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## In vitro toxicological evaluation of mesoporous silica microparticles functionalised 1 with carvacrol and thymol

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3

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### **Abbreviations**

$\Delta\Psi_m$ , Mitochondrial membrane potential; APTES, (3-aminopropyl)triethoxysilane; BHT, di-ter-butyl- methylphenol; CTAB, Hexadecyltrimethylammonium bromide; DFA, Deferoxamine mesylate salt; DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethyl sulfoxide; EOCs, essential oil components; H<sub>2</sub>-DCFDA, 2',7'-dichlorodihydrofluorescein diacetate, IC<sub>50</sub>, Mean inhibition concentration; LDH, Lactate dehydrogenase; LPO, lipid peroxidation; MCM-41, Mobile crystalline material-41; MDA, Malondialdehyde; MTT, thiazolyl blue tetrazolium bromide; NBCS, Newborn calf serum; PBS, phosphate buffered saline; PI, Propidium Iodide; ROS, Reactive oxygen species; TBA, thiobarbituric acid; TBARS, Thiobarbituric acid reactive substances; TEAH<sub>3</sub>, Triethanolamine; TEM, Transmission electron microscopy

11 **Abstract**

12 The cytotoxicity of carvacrol- and thymol- functionalised mesoporous silica  
13 microparticles (MCM-41) was assessed in the human hepatocarcinoma cell line (HepG2).  
14 Cell viability, lactate dehydrogenase (LDH) activity, reactive oxygen species (ROS)  
15 production, mitochondrial membrane potential ( $\Delta\Psi_m$ ), lipid peroxidation (LPO) and  
16 apoptosis/necrosis analysis were used as endpoints. Results showed that both materials  
17 induced cytotoxicity in a time and concentration-dependent manner, being more cytotoxic  
18 than free essential oil components and bare MCM-41. This effect was caused by the cell-  
19 particle interactions and not from degradation products released to the culture media, as  
20 demonstrated in the extract dilution assays. LDH release was seen to be a less sensitive  
21 endpoint than the MTT (thiazolyl blue tetrazolium bromide) assay, suggesting  
22 impairment of the mitochondrial function as the primary cytotoxic mechanism. *In vitro*  
23 tests on specialised cell functions showed that exposure to sublethal concentrations of  
24 these materials did not induce ROS formation within 2-h exposure but produced LPO and  
25  $\Delta\Psi_m$  alteration in a concentration-dependent manner when cells were exposed for 24 h.  
26 Overall, the results found in this study support the hypothesis that carvacrol- and thymol-  
27 functionalised MCM-41 microparticles induced toxicity in HepG2 cells by an oxidative  
28 stress-related mechanism that resulted in apoptosis through the mitochondrial pathway.

29 **Keywords:** mesoporous microparticles; silica; essential oil components; cytotoxicity;  
30 HepG2

31

## 32 **1. Introduction**

33 Consumer awareness of additives and chemicals in their diets has forced the food  
34 industry to search for alternatives to synthetic preservatives to extend the shelf life of their  
35 products (Faleiro and Miguel, 2020; Ribes et al., 2018). In this regard, essential oils and  
36 their constituent components have attracted considerable attention, due to their natural  
37 origin and their well-known antimicrobial and antioxidant activity (Burt, 2004;  
38 Hyldgaard et al., 2012). The monoterpenoids carvacrol and thymol, major components in  
39 essential oils from different plant species such as origanum or thyme, are two of the most  
40 investigated essential oil components (EOCs), due to their strong action against a wide  
41 spectrum of foodborne and food spoilage microorganisms (Abbaszadeh et al., 2014;  
42 Abdelhamid and Yousef, 2021; Čabarkapa et al., 2019; Karam et al., 2019; Tippayatum  
43 and Chonhenchob, 2007; Walczak et al., 2021). The antimicrobial action of these  
44 components has been mainly attributed to the presence of a hydroxyl group and a system  
45 of delocalised electrons in their chemical structure, able to disrupt the cytoplasmic  
46 membrane, leading to leakage of intracellular content and ultimately lysis (Xu et al.,  
47 2008). However, their application in food products presents some challenges, such as high  
48 volatility, low solubility or strong sensory properties (Hyldgaard et al., 2012). In this  
49 regard, grafting of EOCs onto the surface of silica particles has been proposed as a  
50 strategy to increase the antimicrobial activity of these components and to overcome their  
51 drawbacks. These hybrid organic-inorganic particles have efficiently performed as  
52 preservatives in different food matrices (Ribes et al., 2019, 2017) and as filtering  
53 materials for the cold pasteurisation of beverages (García-Ríos et al., 2018; Peña-Gómez  
54 et al., 2020, 2019b, 2019a).

55 Among mesoporous silica materials, Mobil Composition of Matter (MCM)-41 is one  
56 of the most widely employed scaffolds for the synthesis of organic-inorganic hybrid

57 systems, due to the ease of surface functionalisation, large surface area, uniform pore size  
58 and high stability (Diab et al., 2017). Moreover, as a result of the high biocompatibility  
59 and low toxicity reported (Aburawi et al., 2012; Al-Salam et al., 2011; Garrido-Cano et  
60 al., 2021), MCM-41 materials have been studied for the covalent attachment of functional  
61 groups and organic molecules in a number of oral applications (Bagheri et al., 2018; Ros-  
62 Lis et al., 2018). However, as the surface properties of particles are key factors for  
63 determining their interactions with biological systems (Kyriakidou et al., 2020; Puerari et  
64 al., 2019; Vicentini et al., 2017), the analysis of the *in vitro* and *in vivo* behaviour of  
65 surface modified silica structures is crucial to guarantee the safety and innocuousness of  
66 their use for human health.

67 Previous *in vitro* studies have evaluated the stability of different types of synthetic  
68 amorphous silica particles functionalised with carvacrol, eugenol and vanillin under  
69 conditions that represented the human gastrointestinal tract and lysosomal fluid (Fuentes  
70 et al., 2021, 2020). Results showed that functionalisation with these EOCs resulted in  
71 lower dissolution levels than bare MCM-41 microparticles, and therefore increased  
72 stability under both biological conditions (Fuentes et al., 2020). Conversely, the  
73 comparative analysis of the cytotoxic effect of eugenol and vanillin functionalised silica  
74 particles revealed that free EOCs and bare particles had a milder cytotoxic effect on  
75 HepG2 cells than functionalised MCM-41 materials. A relationship between cytotoxicity  
76 and the density of EOC molecules on the surface of the functionalised particles was found.  
77 According to the physico-chemical analysis of the materials, properties such as cationic  
78 nature and hydrophobicity were suggested to enhance the cytotoxic behaviour of the  
79 functionalised silica particles (Fuentes et al., 2021). All together, these results  
80 demonstrate that functionalisation of MCM-41 particles with EOCs derivatives enhances  
81 the stability of these materials under biological conditions, but may increase their

82 cytotoxic behaviour, leading to potential toxicological hazards. However, the molecular  
83 mechanism underlying the cytotoxic behaviour of these materials remains to be  
84 elucidated. Accordingly, in this work we aimed to investigate the cytotoxic effect of  
85 carvacrol and thymol functionalised MCM-41 microparticles and further to elucidate the  
86 related toxicity mechanism. To this end, HepG2 human liver cells were exposed to the  
87 modified silica materials and cell viability, lactate dehydrogenase (LDH) activity,  
88 reactive oxygen species (ROS) production, mitochondrial membrane potential ( $\Delta\Psi_m$ ),  
89 lipid peroxidation (LPO) and apoptotic and necrotic responses were evaluated.

## 90 **2. Materials and methods**

### 91 **2.1. Reagents**

92 Triethanolamine (TEAH<sub>3</sub>), hexadecyltrimethylammonium bromide (CTAB),  
93 carvacrol ( $\geq 98\%$  w/w), thymol ( $\geq 99\%$  w/w), (3-aminopropyl) triethoxysilane (APTES),  
94 thiazolyl blue tetrazolium bromide (MTT), 2',7'-di-chlorodihydrofluorescein diacetate  
95 (H2-DCFDA), Rhodamine 123, thiobarbituric acid (TBA), deferoxamine mesylate salt  
96 (DFA) and di-ter-butyl- methylphenol (BHT) were obtained from Sigma-Aldrich (Spain).  
97 Dimethyl sulfoxide (DMSO) was purchased from Scharlab (Spain).

98 The HepG2 human hepatocarcinoma cell line was obtained from the American Type  
99 Culture collection (ATCC HB-8065). Dulbecco's Modified Eagle Medium (DMEM-  
100 Glutamax™) with high glucose (4.5 g/L), phosphate buffered saline (PBS), newborn calf  
101 serum (NBCS), penicillin, streptomycin, trypsin-EDTA 0.5% and sodium pyruvate were  
102 supplied by Gibco (Life- Technologies, USA).

### 103 **2.2. Preparation of EOCs-functionalised MCM-41 microparticles**

104 The mesoporous silica microparticles MCM-41 were prepared through the 'atrane  
105 route', which is based on the use, under basic conditions, of TEAH<sub>3</sub>, that generates atrane

106 complexes as inorganic hydrolytic precursors and CTAB as structural directing agent  
107 (Cabrera et al., 2000). The procedure of the synthetic process is fully described at (Fuentes  
108 et al., 2020).

109 Once synthesised, the functionalisation of the MCM-41 silica particles with carvacrol  
110 and thymol was performed by a three-stage protocol that includes: (1) synthesis of  
111 carvacrol and thymol aldehyde derivatives by direct formylation with paraformaldehyde;  
112 (2) synthesis of alkoxy silane derivatives by reaction of carvacrol and thymol aldehydes  
113 with APTES; and (3) immobilisation of the alkoxy silane derivatives on the surface of  
114 silica particles (García-Ríos et al., 2018).

