 2014). However, comprehensive i*n vivo* genotoxicity of carvacrol remains to be determined. Azirak and Rencuzogullari (2008) found that carvacrol produced CA in rats. Llana-Ruiz-Cabello et al (2016a) examined the *in vivo* genotoxic potential of carvacrol combining the MN test in bone marrow cells with the comet assay on the stomach and liver cells of rats. Findings from these *in vivo* assays were also contradictory. Although Azirak & Recunzogulari (2008) found that carvacrol (0-70 mg/kg b.w.) was able to produce chromosomal abnormalities in the CA test in rats, in MN test and comet assays a lack of genotoxicity of carvacrol (0-810 mg/kg b.w.) was noted in bone marrow, stomach and liver cells (Llana-Ruiz-Cabello et al. 2016a). This discrepancy might be related to different endpoints measured in each assay. All these studies were carried out after short-term exposure.

Data may differ in exposure to the complete OEO as the interaction of major and minor components of the EO might produce additive, antagonist or synergism responses when the effect of EO is greater than the sum of individual compounds (Burt 2004) and therefore EO need to be considered as substances acting as a whole (Escobar et al. 2012). However, in the present study, the lack of *in vivo* genotoxicity observed in this OEO is in agreement with previous finding obtained with carvacrol (Llana-Ruiz-Cabello et al., 2016a). Hence, taking into account the *in vivo* genotoxicity results and *in vitro* observations, no apparent synergic/antagonistic phenomena occurred.

In summary, both MN and comet assays provided evidence for the first time a lack of potential *in vivo* genotoxicity of this OEO at doses up to 200 mg/kg administered daily to Wistar rats for 90 days, reinforcing the absence of genotoxicity previously demonstrated *in vivo* by the main component carvacrol. Considering the toxicological assessment performed in Wistar rats subchronically treated with this OEO following EFSA guidelines, it appears that OEO may be considered to be safe to be used as food additive.

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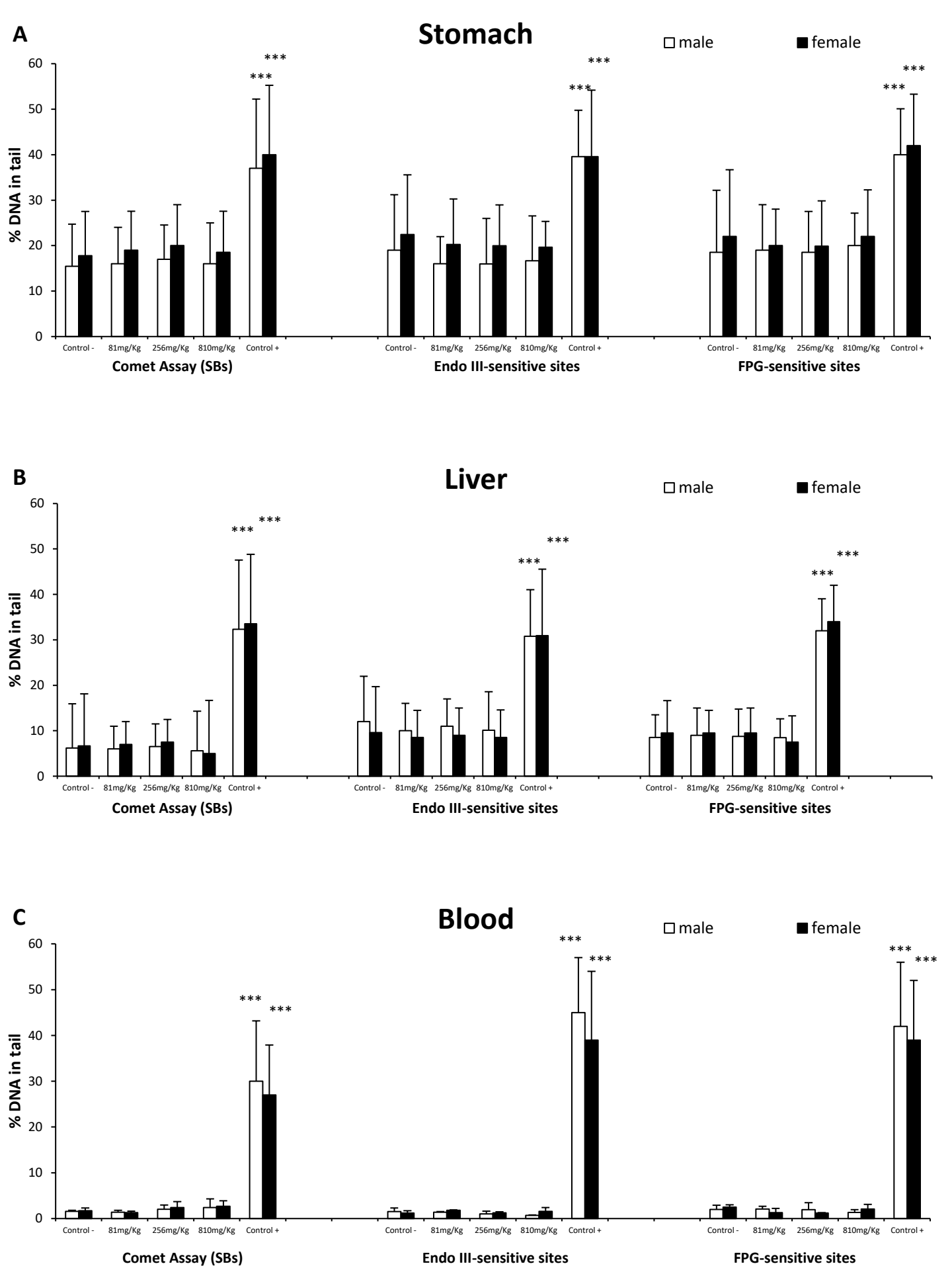
Legend to figure

