Manuscript Number:

Title: Nanoemulsions as delivery systems of hydrophobic silybin from silymarin in foods: effect of oil type on silybin solubility, in vitro bioaccessibility and stability

Article Type: Research Article

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Abstract: The purpose of this study was to investigate the potential of nanoemulsion delivery systems to carry silybin from silymarin. To this purpose, different carrier oils (i.e. sunflower oil, extra virgin olive oil and castor oil) were used to prepare silymarin loaded nanoemulsions. The effect of oil type on the silybin solubility and in vitro bioaccessibility was evaluated. Moreover, the physical and chemical stability of nanoemulsions was studied by measuring particle size, silybin concentration, oxygen consumption and hydroperoxide formation during storage at 20 °C. Results showed that silybin can be successfully incorporated into physically stable nanoemulsions prepared with the different oils. The oil type used slightly affected the silybin in vitro bioaccessibility. On the contrary, the oil nature influenced the nanoemulsion particle size as well as silybin stability during storage: silybin underwent degradation, showing lower stability in extra virgin and sunflower oil than in castor oil. Results also showed that silymarin did not affect the oxidation kinetics of the carrier oils.

Dear Editor,

I would like to submit to your attention the manuscript entitled "Nanoemulsions as delivery systems of hydrophobic silybin from silymarin in foods: effect of oil type on silybin solubility, in vitro bioaccessibility and stability " (Authors: Sonia Calligaris, Piergiorgio Comuzzo, Francesca Bot, Giovanna Lippe, Roberto Zironi, Monica Anese, Maria Cristina Nicoli) for consideration for publication on Food Research International.

The use of the flavolignan silybin from silymarin to treat liver diseases has been well documented. Indeed, in recent years attempts have been made to develop pharmaceutical preparations with increased silybin bioavailability. At the same time no information is available on ways to improve its bioaccessibility in foods. Due to silybin chemical and physical properties, lipid-based delivery systems, such as nanoemulsions, could be effectively used to incorporate this nutraceuticals in functional foods. The choice of the lipid medium appears particularly critical, since the chemical and physical characteristics of the lipid carrier could greatly affect the solubility and the stability of the compound to be delivered.

The aim of this research was to study the potential for nanoemulsion delivery systems to carry silybin from silymarin into foods. To this purpose, different carrier oils (sunflower oil, extra virgin olive oil and castor oil) were used to prepare silymarin loaded nanoemulsions. The physical and chemical stability of nanoemulsions was studied during storage at 20 °C. Also, the effect of oil type on the silybin *in vitro* bioaccessibility was evaluated.

Results showed that silybin can be successfully incorporated into physically stable nanoemulsions prepared with the different oils. The oil type used slightly affected the silybin in vitro bioaccessibility. On the contrary, the oil nature influenced the nanoemulsion particle size as well as silybin stability during storage: silybin underwent degradation, showing lower stability in extra virgin and sunflower oil than in castor oil. Results also showed that silymarin did not affect the oxidation kinetics of the carrier oils.

We would greatly appreciate your comments on the paper.

Best regards
Prof. Monica Anese

Highlights

Silybin was successfully incorporated into nanoemulsions containing different oils

The oil type slightly affected the silybin in vitro bioaccessibility

The oil nature influenced the nanoemulsion particle size and silybin stability

Silymarin did not affect the oxidation kinetics of the carrier oils.

Nanoemulsions as delivery systems of hydrophobic silybin from silymarin in foods: effect of oil type on silybin solubility, *in vitro* bioaccessibility and stability

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13   **Abstract**

14   The purpose of this study was to investigate the potential of nanoemulsion delivery systems

15   to carry silybin from silymarin. To this purpose, different carrier oils (i.e. sunflower oil, extra

16   virgin olive oil and castor oil) were used to prepare silymarin loaded nanoemulsions. The

17   effect of oil type on the silybin solubility and i*n vitro* bioaccessibility was evaluated.

18   Moreover, the physical and chemical stability of nanoemulsions was studied by measuring

19   particle size, silybin concentration, oxygen consumption and hydroperoxide formation during

20   storage at 20 °C.

21   Results showed that silybin can be successfully incorporated into physically stable

22   nanoemulsions prepared with the different oils. The oil type used slightly affected the silybin

23   *in vitro* bioaccessibility. On the contrary, the oil nature influenced the nanoemulsion particle

24   size as well as silybin stability during storage: silybin underwent degradation, showing lower

25   stability in extra virgin and sunflower oil than in castor oil. Results also showed that

26   silymarin did not affect the oxidation kinetics of the carrier oils.

27

28   **Keywords**: silybin, silymarin, delivery, nanoemulsion, stability, bioaccessibility

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1. Introduction

Silymarin is a flavolignans extract of *Silybum marianum*. The flavolignan silybin or silybinin is the most abundant biologically active compound of silymarin. The use of silymarin to treat liver diseases, such as cirrhosis, hepatitis, alcoholic liver disease and toxin exposure has been well documented (Flora, Hahn, Rosen, & Benner, 1998; Fraschini, Demartini, & Esposti, 2002). These biological effects are attributed to the antioxidant, antifibrotic, anti-inflammatory, anti-lipid-peroxidative and anti-carcinogenic activity of silymarin components (Basaga, Poli, Tekkaya, & Ara, 1997; Luper, 1998; Yang, Liu, & Liu, 2004).