### 115 **2.3. Physico-chemical characterisation of MCM-41 microparticles**

116 Bare and functionalised MCM-41 microparticles were analysed by transmission  
117 electron microscopy (TEM), particle size distribution, zeta potential and elemental  
118 analysis. For the morphological analysis of the materials by TEM, particles were  
119 dispersed in dichloromethane and sonicated for 2 min to avoid aggregation. The  
120 suspension was placed onto copper grids coated with carbon film (Aname SL, Spain).  
121 Imaging of the particle samples was done using a JEOL JEM-1010 (JEOL Europe SAS,  
122 France), operating at an acceleration voltage of 80 kV. The zeta potential of the particles  
123 was studied by laser Doppler microelectrophoresis using a Zetasizer Nano ZS (Malvern  
124 Instruments, UK) and the Smoluchowski mathematical model. Particle size distribution  
125 was determined using a laser diffractometer (Mastersizer 2000, Malvern Instruments,  
126 UK) and application of Mie theory (refractive index of 1.45, absorption index of 0.1). For  
127 both zeta potential and particle size distribution analysis, the samples were measured in  
128 triplicate on previously sonicated diluted dispersions in deionised water. Finally, the  
129 elemental composition of the EOCs-functionalised particles was determined by



130 combustion analysis for C, H and N using a CHNS1100 Elemental Analyser (CE  
131 Instruments, UK).

## 132 **2.4. Toxicological evaluation of EOCs-functionalised MCM-41 microparticles**

### 133 **2.4.1. Cell culture**

134 Human hepatocarcinoma (HepG2) cells were cultured in DMEM-Glutamax medium  
135 supplemented with 10% NBCS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells  
136 were maintained in 5% CO<sub>2</sub> humidified incubator at 37°C. Growth medium was changed  
137 every 2–3 days or as required. Cells were subcultured by trypsinisation when about 80%  
138 confluence was reached and were used for experiments under 40 subpassages. Absence  
139 of mycoplasma contamination was examined regularly in cell cultures with the  
140 MycoAlert™ PLUS Myco- plasma kit (Lonza Rockland, USA).

### 141 **2.4.2. Test solutions**

142 Stock solutions of EOCs were prepared in DMSO (2.5 M) and were maintained at -20  
143 °C until used. Particle suspensions were prepared in DMEM supplemented medium and  
144 were sonicated for 10 min immediately prior to use. During the study of the cytotoxic  
145 effect of carvacrol and thymol exposure, the final DMSO concentration in the test  
146 solutions was below 0.1%. Appropriate negative controls containing the same amount of  
147 solvents were included in each experiment. For the analysis of MCM-41 materials, cell-  
148 free particle control samples were included for each particle type and concentration and  
149 were used to correct particle interference from the test wells when necessary.

### 150 **2.4.3. MTT assays**

151 *Direct exposure cytotoxicity assays*

152 The MTT assay is one of the most commonly used colorimetric assays to evaluate cell  
153 viability. In this method, the yellow, positively charged tetrazolium salt enters viable  
154 cells, whereupon it is metabolically reduced to the insoluble blue-violet form of formazan  
155 by components of the respiratory chain (Rampersad, 2012). This assay was used to  
156 determinate the mean inhibition concentration ( $IC_{50}$ ) values of the EOCs and the EOCs-  
157 functionalised silica, and to compare the cytotoxic effect of the functionalised-particles  
158 constituents. Briefly, cells were seeded in 96-well plates at a density of  $1 \times 10^5$  and  $3 \times$   
159  $10^4$  cells/mL for 24-h and 48-h experiments, respectively. After 24 h attachment, cells  
160 were exposed to serial dilutions of carvacrol (0.01-2.5 mM), thymol (0.06-1 mM),  
161 carvacrol- and thymol-functionalised MCM-41 microparticles (0.01-2.5 mg/mL) and to  
162 equivalent concentrations of EOCs, bare and functionalised particles for their  
163 comparative analysis (Table 1). The concentration ranges of EOCs and functionalised  
164 particles were selected according to previous works (Fuentes et al., 2021). In the  
165 comparative studies, concentration ranges were established from the  $IC_{50}$  values found for  
166 carvacrol and thymol, and then the equivalent particle concentrations were calculated  
167 from the EOCs content determined by elemental analysis. After a 24-h or 48-h incubation  
168 period, wells were washed with 100  $\mu$ L PBS and 100  $\mu$ L of 10% MTT stock solution (5  
169 mg/mL in PBS) in supplemented DMEM medium were added per well. Then, plates were  
170 incubated in darkness at 37 °C, 5% CO<sub>2</sub> for 3 h. After this time, the MTT solution was  
171 discarded, wells were washed with PBS and 100  $\mu$ L of DMSO were added to dissolve  
172 formazan crystals. Finally, plates were shaken at 300 rpm for 10 min and absorbance was  
173 measured at 570 nm using a MultiSkan EX ELISA plate reader (Thermo Scientific, USA).  
174 Cell viability was expressed as a percentage in relation to the negative control (unexposed  
175 cells).

176 Exposure to sublethal concentrations of toxicants trigger adaptative cellular responses  
 177 linked to their cytotoxicity mechanism (Severin et al., 2017). For this reason, sublethal  
 178 concentrations ( $<IC_{50}$ ) were used to further investigate the toxicity mechanism underlying  
 179 to EOCs-functionalised particles exposure. To this end, the  $IC_{50}$  values of the particles  
 180 found in the MTT assay were used to calculate the sublethal concentrations ( $IC_{50/2}$ ,  $IC_{50/4}$ ,  
 181  $IC_{50/8}$ ) for the different *in vitro* endpoints: ROS formation,  $\Delta\Psi_m$ , LPO and apoptotic and  
 182 necrotic responses.

183

184 **Table 1.** Concentrations assayed in the comparative study of carvacrol, thymol, and bare  
 185 and EOCs-functionalised MCM-41 microparticles.

|                                 | <b>Concentrations</b> |          |          |          |
|---------------------------------|-----------------------|----------|----------|----------|
|                                 | <b>A</b>              | <b>B</b> | <b>C</b> | <b>D</b> |
| <b>Carvacrol (mM)</b>           | 0.25                  | 0.5      | 1        | 2        |
| <b>Bare MCM-41 (mg/mL)</b>      | 4.5                   | 8.9      | 17.9     | 35.7     |
| <b>Carvacrol MCM-41 (mg/mL)</b> | 4.4                   | 8.8      | 17.7     | 35.4     |
| <b>Thymol (mM)</b>              | 0.25                  | 0.5      | 1        | 2        |
| <b>Bare MCM-41 (mg/mL)</b>      | 3.8                   | 7.7      | 15.3     | 30.6     |
| <b>Thymol MCM-41 (mg/mL)</b>    | 3.7                   | 7.6      | 15.1     | 30.3     |

186

187 *Extract exposure cytotoxicity assays*

188 An extract dilution exposure method was applied to evaluate a possible cytotoxic effect  
 189 due to components leached from the surface of the functionalised particles to the culture  
 190 medium. For this purpose, a stock solution of the carvacrol or thymol-functionalised  
 191 particles in DMEM supplemented medium (2.5 mg/mL) was vigorously stirred, sonicated  
 192 in an ultrasonic bath for 10 min, and maintained at 37 °C for 24 h. After this time, the  
 193 stock solution was serially diluted (0.01-2.5 mg/mL), and dilutions were filtered using

194 0.2 µm cellulose acetate filters in order to remove the particles. Then, cells were exposed  
195 to the filtered solutions and cytotoxicity of the samples was determined by the MTT assay  
196 as described previously.

#### 197 **2.4.4. Lactate dehydrogenase (LDH) activity**

198 The LDH activity was determined using the CyQUANT LDH Cytotoxicity Assay kit  
199 (Thermo Scientific, USA) according to the manufacturer's protocol. Briefly, cells were  
200 seeded in 96-well plates at  $10^5$  cells/mL (100 µL/well) and allowed to attach for 24 h.  
201 Then, cells were treated with 10 µL of functionalised particles concentrations (0.01-2.5  
202 mg/mL) for a 24 h period. After this time, 10 µL sterile ultrapure water (Spontaneous  
203 LDH Activity) or 10 µL lysis buffer (Maximum LDH Release) were added to control  
204 wells and plates were incubated in darkness at 37 °C and 5% CO<sub>2</sub> for 45 min. Then, 50  
205 µL of each sample medium was transferred to a new plate and 50 µL of the reaction  
206 mixture were incorporated. After incubation at room temperature for 30 min, reactions  
207 were ended by adding 50 µL of the stop solution. Absorbance measurements were carried  
208 out in a microtiter plate reader at 490 nm. Results were expressed as LDH release (%) in  
209 the exposed cells relative to spontaneous and maximum LDH controls.

#### 210 **2.4.5. ROS formation**

211 ROS formation was determined as described by Ruiz-Leal and George (2004). Cells  
212 were seeded in black 96-well microplates at  $2 \times 10^5$  cells/mL density. After 24 h of cell  
213 attachment, cells were washed with PBS and 20 µM H<sub>2</sub>-DCFDA dye in culture medium  
214 were added. Following 20 min incubation in darkness at room temperature, the dye  
215 solution was removed and the cells were exposed to different concentrations of the  
216 functionalised particles (18.75, 37.5 and 75 µg/mL). Then, fluorescence was measured  
217 every 15 min for 2 h at 490 nm excitation and 545 nm emission wavelength on a Wallace

218 Victor2, model 1420 multilabel counter (PerkinElmer, Finland). The percentage of ROS  
219 generation was expressed as the percentage of the fluorescence values obtained compared  
220 the negative control (unexposed cells).

#### 221 **2.4.6. Determination of mitochondrial membrane potential**

222 The  $\Delta\Psi_m$  was determined by the uptake of the green-fluorescent dye Rhodamine 123  
223 upon functionalised microparticles exposure. Cells were seeded at a density of  $2 \times 10^5$   
224 cells/mL in black 96-well microplates. After 24 h of cell attachment, cells were washed  
225 with 100  $\mu$ l/well PBS and were exposed to 3 different concentrations of the functionalised  
226 particles (18.75, 37.5 and 75  $\mu$ g/mL) in 10% NCBS-supplemented medium for 24 h.  
227 Following exposure time, cells were washed with 100  $\mu$ l/well of PBS and Rhodamine-  
228 123 (5  $\mu$ M) was added in non-supplemented media. After 15 min of incubation at 37°C  
229 and 5% of CO<sub>2</sub> in darkness, the dye solution was removed, cells were washed twice and  
230 finally resuspended in 200  $\mu$ l/well PBS. The fluorescence ( $\lambda_{excitation}=485$  nm,  
231  $\lambda_{emission}=535$  nm) was measured using a microplate reader Wallace Victor2, model  
232 1420 multilabel counter (PerkinElmer, Finland). The results are presented as the  
233 percentage of fluorescence of Rhodamine 123 dye in the exposed cells compared to the  
234 negative control (unexposed cells).

