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A subchronic 90-day oral toxicity study of *Origanum vulgare* essential oil in rats

M. Llana-Ruiz-Cabello [a], S. Maisanaba [a], M. Puerto [a], S. Pichardo [a, *], A. Jos [a], R. Moyano [b], A.M. Cameán [a]

[a] Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González n°2, 41012 Seville, Spain
[b] Department of Pharmacology, Toxicology and Legal and Forensic Medicine, University of Córdoba, Campus de Rabanales Carretera Madrid-Cádiz s/n, Córdoba, 14071, Spain

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ABSTRACT

Oregano essential oil (*Origanum vulgare L. virens*) (OEO) is being used in the food industry due to its useful properties to develop new active packaging systems. In this concern, the safety assessment of this natural extract is of great interest before being commercialized. The European Food Safety Authority requests different *in vivo* assays to ensure the safety of food contact materials. One of these studies is a 90 days repeated-dose oral assay in rodents. In the present work, 40 male and 40 female Wistar rats were orally exposed to 50, 100 and 200 mg/kg body weight (b.w.) OEO during 90 days following the OECD guideline 408. Data revealed no mortality and no treatment-related adverse effects of the OEO in food/water consumption, body weight, haematology, biochemistry, necropsy, organ weight and histopathology. These findings suggest that the oral no-observed-adverse-effect level (NOAEL) of this OEO is 200 mg/kg b.w. in Wistar rats, the highest dose tested. In conclusion, the use of this OEO in food packaging appears to be safe based on the lack of toxicity during the subchronic study at doses 330-fold higher than those expected to be in contact consumers in the worst scenario of exposure.

1. Introduction

Oregano (*Origanum vulgare L.*) is an aromatic plant with a wide distribution throughout the Mediterranean area and Asia (Wei et al., 2016). The composition of oregano essential oil (OEO) commonly includes carvacrol, thymol, α-terpinene and ρ-cymene among other compounds (Burt, 2004). The traditional applications of OEO are related to the properties against microorganism and oxidation. Recently, OEO has further application since it is recognized as a natural preservative agent with a strong potential for food preservation (Muriel-Galet et al., 2015). In this sense, the antimicrobial effect of OEO on food has been extensively studied. de Medeiros Barbosa et al. (2016) reported antimicrobial activity of OEO combined with rosemary EO at subinhibitory concentrations in fresh leafy vegetables. Also in meat, OEO has demonstrated to display an antimicrobial effect alone (Soultos et al., 2009; Jayasena and Jo, 2013; Pesavento et al., 2015) and in combination with other EO from clove and cinnamon (Radha Krishnan et al., 2014). In addition, OEO has been also useful in cheese not only as antibacterial (Govaris et al., 2011) but also as antioxidant (Asensio et al., 2015). However, the direct addition of OEO may alter the organoleptic characteristics of food and influence negatively in its acceptance. In this sense, Van Haute et al. (2016) reported that the sensorial properties of the meat/fish marinade with OEO and other EOs (thyme and cinnamon) are inevitably affected when the necessary EO concentrations to extend the microbial shelf life are applied. Similarly, a concentration of 4% OEO in active packaging gave rise to unacceptable oregano smell of fresh beef steaks (Camo et al., 2011). Due to the intense aroma of OEO, it can be used in food matrices to provide a balance between sensory acceptability and properties exerted by the spice (Cattelan et al., 2015). In order to avoid the direct incorporation of OEO into food, the active food packaging is a promising trend that allows using OEO that are gradually release from the package to the food (Llana-Ruiz-Cabello et al., 2016a).

The effectiveness of OEO included in food packaging has been confirmed as a preservative in food. Previous experiments carried out in our laboratory have checked the efficacy of polylactic acid

* Corresponding author.
E-mail address: spichardo@us.es (S. Pichardo).

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films containing 5 and 10% OEO as antioxidant and against microorganism (mainly yeast and molds) in ready-to-eat salads (Llana-Ruiz-Cabello et al., 2016a). A concentration of at least 1% oregano extract in the active packaging system was needed to significantly increase beef display life from 14 to 23 days (Camo et al., 2011). In addition, antioxidant and antimicrobial properties of ethylene vinyl alcohol copolymer films containing OEO and green tea extract components were confirmed (Muriel-Galet et al., 2015). In fact, OEO containing gelatine films exhibited higher *in vitro* antimicrobial and antioxidant properties than films incorporating lavender EO (Martucci et al., 2015).