Although results can be hardly summarized, studies on the liver-protective capacity of silymarin (Loguercio & Festi, 2011) and cell oxidation mechanisms (Dehmlow, Murawski, & de Groot, 1996; Zielinska-Przyjemska & Wiktorowicz, 2006) evidenced an important inhibitory effect of silymarin flavonoids on cell enzymes (e.g. lipoxygenase) involved in inflammatory reactions, whereas reaction with $O_2^- \cdot$ occurred to a lesser extent (Dehmlow et al., 1996). Despite this, clinical application and therapeutic efficiency of sylimarin flavolignans are limited due to their poor bioavailability. As known, molecule physical properties may greatly affect its bioavailability. At ambient temperature, silybin is in crystalline state and has low water solubility (Gazak et al., 2004). In recent years, attempts have been made to develop pharmaceutical preparations with increased silybin bioavailability (Jia et al., 2010; Li, Yuan, Huang, Zhou, & Liu, 2010; Javed, Kohli, & Ali, 2011; Parveen, Baboota, Ali, Ahuja, Vasudev, & Ahmad, 2011), while no information is available on ways to improve its bioaccessibility in foods. According to Rao and McClements (2012), lipid-based delivery systems for food application, such as microemulsions, nanoemulsions, liposomes, solid lipid nanoparticles, polymeric nanoparticles, filled hydrogel particles, can be effectively used to incorporate poorly water-soluble nutraceuticals in functional foods. As known, nanoemulsions are thermodynamically unstable colloidal systems containing small lipid droplets dispersed in an aqueous medium (Rao & McClements, 2012). Generally, they show

56 good stability to gravitational separation and particle aggregation becoming a good

57 component to be added to foods that have to be processed and stored under different

58 conditions. Nanoemulsions have been actually proposed to increase the solubility and stability

59 of bioactive molecules, such as quercitin (Pool, Mendoza, Xiao, & McClements, 2013),

60 polymethoxyflavone (Li, Zheng, Xiao, & McClements, 2012) and curcumin (Ahmed, Li,

61 McClements, & Xiao, 2012), to be incorporated into foods. The choice of the lipid medium

62 appears particularly critical, since the chemical and physical characteristics of the lipid carrier

63 greatly affect the solubility of the compound to be delivered. Also, the presence of bioactive

64 molecule crystals may negatively affect nanoemulsion physical stability, leading to possible

65 undesired phase separation during food processing and storage, as well as reduction of the

66 bioavailability of the selected component that may not be adsorbed in this form into the

67 gastrointestinal tract (Giacomelli et al., 2002; Kawabata, Wada, Nakatani, Yamada, & Onoue,

68 2011).

69 The aim of this research was to study the potential for nanoemulsion delivery systems to

70 carry silymarin, and thus silybin, into foods. To this purpose, different carrier oils (sunflower

71 oil, extra virgin olive oil and castor oil) were used to prepare silymarin loaded

72 nanoemulsions. The physical and chemical stability of nanoemulsions was studied during

73 storage at 20 °C. Also, the effect of oil type on the silybin i*n vitro* bioaccessibility was

74 evaluated.

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2. Materials and methods

2.1. Materials

Silymarin extract containing 210 mg/g of silybin, 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH·), lipase from porcine pancreas, porcine bile extract, di-hydrated calcium chloride, Tween 80, sodium azide, ethyl acetate, HPLC grade methanol, isooctane, 2-propanol, 1-butanol, ammonium thiocyanate, cumene hydroperoxide, analytical grade hydrochloric acid and sodium hydroxide were from Sigma Aldrich (St. Luois, MO, USA). Sodium chloride, barium chloride, ferrous sulphate, monosodium dihydrogen phosphate, disodium hydrogen phosphate and 85% w/v phosphoric acid were from Carlo Erba Reagents (Milano, Italy). Analytical standard grade silybin and naringenin-7-*O*-glucoside were from Extrasynthese (Genay, France). Sunflower oil, extra virgin olive oil and castor oil were purchased in a local market.

2.2. Nanoemulsion preparation

The oil phase was prepared by mixing silymarin powder (2.5 mg/g) and Tween 80 (10 mg/g) in sunflower oil, extra virgin olive oil or castor oil. The systems were stirred in the dark until the silymarin was completely dissolved. No recrystallization events were observed before emulsion preparation. The aqueous phase consisted of deionised water added with 0.1 mg/g of sodium azide, to avoid microbial spoilage during the storage experiments. The stock emulsions were prepared by mixing 20% (w/w) oil phase with 80% (w/w) aqueous phase with a high speed blender for 1 min at 9000 rpm (Polytron, PT 3000, Cinematica, Littau, Swiss). Aliquots of 250 mL of the stock emulsions were homogenised at 10 L/h flow rate by two passes at 150 MPa through a two stage high pressure homogeniser provided with cylindrical tungsten carbide homogenising valves (Panda PLUS 2000, Gea Niro Soavi, Parma, Italy). Aliquots of 18 mL of the nanoemulsions were inserted into 20 mL colourless glass vials,

102   sealed with butyl septa and metallic caps and stored at 20 °C in a thermostatic cell for up to

103   50 days.

104   *2.3. Analytical determinations*

105   *2.3.1. Particle size*

106   The mean diameter of emulsion droplets was measured by using the dynamic light scattering

107   instrument Particle Sizer NICOMP^{TM} 380 ZLS (PSS NICOMP Particle Sizing System, Santa

108   Barbara, California, USA). Samples were diluted 1:1000 (v/v) with deionised water prior to

109   the analysis to avoid multiple scattering effects. The angle of observation was 90°. Solution

110   refractive index and viscosity were set at 1.333 and 1.0 cP, respectively, corresponding to the

111   values of pure water at 20 °C. Particle mean diameter corresponding to volume distribution

112   was calculated by NICOMP Distribution Analysis.

113

114   *2.3.2. Silybin solubility*

115   Aliquots of 3 mL of sunflower oil, castor oil, extra virgin olive oil, Tween 80, deionized water

116   or an oil and Tween 80 mixture (1:1 v/v) were introduced in 5.0 mL capacity vials and excess

117   amount of silymarin was added (2% w/w). Samples were kept at a constant temperature

118   (25±1.0 °C) under shaking for 72 h to reach equilibrium (Parveen et al., 2011). The samples

119   were centrifuged at 13100 *g* for 10 min (MiniSpin, Eppendorf, Hamburg, Germany) and the

120   solubilised silybin in the supernatant was then recovered and quantified by HPLC analysis.