#### 235 **2.4.7. Lipid peroxidation assays**

236 The effect of sublethal concentrations exposure to functionalised particles on LPO was  
237 performed by determining the thiobarbituric acid reactive substances (TBARS)  
238 formation, according to the method described by Ferrer et al. (2009). Briefly,  $3 \times 10^4$   
239 cells/well were seeded in six-well plates, allowed to attach for 24 h and then exposed to  
240 functionalised particles (18.75, 37.5 and 75  $\mu$ g/mL). After 24 h exposure, cells were  
241 washed with PBS, homogenised in 150 mM sodium phosphate buffer (NaH<sub>2</sub>PO<sub>4</sub>) pH 7.4

242 and lysate with the Ultra-Turrax T8 IKA®-WERKE for 30 s. Cells samples were mixed  
243 with 0.5% TBA, 1.5mM DFA and 3.75% BHT and heated at 100 °C in a boiling water  
244 bath for 20 min. After cooling for 5 min, samples were centrifuged at 4000 rpm for 15  
245 min to remove the precipitate. The absorbance of the supernatant was then determined at  
246 535 nm. Simultaneously, the protein content of the samples was measured by the Lowry  
247 method using the DC Protein Assay (BIO-RAD Laboratories, USA) at 690 nm  
248 wavelength. Results were expressed as ng of malondialdehyde (MDA) per mg of protein.

#### 249 **2.4.8. Apoptosis/necrosis assays**

250 Apoptosis/necrosis analysis was performed by flow cytometry using the FITC  
251 Annexin V apoptosis detection kit (BD Biosciences, USA). Briefly, cells were seeded in  
252 6-well plates at a density of  $3 \times 10^4$  cells/well. After attachment, cells were exposed to  
253 functionalised particles (18.75, 37.5 and 75  $\mu\text{g}/\text{mL}$ ) for 24 h. Then, cells were trypsinised,  
254 washed twice with ice-cold PBS and resuspended in binding buffer. One hundred  $\mu\text{L}$  of  
255 cells ( $1 \times 10^5$  cells/mL) were stained by adding 5  $\mu\text{l}$  of FITC Annexin V and 5  $\mu\text{l}$  of  
256 Propidium Iodide (PI) and incubated at room temperature in darkness for 15 min. After  
257 this time, 400  $\mu\text{L}$  of binding buffer were added to each tube and samples were analysed  
258 using a BD LSRFortessa flow cytometer (BD Biosciences, USA). Quadrant statistics  
259 were carried out to differentiate between necrotic, early apoptotic and late apoptotic cells.  
260 The percentage of cells in each of the categories was calculated by subtracting the number  
261 of cells in the control group from the number of cells in the treated population.

#### 262 **2.5. Statistical analysis**

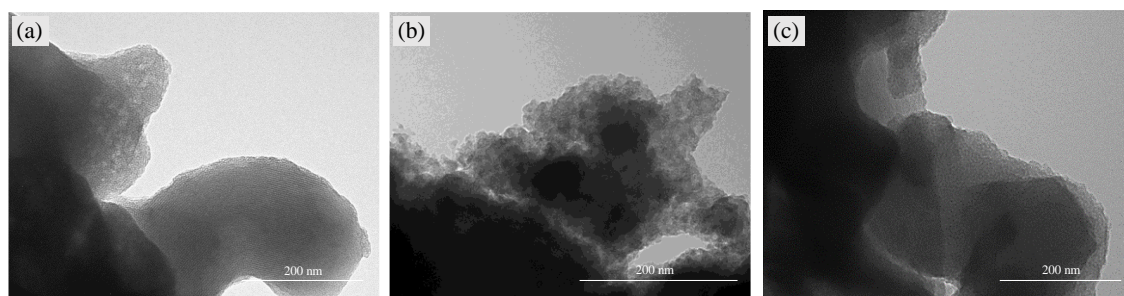
263 Statistical analysis of data was performed using Statgraphics Centurion XVI software  
264 package (Statpoint Technologies, Inc., USA). Data were expressed as the mean (SEM) of  
265 three independent experiments for each endpoint. Data from the cytotoxicity assays were

266 transferred to GraphPad Prism version 8.0.1 (GraphPad Software, USA) to adjust the  $IC_{50}$   
267 curve by using a four-parameter sigmoidal fit. The statistical analysis of the results was  
268 carried out by Student's t-test for paired samples. In the MTT comparative study  
269 differences between groups were analysed statistically with one-way ANOVA followed  
270 by the Tukey HDS *post-hoc* test for multiple comparisons. The difference level of  $p \leq$   
271 0.05 was considered statistically significant.

### 272 3. Results

#### 273 3.1. Physico-chemical characterisation of MCM-41 microparticles

274 To study the morphology and structure of the bare and carvacrol- or thymol-  
275 functionalised MCM-41 particles, TEM analysis was performed. As shown in Figure 1,  
276 the three particle types exhibit an irregular external shape and a hexagonal periodic  
277 structure of internal channels, in the form of alternate black and white parallel lines,  
278 typical of the ordered mesoporous structure of MCM-41 materials (Alothman, 2012;  
279 Meynen et al., 2009). These results confirmed that the synthesis process of the MCM-41  
280 materials was correct and that the functionalisation process did not significantly modify  
281 the characteristic structure of these materials.



282  
283 **Figure 1.** TEM images of bare MCM-41 (a), carvacrol-functionalised MCM-41 (b) and  
284 thymol-functionalised MCM-41 microparticles (c). Scale bar indicates 200 nm.

285

286 The zeta potential values and the particle size distribution of the bare and  
 287 functionalised MCM-41 particles and the carvacrol or thymol content of MCM-41-  
 288 functionalised materials are depicted in Table 2. The negative zeta potential value  
 289 observed for bare MCM-41 is related to negatively charged hydroxyl groups on the silica  
 290 surface. By contrast, functionalised particles displayed positive zeta potential values,  
 291 evidencing the presence of the carvacrol and thymol alkoxysilane derivatives grafted on  
 292 their surface. Particle size distribution analysis showed that the three MCM-41  
 293 microparticles types were under 700 nm. This value was slightly higher for the bare than  
 294 functionalised particles, since the higher number of steps included during the  
 295 functionalisation process has been suggested to reduce the formation of agglomerates  
 296 (Fuentes et al., 2020). Finally, the quantification of carvacrol and thymol grafted on the  
 297 particles surface was determined by elemental analysis. Small differences were found in  
 298 the reaction yield for both types of EOCs, the content of thymol immobilised on the  
 299 surface of MCM-41 functionalised microparticles being slightly higher than carvacrol  
 300 (Table 2). These results were used to establish the equal concentrations of free EOCs and  
 301 functionalised particles for the comparative cell viability assays (Table 1).

302

303 **Table 2.** Zeta potential (ZP) values, particle size distribution ( $d_{0.5}$ ) and EOC content ( $\alpha$ )  
 304 of the different MCM-41 microparticles.

| Type of particle | ZP (mV)       | $d_{0.5}$ ( $\mu\text{m}$ ) | $\alpha$ (g/g <sub>SiO<sub>2</sub></sub> ) |
|------------------|---------------|-----------------------------|--|
| Bare MCM-41      | -33.43 (0.84) | 0.68 (0.00)                 |  |
| Carvacrol-MCM-41 | 27.40 (2.06)  | 0.62 (0.00)                 | 0.0084                                     |
| Thymol-MCM-41    | 21.1 (1.74)   | 0.65 (0.00)                 | 0.0098                                     |

305

ZP and  $d_{0.5}$  values are expressed as mean (SD) (n = 3).



306

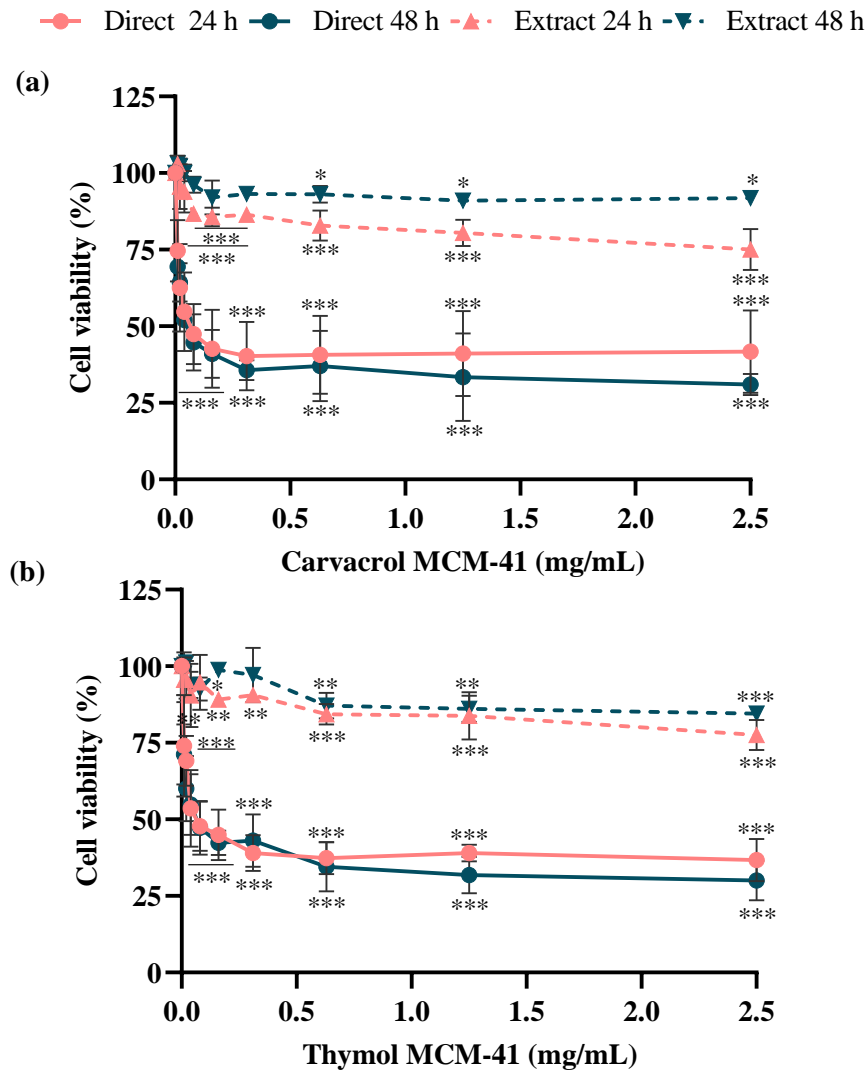
## 307 **3.2. Toxicological evaluation of EOCs-functionalised MCM-41 microparticles**

### 308 **3.2.1. MTT assays**

309 Firstly, the cytotoxic effect of carvacrol and thymol was determined after 24 h and 48  
310 h exposure of HepG2 cells, as determined using the MTT assay. Both components  
311 reduced cell viability in a time and concentration-dependent manner (Fig. S1 and S2).  
312 Carvacrol was slightly less cytotoxic than thymol, when cells were exposed to the EOCs  
313 for 24 h; however, no differences were found after 48 h incubation-period. The  $IC_{50}$   
314 values found after 24 h exposure were 0.45 (0.01) mM and 0.40 (0.03) mM for carvacrol  
315 and thymol, respectively. At 48 h, the  $IC_{50}$  value for both components was similar (0.32  
316 (0.02) mM for carvacrol and 0.32 (0.03) mM for thymol).

