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Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2

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ABSTRACT

Essential oils used as additives in the food industry due to its flavour, antimicrobial and antioxidant properties. Therefore, human can be exposed orally to these compounds through the ingestion of foods. In this sense, the present work aims to assess toxicological effects of oregano essential oil on the digestive tract. In concrete, the cytotoxic effects of two components of the oregano essential oils, carvacrol and thymol, and their mixture, on the intestinal cells line Caco-2 after 24 and 48 h of exposure are studied. The basal cytotoxicity endpoints assayed (total protein content, neutral red uptake and the tetrazolium salt reduction) and the annexin/propidium iodide staining indicated that carvacrol and the mixture carvacrol/thymol induced toxic effects. Moreover, a morphological study was performed in order to determine the ultrastructural cellular damages caused by these substances. The main morphological alterations were vacuolated cytoplasm, altered organelles and finally cell death. In addition, although no cytotoxic effects were recorded for thymol at any concentration and time of exposure, ultrastructural changes evidenced cellular damage such as lipid degeneration, mitochondrial damage, nucleolar segregation and apoptosis.

1. Introduction

Essential oils are aromatic oily liquids obtained from plant materials. They have been traditionally used in food products and perfume preparations because of their flavours. Many species of *Origanum* have a high amount of phenolic contents in their essential oils (Özkan and Erdoğan, 2011). In this sense, carvacrol and thymol have been reported as major phenolic constituents of many of the essential oils of oregano species (Ündeger et al., 2009). Carvacrol (5-isopropyl-2-methylphenol) is the predominant monoterpenic phenol, and it presents antibacterial, antifungal, insecticidal, and antioxidant effects (Akalin and Incesu, 2011). Thymol (2-isopropyl-5-methylphenol) has been widely used as a general antiseptic in the medical practice, agriculture, cosmetics, and food industry (Szentandrassy et al., 2004). In this sense, due to these properties, carvacrol and thymol are being used as alternatives to synthetic chemical products to protect the ecological equilibrium (Bakkali et al., 2008). In addition, the relatively recent of interest of the consumers for in "green and organic" products has lead to a renewal of scientific interest in these substances (Burt, 2004).

On the other hand, a recent packaging technology named "active packaging" is being increasingly developed in the last years (Appendini and Hotchkiss, 2002; Suppakul et al., 2003). This technology consists in the incorporation of active agents into the packaging walls from which they are released to the food surface at a controlled rate (Cerisuelo et al., 2012). In this sense, the incorporation of carvacrol and thymol in food-packaging films allows the controlled release of these active substances into the food reducing the undesirable flavours caused in the case of the direct addition into food (Ramos et al., 2012).

As flavouring, carvacrol and thymol are 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, carvacrol and thymol can be used alone or in combination, for example in the treatment of oral infectious diseases (Ündeger et al., 2009). Therefore, the evaluation of this combination is also required in order to assess its safety. The few *in vivo* toxicity data available in the literature mainly concern acute and subacute effects on different animal species, and suggest that such compounds may not pose a risk to human health (Jenner et al., 1964; Hagan et al., 1964; Domaracky et al., 2007). However, the toxic mechanism of these compounds has not been completely elucidated so far. It seems

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that mitochondrial membranes are first damaged by permeabilization, resulting in a pro-oxidant status and induction of apoptosis thereafter (Bakkali et al., 2008; Deb et al., 2011; Yin et al., 2012).