121

122   *2.3.3. Silybin concentration*

123   Silybin extraction was performed by introducing 1 g supernatant or nanoemulsion into 10 mL

124   Pyrex tubes, added with 5 mL water:methanol mixture (20:80 v/v), and manually shaken for 2

125   min. The tubes were then treated for 15 min in an ultrasonic bath (25 °C) and finally

126   centrifuged at 1000 *g* for 10 min (Labofuge I, Heraeus Christ GmbH, Osterode am Harz,

127   Germany). Samples were then stored overnight at -20 °C, to improve the phase separation.

128 The upper water-methanol phase was filtered on 0.20 μm pore size nylon membranes (Albet-

129 Hahnemühle, Barcelona, Spain), and analysed for silybin concentration by reverse-phase

130 HPLC according to the slightly modified method of Kvasnička, Bìba, Ševčìk, Voldřich, and

131 Kràtkà (2003). Analyses were performed by a LC-2010 AHT liquid chromatographic system

132 (Shimadzu, Kyoto, Japan) equipped with an integrated UV-visible detector. A 4 µm packed

133 150 x 4.6 mm C_{18} column (Synergi Polar, Phenomenex, Torrance, CA), thermostated at 35

134 °C, was used. The elution was in gradient mode using a mixture of 0.5% (v/v) aqueous

135 phosphoric acid (solvent A) and methanol (solvent B) as mobile phase at a flow rate of 1

136 mL/min. Gradient was set as follows: solvent B was held at 36% for the first 5 min, increased

137 to 45% in 1 min and held at this level for 25 min; then 100% solvent B was reached in 2 min

138 and held for 5 min, before to be lowered in 2 min to the initial level (36%). The sample

139 injection volume and the detection wavelength were 10 μL and 288 nm, respectively.

140 Quantitative analysis was carried out by comparing the silybin peak area with the results of a

141 calibration line, obtained by injecting silybin standard solutions (in water:methanol, 20:80

142 v/v). Calibration line was linear ($R^2 = 0.999$) in the 0.5 to 18.0 mg/L concentration interval.

143

144 *2.3.4. Chain breaking activity*

145 The chain-breaking activity was measured following the methodology of Brand-Williams,

146 Cuvelier, and Berset (1995). The bleaching rate of the stable free radical 2,2-diphenyl-1-

147 picrylhydrazyl (DPPH·) was monitored at 515 nm. A volume of 1.85 mL of 6.1×10^{-5} M

148 DPPH· methanol solution was used. The reaction was started by the addition of 150 μL of

149 sample, previously solubilised with methanol. The DPPH· bleaching was followed at 515 nm

150 (Uvikon 860, Kontron Instruments, Milano, Italy) at 25 °C for at least 10 min. In all cases the

151 DPPH· bleaching rate was proportional to the sample concentration added to the medium. The

152 reaction rate of DPPH· bleaching was computed according to the following equation

153 (Manzocco, Anese, & Nicoli, 1998):

154     $\frac{1}{A^3} - \frac{1}{A_0^3} = 3kt$ (eq. 1)

155   where k is the DPPH· bleaching rate, A_0 is the initial absorbance value, and A is the

156   absorbance at increasing time, t. The chain-breaking activity was expressed as the slope (k)

157   obtained from eq. 1 per milligram of dry matter ($A^{-3}/\text{min}\cdot g_{dm}$), assuming that all of the sample

158   dry matter possessed antioxidant capacity.

159

2.3.5. Oxygen concentration

161   Oxygen concentration was measured by an OxySense® fluorimeter (OxySense Inc., Dallas,

162   TX, USA). Aliquots of 18 mL of the nanoemulsions were introduced into 20 mL colourless

163   glass vials. Preliminarily, an oxygen sensitive sensor (O2xyDot®, OxySense Inc., Dallas, TX,

164   USA) was pasted on approximately 1 cm from the bottom edge of the internal surface of the

165   vials, by using an oxygen permeable glue (OxySense Inc.). When the sensor is illuminated by

166   a pulsed blue light, a red fluorescent light is emitted, that is measured by the fluorimeter. The

167   decrease of the O2xyDot® fluorescence lifetime, due to dynamic oxygen quenching, is

168   proportional to the oxygen concentration in the sample. Sample temperature was measured

169   simultaneously, by a sensor located in the reader pen of the fluorimeter (Li, Ashcraft,

170   Freeman, Stewart, Jank, & Clark, 2008). Results were expressed as mg/L of oxygen.

171

2.3.6. Lipid hydroperoxide concentration

173   Lipid hydroperoxide concentration was determined following the methods of Shantha and

174   Decker (1994) and Katsuda, McClements, Migioranza, and Decker (2008). In particular, 3 mL

175   nanoemulsion were mixed three times with 15 mL isooctane:2-propanol (3:1 v/v) solution and

176   vortexed for 10 s. After centrifugation for 2 min at 2000 g, the clear upper layer was collected

177   (0.20 mL) and mixed with 2.8 mL methanol:1-butanol (2:1 v/v) solution, 15 µL of 3.94 mol/L

178   ammonium thiocyanate solution and 15 µL of 0.0072 mol/L ferrous ion solution (prepared

179   through the mixture of 0.132 mil/L $BaCl_2$ and 0.144 mol/L $FeSO_4$). After 20 min incubation

8

180 at room temperature, absorbance was measured at 510 nm with a spectrophotometer

181 (Shimadzu, UV-2501PC, Japan). Hydroperoxide concentration was determined using cumene

182 hydroperoxide standard curve.

183

184 *2.3.7. Silybin* in vitro *bioaccessibility*

185 *In vitro digestion.* A dynamic *in vitro* digestion model was used to study the influence of

186 emulsion composition on silybin bioaccessibility. The methods of Zangerberg, Mullertz,

187 Kristensen, and Hovgard (2001a and 2001b) and Mun, Decker, Park, Weiss, and McClements

188 (2006) were followed with some modifications. 0.75 mL nanoemulsion was mixed with

189 phosphate buffer (pH 7) and heated at 37 °C for 10 min in a water bath. Then, the pH of the

190 sample was adjusted to 7.00 with 2 M NaOH and 4 mL bile extract (46.9 g/L in phosphate

191 buffer at pH 7) and 1 mL calcium chloride (110 g/L in deionized water) were added. The pH

192 was adjusted to 7.00 if necessary. Finally, 2.5 mL of freshly prepared lipase suspension (24

193 g/L in phosphate buffer at pH 7) was added to the mixture. The pH of the mixture was

194 monitored and maintained at 7.00 by adding 0.1 M NaOH. The volume of NaOH added to the

195 sample was recorded and used to calculate the concentration of free fatty acids (FFA)

196 generated during lipolysis. The extent of lipolysis was determined as follows:

197

198 $$FFA\ released\ (\%) = \frac{NaOH\ amount\ consumed}{theoretical\ NaOH\ amount\ for\ complete\ lipolysis} \cdot 100 \quad \text{(eq. 2)}$$

199

200 To calculate the amount of NaOH required for complete lipolysis, it was assumed that 1

201 molecule of sunflower oil, extra virgin olive oil or castor oil consumed 2 molecule of NaOH

202 (Yu & Huang, 2012), each oil molecule being hydrolysed by pancreatic lipase into two free

203 fatty acids and one monoacylglycerol molecule.