317 Figure 2 displays the cytotoxicity-response curves for carvacrol and thymol-  
318 functionalised MCM-41, either directly added to the culture medium or in an extract  
319 dilution form. Both materials reduced cell viability in a concentration-dependent manner  
320 (Fig. S3) when HepG2 cells were directly exposed to the functionalised particles for 24 h  
321 and 48 h, as measured by the MTT assay. The  $IC_{50}$  values found for carvacrol-  
322 functionalised MCM-41 were 0.15 (0.01) mg/mL and 0.09 (0.04) mg/mL for 24 h and 48  
323 h, respectively. Thymol-functionalised microparticles showed an  $IC_{50}$  value of 0.15 (0.08)  
324 mg/mL after 24 h of cells' exposure and 0.11 (0.08) mg/mL after 48 h. However, when  
325 cells were treated with the filtered medium which previously contained each type of  
326 particle, cell viability was significantly higher. A cell viability of 75% and 78% for  
327 carvacrol- and thymol-functionalised silica, respectively, were observed after 24 h  
328 exposure to the highest concentrations tested (2.5 mg/mL). After 48 h of treatment, these  
329 percentages were 92% in the case of carvacrol-functionalised particles, and 85% in the

330 case of thymol-functionalised silica. Indeed, when cells were exposed to the filtered  
 331 solutions equivalent to the IC<sub>50</sub> values found for both types of functionalised particles,  
 332 cell viability remained around 100%.



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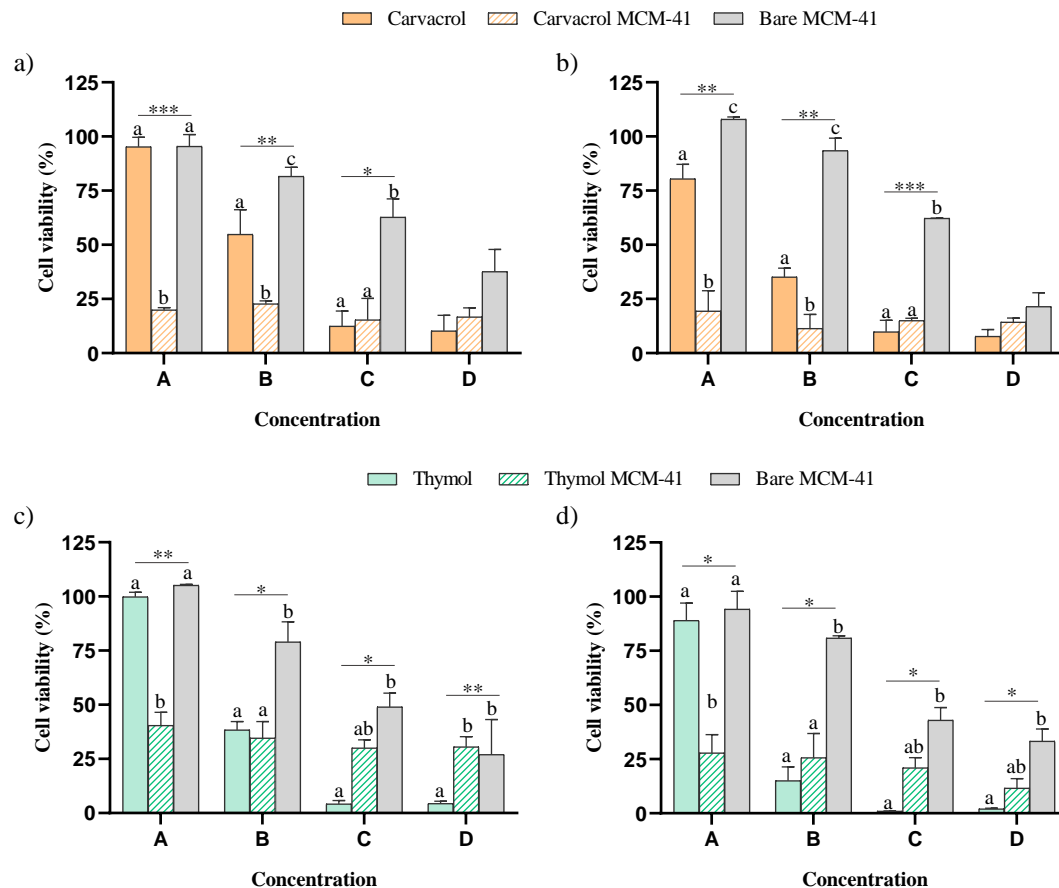
335 **Figure 2.** Concentration-cell viability plots of HepG2 cells exposed either directly or in  
 336 an extract dilution form to carvacrol-functionalised MCM-41 (a); and thymol-  
 337 functionalised MCM-41 (b) for 24 h and 48 h by the MTT assay. Each bar represents  
 338 the mean (SEM) of three independent assays, each performed 6-fold. (\*)  $p \leq 0.05$ ; (\*\*)  $p$

339  $\leq 0.01$ ; (\*\*\*)  $p \leq 0.001$  indicates significant differences compared to the control according  
340 to the Student's t-test.

341

342 The  $IC_{50}$  values previously found for carvacrol and thymol were used to define the  
343 concentration range for the comparative analysis of the cytotoxic effects free EOCs, EOC-  
344 functionalised MCM-41 and bare MCM-41 microparticles. The results of the comparative  
345 analysis are shown in Figure 3. Free carvacrol was significantly less cytotoxic than  
346 equivalent concentrations of carvacrol anchored on the surface of silica microparticles at  
347 the two lowest concentrations assayed (0.25 and 0.5 mM). Differences in cell viability  
348 between free carvacrol and carvacrol-functionalised MCM-41 ranged from 75% to 32%  
349 and from 61% to 24% after 24 h and 48 h exposure, respectively. Bare MCM-41  
350 microparticles also showed a lower cytotoxic response than equivalent concentrations of  
351 functionalised materials at the three highest concentrations tested for both times of  
352 exposure. Differences in cell viability between bare MCM-41 and carvacrol-  
353 functionalised MCM-41 range from 75% to 47% and from 89% to 47% after treating cells  
354 for 24 h and 48 h, respectively.

355 Regarding thymol, the free compound was significantly less cytotoxic than the  
356 equivalent concentration of immobilised compound on MCM-41 microparticles at the  
357 highest concentration tested (0.25 mM). At this concentration, differences in cell viability  
358 between free thymol and thymol-functionalised MCM-41 were of 59% and 61% after  
359 24 h and 48 h exposure, respectively. Bare MCM-41 was found less cytotoxic than  
360 thymol-functionalised MCM-41 at the 0.25 mM and 0.5 mM concentrations. At these  
361 concentrations, differences in cell viability between both types of particles ranged from  
362 65 % to 44 % and from 61 % to 55 % after 24 h and 48 h exposure, respectively.



363

364 **Figure 3.** Concentration-cell viability plots of HepG2 cells exposed to equivalent  
 365 concentrations of carvacrol, carvacrol functionalised MCM-41 and bare MCM-41 for 24  
 366 h (a) and 48 h (b) or to equivalent concentrations of thymol, thymol functionalised MCM-  
 367 41 and bare MCM-41 for 24 h (c) and 48 h (d). Each bar represents the mean (SEM) of  
 368 three independent assays, each performed 6-fold. Significant differences (\*\* $p \leq 0.01$ ,  
 369 \*\* $p \leq 0.01$ , \* $p \leq 0.05$ ) are indicated by different letters (a–c).

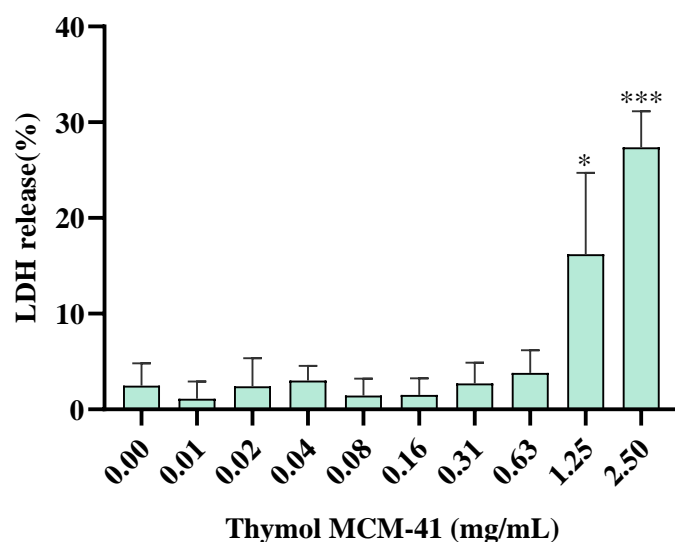
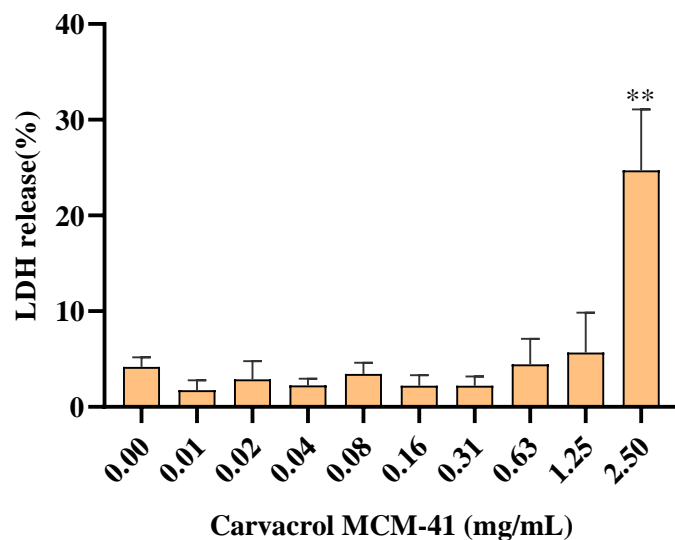
370

### 371 3.2.2. LDH assay

372 The LDH assay measures the activity of LDH released in cell culture medium after  
 373 exposure to cytotoxic substances, as an indicator of irreversible cell death due to cell  
 374 membrane damage (Aslantürk, 2018). Therefore, higher LDH values in the medium

375 indicate higher toxicity levels. Figure 4 depicts the effect of EOCs-functionalised  
376 particles on LDH release into the medium after 24 h exposure. As shown in this figure,  
377 carvacrol and thymol-functionalised silica exposure resulted in a significant increase of  
378 LDH release compared to controls at the highest concentrations tested. Exposure to 2.5  
379 mg/mL of carvacrol-functionalised silica increased the LDH release more than 10%  
380 compared to the control. For thymol-functionalised silica at the two highest  
381 concentrations tested (1.25 and 2.5 mg/mL), the LDH leakage into the culture medium  
382 increased by 14 % and 16%, respectively. No differences were observed at all the other  
383 tested concentrations respect to the control.