Figure 1.DNA damage in stomach (A), liver (B)and blood (C)of male and female rats: formation of strand breaks detected by the standard comet assay and oxidative DNA damage as Endo III-sensitive sites and FPG-sensitive sites by the modified comet assay (from the left to right). The levels of DNA strand-breaks (SBs) and oxidised pyrimidines/purines are expressed as % DNA in tail. All values are expressed as mean ± SD. *Significantly different from control ($p< 0.05$).

Table caption

Table 1. Micronucleus assay results. Bone marrow cytotoxicity expressed as micronucleated polychromatic erythrocytes (PCE) among total erythrocytes; PCE among total erythrocytes (normochromatic erythrocytes (NCE) + PCE) ratio; and the micronuclei induction expressed as % MN. The values are expressed as mean±SD. * Significant from control $*p < 0.05$

Groups	N	Doses	Sex	PCE/Total	PCE/NCE	% MN
Negative Control	5	-	Male	0.49±0.02	0.98±0.07	0.34±0.10
	5		Female	0.50±0.02	1.00±0.10	0.23±0.06
Positive Control (EMS)	3	200 mg/Kg	Male	0.31±0.03*	0.45 ±0.07*	8.17±1.33*
	3		Female	0.31±0.03*	0.46±0.06*	7.55±0.20*
Oregano essential oil	5	50 mg/Kg	Male	0.50±0.01	0.99±0.04	0.33±0.13
	5		Female	0.49±0.03	0.98±0.13	0.21±0.11
	5	100 mg/kg	Male	0.45±0.03*	0.83±0.09*	0.27±0.20
	5		Female	0.47±0.04	0.88±0.15	0.28±0.18
	5	200 mg/kg	Male	0.41±0.02*	0.75±0.12*	0.35±0.21
	5		Female	0.40±0.02*	0.68±0.06*	0.24±0.07


A
Stomach
male
female
% DNA in tail
0
10
20
30
40
50
60
***
Control -
81mg/Kg
256mg/Kg
810mg/Kg
Control +
Comet Assay (SBs)
Endo III-sensitive sites
FPG-sensitive sites
B
Liver
C
Blood