204 In vitro *bioaccessibility determination.* The *in vitro* bioaccessibility of silybin was evaluated

205 after the *in vitro* digestion was completed. The digest was immediately centrifuged (XL-70

9

206 Ultracentrifuge, Beckman, Palo Alto, CA, USA) at 165000 g at 4 °C for about 70 min. After

207 centrifugation, the sample was separated into an opaque sediment phase (pellet) and a clear

208 phase containing the mixed micelles (supernatant). Silybin was extracted from both the digest

209 and the micelles by liquid-liquid extraction. Briefly, 20 mL digest was introduced in 50 mL

210 Pyrex tubes and mixed with 200 µL of 218.8 g/L hydrochloric acid and 100 µL of naringenin-

211 7-O-glucoside methanol solution (0.73 g/L). 10 mL ethyl acetate was then added and the

212 tubes were subjected to manual shaking (5 min) followed by immersion in ultrasonic bath (1

213 h, 40 °C). The sample was then centrifuged (1000 g, 10 min) and the organic phase separated.

214 Manual extraction and ultrasound treatment were repeated twice and the ethyl acetate extracts

215 concentrated to a final volume of approximately 1 mL, in a vacuum centrifuge (Univapo 100

216 H, UniEquip GmbH, Freital, Dresden, Germany). Silybin quantification in the concentrated

217 extracts was carried out by HPLC as reported above (Paragraph 2.3.3.). The bioaccessibility

218 (%) was defined as the percentage ratio between silybin concentration in the mixed micelles

219 and the silybin concentration in the digest. In addition, to allow a better traceability of the

220 repartition of the active compound between the pellet and micelles, the pellet was also

221 analysed for silybin content. To this purpose, the sediment was re-suspended in 5 mL

222 methanol and 100 µL 218.8 g/L hydrochloric acid. Extraction was performed by manual

223 shaking (5 min) followed by immersion in ultrasonic bath (40 °C for 1 h). Samples were

224 finally filtered on 0.20 µm pore size nylon membranes (Albet-Hahnemühle, Barcelona,

225 Spain), and silybin content was determined by HPLC as reported above (Paragraph 2.3.3.).

226 Mass balance of silybin in pellet and micelles evidenced the complete recovery of the

227 bioactive compound.

228

229 *2.4. Statistical analysis*

230 The results are averages of at least three measurements taken from different samples and are

231 reported as means ± SD. Analyses of variance (ANOVA) was performed with significance

232 level set at p<0.05 (Statistica for Windows, ver. 5.1, Statsoft Inc. Tulsa, USA, 1997). The

233 Tukey procedure was used to test for differences between means.

234 **3. Results and discussion**

235

236 *3.1. Silybin solubility*

237 The solubility of silybin in water, selected oils and Tween 80 is shown in Table 1. In

238 accordance with the literature, the solubility of silybin in water was negligible (Gazak et al.,

239 2004; Yang et al., 2013). Silybin presented the highest solubility in castor oil followed by

240 extra virgin olive oil and sunflower oil. As known, among the selected oils, castor oil is the

241 most polar oil due to its high content of ricinoleic acid. This result is consistent with the data

242 reported by Yang et al. (2013) and seems to indicate that a certain degree of polarity of the oil

243 phase might favour silybin solubility. Data on the surfactant Tween 80 support this hypothesis

244 showing silybin solubility one order of magnitude higher than in the oils. As reported in the

245 literature, Tween 80 is actually used as surfactant in different nanoemulsions for drug delivery

246 (Parveen et al., 2011; Yang et al., 2013). Furthermore, silybin solubility was determined in

247 mixes of sunflower oil, extra virgin olive oil or castor oil, and Tween 80 in 1:1 (v/v) ratio.

248 Results showed that in all mixtures the silybin solubility had a value comparable to that found

249 in the Tween 80 alone.

250 To compare the performances of different nanoemulsions to carrying silybin, before emulsion

251 preparation 2.50 mg/g silymarin, corresponding to 0.525 mg/g silybin, was added to

252 sunflower oil, extra virgin olive oil or castor oil containing 10 mg/g Tween 80. At this level,

253 silybin was completely solubilized in the mixture.

254

255 *3.2. Physical properties of silymarin enriched nanoemulsions*

256 Table 2 shows the mean particle diameter of sunflower oil, extra virgin olive oil and castor oil

257 based nanoemulsions enriched with silymarin. Control samples prepared without silymarin

258 showed results not significantly different from the bioactive enriched counterparts (data not

259 shown). Although the samples had relatively small mean particle diameters, appreciable

260 differences in the values among the three types of nanoemulsions can be observed, the one

261 prepared with castor oil showing higher values. As emulsion properties are greatly affected by

262 the nature of the oil used (McClements, 2005), these results can be attributed to differences in

263 chemical properties among the oils considered. In particular, the higher particle size of

264 nanoemulsion with castor oil can be due to higher viscosity and polarity of castor oil in

265 comparison with extra virgin olive oil and sunflower oil. Neither significant increase in

266 nanoemulsions particle size nor visible sediments at the bottom of the test vials were found

267 during storage at 20 °C for up to 50 days. These results suggest that silymarin did not undergo

268 separation and re-crystallisation phenomena during storage.