384



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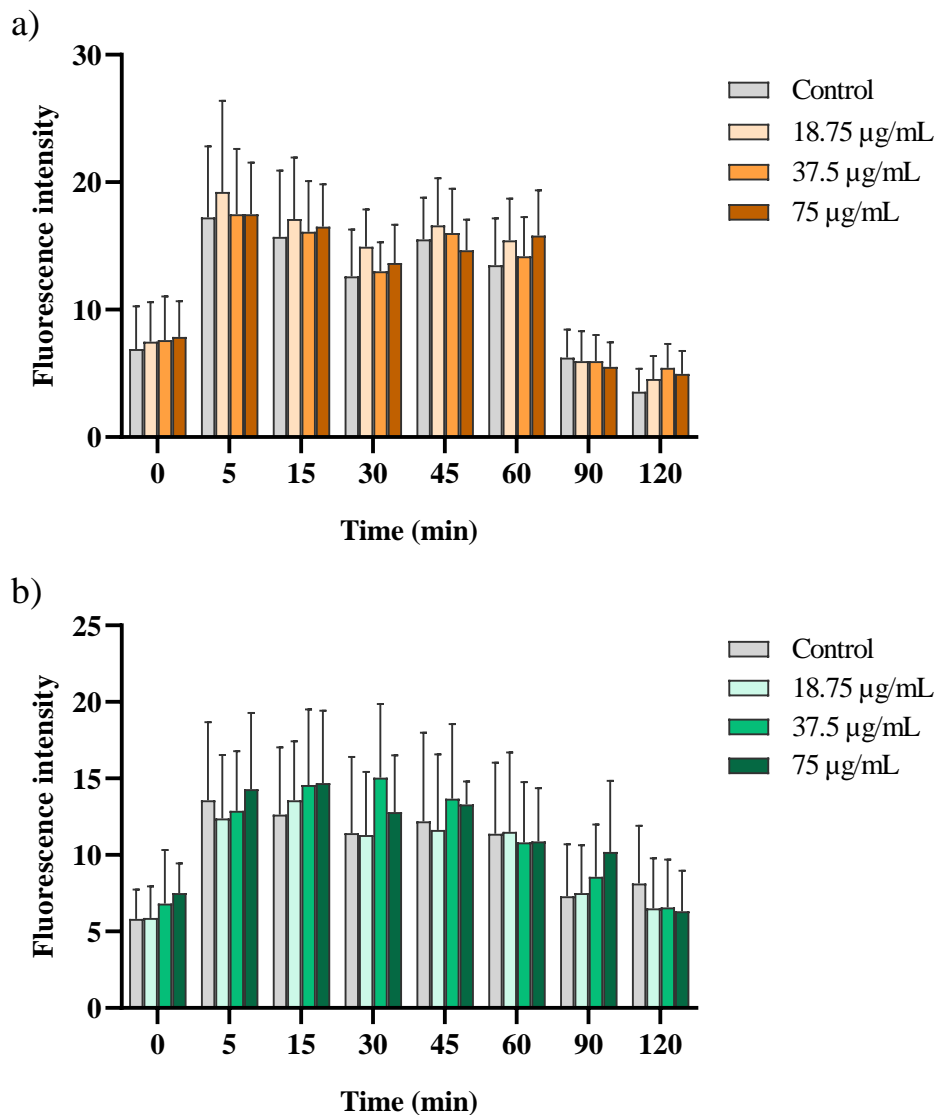
386 **Figure 4.** LDH activity in HepG2 cells exposed to carvacrol-functionalised MCM-41  
 387 microparticles (a) and thymol-functionalised MCM-41 microparticles (b) for 24 h.  
 388 Results are expressed as mean (SEM, n=3). (\*)  $p \leq 0.05$ ; (\*\*)  $p \leq 0.01$ ; (\*\*\*)  $p \leq 0.001$   
 389 indicates significant differences compared to the control according to the Student's t-test.

390

### 391 3.2.3. ROS formation

392 The formation of ROS was studied as an indicator of oxidative stress using the  
 393 fluorescein derivative H<sub>2</sub>DCF-DA. Figure 5 shows ROS production on HepG2 cells

394 exposed to 18.75, 37.5 and 75  $\mu\text{g/mL}$  of carvacrol- and thymol- functionalised MCM-41  
395 microparticles over 120 min post exposure. As can be observed, exposure to the three  
396 concentrations of both types of functionalised particles did not induce the formation of  
397 ROS over this time period, since no significant differences in the DCFDA dye  
398 fluorescence intensity was observed compared to the control cells.  
399



400

401 **Figure 5.** ROS induction as a function of time (0-120 min) in HepG2 cells exposed to  
402 sublethal concentrations of carvacrol-functionalised MCM-41 microparticles (a) and

403 thymol-functionalised MCM-41 microparticles (b). Results are expressed as mean (SEM,  
404 n=3). No significant differences were found between the different test solutions and the  
405 control.

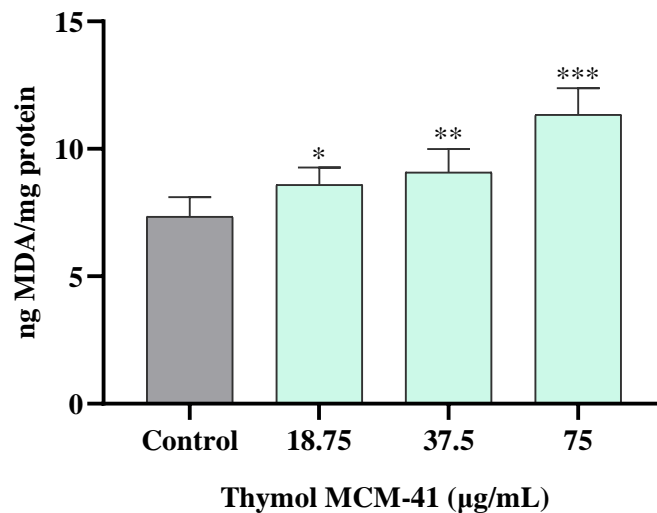
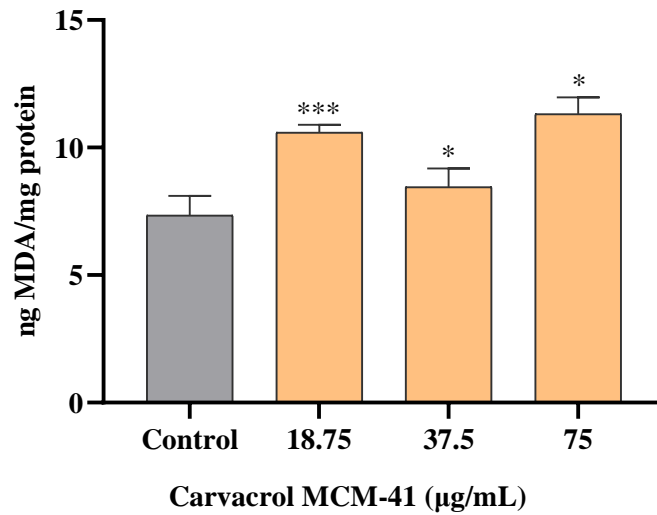
406

#### 407 **3.2.4. Lipid peroxidation assays**

408 The MDA levels were measured as an indicator of LPO and oxidative stress using the  
409 TBARS assay. The LPO production on HepG2 cells in the presence of carvacrol and  
410 thymol-functionalised silica at 18.75, 37.5 and 75 µg/mL can be observed in Figure 6.  
411 The results obtained demonstrated that 24 h of exposure to carvacrol-functionalised  
412 particles caused a significant increase in MDA production by 44% (18.75 µg/mL), 15%  
413 (37.5 µg/mL) and 54% (75 µg/mL) with respect to control cells. Similarly, 24-h exposure  
414 to thymol-functionalised particles significantly increased MDA levels in a concentration-  
415 dependent manner (Fig. 6). Exposure to 18.75, 37.5 and 75 µg/mL of thymol-  
416 functionalised MCM-41 for 24 h resulted in an increase of 17%, 24% and 55%,  
417 respectively, compared to the control.