Various *in vitro* models have been used to assess the toxicity of these essential oils and its constituents. In this sense, time and dose-dependent cytotoxic effects and morphological alterations on a mouse skeletal muscle cell line, CO25, exposed to carvacrol have been reported (Akalin and Incesu, 2011). Horváthová et al. (2006) also observed cytotoxic effects on the human cell lines HepG2 and Caco-2, which were not associated with a DNA-damaging effect. Bostancioglu et al. (2012) reported that three concentrations of *Origanum onites* essential oil (125, 250 and 500 μg/mL) could markedly inhibit cell viability and induced apoptosis of rat adipose tissue endothelial cells (RATECs) and rat embryonic fibroblasts (5RP7). Furthermore, cytotoxic effects and apoptosis induction were observed in human cervical cancer cell lines (HeLa and SiHa) exposed to carvacrol at the concentration of 50 mg/L after 48 h of exposure (Mehdi et al., 2011). In this sense, several authors have suggested that carvacrol induces apoptosis by direct activation of the mitochondrial pathway on HepG2 cells (Yin et al., 2012) and on human metastatic breast cancer cells (Arunasree, 2010). This mechanism of toxicity has been also found for thymol. Deb et al. (2011) reported apoptosis induced by thymol in the promyelotic cancer cell line HL-60 at concentrations of 75 and 100 μM after 24 h of exposure. Similarly, Hsu et al. (2011) found that human glioblastoma cells exposed during 24 h to 200, 400 and 600 μM of thymol died by an apoptotic pathway. However, García et al. (2006) found that thymol did not affect cellular viability in primary cultures of mouse cortical neurons at concentrations ranging from 0 to 1 mM.

A limited number of studies on tissue damages and alterations in cellular models exposed to carvacrol and thymol have been reported. In this sense, no studies regarding the ultrastructural alterations have been performed so far. The morphological alterations induced by carvacrol that have been observed include: inhibition in the cellular growing (Bostancioglu et al., 2012); rounding (Akalin and Incesu, 2011; Koparal and Zeytinoglu, 2003); cytoplasmic blebbing and detachment from the disk (Koparal and Zeytinoglu, 2003); and cell death mainly by apoptosis (Stammati et al., 1999; Yin et al., 2012; Bostancioglu et al., 2012).

Therefore, considering the increasing use of carvacrol and thymol in the food industry, like for instance in active food packaging, the use of the permanent intestinal cell line Caco-2 (a commonly used enterocytic model established from a human colon carcinoma) to assess the safety of these compounds is of great interest. Therefore, the present work aims to assess, for the first time, the cytotoxic effects caused by carvacrol, thymol and their mixture in the Caco-2 cell line. Moreover, a thorough morphological study was performed in order to determine ultrastructural cellular damages that could help to explain the mechanism of action of these substances.

2. Materials and methods

2.1. Supplies and chemicals

Culture medium, fetal bovine serum and cell culture reagents were obtained from BioWhittaker (Spain). Chemicals for the different assays were provided by Sigma–Aldrich (Spain) and VWR International Eurolab (Spain). Protein reagent assay was obtained from BioRad (Spain).

2.2. Model systems

Caco-2 cell line derived from human colon carcinoma (ATCC® HTB-37) was maintained at 37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity (CO_2 incubator, NuAire®, Spain), in a medium consisting of Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 50 μg/ml gentamicin, 1.25 μg/ml fungizone, 2 mM L-glutamine and 1 mM pyruvate. Cells were grown near confluence in 75-cm^2 plastic flasks and harvested weekly with 0.25% trypsin. They were counted in an improved Neubauer haemocytometer and viability was determined by exclusion of Trypan Blue. Caco-2 cells were plated at density of 7.5×10^5 cells/ml to perform the experiments.

2.3. Test solutions

The range of carvacrol and thymol concentrations for the tests was selected considering the content of these active compounds to be incorporated in the packaging materials and the possible migration to the food. In this sense, the maximum concentration was calculated considering that the active compounds completely migrate from the active package into the food (worst scenario of exposure).

Following that a 10% of the oregano essential oil (El Jarpil S.L., Almería, Spain) will be incorporated in 5.5–6.5 g of packaging film needed to pack 1 kg of food, and following that this oregano essential oil contains a 56% of carvacrol and 5% thymol (proportion around 10:1), the potential concentration released to the food will be around 2500 μM and 250 μM for carvacrol and thymol, respectively.

Serial test solutions of carvacrol (0–2500 μM), thymol (0–250 μM), and their combination in the proportion 10:1 (0–2500:0–250 μM), were prepared from both stock solutions of carvacrol (2.5 M) and thymol (0.5 M) in DMSO, being the final concentration in DMSO bellow 0.1%.