269

270 *3.3. Silybin* in vitro *bioaccessibility of silymarin enriched nanoemulsions*

271 The effect of carrier oil on the silybin *in vitro* bioaccessibility in nanoemulsions was studied.

272 As well known, silymarin carrying triacylglycerols have to be decomposed into free fatty

273 acids and monoglycerides to allow the bioactive molecule to be released and subsequently

274 incorporated into the mixed micelles, i.e. made of bile salts and lipolytic products, to be

275 transferred to the epithelium cells (Hofmann & Borgstrom, 1964). To monitor the rate and

276 extent of lipid digestion, the formation of free fatty acids from the nanoemulsions during

277 simulated small intestine digestion was measured (Figure 1). The free fatty acids release

278 steadly increased in the first minutes of the digestion, suggesting that the lipase promptly

279 attached to the oil droplets surface due to an efficient displacement of the surfactant layer by

280 the bile salts (Qian, Derek, Xiao, & McClements, 2012). In our experimental conditions, the

281 free fatty acids release during the *in vitro* digestion was not affected by the carrier oil type,

282 which is consistent with previous results (Hur, Joo, Lim, Decker, & McClements, 2011). The

283 oils used were actually all composed of long chain fatty acids, the chain length being an

284   influencing factor of the extent and rate of *in vitro* lipolysis. Moreover, our results clearly

285   show that in all cases the free fatty acids release was almost complete (around 80-90%

286   digestion). The silybin concentration in the mixed micelles (i.e. silybin bioaccessibility) and

287   precipitated pellets obtained from the digest was then measured (Table 3). Results showed

288   that the carrier oil only slightly influenced the silybin *in vitro* bioaccessibility. In fact,

289   independetly of oil type, silybin concentration in the micelles was approximately 25-30%,

290   indicating that most of the bioactive compound incorporated into the nanoemulsions was not

291   available for absorption.

292

293   *3.4. Chemical properties of silymarin enriched nanoemulsions*

294   Despite a huge number of papers deal with the mechanisms of silymarin biological activity, to

295   our knowledge the effect of flavonoids from silymarin on food lipid oxidation has not been

296   well investigated. Figure 2 shows the changes in oxygen concentration of sunflower oil, extra

297   virgin olive oil and castor oil containing nanoemulsions enriched with silymarin as a function

298   of storage time at 20 °C. Nanoemulsions not containing silymarin were used as controls.

299   Oxygen concentration decreased faster in nanoemulsions containing sunflower oil, followed

300   by nanoemulsions with extra virgin olive oil and castor oil. Such a reactivity rank is consistent

301   with the unsaturation degree of the incorporated oils, sunflower oil having a higher value than

302   extra virgin olive oil, which in turn has a greater number of carbon-carbon double bonds than

303   castor oil. No significant differences in oxygen concentration were found between

304   nanoemulsions with and without silymarin. This result suggests that silymarin did not act as

305   an oxygen scavenger, in agreement with the pionering data reported by Dehmlow et al.

306   (1996), who found a negligible capacity of silybin to react with $O_2^-\cdot$ species. Figure 3 shows

307   the changes in hydroperoxide concentration of nanoemulsion with or without silymarin during

308   storage. It can be observed that the hydroperoxide concentration changed during storage with

309   different kinetics depending on the nature of the carrier oil, the sunflower oil and castor oil

13

310   containing emulsions being the most and the least susceptible to oxidation, respectively.

311   However, also in this case, the evolution of hydroperoxides in the nanoemulsions enriched

312   with silymarin did not significantly differ from those of the respective nanoemulsions without

313   the bioactive molecule. These results further indicate that silymarin incorporation into

314   nanoemulsions did not influence the pathway of the oxidative reactions occuring in oils.

315   However, they contrast with data on the liver protective effect of silymarin, that is generally

316   attributed to the antioxidant activity of the bioactive molecule (Shaker, Mahmoud, & Mnaa,

317   2010). To have insights on the chain breaking activity of silymarin extract used in this study,

318   the DPPH· assay was performed. Only a weak ability of silymarin to scavenge the DPPH·

319   radical was measured (i.e. 0.14 ± 0.06 A^{-3}/min·g_{dm}). Such a value was much lower than the

320   chain breaking activity of α-tocopherol and Trolox, that were 0.94 ± 0.24 and 8.47 ± 0.45 A^{-}

321   ^{3}/min·g_{dm}, respectively. These results are in agreement with the data of the literature (Gazak et

322   al., 2004; Henning et al., 2014). In particular, Henning et al. (2014) reported that the *Silybum*

323   *marianum* extracts had antioxidant properties lower than other dietary supplements, such as

324   pomegranate, resveratol and green tea. It is noteworthy that other authors found that silymarin

325   had high *in vitro* radical scavenging activity (Koksal, Gulcin, Beyza, Sarikaya, & Bursal,

326   2009).

327   Despite the results described above, silybin concentration in nanoemulsions greatly decreased

328   during storage (Figure 4). Such a decrease was greater in sunflower oil based nanoemulsions

329   followed by extra virgin olive oil and castor oil containing samples. In particular, at 50 days

330   storage, silybin losses were 60% and 55% in nanoemulsions containing sunflower oil and

331   extra virgin olive oil, respectively, whereas 25% reduction of silybin content was found in the

332   nanoemulsion with castor oil. These discrepancies in silybin degradation kinetics might be

333   brought back to differences in the bioactive reactivity in the media considered. It can be

334   inferred that in sunflower oil and extra virgin olive oil containing nanoemulsions, silybin

335   could be preferably located at the interface, near the emulsifier layer, where a higher polarity

14

336 would favour its degradation. On the contrary, when castor oil was used as a carrier, silybin

337 would be preferably located inside the oil droplets, being thus less prone to degradation.

338 Possible silybin degradation by reactive species generated from oil oxidative reactions may

339 not be ruled out. Gazak et al. (2004) actually described a number of oxidized derivatives of

340 silybin. These compounds were obtained under strong oxidative conditions (e.g., H_2O_2 in

341 NaHCO$_3$, iodine in glacial acetic acid, high temperature). In our experimental conditions, the

342 greater the oil susceptibility to oxidation, and thus the formation of oxidation products, the

343 greater the silybin degradation. It is a matter of fact that due to the scarcity of information

344 available in the literature, further experiments are needed to understand the fate of silybin in

345 food related environments.

346

347 **Conclusions**

348 The present study is a first attempt to develop delivery systems for incorporating silymarin

349 into functional foods. Results showed that silymarin can be successfully incorporated into

350 physically stable nanoemulsions prepared with different carrier oils. The oil type used slightly

351 affected the *in vitro* bioaccessibility of the main bioactive compound of silymarin, i.e. silybin.