418





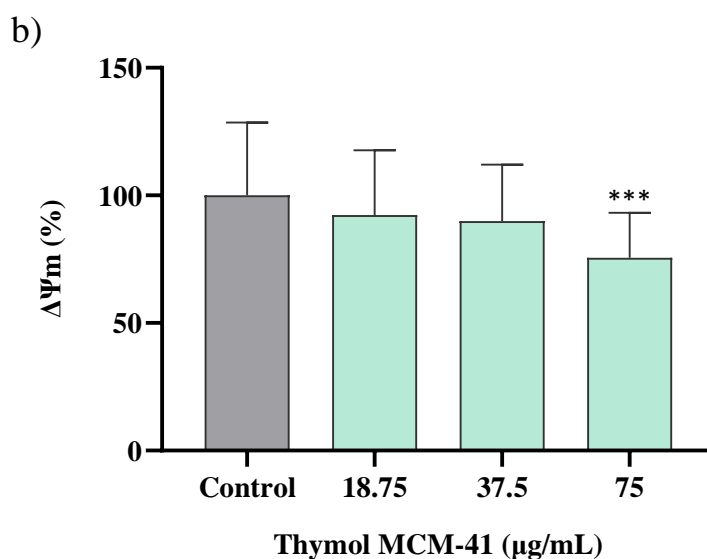
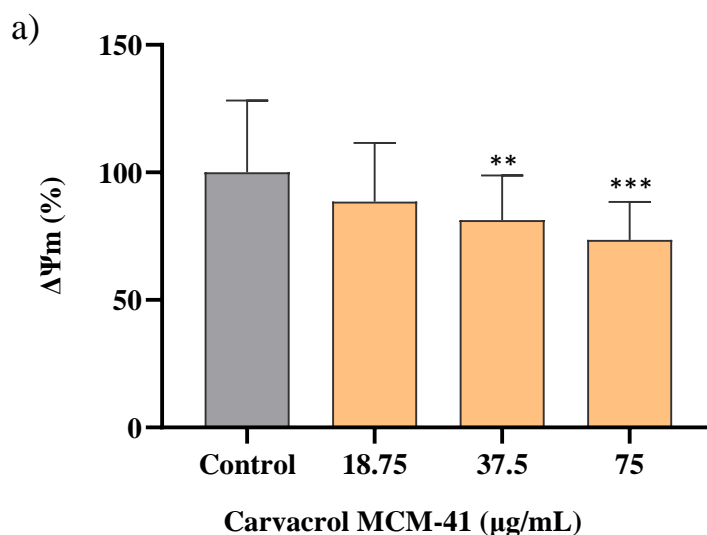
419

420 **Figure 6.** Effect on LPO as measure by MDA production after HepG2 cells exposure to  
 421 sublethal concentrations of carvacrol-functionalised MCM-41 microparticles (a) and  
 422 thymol-functionalised MCM-41 microparticles (b) for 24 h. Results are expressed as  
 423 mean (SEM, n=3). (\*)  $p \leq 0.05$ ; (\*\*)  $p \leq 0.01$ ; (\*\*\*)  $p \leq 0.001$  indicates significant  
 424 differences compared to the control according to the Student's t-test.

425

426 **3.2.5. Determination of  $\Delta\Psi_m$**

427 To assess whether functionalised particles exposure affected mitochondrial function,  
428 potential changes in  $\Delta\Psi_m$  were analysed by employing the mitochondria fluorescent dye  
429 Rhodamine 123. As shown in Figure 7, exposure to both materials for 24 h induced  
430 significant  $\Delta\Psi_m$  decrease in a concentration-dependent manner. This effect was higher  
431 for carvacrol-functionalised particles that decreased  $\Delta\Psi_m$  at the two highest  
432 concentrations tested, 37.5  $\mu\text{g}/\text{mL}$  and 75  $\mu\text{g}/\text{mL}$ , by a 19% and 28% with respect to the  
433 control, respectively. Thymol-functionalised particles in a 75  $\mu\text{g}/\text{mL}$  concentration  
434 resulted in a significant decrease of  $\Delta\Psi_m$  by a 24% when compared to untreated control  
435 cells.

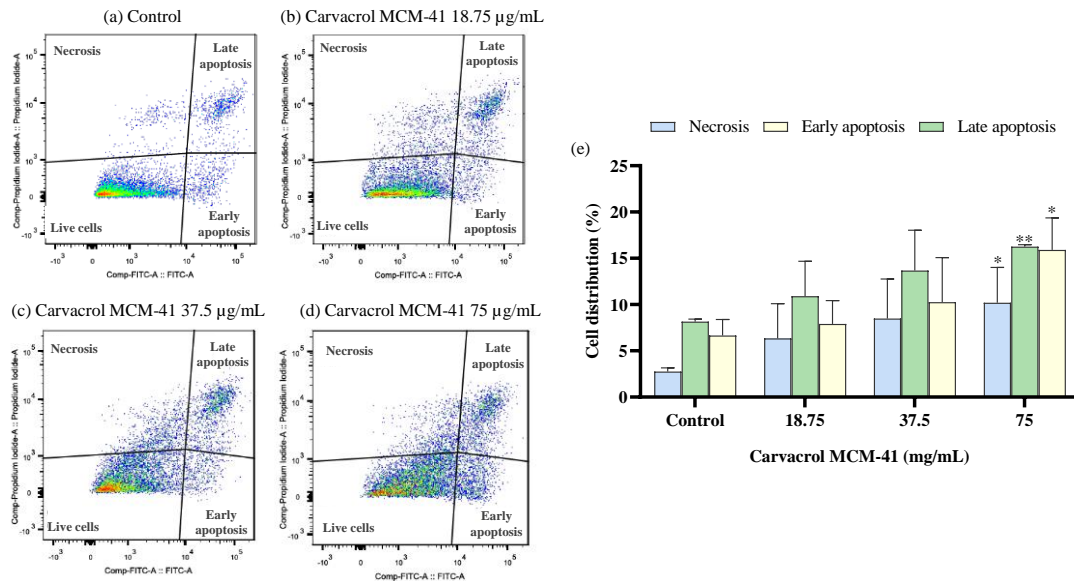


437 **Figure 7.** Effect on mitochondrial membrane potential ( $\Delta\Psi_m$ ) after HepG2 cells exposure  
438 to sublethal concentrations of carvacrol-functionalised MCM-41 microparticles (a) and  
439 thymol-functionalised MCM-41 microparticles (b) for 24 h. Results are expressed as  
440 mean (SEM, n=3). (\*\*)  $p \leq 0.01$ ; (\*\*\*)  $p \leq 0.001$  indicates significant differences  
441 compared to the control according to the Student's t-test.

442

### 443 **3.2.6. Apoptosis and necrosis assays**

444 Flow cytometry analysis was applied with the aim to determine the related death  
445 mechanism underlying the cytotoxic effect observed for functionalised materials. The  
446 fluorescein Annexin V-FITC/PI double staining was used to distinguish and quantify the  
447 percentage of necrotic, early apoptotic and late apoptotic cells after exposure to sublethal  
448 concentrations of carvacrol and thymol-functionalised MCM-41 (Fig. 8 and Fig. 9,  
449 respectively). The results revealed an increase in the percentage of necrotic, early  
450 apoptotic and late apoptotic cells following treatment with increasing concentrations of  
451 both functionalised particles. However, only significant differences were found between  
452 control cells and cells exposed to the highest concentrations tested of both materials (75  
453  $\mu\text{g/mL}$ ). The basal necrotic population in the control was 2.76 (0.40) %. After treatment  
454 with 75  $\mu\text{g/mL}$  of carvacrol and thymol-functionalised particles for 24 h, the necrotic rate  
455 raised to 10.20 (3.82) % and 8.63 (3.54) %, respectively. The percentage of early  
456 apoptotic HepG2 cells underwent an increase from 6.65 (1.73) % in control unexposed  
457 cells to 15.90 (3.46) % and 13.31 (1.83) % for carvacrol and thymol-functionalised MCM-  
458 41 in that order. Similarly, the percentage of late apoptotic cells augmented from 8.16  
459 (0.26) % in the untreated culture to 16.27 (0.20) % and 13.98 (2.18) % for carvacrol and  
460 thymol-functionalised particles exposed cells, respectively.

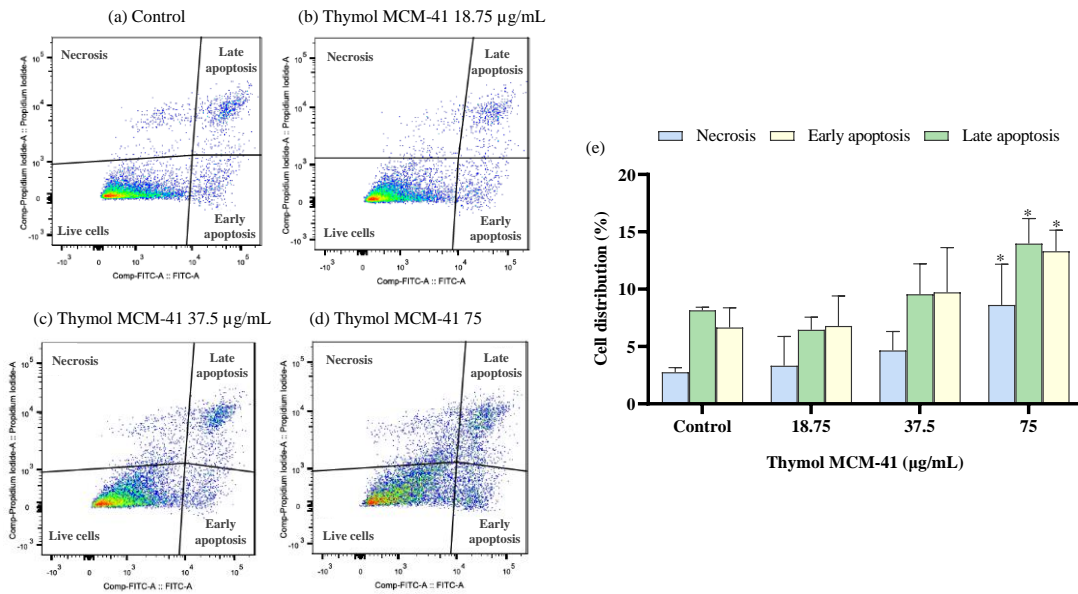


461

462 **Figure 8.** Flow cytometry analysis of apoptotic and necrotic HepG2 cells exposed to  
 463 sublethal concentrations of carvacrol-functionalised MCM-41 microparticles using  
 464 Annexin V-FITC/PI double staining. Representative two-dimensional dot plot diagrams  
 465 of three independent experiments for (a) untreated cells; (b) cells treated with 18.75  
 466 µg/mL; (c) 37.5 µg/mL; (d) and 75 µg/mL of carvacrol-functionalised MCM-41  
 467 microparticles. The upper left quadrant (PI+/Annexin V-FITC-) represents necrotic cells,  
 468 the left lower quadrant (PI-/Annexin V-FITC-) represents live cells, the upper right  
 469 quadrant (PI+ /Annexin V-FITC+) represents late apoptotic cells and the lower right  
 470 quadrant (PI-/Annexin V-FITC+) represents early apoptotic cells. (e) Percentage of early  
 471 apoptotic, late apoptotic and necrotic cells. Results are expressed as mean (SEM, n=3).  
 472 (\*)  $p \leq 0.05$ ; (\*\*)  $p \leq 0.01$  indicates a significant difference compared to the control  
 473 according to the Student's t-test.