Along with the usefulness of OEO, included in active food packaging as preservative, its safety should be also confirmed. In this regard, OEO is categorised as 'generally recognized as safe' (GRAS) by the Food and Drug Administration (Manso et al., 2014) and it is classified as a food additive by the European Union (Muriel-Galet et al., 2015). As flavouring, OEO is normally used in foods at low concentrations. However, the use of these compounds in other applications such as in active packaging may require higher doses that will increase the concern regarding exposure to these compounds (Stammati et al., 1999). In addition, according to the Commission Regulation (EC) No 450/2009, only substances that are included in the Community list of authorised substances may be used in components of active packaging. However, no substance has been included in the list so far. Therefore, a toxicological assessment is needed. The Guidelines of the Scientific Committee on Food for safety assessment of substances used in food contact materials (EFSA, 2016) recommend genotoxicity and subchronic studies in the core set of tests. Genotoxicity studies of OEO and its components are very scarce (EFSA, 2008). However, in the case of the OEO used in the present study the genotoxicity has been evaluated. Results obtained in our laboratory have indicated absence of genotoxic effects of this OEO in rats exposed up to 200 mg/kg body weight (b.w.) (Llana-Ruiz-Cabello et al., 2016c). Carvacrol and thymol, two of the main components of OEO have been also studied. Most of the studies have reported that thymol was neither mutagenic nor genotoxic using *in vitro* assays (Azizan and Blevins, 1995; Stammati et al., 1999; Horvathova et al., 2006; Buyukleyla and Rencuzogullari, 2009; Llana-Ruiz-Cabello et al., 2014a; Maisanaba et al., 2015). However, contradictory results have been obtained for carvacrol using *in vitro* test. It exhibited mutagenic potential and oxidative damage in DNA in the comet assay (Llana-Ruiz-Cabello et al., 2014a). Also, carvacrol showed a weak genotoxic potential on L5178Y/Tk ± cells (Maisanaba et al., 2015). Similarly, in mammalian cells, a positive response was obtained in human lymphocytes through the standard comet assay (Aydin et al., 2005a,b). However, other authors have also observed negative results (Stammati et al., 1999; Ündeger et al., 2009; Aydin et al., 2014). In order to complete the toxicological assessment of OEO and its components *in vivo* studies are needed, but those dealing the toxicity of OEO are very scarce. In the case subchronic studies, no previous experiments have been conducted by the moment as far as we know.

Considering all this background, the aim of the present work was to study for the first time the subchronic toxicity of OEO, containing carvacrol/thymol (10:1), intended to be used in active packaging for food applications, in Wistar rats orally exposed to different concentrations of this extract for 90 days. According to the OECD 408 guideline (OECD, 1998), body weight and food and water consumption were recorded. Moreover, clinical observation, haematological and biochemistry parameters, gross and microscopic pathology were performed.

2. Materials and methods

2.1. Supplies and chemicals

Commercial powder neutral gelatine from pork protein (Jesus Navarro S.A., Alicante, Spain) was used as the vehicle for the test item in the dosed groups and control group in the 90-days study. The rest of the chemicals were purchased from Sigma-Aldrich (Madrid, Spain).

2.2. Oregano essential oil analysis

Oregano essential oil was acquired from El Jarpil® (lot number OR2015) (Almería, Spain). It was analysed according to NF ISO 11024 using a Hewlett Packard 5890 chromatograph interfaced to a Hewlett Packard 5970 Mass selective detector (Hewlett Packard, Palo Alto, USA). The gas chromatograph was equipped with a polar column HP INNOWAX, of 60 m × 0.25 mm x 0.5 μm film thickness. The oven temperature was held at 60 °C for 6 min, raised to 250 °C at 2 °C min^{-1}, and maintained at 250 °C for 10 min. Helium was used as carrier gas at 22 psi and the injection volume was 1 μL. Compound assignment was achieve by single ion monitoring for various homologous series and via comparison with published and stored data (NKS Library). In Table 1 the components found in a percentage above 1% have been listed, being the main components carvacrol (55.82%), ρ-cimene (16.39%), thymol (5.14%), γ-terpine (4.71%) and β-cariophyllene (2.40%).

2.3. Diets

In order to select the doses for the 90-day study, the acute oral toxicity study "Up and Down Procedure", OECD 425 (OECD, 2008) was carried out in Wistar rats. No animal died after dosage up to 2000 mg/kg b.w. of this OEO administered by gavage, so the median lethal dose (LD50) was set above this dose. In addition, a preliminary palatability study evidenced that the maximum dose that was accepted by animals when added to neutral gelatine was 200 mg/kg. Therefore, the selected doses were calculated by dividing this data by a factor of 2, being the doses 50, 100 y 200 mg/kg b.w./day (d). Dietary dose individual formulations were prepared according to Mellado-García et al. (2016).

2.4. Animal and experimental design

The 90-day toxicity study was performed at the Central Service of Experimental Animals from the University of Córdoba (SAE, Córdoba, Spain), in accordance with the OECD Guideline 408

Table 1
Main components of *Origanum vulgare L.* essential oil.

RT (min)	Compound	%
10,6	α-PINENE	1,10
10,8	α-THUYENE	1,69
17,9	β-MYRCENE	1,52
19,2	α-TERPINENE	1,62
23,5	γ-TERPINENE	4,71
25,3	p-CYMENE	16,39
36,8	1-OCTEN-3-OL	1,50
47,4	TERPINENE-4-OL	1,33
47,5	β-CARYOPHYLLENE	2,40
79,8	THYMOL	5,14
81,5	CARVACROL	55,82

RT: retention time. Only the main components were reported. (>1%).