352 Although silymarin incorporation resulted not to affect oil oxidative kinetics, silybin

353 concentration decreased during storage. Such a decrease was greater in extra virgin and

354 sunflower oil than in castor oil. This instability rank is consistent with that relevant to the

355 susceptibility to oxidation of the carrier oils considered. Although additional studies should be

356 accomplished to fully elucidate the mechanism of silybin degradation in lipid carriers, the

357 information acquired represents an important contribution for the design and fabrication of

358 silymarin delivering nanoemulsions.

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471 **Figure captions**

472 Figure 1. Free fatty acids (FFA) release from sunflower oil, extra virgin olive oil and castor

473 oil based nanoemulsions enriched with silymarin.

474 Figure 2. Percentage of residual oxygen in sunflower oil, extra virgin olive oil and castor oil

475 based nanoemulsions with and without silymarin, as a function of storage time at 20 °C.

476 Figure 3. Changes in hydroperoxide concentration in sunflower oil, extra virgin olive oil and

477 castor oil based nanoemulsions with and without silymarin, as a function of storage time at 20

478 °C.

479 Figure 4. Silybin concentration in sunflower oil, extra virgin olive oil and castor oil based

480 nanoemulsions enriched with silymarin, as a function of storage time at 20 °C.

481 **Table captions**

482 Table 1. Silybin solubility in water, sunflower oil, extra virgin olive oil, castor oil and Tween

483 80.

484 Table 2. Mean particle diameter of sunflower oil, extra virgin olive oil and castor oil based

485 nanoemulsions enriched with silymarin.

486   Table 3. Silybin concentration (%) in mixed micelle (i.e. *in vitro* bioaccessibility) and pellet

487   after *in vitro* digestion of sunflower oil, extra virgin olive oil and castor oil based
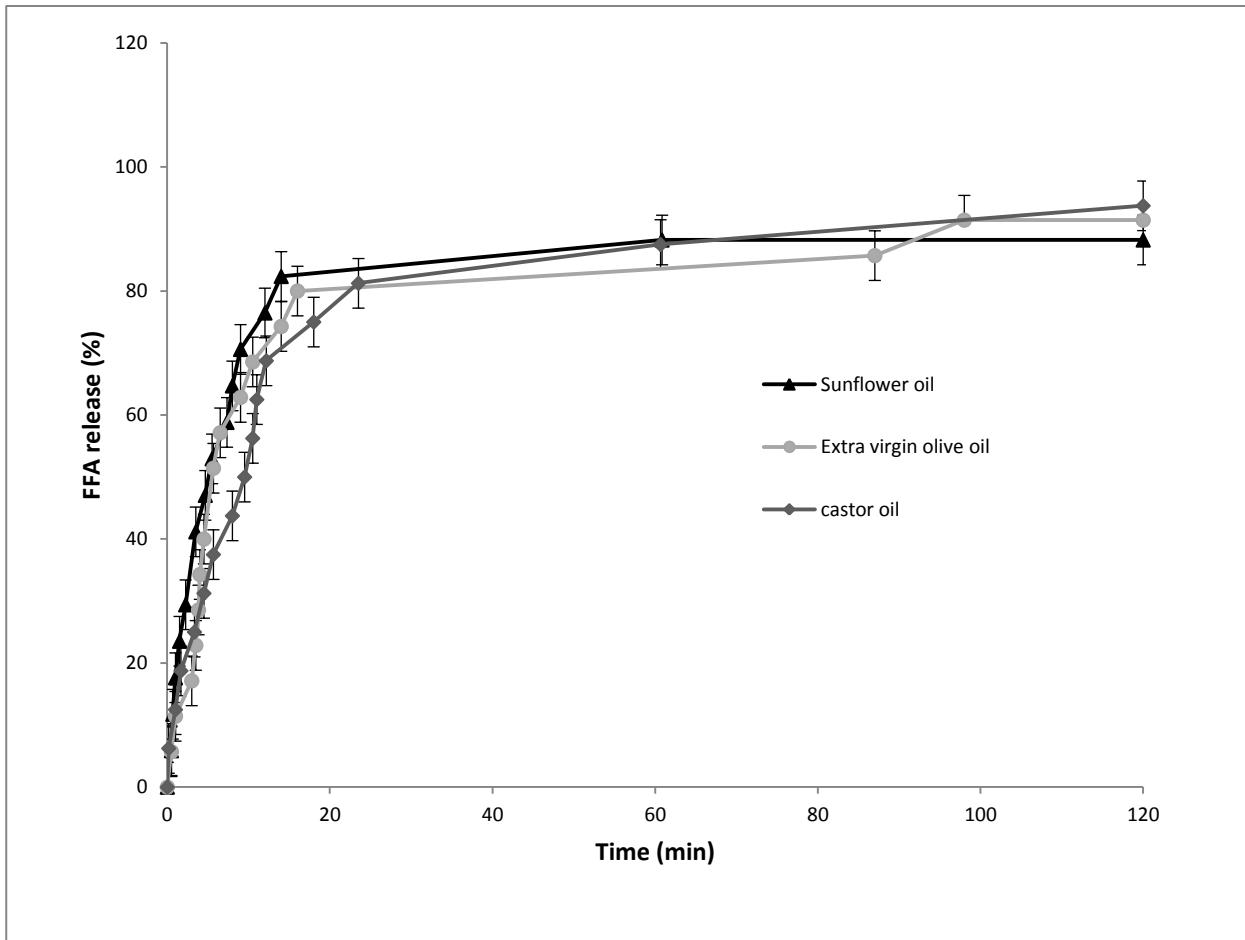
488   nanoemulsions enriched with silymarin.