474

475



476

477 **Figure 8.** Flow cytometry analysis of apoptotic and necrotic HepG2 cells exposed to  
 478 sublethal concentrations of thymol-functionalised MCM-41 microparticles using  
 479 Annexin V-FITC/PI double staining. Representative two-dimensional dot plot diagrams  
 480 of three independent experiments for (a) untreated cells; (b) cells treated with 18.75  
 481 µg/mL; (c) 37.5 µg/mL; (d) and 75 µg/mL of thymol-functionalised MCM-41  
 482 microparticles. The upper left quadrant (PI+/Annexin V-FITC-) represents necrotic cells,  
 483 the left lower quadrant (PI-/Annexin V-FITC-) represents live cells, the upper right  
 484 quadrant (PI+ /Annexin V-FITC+) represents late apoptotic cells and the lower right  
 485 quadrant (PI-/Annexin V-FITC+) represents early apoptotic cells. (e) Percentage of early  
 486 apoptotic, late apoptotic and necrotic cells. Results are expressed as mean (SEM, n=3).  
 487 (\*)  $p \leq 0.05$  indicates a significant difference compared to the control according to the  
 488 Student's t-test.

489

#### 490 4. Discussion

491 The immobilisation of natural EOCs on the surface of silica particles has emerged as an  
 492 innovative technology to enhance their antimicrobial and antioxidant properties.

493 However, due to their possible application in food or food contact materials, their safety  
494 needs to be addressed. For this purpose, the potential health hazards derived from  
495 exposure to these new materials for consumers' health should be thoroughly investigated  
496 at the cellular level. The use of cell cultures is a relevant tool in toxicity testing to enhance  
497 our understanding of hazardous materials and to predict their effects on human health  
498 (Eisenbrand et al., 2002). Assays to determine basal cytotoxicity measure cell viability or  
499 cell death as a consequence of damage to basic cellular functions, and allow for a rapid  
500 identification of toxic compounds. Moreover, *in vitro* tests on specialised cell functions  
501 and metabolic endpoints give insight into the pathways and mechanisms of action  
502 involved in chemically induced toxicity at both the molecular and the cellular level. This  
503 study examines the *in vitro* toxic effect of carvacrol and thymol-functionalised MCM-41  
504 silica particles on HepG2 cells as model cell line. The aim was to evaluate their potential  
505 toxicity and to fully understand the associated mechanism involved.

506 First, a comparative analysis of the functionalised-particles and their constituents was  
507 carried out, revealing that free EOCs and bare MCM-41 microparticles exhibited  
508 significantly milder cytotoxic effects than equivalent concentrations of EOC-  
509 functionalised silica. Similar results were previously found for eugenol and vanillin-  
510 functionalised MCM-41 microparticles (Fuentes et al., 2021). In that study, the stronger  
511 cytotoxic effect observed for the EOCs-functionalised silica was attributed to physico-  
512 chemical properties, such as surface charge and hydrophobicity that could be responsible  
513 for promoting interactions of EOCs with cell membranes. Also, Chen et al. (2009) found  
514 that functionalisation with carvacrol increased the cytotoxicity of chitosan nanoparticles  
515 in a 3T3 mouse fibroblast cell line. The  $IC_{50}$  value observed for carvacrol-grafted chitosan  
516 nanoparticles was around 1 mg/mL, whereas at a 2 mg/mL concentration of unmodified  
517 chitosan nanoparticles cell viability was still higher than 80%, as measured by the MTT

518 assay. However, carvacrol-modified chitosan nanoparticles were significantly less  
519 cytotoxic towards mammalian cells than free carvacrol. As previously reported, this  
520 discrepancy may be the result of differences in the starting material, the cell type  
521 employed for the cytotoxicity assays or the lesser degree of grafting achieved for the silica  
522 particles (Fuentes et al., 2021).

523 Cytotoxicity data may serve to predict acute systemic toxicity *in vivo* and also to define  
524 the concentration range for mechanistic toxicity studies (Ciappellano et al., 2016; Severin  
525 et al., 2017). With this twofold objective, the cytotoxicity of carvacrol- and thymol-  
526 functionalised microparticles was analysed by two methods based on different  
527 physiological endpoints; the MTT and the LDH release assays. As measured by the MTT  
528 method, 24 h exposure resulted in an IC<sub>50</sub> value of 0.15 mg/mL for both functionalised  
529 materials, and this value decreased to 0.09 mg/mL and 0.11 mg/mL after 48-h treatment,  
530 for carvacrol- and thymol-silica, respectively. In a previous work, Fuentes et al., 2021  
531 determined the cytotoxic effect of bare MCM-41 silica microparticles on HepG2 cells by  
532 the MTT assay and confirmed the biocompatibility reported for calcined mesoporous  
533 silica (Aburawi et al., 2012; Al-Salam et al., 2011; Samri et al., 2012; Shamsi et al., 2010).  
534 Exposing cells to bare MCM-41 silica for 24 h and 48 h resulted in IC<sub>50</sub> values of 18.90  
535 mg/mL and 15.82 mg/mL, respectively (Fuentes et al., 2021). By comparison with these  
536 results, herein we found that functionalisation of MCM-41 microparticles with carvacrol  
537 or thymol increase cytotoxicity of the starting material approximately 100-fold. However,  
538 when cells were exposed to the filtered medium which previously contained the particles  
539 during the extract dilution assays, we found that at the IC<sub>50</sub> values calculated for both  
540 materials, the cell viability remained around 100%. These results may be interpreted as a  
541 confirmation of a direct interaction of cells with the particles being responsible for the  
542 cytotoxic behaviour found for both, carvacrol- and thymol-functionalised particles, while

543 an indirect cytotoxicity effect due to components leached from the surface of the  
544 functionalised particles or by means of depletion of nutrients from the culture medium  
545 (Casey et al., 2008) is not expected.

546 Cytotoxic effects assessed by the LDH assay were observed at higher particle  
547 concentrations than with the MTT assay, thus, demonstrating that the MTT assay was  
548 more sensitive than the LDH assay for determining cell viability after EOCs-  
549 functionalised microparticles exposure. The sensitivity of the different cytotoxicity assays  
550 has been demonstrated to differ depending on the mechanisms that lead to cell death  
551 (Weyermann et al., 2005). The MTT method determines mitochondrial metabolic activity  
552 of viable cells, while LDH assay measures cell death due to cell membrane damage. In  
553 this sense, the differences observed between both assays concerning their sensitivity may  
554 suggest that impairment of mitochondrial function may precede disruption of the  
555 membrane integrity and cell lysis in cells exposed to carvacrol and thymol-functionalised  
556 microparticles. Moreover, these results support the widespread consensus that more than  
557 one cell viability assay should be used in order to increase the reliability of the results  
558 during *in vitro* studies (Aslantürk, 2018; Eisenbrand et al., 2002; Fotakis and Timbrell,  
559 2006).

560 It is worth mentioning that different studies have described particle interference when  
561 testing cytotoxicity with both methods (Holder et al., 2012; Kroll et al., 2012). Different  
562 factors have been proven to limit the sensitivity of the MTT method, including pH, optical  
563 activity or surface reactivity of the particles (Abbasi et al., 2021; Laaksonen et al., 2007).  
564 In the LDH assay, different inorganic particles have been demonstrated to interfere with  
565 this assay by either adsorbing or inactivating the LDH protein, both mechanisms causing  
566 a decreased absorbance in the LDH assay and resulting in a false indication of a nontoxic  
567 response (Holder et al., 2012). Moreover, Korhonen et al. (2016) used the LDH assay to



568 evaluate the cytotoxic effect of mesoporous silica microparticles in human corneal  
569 epithelial (HCE) and retinal pigment epithelial (ARPE-19) cells and found an increased  
570 or decreased reactivity in the LDH assay depending on the cell culture medium used.  
571 Herein, the MTT and LDH viability assays were also performed under cell-free conditions  
572 in order to evaluate interference of the functionalised particles with both assays. At low  
573 concentrations, EOCs-functionalised materials did not induce any non-specific response  
574 in the MTT and LDH viability assays, while at concentrations higher than 0.31 mg/mL  
575 of particles, significantly increased absorbance was observed for both cell-free assays.  
576 Consequently, data were corrected to avoid any particle interferences by subtracting the  
577 absorbance of the cell-free controls from that of the test wells.

578 In order to gain insight into the cytotoxicity mechanism induced by these materials,  
579 different endpoints related to oxidative stress, mitochondrial dysfunction and cell death  
580 pathway were investigated. In this aspect of the work, the IC<sub>50</sub> values found for both  
581 materials by the MTT assay were used to define the concentration range for further assays.

582 Oxidative stress is a major mechanism involved in toxicity induced by many xenobiotics  
583 (Zhang, 2018). It is a result of an imbalance between the production of oxidising  
584 molecular species and the protective mechanisms produced by the cells for their removal.  
585 ROS are oxygen-containing chemically-reactive molecules that, under normal conditions,  
586 are produced by the cells as a consequence of aerobic metabolism (Ray et al., 2012).  
587 However, the over production and accumulation of ROS due to interactions of cells with  
588 toxic agents may lead to dysfunction of the antioxidant system and oxidative damage to  
589 cellular macromolecules such as lipids, proteins or nucleic acids, causing severe cell  
590 toxicity (Eisenbrand et al., 2002). To evaluate whether oxidative stress was involved in  
591 carvacrol- and thymol-functionalised MCM-41 cytotoxicity, two different biomarkers  
592 were used: ROS production and LPO generation. Results showed that exposure to

593 sublethal concentrations of both materials did not induce early ROS formation as  
594 measured by the DCFDA assay. However, a significant increase in MDA levels was  
595 found when cells were exposed to the particles for 24 h, indicating that oxidative stress  
596 occurred through LPO.