2.4. Cytotoxicity assays

Culture medium with 0.1% DMSO was used as control group. After replacing the previous medium, the exposure solutions were added to the systems, and incubated at 37 °C for 24 and 48 h according to INVITOX cytotoxicity protocols. The basal cytotoxicity endpoints were protein content (PC), supravital dye neutral red cellular uptake (NR), and tetrazolium salt reduction (MTS).

Protein content (PC) is a very useful endpoint to assess cytotoxicity, since it gives data about cell damage in independence of the toxic mechanism involved (Pichardo et al., 2007). PC was analysed *in situ*, according to the procedure given by Bradford (1976), using Coomassie Brilliant Blue G-250 (BioRad, Madrid, Spain) in the same 96-well tissue culture plates in which exposure originally took place, in order to determine the percentage of cells present in the culture in comparison to the control group.

NR uptake is a suitable endpoint to determine viable cells, because this dye is taken up by viable lysosomes. This assay was performed according to Borenfreund and Puerner (1984). Briefly, neutral red (NR) (Sigma–Aldrich, Madrid, Spain) in medium is absorbed and concentrated in lysosomes of cells. NR uptake is proportional to the concentration of the NR solution and the numbers of viable cells. The NR can be extracted from lysosomes for quantitative measurement of cells viability and cytotoxicity of xenobiotics (Zhang et al., 1990).

MTS reduction is carried out by dehydrogenases enzymes present in mitochondria, being this endpoint a good biomarker of the damage induced in this organelle. MTS reduction was measured according to the procedure of Baltrop et al. (1991). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) tetrazolium compound (Promega Biotech Ibérica, Madrid, Spain) added to the medium is bioreduced by cells into a coloured formazan product soluble in culture medium and is directly measured spectrophotometrically at 490 nm after 3 h of incubation in the dark.

The concentrations used in further assays were calculated based on the cytotoxicity study. The EC_{50} values obtained in the most sensitive endpoint for carvacrol and its mixture with thymol at 24 h of exposure, MTS reduction in both cases, were chosen as the highest exposure concentration for the detection of apoptosis and the morphological study, along with the fractions $EC_{50}/2$ and $EC_{50}/4$. In the case of thymol, since no cytotoxic effect was recorded, the highest concentration used and its fractions were maintained for further studies.

2.5. Detection of apoptosis by flow cytometry

The detection of apoptosis was performed following the method described by Cárdeno et al. (2013) with modifications. Briefly, Caco-2 cells (7.5×10^5 cells/mL) were seeded in 6-well plates. After 24 h of incubation, cells were treated in presence of different concentrations of carvacrol (115, 230 and 460 μM), thymol (62.5, 125, and 250 μM), and their mixture in the selected proportion (75:7.5, 150:15, and 300:30 μM). Moreover, a negative control without the presence of any compound, a positive control with the presence of curcumin 40 μM (Sigma–Aldrich, Madrid, Spain), and a 1% DMSO control were prepared. After 24 h of exposure, media were collected and cells were detached by trypsinization (0.05% Trypsin–EDTA; Gibco, Paisley, United Kingdom) and collected in 0.5 mL of MEM without serum. Afterwards, cells were centrifuged at 4500 rpm during 3 min at 4 °C, resuspended and washed with ice-cold PBS, centrifuged, and resuspended in ice-cold 1× binding buffer (BB) to 5×10^5 cells/mL. Cells were incubated with 25 μL/mL Annexin V-FICT and 20 μg/mL propidium iodide (PI) solution (Annexin V-FICT Apoptosis Detection Kit, eBioscience, Vienna, Austria). Four different groups of cells were obtained based on their stainability: those unstainable with annexin V or PI [annexin(−)/PI(−)]: viable cells (quadrant E3); those stainable with annexin V but unstainable with PI [annexin(+)/PI(−)]: early apoptotic cells (quadrant E4); those