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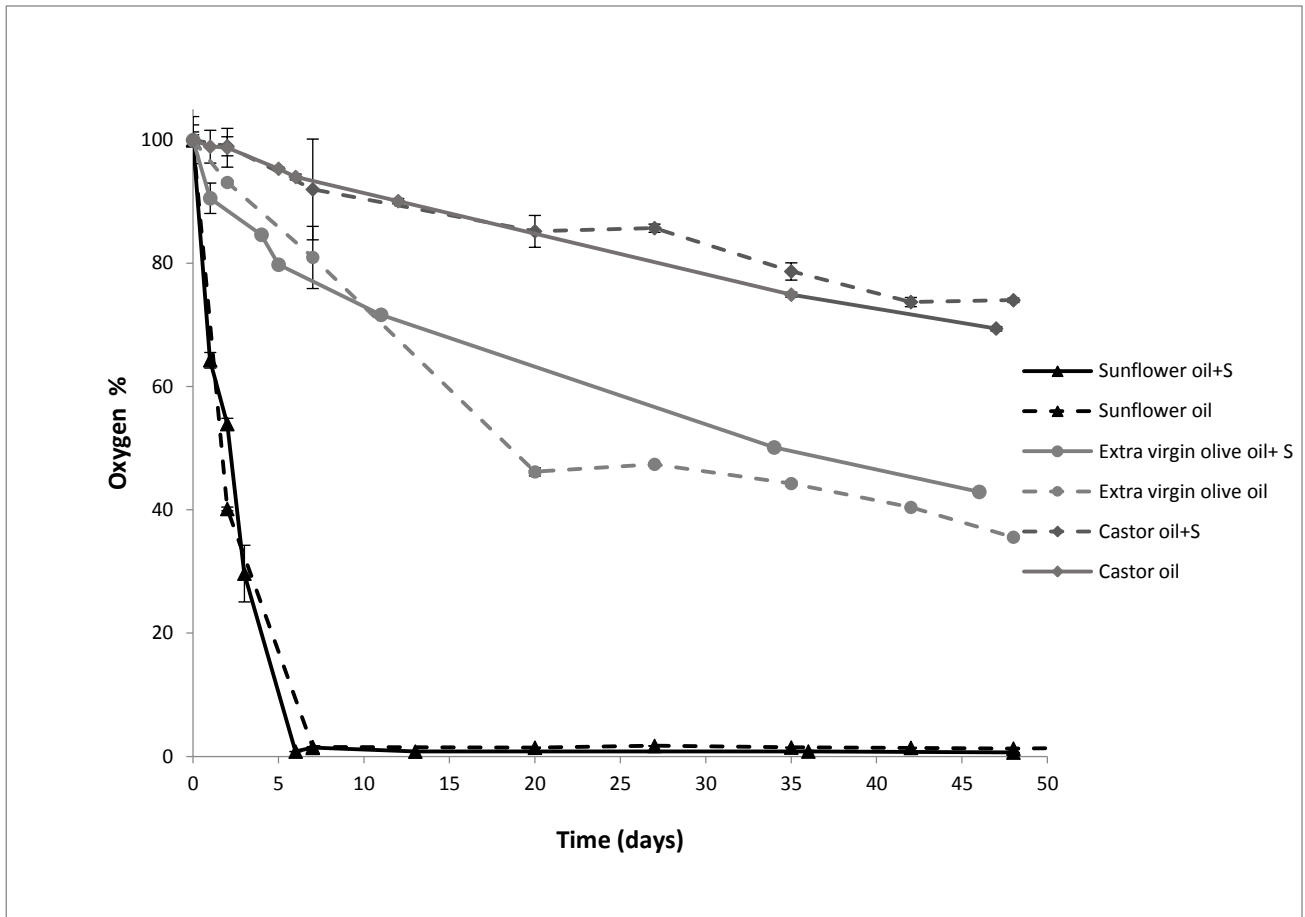
Figure