597 High levels of ROS that persist for a long period are thought to be the major factor  
598 responsible for reacting with the polyunsaturated fatty acids of lipid membranes and  
599 inducing LPO (Barrera, 2012). The absence of ROS in this study may be explain by  
600 differences in the exposure times employed between both assays. The ROS formation  
601 was measured within 2 h after exposure to sublethal concentrations of the functionalised  
602 silica, while LPO was determined when cells were treated with these materials for 24 h.  
603 Accordingly, Santos et al. (2010) evaluated ROS production followed exposure to  
604 different mesoporous silicon microparticles in the human colon carcinoma cell line Caco-  
605 2 using the DCFDA assay. These authors found no significant increases in hydrogen  
606 peroxide concentration or mitochondrial superoxide after 3 h-incubation time but a  
607 significant increase in hydrogen peroxide formation after 24 h exposure. Longer exposure  
608 times than those usually employed with this method have also been found necessary to  
609 detect oxidative stress caused by other toxic insults (Aranda et al., 2013). Some authors  
610 also suggest that, although the DCFDA probe has been extensively employed as a  
611 biomarker for oxidative stress and is supposed to reflect the overall oxidative status of the  
612 cell, it can only detect hydrogen peroxides, peroxy radicals and peroxy nitrite anions and  
613 not all the different types of ROS (Herzog et al., 2009).

614 Toxic agents can generate ROS by directly interacting with the electron-transport chain  
615 complexes in the inner mitochondrial membrane (Boelsterli, 2007). Moreover cell-  
616 particle interactions can induce ROS formation by a surface- catalysed reaction (Lehman  
617 et al., 2016). Indeed, silica particles have been demonstrated to induce ROS formation by

618 both mechanism; by direct contact of cell membrane with the particles surface and by  
619 triggering cell-signalling pathways that initiate cytokine release and apoptosis within the  
620 cells (Hamilton et al., 2008). Different phenomena including hydrophobic or hydrophilic  
621 interactions, active electron configurations, redox potential or semiconductor and  
622 electronic properties may be responsible for ROS generation upon interactions of  
623 particles with biological systems (Santos et al., 2010). In line with this, Lehman et al.  
624 (2016) studied the free radical species generated from the surface of non-porous and  
625 mesoporous nanoparticles by electron paramagnetic resonance spectroscopy. These  
626 authors found a correlation between ROS released from the nanoparticle surface,  
627 intracellular ROS and cellular toxicity in the murine macrophage cell line RAW 264.7.  
628 Moreover, amine-functionalisation reduced the amount of free radical generated at the  
629 solid–liquid interface by non-porous nanosilica and, as suggested by the authors, this  
630 would mitigate their toxic behaviour. Similarly, Santos et al. (2010) also found surface  
631 chemistry as a determinant factor that determines ROS production and cell–particle  
632 interactions. According to their work, thermally carbonised particles induced toxicity as  
633 a result of stimulation of ROS production on Caco-2 cells, while thermally oxidised  
634 particles did not induce significant ROS formation and resulted in less damage to cells as  
635 a result of weak cell–particle interactions. In our work, the increased cytotoxicity found  
636 for the EOCs-functionalised compared to the native microparticles may be attributed to  
637 differences in the surface properties between bare and EOCs-functionalised particles,  
638 since no differences of size and shape were observed. Cationic nature and hydrophobic  
639 surfaces have been demonstrated to increase *in vitro* toxicity and the number of apoptotic  
640 cells as a result of strong cell–particle interactions (Saei et al., 2017; Santos et al., 2010).  
641 These properties may therefore be related to the increased cytotoxicity found for the  
642 functionalised materials in our study.

643 A close relationship exists between ROS formation and mitochondria, since these  
644 organelles are considered the main source of ROS in the cell and at the same time  
645 mitochondrial damage by ROS formation is a main mechanism of toxicant-induced  
646 cytotoxicity (Zhang, 2018). Accordingly, mitochondrial dysfunction is one of the most  
647 sensitive indicators of adverse cell effects and it can be evaluated by monitoring changes  
648 in  $\Delta\Psi_m$  of exposed cells (Xu et al., 2004). In this study, a depletion of the  $\Delta\Psi_m$  was  
649 observed after treatment of HepG2 cells with carvacrol and thymol-functionalised silica.  
650 The  $\Delta\Psi_m$  generated is an essential component in a range of processes including energy  
651 storage during oxidative phosphorylation, calcium homeostasis or cellular differentiation.  
652 Moreover, disruption of mitochondrial integrity has been described as one of the early  
653 events leading to apoptosis and may serve as a biomarker for apoptotic cell death (Jeong  
654 and Seol, 2008).

655 Exposure to cytotoxic agents can lead to cell death mainly by two major mechanisms:  
656 apoptosis and necrosis. In this work, the death mechanism related to the cytotoxic effects  
657 induced by modified-MCM-41 exposure was evaluated using the Annexin V-FITC/PI  
658 double staining and flow cytometry analysis. This method allows to discriminate between  
659 healthy, early apoptotic, late apoptotic and necrotic cells. We found that all three, early  
660 apoptotic, late apoptotic and necrotic rates significantly increased after treating HepG2  
661 cells to the highest sublethal concentration of both carvacrol and thymol-functionalised  
662 silica for 24 h. According to these results, both mechanisms of cell death are involved in  
663 the cytotoxicity induced by EOCs-functionalised MCM-41.

664 Apoptosis or programmed cell death is a slow form of cell death that can occur under  
665 normal physiological conditions or may be induced by apoptotic compounds. There exist  
666 two main pathways that lead to apoptosis: the extrinsic or death-receptor pathway that is  
667 activated from outside the cell by ligation of transmembrane death receptors, and the

668 intrinsic or mitochondrial pathway that begins with the permeabilisation of the  
669 mitochondrial outer membrane triggered by different signals such as DNA damage,  
670 ischemia or oxidative stress (Wang and Youle, 2009). Depletion of  $\Delta\Psi_m$  causes the  
671 release of mitochondrial intermembrane space proteins into the cytoplasm, including  
672 cytochrome c, that consequently triggers other apoptotic factors such caspases activation  
673 or chromosome fragmentation, leading to apoptosis through the mitochondrial or intrinsic  
674 pathway apoptotic death pathway (Tait and Green, 2013). Therefore, loss of  $\Delta\Psi_m$  serves  
675 as a biomarker for apoptotic cell death.

676 In contrast to apoptosis, necrosis consists in a rapid form of cell death that is induced by  
677 external injuries such as hypothermia, radiation, hypoxia or chemicals that damage the  
678 cell membrane (D'Arcy, 2019). Destruction of the plasma membrane or the biochemical  
679 supports of its integrity leads to the release of intracellular material, local inflammatory  
680 responses, and cell swell and lysis (Miret et al., 2006). In consequence, necrosis can be  
681 measured by the presence of the cytoplasmic content in the extracellular fluid i.e. by  
682 measuring the activity of enzymes such as LDH. As already explained, the LDH assay  
683 was far less sensitive than the MTT assay for evaluating the basal cytotoxicity on HepG2  
684 cells as a consequence of the microparticles exposure, suggesting impairment of the  
685 mitochondrial activity rather than cell membrane disruption. As a result, we hypothesise  
686 that apoptosis is the most likely mechanism of cell death after EOCs-functionalised  
687 particles exposure.

688 According to our results, the mechanism underlying the cytotoxic effect of carvacrol and  
689 thymol-functionalised silica microparticles on HepG2 cells involves induction of  
690 oxidative stress that will cause mitochondrial dysfunction and lead to activation of the  
691 apoptotic death pathway. This mechanism of toxic action bears similarities with the  
692 mechanism described for their constituents. Essential oils and their components have been

693 demonstrated to induce toxicity in eukaryotic cells due to a phenolic-like prooxidant  
694 mechanism (Bakkali et al., 2008). These components will penetrate cells and permeabilise  
695 cytoplasmic and especially mitochondrial membranes. Then, damaged mitochondria  
696 produce ROS, generating reactive phenoxy radicals with prooxidant potential that may  
697 oxidise EOCs. Ultimately, this sequence of events leads to cell death by apoptosis  
698 (Bakkali et al., 2008). For their side, different size MCM-41 and SBA-15 microparticles  
699 induced ROS formation, especially  $O_2^-$ , at concentrations over 1 mg/mL after 3 h  
700 incubation on Caco-2 cells, overwhelming the antioxidant defences and causing  
701 mitochondrial dysfunction and increasing the apoptotic signalling (Heikkilä et al., 2010).  
702 Moreover, as found herein for the EOCs-functionalised silica, metabolic activity was a  
703 more sensitive endpoint as measured by ATP depletion, than cell membrane integrity  
704 (Heikkilä et al., 2010). However, both bare MCM-41 microparticles and EOCs exhibit  
705 cytotoxic effects and ROS generation at much higher concentrations than those found for  
706 the functionalised particles object of this study. Our results suggest that either a  
707 synergistic effect by the presence on the functionalised particles surface of both, silanol  
708 groups from the bulk material and EOCs derivatives from the functionalisation process,  
709 either a boosting effect of the EOCs as a consequence of their higher density or reduced  
710 volatility, increasing EOCs-cell membrane interactions (Fuentes et al., 2021). Another  
711 possible explanation is that EOCs in their free form can be partly metabolised by cells  
712 and this phenomenon is not possible for immobilised compounds. However, the cellular  
713 uptake of the microparticles by HepG2 cells would still need to be confirmed by confocal  
714 microscopy analysis in the future.

715 Alternatives to synthetic preservatives for food applications are not free from potential  
716 toxicological hazards. As observed in this work, toxicity studies are necessary for the  
717 understanding of the interactions of new materials with biological systems and to

718 guarantee their safety for human health. In summary, our results show that  
719 functionalisation of silica MCM-41 microparticles with the natural EOCs carvacrol and  
720 thymol increased the cytotoxic potential of these materials compared to their free  
721 constituents. Both particle types behaved similarly in regard to their cytotoxic effects that  
722 were proven to emerge from the microparticles themselves and not from degradation  
723 products released to the culture media. Overall, the results found in this study support the  
724 hypothesis that carvacrol- and thymol-functionalised MCM-41 induced toxicity on  
725 HepG2 cells by an oxidative stress-related mechanism. A direct physical interaction  
726 between the particles surface and cell membranes could be responsible to induce ROS  
727 overproduction. The oxidative stress would lead to oxidation of different cellular  
728 components such as lipids and to  $\Delta\Psi_m$  function that in turn would trigger apoptosis  
729 signalling through mitochondrial pathway, ultimately leading to cell death by both the  
730 proteolytic cascade of pro-apoptotic enzymes and the damage caused to the mitochondrial  
731 function. These results should be considered when designing new hybrid materials for  
732 food-industry applications.

733

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737

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