Figure 2. Percentage of residual oxygen in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions with and without silymarin, as a function of storage time at 20 °C.
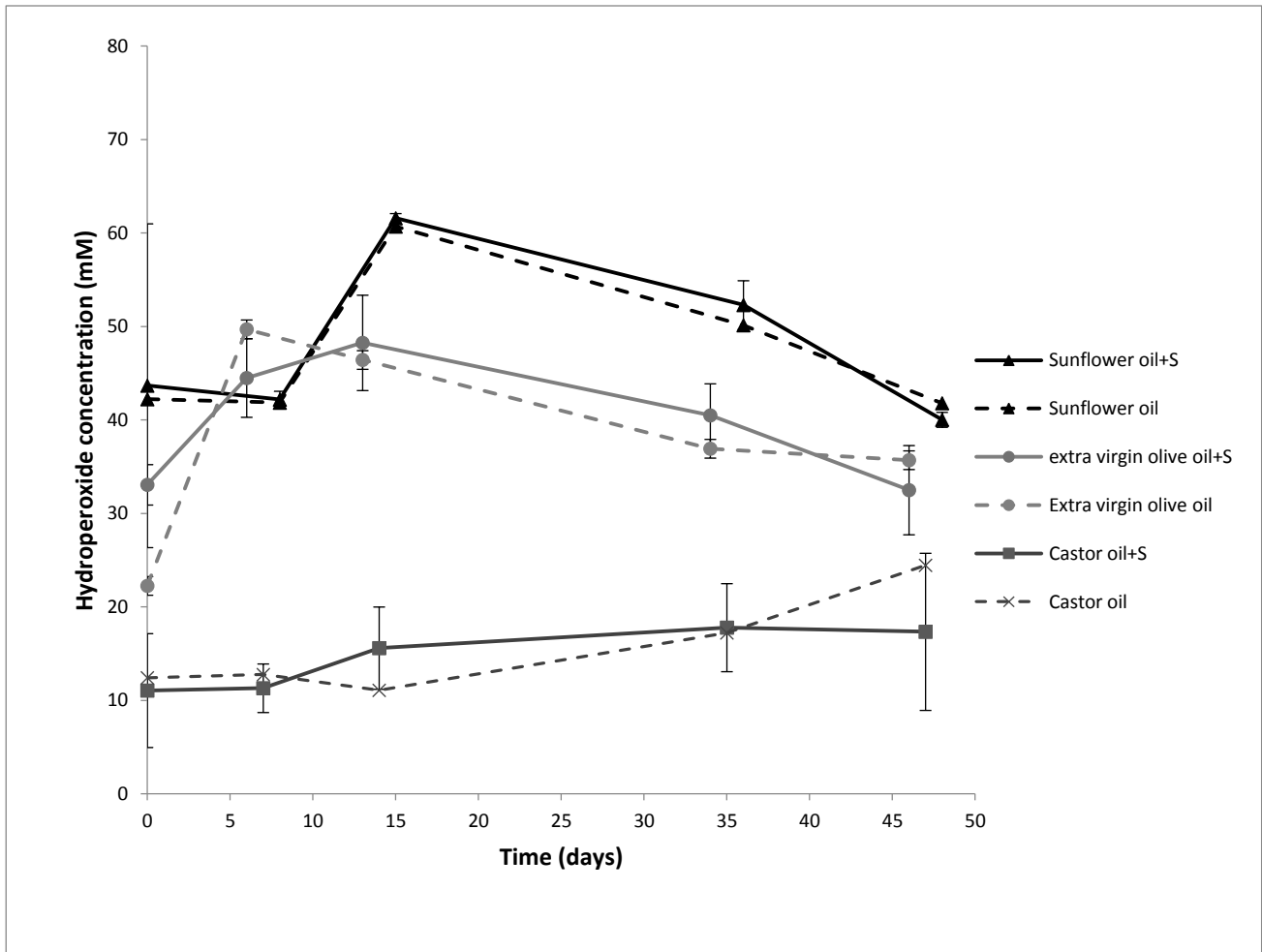
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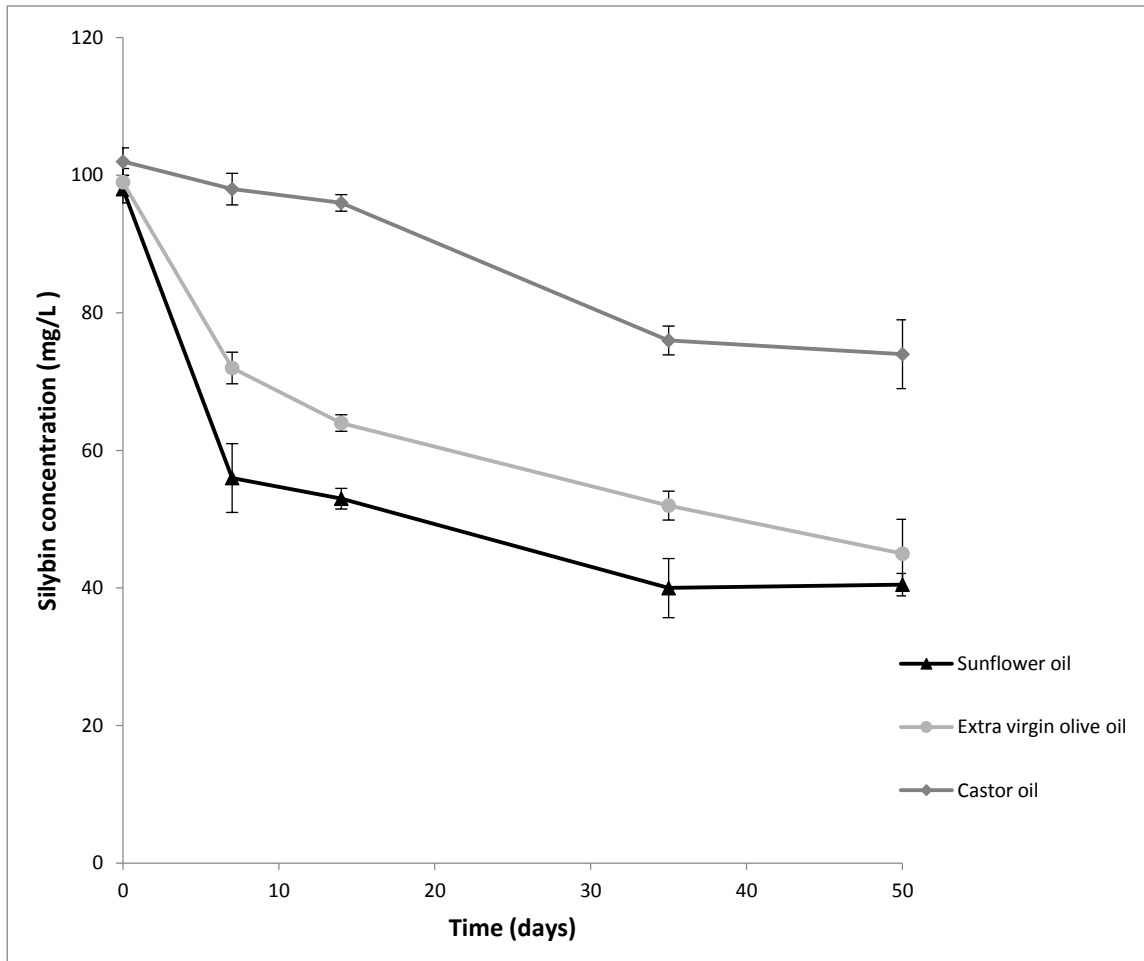
Figure

Figure 4. Silybin concentration in sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin, as a function of storage time at 20 °C.

Table

Table 1. Silybin solubility in water, sunflower oil, extra virgin olive oil, castor oil and Tween 80.

Medium	Silybin (mg/g)
Water	nd
Sunflower oil	0.028 ± 0.006^{b}
Extra virgin olive oil	0.009 ± 0.002^{a}
Castor oil	0.668 ± 0.072^{c}
Tween 80	2.061 ± 0.110^{d}

[a-d]*: means with different letters are significantly different (p < 0.05)*

nd: not detectable

Table

Table 2. Mean particle diameter of sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin.

Storage time (days)	Mean particle diameter (nm)		
	Sunflower oil	Extra virgin olive oil	Castor oil
0	208 ± 39^a	241 ± 46^a	307 ± 52^b
15	208 ± 37^a	229 ± 39^a	309 ± 53^b
36	208 ± 38^a	235 ± 48^a	329 ± 6^b
50	232 ± 49^a	235 ± 47^a	323 ± 62^b

[a-b] *means with different letters in the same row are significantly different (p<0.05)*

Table

Table 3. Silybin concentration (%) in mixed micelle (i.e. *in vitro* bioaccessibility) and pellet after *in vitro* digestion of sunflower oil, extra virgin olive oil and castor oil based nanoemulsions enriched with silymarin.

Carrier oil	Silybin (%)	
	Micelle	Pellet
Sunflower oil	25.3 ± 2.1^{b}	78.1 ± 6.5^{a}
Extra virgin olive oil	29.1 ± 0.7^{a}	71.1 ± 4.5^{ab}
Castor oil	29.6 ± 1.6^{a}	68.0 ± 0.6^{b}

[a-b] *means with different letters are significantly different ($p < 0.05$)*